Assimilation mechanisms of *Bacillus subtilis*

Man Wai Chow

A thesis submitted in partial fulfilment of the requirements
of the regulation for the degree of **Doctor of Philosophy**

Newcastle University

Faculty of Medical Sciences

Biosciences Institute

March 2023

Abstract

Transport systems in bacteria enable uptake of nutrients and release of useless or even toxic material out of the cell but currently these cellular processes remain poorly characterised. Despite establishing the whole genome sequence of *Bacillus subtilis*, the use of bioinformatics analyses to identify transport systems has not been effective as expected as *in vivo* experiments are necessary to demonstrate the expression and the specificity of transporters. This thesis set out to develop methods to identify the substrates of transporters and to characterise their regulation and role in cellular metabolism. In this pilot work amino acids were chosen as the nutrients to study and develop the methods to look at uptake of other molecules. The initial focus was to understand how *B. subtilis* utilises available nutrients and for this work it was necessary to develop a defined medium that was compatible with both analytical methods and permitted good growth of the bacterial strains. Through this work an understanding of which amino acids are preferentially taken up by *B. subtilis* and how specific auxotroph's altered assimilation of amino acids were obtained. Combining with other work, alanine metabolism was indicated poorly defined, and consequently this became a focus of both genetic and phenotypic analyses. The work also indicated that a conventional genetic approach would not permit the identification of transporters for other amino acids and so transcriptional approach was used to try and define genes that were up-regulated in strains starved for specific amino acids. It was expected that some would be involved in the uptake of the limited amino acid. But the results indicated a more complex picture of transcriptional regulation that implies that uptake systems are potentially constitutive and starvation for one specific amino acid tends to result in a global stress response rather than the up-regulation of discrete set of genes to resolve the problem.

The work presented details of methods to look at the nutritional priority of bacterial strain using *B. subtilis* as a model system. The work also clarifies the metabolism of alanine at the level of synthesis, extracellular assimilation and degradation I also showed that specific amino acid starvation did not directly result in the stringent response as might be expected. In summary, this work provides a new perspective on bacterial growth in rich media and the potential factors that limit growth as well as provides an insight into the uptake repertoire of *B. subtilis* with respect to amino acids. An understanding of which has application in synthetic biology with respect to harnessing of bacterial metabolism for the production of specific bio-molecules.

Acknowledgements

Firstly, I would like to express my gratitude to my supervisor, Dr. Richard Daniel, for his continuous help on everything. I would also like to express my gratitude to Prof. Yun Chung Leung from Hong Kong Polytechnic University, for his kind advice when I was in Hong Kong for thesis writing. I am grateful to Mr. John Casement from Bioinformatics Unit at Newcastle University who helped on assessing data quality and processing the RNA sequencing data from Novogene.

I am also grateful to Dr. Ling Juan Wu, who always offers generous and kind advice on life outside of the lab. Also, I want to thank colleagues at Centre for Bacterial Cell Biology for their help on lab duties, especially Mr Ian Selmes and Ms Frances Davison, our two dear laboratory managers.

Finally, I would like to thank my master and parents for their unconditional support in this journey. And I would like to thank my friends who encouraged me at difficult times. Last but not least, my plushies have given the strongest psychological support through my ups and downs during the journey.

Table of Contents

Abstract....ii

Acknowledgements....iv

Chapter 1 Introduction 1

1.1 Cell envelope 1

1.1.1 Cell wall 2

1.1.2 Cytosolic membrane 6

1.2 Cellular metabolism 10

1.3 Network of adaptational responses in *B. subtilis* 15

1.4 Aims and objectives 18

Chapter 2 Materials and method 20

2.1 Strains maintenance and culture 20

2.2 Markerless deletion of genes in *B. subtilis* strains 20

2.3 Growth measurement on plate reader 21

2.4 *B. subtilis* transformation 21

2.5 *E. coli* transformation 21

2.6 Sequencing 21

2.7 Oligonucleotides 21

2.8 Gel electrophoresis 22

2.9 Plasmid DNA preparation 22

2.10 Chromosomal DNA preparation 22

2.11 Polymerase chain reaction (PCR) 22

2.12 Restriction digestion and ligation 22

2.13 Site-directed mutagenesis PCR 23

2.14 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) 23

2.15 Quantitative analysis of amino acids from HPLC chromatograms 24

2.16 Sample collection and processing for RNA sequencing 24

2.17 RNA sequencing analysis 25

2.18 Blue-white colony screening 26
2.19 Strains table 27
Chapter 3 Amino acid assimilation in *B. subtilis* 35
3.1 Introduction 35
3.2 Results 38
3.2.1 Developing defined growth medium for amino acid assimilation analysis 38
3.2.2 Determination of the methods suitable of amino acid quantification in defined culture medium 42
3.2.3 Amino acid assimilation profile under gluconeogenic or glycolytic growth conditions 46
3.2.4 Amino acid assimilation profile of amino acid auxotrophs 52
3.3 Discussion 56
3.3.1 Assessment of indirect approach for amino acid assimilation profiling ..57
3.3.2 The assimilation of amino acids during glycolytic growth of B. subtilis wild type strain in the defined media with glucose as carbon source 60
3.3.3 Gluconeogenic growth in defined medium with malate, another preferred carbon source of B. subtilis, malate. 67
3.3.4 Amino acid assimilation profile reserved in the manipulation of amino acids biosynthetic pathways 71
3.3.5 Speculation of the preference of amino acid assimilation 71
Chapter 4 alanine metabolism 74
4.1 Introduction 74
4.2 Results 77
4.2.1 Phenotypic characterisation of an alaT mutant 77
4.2.2 Construction of an alanine auxotroph 79
4.2.3 Complementation of *alaT* and *dat* 80
4.2.4 Alanine utilisation 82
4.2.5 *In vivo* characterisation of Dat 85

4.2.6 Isoform specificity of Dat in genetic context 88
4.2.7 AlaP transporter shared by both isoforms of alanine 90
4.2.8 Growth characteristics of strains defective in alanine uptake and/or biosynthesis in defined medium 92
4.2.9 *In vivo* analysis of the specificity of AlaP 97
4.3 Discussion 100
4.3.1 Alanine metabolism 101
4.3.2 Chacterisation of alanine transporter AlaP 105
Chapter 5 Transcriptional response to amino acid starvation 109
5.1 Introduction 109
5.2 Results 111
5.2.1 Global transcriptional profile of *B. subtilis* in response to specific amino acid starvation 111
5.2.2 Validation of the transcriptional data using a *lacZ* reporter system 130
5.2.3 Characterisation of *yvyD* reporter system for amino acid transporter screening 133
5.2.4 Specificity of tRNA synthetases in response to specific amino acid starvation 135
5.2.5 Testing of the reporter system using T-box switch from *tyrS* on positive control model of transporter screening 139
5.2.6 Improvement of reporter system with specifier codon targeting the amino acid 140
5.2.7 Use of transcriptomic data to screen for possible specific transporters 144
5.3 Discussion 149
5.3.1 Global transcriptomic analysis on specific amino acid starvations 150
5.3.2 Stringent response genes in response to amino acid starvation 154
5.3.3 Ribo-switch tyrS response in amino acid starvation 156
5.3.4 Using RNA sequencing filtering specific amino acid transporters 160
5.3.5 Proposed transcriptional response to specific amino acid starvation .. 165

Chapter 6 Final discussion and future work.....167
6.1.1 Final discussion.....167
6.1.2 Proposal of specific amino acid transporters screening.....169
6.1.3 Summary.....170
Chapter 7 References.....172
Chapter 8 Appendix.....201
Chapter 9 Publication.....220

List of Figures

Figure 1.1: A schematic diagram of metabolic pathways of amino acids with glycolysis and TCA cycle.. 12
Figure 3.1: Growth of strain 168+ in different medium measured in plate reader. 40
Figure 3.2: Detection and quantification of amino acids using RP-HPLC. 45
Figure 3.3: Growth of *168*+ in Gaa, Maa and GMaa and amino acid assimilation profile of *168*+ in Gaa.. 47
Figure 3.4: Graphs of genetically manipulated strains growing in defined medium (GaaP) compared with wild type strain... 55
Figure 3.5: Schematic diagram of amino acid metabolism of *B. subtilis*. 64
Figure 4.1: Growth characteristics of *ΔalaT::erm* (MC2) on plate GMP with or without L- or D-alanine. 78
Figure 4.2: Growth characteristics of *ΔalaT Δdat::erm* (MC4) on plate GMP with or without L-alanine. 80
Figure 4.3: Complementation of *ΔalaT::kan pAG-alaT(erm)* (RD220) and *ΔalaT::kan pAG-dat(erm)* (RD226) on plate GMP 82
Figure 4.4: Ald was required to utilise alanine as sole carbon source and sole nitrogen source.. 84
Figure 4.5: *in vivo* characterisation of D-alanine dependent auxotroph. 86
Figure 4.6: Complementation of *ΔalrA::zeo pAG-dat(erm)* (RD222) and *ΔalrA::zeo pAG-dat(erm)* (RD223) on minimal plate (GMP).. 89
Figure 4.7: AlaP transporter was shown required for alanine uptake (either isoform) from only specific medium condition.. 91
Figure 4.8: Quantitative growth measurement of alanine auxotrophs with or without deletion of *alaP*.. 94
Figure 4.9: Quantitative growth measurement of alanine auxotrophs with or without deletion of *alaP*. 95
Figure 4.10: Quantitative growth measurement of alanine auxotrophs with or without deletion of *alaP*.. 96
Figure 4.11: *in vivo* analysis of the specificity of AlaP. 98
Figure 4.12: A schematic diagram of alanine assimilation in *B. subtilis*.. 104
Figure 5.1: Growth curves of WT and the selected auxotrophs. 113
Figure 5.2: Schematic flow chart of data processing of the RNA sequencing result.. 120

Figure 5.3: Characterisation of the expression of selected stringent response related genes. 133
Figure 5.4: Characterisation of the *yvyD* reporter system on positive control model of amino acid transporter screening on plate.. 135
Figure 5.5: Characterisation of the reporter systems controlled by specifier codon of riboswitch controlling transcription of *tyrS* targeting tRNA of different amino acids on plate. 137
Figure 5.6: Characterisation of the reporter system of specifier codon of *tyrS* targeting tRNA on positive control model of amino acid transporter screening on plate.. 140
Figure 5.7: Characterisation of the reporter system targeting tRNA of two different amino acids on positive control model of amino acid transporter screening on plate.. 143
Figure 8.1: Chromatogram of amino acids content in defined medium of culture taken during time course experiment for quantitative analysis. 201

List of Tables

Table 1.1: Transporters encoded in *B. subtilis* genome. 9
Table 1.2: Amino acids transporters or transporter systems identified in *B. subtilis*. 10
Table 2.1: strains used in this project. 27
Table 2.2: Plasmids used in this project..... 32
Table 2.3: primers used in this project. 33
Table 3.1: Relative abundance of amino acids of *168+* growing in Gaa medium at different time points. 48
Table 3.2: Depletion rates of amino acids from defined medium of growing *168+*.... 50
Table 3.3: Tables of assimilation efficiency of the amino acid specified for different auxotrophs and the wild type strain. 56
Table 5.1: Up-regulated genes recognised as response to specific amino acid starvation using approach 1. 121
Table 5.2: Genes that were up-regulated in both comparison PP2 VS PP1 and LL2 VS LL1. 124
Table 5.3: Up-regulated genes recognised as response to specific amino acid starvation using approach 2. 127
Table 5.4: Genes that were up-regulated in both comparison PP2 VS WP2 and LL2 VS WL2..... 128
Table 5.5: Fold change of selection of genes in response to specific amino acid starvations using approach 1. 131
Table 5.6: Fold change of selection of genes in response to specific amino acid starvations using approach 2. 131
Table 5.7: Possible gene candidates for transporters screening of lysine. 146
Table 5.8: Possible gene candidates for transporters screening of phenylalanine. 147
Table 5.9: Transcription expression of different tRNA synthetases which were regulated by T-box in response to leucine and phenylalanine specific amino acid starvation. 158
Table 5.10: Transcription expression of different tRNA synthetases which were regulated by T-box in response to leucine and phenylalanine specific amino acid starvation. 159
Table 8.1: Relative abundance of amino acids of *168+* growing in Gaa medium at different time points. 201
Table 8.2: Depletion rates of amino acids from defined medium of growing *168+*.. 202

Table 8.3: Tables of assimilation efficiency of the amino acid specified for different auxotrophs and the wild type strain. 203
Table 8.4: Table of upregulated genes in lysine auxotroph grown in limited lysine compared to that of grown in excess. 203
Table 8.5: Table of upregulated genes in lysine auxotroph and wild type strain grown in limited lysine compared to that of grown in excess. 206
Table 8.6: Table of genes upregulated in phenylalanine auxotroph grown in limited phenylalanine compared to that of grown in excess. 206
Table 8.7: Table of genes upregulated in phenylalanine auxotroph and the wild type strain grown in limited phenylalanine compared to that of grown in excess. 211
Table 8.8: Table of genes up-regulated in lysine auxotroph relative to the wild type strain grown in limited lysine 212
Table 8.9: Table of genes up-regulated in lysine auxotroph relative to the wild type strain grown in limited lysine as well as that of grown in excess lysine. 216
Table 8.10: Table of genes up-regulated in phenylalanine auxotroph relative to the wild type strain grown in limited phenylalanine. 216

Chapter 1 Introduction

Bacillus subtilis is a Gram-positive bacterium that is often found in diverse and often hostile environments. The cells are rod-shaped in vegetative growth with the aqueous cytosol enclosed by a lipid bilayer that in turn is enclosed by a cell envelope. The cell envelope consists of a cytoplasmic membrane and a multi-layered cell wall composed of a proteo-glycan polymer with additional anionic polymers. This structure, works to both protect the cell from the external environment and to act as a gate to control material entering and leaving the cytosol for optimised growth (Dijkstra and Keck, 1996). To survive through the ever-changing, quite often extreme conditions, the bacterium is a prototroph that can effectively utilise diverse extracellular nutrients to permit the synthesis of the precursors for growth. It has also evolved to be adaptive to extreme conditions with the help of a sophisticated regulatory system that acts to fine-tune the cellular metabolism and respond to nutrient depletion in various ways, *e.g.*, becoming motile or by entering a dormant state (sporulation) under extreme conditions.

The essential components for survival include carbon and nitrogen sources to provide the energy for metabolism and basic building blocks for both protein and carbohydrates necessary for growth. Phosphorus and sulphur as well as various metal ions are also required for the synthesis of other key precursors and act as cofactors for enzyme catalysis. These components are essential to feed cellular metabolism and permit enzyme activity (Sonenshein, 2007). However, all must be captured by the cell, transported through the cell envelope, and transported across the cell membrane to where they can be utilised. Thus, efficient systems must exist to ensure uptake of the necessary material can occur that is capable of being selective and has high affinity for diverse substrates.

1.1 Cell envelope

B. subtilis has a cell envelope which can be considered to be relatively simple two layered structure. The outermost layer consists of a complex matrix like structure that is the generally referred to as the cell wall which encloses the cytoplasmic membrane. These two components have various properties that can be modified in various ways that alter their properties and in combination seem to play different roles in transport of material into and out of the cell (van Wely *et al.*, 2001; Silhavy, Kahne and Walker, 2010) (explain later). It is also clear that the components of them

vary between different bacterial species, but the basic principles seem to be common. Consequently, for this introduction and this project will only focus on characteristics specific to *B. subtilis* and only briefly mention specific differences identified in other species where they are relevant.

1.1.1 Cell wall

The cell wall is a mesh-like complex and dynamic structure made of two major polymeric components, peptidoglycan (PG) and teichoic acids, including lipoteichoic acid (LTA) and wall teichoic acid (WTA).

The peptidoglycan consists of a network of rigid linear glycan strands, which are made of N-acetylglucosamine and N-acetylmuramic acids that are joined by β-(1-4) glycosidic bonds, together forming a multi-layered structure (Heijenoort, 2001). For *B. subtilis*, the average strand length is about 96 disaccharides (Ward, 1973), with chances of very long glycan strands of up to 5000 disaccharides being present in the cell wall (Hayhurst *et al.*, 2008; Kern *et al.*, 2010). The layers of glycan strands were cross-linked by short and elastic peptide stems containing L-alanine, D-glutamate and D-alanine dimers (Heijenoort, 1994; Vollmer, Blanot and de Pedro, 2008; Turner, Vollmer and Foster, 2014). Interestingly, the use of D-amino acids in the stem peptides of the wall seems to be an important feature of the peptidoglycan and the enzymes involved specifically require this isomeric form of the amino acid. Since D-amino acids are not generally available they have to be synthesized *de novo* or by conversion of the corresponding L-isoform by an amino acid racemase or D-amino acid aminotransferase (Yoshimura and Esak, 2003; Radkov and Moe, 2014). The reactions were reversible and would be explained in later sections, focusing on alanine. There are also some interesting exchange reactions that occur for certain amino acids in synthesized PG where D-alanine can be replaced by other D-amino acids, glycine and even D-lactate to varying degrees (Atrih *et al.*, 1999; Cava *et al.*, 2011).

Teichoic acids are made up of repeating units of anionic monomers (glycerol-phosphate or ribitol-phosphate) joined by phosphodiester linkages. Two distinct forms are generally present, one linked to the cell membrane (lipoteichoic acids, LTA) and the other covalently linked to the PG (wall teichoic acids, WTA) (Armstrong, Baddiley and Buchanan, 1959; Neuhaus and Baddiley, 2003). These are proposed to thread through the cell wall (Vollmer, Blanot and de Pedro, 2008; Vollmer and Seligman, 2010). The structure of these polymers is determined by the nature of the

glycosyl substituents (if present), the D-alanyl esters and the polymer chain. The major component of WTA is D-alanyl- [α-D-glycosylated poly 1,3-glycerol-phosphates (Gro-P)]. WTA is covalently bonded to the peptidoglycan with a chain length of 45-60 residues (Birdsell, Doyle and Morgenstern, 1975). Even if the bacterium is grown in phosphate-limited condition where teichoic acids synthesis would not be favoured, teichuronic acid, a negatively charged phosphorus-free polysaccharide, would be produced. This emphasized the importance of the negative charged nature of the wall, implying that this could be more important than the actual chemical composition of teichoic acid or teichuronic acid (Ward, 1981). On the other hand, LTA is made of poly (Gro-P) with a glycolipid anchor tethered to the cytosolic membrane whereas its poly (Gro-P) repeating units are suggested to be penetrating into the peptidoglycan (Neuhaus and Baddiley, 2003).

Both the peptidoglycan and teichoic acids can be modified by the addition of various molecules. D-alanylation is the best characterised and this modification is proposed to be highly dependent on the growth conditions, specifically temperature, acidity (pH) and the concentration of NaCl. For instance, a loss of D-alanylation was observed when the bacterial culture was incubated at 55°C instead of 37°C (Neuhaus and Baddiley, 2003; May *et al.*, 2005). This has been suggested to result in the modulation of the net charge within the wall and probably has significance for the activity of enzymes acting in the PG matrix and for the passage of compounds through the cell wall. Thus, this modification of the wall may act to match the extracellular conditions of the cell and impact on cell wall turnover (Perego *et al.*, 1995; Neuhaus and Baddiley, 2003). D-alanylation occurs to both types of TA in that around 9 % of glycerophosphate residues in WTA and 44 % of the residues in LTA are subject to the substitution of D-alanine esters (Perego *et al.*, 1995). The process is mediated in a single D-alanine incorporation system which is encoded by a dispensable *dlt* operon. Thus D-alanylation is not important to cell viability and the absence of D-alanylation was suggested to have no detectable effect on cell growth and basic metabolism despite a level of cell autolysis (Perego *et al.*, 1995; Wecke, Madela and Fischer, 1997; Hyyrylainen *et al.*, 2000).

Together the peptidoglycan and teichoic acids build a matrix-like, thick cell wall which is estimated to be a minimum of about 30 nm thick (Beveridge and Murray, 1979; Smith, Blackman and Foster, 2000). This is dynamic structure which is continually processed to permit growth (Foster and Popham, 2002; Hayhurst *et al.*, 2008). There

are several proposed architectural models of the wall. The general view being that the glycan strands are polymerised to form horizontal layers going around the cylindrical part of the cell and in parallel with the plasma membrane (Höltje, 1998; Vollmer and Höltje, 2001) on which the WTA are attached and potentially project up through the matrix. This concept with minor difference is supported by cryotomography (Beeby *et al.*, 2013) and atomic force microscopy (Turner, Hobbs and Foster, 2016).

Collectively, the wall components are made and progressed to move outwards following an inside-to-outside growth model even though the cell elongation is still poorly characterised (Hayhurst *et al.*, 2008; Vollmer, Blanot and de Pedro, 2008). Hypothetically the wall components might be pushed outwards and become stretched as the cells elongate. This potentially results in stretched wall on the outside surface which then become the substrate for the autolytic enzymes (Glaser and Lindsay, 1977; Cheung, Vitković and Freese, 1983; Mobley *et al.*, 1984). This resulting asymmetric density characteristic was observed in microscopic studies with labelled probes (Anderson *et al.*, 1978). Finally, it is presumed that degraded wall materials are then released outside of the cells (Koch and Doyle, 1985). The percentage of old wall materials released per generation was calculated to be around 25 – 50 %, depending on bacterial species (Mauck, Chan and Glaser, 1971; Pooley, 1976; Goodell and Schwarz, 1985; Park and Uehara, 2008). This whole idea was depicted in a cryo-TEM study in which the complicated biosynthesis of peptidoglycan cell wall was shown using heavy metal stain and detected denser by mass in frozen hydrated sacculi in which the newly added material is densely packed. This complex turnover seems to be provided by a diverse set of wall degrading enzymes encoded by the genome (more than 41 genes related to peptidoglycan hydrolases can be identified). The large number of autolysins and their functional redundancies have restricted our ability to precisely suggest physiological role of some autolysins (Smith, Blackman and Foster, 2009). But cell wall turnover was observed to be reduced in autolysin deficient cells (Mobley *et al.*, 1984). Although the mechanism is not clear yet, the cell wall synthesis and turnover are believed to be linked in their regulation (Mauck, Chan and Glaser, 1971).

Cell wall turnover results in the release of fragments of cell wall continuously in actively growing cells. Since the cell wall accounts for more than 20 % of total cell mass (Litzinger *et al.*, 2010), this would be a significant drain of cellular resources

unless the released materials can be recycled. The cell wall recycling pathways have been identified in several bacteria species including *B. subtilis* (Park and Uehara, 2008; Litzinger *et al.*, 2010; Reith and Mayer, 2011; Johnson, Fisher and Mobashery, 2013). However, the loss of degraded cell wall material has been historically reported in *Bacillus spp.* (Chaloupka et al, 1962, Muack et al, 1971, Blackman et al, 1998). The rate and necessity of wall recycling is determined by nutritional status in the surrounding environment, so the bacterial cell wall recycling may be repressed in rich media (Park and Uehara, 2008; Reith and Mayer, 2011). The peptidoglycan hydrolases might generate different sorts of peptidoglycan turnover products in *B. subtilis*. Muropeptide recycling pathway have been identified in *B. subtilis*. Peptides are cleaved with secreted enzymes and detached peptides are probably recycled through two oligopeptide permeases, Opp (LeDeaux, Solomon and Grossman, 1997) and App (Koide and Hoch, 1994). The *opp* gene is expressed in exponential phase and *app* gene is induced at beginning of stationary phase. *Opp* and *app* are specific for tetra- and penta-peptides but *opp* can also transport tripeptides (Koide, Perego and Hoch, 1999). The accumulation of different D-amino acids was reported in stationary culture supernatant of *B. subtilis* (Lam *et al.*, 2009; Kolodkin-Gal *et al.*, 2010). As of the functions of cell wall, firstly, the layers of long glycan strands cross-linked by elastic peptide stems provide rigidity to the wall but also permit a degree of flexibility (Vollmer, 2008). This was evident in the deformation of the cell wall when transient force was applied (Amir *et al.*, 2014). Thus, the rigidity contributes to the mechanical strength necessary to withstand the turgor pressure and so preserve the cell integrity as well as maintain the cells shape and the flexibility presumably acts to permit growth. In the absence of wall teichoic acids cells become morphologically deformed (Boylan *et al.*, 1972; Birdsell, Doyle and Morgenstern, 1975) implying integration of teichoic acids contributes in same way to the rigidity either directly as a component of the wall or by modulating the activity of wall modifying enzymes. Secondly the complex wall structure allows proteins with a wall-binding domain to be anchored in the matrix (Hayhurst *et al.*, 2008). These proteins include some penicillin-binding proteins for synthesis or modification of peptidoglycan, hydrolases for wall degradation, proteases for degrading mis-folded or defective proteins as well as DNases and RNases (Silhavy, Kahne and Walker, 2010).

Given that the wall structure is porous and negatively charged, combined with the presence of teichoic acids it is possible that material is transported by a charged

gradient, although this would only work for cations or cationic proteins (Birdsell, Doyle and Morgenstern, 1975). *In vitro* analysis of the wall architecture presumably reflecting both thickness and the level of cross-linkage between glycan strands provides an estimate of the pore radius of the wall of about 2 nm, allowing molecules up to maximum of 50 kDa to pass through under normal growing conditions (Demchick and Koch, 1996; Chow and Daniel, unpublished). This limitation in permeability and the presence of degradative enzymes *e.g.,* proteases, suggests that large molecules transport is restricted, both into and out of the cell. It is also conceivable that the combined properties of the envelope and bound enzymes acts to protect cells from 'active' extracellular enzymes that are harmful to the cells and/or toxic substances.

1.1.2 Cytosolic membrane

The membrane is made up of fatty acid esters linked to sn-glycerol-3-phosphate, structurally forming a bi-layer structure. The ester length, modification of their acylated fatty acids as well as composition of their headgroups are highly variable, allowing a range of different physiological properties in the nature of the membrane fluidity and surface charge (Silhavy, Kahne and Walker, 2010). For *B. subtilis*, the membrane mainly consists of anionic phospholipid phosphatidyglycerol (PG), which is the only essential component and zwitterionic phosphatidylethanolamine (PE) with the involvement of neutral glycolipids (GL), positively charged lysylphosphatidylglycerol (L-PG) and anionic cardiolipin (CL). This composition makes a hydrophobic and negatively charged dynamic membrane, contributing to cell adaptability in extreme environments. With the exception of PG, removal of other components of the membrane results in distorted cell morphology or abnormal cell separation but the growth is still maintained (Salzberg and Helmann, 2008). The end result of the assembly of these components is the formation of a hydrophobic bilayer barrier protecting the cellular cytoplasm from the outside. This provides a platform for proteins with trans-membrane domain(s) or lipobox attachment sites to pass through the membrane or be directly attached to the membrane and actively participate in different cellular activities for growth. Other proteins or assemblies of proteins that traverse the membrane can act to transport material across membrane (Yamane, Bunai and Kakeshita, 2004) and so allow for the import and export of substances.

Passive diffusion of amino acids might be possible and tryptophan was found to be in this category (Sarsero, Merino and Yanofsky, 2000). However, as the difference in

the chemical nature of amino acids are different from that of the cell membrane, specific transporters for them are more likely, allowing a more controllable uptake. Bioinformatic analysis has suggested a total of 285 transporters are encoded in the genome of *B. subtilis* (Saier *et al.*, 2002). This includes a wide variety of transport systems for different kinds of substrates and they have been categorised according to 'possible' substrate specificity (Table 1.1). In this thesis, the focus has been on amino acid transport and so this introduction will primarily describe what is known for the transport of these substrates. Amino acids can be actively taken up by bacteria through different types of transport systems, including ATP binding cassette (ABC)-type uptake system (primary transport system) or secondary carriers (secondary transport system) (Hosie and Poole, 2001). Nineteen families of the ABC-type transporters are present in prokaryotes, of which only two families are related to amino acids, either polar or non-polar (hydrophobic) amino acids. However, 9 out of 11 families of the secondary carriers for transporting amino acids and their derivatives have been found in bacteria. Therefore, most of the amino acid transporters are expected to be secondary carriers (permeases) (Saier, 2000). ABC-type uptake systems are assembled by the products of more than one gene, and are composed of two integral membrane proteins (channel proteins), two cytoplasmic ATP binding cassette subunits and one, or rarely two, solute specific binding protein(s) (Hosie and Poole, 2001; Scott and Barnett, 2006). The system depends on adenosine triphosphate (ATP) as an energy source for transporting of amino acids (Saier, 2000). In contrast, secondary carriers (permeases) usually comprise of a single polypeptide chain, which is integrated into the plasma membrane through its transmembrane alpha helical domains and arranged either as monomer or oligomer. The secondary carriers transport amino acids by electrochemical energy (sodium or proton-motive force) accumulated in the gradients of sodium or hydrogen ions (Saier, 2000; Scott and Barnett, 2006). Depending on the direction of gradients, secondary carriers are uniporter, symporter, or antiporter. The uniporters usually transport amino acids when the substrates own gradient is directed inwards. The amino acid/cation (H^+ or Na^+) symporters are the most frequent secondary carriers in bacteria. These symporters couple either H^+ or Na^+ with the amino acids during transport across the cell membrane. In contrast, the antiporters exchange two solutes across membrane (Krämer, 1994). The active transport systems of the amino acids are highly substrate specific in bacteria (Cohen and Monod, 1957). However, structurally related amino acids may be transported by a single transporter. For

instance, kinetic studies showed that alanine, glycine and serine have a common uptake system in bacteria (Oxendeb, 1972; Clark and Young, 1974) which is termed as substrate heterogeneity or functional redundancy. Out of 285 transporters, approximately 40 of them are predicted to be transporters for amino acids and their derivatives, yet only a small number of amino acids transporters have been characterised, either genetically or biochemically, to confirm the predicted functions (Table 1.2).

Collectively the literature on identified transporters in *B. subtilis* has shown some features in common. Firstly, some identified transporters were shown to be able to recognise certain other amino acids (Lorca, Winnen and Saier, 2003; Burguière *et al.*, 2004; Reig *et al.*, 2007; Belitsky, 2015). For instance, YveA was identified as a L-aspartate transporter but its uptake could be inhibited by the presence of other amino acids in high concentration such as asparagine and serine (Lorca, Winnen and Saier, 2003). This suggested the existence of specific amino acid transporters that could recognise other amino acids although possibly at a lower affinity. Secondly amino acids could be transported by more than one transporter *e.g.*, cystine, where three systems were identified with different properties (Burguière *et al.*, 2004). Together these two observations have shown the existence of transporters with a degree of specificity for their amino acids yet they also could recognise a number of other amino acids in extreme conditions.

Given the large number of transporters encoded in the genome, presumably a tight regulatory system would be necessary to control the expression of them in the different, often hostile environments encountered by bacteria. However, the regulation of the expression of transporters seems to be complex network that is poorly understood, potentially suggesting that they are either generally constitutively present in the membrane. Some transporters, such as BcaP and BraB, which were suggested to be responsible for branched-chain amino acids uptake, were shown regulated by CodY (a regulator in response to branched-chain amino acid limitations) at the transcriptional level (Belitsky, 2015). Apart from CodY, there were also other regulatory proteins modulating the expression of amino acid transporters, for example the proposed transporters for aspartate and glutamate, YveA and GltP, were shown to be regulated in response to different carbon source utilised for growth (Nicolas *et al.*, 2012; Chubukov *et al.*, 2013). At the same time, another transporter for the uptake of these two amino acids, GltT was shown regulated by a different

regulator, CodY (Belitsky and Sonenshein, 2013). However, these seem to be exceptions as most of the transporters remain uncharacterised and seem to be constitutively transcribed (Nicolas *et al.*, 2012), suggesting transcriptional analysis is not enough to understand the regulatory network acting on the transporters. In some cases such as MetNP and YhaG, they have been shown to be regulated to some degree at the translational level (Hullo *et al.*, 2004; Yakhnin *et al.*, 2004).

Table 1.1: Transporters encoded in *B. subtilis* genome.

Types of substrates	**Number**
Carbon compounds	58
Amino acid and their derivatives	40
Peptides	5
Cations / anions	37
Others (*e.g.* multi drugs / metabolites)	91
Unknown	54

Reference from Saier *et al.*, 2002.

To summarise, there is a general lack of understanding of the role of most of the potential transporters encoded in the genome of *B. subtilis*, most of the analysis that has been done has tended to focus on extreme or artificial conditions. Consequently, in most cases, the biological role of the transporters has not been defined, or has only been characterised under very specific conditions.

Table 1.2: Amino acids transporters or transporter systems identified in *B. subtilis*.

Transporter	Substrate(s)	Possible inhibitors	Reference
SteT	Ser and thr	Phe, tyr, typ	Reig et al., 2007
YbeC	Ser		Klewing et al., 2020
YbxG	Thr	Ser	Klewing et al., 2020
MetNP	Met	N.A	Hullo et al., 2004
GltP	L-glu and L-asp	N.A	Tolner et al., 1995
YveA	L-asp and L-glu	Asn, ser, thr, gln	Lorca et al., 2003
YhcL	Cystine	Leu, val, ala, cys	Burguiere et al., 2004
YtmJKLMN	Cystine	His, met, arg, gln	
YckK	Cystine	N.A	
BcaP	Ile, val and leu	Thr, ser, ala, asn	Belitsky, 2015
BraB	Ile, val and leu	N.A	
BrnQ	Ile and val	N.A	
OpuE	Pro	N.A.	Moses et al., 2012
PutP	Pro	N.A.	
GabP	Pro	N.A.	Zaprasis et al., 2014
GltT	Glu and asp	N.A.	Zaprasis et al., 2015
YhaG	Trp	N.A	Sarsero et al., 2000

Transporters identified (1st column) are shown with its substrates (2nd column) indicated in the corresponding study (4th column). Possible inhibitory amino acids were predicted for some of the transporters (3rd column). However, the prediction was not done to every transporters listed above. N.A = data not available. Amino acids are represented in 3-letter code unless specified.

1.2 Cellular metabolism

In order to survive, *B. subtilis* has a well developed a sophisticated metabolic network to supply the necessary precursor molecules for growth to satisfy a high demand of energy in the form of both ATP and membrane potential (Blencke *et al.*, 2003).

To obtain ATP a carbon source is catabolized in several steps for ATP generation under aerobic conditions through a process termed glycolysis. A range of carbon sources can be utilised by *B. subtilis* including fumarate, malate, citric acid and succinate but glucose is generally considered the preferred carbon source. The uptake of other carbon sources was shown repressed in the presence of glucose, with exception of malate was shown to be co-utilised with glucose (Blencke *et al.*, 2003; Sonenshein, 2007; Kleijn *et al.*, 2010). Glycolysis produces pyruvate, a molecule which stores energy accumulated from previous steps, which is then utilised in tricarboxylic acid (TCA) cycle to produce the energy. In addition, the intermediate compounds in the cycle are also involved in multiple cellular biosynthetic processes (Meyer *et al.*, 2014). From the perspective of this project pyruvate and the intermediates of the TCA cycle can be used for amino acid biosynthesis as well as the utilisation of the amino acids as precursors in the biosynthesis of other amino acids, these are in which depicted in Figure 1.1. Some of the pathways have unique precursors whereas others share the same precursors for synthesis or are inter-converted. The basic functions of amino acids are usually for protein synthesis, energy production, nitrogen metabolism and intercellular communication. All of which contribute to cell propagation and the predominant form of amino acids is the L-isomer (Saier, 2000; Lam *et al.*, 2009). Glutamate was shown the most abundant amino acids intracellularly whereas alanine was shown relatively more abundant than other amino acids, implying their importance to the cell (Tempest, Meers and Brown, 1970; Akashi and Gojobori, 2002).

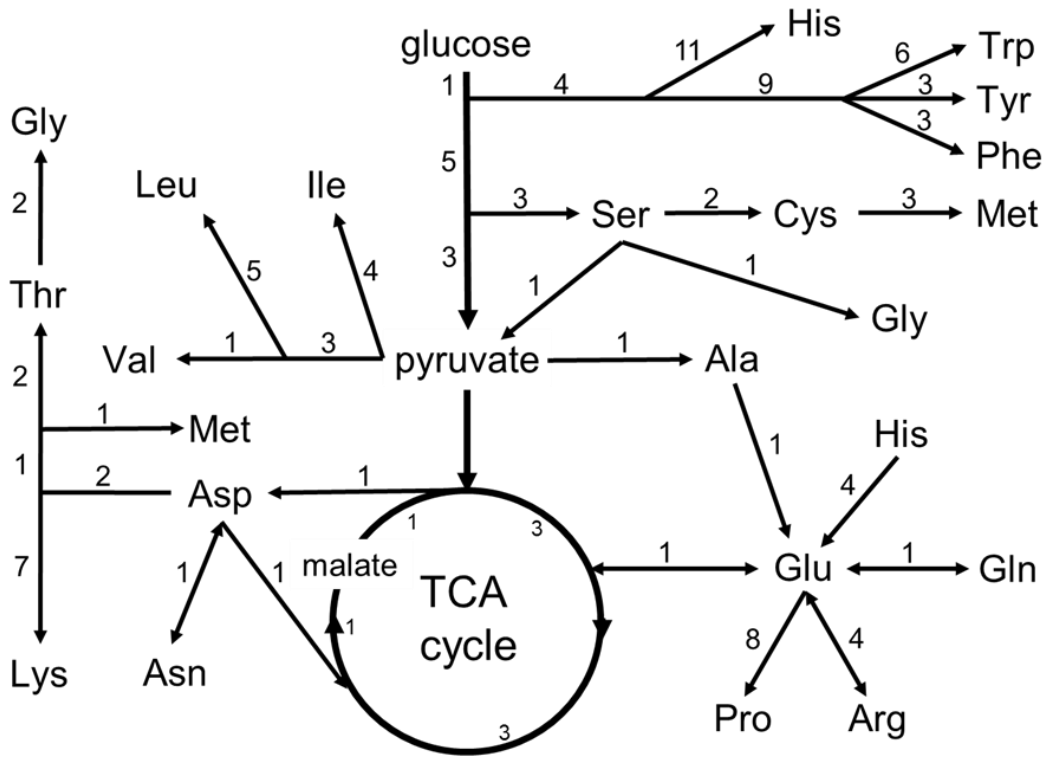


Figure 1.1: A schematic diagram of metabolic pathways of amino acids with glycolysis and TCA cycle. Each glucose molecule is utilised to generate 2 ATP molecules through glycolysis and the product for this process, pyruvate is used in metabolism. Each conjunction represents a metabolite that could be converted into more than 1 metabolite(s). Numbers next to/above the lines represent the number of steps necessary for conversion of one metabolite at last conjunction to the other metabolite at the nearest conjunction. Amino acids are presented as 3-letter code. Ala = alanine; arg = arginine; gly = glycine; thr = threonine; lys = lysine; asn = asparagine; asp = aspartate; met = methionine; val = valine; leu = leucine; ile = isoleucine; his = histidine; trp = tryptophan; tyr = tyrosine; phe = phenylalanine; cys = cysteine; ser = serine; glu = glutamate; gln = glutamine; pro = proline. TCA cycle = tricarboxylic acid cycle. Information adapted from SubtiPathway, a metabolic pathways database specialised for *B. subtilis*.

Glutamate is used for a lot of cellular reactions, in which it acts primarily as the major amino group donor and this amino acid is estimated to be involved in at least 37 transamination reactions. Considering the energy cost of these reactions, the intracellular glutamate concentration was expected to remain high to favour of the occurrence of these reactions (Gunka and Commichau, 2012), something that is supported by quantitative analysis of the intracellular metabolome (Tempest, Meers and Brown, 1970; Fischer and Sauer, 2005; Muntel *et al.*, 2014). Of these reactions the biosynthesis of proline, which is important as it functions as an osmoprotectant for the cell (Zaprasis *et al.*, 2015), is perhaps best studied. Proline has been found to be in high demand under hyperosmotic conditions and where the demand of glutamate would be expected to increase. Thirdly, glutamate is an important

metabolic intersection between carbon and nitrogen metabolism. This is indicated by the characterisation of the complex regulatory system involved maintaining the homeostasis of glutamate (Gunka and Commichau, 2012). Glutamate dehydrogenase was identified one of the key enzymes in the homeostatic control of synthesis as it was found to be actively participating in glutamate metabolism as well as controlling gene expression in response to carbon and nitrogen availability. Deletion of glutamate dehydrogenase was shown to result in severe growth defect that then resulted in adaptive mutagenesis of the glutamate degradation pathway (Belitsky and Sonenshein, 1998). It is also important to note that the cell also utilises glutamate in both the L-isomeric form for protein synthesis and the D-isomeric form in cell wall (Vollmer, Blanot and de Pedro, 2008)(Section 1.1.2), and hence a controlled glutamate metabolism is important in multiple ways to allow growth and for adaptation to different environmental conditions.

Alanine is another amino acid that can be predicted to be in high demand during cell growth as, like glutamate, it is required in both D- and L- monomeric states. With the L-isomer functioning in protein synthesis and D-alanine functioning as the essential substrate for cross-linkage of the peptidoglycan as well as the D-alanylation of teichoic acids in the wall (see Section 1.1.1).

D-amino acids can be synthesised from the corresponding L-isoforms either by stereochemistric inversion of alpha-carbon, which is catalysed by amino acid racemase or epimerase (Yoshimura and Esak, 2003; Radkov and Moe, 2014) or stereospecific amidation of alpha ketoacid, which is catalysed by D-amino acid aminotransferase. These two mechanisms for the production of D-amino acids are essentially reversible (Radkov and Moe, 2014). For *B. subtilis*, D-alanine is synthesized from L-alanine in a bidirectional reaction catalysed by alanine racemase (Walsh, 1989). There are two alanine racemases in the *B. subtilis* genome, encoded by *alrA* and *alrB* which are expressed in vegetative and sporulation growth respectively (Ferrari, Henner and Yang, 1985; Pierce, Salifu and Tangney, 2008). D-glutamate was later found to be synthesized from L-glutamate by at least two glutamate racemases, RacE and YrpC (Ashiuchi *et al.*, 1998; Ashiuchi, Soda and Misono, 1999). The *racE* is expressed in both rich and minimal media whereas *yrpC* only expressed in minimal media. On the other hand, *B. subtilis* utilise D-amino acid aminotransferase in both D-alanine and D-glutamate biosynthesis and its enzyme activity can be detected in the cellular extracts of *B. subtilis* (Thorne, Gomez and

Housewright, 1955) and was purified and characterised (Martinez-Carrion and Jenkins, 1963). The enzyme was shown to catalyse the transamination from D-alanine and D-glutamate (amino donors to alpha-ketoglutarate and pyruvate (amino acceptors) respectively (Yonaha *et al.*, 1975). The putative D-alanine aminotransferase is encoded *dat* in the genome of *B. subtilis*, which is 42 % similar to Dat in *B. sphaericus* (Fotheringham, Bledig and Taylor, 1998). This putative protein Dat, was first thought to be involved in D-glutamate synthesis but this hypothesis was rejected because the presence of this gene was not able to recover the growth of a strain deleted with both D-glutamate racemases (Kimura, Tran and Itoh, 2004). Whilst the physiological roles of D-alanine and D-glutamate racemases have been understood, the role of D-amino acid aminotransferases remains to be explored.

In general *B. subtilis* has developed a network of dynamic metabolic pathways to survive in a wide variety of physical conditions. These pathways function to ensure the bacteria to maintain a balanced supply of different essential resources for growth. Unfortunately, these pathways are not clearly defined because of insufficient characterisation of the functionality of the enzymes involved and the existence of uncharacterised secondary pathways. Therefore, it is difficult to obtain a complete picture of the metabolism of *B. subtilis*.

Apart from being metabolised in some cellular pathways, amino acids are mainly used in protein synthesis as they are the essential building blocks of proteins. For protein synthesis, a specific tRNA synthetase is present for each amino acid which mediate specific binding of the corresponding free monomeric amino acid and an ATP molecule at its active site and then the appropriate specific tRNA that has an anticodon corresponding to that of the amino acid associates with the active site of the aminoacyl tRNA synthetase and a strong covalent bond is formed between the amino acid and the tRNA to produce a charged tRNA molecule (Yamane, Bunai and Kakeshita, 2004). This molecule will then be released and is used in the translation process mediated by the ribosome and mRNA in the protein synthesis.

When glucose is replaced by other other carbon sources to support bacterial growth, glucongeogensis could occur and the metabolic flux could be in opposite direction compared to that of grown in glucose (Meyer *et al.*, 2014). On some rare occasions, overflow metabolism could occur in such conditions in which it often is associated with the secretion of acetate. As acetate could inhibit bacterial growth, the understanding of the rationale behind overflown metabolism remained to be

elucidated (Pinhal *et al.*, 2019). Although amino acids were unlikely found remained in the cellular culture for *B. subtilis*, valine and leucine were once shown released into the culture medium and then reassimilated when pyruvate was exhausted (Lam *et al.*, 2009; Meyer and Stülke, 2013; Meyer *et al.*, 2014).

1.3 Network of adaptational responses in *B. subtilis*

To have a sophisticated cellular mechanism enabling *B. subtilis* to survive in everchanging hostile conditions, one of the crucial components of an adaptive nature of the cells is to coordinate the genes well for a variety of extracellular situations. *B. subtilis* possesses overlapping regulatory systems that detect intracellular pools of metabolites as well as a well-organised global transcriptional coordination within itself for rapid modulation of the cellular metabolism for adaptation to dynamic environment. One of the examples of transcriptional regulation in *B. subtilis* is sigma factor, σB, which is responsible for general stress response, is usually triggered by stress or energy depletion but not amino acid starvation and it is independent of stringent response (see later) (Maul *et al.*, 1995; Voelker *et al.*, 1995; Eymann *et al.*, 2002). Here we only focus on the regulations related to amino acid starvations.

One of the mechanisms that *B. subtilis* might trigger in response to amino acid starvation is the stringent response of which its mechanism is dependent on (p)ppGpp (guanosine tetra- and pentaphosphate) has been recognised to play an important role on regulating genes for adapting to different stress or starvation conditions that the cells subjected to. In *B. subtilis*, there are supposedly three active (p)ppGpp synthases but *relA* was found encoding the major synthetase that catalyses the transfer of a pyrophosphoryl group from ATP to the 3’-hydroxyl group of GTP. RelA was also thought to be a sensor of amino acid starvation because the protein was found activated by the arrival of an uncharged tRNA at the ribosome, showing higher activity in response to amino acid deprivation (Cashel *et al.*, 1996; Sonenshein, 2005). Deletion of *relA* gene would cause impaired growth phenotype to the strain because of its inability to decrease the GTP level during the transition state, causing defective sporulation and development of genetic competence (Lopez, Dromerick and Freese, 1981). There were two more identified genes, yjbM and ywaC, encoding proteins of two other (p)ppGpp synthases in which are smaller and less understood (Nanamiya *et al.*, 2007). And the defects observed from a *relA* null mutant could be partially rescued by additional deletion of *yjbM* or *ywaC*, identified genes encoding proteins of potentially similar functions (Natori *et al.*, 2009).

Unfortunately the regulation of mechanisms of all three remained to be elucidated. Also, this protein might be involved in glucose, amino acid or even oxygen starvation responses (Nishino *et al.*, 1979; Hecker *et al.*, 1987; Wendrich and Marahiel, 1997).

The importance of GTP pool has also been emphasized in *B. subtilis* in response to nutrient limitations in that CodY, the global transcriptional repressor was shown to sense nutrient availability with the help of GTP and branched-chain amino acids (BCAAs) as effector molecules (Ratnayake-Lecamwasam *et al.*, 2001; Molle *et al.*, 2003; Shivers and Sonenshein, 2004; Handke, Shivers and Sonenshein, 2008; Brinsmade *et al.*, 2010). CodY was shown as a transcriptional repressor during exponential growth of the bacteria and GTP and BCAA were shown as effector molecules that facilitate the binding of CodY on its gene target to inhibit RNA-polymerase activity. The abundance of GTP and BCAA was high when the bacteria were grown in rich nutritious condition. When the bacterial growth is entering transition state, or near entry point of stationary phase, the stringent response would impose a drop of the abundance of intracellular GTP and BCAA. These act as primarily effectors for more efficient binding of CodY on its targeting genes, leading to the release of CodY from binding DNA and thus derepress the genes it targets (Handke, Shivers and Sonenshein, 2008; Geiger and Wolz, 2014). Concomitantly GTP itself also would regulate its gene targets (Krásný *et al.*, 2008; Tojo *et al.*, 2008, 2010; Sojka *et al.*, 2011). The drop of GTP could occur within minutes after starvation, allowing immediate physiological effects of *B. subtilis* to overcome struggles living in extreme conditions (Sojka *et al.*, 2011). The drop of the abundance of intracellular GTP and BCAA was correlated with the derepression of CodY on its gene targets, usually near transient state or near entry point of stationary phase (Mitani, Heinze and Freese, 1977; Lopez, Marks and Freese, 1979; Lopez, Dromerick and Freese, 1981; Ochi, Kandalas and Freese, 1981; Tomoyoshi Soga *et al.*, 2003). Genes targeted directly or indirectly by CodY encode extracellular degradative enzymes, transport systems, catabolic pathways, genetic competence, antibiotic production, flagellin, early sporulation (Slack *et al.*, 1991, 1995; Ferson, Lewis V. Wray and Fisher, 1996; Fisher, Rohrer and Ferson, 1996; Serror and Sonenshein, 1996; Wray, Ferson and Fisher, 1997; Inaoka *et al.*, 2002; Bergara *et al.*, 2003; Molle *et al.*, 2003; Shivers and Sonenshein, 2004). A genome wide screening of CodY binding sites in *B. subtilis* has shown about 90 out of 323 strongest CodY binding regions they found were associated with nitrogen

metabolism. Also the CodY activity as a global transcriptional regulator was suggested highest when the bacteria is grown in excess amino acids (Belitsky and Sonenshein, 2013).

However, apart from nitrogen metabolism, as mentioned in Mader *et al*., 2002, CodY regulations can be related to different physiological activities of the bacterial cells, including competence development, motility and sporulation (Slack *et al.*, 1995; Ferson, Lewis V. Wray and Fisher, 1996; Fisher, Rohrer and Ferson, 1996; Serror and Sonenshein, 1996; Wray, Ferson and Fisher, 1997; Kroos and Yu, 2000; Mirel *et al.*, 2000).

There are also other transcriptional regulations in *B. subtilis* that control expression of independent operons and this type of regulation might result in a response towards starvation of only a particular amino acid. For instance, T-box families which control gene expression through transcriptional antitermination mechanism was found regulating the transcription of most of the aminoacyl-tRNA synthetase genes (Grundy and Henkin, 1994; Pelchat and Lapointe, 1999). One of the tRNAs studied most is *tyrS* in that it is regulated by the read-through of a leader region transcriptional terminator which is upstream of the start of the coding sequence of mRNA and the 5' side of it can form an antiterminator structure by pairing with T-box (Grundy *et al.*, 1997). When there is pairing of anticodon of cognate uncharged tRNA with the specifier sequence of the leader region, a read-through is induced. In other words, if there is amino acid limitation or insufficient levels of the aminoacyl-tRNA synthetases, expression of the corresponding gene would be up-regulated (Grundy, Rollins and Henkin, 1994; Putzer *et al.*, 1995). However, when there are sufficient amino acids, the mentioned pairing of uncharged tRNA with the specifier sequence might not be possible as the presence of charged tRNA would block the uncharged ones (Garrity et al., 1994, Grundy et al., 1994, Putzer et al., 1995). Thus the control of gene expression is through a switch between the antiterminator and terminator forms of the transcript which is at the level of read-through which can be understood as dictated by codon-anticodon pairing of mRNA-tRNA interaction. Some amino acid biosynthetic operons were also regulated by this mechanism, like the *ilv-leu* operon, *cysES* operon, the *proBA* operon, the S-box regulon which was supposed to be BCAAs, cysteine, proline and methionine biosynthetic genes respectively (Grandoni, Zahler and Calvo', 1992; Gagnon *et al.*, 1994; Grundy, Turinsky and Henkin, 1994; Marta, Ladner and Grandoni, 1996; Grundy *et al.*, 1997; Chopin, Biaudet and Ehrlich,

1998; Pelchat and Lapointe, 1999). One of the regulation systems on the tryptophan biosynthesis was T-box dependent as well (Babitzke and Gollnick, 2001). Also, lysine biosynthesis pathway was found regulated by the availability of lysine through antitermination system whilst biosynthesis of arginine was shown regulated by AhrC regulatory protein in which its activity was dependent on arginine availability (Czaplewski *et al.*, 1992; Kochhar and Paulus, 1996).

1.4 Aims and objectives

Decades ago, the advantages of using *B. subtilis* for protein production have been noticed and hence it gained a role as a workhorse in the protein production industry. Not only protein production, but also amino acids and vitamins production is becoming important for the food industry and clinical medicine. For instance, L-alanine could be used as a pre- and post-operative nutrition therapy (Zhang *et al.*, 2007). On the other hand, it could be used as a food additive in the food industry (Hols *et al.*, 1999; Lee *et al.*, 2004; Liu, Xu and Zhang, 2021). To promote *B. subtilis* productivity in the industry, research studies needed to focus on improving the rate-limiting steps production. Although the studies were extensive, they did not address basic cellular metabolism, rather relying on empirical selection of improved strains. However, an optimal nutrient provision/intake for a boosted growth and production of *B. subtilis* is equally important and yet poorly understood. This is likely due to the lack of physiological studies done using relatively ill defined medium formulations. For this purpose, a simple yet defined rich medium is more suitable rather than commercially available culture media that despite having a theoretically high nutritional value are not utilised fully by the bacterial strain. Also, a better understanding in the cellular metabolism of cells and how the cells control metabolic pathways through its sophisticated regulatory systems in such physiological conditions is also necessary.

Hence in this project, to address basic cellular metabolisms, we first established a profile of amino acids preferences of B. subtilis during its exponential growth. This helped us to identify the essential requirements for the cells to maintain its active growth and the factors that are used to determine growth potential. We aimed to further explore amino acid metabolism in depth for de novo synthesis and utilisation pathways as well as assimilation from outside environment. Hence we focused on alanine, as it is one of the less studied but important amino acids for different cellular functions. Given the well known flexibility of cellular metabolism of B. subtilis, we also aim to understand the regulatory responses of the bacterial cells when they are

starved of an amino acid. Therefore, this study would also focus on the transcriptional response of B. subtilis towards our defined specific amino acid starvation condition. Together, these would allow a more developed and hopefully complete picture about the connections between growth phenotypes, medium composition and cellular metabolism which could be beneficial to the optimisation of industrial protein production using B. subtilis.

Chapter 2 Materials and method

2.1 Strains maintenance and culture

Nutrient agar (Oxoid) and Lysogeny Broth (LB, from Oxoid) were used for routine media for both selection and maintenance of strains. Antibiotics were added when necessary at required concentration: chloramphenicol (5 µg/ml), erythromycin (1 µg/ml), spectinomycin and ampicillin (200 µg/ml). The defined media was prepared from amino acids stocks (Duchefa Biochemie, Sigma-Aldrich), D-glucose (Sigma-Aldrich) and malic acid (Sigma-Aldrich) diluted in SMM (Spizizen minimal medium; 0.2 % ammonium sulphate; 1.4 % dipotassium phosphate; 0.6 % potassium dihydrogen phosphate; 0.1 % sodium citrate dihydrate; 0.02 % magnesium sulphate) for experimental use unless specified (Anagnostopoulos and Spizizen, 1961; Young and Spizizen, 1961).

Strains were stored in glycerol stock at - 80°C.

In amino acid assimilation profiling experiments, *168+* was grown in PTM (2.5 % D-glucose; 0.02 mg/ml tryptophan; 0.01 M magnesium sulphate; 1 mM calcium chloride; 0.65 mM manganese sulphate; 0.4 % CAA dissolved into SMM) overnight at 37°C and it was grown in the defined media containing 3 g/l glucose and 1.25 mM amino acid mixture in SMM (or specified otherwise) the next morning from OD_{600} 0.2 incubated at 37°C with shaking. Bacterial growth was monitored and samples were taken during the exponential growth of the strain.

2.2 Markerless deletion of genes in *B. subtilis* strains

Strains of *B. subtilis* containing single deletion of most of the identified genes in the whole genome could be purchased and they are termed as the BKE collection as these strains are erythromycin resistant (Koo *et al.*, 2017). The gene deletion in the *B. subtilis* strains in the BKE collection purchased were first transformed into *168+* or *168CA* background. These strains were then made markerless using plasmid pDR244 (Koo *et al.*, 2017). The plasmid was transformed into the target strains and the resulting transformants were then streaked onto a fresh LB plate incubated at 42°C overnight in order to remove the temperature sensitive plasmid which is spectinomycin resistant. Hence, colonies were then screened of the resistance to the two mentioned antibiotics by growing them in LB plate of containing either of the

corresponding antibiotics. A colony losing resistance to both antibiotics is recognised as a clean markerless deletion of a single mutant desired.

2.3 Growth measurement on plate reader

168+ was grown in PTM overnight at 37°C and diluted in fresh minimal media (2 g/l ammonium sulphate; 14 g/l dipotassium phosphate; 6 g/l potassium dihydrogen phosphate; 1 g/l sodium citrate dehydrate; 0.2 g/l magnesium sulphate; 0.6 % D-glucose; 0.05 mM calcium chloride; 0.02 g/l tryptophan) incubated at 37°C for approximately an hour. This allows the bacteria to consume its nutrient storage when it is growing in a less nutritious medium. The culture was then added to different media of interest and the bacterial growth in 20 µl in different media prepared in 96 well plate was monitored on a plate reader. The reading OD_{600}, was taken every 5 minute using spectrophotometer (Beckman DU650).

2.4 *B. subtilis* transformation

The protocol was used according to the method developed by Anagnostopoulos and Spizizen (1961) and then optimised by Young and Spizizen (1961) in the 1960s (Anagnostopoulos and Spizizen, 1961; Young and Spizizen, 1961).

2.5 *E. coli* transformation

The protocol was obtained and slightly modified from Current Protocol in Molecular Biology (Ausubel *et al.*, 2003). 50 µl of *E. coli* competent cells (*DH5α*) were mixed with mixture of ligation reaction or plasmid DNA (10 ng) and left on ice for 30 minutes. The mixture was then heat shocked at 42°C for 90 seconds followed by an incubation on ice for at least 2 minutes. 1 ml of LB media was then added and the tube incubated at 37°C for one hour for recovery. The transformants were selected on ampicillin containing plates incubated at 37°C overnight.

2.6 Sequencing

DNA samples were sent to sequencing service available at Dundee University for sequencing.

2.7 Oligonucleotides

Primers were designed using Clone Manager software and the sequence was then sent to Eurogentec Kaneka Corporation for synthesis. Stock solutions in MilliQ water (100 µM) were usually stored at - 20°C.

2.8 Gel electrophoresis

Gel electrophoresis was carried out to visualise the DNA fragments by separating the DNA according to its size in the electrophoresis. 1 % agarose (Sigma-Aldrich) was commonly prepared in 1 X TAE (Tris-acetate-EDTA) buffer (laboratory made). Sample is loaded into the gel with diluted SYBR™ Gold DNA stain (Thermo Fisher) with loading dye (Orange G). The electrophoresis was run in 1 X TAE buffer at 100 V for approximately an hour. The stained DNA in the gel was visualised in the G: Box, the UV transilluminator using the programme GeneSnap 6.07 (SynGene) at a common exposure time 300 ms. 1 kb ladder (New England Biolabs (N.E.B)) was used for the DNA size references.

2.9 Plasmid DNA preparation

E. coli (*DH5α*) strains containing plasmid were grown in 5 ml LB medium with 100 µg/ml ampicillin at 37°C overnight. The plasmid DNA was extracted using QIAprep Spin Miniprep kit (Qiagen) following the manufacturer's protocol. The plasmid DNA extracted would be stored at - 20°C.

2.10 Chromosomal DNA preparation

For sequencing and PCR, GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich) was used and the protocol was provided by the manufacturer.

For bacterial transformation, chromosomal DNA was extracted following a method from (Ward and Zahler, 1973).

2.11 Polymerase chain reaction (PCR)

The Q5 High-Fidelity DNA polymerase (N.E.B) was used for standard cloning. This was done following manufacturer's protocol.

2.12 Restriction digestion and ligation

Products of DNA fragments amplified from PCR and targeted plasmid would be digested at the restriction enzyme binding site(s). Restriction enzymes (New England Biolabs or Promega) were used according to manufacturer's protocol. The digested DNA fragment would then be ligated into digested plasmid with the same restriction enzymes. T4 DNA ligase (Roche or N.E.B) was used according to manufacturer's protocol. The ligation reactions were incubated at 4°C overnight.

2.13 Site-directed mutagenesis PCR

PCR using Q5 High Fidelity DNA polymerase (N.E.B) with designed primers (Table 2.3) was performed to introduce 3 base-pair codon mutation into the vector pMC5 containing *tyrS* regulatory region. Approximately 1 ng parent plasmid DNA was subjected to the PCR. Reaction mixture was prepared following standard protocol with slight modifications. Firstly, the extension time was set as 40 s/kb. Secondly, the number of cycles of amplification was reduced from 25 to 10 to reduce the semi-methylated products (unwanted) whilst amplifying enough desired products for downstream processes. The PCR products were then digested with 1 µl DpnI (N.E.B) at 37°C for 1 to 2 hours for methylated DNA digestion followed by the denaturation of the enzyme in 80°C for 20 minutes before transformation into *DH5α E. coli* competent cells. Sequence checked to confirm the presence of mutation.

2.14 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was used to assess the amino acid content in media in this project. Samples of bacterial culture taken during the time course of bacteria growing in different variations of the defined medium mentioned in Section 2.1 were first centrifuged. The resultant supernatant was then filtered in that only molecules below 3000 Da was to be analysed by the RP-HPLC. This was to avoid active proteins or big molecules that potentially damage the HPLC column. The derivatization step of the filtered samples was done automatically. Amino acid peaks were quantified on ChemoStation (see also Section 2.15). The setting up of the RP-HPLC generally was following the protocol provided by Agilent Ltd (Henderson et al.) A 1100 Series HPLC (Agilent) was used for the amino acid analysis. The HPLC consists of 5 main components including a degasser (Degasser G1322A), a pump for quaternary pump (QuatPump G1311A), an autosampler (ALS G1313A), a column compartment (Col Comp G1316A) and signal detector (VWD G1314A). The protocol for HPLC analysis of amino acids was adapted from the manufacturer. Samples were first derivatized with OPA (o-phthalaldehyde) using 3-mercaptopropionic acid and FMOC (9-fluorenylmethyl chloroformate) using the autosampler. The reaction mixture was buffered in 0.4 M borate buffer solution (Agilent) at pH 10.2. The whole process was done automatically following the settings in the injector programme. Each 0.5 µl sample was first mixed with 2.5 µl borate buffer in which 0.5 ul OPA and FMOC was added to the reaction mixture diluted with 32 µl MilliQ. Here OPA reacts specifically with primary amines whilst FMOC reacts with primary and secondary amines. The

derivatized amino acids would then be separated by mainly their hydrophobicity in a silicone coated column. The separation column contains reversed-phase material (Poroshell 120 EC-C18 4.6 x 150 mm, 4 µm) allowing the amino acids eluted according to their hydrophobicity at a column temperature of 40°C. The components of the mobile phases to elute the amino acids are annotated as buffer A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2) and B (45 % v/v acetonitrile, 45 % methanol, 10 % MilliQ H_2O). All the components of the buffers should be HPLC grade in which they were pumped using the quaternary pump at a flow rate of 2 ml/min for a 26 min run in linear gradient. A 100 % buffer A was run for the first 1.9 min followed by a 43% buffer A and 57 % buffer B from 18.1 min for 5 min and then a wash for the column with 100% buffer B to elute everything. The run would be finished using 100% buffer A from 23.2 min until the end. The UV signal of the derivatized amino acids would then be monitored at 338 nm and shown in a chromatogram for each sample. Both HPLC experimental condition settings and analysis of the chromatograms can be done on ChemStation (Agilent).

2.15 Quantitative analysis of amino acids from HPLC chromatograms

Quantification of peak areas was done on ChemStation automatically. The value of each peak is directly proportional to the concentration of amino acids in the sample correspondingly. Conversion of peak area into amino acid concentration (mM) was done according to the standard curves produced for each amino acid (Henderson *et al.*, no date) (Figure 3.1E). The amino acid concentration was converted into percentages where the concentration of each amino acid at T_0 was set as 100 %. Hence the calculated percentages of amino acids in samples collected in the following time points represents the relative abundance of the amino acids. For comparing assimilation of strains with different growth rates, the assimilation rate of each amino acid was calculated as the change of the calculated percentage change over OD_{600} and defined as the efficiency of assimilation.

2.16 Sample collection and processing for RNA sequencing

Bacterial cells were harvested at a specific time point (when OD_{600} was around 2) during its exponential growth. The cells were spun down at maximum speed for 5 minutes and the cell pellet was then stored in 0.5 ml RNAlater solution (Thermo Fisher Scientific) to protect the cellular RNA of the samples in intact form and the sample was stored at - 20°C for later processing. Total RNA was extracted next day

using FastRNA® Pro Blue kit (MP Biomedicals) according to the protocol supplied. The extracted total RNA was further cleaned up using RNeasy Mini Kit (Qiagen) according to the RNA Cleanup protocol supplied. The RNA quality was assessed using Agilent RNA 6000 Nano Kit (Agilent Technologies) and the quantity was assessed using NanoDrop®.

2.17 RNA sequencing analysis

The total RNA was sent to Novogene company in Cambridge for further processing and sequencing. Initial data processing including quality check of RNA sequencing result was done by both Novogene and the Bioinformatics unit at Newcastle University. The Bioinformatics unit further helped with the statistical analysis using principal component analysis to check the significance of data and provided us the normalized data as number of reads per gene for our data analysis downstream.

To start with, the total number of reads for all genes per sample was calculated. The sum was then interpreted as 1 (as a whole) and the number of reads per gene was calculated as number of reads divided by the number of sum of reads. Genes with no reads (0) or low number of reads (reads of the least 10 % of each biological sample) were neglected for integrity of sequencing result. It was also to meet the criteria for selecting genes candidates in reporter system for amino acid transporters screening. There are two biological replicates in each set of comparison of the transcriptome profile. For example, in the comparison of LL2 VS LL1 transcriptome profile, sample LL2 from set 1 was compared to sample LL1 from same set whereas sample LL2 from set 2 was compared to LL1 of same set independently. Each of the comparison was the number of fold change of one gene in one sample compared to that of in the other sample from the same experiment. In comparison of LL2 VS LL1, the number of reads of one gene in LL2 would be divided by that of the same gene in LL1. If the number of reads in two samples were similar, the number of fold change calculated would be close to 1, which is considered insignificant difference in expression of this gene. When looking at upregulation of a gene, the higher the number of fold-change, the more significant the gene to be upregulated and the minimum number of fold change considered to be of significant in this project was 5 in order to select more promising gene candidate(s) for developing reporter system of transporters screening. The calculation of comparison pair for independent experiments in parallel would result in two numbers for the comparison of same strains in the same conditions. Only genes up-regulated by 5 or more fold on its transcriptional

expression in both biological duplicate sets of experiment would be considered as a significant change.

2.18 Blue-white colony screening

Strains were either directly streaked onto the plate for incubation at 37°C. Or the strains were grown to exponentially growing culture to an $O.D._{600}$ 0.2. The growing culture would then be washed and resuspended in minimal medium without amino acid mixture. 10 ul of resuspended cells would then be spotted onto agar plates with X-gal for screening. β-galactosidase, encoded by *lacZ*, hydrolyses X-gal to form 5-bromo-4-chloro-indoxyl. Dimerization of the molecules instantly occurs and form an insoluble blue pigment 5,5'-dibromo-4,4'-dichloro-indigo. Plates were made with defined media added with 0.02 % X-gal and 5 µg/ml chloramphenicol. Colonies turned blue represent the presence of functional *lacZ* gene in the vector. The bluer the colony, the higher the transcriptional expression of *lacZ*.

2.19 Strains table

Table 2.1: strains used in this project.

Strains	Genotype	Reference
168CA	*trpC2*	Laboratory collection
168+	*Wild type strain*	Richard Daniel's collection
RD180	*trpC2 ΔalrA::zeo*	
KS78	*trpC2 ΔalrA::zeo Δdat::erm*	Karzan Sidiq's collection
BKE30530	*trpC2 ΔalaP::erm*	Bacillus genetic stock centre
BKE28280	*trpC2 ΔleuA::erm*	
BKE31400	*trpC2 ΔalaT::erm*	
BKE09670	*trpC2 Δdat::erm*	
BKE31930	*trpC2 Δald::erm*	
BKE18120	*trpC2 ΔalsT::erm*	
BKE02850	*trpC2 ΔznuA::erm*	
BKE23070	*trpC2 ΔserA::erm*	
BKE27900	*trpC2 ΔpheA*	
BKE23380	*trpC2 ΔlysA*	
BKE22610	*trpC2 ΔtyrA*	
MC1	*ΔalaT::erm*	This work: gDNA of BKE31400 transformed into 168+.
MC1.1	*ΔalaT::kan*	This work: gDNA of BKK31400 transformed into 168CA.
MC1.2	*ΔalaT::kan pAG-alaT*	pAG-alaT introduced into MC1.1.
MC1.3	*ΔalaT::kan pAG-dat*	pAG-dat introduced into MC1.1.

MC2	Δ*alaT*	This work: Erythromycin marker was removed using pDR244.
MC3	Δ*dat::erm*	This work: gDNA of BKE09670 transformed into 168+.
MC4	Δ*alaT* Δ*dat::erm*	This work: gDNA of BKE 09670 transformed into MC2.
MC4.1	Δ*alaT* Δ*dat::kan*	This work: gDNA of BKK 09670 transformed into MC2.
MC4.2	Δ*alaT* Δ*dat::kan pAG-alaT*	pAG-alaT introduced into MC4.1.
MC4.3	Δ*alaT* Δ*dat*	This work: Erythromycin marker was removed using pDR244.
MC5	Δ*ald::erm*	This work: gDNA of BKE 09670 transformed into 168+.
MC6	Δ*alaT* Δ*ald::erm*	This work: gDNA of BKE 09670 transformed into MC2.
MC7	Δ*alaP::erm*	This work: gDNA of BKE 09670 transformed into 168+.
MC8	Δ*alaT* Δ*alaP::erm*	This work: gDNA of BKE 09670 transformed into MC2.
RD220	Δ*alaT::kan pAG-alaT (erm)*	Richard Daniel's collection
RD221	*ΔalrA::zeo Δdat::kan*	
RD222	Δ*alrA::zeo pAG-dat(erm)*	
RD223	Δ*alrA::zeo* Δ*dat::kan pAG-dat(erm)*	
RD226	Δ*alaT::kan pAG-dat (erm)*	

DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England BioLabs®
MC9	*Δdat*	This work: Erythromycin marker was removed using pDR244.
MC10	*Δdat Δald::erm*	This work: gDNA of BKE31930 transformed into MC9.
MC11	*ΔalaT Δdat ΔalaP::erm*	This work: gDNA of BKE30530 transformed into MC4.3.
MC12	*ΔalsT::erm*	This work: gDNA of BKE18120 transformed into 168+.
MC13	*ΔalaT ΔalsT::erm*	This work: gDNA of BKE18120 transformed into MC2.
MC14	*ΔznuA::erm*	This work: gDNA of BKE02850 transformed into 168+.
MC15	*ΔalaT ΔznuA::erm*	This work: gDNA of BKE02850 transformed into MC2.
MC16	*ΔserA::erm*	This work: gDNA of BKE23070 transformed into 168+.
MC18	*ΔpheA::erm*	This work: gDNA of BKE27900 transformed into 168+.
MC19	*ΔlysA::erm*	This work: gDNA of BKE23380 transformed into 168+.
MC21	*PgspA-GspA-RBS-lacZ cat*	This work: pMC1 transformed into 168+.
MC22	*PtnrA-TnrA-RBS-lacZ cat*	This work: pMC2 transformed into 168+.
MC23	*Pvpr-Vpr(RBS)-lacZ cat*	This work: pMC3 transformed into 168+.

MC24	*PyvyD-YvyD(RBS)-lacZ cat*	This work: pMC4 transformed into 168+.
MC29	*ΔalaT::erm PyvyD-YvyD(RBS)-lacZ cat*	This work: pMC4 transformed into MC1.
MC30	*ΔalaT ΔalaP::erm PyvyD-YvyD(RBS)-lacZ cat*	This work: pMC4 transformed into MC8.
MC31	*PtyrS-TyrS(RBS)-lacZ cat*	This work: pMC5 transformed into 168+.
MC32	*PtyrS-TAA-TyrS(RBS)-lacZ cat*	This work: pMC6 transformed into 168+.
MC33	*PtyrS-TGC-TyrS(RBS)-lacZ cat*	This work: pMC7 transformed into 168+.
MC34	*PtyrS-GGC-TyrS(RBS)-lacZ cat*	This work: pMC8 transformed into 168+.
MC35	*PtyrS-GAG-TyrS(RBS)-lacZ cat*	This work: pMC9 transformed into 168+.
MC36	*ΔalaT::erm PtyrS-TyrS(RBS)-lacZ cat*	This work: pMC5 transformed into MC1.
MC37	*ΔalaT::erm PtyrS-TAA-TyrS(RBS)-lacZ cat*	This work: pMC6 transformed into MC1.
MC38	*ΔalaT::erm PtyrS-TGC-TyrS(RBS)-lacZ cat*	This work: pMC7 transformed into MC1.
MC39	*ΔalaT::erm PtyrS-GGC-TyrS(RBS)-lacZ cat*	This work: pMC8 transformed into MC1.
MC51	*ΔalaP::erm PtyrS-TyrS(RBS)-lacZ cat*	This work: pMC5 transformed into MC7.
MC52	*ΔalaT ΔalaP::erm PtyrS-TyrS(RBS)-lacZ cat*	This work: pMC5 transformed into MC8.

MC53	*ΔalaT ΔalaP::erm PtyrS-TAA-TyrS(RBS)-lacZ cat*	This work: pMC6 transformed into MC8.
MC54	*ΔalaT ΔalaP::erm PtyrS-TGC-TyrS(RBS)-lacZ cat*	This work: pMC7 transformed into MC8.
MC55	*ΔalaT ΔalaP::erm PtyrS-GGC-TyrS(RBS)-lacZ cat*	This work: pMC8 transformed into MC8.

gDNA = genomic DNA

Table 2.2: Plasmids used in this project.

Plasmid	Relevant features	Constructions	Reference
pRD62	possible *yllC* promotor fragment with a synthetic RBS and start codon inserted into pPS1326		Daniel et al., 1996, pPS1326 was gpr'-lacZ cat fusion for integration at the amyE locus
pMC4	possible *yvyD* promotor fragment and RBS binding site with a synthetic start codon inserted into pRD62	Insertion of possible *yvyD* promotor and RBS binding site DNA fragment into pRD62	This work.
pMC1	possible *gspA* promotor fragment and RBS binding site with a synthetic start codon inserted into pRD62	Insertion of possible *gspA* promotor and RBS binding site DNA fragment into pRD62	This work.
pMC3	possible *vpr* promotor and RBS binding site fragment with a synthetic start codon inserted into pRD62	Insertion of possible *vpr* promotor and RBS binding site DNA fragment into pRD62	This work.
pMC2	possible *tnrA* promotor and RBS binding site fragment with a synthetic start codon inserted into pRD62	Insertion of possible *tnrA* promotor and RBS binding site DNA fragment into pRD62	This work.
pMC5	possible *tyrS* promotor and RBS binding site fragment with a synthetic start codon inserted into pRD62	Insertion of possible *tyrS* promotor and RBS binding site DNA fragment into pRD62	This work.
pMC6	Stop codon at specifier codon of possible *tyrS* promotor and RBS binding	Site mutagenesis of specifier codon of T-	This work.

	site fragment with a synthetic start codon inserted into pRD62	box switch of *tyrS*. V(-)-.	
pMC7	Alanine specifier codon of possible *tyrS* promotor and RBS binding site fragment with a synthetic start codon inserted into pRD62	Site mutagenesis of specifier codon of T-box switch of *tyrS*. V(-)E for binding leucine tRNA	This work.
pMC8	Alanine specifier codon of possible *tyrS* promotor and RBS binding site fragment with a synthetic start codon inserted into pRD62	Site mutagenesis of specifier codon of T-box switch of *tyrS*. V(-)C for binding first alanine tRNA	This work.
pMC9	leucine specifier codon of possible *tyrS* promotor and RBS binding site fragment with a synthetic start codon inserted into pRD62	Site mutagenesis of specifier codon of T-box switch of *tyrS*. V(-)G for binding second alanine tRNA	This work.

Table 2.3: primers used in this project.

primer name	**sequence**
MW pRD62-tyrS FPv2	GGCTACTATTCGACAGTTTGTACTCTTAAGCATGC GGCTCCGCGTGAGATCG
MW pRD62-tyrS RPv2	ACGACGTTGTAAAACGACGGGATCGCCAAGCTTC CATTCGGATACGATGTCAG
gspA FP trial 1	GCGAATTCGGCTTTATCGCATCTTC
gspA RP trial 1	GGCAAGCTTCATGATTTCATCTTCCTCAATGGTATT CATCTCCC
tnrA FP trial 1	GCGAATTCCTCATAGCGTCCTCCTTCATTTTGAGG TATCC

tnrA RP trial 1	GGTAAGCTTCATTTTTCCACCCCTGGATGTC
vpr FP trial 1	GACCGTGGAAAGAGAATTCTTTGTCACAATATGAG TG
vpr RP trial 1	GGCAAGCTTGACGAAACTTACAAGCAGAAAGCGA ATG
yvyD reverse RP trial1 (FP)	GCGAATTCCTATTCATCAATGTATGGAACCC
yvyD reverse FP trial1 (RP)	GGCAAGCTTTCCTCTGATGTTATAGTTCATCAAAG AACGCCTCCC
tyrS_GTA_TGC_FP	GCGGTTCATGAGTTGCTTCGATATATCCG
tyrS_GTA_TGC_RP	CGGATATATCGAAGCAACTCATGAACCGC
tyrS_GTA_GGC_FP	CGGTTCATGAGTGGCTTCGATATATCC
tyrS_GTA_GGC_RP	GGATATATCGAAGCCACTCATGAACCG
tyrS_GTA_TAA_FP	GCGGTTCATGAGTTTATTCGATATATCCG
tyrS_GTA_TAA_RP	CGGATATATCGAATAAACTCATGAACCGC
tyrS_GTA_GAG_FP	GCGGTTCATGAGTGAGTTCGATATATCCG
tyrS_GTA_GAG_RP	CGGATATATCGAACTCACTCATGAACCGC
alaT check FP	GGAACGGATTTACCGATTCCAAGAGG
alaT check RP	CAGGTTTATGCACAGCAAAAAGGGGG
dat check FP	GACGAGGGAATCGGAATGACAGAAG
dat check RP	GCTCATTTGGAGCCGAGTCAGC
ald check FP	GCGGACAGCAACGTGAATACCGC
ald check RP	CAGATTCCTCAGAACTGGAC
ytnA check FP	CGGACAAGGCTGATAACAG
ytnA check RP	CCATCGTTAACCCGGACGTC

Chapter 3 Amino acid assimilation in *B. subtilis*

3.1 Introduction

B. subtilis as a prototroph, can either synthesize amino acids within its complex cellular metabolism or assimilate from the environment. The bacterium can sense the nutritious level of its surrounding, the abundance of amino acids pool as well as the metabolic fluxes to control its assimilation of nutrients from the environment (Fisher and Sonenshein, 1991).

For uptake of materials in general, bacteria, including *B. subtilis*, tend to utilize those which could be metabolized easily and the reduced nitrogen is preferred as a nitrogen source because it is the form when utilized inside the cells (Fisher and Sonenshein, 1991). In *B. subtilis*, the preferred candidate is glutamine because of its role as the precursor of all nitrogen-containing compounds but arginine and ammonium have also been suggested as the preferred nitrogen sources (Fisher and Sonenshein, 1991; Tam *et al.*, 2006). Efficient assimilation of other amino acids, including serine, asparagine, branched-chain amino acids have been shown when the cells were grown in minimal media or other similar condition (Darmon *et al.*, 2002).

However, little is known about amino acid transport on the molecular level. One of the problems suggested was the degeneracy of amino acid transporters. Around 40 transporters were suggested to be responsible for amino acid transport (Section 1.1.2). Hence presumably there should be enough transporters allowing high specificity for all 20 standard amino acids. However, one transport system could be found responsible for the transport of a few amino acids and some amino acids can be transported through more than one transport system (Section 1.1.2). Therefore, this complexity greatly increases difficulties in the identification of specific amino acid transporters from screening a pool of transporters encoded in *B. subtilis* genome. On the other hand, when the bacteria is grown in a medium with abundant amino acids, global regulatory system in *B. subtilis* is expected to be adjusted in order to assimilate those that can be metabolized efficiently (Fisher, 1999)(Section 1.2). With that said, the biological roles of transporter(s) may be dependent on the media conditions. However, a number of amino acid transporters were identified by growing the bacterial cells in a rather extreme growth condition, minimal media (Section 1.1.2) instead. Although the clearer media (a simpler media composition) allowed a more

straightforward evidence of the transport of a particular amino acid, the biological role(s) of the identified amino acid transporters might be overlooked because of possible differences in the global cellular metabolism of the cells grown in different media (Section 1.1.2). In other words, transporters that are active during exponential growth of *B. subtilis* grown in rich media might not be the same as those active when it is grown in nutrient restrictive condition like minimal media. Also the amino acid uptake in rich media during the exponential growth of *B. subtilis* has not been well understood and there is no clear understanding on the molecular level.

In order to understand the cellular metabolism in nutrient uptake, reliable physiological studies would be necessary and these often heavily rely on the stable and consistent supply of medium for growing the bacteria of interest. As of today, there are many types of commercially available rich bacterial growth culture media, *e.g.*, Luria-Bertani broth (LB) and Nutrient broth (NB). However, they might not contain all amino acids and the amino acids might be at different concentrations. Also, the chemical composition of these media could vary from batch to batch due to the complicated chemical processing during production which stated in the technical sheet of these products from different companies. A medium without a definite composition might not be ideal for physiological studies on assimilation of material which are related to cellular metabolism of the bacteria. Also, some of these media might not be ideal for bacterial growth due to the presence of extremely low amount or even absence of the preferred carbon source, such as glucose (Fisher and Sonenshein, 1991; Stülke and Hillen, 2000). For instance, LB, as it is currently used, does not contain glucose, which is a preferred carbon source for bacteria.

With that said, it was necessary to establish a reliable and consistent media recipe for this project and the recipe is required to meet four criteria. Generally, essential components for a bacterial growth are carbon and nitrogen sources supplemented with ionic salts, especially phosphates. Therefore firstly, the defined media should contain all three components. Secondly the media should be simple and easy to prepare in order to minimise variations from preparation between different batches. The media recipe is expected to be easy to manipulate in the future analyses. Thirdly the media was preferably easy to chemically analyse, particularly the amino acid content and the components of media were ideally relatively stable for media composition analysis. Last but not least, the media should be able to support the growth of bacterium of interest which is expected to be comparable to the growth

obtained when the bacterium is grown in conventional rich media. This would be judged by the growth rate and the final optical density obtained.

With reference to the composition of different media, pre-transformation media (PTM) (Anagnostopoulos and Spizizen, 1961; Young and Spizizen, 1961) which consists of glucose, Casamino acids (CAA) and a mixture of ions in ammonium and phosphate salt solution (SMM), is the media which best fit the criteria listed. The glucose is used as the carbon source whereas the CAA acts as the nitrogen source with the presence of phosphate, satisfying the first criteria stated above and the recipe is easy to manipulate. More importantly it is well known to be good for *B. subtilis* growth in which the good growth was contributed by the added hydrolysed casein (Anagnostopoulos and Spizizen, 1961; Young and Spizizen, 1961). However, CAA is made of hydrolysed casein in which the actual abundance of each amino acid may vary between different batches, possibly leading to a larger variation in a quantitative analysis of amino acids. Its poor definition of content might as well suggest the presence of small peptides which could be problematic to amino acid content analysis.Also, two amino acids were not detected in CAA according to the technical analysis by Difco. Therefore, in order to identify the preferred amino acids for assimilation in *B. subtilis*, the CAA was replaced by a mixture of 20 amino acids as the nitrogen sources in the process of creating the defined medium.

Hence this chapter started with an optimisation of PTM to be a defined medium suitable for the physiological study in this project (Section 3.2.1). It was then followed by an establishment of an analysis involving easy amino acid quantification of media (Section 3.2.2). A robust protocol with an equipment satisfying level of sensitivity of amino acid detection and reasonable reproducibility is required in the quantification of amino acids in the culture media. By monitoring bacterial growth in a defined media with a quantitative amino acid analysis of the media during its exponential growth, together these could serve as an estimation of amino acid uptake indirectly (Section 2.1 , 2.14 and 2.15) (Jung, Branciamore and Martini, 2000). This measurement then allowed the characterisation of the preferences (if any) of amino acids assimilation of *B. subtilis* in various conditions (Section 3.2.3). The possibility of modulating the amino acid assimilation profiles was also explored to better understand amino acid metabolism. One was thought to be done through genetic manipulation of amino acid biosynthetic pathways whilst the other one was the alteration of carbon source(s) (Section 3.2.3 and 3.2.4). Here the preference of amino acids for *B. subtilis* during

exponential phase growth in relatively rich media is explored in order to have a better idea of the participation of amino acids in maintaining bacterial growth. Together these growth studies with the profile of amino acid assimilation of *B. subtilis* grown in different tested conditions, focusing exponential phase helped to outline a potential for developing an optimal media for bacterial growth or ultimately for amino acid transporters screening, by adjusting the media for specific purposes.

3.2 Results

3.2.1 Developing defined growth medium for amino acid assimilation analysis

PTM was chosen to be optimised to become the defined medium for amino acids assimilation profiling in this project because it is the closest possible choice of medium to the criteria we set (Section 3.1). PTM essentially consists of glucose, Casamino acids (CAA), and salts mixed in ammonium salt buffer where the salts are magnesium, calcium, and manganese. However, nature of CAA could be a problem to quantify the amino acid content in the medium. Therefore, a modification of PTM was necessary.

To determine which component(s) is/are useful for efficient bacterial growth and which might be limiting growth, the growth of 168+ , that was isogenic to the well characterised model 168CA, but corrected for its tryptophan auxotrophy, in various permutations of PTM was monitored at 37°C in a plate reader as described in Section 2.3 . When 168+ was grown in complete PTM, its exponential growth lasted for about 250 minutes followed by a gradual reduction in growth rate until it reached its maximum optical density at around 500th min and flattened, presumably entering its stationary phase (dark grey 'FULL' curve in Figure 3.1A). When glucose was omitted from the medium, the wild type strain was grown at a slower growth rate and declining in growth after it reached its highest optical density entering stationary phase in 600 minutes of incubation at 37°C (light dotted '- glucose' curve in Figure 3.1A). This highlighted the importance of glucose as the carbon source for boosting bacterial growth. When CAA was omitted from the medium (dark grey dashed '- CAA' curve in Figure 3.1A), the strain adapted to an even lower exponential growth rate relative to that of grown in either PTM without glucose (light dotted '-glucose' curve in Figure 3.1A). But its exponential phase was shown longer than 600 minutes and was presented a trend of maintaining the exponential phase of growth (dark grey dashed '– CAA' curve in Figure 3.1A). In addition to this, when CAA was absent in the PTM,

the maximum optical density achieved by the wild type strain was about 66 % lower than that of growing in full PTM within 600 minutes of growth ('– CAA' curve compared to 'FULL' curve in Figure 3.1A). The estimated average amino acid concentrations in CAA was around 1 – 1.5 mM amino acid according to the manufacturer. Replacing the CAA with the 1.25 mM amino acid mixture, the wild type strain grew at a relatively slower growth rate but presented a similar growth pattern (light grey 'CAA →AAmix' curve in Figure 3.1A) resembling that of grown in a complete PTM (dark grey 'FULL' curve in Figure 3.1A). It also reached a final optical density similar to that of grown in PTM. These suggested that the individual amino acid mixture was a suitable substitute for CAA.

The concentration of glucose in PTM was suggested to be 10 g/l, which was the concentration that might be more than sufficient for bacterial growth. The possibility of the bacterial cells relying on glucose for growth was therefore higher and the reliance on the uptake of other nutrients might then be lower. For the purpose of an indicative amino acid assimilation analysis, a glucose concentration where both detectable amino acid assimilation and a good growth maintained would be preferred. To determine an optimal glucose concentration in defined medium, strain *168*+ was grown in concentrations of glucose ranging from 10 g/l down to 1.25 g/l in the presence of 1.25 mM amino acid mixture in SMM salt buffer (Section 2.1). When the strain was grown in 10 g/l glucose, the strain was grown at a faster exponential growth rate in the first 300 minutes which was followed by a post-exponential growth with a slightly decreased growth rate from 300 minutes onwards until the end of the time course (blue curve in Figure 3.1B). Its growth had reached highest optical density compared to other three tested concentrations by the end of the time course (blue curve in Figure 3.1B). When the strain was grown in 1.25 g/l glucose, which was almost 10 times less than the suggested glucose concentration in PTM, the growth reached slightly lower final optical density (purple curve in Figure 3.1B). Nevertheless, the exponential growth rate for all four tested glucose concentration was similar to each other (Figure 3.1B).

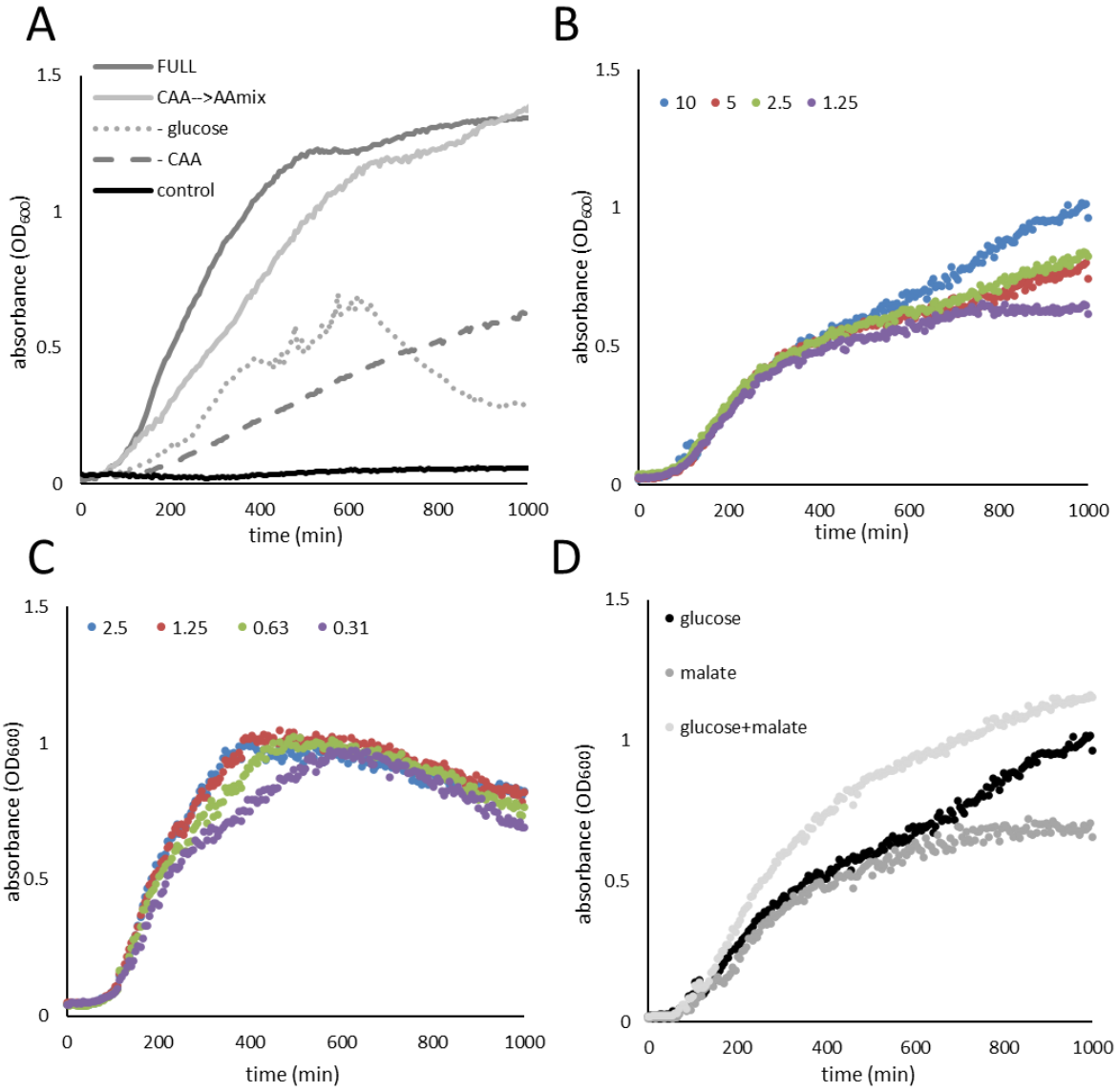


Figure 3.1: Growth of strain 168+ in different medium measured in plate reader. (A) Growth curves in different variations of PTM. PTM essentially contains glucose, CAA and solution P (magnesium, calcium and manganese salt) in SMM salt buffer. Strain 168+ was grown in different variation of PTM omitted with one of the components. FULL = PTM without any components omitted; CAA→AAmix = PTM with CAA replaced by mixture of 1.25 mM amino acids; glucose = PTM without glucose; CAA = PTM without CAA; control = SMM only. (B) Growth curves in different concentrations (in g/l) of glucose in the presence of 1.25 mM amino acid mixture. (C) Growth curves in different concentrations (in mM) of amino acid mix (AAmix) in the presence of 5 g/l glucose in SMM buffer. (D) Growth curves in different carbon source(s) in the presence of 1.25 mM amino acid mixture. Glucose = 0.5 % glucose; malate = 0.5 % malate; glucose + malate = 0.25 % glucose and malate. The experiment has been repeated once and the data above come from a single experiment.

According to the technical analysis by Difco, the approximate concentration of each amino acid present in the CAA is around 1 – 1.5 mM. To determine an optimal concentration of amino acids for the defined medium, strain 168+ was grown in a four

different concentrations of amino acid mixture ranging from 0.3 to 2.5 mM in the presence of 5 g/l glucose and salts in SMM salt buffer. The growth was monitored using a plate reader (Section 2.3). The growth rates of all four conditions were similar to each other in the first 200 minutes (blue, red, green and purple curves in Figure 3.1C). But only the growth curves of the wild type strain grown in either 1.25 mM or 2.5 mM strain maintained the growth rate (blue and red curves in Figure 3.1C). At about 400th minute growth, the strain reached the highest optical density in both conditions and slowly decreased in optical density. The depreciation rate was slightly slower when the strain was grown in 1.25 mM amino acid mixture compared to that of in 2.5 mM amino acid mixture (red compared to blue curves in Figure 3.1C). The strain growing in lower amino acid concentrations slightly decelerated in growth from 200th minute onwards but reached similar highest optical density as that of grown in higher amino acid concentration (green and purple curves in Figure 3.1C).

An alternative way to alter bacterial growth would be to change the carbon source as it has been demonstrated that the laboratory strain has limited ability to utilise single carbon sources under certain conditions. To test this, growth was analysed using the basic PTM medium with carbon sources that would require either glycolysis and glucogenesis or both to function. From this test malate was used alone or in combination with glucose (Kleijn *et al.*, 2010). To assess if a different carbon source would improve the growth, growth of strain 168+ in medium with final concentrations of 5 g/l glucose, 5 g/l malate or a combination both at 2.5 g/l at 37°C was monitored using a plate reader (Section 2.3). Each of them was supplemented with 1.25 mM amino acid mixture.

When 168+ was grown in medium containing 5 g/l glucose, it had an exponential phase for the first 400 minutes approximately followed by a slower growth for the next 600 minutes (glucose curve in Figure 3.1D). When the strain was grown in medium containing 5 g/l malate, its growth rate was similar to that of grown in glucose and the exponential phase also lasted for about 400 minutes before it propagated at a much slower rate (malate curve in Figure 3.1D). The deviation of growth rate was found to be more significant than that of curve glucose at the similar time point (malate curve in Figure 3.1D). When glucose and malate were used together (2.5 g/l glucose and 2.5 g/l malate), the growth rate of 168+ was the highest among the three tested conditions during exponential phase (glucose + malate curve in Figure 3.1D). After about 250 minutes, the growth rate slightly reduced but still

higher than that of grown in either one of the carbon sources and the growth continued for another 400 minutes approximately until the strain entered stationary phase (glucose + malate curve in Figure 3.1D).

These growth curves together suggested the initial composition of the defined growth medium to be 1.25 mM amino acid mixture in SMM buffer with 5 g/l single carbon source (glucose or malate) or 2.5 g/l of both carbon sources (glucose and malate).

3.2.2 Determination of the methods suitable of amino acid quantification in defined culture medium

To determine whether quantification of amino acids was possible for bacterial culture medium initial analysis was focused on adapting well established analytical method for amino acid analysis on RP-HPLC. As a starting point, we used a standard 20 amino acids (except selenocysteine and pyrrolysine) in their monomeric L-form were dissolved in MilliQ water diluted into different concentrations ranging from 0.3 to 1.25 mM. The different concentrations of amino acid mixtures were submitted to RP-HPLC analysis using a protocol from Agilent Ltd (Henderson *et al.*, no date). The samples were first derivatized with OPA and FMOC reagents then resolved and by HPLC detecting the eluting material by absorption at 338 nm. This generated a chromatogram that is representative of the amino acids present (Figure 3.2A). Running samples with different amino acid concentrations allowed identification of each peak and an understanding of the strength of the signal obtained relative to the concentration of each amino acid present. Using a modified protocol (Section 2.14), it was possible to have peaks corresponding to all of the amino acids in the standard except proline as detection of this derivatised amino acid is only possible at 242 nm. However, the available equipment was unable to operate in dual wavelength detection mode. It was also found that the chromatogram generated at this wavelength did not show a reproducible distinctive peak in the expected position. Hence for technical reasons, quantification of proline was not possible and was excluded from the analysis, although it was always present in the medium preparations (except where specifically omitted).

To determine the sensitivity and the reproducibility of quantitative analysis using RP-HPLC, standard curves for each amino acid were generated in which the peak area of each amino acid detected (y-axis) is plotted against the concentrations of amino acid mixture analysed (x-axis) (Figure 3.2E). All standard curves are determined to be essentially linear for the concentrations tested, indicating that the peak area of an

amino acid is directly proportional to the amino acid concentration of the mixture analysed and the detectable range could be low as 0.3 mM before reliable identification of the peak became problematic due to low signal to noise ratio in the chromatogram. Repeated analyses were found to give reliably reproducible data both in terms of the chromatograms obtained and the standard curves derived from the plots. This is shown in Figure 3.2E by the standard deviations for each concentration as error bars for each amino acid.

From this preliminary work, it was clear that the quantification was possible for amino acids dissolved in water and that the concentration of each amino acid could be determined using the standards as a reference. However, the variations in the calculated concentration of some amino acids (*e.g.*, cysteine and histidine; dark green and green curves in Figure 3.2E) were likely to be less accurate. This was because the peak area relative to concentration change was relatively small. due to the insignificant standard deviations shown. However, there was no universal standard curve formula available for the quantification of all participating amino acids because each standard curve has its own gradient (Figure 3.2E). Therefore, the quantification of each amino acid would be independent from each other. Moreover, standard curve with a smaller gradient means the ratio of peak area to concentration of the corresponding amino acid is smaller. Hence there would be larger technical variations in the calculated concentration of the corresponding amino acids in the samples analysed. For instance, cysteine and histidine might be difficult to quantify because of their standard curves of smaller gradient (dark green and green curves in Figure 3.2E). In this case, a difference in concentration detected between samples might then be more difficult to be justified as biologically relevant. Therefore, extra caution was necessary for analysing the changes in cysteine and histidine in that the observed changes (if any) have to be significant and in a trend or pattern.

These results indicated that the analytical methodology was possible, but the analysed samples were not representative of a culture medium. Hence the analysis was repeated using Gaa, the initially defined medium containing 1.25 mM amino acid mixture and glucose mixed in SMM buffer. The pattern of the chromatogram obtained (Figure 3.2C) was almost identical to that of obtained using the amino acid mixture in water (Figure 3.2A) with negligible peak shifting. This indicated that the hydrophobicity of the derivatised amino acid monomers dissolved in MilliQ water was not significantly different from that of dissolved in solution added with glucose and

ammonium salts buffer. However, a new overlapping peak could be identified in the chromatogram for the defined medium, close to the methionine peak in the defined medium (at about 12 minute post injection into the column; Figure 3.2C). A magnified version of the plot is shown in Figure 3.2D. This peak was also evident in analyses of the salt solution that was used as the basis of the medium, SMM salt buffer (Figure 3.2B), at almost the same position as the methionine peak in Figure 3.2C. Although there are a number of components in SMM, the most probable “reactant’’ would be the ammonium salt that was present but this was not directly tested as it was present as the source of nitrogen in the medium and was not something that could be easily removed. Thus, for all the analyses detailed in this work, this ‘extra peak’ was present and may have some impact on methionine quantification.

In summary, the quantification of amino acids present in the defined medium Gaa was feasible within the tested range of concentrations. However, analysis of proline was not attempted and the absolute values obtained for methionine, cysteine and histidine have limited accuracy.

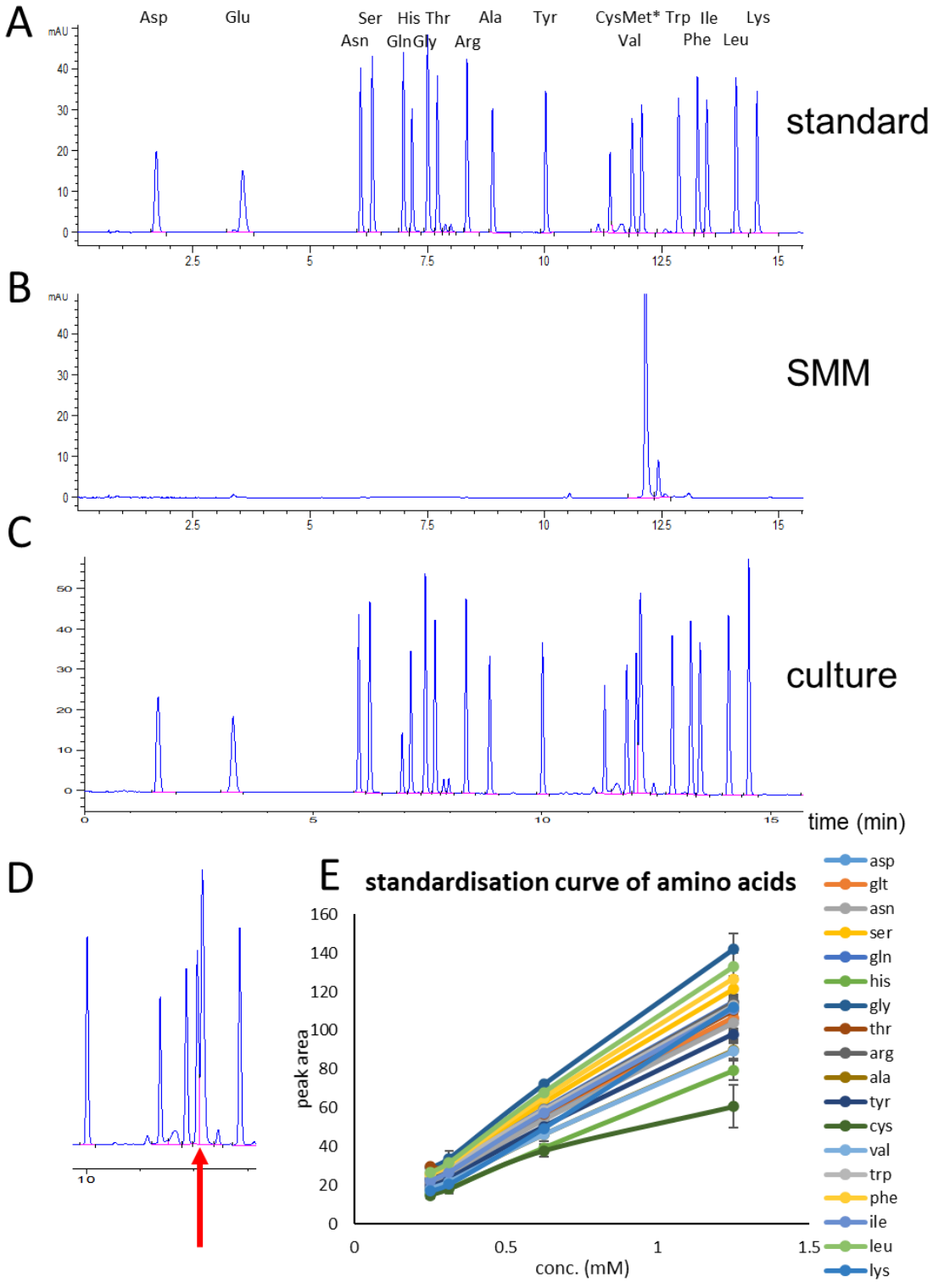


Figure 3.2: Detection and quantification of amino acids using RP-HPLC. (A) Chromatogram of 1.25 mM standard amino acid mixture; (B) Chromatogram of SMM; (C) Chromatogram of defined medium (Gaa); (D) a close-up of the chromatogram of (C) in the region between 10 – 12 min (red arrow) showing the single peak which resulted from the overlap of two peaks; (E) Standard curves for detected peak area for different amino acids at 4 concentrations. (Mean values from 4 replicates. Standard deviations for each are shown by error bars.)

3.2.3 Amino acid assimilation profile under gluconeogenic or glycolytic growth conditions

Having defined the analytical methodology, it was then possible to determine the abundance of free amino acids in a defined growth medium and if the amino acids were assimilated during the growth of *B. subtilis* in the medium. The defined medium from Section 3.2.1 was used as a growth medium for a strain of *B. subtilis* that was isogenic to the well characterised model *168CA* but corrected for its tryptophan auxotrophy (168+). To assess how the amino acid assimilation profile of wild type strain (168+) might change when the cells require glycolytic or glucogenic metabolism, cultures were analysed where glucose (G), malate (M), or both glucose and malate (GM) were provided as the carbon source. These media were named Gaa, Maa and GMaa respectively when the carbon source(s) was mixed with 1.25 mM amino acids (aa) in SMM buffer.

The growth of the strain in different conditions was monitored over time from initial inoculation to deviation from exponential growth at 37°C with shaking as determined by the changes in optical density. During this time samples were taken at regular intervals and the cells were removed to permit the changes in the amino acid composition of the medium to be determined by RP-HPLC (Section 2.14 and 2.15). Also, given the presence of amino acids at equal concentration and the quantitative analysis protocol, preferred amino acids (if any) to be assimilated in the given medium condition during its exponential growth could be identified.

Analysis of the growth of the strain in this defined medium with glucose as the carbon source (Gaa) showed that after the initial inoculation of the medium (with exponentially growing cells; see Section 2.1 and 2.14), the optical density remained constant for about 100 minutes. After this initial ‘lag’ phase, a period of rapid exponential growth was observed, reaching OD_{600} 1.3 after about 600 minutes and the growth was then gradually slowing down (G curve in Figure 3.3A). This indicated that the medium was suitable for the growth of the strain, but period of exponential growth was not long, suggesting something was limiting the growth of the bacteria. However, for the purpose of this study, it was sufficient to indicate amino acid assimilation during vegetative growth and it was potentially advantageous to avoid complications of high cell densities and post exponential growth developmental processes where cell lysis or alternative metabolic activities might complicate the results.

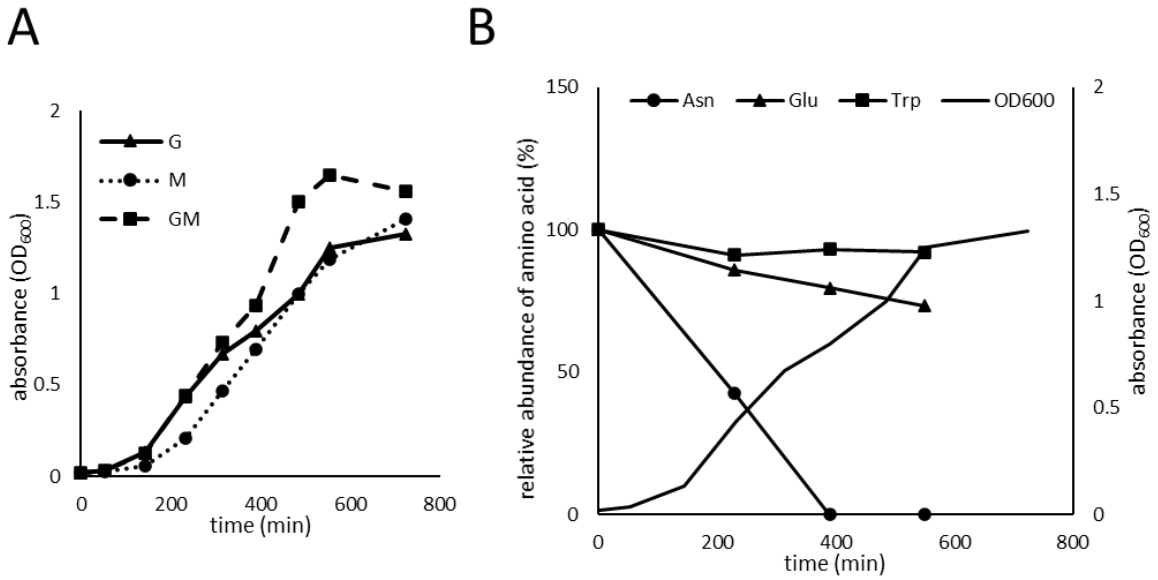


Figure 3.3: Growth of *168+* in Gaa, Maa and GMaa and amino acid assimilation profile of *168+* in Gaa. (A) Growth in Gaa, Maa and GMaa in which the growth was measured using spectrophotometer at wavelength 600 nm plotted against time. G = growth in Gaa; M = growth in Maa; GM = growth in GMaa. (B) Amino acid assimilation profile of *168+* in Gaa during exponential growth. Optical density of the culture was G curve in A. The relative abundance of amino acids in percentage during the time course is shown using representatives from each group of amino acids (Table 3.1). Asparagine and glutamate are used as the representative from groups 1 and 2 respectively which were efficiently assimilated ones. Tryptophan is used as the representative for amino acids from group 3 without obvious depletion and increased in abundance. Grouping criteria refers to text. Amino acids are presented as 3-letter code. Asn = asparagine; Glu = glutamate; Trp = tryptophan; OD_{600} = *168+* growth. The experiment has been repeated once and the data above come from a single experiment.

Using different carbon sources in the defined medium, growth curves were obtained and they were similar to that obtained when it was grown in microtitre plate (Figure 3.3A compared to Figure 3.1D). 168+ was found to grow most rapidly when both glucose and malate were present in the medium (GMaa) with its best growth rate and highest optical density reached (GM curve in Figure 3.3A). Interestingly, the growth of the strain in medium added with only glucose (Gaa) exhibited a similar growth rate as that of in medium with both glucose and malate (GMaa) but a slightly lower final optical density (G curve compared to GM in Figure 3.3A). Although the growth rate of the wild type strain in the medium added with only malate (Maa) was the slowest (M curve in Figure 3.3A), here its maximum optical density reached was similar to that of when the strain was grown in medium added with only glucose (G curve in Figure 3.3A).

Table 3.1: Relative abundance of amino acids of *168+* growing in Gaa medium at different time points.

	Relative level of amino acid (percentage to T_0 at T_{230})	Relative level of amino acid (percentage to T_0 at T_{390})	Relative level of amino acid (percentage to T_0 at T_{550})
Asparagine	42.6	N.D	N.D
Glutamine	61.8	25.7	N.D
Serine	74.4	40.1	14.6
Aspartate	84.7	74.7	58.6
Alanine	81.3	71.9	65.7
Glutamate	85.8	79.5	73.4
Leucine	88.0	84.0	74.2
Isoleucine	87.2	82.5	74.5
Lysine	89.6	85.9	75.1
Valine	86.8	83.0	76.8
Phenylalanine	90.3	90.8	89.0
Arginine	91.0	91.0	90.6
Tyrosine	88.1	92.5	91.6
Tryptophan	91.2	93.1	92.1
Threonine	91.4	93.7	92.8
Glycine	95.8	101.5	111.9

The relative abundance of each amino acid at each time point (T_{230}, T_{390} and T_{550} from left to right) was calculated in percentage relative to their initial concentration correspondingly and the numbers were corrected to 1 decimal place. Amino acids were divided into four groups and presented in shades of grey according to their depletion efficiency (Darkest grey: most efficient; white: least efficient). Group 1 were amino acids with less than 15 % abundant at T_{550} whilst group 2 includes those between 55 to 80 %. They were coloured deep grey and grey respectively. Group 3 were amino acids depleted for less than 10 % and glycine was of group 4 as there was an increase in abundance in medium. They were coloured as light grey and

white respectively. Data shown is from single experiment. This experiment was repeated once from sample collection to data analysis and similar trend of amino acid assimilations were obtained. N.D = amino acid concentration was below detectable range.

Analysis of the culture medium sampled over the duration of the growth for the amino acids present showed that the amino acids were depleted from the medium at different rates, a summary of the determined rates is shown in Table 3.1 where they have been sorted according to the rate of depletion (fastest first). Examples of chromatograms for amino acid quantification are shown in Appendix (Figure 8.1). The amino acids in the Gaa were depleted at different rates and they were ranked according to their relative abundance at T_{550} (Table 3.1). In this table, the abundance of the amino acid is expressed as a function of the initial amount. Doing this it was clear that the amino acids could be divided into four groups by their apparent depletion rates. The amino acids that decreased to below 15 % by 550-minute incubation were categorised as group 1 (highlighted dark grey in Table 3.1). Of these, asparagine dropped dramatically very quickly whereas the other 2 in this class, serine and glutamine were assimilated less rapidly. The amino acids in group 2 tended to reduce to between 55 and 80 % by the last sample (grey in Table 3.1). For the other amino acids there were relatively minor changes in their abundance over the duration of the experiment. However, they could be divided into two sets based on their abundance at the end of the time course. Amino acids that were depleted less than 10 % by 550^{th} minute from the start of the time course were put into group 3 (light grey in Table 3.1) which included phenylalanine and tryptophan. The numerical values of their abundance in medium have barely changed over time. Alternatively, the amino acids could be assimilated and released at similar rates. But it is also possible that the change was not detected because of the technical limit of the equipment. Glycine was shown a 10 % increase in abundance in the medium and was categorised as group 4 (white in Table 3.1).

This assimilation data is summarised in Figure 3.3B by plotting the change in amino acid abundance in the culture medium for a representative amino acid for each of the four groups identified with the growth curve of the strain.

Table 3.2: Depletion rates of amino acids from defined medium of growing *168+*.

	Gaa	Maa	GMaa
Asparagine	-52.4	-51.1	-53.8
Glutamine	-76.1	-78.7	-49.9
Serine	-73.6	-87.0	-57.5
Aspartate	-32.1	-37.1	-65.0
Alanine	-19.1	28.8	1.3
Glutamate	-15.2	-45.1	-75.4
Leucine	-17.0	-18.1	-11.9
Isoleucine	-15.6	-20.9	-4.1
Lysine	-17.9	-21.6	-3.1
Valine	-12.3	-17.1	11.7
Phenylalanine	-1.7	-8.7	4.5
Arginine	-0.5	-9.6	4.1
Tyrosine	4.4	-3.2	17.4
Tryptophan	1.2	-6.2	7.2
Threonine	1.6	-11.2	-5.7
Glycine	19.9	2.0	14.7

The depletion rates (% decrease per OD_{600}) were calculated as the percentage change of the relative abundance of amino acids per change of optical density. Column Gaa represents the relative abundance of amino acids recorded when the strain was grown in Gaa whilst column Maa represents those when the strain was grown in Maa. Column GMaa shows the relative abundance of amino acids when the strain was grown in GMaa. The order of amino acids follows that of Table 3.1. This experiment was repeated once from sample collection to data analysis and similar trend of amino acid assimilations were obtained.

To compare the depletion of amino acids in different medium condition, the change in amino acid abundance as a percentage was divided by change in optical density

during exponential growth (approximately from 230 min to 550 min) and the comparison was tabulated in Table 3.2. The more negative the numerical value in the table, the more efficient depletion of amino acids from the medium. When the strain was grown in the presence of glucose (Gaa), asparagine, glutamine and serine from group 1 were depleted efficiently where the depletion rates were between - 52 to - 76 %/OD_{600} (Column GMaa in Table 3.2). The depletion rates of these amino acids were more negative than those of other amino acids, suggesting they were more efficiently assimilated than the others. The depletion rates of aspartate, glutamate, lysine, alanine and the three branched-chain amino acids (isoleucine, leucine and valine) from group 2 were in general lower than those from group 1 in which they were moderately depleting at -12 to -32 %/OD_{600} (Column Gaa in Table 3.2). The depletion rate of amino acids from group 3 was within plus or minus 10 relative to zero in change, indicating insignificant changes of the abundance of these amino acids in the medium (Column Gaa in Table 3.2). Glycine was shown with an approximately 20 % increase in depletion rate suggesting the amino acid to be released into the medium (Column Gaa in Table 3.2). All of these largely matched with the analysis done by sorting the percentage changes of the relative abundance of amino acids at different time points in Table 3.1.

When 168+ was grown in malate (Maa), the amino acid assimilation profile was slightly different from that seen when the strain was grown in glucose (Gaa) (Column Maa compared to Gaa in Table 3.2). Firstly, there was a slightly more obvious depletion of glutamate in which there was an almost three times more significant depletion rate of glutamate when the strain was grown in malate (Maa) compared to that of in glucose (Gaa) (Column Maa compared to Gaa in Table 3.2). Unlike the profile of the strain grown in glucose (Gaa), here alanine was shown increased in abundance in the medium in that a 28 % of increase of the amino acid was observed during exponential phase (Column Maa compared to Gaa in Table 3.2). Thirdly, glycine accumulation was not seen when the strain was grown in malate (Maa) but it remained rich in medium by the end of the time course (Column Maa in Table 3.2). For the remaining amino acids, their depletion rates were insignificantly different from those of grown in medium containing glucose (Gaa).

The amino acid assimilation profile of 168+ growing in the medium with both glucose and malate added (GMaa) was surprisingly different from that of grown in either medium G or M (Column GMaa in Table 3.2). Glutamate and aspartate were

depleted significantly more efficiently, which was not seen in other two conditions. However, the depletion rate of glutamine and serine from medium GMaa was slower compared to that of when the strain was grown in media containing glucose (medium Gaa) or malate (Maa) (Table 3.2). The depletion rate of the two amino acids were almost a double or even triple of the rates obtained when the strain was grown in glucose (Gaa) (Column GMaa compared to Gaa in Table 3.2). Unlike the accumulation of alanine observed when the strain was grown in malate (Maa) or the efficient depletion of this amino acid in Gaa, alanine remained abundant when the strain was grown in the presence of both glucose and malate (GMaa, Column GMaa in Table 3.2). Tyrosine and glycine were suggested to be released into the medium at a rate of over 10 % per OD_{600}. A lot of quantified amino acids were detected of less than 15 % change from the initial amount of amino acids detected in the culture medium (Column GMaa in Table 3.2). This suggested that the growth of the strain in this medium might be independent on the amino acids supplied, except the six efficiently assimilated ones. The observation could be due to the compensation of recycling amino acids from the overflown of metabolism.

3.2.4 Amino acid assimilation profile of amino acid auxotrophs

As seen in Section 3.2.3, the uptake of some amino acids was not evident, *e.g.,* tryptophan which suggested the cells ignoring these amino acids. Or this could be due to a lower cellular demand of the amino acid which made the assimilation undetectable. However, the assimilation of some other amino acids was significantly, *e.g.,* leucine which might be due to its involvement in metabolism. Thus, analysis of assimilation was done using specific auxotrophs. Here, the assimilation of the corresponding amino acid was expected to increase as a reflection of the need of this amino acid to the cell. Alternatively, the assimilation of an amino acid in an auxotroph could decrease if the mutation prevented reverse metabolism, *e.g.,* serine.

In order to test this hypothesis, the amino acid assimilation of auxotrophs of the corresponding amino acids was compared with the wild type strain. According to the previous assimilation profiles generated based on the wild type strain grown in glucose, tryptophan remained rich in the culture medium leucine was gradually depleted, whereas with serine and alanine efficiently depleted from growth medium (see Section 3.2.3). Therefore strains with a deletion of *trpC2*, *leuA*, *alaT* or *serA*, to result in tryptophan, leucine, alanine and serine auxotrophs respectively (168CA, MC17, MC2 and MC16), were used to grow in parallel with the wild type strain *168*+

at 37°C with agitation (Section 2.1 , 2.4 , 2.10 , 2.14 and 2.15). The growth medium contained glucose and a mixture of 20 amino acids with ion salts in SMM buffer (GaaP). The addition of P, containing a mixture of ion salts, was to provide a longer exponential growth period for observation. The assimilation of the corresponding amino acid that the strain needed for growth was compared to that of the wild type strain. Growth measurement and amino acid assimilation sampling were as previously described.

In this experiment the growth curve of 168+ (wild type) exhibited exponential growth for the first 200 minutes. This growth curve is used in all panels A - C and is used to compare with the other strains of interest and the whole set was done in a single experiment (dashed line in Figure 3.4A - D). The growth pattern of all auxotrophs involved in this analysis (MC16, MC17, MC2 and 168CA) was similar to that of the wild type strain (black lines in Figure 3.4A - D). For amino acid assimilation, there was no observable significant difference in the depreciation of the specified amino acids in each graph (Figure 3.4A - D). The assimilation efficiency of the amino acid was further calculated as the percentage change of the amino acid per OD_{600}, similar to that of in previous section. The more negative the number, the more efficient assimilation of the amino acid had taken place. The overall depletion rate of serine was roughly two times more efficient than that of the alanine and leucine during exponential growth of the wild type strain (Table 3.3A - C). The overall depletion rate of tryptophan was the least efficient among the four selected amino acids (Table 3.3D). This matched with the deduction from Section 3.2.3 where serine and tryptophan was shown one of the most efficiently assimilated amino acids and least efficiently assimilated ones among the detectable ones respectively. Compared to the wild type strain, the overall rate of depletion of alanine by the alanine auxotroph (MC2) was only slightly higher than that of the wild type strain (Table 3.3B). However, the depletion rate of alanine from culture medium for the alanine auxotroph (MC2) in the first half and second half of the exponential growth period was similar to that of the wild type strain (168+) correspondingly (row 0 – 100 and 100 – 160 in Table 3.3B). This suggested an insignificant difference of the assimilation efficiency of alanine between the two strains during exponential growth. Despite the similar overall depletion rate of leucine from culture medium obtained between the leucine auxotroph (MC17) and the wild type strain (168+), the depletion rate of leucine of the leucine auxotroph (MC17) for the first 100 minute was near 1.5 times less efficient

than that of the wild type strain (168+) (row 0 – 100 in Table 3.3C). There was no significant difference of the depletion rate of leucine in the later period of exponential growth (row 100 – 160 in Table 3.3C). This suggested a more efficient assimilation of leucine for the wild type strain (168+) than that of the leucine auxotroph (MC17). A similar observation was seen when the depletion of serine of the serine auxotroph (MC16) was compared to the wild type strain (168+) (Table 3.3A). Despite the depletion rate of serine of the auxotroph for the first 100 minutes was almost half of that of the wild type strain, this, however, was not reproducible in a separate experiment. The depletion rate of serine in the serine auxotroph (MC16) in the separate experiment was shown more significant than that of the wild type strain (Appendix). Nevertheless, the overall depletion rate of serine and that of during 100 – 160th minute exponential growth of the serine auxotroph was similar to that of the wild type strain (Table 3.3A). In contrast, depletion of tryptophan of the tryptophan auxotroph was shown around two times more efficient than that of the wild type strain during first half of exponential growth (row 0 – 100 in Table 3.3D). However, this was also not reproducible in its separate experiment. The expectation that phenotype of auxotrophs would assimilate the amino acid it needed for growth slightly more efficiently than the wild type strain was not necessarily true and it seemed to be auxotroph-specific. This suggested the possibility of dynamic changes in metabolism to adjust cellular demand of an amino acid. Alternatively, the expected phenotype was not detected due to technical limits.

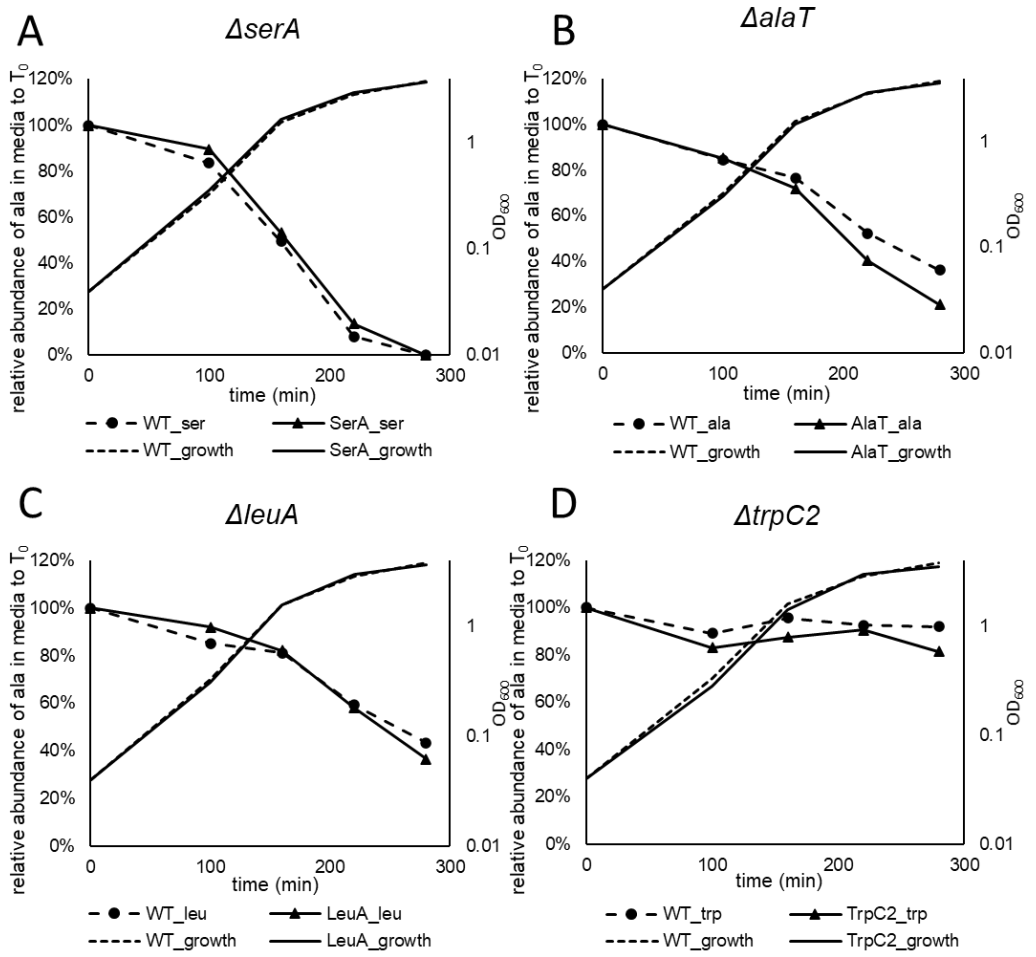


Figure 3.4: Graphs of genetically manipulated strains growing in defined medium (GaaP) compared with wild type strain. Each graph shows the growth curve of the auxotroph strain and the wild type strain (WT). For amino acid assimilation, each graph only shows the relative abundance of the particular auxotroph-depending amino acid in the medium relative to T_0 correspondingly. The growth of WT is shared between the three graphs above. The first one (top left) shows comparison between serine auxotroph (MC16) and wild type strain. The curve annotated XX_ser refers to the relative percentage of serine for the strain XX. E.g WT_ser means the relative abundance of serine for the wild type strain. The curve annotated XX_growth represents the growth measured in OD_{600} of strain XX. E.g WT_growth shows the OD_{600} measured for the wild type strain. The second graph (top right) shows comparison between alanine auxotroph (MC2) and WT. The third one (bottom left) shows leucine auxotroph (MC17) and WT whilst the forth one (bottom right) shows comparison of tryptophan auxotroph (168CA) with WT. The experiment has been repeated once and the data above come from a single experiment.

Table 3.3: Tables of assimilation efficiency of the amino acid specified for different auxotrophs and the wild type strain.

A Serine

Time (min)	Wild type strain	Auxotroph (*ΔserA*)
0-160	-32.9	-28.6
0-100	-56.8	-33
100-160	-27	-28

B Alanine

Time (min)	Wild type strain	Auxotroph (*ΔalaT*)
0-160	-8.07	-19.5
0-100	-26.8	-55.2
100-160	-4	-11

C Leucine

Time (min)	Wild type strain	Auxotroph (*ΔleuA*)
0-160	-6.57	-11.7
0-100	-41.7	-30
100-160	1.6	-8

D Tryptophan

Time (min)	Wild type strain	Auxotroph (*ΔtrpC2*)
0-160	-10.5	-9.04
0-100	-43.2	-70.5
100-160	-3	3.9

The strains were grown in defined medium (GaaP) containing equal concentrations of 20 amino acids. Each table shows the assimilation efficiency rate calculated as percentage change per OD_{600} of the amino acid that the auxotroph required for growth for the auxotroph compared to the wild type strain during exponential growth over three time points. The first row of each table shows the overall rate during exponential growth. The second row shows the first 100 minute of the assimilation efficiency of the specified amino acid whilst the third row shows the following 60 minute of assimilation efficiency of the amino acid. The more negative the value in each table, the higher assimilation efficiency of the specified amino acid. A: comparison of assimilation efficiency of serine between serine auxotroph (MC16) and wild type strain. B: comparison of assimilation efficiency of alanine between leucine auxotroph (MC2) and wild type strain. C: comparison of assimilation efficiency of leucine between leucine auxotroph (MC17) and wild type strain. D: comparison of assimilation efficiency of tryptophan between tryptophan auxotroph (*168CA*) and wild type strain. The experiment has been repeated once and the data above come from a single experiment.

3.3 Discussion

In this chapter, a standardised protocol was developed for the physiological study of amino acid assimilation in this project. The amino acid assimilation profiling of *B. subtilis* was an indirect approach to measure amino acid requirements in growth culture using RP-HPLC. To begin with, a defined rich medium was decided specifically for this project studying the assimilation of amino acids (Section 3.2.1). Concomitantly, the applicability of the approach was considered in terms of technical feasibility and the credibility of result (Section 3.2.2). The protocol was then further applied to study the amino acids assimilation for glycolytic growth as well as gluconeogenic growth conditions. In these analyses, some of the amino acids were apparently less prioritised for assimilation or were maintained by a dynamic flow into and outside of the cells at similar rates (Section 3.2.3). Hence, the assimilation of

some of the amino acids were selected for analysis in its auxotrophic background (Section 3.2.4). Surprisingly, the assimilation of amino acids seemed to follow similar patterns regardless of the growth conditions or the background of strains tested.

3.3.1 Assessment of indirect approach for amino acid assimilation profiling

As mentioned in Section 3.1 , creating defined medium was the preferred route to perform physiological studies. And a mixture of amino acids at an equal and definite concentration would also benefit the quantification of the abundance of these components in the media. PTM, essentially a combination of glucose, hydroxylated casein (CAA) and ion salts in ammonium salt buffer, was traditionally useful in boosting the growth of *B. subtilis* with the presence of CAA (Anagnostopoulos and Spizizen, 1961). This was also shown in the significant growth defect when CAA was removed in PTM (Figure 3.1). However, the nature of CAA as a hydrolysed casein without accurate and even distribution of amino acids contents during production made it a possible concern for doing physiological studies (Section 3.1 and 3.2.1). The optimisation of PTM was to replace the CAA with amino acid mix for the purpose of our project as well as fine-tuning the composition of the medium. With a similar growth pattern and final optical density reached, replacement of CAA with the 1.25 mM amino acid mixture was shown possible despite a relatively slower growth rate (Section 3.2.1). Due to the similar growth of the strain observed within the tested range of glucose concentrations, a slightly lower concentration of glucose was selected for initial trial of amino acid assimilation analysis in order to shift the dependence of growth of a strain from glucose to amino acids as nutrients. Whilst the shift was aimed to increase the possibilities of detecting any changes in the amino acid assimilation profile during the exponential growth of the strain, maintaining a good bacterial growth was also important. Therefore, glucose was not removed or being minimised to the lowest concentration possible in order to ensure a sufficient supply of carbon source for a good exponential growth. The bacterial growth in the absence of glucose from PTM was shown poor with decreased growth rate and low final optical density reached despite the presence of rich nitrogen source (Figure 3.1). This was also in agreement with the growth of *E. coli* with short exponential growth when it was grown in LB where glucose was absent (Sezonov, Joseleau-Petit and D'Ari, 2007). Although the growth curve of the strain grown in 2.5 g/l glucose apparently to be slightly better than that of grown in 5 g/l glucose, the strain demonstrated the trend of better growth curves in the presence of higher

concentration of glucose in general (Figure 3.1B). Therefore the concentration of glucose in the defined medium in future analysis was set to be 5 g/l. On the other hand, as the strain was grown very similarly in two tested concentrations of amino acids, the lower concentration was selected in order to avoid saturation of detection signal in the RP-HPLC analysis (if any). Such initial defined rich medium was later termed as Gaa.

For technical feasibility of RP-HPLC analysis, the measurement of amino acid content in the defined media has been shown reliable through the distinctive peak in the chromatogram for each amino acid and their corresponding standard curves for quantification (Section 3.2.2). For distinctive peaks, the derivatising reagents have added extra specificity to amino acids for detection which increases sensitivity of the equipment detecting amino acids. The signal to noise ratio was maximised and distinctive peaks were shown for easier peak identification (Section 3.2.2). For quantification, the straight lines of amino acids standard curves with insignificant standard deviations indicated that the detected peak area is directly proportional to the concentration of the corresponding amino acids (Section 3.2.2). This allowed easier quantification of amino acids with simple mathematic formulas. However, proline, histidine, cysteine and methionine were avoided for quantification due to various technical limitations (Section 3.2.2). As for the presence of methionine giving an overlapping peak to the peak representing the presence of ammonium , one of the possible solutions was to remove ammonium salts from SMM buffer given the assumption that a mixture of amino acids should provide good nitrogen source for growth. Yet the removal of ammonium salts might introduce significant changes to the cellular metabolism as ammonium has been shown to be important for bacterial growth and might be responsible for metabolic regulation (Tam *et al.*, 2006). Therefore, the recipe of SMM was not modified for the assimilation analyses. Although histidine and cysteine were detectable, the gradient of the standard curves of these amino acids was small with higher standard deviation than those of others (Section 3.2.2). This means a decrease in accuracy when quantifying peak representing histidine. Histidine remained abundant in the initial trial and its uptake was previously suggested to be repressed in the presence of a mixture of 16 amino acids (Atkinson, Wray and Fisher, 1990). Hence quantification of histidine assimilation was not done in later trials although histidine transporter during exponential growth has yet been experimentally verified. Cysteine was shown

progressively assimilated in the initial trial. However due to the low gradient standard curve, this amino acid is not included in the quantification analysis as the values obtained were too unreliable.

For the credibility of results, one of the concerns was whether the detection was sensitive enough for measuring the assimilation of amino acids by bacterial cells. As reference to the tested range of amino acid concentrations in Section 3.2.2, the number of moles per sample analysed was around 125 – 600 pmole amino acids. To assess whether this level of sensitivity was good enough for measuring changes of amino acid abundance in each sample, the cellular demand for amino acid was also roughly calculated. Based on the assumption that amino acids are only used in protein synthesis and an equal distribution of amino acid usage for this purpose, the change of amino acid concentration in cells for 3 generations (from OD_{600} 0.1 to OD_{600} 0.8) was estimated to be in about 1 to 100 pmole. Hence the bacterial growth has to reach around OD_{600} 1 at least to ensure a detectable change in the concentrations of amino acid might not be detectable if the bacterial growth only reached the final optical density of 0.8. As the growth of the wild type strain had been higher than OD_{600} 1 in all analyses, changes, if there was any, should have been detected (Section 3.2.3).

Another question would be on the electrostatic charges of amino acids in the defined media because amino acids are zwitterions which can be both positively and negatively charged within a certain pH range. A quick pH measurement of the defined media was shown to be approximately pH 7 or 8, suggesting most of them would be of a net charge of zero in the media according to the pKa value of amino acids with only a few exceptions. Aspartate and glutamate were expected to be negatively charged whilst lysine, arginine and histidine should be positively charged. Given that the cell wall is negatively charged, the positively charged amino acids might adhere to the cell surface by electrostatic attraction and the negatively charged ones to be repelled from the cells. This should be reflected in the quantitative analyses of the culture supernatant in which the relative abundance of positively charged amino acids are expected to be greatly reduced in reasonably efficient manner. However negatively charged amino acids, glutamate and aspartate, were consistently shown to be depleted from media efficiently and they seemed to be preferred for assimilation than the positively charged ones *e.g.,* lysine and histidine. Thus, the issue raised was proven less likely accountable for the observations

obtained. Technically possibility of chemotaxis driving preferential assimilation can also be excluded as the medium used was rich and efficiently mixed and so the medium should have been homogeneous. Moreover, the efficiency was found uncorrelated to the nutritional value of the amino acids to the cells (Yang *et al.*, 2015). Cell wall turnover and proteolysis would occur during growth in which the material would be released into the medium. However, the amount accounted for these activities were suggested trivial (Pine, 1967), except perhaps in the case of alanine (see Chapter 4).

In the current indirect approach of amino acid analysis, the amino acid depletion in the media was interpreted as an overview of the amino acid net assimilation by *B. subtilis* which was calculated by comparing the difference in percentage of relative abundance between samples collected from different time points (Jung, Branciamore and Martini, 2000). In other words, the flow of amino acids which in realistic term being transported efficiently in and out of the cells dynamically would be difficult to track. Therefore, the relative abundance of an amino acid is an account of both absorption and release of the amino acid. Also, the enantiomers of any amino acids cannot be differentiated using the current analytic protocol. Hence each amino acid peak represents a total amount of both L- and D-amino acid correspondingly. However, this should not be a major concern as the naturally occurring amino acid is usually in its L-isoform. Also, there was surprisingly low amount of D-amino acids to be detected in the culture supernatant for *B. subtilis* (Lam *et al.*, 2009).

Nevertheless, this approach has been shown as an easy method to identify the order of preference of amino acids to be assimilated in *B. subtilis* by efficiently assessing the concentrations of most of the amino acids in each sample. Hence the possible complicated calculation of amino acid assimilation if the growth of the strains of interest is different from each other could be avoided.

3.3.2 The assimilation of amino acids during glycolytic growth of B. subtilis wild type strain in the defined media with glucose as carbon source

The amino acid assimilation analysis focused on exponentially growing *B. subtilis*, where the expected change(s) of amino acid concentration during the time course might be maximum. In the glycolytic growth condition which was defined as using glucose as the carbon source, the amino acids were divided into four groups by ranking the relative abundance of each of them by the end of exponential growth.

Potentially *B. subtilis* has a preference towards only asparagine, glutamine and serine (Table 3.1). The complete assimilation of asparagine within vegetative growth of *B. subtilis* agreed to that obtained using a different analytical method (Jung, Branciamore and Martini, 2000; Yang *et al.*, 2015) and it was suggested to be the most important amino acid serving as the main nitrogen source for *B. subtilis*. This is presumably because it can be converted to aspartate then fumarate or used for synthesizing other amino acids (Fisher and Sonenshein, 1991). The efficient assimilation of glutamine matched with the common understanding of glutamine as a preferred nitrogen source of *B. subtilis* as it is used as the precursor for about 15 % of the nitrogenous molecules per cell (Fisher, 1999). Glutamate is important as it serves as a precursor in different cellular processes and the maintenance of the abundance of this amino acid can also be helped by glutamine in the glutamate homeostasis (Section 1.2). Serine had been suggested toxic if the bacterium was grown in minimal media (medium without other amino acids) containing high concentration (as high as 1 mM) of this amino acid (Klewing *et al.*, 2020). But the efficient depletion of serine was also detected using when it was grown in the presence of all 20 standard L-amino acids but without additional carbon sources (Yang *et al.*, 2015). Here this amino acid was shown assimilated for at least 80 % level when the strain was grown in the defined rich medium (Gaa) (Section 3.2.3). The important role of serine as a precursor for a lot of cellular reactions, *e.g.,* to be metabolised into pyruvate for growth, might be the reason for the observed efficient assimilation of this amino acid in the analysis (Ponce-de-Leon and Pizer, 1972; Stauffer, 2004). Also, the presence of other amino acids seemed to eliminate its toxicity on *B. subtilis* growth although the mechanism remained unclear.

Assimilation of aspartate, alanine and glutamate was also relatively more efficient than other amino acids. The importance of aspartate was highlighted in peptidoglycan synthesis and the synthesis of other amino acids, including lysine and threonine (Daniel and Errington, 1993; Zhao, Roistacher and Helmann, 2018). At the same time, aspartate could be converted into asparagine for use as asparagine was shown to be depleted by the middle of exponential growth (Table 3.1). And alanine is used in cell wall biosynthesis and metabolised into glutamate or ammonium through different pathways (detailed in Section 1.2). Interestingly, all these amino acids mentioned above serve as one of the metabolites that were, at most, only three steps of reactions away from the pathways involved in the glycolysis and TCA cycle

according to SubtiPathway (highlighted red in Figure 3.5). This suggested that these amino acids were likely assimilated for maintaining growth through supporting metabolites for the central metabolism. This also matched with the suggestion of the presence of amino acids in medium for better bacterial growth (Anagnostopoulos and Spizizen, 1961). Interestingly, the rapid consumption of serine, aspartate, glutamate, alanine and asparagine was also recorded in *E. coli* (Selvarasu *et al.*, 2009).

Apart from the efficiently assimilated amino acids, lysine and branched-chain amino acids (BCAA) including isoleucine, leucine and valine were readily assimilated (Table 3.1). The intracellular pool of BCAA was known to be important for the regulation of CodY, the global nitrogen regulator, in response to nutrient starvation (Shivers and Sonenshein, 2004; Handke, Shivers and Sonenshein, 2008; Villapakkam *et al.*, 2009; Brinsmade *et al.*, 2010; Levdikov *et al.*, 2017). The presence of BCAAs could enhance the binding of CodY some of its target DNA fragments to either repress or derepress the expression of the expression of proteins downstream of the targeted fragments (Handke, Shivers and Sonenshein, 2008). For instance, the accumulation of the BCAAs was once shown to induce *bkd* operon for synthesizing branched chain fatty acid synthesis and hyperrepressing *yhdG* gene expression (Brinsmade *et al.*, 2010). Although transporters of BCAA were also found repressed by CodY in normal condition, the uptake of these amino acids observed seemed less likely due to a derepression of CodY in response to nutrient starvation as the assimilation was gradual and continuous during the time course (Molle *et al.*, 2003; Brantl and Licht, 2010; Geiger and Wolz, 2014; Belitsky, Brinsmade and Sonenshein, 2015). Lysine is one of the amino acids within the synthesis of the aspartate family in *B. subtilis* (Zhao, Roistacher and Helmann, 2018). The efficient assimilation of this amino acid might be a result of the more efficiently assimilated aspartate and asparagine, in which one of them had been completely depleted from medium in the early exponential growth (Table 3.1). Putative lysine transporter was suggested in *B. subtilis* which is encoded by *yvsH* in which the gene was suggested to be regulated through L-box riboswitch depending on the presence of lysine (Wilson-Mitchell, Grundy and Henkin, 2012).

The other amino acids were assimilated slowly, including the aromatic amino acids (phenylalanine, tryptophan and tyrosine), arginine and threonine. Interestingly, arginine has been one of the preferred nitrogen sources commonly recognised thus it was expected to be assimilated as efficiently as glutamine, another proposed

preferred amino acid of *B. subtilis* (Belitsky and Sonenshein, 1998; Fisher, 1999; Gunka and Commichau, 2012). Here the slower assimilation of this amino acid (compared to others) could have been due to the presence of glucose in media. The expression of putative arginine permease encoded by *rocE* could be induced by the presence of arginine but was also found dependent on CcpA regulation in which the *rocE* expression could be inhibited in the presence of glucose (Gardan, Rapoport and Débarbouillé, 1995; Belitsky and Sonenshein, 1998; Fisher, 1999; Moreno *et al.*, 2001; Gunka and Commichau, 2012). For threonine, it could be metabolised into pyruvate which is one of the important metabolites in the core metabolism of *B. subtilis* (Willetts and Turner, 1970). But the assimilation of threonine was detected less significant compared to a lot of other amino acids (Section 3.2.3). As serine and threonine could share same transporter(s), the transport of threonine might be slightly delayed as serine being assimilated efficiently during exponential growth (Klewing *et al.*, 2020). Also it could be suggesting the preference of serine over threonine for the transport system responsible for transferring these two amino acids. *B. subtilis* has been reported to be intolerant to 1 mM threonine in minimal media toxicity (Lambt and Bott, 1979; Lachowlcz *et al.*, 1996). However, similar to serine, the presence of amino acids mixture seemed to have eliminated the inhibition effect of threonine on the growth of the wild type strain even though the concentration of threonine provided was 1.25 mM. Together with the absence of serine inhibition, the components in the media might have hindered the shared uptake system of serine and threonine or repressed the expression of the corresponding uptake system hence reducing the toxicity of both of them suggested previously. For the aromatic amino acids, phenylalanine, tryptophan and tyrosine, they were less likely involved in the central metabolic pathways (glycolysis or TCA cycle) for generating ATP for growth. Also the metabolites necessary for synthesizing either of these amino acids were not related to the central metabolism. According to SubtiPathway, the number of steps required to convert metabolites in the central metabolism to generate these aromatic amino acids were of more than ten (Figure 3.5).

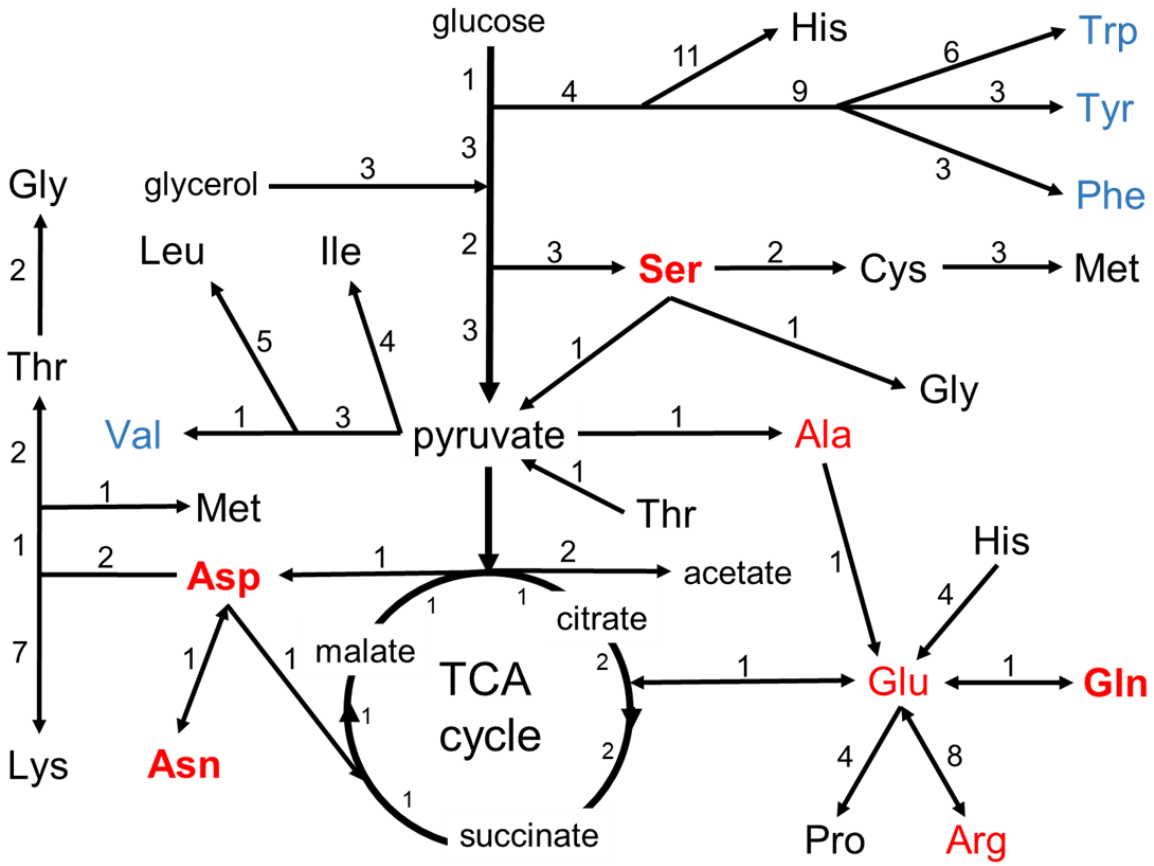


Figure 3.5: Schematic diagram of amino acid metabolism of *B. subtilis*. Pathways are referred to SubtiPathway and this is a simplified version only includes metabolites (amino acids and carbon sources) mentioned in this chapter. Each conjunction of two lines indicates the presence of an unnamed metabolite. The number indicates the number of reactions involved in the nearest straight line from one metabolite converting to another. Amino acids are annotated as 3-letter code. Amino acids labelled in red are the ones shown preferred in amino acid assimilation profiles generated in this chapter although with some exceptional media conditions. The red and bolded ones are the ones that were shown preferred in all tested media conditions. The blue ones are the less prioritised amino acids suggested from this chapter. Ala = alanine; arg = arginine; gly = glycine; thr = threonine; lys = lysine; asn = asparagine; asp = aspartate; met = methionine; val = valine; leu = leucine; ile = isoleucine; his = histidine; trp = tryptophan; tyr = tyrosine; phe = phenylalanine; cys = cysteine; ser = serine; glu = glutamate; gln = glutamine; pro = proline.

Glycine was of most abundant in the media during early exponential growth of the strain in that the relative abundance of it went slightly higher than that of its starting concentration when the strain was grown in early exponential growth (Table 3.1). But the amino acid was later slowly depleting from media in the later stages of growth (Table 3.1). Glycine assimilation might be blocked during early stage of growth when there were other more preferred amino acids in media for growth. Once these amino acids have been depleted, glycine was then slowly assimilated. The detected glycine could be a result of digestion of released material by extracellular aminopeptidase(s).

When extracellular proteases degrade the released proteins into peptides or amino acids by hydrolysing peptide bonds in the proteins, some residues might appear as simplest form of amino acid, glycine, but this remains to be confirmed.

The overall abundance of intracellular amino acids pool has often been quantified and suggested to remain stable under different conditions (Majumdar and Bose, 1958; Bernlohr, 1967; U *et al.*, 1996; Akashi and Gojobori, 2002; Tännler *et al.*, 2008; Kleijn *et al.*, 2010; Meyer *et al.*, 2014). One of the ways to maintain the equilibrium state of the pool was expected to be assimilating extracellular free amino acids for cellular needs and modulating biosynthesis. So the assimilation of amino acids might serve as a reflection of what amino acids to be higher demand in the cells (Meyer *et al.*, 2014). As of the amino acid assimilation profile of the wild type strain found in Section 3.2.3, it showed a few similarities with the intracellular amino acids pool. For instance, the most abundant intracellular amino acid glutamate was also one of the most efficiently assimilated amino acids in the analysis. Other relatively abundant amino acids in the cells, including alanine, were also shown efficiently assimilated (Section 3.2.3). However, assimilation could not act as the reflection of the intracellular abundance of amino acids. For instance, glycine, another amino acid of higher abundance in the cells, was recorded as increased in abundance in the medium (Section 3.2.3). Serine, one of the most efficiently assimilated amino acid, was not reported abundant in the cells (Section 1.2). Hence, the assimilation characteristics might have suggested amino acids like glycine, possibly with an economic synthetic pathway in which the energy cost of synthesising this amino acid was lower than that of assimilation from the outside environment. GlyA was suggested to be responsible for synthesising glycine from serine but biochemical evidence of the protein from *B. subtilis* is still lacking (Grundy and Henkin, 2014). And for amino acids like serine, they could be efficiently assimilated and then metabolised rapidly. Overall, the preference of amino acids for assimilation seemed to directly correlate with the likelihood of the amino acids being involved in the glycolysis or TCA cycle. This suggested that *B. subtilis* preferred to assimilate amino acids that could be metabolised into other important precursors involved in central metabolism for growth, implying the assimilation was mainly for growth maintenance or enhancement. This agreed to the suggested importance of CAA for boosting growth (Figure 3.1) (Anagnostopoulos and Spizizen, 1961). It also matched with the general understanding of the bacteria utilisation preference towards nutrients that could be

metabolised rapidly for an economically efficient growth (Fisher and Sonenshein, 1991; Yang *et al.*, 2015). The assimilation could also be a reflection of the repressed transcriptional expression of the enzymes involved in the TCA cycle by the presence of glucose, ammonium, glutamine and glutamate in the defined rich medium (Jourlin-Castelli *et al.*, 2000; Sonenshein, 2002; Blencke *et al.*, 2006). Hence these preferred amino acids could be metabolised rapidly into the TCA cycle to generate ATP for growth. Amino acids are also for protein synthesis in that they act as the building blocks of proteins. However, the correlation of the preference of amino acids usage with the highly expressed proteins during exponential growth remained to be elucidated. Nevertheless, with the assumption of equal usage of amino acids in protein synthesis, the proportion of amino acids assimilated for protein synthesis could be negligible as some of the amino acids were shown abundant throughout the time course (Section 3.2.3). For instance, tyrosine was remained abundant in the medium (Section 3.2.3). The excreted tyrosine was suggested to be directly used for protein synthesis when it was assimilated back into the cells (Champney and Jensen, 1970). This might have suggested that the metabolic flux in the cells in the experimental condition was not as efficient as expected.

As the assimilation of amino acids were suggested to be used in central metabolism for maintaining growth, the energy cost of the assimilation was hence indirectly suggested to be lower than that of biosynthesising them given the assumption of an economical efficient growth was maintained. In the calculation of the energy cost, the biosynthesis of an amino acid could be defined as the sum of the energy lost in the diversion of chemical intermediates from the reactions within central metabolism and that of for the conversion of them into amino acids (Akashi and Gojobori, 2002). And the energy for uptake of an amino acid usually refers to the ATP required for substrate binding only. The consideration of energy consumption also raised the question on the nature of transportation, whether the uptake system is actively selective or driven by passive uptake for maintaining cellular metabolism. If the transport of amino acids is passive, no ATP is required as the amino acid would be transported down the concentration gradient into the cell. But the intracellular concentration of glutamate was suggested to be high (mM) in most conditions (Tempest, Meers and Brown, 1970; Muntel *et al.*, 2014). Therefore, at least the glutamate assimilation would be less likely to be passive. Identified glutamate transporters in certain medium conditions were suggested to be active transporters

(Tolner *et al.*, 1995; Lorca, Winnen and Saier, 2003; Zaprasis *et al.*, 2015). On the contrary, an active transport of amino acids might be a more logical condition to explain the more efficient assimilation of the preferred amino acids to be in correlation to a higher cytosolic need of amino acids. According to the previous statistical analysis on bacterial transporters, amino acid transporters had been suggested to be mostly secondary carriers in which the amino acids could be assimilated by utilising electrochemical energy instead of ATP (Saier *et al.*, 2002). Therefore, the uptake systems responsible for the efficient assimilation of the preferred amino acids could be both active and specific, which agreed to the previous speculation of the nature of amino acids transport (Krämer, 1994). And such condition would allow the deduction above on the correlation of the preference of amino acids to be assimilated with the importance of amino acids to central metabolism to maintain exponential growth. Nevertheless, direct evidence of the deduced relationship is preferred hence the identification of related transporters and their own regulation is necessary.

3.3.3 Gluconeogenic growth in defined medium with malate, another preferred carbon source of B. subtilis, malate.

With reference to the growth in Gaa where glucose was supplied as the carbon source for a glycolytic growth, the growth rate of the wild type strain in malate (Maa) as a gluconeogenic growth was shown to be similar to that of grown in glucose (Gaa). The use of both carbon sources (GMaa) resulted in the highest growth rate of the strain (Figure 3.3). This agreed to the suggested function of malate as an alternative carbon source to *B. subtilis* (Meyer and Stülke, 2013). The result also matched with the findings of separate specific uptake system(s) for both carbon sources (Gonzy-Tréboul *et al.*, 1991; Tanaka, Kobayashi and Ogasawara, 2003). Glucose was found transported into cells through the phosphoenolpyruvate:glucose phosphotransferase system (glucose PTS) whilst malate was found transported through malate transporters, MaeN and YflS. Both types of transporters were found up-regulated independently in the presence of the carbon source they are transporting (Sutrinas *et al.*, 1990; Gonzy-Tréboul *et al.*, 1991; Tanaka, Kobayashi and Ogasawara, 2003). Hence the uptake of glucose and malate was unlikely to interfere with each other which agreed to the proposed co-utilisation of glucose and malate as carbon sources by *B. subtilis* (Zalieckas, Wray and Fisher, 1999; Kleijn *et al.*, 2010). But even though malate was suggested to be probably the only

gluconeogenic carbon source that was not repressed for utilization by glucose, glycolytic growth seemed to be more efficient and likely to generate more ATP as the dependence on amino acid for assimilation was lower when the strain was grown in glucose compared to that in malate (Section 3.2.3).

Although there was evidence of a different metabolic flux when a gluconeogenic carbon source was utilised compared to that of utilising glycolytic carbon source, the amino acid assimilation profile was largely similar with only few differences (Column Maa in Table 3.2). Amino acids with insignificant changes in assimilation, including aromatic amino acids, were likely metabolised for cellular activities independently of glycolysis and the TCA cycle. Also these cellular activities were likely less sensitive to growth rate. However, significant differences were observed and the first one was the increase of glutamate assimilation in gluconeogenic growth (Section 3.2.3). This could be due to a reversed glycolytic flux which was suggested to occur in the presence of malate (Kleijn *et al.*, 2010). As malate was assimilated, the gluconeogenic flux was directed upward towards pyruvate and little was converted to metabolites in the TCA cycle (Kleijn *et al.*, 2010). Here the first few enzymes of the glycolysis pathway could be repressed and hence the assimilated glutamate could be used to fuel the TCA cycle to generate ATP. Another possible cause could be the increase of intracellular glutamine under no glucose conditions which was suggested in Kleijn et al., 2010. As glutamine was more efficiently assimilated, glutamate assimilation could increase in order to support the increased cellular demand of glutamine. The second was the more significant assimilation of arginine (Table 3.2). This supported of the idea that glucose inhibited arginine uptake mentioned by Fujita in 2009, although the regulation mechanism has not been explored (Fujita, 2009). Interestingly, alanine was found more abundant than the starting concentration (Column Maa in Table 3.2). This observation was also suggested in other articles which had been interpreted as a sign of the overflow of metabolites (Kleijn *et al.*, 2010; Meyer and Stülke, 2013). Malate was suggested to be metabolised by malic enzymes, MalS as the major one, giving pyruvate which could then be converted as alanine, resulting in an overflown of metabolites into the medium (Doan *et al.*, 2003; Lerondel *et al.*, 2006; Kleijn *et al.*, 2010; Meyer and Stülke, 2013; Meyer, Weidmann and Lalk, 2013; Meyer *et al.*, 2014; Hörl, Fuhrer and Zamboni, 2021). If alanine was one of the overflow metabolites, the absence of the decrease in abundance of alanine in the medium suggested there was no reassimilation of the metabolite,

indicating unfinished metabolism of malate though a malate assay of the medium was necessary to confirm (Kleijn *et al.*, 2010; Meyer *et al.*, 2014). However, as the current analysis could not distinguish between L- and D-alanine, the increased abundance of alanine could be a mixture of the two isoforms of alanine. Whilst the overflown of metabolites could account for the increased abundance of alanine, the D-alanine released from cell wall turnover could be a contributing factor. It was not clear whether there was any increase of cell wall turnover if the strain was grown in the presence of malate. On the other hand, the conversion between glutamate and alanine was suggested reversible (See Section 1.2). But the increased assimilation of glutamate and increased secretion of alanine suggested the reaction likely to be unidirectional in which alanine was less likely to be converted into glutamate (this aspect is further explored in Chapter 4. Finally, the disappearance of glycine accumulation in the medium could be related to the decrease in intracellular abundance of serine which was shown when *B. subtilis* strain was grown in the presence of malate (Kleijn *et al.*, 2010). Serine could be metabolised into glycine in one step hence the abundance of serine could be directly proportional to the abundance of glycine. However, increased rate of serine assimilation did not increase the release of glycine into the medium (Figure 3.5). This could be due to the conversion of glycine into something else for growth as the growth was shown slightly weaker when the strain was grown in malate (Figure 3.5). However, the metabolism of serine and glycine in *B. subtilis* is not well understood (Grundy and Henkin, 2014).

And when glucose and malate were both present in the medium, a co-existence of both glycolytic and gluconeogenic flux might result in the overflow of metabolites (Kleijn *et al.*, 2010). It is a typical phenomenon that allows the exchange of nutrients between microbes in soil or plant roots (Moe, 2013). Our data showing a lot of amino acids remained abundant in the medium seems to agree with this idea (Column GMaa in Table 3.2). These abundant amino acids could be dynamically flowing into and out of the cells. Or they could simply be ignored as the cellular need for these amino acids is reduced. The overflow metabolites could also be the sign of an adequate ATP supply in the cells for growth. Hence the improved growth of the strain observed when growing in the presence of both glucose and malate (Figure 3.3). Interestingly, aspartate and glutamate were both more significantly depleted from medium in which the increased assimilation of glutamate was previously seen when

the strain was grown in the presence of malate (Column Maa in Table 3.2). These two amino acids were likely the metabolites closely related to those involved in the TCA cycle (Figure 3.5). Apart from the impact of gluconeogenic flux that only contributed to a low level of flux into TCA cycle, the co-existance of the two fluxes could result in a synergistic effect on the TCA cycle. Here the enzymes involved in the cycle were transcriptionally repressed (Ohne, 1974; Melin, Rutberg and Von Gabain', 1989; Resnekov *et al.*, 1992; Tobisch *et al.*, 1999; Schilling *et al.*, 2007; Kleijn *et al.*, 2010). In contrast, the two enzymes in the cycle that generate succinyl CoA and fumarate respectively were suggested to have high expression in rich medium despite the presence of glucose These two conditions might bring a more significant demand of the two amino acids as metabolites closely related to the TCA cycle in order to generate ATP to maintain growth. As the rate of protein synthesis was suggested directly proportional to the growth and the possible variation in gene expression depending on growth rate, it was difficult to determine whether the increased assimilation of the two amino acids was the result of increased demand of ATP generation for growth or any change in the metabolic pathways due to the additional glycolytic flux (Scott *et al.*, 2010; Borkowski *et al.*, 2016). The decrease of glutamine and serine could be due to a reduction of cellular demand of these two amino acids. And the decreased assimilation of glutamine could be a result of the increased assimilation of glutamate, which could be metabolised into glutamine in one step (Figure 3.5) (Belitsky, 2002). For alanine, it was likely to be one of the overflow metabolites in the presence of malate as it remained abundant in the medium (Table 3.2). However, the reduced alanine secretion compared to that of grown in only malate (Maa) could be a result of the increased demand of alanine in glycolytic growth with the increased growth rate. And for arginine, the inhibition of the uptake by glucose was observed again. This suggested that the inhibition was likely independent of malate and the presence of malate was less likely able to counteract the inhibition. Moreover, the increased abundance of glycine detected matched with that of detected when the strain was grown in only glucose (Gaa). Hence the excretion of glycine was likely caused by glucose although the mechanism was not clear. Similar to glycine, the increased abundance of tyrosine detected was likely caused by glucose as well. However, the excretion could be directly proportional to the growth rate (Table 3.2). This matched with the suggested growth rate dependent tyrosine excretion although the reason of the excretion remained unclear (Champney and Jensen, 1970). The proposed function of the excretion was an expandable pool

of tyrosine in the medium for assimilation when necessary for protein synthesis (Champney and Jensen, 1970).

3.3.4 Amino acid assimilation profile reserved in the manipulation of amino acids biosynthetic pathways

The largely unchanged amino acid assimilation profile of the wild type strain grown in different conditions has suggested the flexibility of the cellular mechanism to adapt to the surrounding nutrients availability. This led to the question whether specific amino acid assimilation might change when the strain was genetically manipulated to be auxotrophic to the amino acid. However, the manipulation of biosynthetic pathway of an amino acid, regardless of the preference of *B. subtilis* towards that amino acid, was not seen to have significantly changed the assimilation of the amino acid (Section 3.2.4). In other words, it suggested alternative minor biosynthetic pathways for those amino acids chosen in which the pathway was efficient enough to supply cellular usage of the corresponding amino acid. It might also have further suggested the cellular need of the amino acids was low or the amino acids were not essential to maintain growth. Alternatively, the cellular usage of the amino acids tested have been mainly relying on the assimilation from the environment regardless of the presence of the biosynthetic pathways for the amino acids. In this scenario, small difference in the assimilation of the corresponding amino acids might not be detectable using the current protocol. But this might not be applicable to explain the observation for those efficiently assimilated amino acids in our set conditions by the wild type strain, *e.g.,* serine. On the other hand, a serine starvation could result in ribosome pausing on selective serine codons which might have reduced the use of serine in the cells (Subramaniam *et al.*, 2013). This might also be a reason of the insignificant difference in the assimilation of serine between the serine auxotroph and the wild type strain.

3.3.5 Speculation of the preference of amino acid assimilation

Overall, the amino acid assimilation profile was largely agreed in the chemotaxis or metabolomics studies on *B. subtilis* which conducted with a variation of media conditions in different studies (Ordal and Gibson, 1977; Ordal, Villani and Gibson, 1977; Kleijn *et al.*, 2010; Meyer *et al.*, 2014; Yang *et al.*, 2015). Every analysed amino acid was shown assimilated during exponential growth, confirming the existence of transporters of them although the identity and specificity of amino acid transporters remained to be explored. Based on the amino acid assimilation profiles

generated in the previous sections, the profiles suggested that *B. subtilis* has a preference towards some amino acids over the others in a lot of conditions. Interestingly, even when the strain was grown in malate which should result in the gluconeogenic growth, the preference of amino acids assimilation was largely the same despite the opposing metabolic flux with slightly slower growth (Section 3.2.3). Also there are also other changes of the components in the media left unexplored whether they might induce changes of the amino acid assimilation preferences of *B. subtilis*. However, few of the amino acids were left out of the assimilation profiling analysis due to technical limitations. Hence the amino acid analysis protocol needs to be refined.

Although the preferred amino acids were suggested to feed the central metabolism for growth, the regulation of their metabolism seemed to be independent of the change of metabolic flux in the central metabolism pathways. The dependence on amino acids, as determined by efficiency of assimilation, seemed more significant in gluconeogenic growth compared to that of in glycolytic growth (Section 3.2.3). And in the presence of ‘’co-exist flux’’ provided by co-utilisation of glucose and malate, total amino acid assimilation reduced (Section 3.2.3). Hence, the efficiency in generating ATP for cellular activities seemed to be inversely proportional to the assimilation of amino acids from medium. Regardless of the difference in growth efficiency, the anabolic activities for growth were largely similar despite the change of metabolic flux in the central metabolism. Also, the insignificant difference of auxotroph in the assimilation of the amino acid it needed for growth compared to the wild type grown in same medium (Section 3.2.3). This indirectly suggested that the changes in metabolism of metabolites were likely to be dependent on the metabolism of the concerned amino acids rather than the metabolic flux. In fact, the assimilation of amino acids profile observed was similar to that of obtained in an amino acid consumption study on *B. subtilis* grown in the absence of carbon source. The more efficiently assimilated amino acids in the tested coniditions were still asparagine, glutamine, glutamate, serine, aspartate and alanine (Yang *et al.*, 2015).

Alternatively, the uptake of different amino acids has been shown to be under separate regulation or interplay of regulators. For instance, the CodY regulated transporters and the regulation acting on transporters discussed in Section 1.3 . Also the assimilation of arginine was suggested to be under specific regulation (Calogero *et al.*, 1994; Gardan, Rapoport and Débarbouillé, 1995). Apart from these, histidine

uptake was previously suggested to be repressed in the presence of a mixture of 16 amino acids although histidine transporter has yet been identified (Atkinson *et al.*, 1990). These together raised the question of the expression of the amino acid transporters in the defined media. Also, if the transporter(s) were transporting more than one amino acid at a different specificity, the amino acid mixture might hinder the uptake of the ones having less affinity with the transporters. These hypotheses are all difficult to easily test for now and individual analysis of the nature of each identified transporter is necessary. And, if possible, a more focused study of the pathways involved in amino acid metabolism is encouraged for developing an amino acid transporter screening.

In the search of transporters, we could focus on the amino acids that were assimilated efficiently as an initial priority. This project allowed us to have a more complete picture of amino acids uptake and a more stringent control of amino acid concentrations in the media or genetic manipulation might be necessary. Our lack of understanding of the cellular metabolism also presented a complication of preparing the media for transporter screening. Apart from the extracellular amino acid content analysis, a better understanding of the intracellular amino acids pool in the defined media in this project is another possible direction although the intracellular pool of metabolome was suggested to be maintained constant in different growth conditions (Tempest, Meers and Brown, 1970).

Chapter 4 Alanine metabolism

4.1 Introduction

In the last chapter, one situation was described in which several amino acids were assimilated more efficiently than the rest in *B. subtilis* during its exponential growth. As these amino acids or their metabolites were thought to be involved in the glycolysis or TCA cycle for maintaining growth, the metabolism of these amino acids is of higher interest in order to better understand how the metabolites were regulated to feed cellular needs. And the difference in the assimilation rate of alanine when the strain was grown in glucose comparing to that of in malate made alanine to be of particular interest.

Although glutamate was far more abundant amino acids in the cytosol compared to the rest of them, alanine was also known to be relatively more abundant in the intracellular content of *B. subtilis* (Tempest, Meers and Brown, 1970; Gunka and Commichau, 2012). Its high abundance could be accounted for its important role in cell wall synthesis where both isoforms, L- and D-alanine are key components of the bacterial cell wall. D-alanine was shown involved in the building of peptidoglycan cell wall and also in the D-alanylation of teichoic acids (Section 1.2). In the peptidoglycan precursor synthesis, each stem peptide in the cross-linkages of approximately 10-20 layers of peptidoglycan strands in the cell wall consists of both L- and D-alanine in which the cross-linkages were important in maintaining the integrity of the peptidoglycan wall (Dul and Young, 1973; Heijenoort, 1994; Foster and Popham, 2002; Vollmer, Blanot and de Pedro, 2008; Turner, Vollmer and Foster, 2014). Also, D-alanine is also a source for D-alanylation of teichoic acids (TA) (Dul and Young, 1973; Höltje, 1998; Hyyrylainen *et al.*, 2000). The loss of D-alanyl ester in teichoic acids might result in enhanced cell autolysis and increased susceptibility to methicillin (Wecke, Perego and Fischer, 1996; Wecke, Madela and Fischer, 1997). For L-alanine, apart from building cell wall, it is used in protein synthesis as the building blocks of proteins. It might be used in maintaining cellular metabolism for growth as it might produce pyruvate and ammonia (Wiame and Pierard, 1955; Pozo *et al.*, 1989; Siranosian, Ireton and Grossman, 1993). Despite the suggested involvement of alanine in maintaining glycolysis pathway and TCA cycle as well as the known functions of the two isomers in cell wall, the alanine metabolism is not well characterized. There was not any single mutant(s) showing an alanine auxotrophic

phenotype reported so far in *B. subtilis* and one of the problems was the poor annotation of amino acid racemase and aminotransferase genes (Radkov and Moe, 2014).

Regarding D-alanine metabolism, *B. subtilis* has two alanine racemases which are encoded by *alrA* and *alrB* and expressed during exponential growth and sporulation respectively (Ferrari, Henner and Yang, 1985; Pierce, Salifu and Tangney, 2008). For AlrA, D-alanine was found synthesized from its L-isoform by alanine racemase, encoded by *alrA* and the reaction was shown to be reversible (Walsh, 1989). An *alrA* deleted strain was shown unable to grow in Lysogeny broth (LB medium) due to its composition devoid of D-alanine (Ferrari, Henner and Yang, 1985). The absence of D-alanine could result in the inability of synthesizing cell wall hence cells lyse. Interestingly, the D-alanine auxotroph was shown able to grow in minimal medium which was also free of D-alanine (Ferrari, Henner and Yang, 1985; Sidiq, 2016). Here it was believed that the presence of an enzyme to be supplying D-alanine for growth. And when it was grown in minimal medium condition, addition of L-alanine could inhibit its growth (Ferrari, Henner and Yang, 1985). A colleague in our group had further characterised D-alanine metabolism in *B. subtilis* in which Dat was suggested to be responsible for the growth of *alrA* null mutant in minimal medium (Sidiq, 2016).

With reference to the complete genome sequence of *B. subtilis*, *dat* (*yheM*) was suggested to encode a putative D-ala aminotransferase in which 42% of its sequence was shown to be similar to a Dat protein in *B. sphaericus* (Kunst *et al.*, 1997; Fotheringham, Bledig and Taylor, 1998). Also, an aminotransferase which is encoded by *dal* in *L. monocytogenes* was suggested to produce D-alanine (Thompson *et al.*, 1998). In contrast to AlrA, Dat, an D-alanine aminotransferase, has been suggested to produce D-alanine but a deletion of *dat* would not result in an alanine auxotrophic phenotype. The aminotransferase activity was first detected in cellular extract of *B. subtilis* which was followed by another *in vitro* study where D-alanine-D-glutamate aminotransferase was purified and characterized in *B. subtilis* (Thorne, Gomez and Housewright, 1955; Martinez-carrion and Terry Jenkins, 1965). For a Dat involved reaction, D-alanine and D-glutamate could act as the amino donors, being catalyzed by the aminotransferase of its transamination into alpha ketoglutarate and pyruvate (as amino acceptors) respectively (Martinez-carrion and Terry Jenkins, 1965; Yonaha *et al.*, 1975). It was suggested to convert D-alanine and alpha-ketoglutarate into D-glutamate and pyruvate in a reversible manner (Radkov and Moe, 2014). Although

the reaction involving Dat was suggested reversible *in vitro*, the preference of Dat could be making glutamate rather than alanine in that L-alanine being converted to D- or L-glutamate rather than making D-glutamate into D-alanine (Thorne, Gomez and Housewright, 1955). At the same time, a less likelihood of Dat to be involved in D-glutamate synthesis was also suggested from another study (Kimura, Tran and Itoh, 2004). Therefore, the physiological role and preference of substrates of Dat remained to be investigated. Another alanine aminotransferase in *B. subtilis* is encoded by *alaT* was suggested to have the ability to convert pyruvate and glutamate into L-alanine and alpha-ketoglutarate although experimental evidence was not shown (Belitsky, 2002).

Alanine dehydrogenase, which is encoded by *ald* (or *spoVN*) was suggested to be responsible for alanine utilization. The enzyme was also shown to be required for utilizing alanine as the sole carbon or nitrogen source (Freese and Oosterwyk, 1963; Freese, Park and Cashel, 1964; Berberich *et al.*, 1968). The enzyme was suggested to be most active when L-alanine was supplied as substrate comparing to D-alanine or its analog in substrate specificity studies (Yoshida and Freese, 1965). But it was also suggested to be able to catabolize reversible reactions synthesizing alanine when the two metabolically important substrates (ammonia and pyruvate) are present (Wiame and Pierard, 1955; Freese, Park and Cashel, 1964; Yoshida and Freese, 1964; Grimshaw and Cleland, 1981). Given there were three major L-alanine transaminases identified in *E. coli* for synthesizing L-alanine in which the auxotrophic phenotype was only achieved when all three of the genes responsible were deleted (Kim, Schneider and Reitzer, 2010; Yoneyama *et al.*, 2011; Peña-Soler *et al.*, 2014). Hence the involvement of more than one enzyme contributing to alanine synthesis in *B. subtilis* is possible.

Despite the presence of D-alanine was shown in cell wall in which the material would be cleaved and released into medium, no accumulation of D-alanine was detected in stationary culture supernatant of *B. subtilis*. This suggested the existence of recycling the material released outside of the cells for *B. subtilis* (Lam *et al.*, 2009; Kolodkin-Gal *et al.*, 2010). Our previous colleague had identified a D-alanine transporter, encoded by *ytnA* (later renamed as *datA* then *alaP*) and it was characterized as a permease bioinformatically (Poolman and Konings, 1993). In the genetic context, homologs of AlaP were found usually the putative or characterized amino acid transporters in different bacterial species transporting different amino acids or their

derivatives (Sidiq, 2016). AlaP was found with good percentage (approximately 40 and 35 %) in amino acid sequence identical to its homologue, CycA in *E. coli* and *Mycobacterium bovis* BCG respectively (Wargel, Shadur and Neuhaus, 1970; Wargel, Hadur and Neuhaus, 1971; Robbins and Oxender, 1973; Sidiq, 2016). These homologues in other bacteria were suggested to be D-alanine, D-serine, D-cycloserine and glycine permease (Wargel, Shadur and Neuhaus, 1970; Wargel, Hadur and Neuhaus, 1971; Robbins and Oxender, 1973). A deletion of *alaP* resulted in the loss of D-alanine uptake (Sidiq, 2016). Hence in genetic context, AlaP was likely a D-alanine specific transporter responsible for D-alanine recycling in *B. subtilis* (Sidiq, 2016). Amino acid competition assay also revealed that the transporter could not transport other similarly structured amino acids or their derivatives (Sidiq, 2016). Preference of AlaP towards which isomeric form of alanine was briefly explored but conclusions could not be made. On one hand, only L-alanine was suggested to compete with D-alanine for uptake compared to other structurally similar amino acids, suggesting a shared transporter system of the alanine isomers (Sidiq, 2016). On the other hand, L-alanine was thought to be assimilated through a separate transport system in that an *alaP* deletion carrying alanine auxotroph was found able to grow in the presence of L-alanine (Sidiq, 2016).

In this chapter, some of the long-standing questions are to be addressed for alanine metabolism as it was explored focusing on its L-isomer, both biosynthetic and utilization pathways through growth tests and *in vivo* analysis. With the understanding of alanine metabolism, AlaP was further characterized in terms of its specificity of alanine transport.

4.2 Results

4.2.1 Phenotypic characterisation of an alaT mutant

As mentioned Section 4.1 , alanine metabolism in *B. subtilis* has not been well understood and no single mutant(s) showing an alanine auxotrophic phenotype have been reported so far (detailed in Section 4.1). The possible alanine metabolism-related enzymes included alanine transaminase (AlaT), D-alanine aminotransferase (Dat) and alanine dehydrogenase (Ald) (Section 4.1). To characterise the biosynthesis of alanine, the first step was to introduce a deletion of the genes *alaT*, *dat* and *ald* independently into the wild type strain (*168*+), resulting in ***ΔalaT::erm*** (MC1), ***Δdat::erm*** (MC3) and ***Δald::erm*** (MC5) respectively. These strains inoculated

in parallel with the wild type strain (*168+*), onto plates containing GMP containing glucose and ionic salts added in SMM (GMP), with or without 1 mM L- or D-alanine incubated at 37°C for 48 hours (Figure 4.1, Section 2.1). Of the four strains, only *ΔalaT::erm* (MC1) was found growing poorly where no alanine was present (top left quadrant in Figure 4.1B) but was able to grow on the plates with the presence of either isoform of alanine (top left quadrant in Figure 4.1C and D). The other strains were able to grow normally on all three media. This suggested that AlaT was a major enzyme responsible for alanine synthesis for *B. subtilis*. But the presence of small and thin colonies implied that another less efficient biosynthetic pathways could be present that permitted limited alanine synthesis.

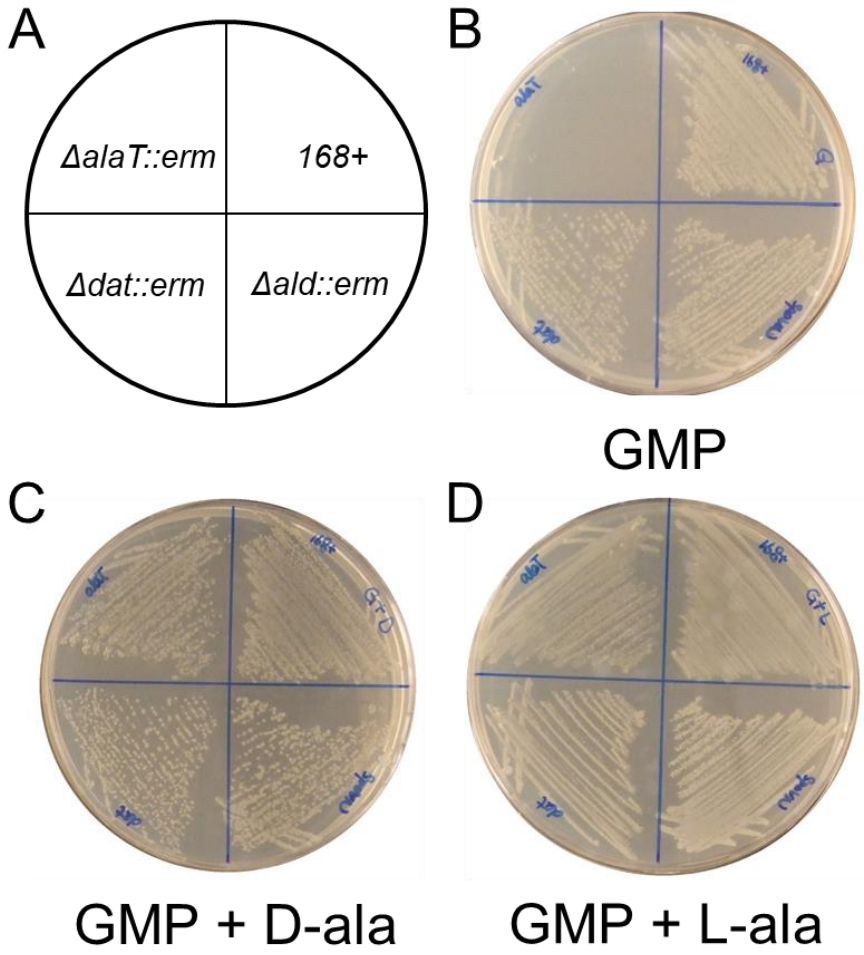


Figure 4.1: Growth characteristics of *ΔalaT::erm* (MC2) on plate GMP with or without L- or D-alanine. *ΔalaT::erm* (MC1), *Δdat::erm* (MC3) and *Δald::erm* (MC5) grown in parallel with the wild type strain (168+) on plates GM with or without 1mM L- or D-alanine at 37°C for 48 hours. Medium composition is indicated by a letter code: G means glucose, M means SMM buffer and P indicates the addition of metal salts. (A) Schematic diagram of strains position on each plate correspondingly. (B) Plate GMP represents medium GMP. (C) Plate GMP with 1 mM D-alanine. (D) Plate GMP with 1 mM L-alanine.

4.2.2 Construction of an alanine auxotroph

As reactions involving Dat and Ald were suggested to be reversible reactions in *in vitro* studies, these two enzymes were prioritized as candidates for further characterization of alanine metabolism. To determine if either of them could be involved in alanine synthesis, double mutants were created by deleting erythromycin marker using a Cre-lox recombinase to make the parent strain, MC1 in this case, to become a markerless deletion mutant MC2 (Section 2.2). This would allow progressively more deletion of gene(s) to be introduced into the strain with the same antibiotic marker. The deletion of *dat* and *ald* were transformed into *ΔalaT* (MC2) resulting in strains *ΔalaT Δdat::erm* (MC4) and *ΔalaT Δald::erm* (MC6). Also *Δdat::erm* (MC3) was made markerless (MC9) and gDNA of *Δald::erm* (BKE31930) was transformed into it to create a double mutant *Δdat Δald::erm* (MC10). The three double mutants were grown on the GMP plates with or without L-alanine in parallel with MC1 incubated at 37°C for 48 hours. Whilst *ΔalaT::erm* (MC1) had shown poor growth as expected on plate GMP in the absence of alanine (upper right quadrant in Figure 4.2B). Interestingly, *ΔalaT Δdat::erm* (MC4) failed to show any sign of growth compared to *ΔalaT::erm* (MC1) on plate GMP only, suggesting that the loss of Dat resulted in a fully auxotrophic phenotype. Thus Dat was implicated in L-alanine synthesis likely as a minor role (bottom right quadrant in Figure 4.2B). *ΔalaT Δald::erm* (MC6) showed similar growth as *ΔalaT::erm* (MC1) (bottom left quadrant in Figure 4.2B), implied that Ald might not have a role in the synthesis of L-alanine. *Δdat Δald::erm* (MC10) was seen to grow healthily in both medium conditions (top left quadrant in Figure 4.2B and C) showing that the absence of *dat* and *ald* had no direct effect on the ability of the strain to grow in the absence or presence of L-alanine.

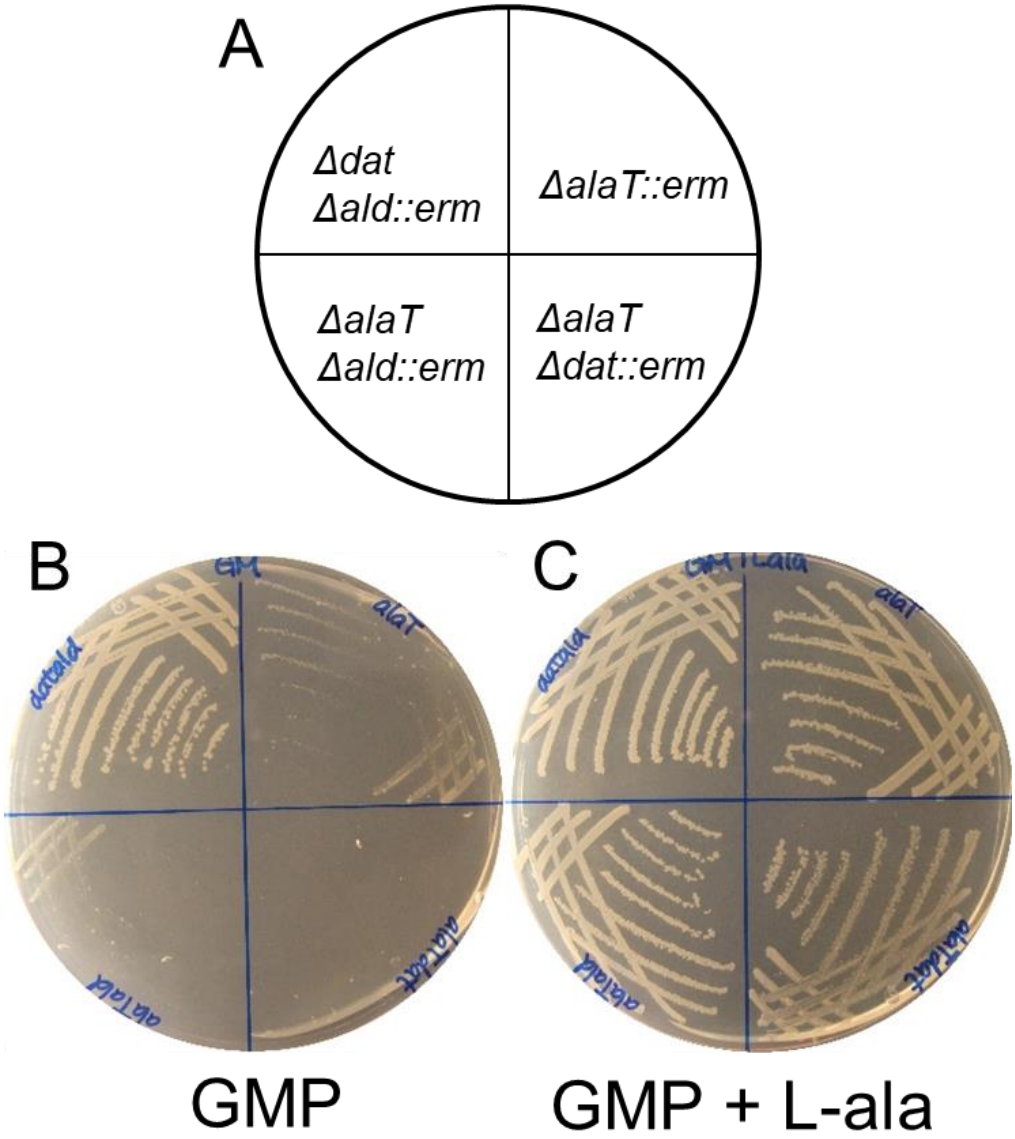


Figure 4.2: Growth characteristics of *ΔalaT Δdat::erm* (MC4) on plate GMP with or without L-alanine. *ΔalaT Δdat::erm* (MC4), *ΔalaT Δald::erm* (MC6) and *Δdat Δald::erm* (MC10) were grown in parallel with *ΔalaT::erm* (MC1) on plates GMP with or without 1mM L-alanine which were incubated at 37°C for 48 hours. Plates are annotated as in Figure 4.1. (A) Schematic diagram of strains position on each plate correspondingly. (B) Plate GMP. (C) Plate GMP with 1 mM L-alanine in medium GMP.

4.2.3 Complementation of alaT and dat

To confirm the roles of AlaT and Dat in alanine biosynthesis suggested in the above growth tests (Section 4.2.1 and 4.2.2), complementation of *alaT* and *dat* in alanine auxotroph strain was used. For this the coding sequence of each gene along with the ribosome binding site (rbs) and the stop codon was cloned into a low copy, self-replicating plasmid pAG-08 downstream of a constitutive promotor creating pAG-*alaT* and pAG-*dat* respectively. This allowed a constitutive expression of *alaT* or *dat*. The two plasmids were then transformed into *ΔalaT::kan* (MC1.1) to create *ΔalaT::kan*

pAG-*alaT(erm)* (RD220) and *ΔalaT::kan* pAG-*dat(erm)* (RD226) and grown in parallel with *ΔalaT* (MC2) and *ΔalaT Δdat::kan* (MC4.1) on plate GMP with or without 1 mM L-/D-alanine (Figure 4.3). Complementation of the *alaT* null mutation (*ΔalaT::kan* pAG-*alaT(erm)* (RD220)) was shown to restore growth of the auxotroph grown in medium GMP in the absence of alanine (bottom left quadrant in Figure 4.3). Interestingly, it was observed that the growth of *ΔalaT::kan* pAG-*alaT(erm)* (RD220), compared to other control strains, was reduced, when it was grown in the presence of either L- or D-alanine (bottom left quadrant in Figure 4.3C and D respectively). This confirmed that it was the expression of *alaT* that was needed for the strain to grow in the absence of alanine and further supported the idea that AlaT is probably the main enzyme acting in alanine biosynthesis.

To test if Dat was involved in the biosynthesis of alanine, the overexpression of *dat* (*ΔalaT::kan pAG-dat(erm)* (RD226)) was also shown to restore growth of the alanine auxotroph (bottom right quadrant in Figure 4.3B). The auxotroph *ΔalaT Δdat::kan* (MC4.1) was not able to grow without alanine supplemented in the medium (top right quadrant in Figure 4.3B). Unlike *ΔalaT::kan* pAG-*alaT(erm)* (RD220), there was no observable inhibitory effect of the overexpression of Dat when *ΔalaT::kan pAG-dat(erm)* (RD226) was grown in the presence of alanine (bottom right quadrant of Figure 4.3C and D). This demonstrated that Dat was able to synthesize alanine but its efficiency was either lower than AlaT or it was expressed poorly. It was also not clear whether Dat was generating L-alanine directly or generating D-alanine that was converted to L-alanine by the racemase AlrA. This latter possibility is suggested by the fact that *dat* had previously been shown to act in the synthesis of D-alanine in both *B. subtilis* (Thompson *et al.*, 1998; Sidiq, 2016).

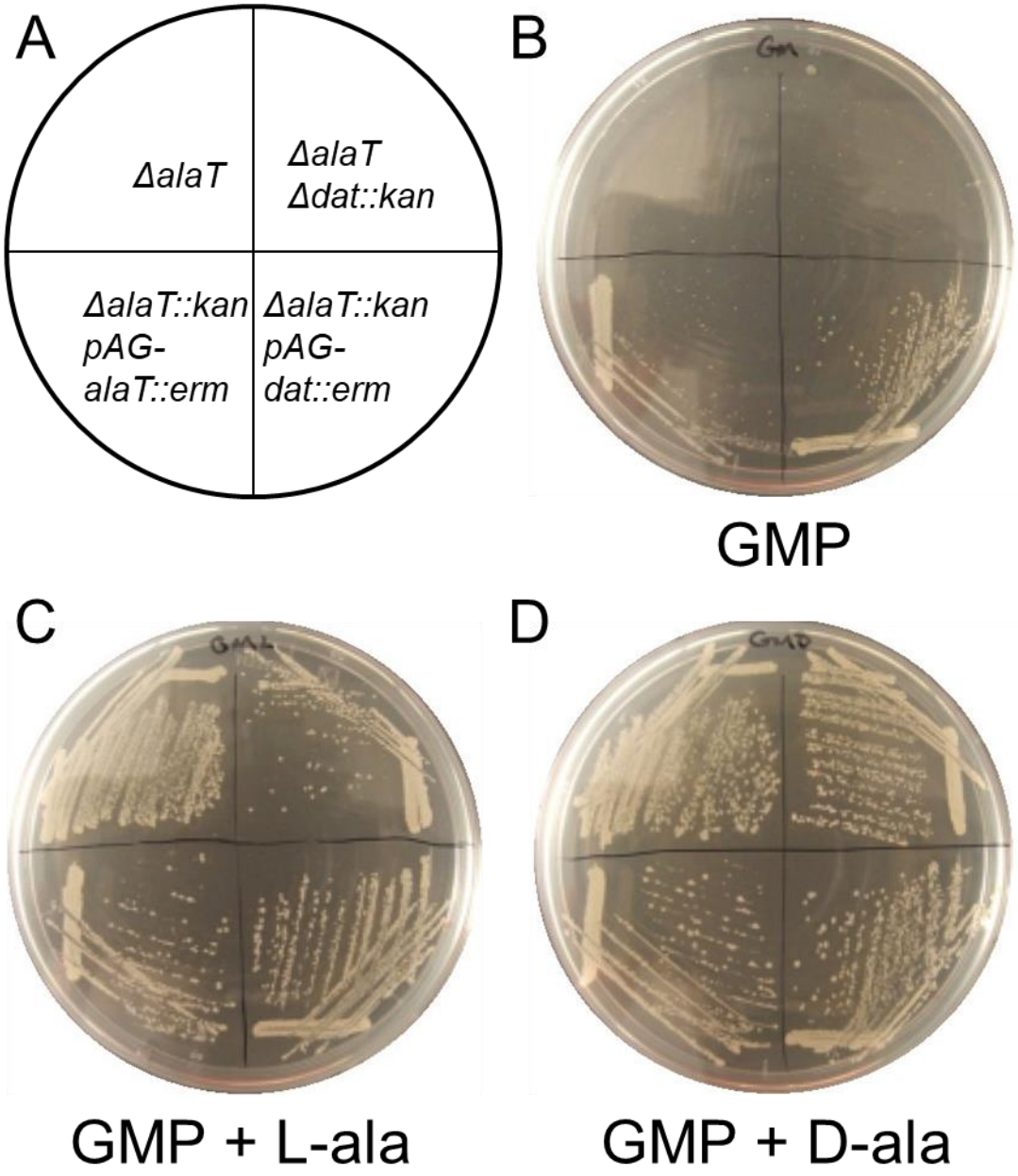


Figure 4.3: Complementation of *ΔalaT::kan pAG-alaT(erm)* (RD220) and *ΔalaT::kan pAG-dat(erm)* (RD226) on plate GMP. *ΔalaT* (MC2), *ΔalaT Δdat::kan* (MC4.1), *ΔalaT::kan pAG-alaT(erm)* (RD220) and *ΔalaT::kan pAG-dat(erm)* (RD226) were grown on plates GMP with or without 1mM L- or D-alanine which were incubated at 37°C for 24 hours. Plates are indicated as in Figure 4.1. (A) Schematic diagram of strains position on each plate correspondingly. (B) Plate GMP. (C) Plate GMP with 1 mM L-alanine. (D) Plate GMP with 1 mM D-alanine.

4.2.4 Alanine utilisation

As indicated in the introduction, in contrast to other amino acids, alanine biosynthesis and degradation is not well defined in *B. subtilis*.

The products of only two genes were suggested to have roles in alanine metabolism, Dat and Ald, both presumed to act in the utilization of alanine as either a carbon or

nitrogen source (Thorne, Gomez and Housewright, 1955; Freese and Oosterwyk, 1963; Freese, Park and Cashel, 1964; Berberich *et al.*, 1968). However, both are suggested to catalyse reversible reactions as well (Section 4.1) (Wiame and Pierard, 1955; Yoshida and Freese, 1965; Berberich *et al.*, 1968; Pozo *et al.*, 1989; Mortuza *et al.*, 2018). To test if either of them, and AlaT were required for alanine utilisation, strains of *ΔalaT* (MC2), *Δdat::erm* (MC3) and *Δald::erm* (MC5) were created to permit their growth characteristics to be determined in different conditions (Section 2.4 and 2.10).

In order to avoid the insufficient nutrients for growth giving false negative result of bacterial strain grown on the plate, 25 mM alanine was a crude estimation of the sum of 20 amino acids mixture used in defined medium. To test if the mutants could utilise alanine as the sole carbon source, they were grown on plates, containing only 25 mM L-or D-alanine as a carbon source in SMM buffer with ammonium salts at 37°C for 72 hours (bottom panel in Figure 4.4). Of the strains tested, only *Δald::erm* (MC5) failed to grow on L- or D-alanine plates (top left sector of plate M L-ala and M D-ala in Figure 4.4). The other two strains were found to grow (top right and bottom sectors of plate M L ala and M D ala in Figure 4.4). This indicated that alanine, regardless of its isoforms, could be utilized as sole carbon source provided *ald* was functional. This confirmed previous work that suggested *ald* had a major role in alanine utilization. However, it did not suggest the preference (if any) of alanine isoforms for Ald and the preference could not be deduced due to the presence of AlrA which could interconvert L- and D-alanine in a reversible manner inside the cells.

Similarly, to test if the mutants could use alanine as sole nitrogen source, the strains were grown on plates GM-NH_3, which contain glucose with 25 mM L- or D-alanine mixed in SMM buffer made without ammonium salts at 37°C for 48 hours. Again only *Δald::erm* (MC5) was unable to grow after prolonged incubation (≥ 48 hours) and the observation was similar in both plates (top left sector of plate GM-NH_3 + L-ala and GM-NH_3 + D-ala in Figure 4.4). The other two strains were growing well and the growth was similar to each other (top right and bottom sectors of plate GM-NH_3 + L-ala and GM-NH_3 + D-ala in Figure 4.4).

Together, as both Dat and AlaT were not shown involved in utilizing alanine, this suggested that the in vivo reactions of both are less likely reversible.

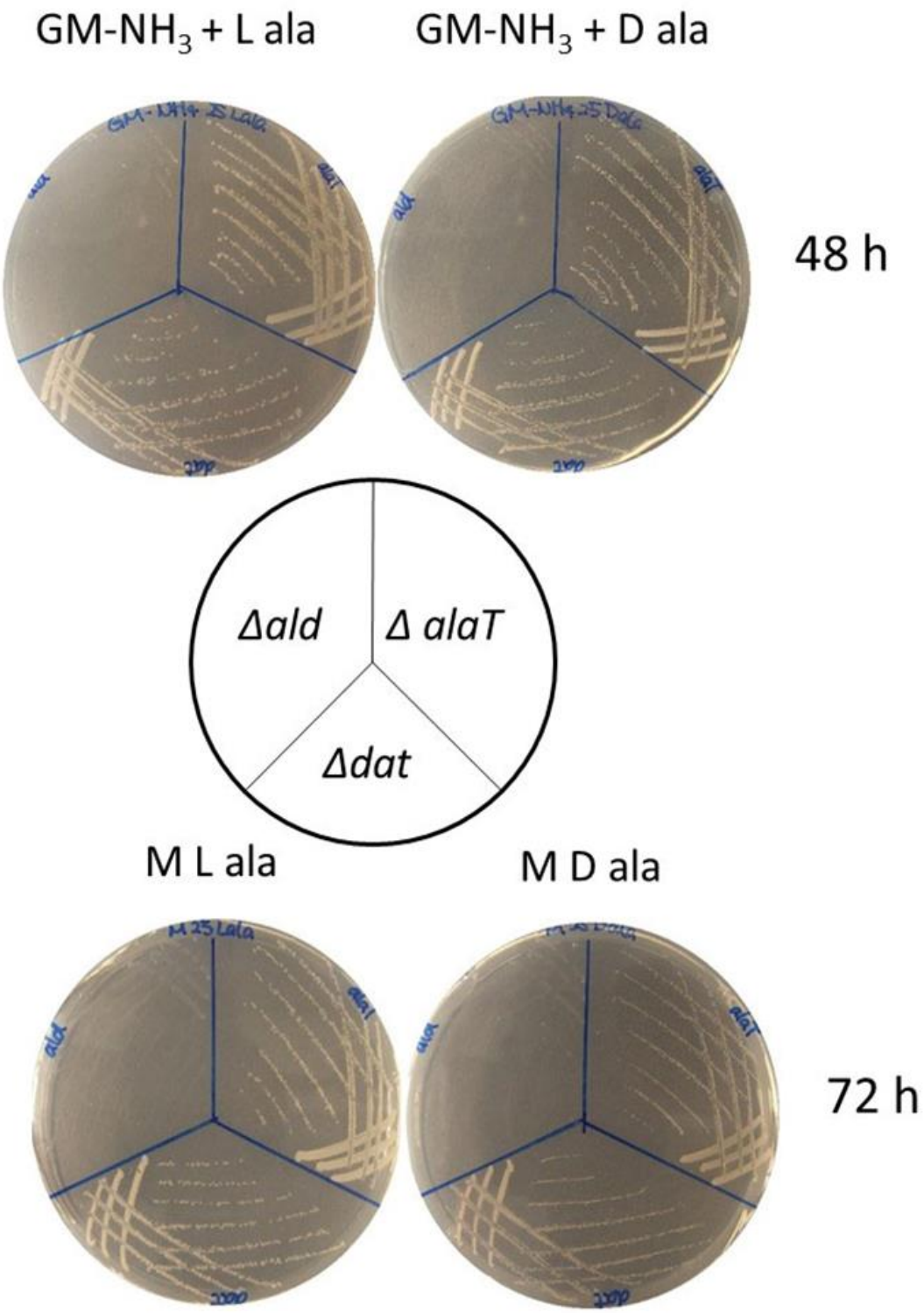


Figure 4.4: Ald was required to utilise alanine as sole carbon source and sole nitrogen source. *ΔalaT* (MC2), *Δdat::erm* (MC3) and *Δald::erm* (MC5) were grown on plates with L- or D-alanine served as either carbon or nitrogen source which were incubated at 37°C with different incubation hours specified on the right. A schematic diagram of the positions of strains is shown in the middle. Plates are annotated as in Figure 4.1 with M-NH_3 means SMM buffer without ammonium salts. Plates of medium GM-NH_3 contained 25 mM L- or D-alanine as indicated and served as sole nitrogen source for bacterial growth (top row). Plates of M contained 25 mM L- or D-alanine serves as sole carbon source (bottom row).

4.2.5 In vivo characterisation of Dat

A colleague in my group had further characterised the D-alanine auxotrophic phenotype of *ΔalrA::zeo* (RD180) and suggested the participation of Dat in the biosynthesis of D-alanine (Sidiq, 2016). On the other hand, earlier analysis (Section 4.1) suggested that Dat could function in L-alanine synthesis in *B. subtilis*. To determine if which was the case (or both), genetic experiments were devised to test if Dat functioned in the formation of both isomeric forms of alanine. In a preliminary test the previous observations were confirmed using the defined medium (GMaaP) (See Section 4.2.2 and 4.2.3 and methods in Section 2.1 – 2.3) as the earlier work was done using LB medium. This would allow the abundance of both isomeric forms of alanine to be manipulated and detected, if necessary.

ΔalrA::zeo (RD180) was grown in medium GMaaP-ala where 1.25 mM of all amino acids were supplemented except alanine (GMaaP-ala) at different concentration of L-/D-alanine or both in parallel with the wild type strain 168CA. The growth of the two strains in different medium condition was measured in a microplate reader (Section 2.3). When 0.5 or 1 mM D-alanine was added to the medium GMaaP-ala, both the wild type and *ΔalrA::zeo* (RD180) shared similar growth pattern in terms of growth rate and final optical density reached (red and green curves in Figure 4.5A and B). When there was no alanine added, *ΔalrA::zeo* (RD180) was shown unable to grow one hour after starting the time course measurement (Figure 4.5H and purple curve in B). This indicated that the growth of *ΔalrA::zeo* (RD180) was shown dependent on the presence of D-alanine in the presence of glucose and other amino acids (GMaaP-ala). The medium GMaaP-ala with 1 mM D-alanine was modified where different concentrations, 5, 10, 20 and 40 mM L-alanine was further supplemented and the growth of the two strains in the same condition was monitored (Figure 4.5D and E). The growth of *ΔalrA::zeo* (RD180) was similar to that of the wild type strain when there was only 5 or 10 mM L-alanine added to the medium (green and purple curves in Figure 4.5E). But when 20 mM L-alanine was added with the presence of 1mM D-alanine in the presence of glucose and other amino acids (GMaaP-ala), the growth of *ΔalrA::zeo* (RD180) was impaired in that both of its growth rate and final OD was lower than that of growing in lower concentration of L-alanine (red curve in Figure 4.5E). The strain could not grow when 40 mM L-alanine was present in the medium (blue curve in Figure 4.5E). In contrast, the growth of the wild type strain seemed unaffected by the addition of L-alanine in the medium (Figure 4.5A, D and

G). This suggested a concentration dependent inhibitory effect of L-alanine on the growth of *ΔalrA::zeo* (RD180), an effect seemed to occur about one hour after the start of the time course, similar to that of when the strain was grown without alanine (purple curve in Figure 4.5B and red and blue curves in Figure 4.5E respectively).

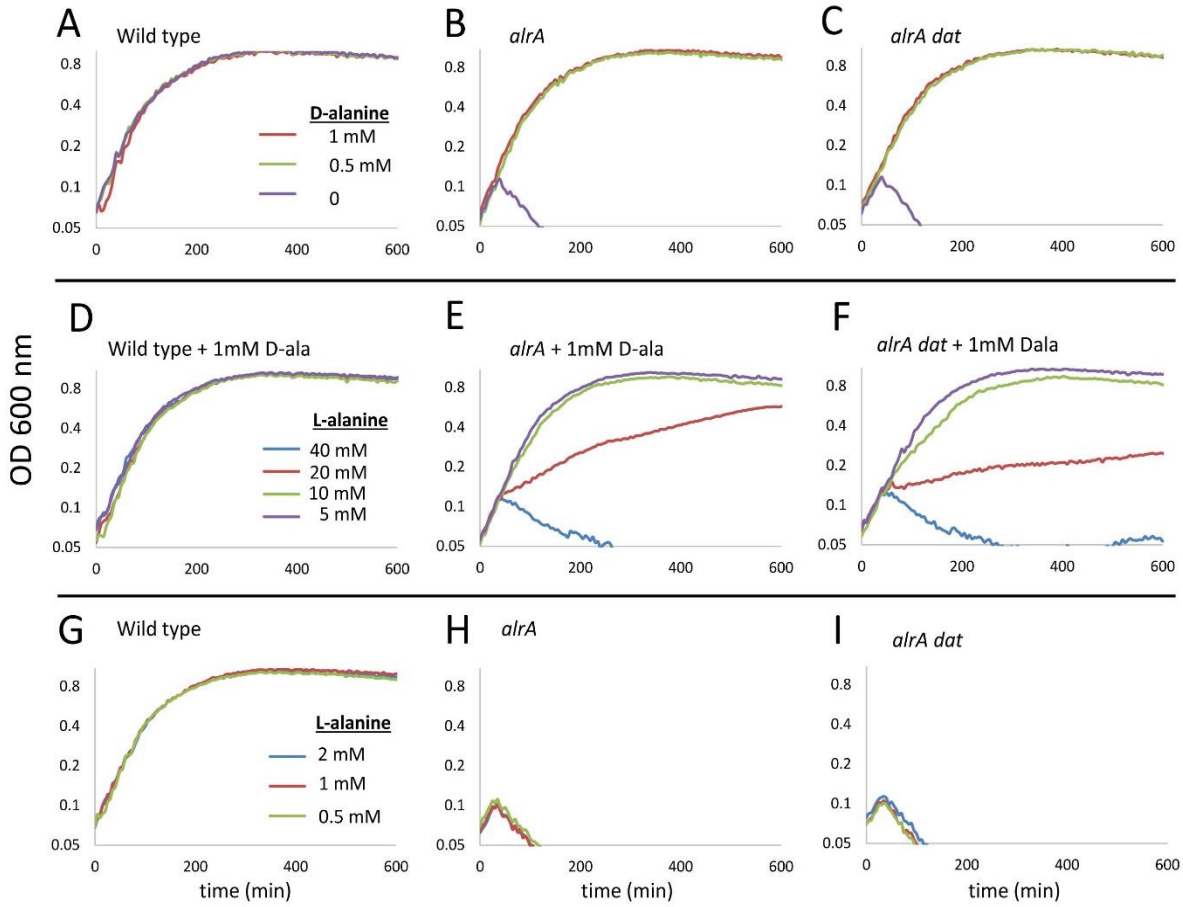


Figure 4.5: *in vivo* characterisation of D-alanine dependent auxotroph. The growth of *ΔalrA::zeo* strain (RD180), *ΔalrA::zeo Δdat::erm* (KS78) and the wild type strain which were grown at 37°C in medium GMaaP-ala added with a variation of L- or D-alanine at different concentrations were measured using plate reader. Medium GMaaP-ala represents the medium of glucose and an amino acid mixture with varied concentration of alanine. The strain genotype is shown on top of each graph and the addition of alanine was indicated in molarity (mM). Panel A-C showed the growth of the strains in medium GMaaP-ala added with 0, 0.5 or 1 mM D-alanine. For panel D-F, the medium was of a fixed concentration 1 mM D-alanine and different concentrations of L-alanine including 5, 10, 20 and 40 mM. For panel G-I, the medium was of 0.5, 1 or 2 mM L-alanine. The experiments were carried out twice independently. The graphs above are a representative of the two sets of experiments.

To confirm if Dat was involved in the biosynthesis of D-alanine, the double mutant, *ΔalrA::zeo Δdat::erm* (KS78) was grown in parallel with its parent strain, strain RD180 (*ΔalrA::zeo*) in different concentrations of L-alanine in the presence of 1 mM D-alanine (Figure 4.5E and F). The growth of *ΔalrA::zeo Δdat::erm* (KS78) was

similar to that of its parent strain *ΔalrA::zeo* (RD180) when there was 5 mM or less L-alanine added to the medium (purple curve in Figure 4.5E and F). However, when 10 mM L-alanine was added, the growth of *ΔalrA::zeo Δdat::erm* (KS78) was slightly deviated in that the growth rate was relatively slower from about 100 min and the final OD was little lower compared to the growth in 5 mM L-alanine with 1 mM D-alanine (green curve compared to purple in Figure 4.5F). It was also considered slightly deviated when it was compared to the growth of *ΔalrA::zeo* (RD180) grown in the same condition (green curve in Figure 4.5F compared to E). When the concentration of L-alanine was increased to 20 mM, the growth of *ΔalrA::zeo Δdat::erm* (KS78) was perturbed more significantly from around 1 hour after beginning of time course in terms of both growth rate and final OD than its parent strain *ΔalrA::zeo* (RD180) (red curve in Figure 4.5E and F). Here the growth of *ΔalrA::zeo Δdat::erm* (KS78) was also slower and reached significantly lower final optical density compared to that of its parent strain, *ΔalrA::zeo* (RD180). When 40 mM L-alanine with 1 mM D-alanine was added in the medium, the growth of *ΔalrA::zeo Δdat::erm* (KS78) was essentially blocked in a similar way to that observed for *ΔalrA::zeo* (RD180)(blue curve in Figure 4.5F). *ΔalrA::zeo Δdat::erm* (KS78) was shown growing normally when there was 0.5 or 1 mM D-alanine added in the medium with glucose and other amino acids (GMaaP-ala)(red and green curves in Figure 4.5C). Also it was shown unable to grow without D-alanine (Figure 4.5I and purple curve in C).

Together these observations suggested that the double mutant was also a D-alanine auxotroph which was more sensitive to the inhibiting effect of L-alanine than its parent strain but the starting point of inhibition was similar to each other. This implied that Dat could also synthesize in D-alanine which agreed with the previous suggestion (Sidiq, 2016). It was possible that D-glutamate was one of the substrates of Dat to generate D-alanine. And it was also possible that L-glutamate was converted into D-glutamate and in turn acted as a substrate for Dat to generate D-alanine. However, the reasons of the inhibitory effect of L-alanine on the double mutant KS78 remained unclear. L-alanine in the medium could be competing with D-alanine for uptake by the cells. Alternatively, it could be inhibiting the expression of Dat or the kinetic reaction of Dat producing alanine. The preference of Dat towards which isoform of alanine to produce remained unclear.

4.2.6 Isoform specificity of Dat in genetic context

To confirm the role of Dat in D-alanine biosynthesis suggested by Sidiq, 2016 and Section 4.2.5, a D-alanine auxotroph strain was constructed with the chromosomal copy of *dat* deleted and a complementing copy of the gene provided by the plasmid pAG-*dat* was grown in minimal medium (GMP) with or without L-/D-alanine (Figure 4.6). This aimed to eliminate the possibilities of the observed phenotype caused by polar effects of the knockout and provide a way to ensure the expression of *dat* was subject to transcriptional regulation. For this the pAG-*dat* vector was transformed into *ΔalrA::zeo* (RD180) and *ΔalrA::zeo Δdat::kan* (RD221) to create *ΔalrA::zeo* pAG-*dat(erm)* (RD222) and *ΔalrA::zeo Δdat::kan* pAG-*dat(erm)* (RD223) respectively. The strains were grown in parallel with their parent strains *ΔalrA::zeo* (RD180) and *ΔalrA::zeo Δdat::kan* (RD221) on minimal plates (GMP) with or without 1 mM L- or D-alanine (Figure 4.6). Complementation of *dat* in both strains *ΔalrA::zeo* pAG-*dat(erm)* (RD222) and *ΔalrA::zeo Δdat::kan* pAG-*dat(erm)* (RD223) was shown to restore growth of the auxotroph grown on minimal (GMP) plates in the absence of D-alanine (bottom half in Figure 4.6B). In contrast, the D-alanine auxotrophs *ΔalrA::zeo* (RD180) and *ΔalrA::zeo Δdat::kan* (RD221) were unable to grow without alanine in the time given (top half in Figure 4.6B). It was also evident that the plasmid (pAG-*dat*) improved the growth of the D-alanine auxotrophs *ΔalrA::zeo* (RD180) and *ΔalrA::zeo Δdat::kan* (RD221) in the presence of 1 mM D-alanine (bottom half compared to top half in Figure 4.6D). However, all the four strains were unable to grow in presence of 1 mM L-alanine (Figure 4.6C). Together these confirmed that Dat was responsible for the biosynthesis of D-alanine and that L-alanine probably alters the activity of Dat.

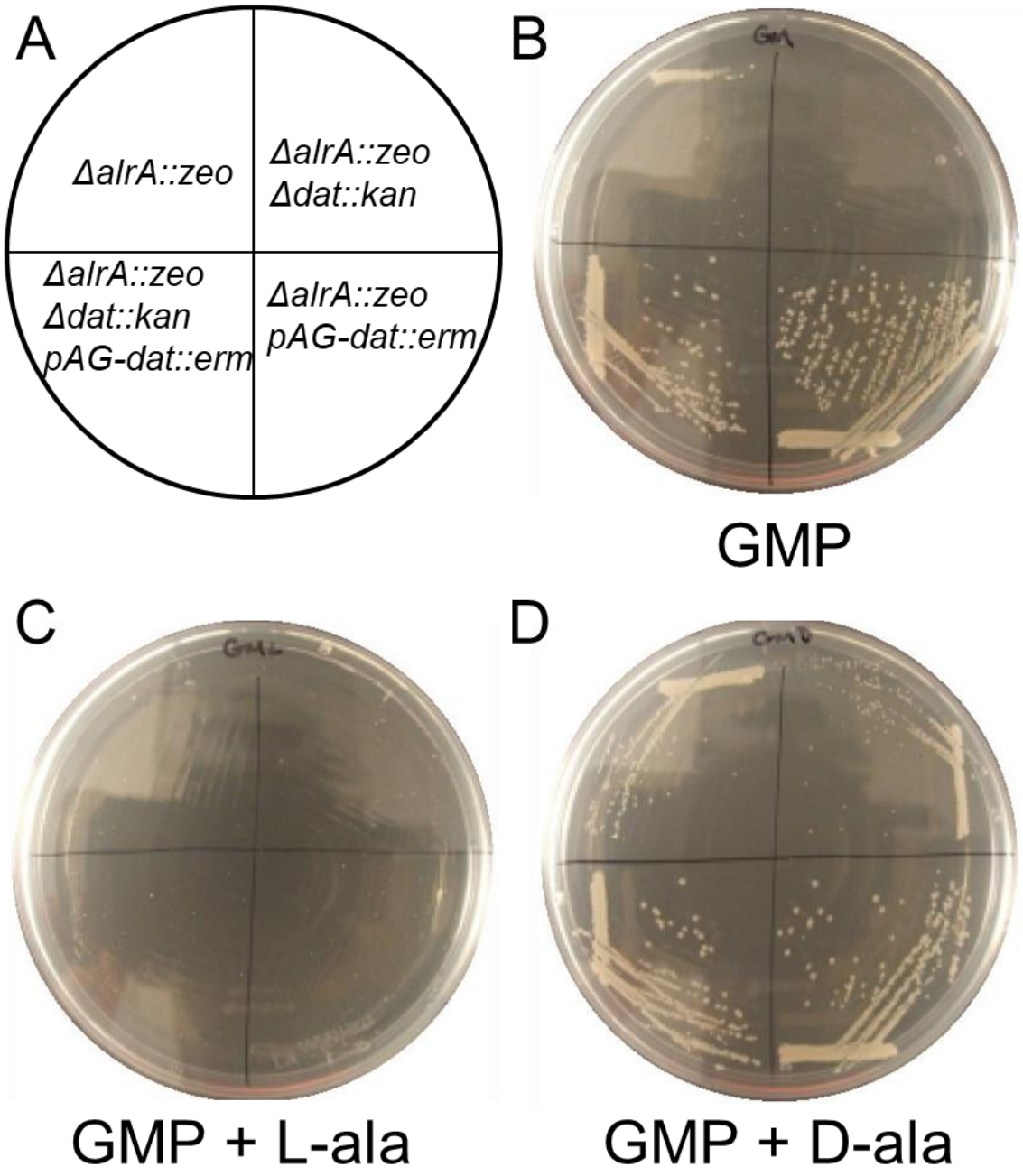


Figure 4.6: Complementation of *ΔalrA::zeo pAG-dat(erm)* (RD222) and *ΔalrA::zeo pAG-dat(erm)* (RD223) on minimal plate (GMP). *ΔalrA::zeo (RD180)*, *ΔalrA::zeo Δdat::kan* (RD221), *ΔalrA::zeo pAG-dat(erm)* (RD222) and *ΔalrA::zeo Δdat::kan pAG-dat(erm)* (RD223) were grown on plates GMP with or without 1mM L- or D-alanine which were incubated at 37°C for 24 hours. Plates of each medium condition is annotated as in Figure 4.1. (A) Schematic diagram of strains position on each plate correspondingly. (B) Plate GMP represents minimal medium (GMP). (C) Plate GMP with 1 mM L-alanine. (D) Plate GMP with 1 mM D-alanine.

4.2.7 AlaP transporter shared by both isoforms of alanine

Previously, our colleague has identified AlaP was responsible for D-alanine uptake (Sidiq, 2016). But the uptake of L-alanine is not known and L-alanine was shown possible to compete with D-alanine for uptake in last section.

To test if the AlaP transporter was required for L-alanine uptake in *B. subtilis*, a deletion of *alaP* was transformed into the alanine auxotroph strains, *ΔalaT::erm* (MC1) and *ΔalaT Δdat::erm* (MC4), creating *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11) respectively. The growth of these strains were compared with the wild type strain 168+ grown in two types of medium supplemented with 0.5 mM L- or D-alanine. One type of medium would be the defined rich medium that contains glucose and other amino acid (except alanine) (GMaaP-ala). The other type was not supplemented with amino acids mix and so was essentially a minimal medium containing glucose but not the other amino acid (GMP). To both types of plates, 0.5 mM L- or D-alanine was added and the strains together with the wild type strain 168+ were then inoculated and the plates were incubated at 37°C for 36 hours (Figure 4.7, Section 2.1 , 2.2 , 2.4 and 2.10).

In the presence of low concentration of alanine (0.5 mM) and the mixture of other amino acids, strains deleted for *alaP* (*ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm*) (MC11), exhibited poor growth (top and bottom left in Figure 4.7C) in the presence of D-alanine when compared to the parent strains, *ΔalaT::erm* (MC1) and *ΔalaT Δdat::erm* (MC4)(middle left and right in Figure 4.7C) as expected. This confirmed AlaP played a major role in transporting D-alanine which was consistent with previous studies (Sidiq, 2016). A few colonies appeared for strain *ΔalaT ΔalaP::erm* (MC8) (bottom left in Figure 4.7C). This was suggested to be a result of spontaneous upregulation of *dat* gene and a further indication that Dat as a minor role synthesising alanine consistent with the previous results (Section 4.2.2 and 4.2.6) as no ‘suppressor’ colonies were evident for strain *ΔalaT Δdat ΔalaP::erm* MC11 (top left in Figure 4.7C). Interestingly, similar results were obtained when L-alanine was supplemented instead of its D-isoform at the same concentration in the presence of the other amino acids (GMaaP-ala) (Figure 4.7D). The other strains grew healthily under the test conditions (Figure 4.7). Also, no observable growth difference obtained for strains growing in either isoform of alanine (Figure 4.7C and D). This seemed to suggest that AlaP was required for the uptake of both isoforms of alanine in *B. subtilis*.

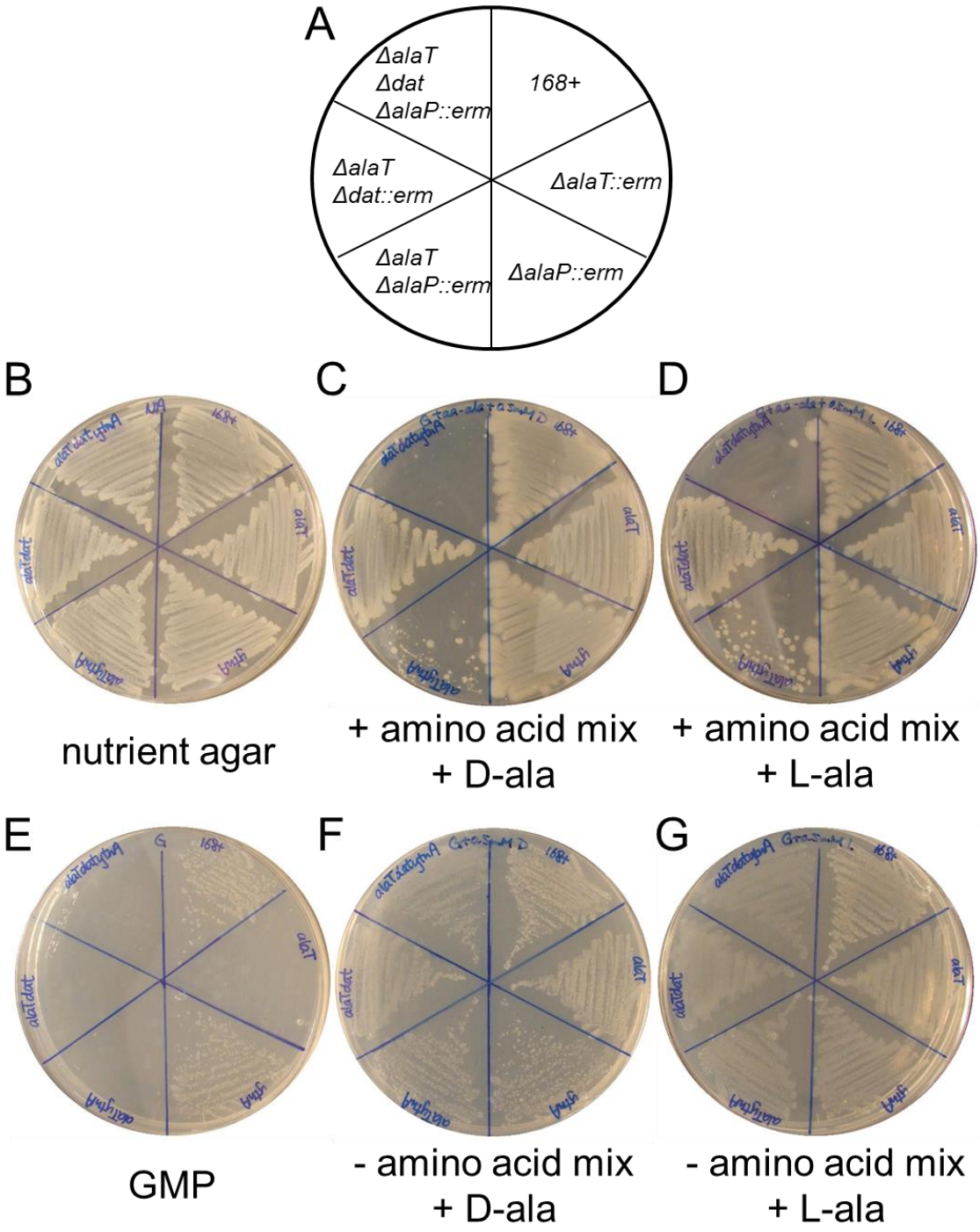


Figure 4.7: AlaP transporter was shown required for alanine uptake (either isoform) from only specific medium condition. Growth of alanine auxotrophs without *alaP*, *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11) were compared *ΔalaT::erm* (MC1), *ΔalaT Δdat::erm* (MC4), *ΔalaP::erm* (MC7) and wild type strain 168+ in different medium conditions grown at 37°C for 36 hours. The annotation above each plate specifies the presence of amino acid mix and/or L- or D-alanine. Nutrient agar plate acted as a positive control. (A) Schematic diagram of strains position on each plate correspondingly. (B) Plate of nutrient agar. (C) Plate with amino acid mix and D-alanine. (D) Plate with amino acid mix and L-alanine. (E) Plate GMP indicates medium GMP only, essentially a minimal medium. (F) Plate with 0.5 mM D-alanine but without amino acid mix. (G) Plate with 0.5 mM L-alanine but without amino acid mix.

Interestingly, for minimal medium (GMP), all strains, including strains with a deletion of *alaP*, were able to grow regardless of the isoforms of alanine supplemented which suggested more than one alanine transporter(s) in *B. subtilis* (Figure 4.7F and G). Since only alanine as an amino acid was present in this medium, the alanine transporter transporting alanine in this condition might be mediating something else in normal conditions.

4.2.8 Growth characteristics of strains defective in alanine uptake and/or biosynthesis in defined medium

To confirm the observations above in a more quantitative manner, the growth of the strains involved was monitored over 10 hours at 37°C using a microplate reader (Section 2.3). The strains were grown in a range of concentrations of L- or D-alanine (0, 0.4, 0.6, 0.8 and 1 mM) added into the defined rich medium containing glucose and other amino acids (GMaaP-ala).

The expected auxotrophic phenotype was shown for *ΔalaT::erm* (MC1) in that its growth rate and final optimal density reached was limited by the available concentration of alanine. This can be best seen by the difference in the growth curves obtained with 0.8 mM D-alanine compared to that of grown at 1 mM (orange curve compared to blue in Figure 4.8B). As for *ΔalaT ΔalaP::erm* (MC8), its growth was similar to that of its parent strain (*ΔalaT::erm* (MC1)) when there was 1 mM D-alanine present in the medium with the amino acid mix (GMaaP-ala) (blue line plot in Figure 4.8C). However, this strain seemed to be more sensitive to lower concentrations of D-alanine. When the concentration of D-alanine slightly reduced to 0.8 mM, the growth was significantly slower and reached lower final optical density compared to the strain with a functional AlaP, MC1 (orange line plot in Figure 4.8B and C). This trend became more significant at 0.6 mM or less D-alanine (grey and purple line plot in Figure 4.8B and C).

Deletion of *dat* in these genetic backgrounds made the concentration effects even more obvious, consistent with its probable role in synthesising a limited amount of alanine. The double mutant auxotroph *ΔalaT Δdat::erm* (MC4) was shown more difficult to grow compared to *ΔalaT::erm* (MC1) when 0.6 mM or lower concentrations of D-alanine was present in the medium (GMaaP-ala). *ΔalaT Δdat ΔalaP::erm* (MC11) was found to be even more sensitive to the reduced concentration of D-alanine present in the medium compared to its parent strain MC4 (*ΔalaT Δdat::erm*) when D-alanine was reduced to 0.8 mM or lower (Figure 4.8D and E). Growth of

strain *ΔalaT Δdat ΔalaP::erm* (MC11) only occurred in the presence of 1 mM D-alanine (Figure 4.8E). This suggested that efficient assimilation of D-alanine was dependent on AlaP, but the uptake was sufficient to support growth when D-alanine was present at 1 mM or higher. *ΔalaP::erm* (MC7) was used as a control and the growth of this strain was independent of alanine supplementation showing that the ability to grow was specifically being limited by alanine synthesis defects in the other strains.

When the growth experiments were repeated, but with D-alanine replaced by L-alanine added into the medium with the amino acid mix (GMaaP-ala) (Figure 4.9), a similar to the result was obtained. The deletion of *alaP* resulted in significant repression of growth when the strains *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11) were compared to their parent strains *ΔalaT::erm* (MC1) and *ΔalaT Δdat::erm* (MC4) grown in the 0.8 mM or lower concentrations of L-alanine in the presence of amino acids mix respectively (Figure 4.9B and C). The repression seemed to be more significant compared to that of with D-alanine (Figure 4.8 and Figure 4.9B - E). These demonstrated that the strains with *alaP* deletion were more sensitive to the abundance of L-alanine when it was grown in the presence of amino acid mix. The growth of all four strain with alanine auxotrophic phenotype (*ΔalaT::erm* (MC1), *ΔalaT Δdat::erm* (MC4), *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11)) suffered a slightly more significant depreciation of growth in the presence of L-alanine than that of its D-isoform given at same concentration (Figure 4.8 and Figure 4.9B-E). This might suggest a better cellular uptake efficiency of D-alanine over L-alanine. Or this could be due to cellular demand of one isoform over another. But it does not suggest any specificity of AlaP towards either isoform. Together these confirmed that AlaP as an alanine transporter for both isoforms of alanine but there could be more than one transporter for taking up alanine when the cells were grown in high concentration of alanine, presumably 1 mM or above. Both *ΔalaT::erm* (MC1) and *ΔalaT Δdat::erm* (MC4) exhibited a L-alanine dependent phenotype as expected and *ΔalaP::erm* (MC7) acted as the control (Figure 4.9A, B and D).

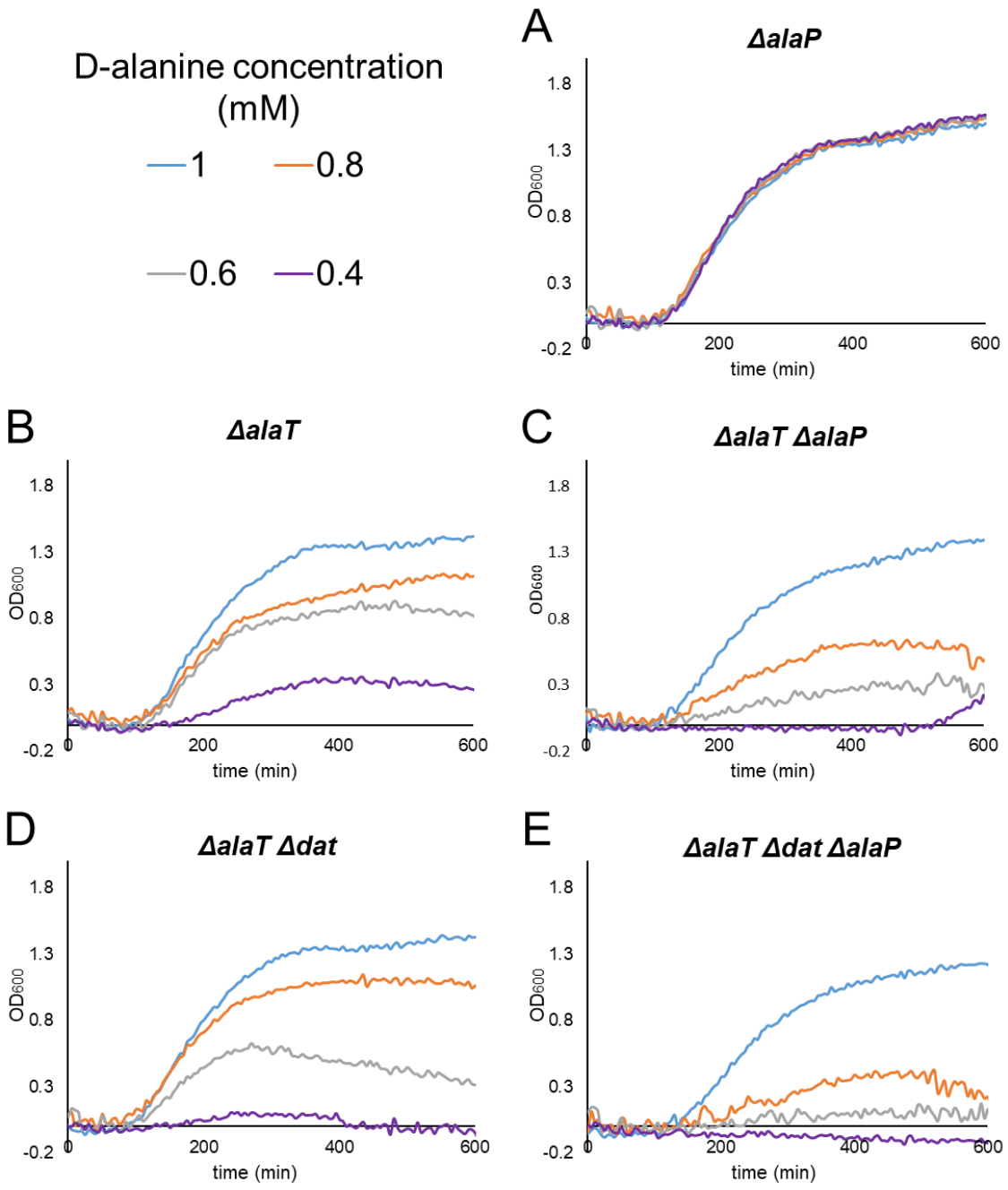


Figure 4.8: Quantitative growth measurement of alanine auxotrophs with or without deletion of *alaP*. Growth of alanine auxotrophs without *alaP*, *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11) were compared with *ΔalaT::erm* (MC1), *ΔalaT Δdat::erm* (MC4) and *ΔalaP::erm* (MC7) grown in medium GMaaP-ala supplemented with different concentrations of D-alanine at 37°C for 10 hours. The tested concentrations include 0.4, 0.6, 0.8 and 1 mM in which the curves were coloured purple, grey, orange and blue respectively. Each graph shows the growth of strain indicated in different concentrations of D-alanine as colour coded by the curves with reference. (A) Growth of MC7. (B) Growth of MC1. (C) Growth of MC8. (D) Growth of MC4. (E) Growth of MC11. The experiment was replicated twice and these are the representative graphs of one experiment.

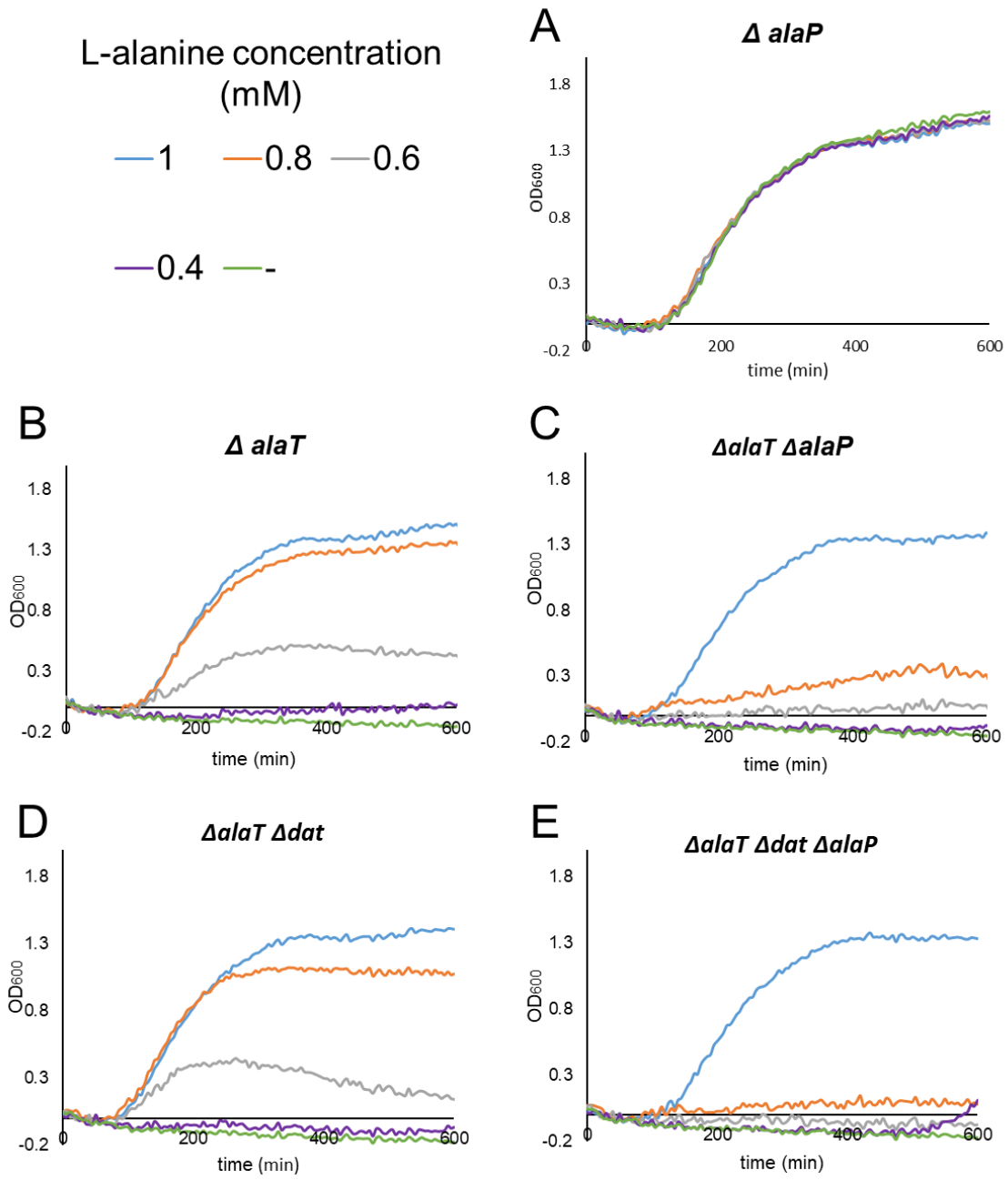


Figure 4.9: Quantitative growth measurement of alanine auxotrophs with or without deletion of *alaP*. Growth of alanine auxotrophs without *alaP*, *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11) were compared with *ΔalaT::erm* (MC1), *ΔalaT Δdat::erm* (MC4) and *ΔalaP::erm* (MC7) grown in GMaaP-ala supplemented with different concentrations of L-alanine at 37°C for 10 hours. The tested concentrations include 0, 0.4, 0.6, 0.8 and 1 mM in which the curves were coloured green, purple, grey, orange and blue respectively. Each graph shows the growth of strain indicated in different concentrations of L-alanine as colour coded by the curves with reference. (A) Growth of MC7. (B) Growth of MC1. (C) Growth of MC8. (D) Growth of MC4. (E) Growth of MC11. The experiment was done together as one with Figure 4.8 twice and these are the representative graphs from one experiment.

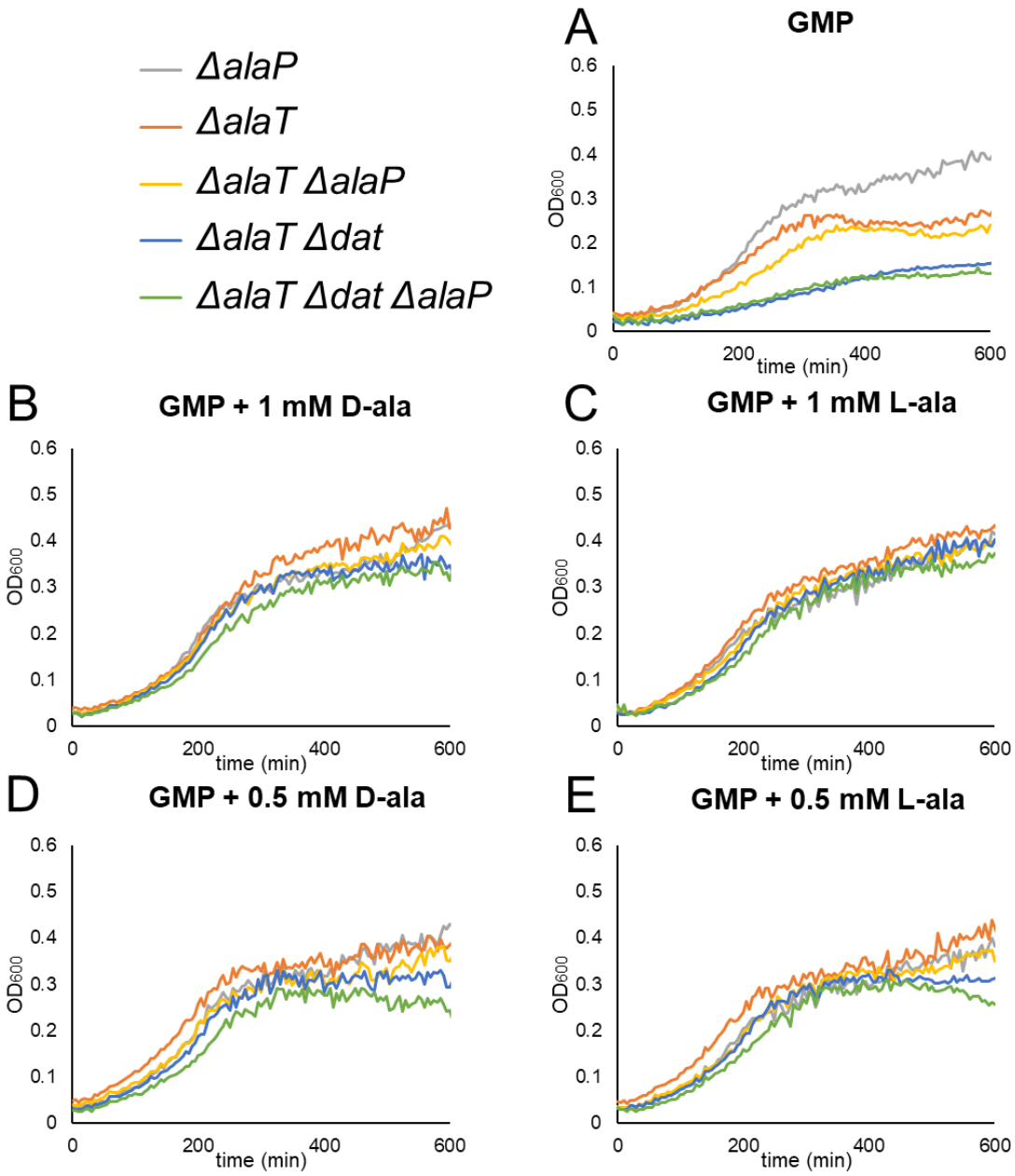


Figure 4.10: Quantitative growth measurement of alanine auxotrophs with or without deletion of *alaP*. Growth of alanine auxotrophs without *alaP*, *ΔalaT ΔalaP::erm* (MC8) and *ΔalaT Δdat ΔalaP::erm* (MC11) were compared with *ΔalaT::erm* (MC1), *ΔalaT Δdat::erm* (MC4) and *ΔalaP::erm* (MC7) grown in GMP supplemented with two concentrations (0.5 and 1 mM) of L-/D-alanine at 37°C for 10 hours. 1 mM alanine was defined as high concentration and 0.5 mM was defined as a low one. Each graph shows the growth of strains colour coded by the curves as in reference. (A) Growth of strains in medium GMP. (B) Growth of strains in medium GMP added with 1 mM D-alanine. (C) Growth of strains in medium GMP added with 1 mM L-alanine. (D) Growth of strains in medium GMP added with 0.5 mM D-alanine. (E) Growth of strains in medium GMP added with 0.5 mM L-alanine. The experiments were carried out twice independently. The graphs above are a representative of the two sets of experiments.

A deletion of *alaP* did not result in significant reduction of growth of alanine auxotrophs when it was grown in the absence of amino acids in Section 4.2.7. Hence

the experiment was repeated in a more sensitive growth measurement using plate reader (Section 2.3). Here the strains were grown in either 0.5 or 1 mM L- or D-alanine added into the medium containing only glucose, solution P but without the mix of other amino acids (GMP) at 37°C for 10 hours.

When the strains were grown in minimal medium (GMP), all strains were having poor growth (Figure 4.10). Among the strains involved, *ΔalaP::erm* (MC7) reached a highest optical density but it was only 0.4 after 10 hours of growth (grey line plot in Figure 4.10A). When the strains were grown at 1 mM (high) concentration of L- or D-alanine, they were sharing similar growth rate and final optical density (Figure 4.10B and C). Even when the strains were grown in GMP with low concentration of alanine (0.5 mM) supplemented, there was not much difference in their growth (Figure 4.10D and E). Although the growth rate of *ΔalaT Δdat ΔalaP::erm* (MC11) appeared to be slightly slower than its parent strain, *ΔalaT Δdat::erm* (MC4), when there was only 0.5 mM L- or D-alanine in minimal medium (GMP), their final optical density was close (blue and green line plot in Figure 4.10D and E). The same observation also could be applied to the comparison of *ΔalaT ΔalaP::erm* (MC8) and its parent strain, *ΔalaT::erm* (MC1), grown in 0.5 mM L- or D-alanine added to minimal medium (GMP) (orange and yellow line plot in Figure 4.10D and E). This once again suggested that alanine could be taken up by other transporter(s), reconfirming the findings in last section.

4.2.9 In vivo analysis of the specificity of AlaP

Since the growth analysis of strains deleted for AlaP indicated that it was involved in alanine uptake, a different approach was employed to determine if this transporter was more efficient in the assimilation of L- or D-alanine. Here, rather than focusing on the ability of the strains to grow, the abundance of alanine in the growth medium was determined using growth of the strains. Previously D-alanine was found released by strains deleted for *alaP* as they grew on agar plates and this was sufficient to support the growth of a D-alanine auxotroph (Sidiq, 2016, Section 4.2.8). Hence, a more biochemical approach studying the specificity of AlaP seemed feasible by determining the consumption of alanine in the culture supernatant.

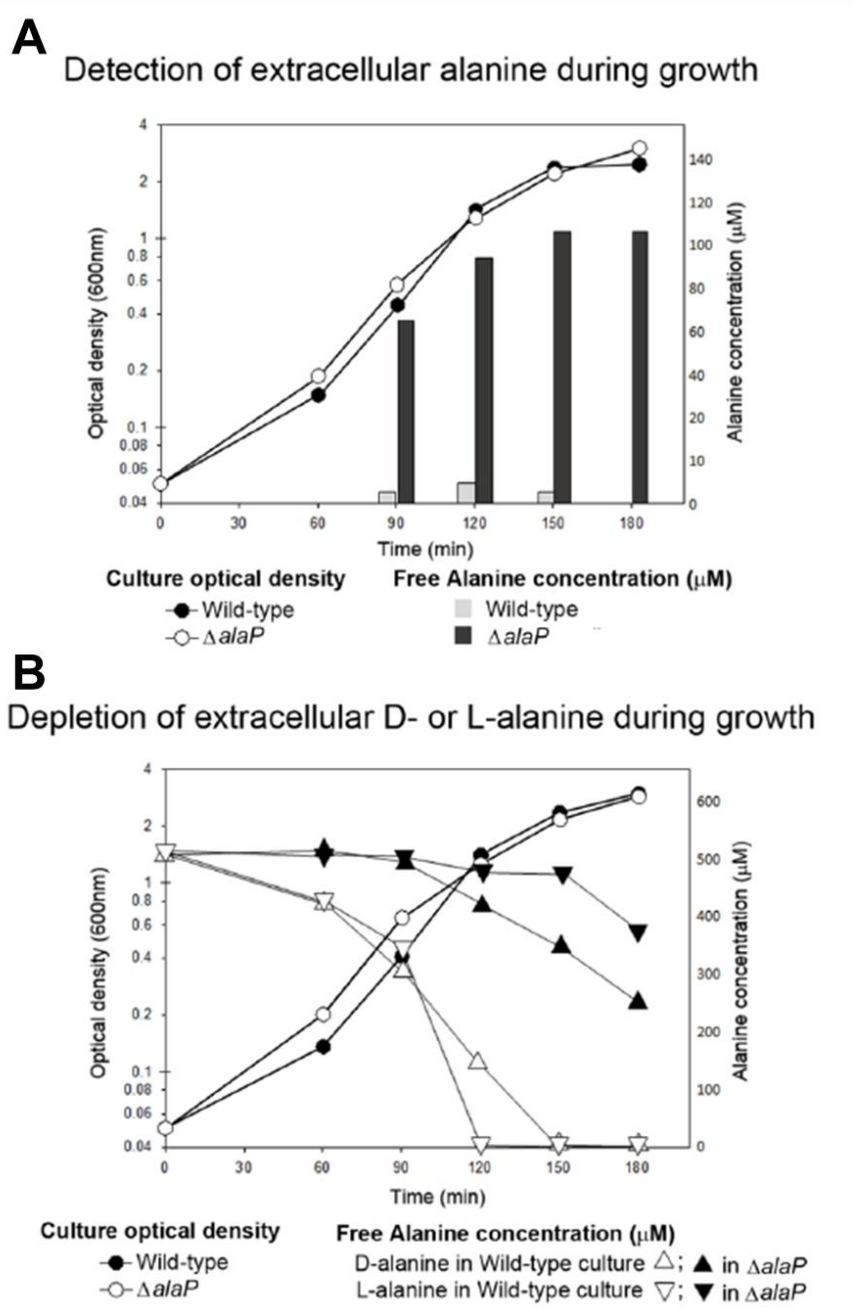


Figure 4.11: *in vivo* analysis of the specificity of AlaP. Growth of *ΔalaP::erm* (MC7) and the wild type strain growing in GMP opt supplemented with L- or D-alanine at 37°C for 180 minutes. (A) Concentration of free alanine (μM) released into medium. The line curves show the growth of the two strains expressed as OD_{600} whilst the concentration of alanine of culture medium of each strain are expressed as columns. (B) Growth and the concentration of alanine of the culture medium containing L- or D-alanine (0.5 mM) of the two strains. The line curves (with dots) were the growth curves of the two strains measured at OD_{600}. Only growth curves of strains in D-alanine are shown as the growth curves of the strains in L-alanine were essentially the same. Plots with triangle data points represent the free alanine in the culture medium the strains that were given L-alanine whereas inverted triangles represent the free alanine in the culture medium the strains where D-alanine was present. The experiments were carried out twice independently. The graphs above are a representative of the two sets of experiments.

To confirm the release of D-alanine in the growth culture, the *alaP* null mutant, *ΔalaP::erm* (MC7) and the wild type strain, 168+, were grown in a defined minimal medium (GM opt) at 37°C. This modified minimal medium contained glucose, solution P and 0.5 mM serine, glutamine, asparagine and glutamate with 0.25 mM of the other amino acids except alanine. This allowed good growth of the strains and was easier to analyse by HPLC as there were fewer labelled products in the sample (see section 2.1). The growth was monitored and the culture samples were taken over the time course for quantifying the abundance of alanine using RP-HPLC (Section 2.14 and 2.15). However, due to the technical limitations of OPA as the derivatising agent, it was not possible to differentiate between D- or L- isomeric forms. Nevertheless, as most of the assays were looking at the assimilation of alanine specifically added to the culture medium, this only became an issue when the alanine abundance increased.

Growth of the wild type and a corresponding *alaP* mutant exhibited similar growth characteristics during the time course (line plots in Figure 4.11A). For the *alaP* null strain, *ΔalaP::erm* (MC7), 90 minutes after diluting the culture into fresh medium, alanine was detectable in the culture medium at just over 60 µM (Figure 4.11A). This was significantly higher than that of detected in the culture supernatant of the wild type strain (168+) where less than 5 µM alanine detected (Figure 4.11A). This reconfirmed that the recycling of synthesized and released alanine was dependent on AlaP. The amount of alanine in the culture of the *alaP* null mutant slowly increased until the strain entered stationary phase, reaching about 100 µM alanine in the supernatant (Figure 4.11A). In contrast, the amount of alanine in the culture of wild type strain (168+) remained very low and was barely detectable by the end of the time course.

As the experiment showed that it was possible to assay alanine in the culture medium and gave results comparable to that previously determined (Sidiq, 2016), a variation of the experiments was devised to determine the specificity of AlaP. For this, the two strains were also grown in defined minimal medium (GM opt) to which was added 0.5 mM L- or D-alanine along with, parallel cultures without addition of alanine. These were incubated at 37°C and the growth was monitored in optical density together with samples being taken to determine the abundance of alanine in the culture medium. The strains were found to grow at similar rates, reaching similar final optical density (line plots with dots in Figure 4.11B). For the assimilation of wild type

strain when D-alanine was supplied, the abundance of it in the medium decreased gradually in the first 60 minutes followed by an accelerated depletion of alanine until 150th minute where alanine was completely depleted (white triangle in Figure 4.11B). The depletion rate was slightly more efficient from 60th minute onward when L-alanine was supplied instead of its D-isomers for growth (inverted white triangle in Figure 4.11B). L-alanine was shown completely depleted in the next 60 minutes (inverted white triangle in Figure 4.11B). Alanine, regardless of its isomeric forms, was completely assimilated before the end of exponential growth in the wild type strain (white triangle plots in Figure 4.11B). For the culture of the *alaP* null mutant (*ΔalaP::erm* (MC7)) grown in medium added with D-alanine, the abundance of alanine remained abundant in the medium until 90-minute incubation (triangle plots in Figure 4.11B). Alanine was then gradually depleted where only 50 % remained in the medium by the end of the time course (black triangle plot in Figure 4.11B). Interestingly, when the strain was supplied with L-alanine, it remained abundant in the medium until near end of exponential phase (inverted black triangle plot in Figure Figure 4.11B). It remained relatively abundant in the medium by the end of the time course in that around 70 % of the initial amount of alanine left in the medium (inverted black triangle in Figure 4.11B). Together, these suggested the existence of other alanine transporters but likely inefficient compared to AlaP. As the deletion of *alaP* resulted in a more significant reduction of assimilation of L-alanine compared to that of the D-isoform, this seemed to indicate the preference of AlaP could be L-alanine. But the difference could also be due to the preference of the other inefficient alanine transporter towards D-alanine. Hence, it remained difficult to identify the difference of the uptake between the two isomers.

4.3 Discussion

This chapter is an extension of a study previously done by my colleague on D-alanine metabolism (Sidiq, 2016) with the aim of providing a more complete understanding of alanine metabolism in *B. subtilis*. This study has been more focused on L-alanine metabolism by characterising the alanine related enzymes. Additionally, with a better understanding of the metabolism of alanine in both isoforms, the previously identified D-alanine specific transporter, AlaP has been further characterised using the available alanine auxotrophs (Sidiq, 2016).

4.3.1 Alanine metabolism

To begin with, an L-alanine auxotroph of *B. subtilis* has been identified. AlaT was shown to play a major role in L-alanine biosynthesis which agreed with the suggestion from Belitsky, 2002 and provided experimental evidence to it (Section 4.2.1). The responsibility of alanine biosynthesis relying majorly on one enzyme in *B. subtilis* was different from that of in *E. coli* where the responsibility was shared between three enzymes (Section 4.1) (Kim, Schneider and Reitzer, 2010; Yoneyama *et al.*, 2011; Peña-Soler *et al.*, 2014). Whilst deletions of all three enzymes were necessary to create an alanine auxotroph in *E. coli*, a single deletion of *alaT* in *B. subtilis* have resulted in a partial alanine auxotroph (Section 4.2.2). The role of AlaT in synthesising alanine was confirmed in the overexpression of AlaT in an alanine auxotroph strain grown in the minimal medium (Section 4.2.3). Here *alaT* was constitutively expressed through pAG-*alaT* and this allowed growth of the alanine auxotroph (Section 4.2.3). However, the overexpression of *alaT* seemed to inhibit growth of the alanine auxotroph when it was grown in the presence of either L- or D-alanine. This inhibition of growth was not observed in the overexpression of Dat in the alanine auxotroph (Section 4.2.6). This is presumed to be the overexpression of *alaT* in pAG-*alaT* was higher than that normally expressed level and resulted in the cellular metabolism being imbalanced.

The small colonies developed in prolonged incubation of *alaT* null mutant suggested additional pathway of L-alanine synthesis (Section 4.2.1). The characterisation of the alanine auxotroph identified *dat* as playing a minor role in L-alanine synthesis and its role was then confirmed in the overexpression of Dat in *alaT* null background strain (Section 4.2.2). Although Dat was likely less efficient compared to AlaT, the existence of this could permit limited bacterial growth in the absence of alanine (Section 4.2.3). Given Dat was also identified as functioning to convert D-glutamate into D-alanine, the observation could be a reflection of Dat converting D-glutamate into D-alanine then AlrA acting to convert D-alanine into its L-isoform (Thorne, Gomez and Housewright, 1955; Ferrari, Henner and Yang, 1985; Sidiq, 2016)(Section 4.2.5). Or Dat might directly convert L-glutamate into L-alanine (Freese, Park and Cashel, 1964) but this might contradict to the *in vitro* study showing the specificity of Dat reaction with D-amino acids, mainly D-alanine and D-glutamate (Thorne, Gomez and Housewright, 1955).

The role of Dat deduced might also contradict to a suggestion in which Ald could be involved in synthesizing alanine (Belitsky, 2002). But Ald was later confirmed its role in utilizing alanine where a deletion of *ald* in *B. subtilis* was not able to grow when alanine (regardless of isoform) was provided as sole carbon or nitrogen source (Section 4.2.4). This agreed to the *in vitro* study where L-alanine was shown to be converted into ammonium by Ald (Yoshida and Freese, 1964). Although the Ald-involved reaction was suggested reversible repeatedly in *in vitro* studies, Ald was shown unlikely involved in alanine synthesis as it is clearly expressed, but does not contribute to growth of the auxotroph, thus the reaction *in vivo* is most likely uni-directional (Section 4.1 and 4.2.4) (Wiame and Pierard, 1955; Yoshida and Freese, 1965; Berberich *et al.*, 1968; Pozo *et al.*, 1989). Although Ald was shown to be specific to L-alanine, the preference of its specificity for the isoform of alanine was not explored in a genetic context in this project (Wiame and Pierard, 1955; Yoshida and Freese, 1965; Berberich *et al.*, 1968; Martínez del Pozo *et al.*, 1989). On the other hand, deletion of *alaT* and *dat* did not result in a growth defect when alanine was provided as sole carbon or nitrogen source (Section 4.2.4). Thus, the two enzymes were less likely reversible. This also matched with the deductions where AlaT and Dat were responsible for L-alanine synthesis, directly or indirectly, in previous sections and reinforced the idea that the reactions were more likely uni-directional. Hence Dat in *B. subtilis* seemed to be different from that of the homologue in *Mycobacterium smegmatis* in which its Dat involved reaction was identified as being reversible and capable of synthesising D-glutamate (Mortuza *et al.*, 2018). However, this might be simply because it is not sufficiently active to provide the quantity necessary for the normal growth rate of *B. subtilis*, considering that *M. smegmatis* is growing significantly slower.

Apart from L-alanine synthesis, Dat was suggested to synthesize D-alanine previously (Sidiq, 2016) and it was confirmed when the experiment was repeated in a more quantitative growth medium (Section 4.2.5). The growth of both strains *ΔalrA::zeo* (RD180) and *ΔalrA::zeo Δdat::erm* (KS78) were shown D-alanine dependent (Section 4.2.5). Interestingly, the inhibitory effect of L-alanine on the D-alanine auxotrophs was shown and the double mutant was more sensitive to the inhibitory effect compared to its parent strain *ΔalrA::zeo* (RD180). This resembled an aminotransferase in *Listeria monocytogenes* which was shown to synthesize D-alanine when its racemase was deleted but there was no reported inhibitory effect of

L-alanine (Thompson *et al.*, 1998). L-alanine was later suggested to be likely inhibiting the activity of Dat because the double mutant *ΔalrA Δdat* was more sensitive to the inhibitory effect (Section 4.2.5). Even though pAG-*dat* was shown to have restored the growth of D-alanine auxotroph in the absence of D-alanine, the presence of L-alanine inhibited the growth of that strain (Section 4.2.6). This could be suggesting that Dat has a preference for L-alanine as substrate in the reaction making L- or D-glutamate rather than L- or D-glutamate to make L- or D-alanine (Thorne, Gomez and Housewright, 1955). However, this would contradict to the results from other sections suggesting a more uni-directional Dat reaction producing L- and D-alanine (Section 4.2.2 and 4.2.4). On the other hand, Dat alone was shown to be able to restore growth in D-alanine auxotroph *ΔalrA::zeo* (RD180), which was similar to that of restoring growth of the *alaT* null mutant. In addition, it was indicated by the creation of the double mutant *ΔalaT ΔalrA* as here, theoretically isomerisation of alanine should not occur and hence Dat seems to mediate the formation of both isoforms (Sidiq *et al.*, 2021).

Combining the work from my colleagues, the results in this chapter have addressed some past confusions in alanine metabolism, and the current view is summarised in Figure 4.12. Here, *B. subtilis* has a primary enzyme responsible for the synthesis of L- and D-alanine with Dat identified to play a minor role in making both isomers of alanine (Section 4.2.2 and 4.2.5). Although some of the literature has suggested the reversibility of reactions involving either Dat or Ald in *in vitro* studies, the results in this chapter collectively have suggested these reactions tend to be unidirectional instead *in vivo*.

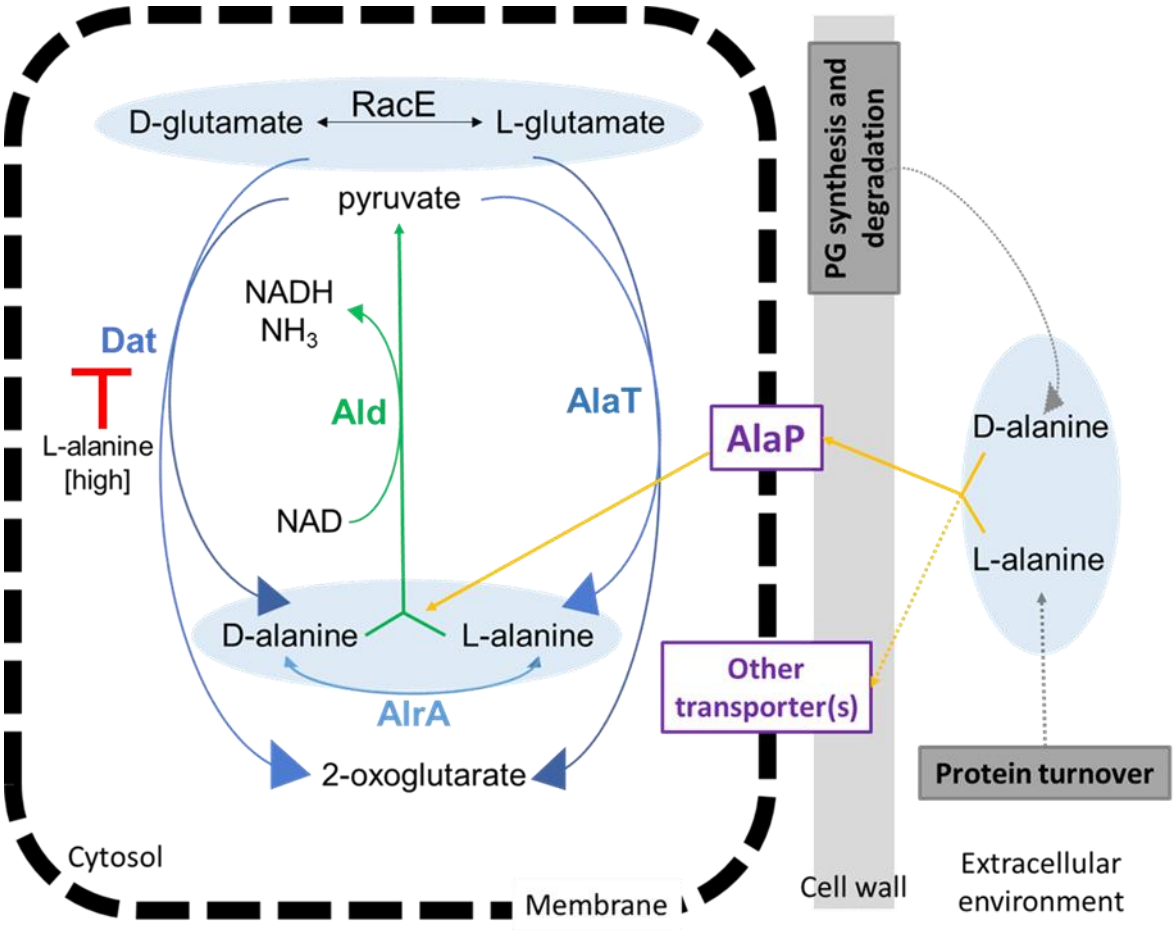


Figure 4.12: A schematic diagram of alanine assimilation in *B. subtilis*. The result in this chapter and colleagues' work (Sidiq, 2016) together proposed the model of alanine assimilation in addition of the understanding of alanine biosynthesis in the previous chapter. The uptake of alanine is indicated by yellow arrows and the release of alanine into media is indicated by grey arrows. Blue arrow suggested synthesis of alanine whilst green arrow suggested utilsation of alanine. Proteins specifically characterized in both chapters are indicated in bold and the substrates involved are indicated at either end of arrows.

Hence a possible scenario of mechanism during normal cell growth would be L-alanine can be made by AlaT whereas D-alanine to be made by AlrA. Ald would be useful in utilising alanine and converting it into ammonia and pyruvate (Siranosian, Ireton and Grossman, 1993). Dat would fine-tune the relative abundance of the two isoforms to satisfy the cell's demand and it might be done by L-alanine acting as an inhibitor of its activity. This might also have consequences for the glutamate metabolism with respect to the balance of L- or D-isomers. What is unclear at this point is how the activity of Ald, converting alanine into pyruvate, is controlled to prevent futile competition with AlaT (Figure 4.12). More work has to be done in studying kinetic activities of these involved enzymes, particularly Dat. Nevertheless, the biosynthesis of alanine and glutamate for both isoforms could be interdependent

and the enzymes in this complex network and are most likely to be regulated by the availability of substrates, which seemed logical as a way to balance synthesis with needs.

The alanine metabolism is even more difficult to envisage as a whole picture in *B. subtilis* when taking regulation mechanism of these enzymes into account. The regulation of Ald by alanine during exponential growth was suggested which seemed logical given the utilization of alanine by Ald in *B. subtilis* (Freese and Oosterwyk, 1963; Siranosian, Ireton and Grossman, 1993) (Section 4.2.4). In the case where the activity of Ald to be inducible by D-alanine, a model was proposed in which D-alanine regulates its own biosynthesis by limiting the cytosolic abundance of L-alanine, the substrate of AlrA to convert into its D-counterpart (Berberich *et al.*, 1968). Given the evidence of only Ald to be utilizing alanine as sole carbon or nitrogen source instead of AlaT and Dat (Section 4.2.4), the regulation might be interpreted as generating more D-alanine for cell wall synthesis as well as to upregulate the expression of *ald* hence converting alanine into ammonium and pyruvate. As *alaT* and *dat* were shown to be inducible by ammonium starvation instead of *ald*, the up-regulation of *alaT* and *dat* would in turn increase abundance of L-alanine and then converted by AlrA to D-alanine as suggested previously (Tam *et al.*, 2006). Concomitantly, the upregulation of Ald might also in turn increase the activity of AlaT and Dat by supplying more pyruvate, the substrate of the AlaT and Dat reaction producing alanine and boosting growth as pyruvate was an important metabolite during glycolysis. Unfortunately, little has been known about the regulation of AlaT except the regulation of the operon of this gene was only suggested to be independent of the availability of alanine (Belitsky, 2002). However, this work suggests that the activity of Dat is regulated by the presence of L-alanine (Section 4.2.5), Dat was also shown upregulated in proteomics study in response to a starvation of a mixture of many amino acids and peptides (Mäder *et al.*, 2002). Due to the complexity of the network, a more detailed study on the expression levels of these enzymes is necessary in the future work. Together these might help building a whole picture of the kinetic activities and how they are controlled and to understand the cooperation between the four proteins in order to maintain the balance of the isoforms of alanine or even glutamate.

4.3.2 Chacterisation of alanine transporter AlaP

The inhibitory effect of L-alanine on the *alrA* mutants growth was once suspected to be a result of the competition of the two isomers for uptake through a shared

transporter system (Section 4.2.5). The available alanine auxotrophs became useful to the characterisation of the AlaP alanine transporter in terms of its specificity (Section 4.2.7).

To begin, the specificity of AlaP towards which isoform of alanine was explored in both phenotypical and biochemical approach (Section 4.2.7). Firstly in the phenotypical approach, AlaP was shown to be able to transport both isoforms of alanine (Section 4.2.7). In the defined rich medium where glucose and a mixture of 19 other amino acids were present, AlaP seemed to be transporting both isoforms at a similar efficiency which was reflected in the pattern of the growth curves (Section 4.2.8). The bacterial growth in rich medium has been more efficient compared to that of in minimal medium and this allowed the appearance of the phenotype. However, the components in the defined rich medium could be hindering the transport of alanine by other minor transporters which might have less priority towards alanine relative to the components in the medium (Section 4.2.8). Nevertheless, given the possibility of sharing AlaP with other amino acids have been eliminated in the previous work done by colleague, AlaP could be recognised as an alanine specific transporter for both isomers (Figure 4.12, Sidiq, 2016, Clark and Young, 1977). This matched with the proposed model of a highly specific transport systems for L- and D-alanine in *B. subtilis* and such system was also found in *Bacillus sp.* PB3, *M. tuberculosis* and *Methanococcus maripaludis*, which is an archaea bacterium (Sidiq, 2016, Clark and Young, 1974, Kanamori et al., 1999, David, 1971, Moore and Leigh, 2005).

However, in terms of specificity of transporter AlaP, the alanine auxotrophs with a mutated *alaP* were growing slightly better in D-alanine relative to that of in L-alanine supplied at the same concentration (Section 4.2.8). Together with the genetic characterisation of AlaP done by my colleague, they suggested the preference of AlaP towards D-alanine which agreed to the conclusions suggested from the *in vitro* studies (Martinez del Pozo et al., 1989). However, when the ratio of L-alanine to D-alanine was high, the inhibition of growth on the D-alanine auxotroph occurred (Section 4.2.6). On the other hand, no clear indication of preference of AlaP towards D- or L-alanine could be deduced from the *in vivo* analysis of the specificity of AlaP (Section 4.2.8). Nevertheless, the preference of AlaP was likely D-alanine over L-alanine.

The above interpretation is complicated by the presence of other minor alanine transporters, indicated by the absence of the expected phenotype (impaired growth) of the alanine auxotroph with defected AlaP (*ΔalaT Δdat ΔalaP*) when the strains were grown in higher concentration of alanine (1 mM or above) (Section 4.2.8). The high concentration of alanine in the medium might outcompete the other components that the minor transporter(s) might have prioritised for assimilation. The presence of other uptake systems was also verified where the assimilation of *alaP* null mutant was shown minimum during exponential growth but followed by a gradual increase in assimilating alanine (Section 4.2.9). This reflected that AlaP was playing the major role in alanine transport during the exponential growth whilst these unknown transport systems could be of lower expression or kinetic activity. As the later stage of growth quite often could be related to nutrient starvation, the minor transporter(s) could potentially be up-regulated or more active kinetic activity in response to nutrient starvation.

In support of the above, when the strain was grown in media where the only amino acid present was alanine, growth occurred, even at the lower concentrations (Section 4.2.8). This suggested the unknown minor transporter(s) could be more dominant in transporting alanine in this condition compared to AlaP. Similar to the phenotype in the later stage of growth in the defined medium, this could be a result of the difference in expression, either transcriptionally or translationally, between the minor transporter(s) and AlaP. Or it could be due to the absence of its more prioritised substrates. It further suggested a possibility of identifying the amino acid(s) that interferes with the alanine uptake in this medium condition. Nevertheless, the absence of the phenotype could also be possibly due to the slower growth rate of the strains in this minimal medium (GMP) and so reduced the demand of alanine assimilation hence the absence of the expected phenotype. This is best demonstrated by the fact that the highest final optical density reached when the bacterial cells grown in the absence of amino acids (GMP) was only half of that grown in medium with amino acids (GMaaP) (Section 4.2.8). These uptake systems remained to be identified (Section 4.2.8). Interestingly, the unknown alanine transporter(s) were also deduced to be collectively transporting D-alanine more efficiently than L-alanine because of a more significant assimilation of alanine of the strain with deleted *alaP* (Section 4.2.9). Interestingly, this seems to contradict

previous analyses where AlaP was found to be essential for efficient D-alanine uptake.

It is known that the process of cell wall synthesis occurs in concert with cell wall degradation to allow growth (Mauck, Chan and Glaser, 1971; Sidiq, 2016). It was expected that, under normal exponential growth condition, D-alanine is released into the medium during cell wall turnover which involves peptide cleavages from peptidoglycan and teichoic acids (grey arrow in Figure 4.12). But the absence of alanine in the culture medium suggested that it could be recycled back into the cells through AlaP, which presumed to be the primary purpose of the specified transporter (Lam *et al.*, 2009) (yellow arrow in Figure 4.12). On the other hand, L-alanine should be present in the growth medium or from the degradation of secreted proteins with the help of secreted or wall-bound proteases, *e.g*. Vpr and AprE (dotted grey arrow in Figure 4.12). As AlaP was shown to be assimilating both alanine isomers at similar efficiency (Section 4.12), the assimilation of L-alanine by AlaP could be a recycling of released L-alanine as well (yellow arrow in Figure 4.12).

The other minor transporter(s) have not been identified yet but the nature of them has been suggested (dotted yellow arrow in Figure 4.12). Firstly they should be able to transport alanine during exponential growth because alanine was shown to be supplied for growth of alanine auxotroph in the absence of AlaP under some conditions (Section 4.2.9). They are also suggested to be upregulated transcriptionally or translationally in the later stages of growth (stationary phase, Section 4.2.8) because alanine was shown assimilated significantly in the absence of AlaP (Section 4.2.9). However the possibility that the transcriptome of the bacterial cells grown in minimal medium is different cannot be excluded.

Chapter 5 Transcriptional response to amino acid starvation

5.1 Introduction

Bacteria are good at adapting to different conditions for survival. This is generally mediated by sophisticated cellular systems to regulate gene expression, enzyme activity and protein stability. The nitrogen metabolism genes of *B. subtilis* are regulated by the availability of rapidly metabolised nitrogen sources. Thus a specific pool of genes are regulated in response to amino acid starvation (Fisher, 1999). As discussed in Section 1.3 , there are a diverse of control mechanisms for the transcriptional expression of many amino acid biosynthetic pathways. These range between very specific mechanism to modulate a single gene or operon to pleotropic global regulation mechanism, such as the stringent response (Section 1.3).

The overview of the first chapter has briefly suggested the complexity of regulations given the possibility of inhibition by some of the amino acids on transporters or amino acid metabolism related enzymes or glucose repression on some operons (Section 1.3). This was followed by the demonstration from last chapter of a complexity network of enzymes reactions for alanine metabolism in that the enzymes were suggested to be working interdependently. Although regulation of these enzymes is unknown, at least one of them were expected to be regulated by the metabolites relevant to the reactions involved (Section 4.3.1). As the technology advanced, global transcriptomic and proteomic analyses become feasible and insights into the networks of regulatory system have become easier. Interestingly, changes in the expression of the genes encoding these enzymes involved in alanine metabolism were also detected when the cells were stimulated with starvation of different amino acids, specific or general instead of specifically to alanine (Section 4.3.1). For instance, *alaT* was upregulated in response to ammonium starvation whilst *ald* was up-regulated in response to tryptophan starvation and a starvation of isoleucine and leucine (Eymann *et al.*, 2002; Tam *et al.*, 2006). *dat* was up-regulated in response to ammonium and tryptophan starvation as well as a mixture of amino acids (Eymann *et al.*, 2002; Mäder *et al.*, 2002; Tam *et al.*, 2006). Additionally, in the mentioned global transcriptomic studies, genes encoding enzymes responsible for a wide range of cellular functions, including protein synthesis or carbon and nitrogen sources acquisition were recognised as significantly changed in expression. Many of them have been additionally shown significantly changed on the proteomic level. For

instance, transcriptomic analysis of ammonium and tryptophan starvation on *B. subtilis* has shown genes related to amino acid biosynthesis, sporulation and competence development to be recognised as significantly changed in expression (Tam *et al.*, 2006). Whilst the starvation response was suggested to be mediated by CodY, SigH or as a stringent response, there were also genes that were significantly changed but independent of these regulations (Tam *et al.*, 2006). Another similar transcriptomic study regarding the response of *B. subtilis* towards isoleucine and leucine starvation was focused on stringent response also revealed unknown genes that were independent of stringent response (Mäder *et al.*, 2002). but no common global regulator suggested modulated by the amino acid availability in the media (Mäder *et al.*, 2002). Altogether, these demonstrated the complexity of the control of the regulatory system over cellular activities for *B. subtilis* to respond to changes and its sensitivity towards the outside environment. However, no common regulators were found so far for *B. subtilis* when it was subject to a starvation of a specific amino acid. Also the correlation of the transcriptionally changed genes detected in different transcriptomic studies with the acquisition of the amino acid required for growth, either by assimilating or by synthesizing the amino acid seemed to be vague.

The amino acid uptake is believed to be strictly controlled by the carbon and nitrogen metabolism regulators, in addition to some other transcriptional regulators depending on the media conditions (Fisher and Sonenshein, 1991; Fisher, 1999). However, a clear understanding of the molecular mechanism is lacking.

As for amino acid transport across the cytosolic membrane, some of the amino acids might be diffused into the cell but many were relying on transporter systems (Jung, Branciamore and Martini, 2000; Hosie and Poole, 2001). However, one common problem was that the identified transporter(s) were usually evident when the bacteria was grown in minimal media instead of rich media (Section 1.2). *In vivo* studies on the differential expression of the transporter or possible inhibition of other substrates of the transporter might have been overlooked. The other problem could be that the target substrates suggested bioinformatically for putative transporters quite often required further experimental evidence for confirmation (Section 1.2). The characteristics of the identified alanine transporter AlaP from the last chapter could be a suitable example depicting the complexity of the transportation system. The pool of transporters could have overlapping roles (same substrate targets) and concomitantly having a certain level of specificity towards a particular substrate in

certain type(s) of media condition(s). Therefore, it raised a problem in terms of screening transporters, particularly in defining a universal platform for screening (if any).

A live/dead colony screening for specific amino acid starvation was first used to screen for a D-alanine transporter (Sidiq *et al.*, 2021). Initially, no colonies were expected to form if an amino acid auxotroph was unable to assimilate the amino acid it required for growth. However, no possible candidates have been picked in the screening for L-amino acid transporters and one of the possible technical concerns could be technical variations including DNA extraction and transformation efficiency. One of the possible solutions to this was the application of reporter system on the amino acid transporter screening platform. The reporter would be constructed to be containing an insertion of regulatory region of a gene which should be upregulated in response to specific amino acid starvation (in this case) upstream of a promotorless gene *lacZ*. Colonies of a *B. subtilis* strain carrying this vector would turn blue if the strain encountered a condition possibly triggering starvation response to a specific amino acid and this assay is termed blue/white colony screening.

In this final chapter, an attempt was made to utilise transcriptional data to identify potential transporter systems for specific amino acids. The hypothesis that was employed was simply based on the idea that on starvation for a specific amino acid, an expected response would be to up-regulate the expression of genes involved in both the synthesis of the amino acid and extracellular uptake.

5.2 Results

5.2.1 Global transcriptional profile of B. subtilis in response to specific amino acid starvation

This chapter describes the experimental approach used to determine if it was possible to identify genes involved in amino acid uptake through comparison of the transcriptional changes that occur upon starvation for a specific amino acid.

In the previous work, it was notable that the auxotrophs (*Δ alaT* and *Δ alaT Δ dat*) exhibited more limited growth comparing to the wild type strain even when the required amino acid was provided sufficiently (1 mM) (Section 4.2.8). Hence, the auxotroph growing in that condition must be limited in its ability to assimilate the required nutrient. This concept seemed to be supported by the observation that this limited growth was predominantly related to the final optical densities reached

whereas as initial growth rates were essentially identical to that seen for the prototrophic strain. Earlier work also indicated that there were significant differences in the assimilation rates for different amino acids. This could be due to either the fact that some amino acids are more readily used as metabolic precursors or because the uptake systems for the amino acids that were not assimilated significantly are not active or efficient. The later possibility seemed to be excluded by the fact that even when strain auxotrophic for various amino acids were all able to grow when provided with the required amino acid, indicating that uptake was occurring and sufficient to support growth (Section 4.2.8). Alanine auxotroph was shown able to assimilate alanine at relatively lower concentration (Section 4.2.8). This seemed to suggest that the uptake might be transcriptionally regulated and hence offered the possibility that transcriptional analysis of the auxotrophic strains growth might provide a way to identify the transporter(s) required for that specific auxotrophy.

In general, when the auxotrophs were grown in excess of the amino acid that their growth was depending on, they demonstrated very similar growth pattern as the wild type strain regardless of the efficiency of the wild type strain to assimilate that amino acid (Section 3.2.4). But the amino acid auxotrophs exhibited more limited growth comparing to the wild type strain when they were grown in sub-optimal concentration of the amino acid they needed for growth. For instance, when an alanine auxotroph (*Δ alaT* or *Δ alaT Δ dat*) was grown at a lower concentration of alanine in parallel with the wild type strain, the auxotroph suffered a shorter exponential growth in which it progressed into stationary phase earlier than the wild type strain (Figure 4.8 and Figure 4.9). However, the growth rate during its exponential growth was similar to that of the wild type strain. The strain was expected to experience specific amino acid starvation before it entered stationary phase, probably near the time point where its growth diverted from that of the wild type strain. This observation was expected applicable to other amino acid auxotrophs if they encountered similar starvation, triggered by growing them at lower concentration of the amino acid they needed to survive. The wild type strain was suggested to have a preference to certain amino acids when it was grown in the defined rich medium and some others remained relatively abundant in the growth medium (Table 3.2). In order to filter out non-specific general starvation responses and to avoid the complication of inducing sporulation due to the shortage of nutrient, auxotrophs of two less prioritised amino acids suggested from Chapter 3 were chosen to focus. Lysine was shown

moderately assimilated whilst the assimilation of phenylalanine was milder (Table 3.2). Hence the auxotroph for these two amino acids were used in this transcriptome study. A deletion of the genes *pheA* and *lysA* was separately introduced into the wild type strain (168+), creating *ΔpheA::erm* (MC18) and *Δ lysA::erm* (MC19) respectively. Created strains were tested for its auxotrophy where both were not able to grow without phenylalanine and lysine respectively which matched with the literature suggested the gene functions (Brans *et al.*, 2004; Koo *et al.*, 2017).

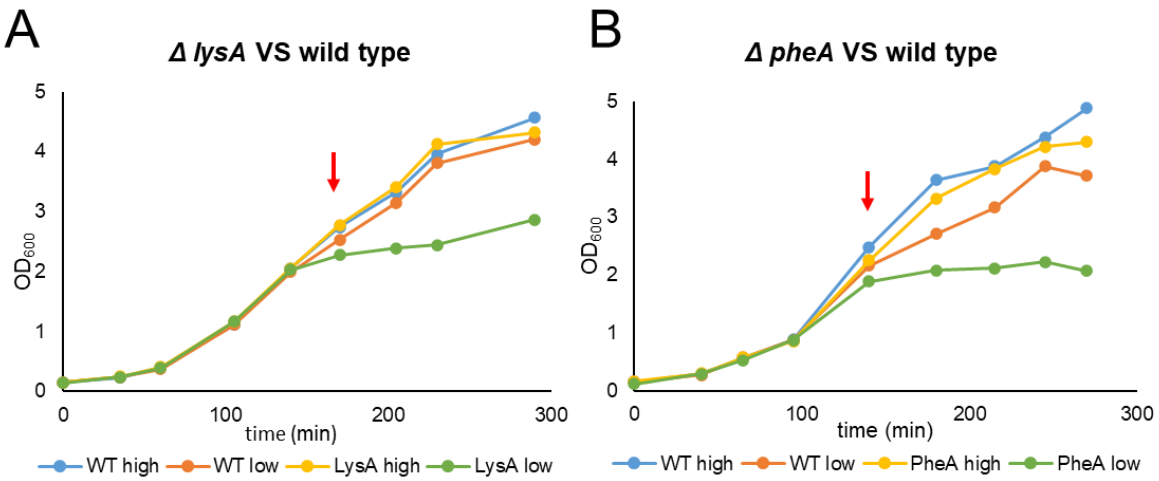


Figure 5.1: Growth curves of WT and the selected auxotrophs. The strains were grown in GMaaP with two different concentrations of the amino acid that the auxotrophs needed to survive accordingly. A: growth curves of wild type strain *168+* and *Δ lysA::erm* (MC19). WT high: growth of *168+* in higher concentration of lysine (1 mM) in GMaaP; WT low: growth of 168+ in GMaaP with sub-optimal concentration of lysine (0.25 mM); LysA high: growth of MC19 in high concentration of lysine in GMaaP; LysA low: growth of MC19 in GMaaP in sub-optimal concentration of lysine. B: growth curves of wild type strain and *Δ pheA::erm* (MC18). WT high: growth of *168+* growing in GMaaP with excess amount (1 mM) of phenylalanine; WT low: growth of WT growing in GMaaP with sub-optimal amount (0.05 mM) of phenylalanine; PheA high: growth of MC18 in GMaaP with excess amount of phenylalanine; PheA low: growth of MC20 in GMaaP with sub-optimal amount of phenylalanine. The experiments were carried out twice independently. The graphs above are a representative of the two sets of experiments.

To determine the conditions necessary for a transcriptional response to specific amino acid starvation, the strains were grown in higher and lower concentration of the amino acid they required in the presence of glucose, amino acid mixture and salts, essentially preparing defined rich medium (GMaaP) where it was possible to alter the concentration of specific amino acids. To test this, the lysine auxotroph, *Δ lysA::erm* (MC19) was grown in parallel with the wild type strain *168+* in two different concentrations of lysine added to the defined medium GMaaP without lysine, one was essentially in excess (1 mM) and the other was sub-optimal (0.25 mM). Similarly,

the same comparison was made between phenylalanine auxotroph, *Δ pheA::erm* (MC18) and the wild type strain by growing them in high (1 mM) and low (0.05 mM) concentrations of phenylalanine added into the defined medium GMaaP without phenylalanine. The cultures were then grown at 37°C in flask and the growth was measured using spectrophotometer (Section 2.1).

The wild type strain exhibited lag phase for about 30 minute and then entered exponential growth which lasted for 200 minutes, followed by a deceleration of the growth rate approaching stationary phase. The growth of this strain in two different concentrations of lysine is very similar to each other (WT high and WT low curves in Figure 5.1A). When *Δ lysA::erm* (MC19) was grown in excess concentration of lysine (1 mM), the growth pattern was almost the same as that obtained from the wild type strain (lysA high curve in Figure 5.1A). But when it was grown in sub-optimal concentration of lysine (0.25 mM), its growth rate was significantly decreased, entering its stationary phase after around 130 minutes, which was about 60 min earlier than other growth curves (lysA low curve in Figure 5.1A). Similarly, the phenylalanine auxotroph *Δ pheA::erm* (MC18) and the wild type strain grown in the two concentrations of phenylalanine, the phenylalanine auxotroph, *Δ pheA::erm* (MC18), shared a similar growth pattern with the wild type strain when they were grown in excess phenylalanine (WT high and PheA high curves in Figure 5.1B). However, when the auxotroph *Δ pheA::erm* (MC18) was grown in limited concentration of phenylalanine (0.05 mM), it suffered from poor growth in that its exponential growth was significantly slowed down entering stationary phase from around 150th minute (PheA low curve in Figure 5.1B). Whereas the wild type strain grown under the same conditions, started its exponential growth from around 50th minute but slightly deviated from this at around 130th minute but continued to grow albeit at a slower pace (WT low in Figure 5.1B). Together, these show that the deviation of growth for auxotrophs *Δ lysA::erm* (MC19) and *Δ pheA::erm* (MC18) from the wild type strain inoculated in sub-optimal concentration of the amino acids (red arrow in Figure 5.1A and B) was possible to achieve with minimal interference with the culture, and that the point where growth deviation could be considered the time point where the strain responded to the specific amino acid starvation. Culture samples were taken at this time point and the total RNA was then extracted (Section 2.16). The experiment was done in duplicate due to budget limits and both growth curves demonstrated similar patterns.

Total RNA from the samples collected for the two auxotrophs and the wild type strain near that time point were extracted and the quality of the RNA samples were assessed using bioanalyser from Agilent (Section 2.16). The RIN (RNA integrity number) and concentration of RNA for samples sent to RNA sequencing service provider met the minimum requirement of the company. The data quality and processing was done by the company and the Bioinformatics unit from our university. For each sample, the number of reads for each gene in each sample were normalised to exclude genes with only few counts (number of reads). To reduce the technical variations in the number of reads between samples, the normalised number of genes in each sample was further adjusted and presented as a fraction of the total number of reads for all normalised genes in each sample (Section 2.17). The fold of change of each gene compared in two conditions was defined by the difference of the adjusted number of reads of the gene of interest (Section 2.17). The comparisons were made by comparing transcriptome profile within single experiment and further verified by their own biological replicate (Figure 5.2A). Genes found up-regulated in one experiment (s1) were verified in another experiment (s2), and only the verified ones were recognised as the up-regulated genes in the comparison unless there was no biological replicate for the samples involved in the comparison available. Due to shipment delay, two samples were lost for sequencing hence there was no biological replicates for the wild type strain grown in excess lysine and phenylalanine. If samples did not have any replicates, the comparison would be made by using the only available data from that sample. In other words, only genes verified in both lists with significant change of expression in the given condition were used for data interpretation unless specified (Table 5.1).

Two approaches for comparison have been made. The first of the approaches (approach 1) was to screen for up-regulated genes in response to phenylalanine limitation, using the transcriptome profile of lysine auxotroph, *Δ lysA::erm* (MC19), grown in lower concentration of lysine to that of the strain grown in excess of the amino acid. This comparison will be referred to as LL2 VS LL1. The same comparison was also made for the wild type strain to act as a background control and this is referred to as WL2 VS WL1 (Figure 5.2B). Similar comparisons of phenylalanine auxotroph *Δ pheA::erm* (MC18), grown in lower concentration of phenylalanine was compared to that of grown in excess phenylalanine, which is annotated as PP2 VS PP1 and the control comparison for the wild type strain grown

in same conditions, referred to as WP2 VS WP1 (Figure 5.2B). To filter the positive candidates, genes might be validated later using a reporter system (Section 5.2.2 – 5.2.6), only genes of 5-fold up-regulation in response to the starvation specific to one amino acid in comparisons PP2 VS PP1 and LL2 VS LL1 but not in their control comparison pairs WP2 VS WP1 and WL2 VS WL1 respectively were selected (Figure 5.2B). However, there were no biological replicates for samples WP2 and WL1 (see above paragraph). Comparisons involving these two samples which used the only available data coming from one set of experiments were marked with asterisk but these were not included in data interpretation in later steps (Table 5.1 and 5.3,

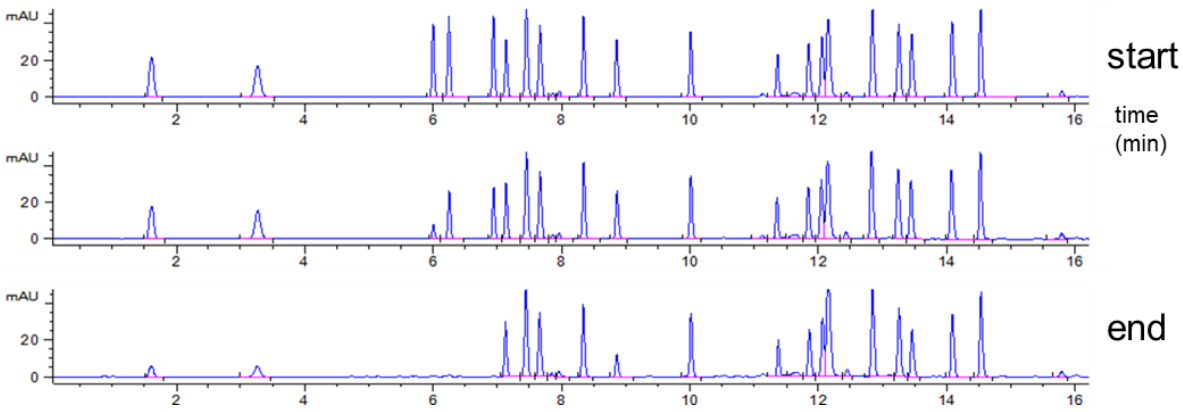


Figure **8.1: Chromatogram of amino acids content in defined medium of culture taken during time course experiment for quantitative analysis.**

Table 8.1: Relative abundance of amino acids of *168+* growing in Gaa medium at different time points.

	Relative level of amino acid (percentage to T0 at T150)	Relative level of amino acid (percentage to T0 at T250)	Relative level of amino acid (percentage to T0 at T400)
Asparagine	19.4	0.0	N.D
Serine	60.4	12.3	N.D
Glutamine	62.6	20.7	N.D
Aspartate	80.5	53.6	25.0
Glutamate	90.0	62.5	33.7
Alanine	84.7	57.5	39.5
Isoleucine	92.4	81.5	73.7
Leucine	92.9	85.3	81.7
Lysine	89.8	86.5	86.6

Valine	95.9	90.8	88.2
Threonine	94.5	90.1	88.6
Arginine	95.2	91.4	90.8
Phenylalanine	96.2	94.2	95.2
Tyrosine	96.3	94.7	96.9
Glycine	98.5	98.3	99.5
Tryptophan	95.1	98.0	99.9

The relative abundance of each amino acid at each time point (T150, T250 and T400 from left to right) was calculated in percentage relative to their initial concentration correspondingly and the numbers were corrected to 1 decimal place. Data shown is from single experiment. The experiment was repeated and similar trend of assimilation profile was obtained. N.D = amino acid concentration was below detectable range.

Table 8.2: Depletion rates of amino acids from defined medium of growing *168+*

	Gaa	**Maa**	**GMaa**
Asparagine	-25.1	-19.3	-22.6
Glutamine	-42.7	-21.7	-20.9
Serine	-18.5	-23.6	-24.2
Aspartate	-17.6	-17.7	-27.3
Alanine	-11.3	1.8	0.5
Glutamate	-17.7	-27.4	-31.7
Leucine	-8.2	-7.5	-5.0
Isoleucine	-8.6	-7.7	-1.7
Lysine	-7.1	-7.7	-1.3
Valine	-6.8	-11.8	4.9
Phenylalanine	-3.3	-3.4	1.9
Arginine	-4.1	-3.8	1.7
Tyrosine	-0.4	-0.4	7.3
Tryptophan	-0.4	-0.6	3.0
Threonine	-5.4	-2.7	-2.4
Glycine	0.4	0.0	6.2

The depletion rates (% decrease per OD600) were calculated as the percentage change of the relative abundance of amino acids per change of optical density. Column Gaa represents the relative abundance of amino acids recorded when the strain was grown in Gaa whilst column Maa represents those when the strain was grown in Maa. Column GMaa shows the relative abundance of amino acids when the strain was grown in GMaa. The order of amino acids follows that of Table 3.2 for easier reference. Data shown is from single experiment. The experiment was repeated and similar trend of assimilation profile was obtained.

Table 8.3: Tables of assimilation efficiency of the amino acid specified for different auxotrophs and the wild type strain.

A Serine

Time (min)	Wild type strain	Auxotroph (*ΔserA*)
0-160	-28.7	-34.9
0-100	-37.1	-55.3
100-160	-26.5	-28.7

B Alanine

Time (min)	Wild type strain	Auxotroph (*ΔalaT*)
0-160	-13.7	-16.9
0-100	-10.2	-14.5
100-160	-14.6	-17.7

C Leucine

Time (min)	Wild type strain	Auxotroph (*ΔleuA*)
0-160	-14.1	-9.5
0-100	-20.8	-4.3
100-160	-12.3	-11

D Tryptophan

Time (min)	Wild type strain	Auxotroph (*ΔtrpC2*)
0-160	-2.9	-1.8
0-100	2.8	8.6
100-160	-4.4	-5.1

The strains were grown in defined medium (GaaP) containing equal concentrations of 20 amino acids. Each table shows the assimilation efficiency rate calculated as percentage change per OD600 of the amino acid that the auxotroph required for growth for the auxotroph compared to the wild type strain during exponential growth over three time points. The first row of each table shows the overall rate during exponential growth. The second row shows the first 100 minute of the assimilation efficiency of the specified amino acid whilst the third row shows the following 60 minute of assimilation efficiency of the amino acid. The more negative the value in each table, the higher assimilation efficiency of the specified amino acid. A: comparison of assimilation efficiency of serine between serine auxotroph (MC16) and wild type strain. B: comparison of assimilation efficiency of alanine between leucine auxotroph (MC2) and wild type strain. C: comparison of assimilation efficiency of leucine between leucine auxotroph (MC17) and wild type strain. D: comparison of assimilation efficiency of tryptophan between tryptophan auxotroph (*168CA*) and wild type strain. The experiment has been repeated once and the data above come from a single experiment

Table 8.4 – 8.6). The other approach (approach 2) was to compare the transcriptome of auxotroph with the wild type strain when they were grown in medium with sub-optimal concentration of amino acid (Figure 5.2B). In the case of lysine auxotroph, *Δ lysA::erm*, the transcriptomic profile of lysine auxotroph grown in lower concentration of lysine was compared to that the wild type strain grown in the same medium (LL2 VS WL2). The control comparison for this pair would be the comparison of the transcriptome of the lysine auxotroph grown in excess lysine compared to that of the wild type strain grown in the same condition (LL1 VS WL1). Similarly, the transcriptomic profile of phenylalanine auxotroph grown in limited phenylalanine was compared to that of the wild type strain grown in the same condition (PP2 VS WP2).

The control comparison was the transcriptome profile of the two strains grown in excess phenylalanine (PP1 VS WP1).

Using approach 1 for lysine auxotroph, *Δ lysA::erm*, the adjusted number of reads of each gene in sample LL2 was compared to that of in sample LL1 (LL2 VS LL1) and the comparison was repeated for two sets of experiment (s1 and s2). The comparisons with data from different sets were marked with asterisks in the table were not used for data intrepretation (Table 5.1). The same comparisons were made for the phenylalanine auxotroph, *Δ pheA:: erm* in which the auxotroph grown in limited phenylalanine was compared to that of grown in excess of this amino acid (PP2 VS PP1). And the wild type strain grown in the same conditions (WP2 VS WP1) acted as the control.

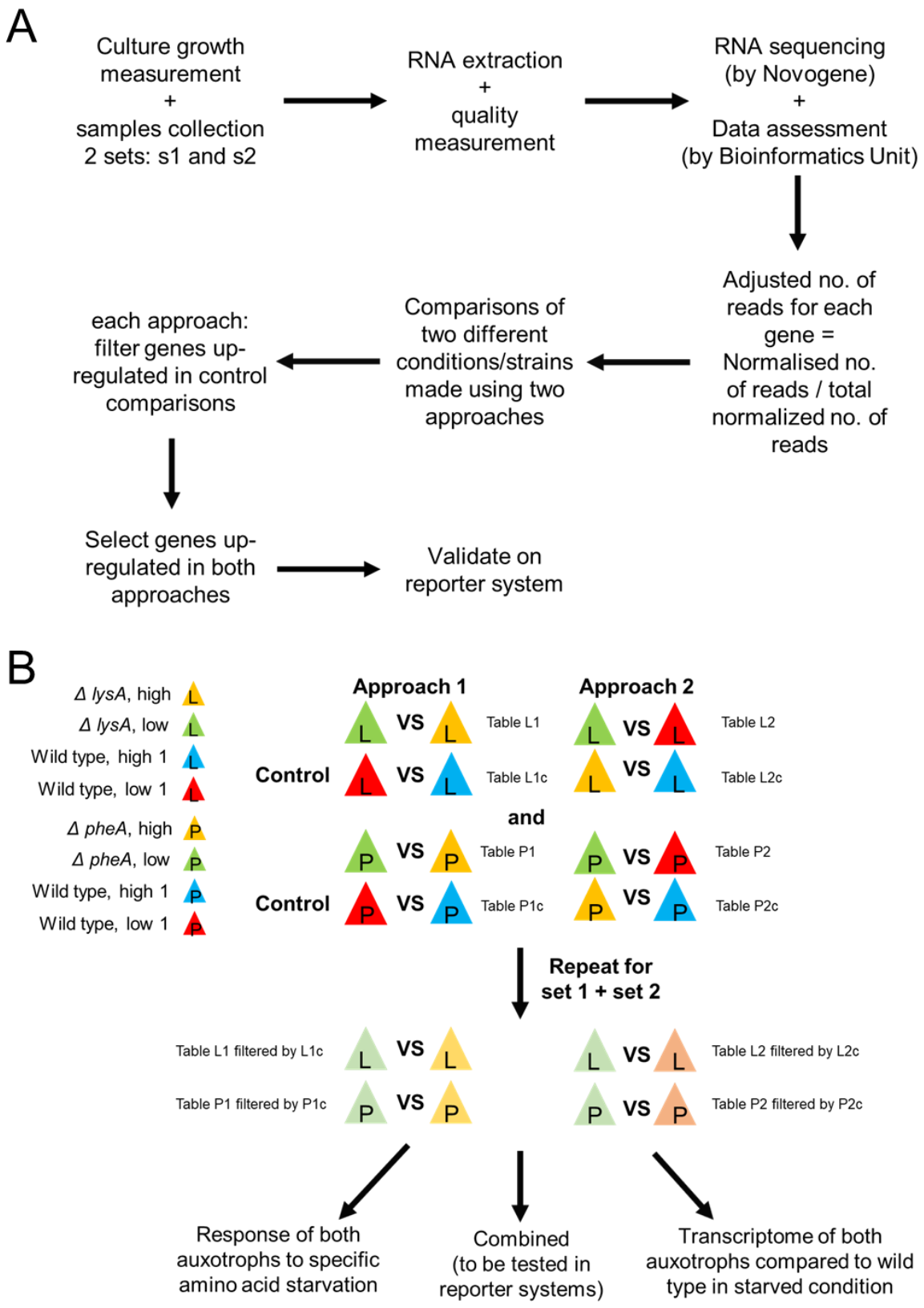


Figure 5.2: Schematic flow chart of data processing of the RNA sequencing result. A: Flowchart of RNA sequencing from samples collection to generating list of up-regulated genes in response to specific amino acid starvation. B: Schematic diagram of comparisons made between samples and selection of up-regulated genes using two approaches. Detailed description is in the text.

Table 5.1: Up-regulated genes recognised as response to specific amino acid starvation using approach 1.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
dctP	59.71	21.36	35.88	53.88	0.91	0.73	4.53	5.17
vpr	56.57	16.26	21.41	7.17	0.63	1.38	4.43	2.62
maeN	37.94	15.83	10.02	10.01	0.49	1.74	3.41	21.25
ydeK	36.63	9.29	7.95	8.44	2.05	1.13	2.53	11.60
ybgJ; glsA	30.45	21.63	13.78	54.03	38.03	0.61	1.66	4.31
yvfV;lutA	24.52	7.99	8.39	7.62	1.07	1.88	4.77	6.73
guaD	24.09	9.40	14.73	13.20	0.89	1.25	4.64	12.48
yvbY;lutC	23.06	5.96	10.67	9.02	0.90	1.55	4.59	5.24
yvfW;lutB	23.05	6.41	9.20	9.45	0.87	1.60	4.52	5.47
yxeL;snaB	22.67	9.13	6.92	23.55	3.63	2.67	2.80	1.94
thrB	22.43	5.84	8.58	6.60	1.08	1.59	4.56	4.63
sapB	21.13	15.45	10.03	7.56	1.16	0.97	3.40	6.50
gabT	19.07	11.02	9.66	24.09	1.97	2.51	3.69	3.90
ycgA	19.02	5.06	14.63	13.33	0.75	1.15	2.89	4.73
gcvT	19.01	6.40	5.14	5.67	1.10	1.51	3.74	8.48
thrC	16.37	6.08	7.59	5.58	1.35	1.79	3.99	4.41
frlM	16.09	8.36	6.61	18.93	0.95	2.83	4.27	9.01
yxeN	15.67	5.30	5.17	8.31	1.55	1.10	2.40	2.36
yubD	15.22	7.57	10.26	10.79	1.07	1.37	2.46	5.38

dppB	15.06	7.77	25.93	18.55	0.43	2.24	3.79	3.93
yjcK	14.87	13.36	17.42	15.58	1.53	2.06	3.40	5.11
yvdA	13.32	10.14	6.80	57.57	1.23	1.31	3.74	2.21
ycgS	12.29	6.51	6.34	6.20	0.91	1.04	3.44	5.83
glnT	12.24	7.83	11.35	31.03	6.62	1.01	3.00	4.60
yxeO	11.79	6.03	5.93	5.05	0.67	0.67	2.88	3.40
ycgN;putC	10.50	6.02	8.50	6.91	1.09	1.07	2.79	2.27
rapA	10.32	6.29	9.36	8.89	0.21	1.47	4.29	4.44
yhaA;sndC	9.56	6.61	5.46	7.06	1.26	1.53	3.42	4.00
aprE	9.29	5.90	8.74	6.13	0.51	1.30	3.95	2.70
yueE	8.67	14.84	7.60	6.67	1.98	1.46	3.87	6.25
yxeK	8.65	7.21	6.55	6.58	1.75	0.88	1.94	2.38
yhdX	8.60	12.61	8.69	8.88	0.17	1.03	4.27	3.47
yczJ	7.70	8.80	5.41	6.96	1.54	1.17	3.64	5.69
licR	7.44	5.69	7.42	7.94	1.75	1.92	2.65	5.17
ynfC	7.10	8.47	7.18	6.33	0.98	1.38	4.58	6.74
yhdJ	6.90	7.25	7.42	6.55	1.09	0.78	2.14	3.08
hisJ	6.66	7.17	5.37	5.52	1.77	1.49	3.67	7.23
yttP;refZ	6.53	7.04	6.33	5.96	1.40	1.21	3.24	5.11
yebD	6.35	30.01	15.90	6.82	1.19	1.13	4.64	5.80
yozE	6.19	17.08	8.79	5.44	2.12	1.16	3.44	4.32
ggt	5.55	7.26	7.39	6.58	0.43	1.60	4.19	3.60
yuiB	5.46	6.00	5.97	8.18	0.68	1.46	3.04	1.99

Genes up-regulated by 5-fold or more in both auxotrophs grown in limited amino acid that each of them required for growth compared to that of grown in excess of the amino acid (LL2 VS LL1, PP2 VS PP1). The genes also up-regulated by 5-fold or

more in the wild type control in the same conditions were excluded (WL2 VS WL1 and WP2 VS WP1). Comparisons marked with * were excluded from data interpretation.

In order to identify candidates suitable for building a reporter system in response to specific amino acid starvation the first approach used (approach 1) focused on genes up-regulated by five-fold or more in both of the auxotroph pairs (LL2 VS LL1 and PP2 VS PP1), but not the wild type strain pair comparisons (WL2 VS WL1 and WP2 VS WP1). These were expected to represent genes up regulated in response to specific amino acid starvation (Table 5.1). Genes of higher number of fold-change of its number of reads in two conditions suggested a more significant increase of gene expression in response to the starvation. The number of fold-change of each gene in the separate experiments (s1 or s2) could vary in that the number of fold-change in one experiment could be two to three times of that of the same gene in its biological replicate (Table 5.1). This might be due to the higher sensitivity of the RNA sequencing analysis for detecting low expression genes which were expected to have lower number of reads. A small change of the number of gene reads of these lowly expressed genes which could be caused by technical variation would then result in a significant number of fold changes. Referring to the 42 genes up-regulated in both comparisons of PP2 VS PP1 and LL2 VS LL1 but not their control comparisons, they were annotated with their functions and regulations with reference to the gene categories suggested by Subtiwiki (Table 5.2, Nicolas et al., 2012). Among these 42 genes, 23 of them related to cellular metabolism and the rest were either of other functions or remained poorly characterised or unknown. Within these 23 genes, more than half of them (12) were amino acid or nitrogen metabolism related, suggesting that they were for biosynthesis or acquisition of amino acids. 3 out of the 12 genes encoded transporters *dppB*, *yxeN* and *yxeO*. DppB has been identified as a peptide transporter whereas YxeN and YxeO were suggested to be transporter for S-(2-succino)cysteine (Table 5.2). Genes encoding proteins for threonine biosynthesis (*thrB* and *thrC*) were also up-regulated in both auxotrophs (Table 5.2). There were about seven genes in the list that encode proteins of carbon related functions including utilisation of organic acids, amino sugars and arabinan (Table 5.2). Also, *aprE* and *vpr*, encoding extracellular proteases were up-regulated. None of them were essential genes in *B. subtilis* genome according to Subtiwiki. In terms of transcriptional regulation, the 45 genes were grouped into 35 different

operons but only 20 of their regulation were suggested. Half of these operons were potentially regulated by CodY, CcpA or ScoC which are recognised as the known transcriptional factors involved in global responses in *B. subtilis*.

Table 5.2: Genes that were up-regulated in both comparison PP2 VS PP1 and LL2 VS LL1.

Gene name	Operon	Regulation	Function
aprE	*aprE*	*sigA, sinR, scoC, abrB, degU, codY*	extracellular alkaline serine protease (subtilisin E) for protein degradation
dctP	*dctP/dctS*	*sigA, ccpA, fur*	C4-dicarboxylate transport protein for uptake of succinate, fumurate, malate and oxaloacetate
dppB	*dppA*	*sigA, codY*	dipeptide ABC transporter (permease)
frlM	*frlB*	*sigA, codY, frlR*	aminosugar ABC transporter (permease) for uptake of sugar amines
gabT	*gabT*	*sigA, gabR*	for utilization of gamma-amino butyric acid
gcvT	*gcvT*	*gly-box*	aminomethyltransferase (glycine cleavage sys protein T) for glycine utilisation
ggt	*ggt*	glucose starvation in M9 medium	gamma-glutamyltransferase for degradation of poly-glutamate capsules
glsA/ybgJ	*glsA*	*sigA, glnL* (induced by presence of glutamine)	glutamine degradation

glnT			glutamine uptake
guaD	*guaD*	*sigA, sigB, pucR*	guanine deaminase for deamination of guanine, purine salvage, interconversion
hisJ	*hisJ*		histidinol phosphate phosphatase for histidine biosynthesis
thrB	*hom*	*codY, tnrA, thrR*	homoserine kinase for biosynthesis of threonine
thrC			threonine synthase for biosynthesis of threonine
licR	*licR*	*sigA*	transcriptional activator, PRD-type for regulation of lichenan utilisation
lutA	*lutA*	*sigA, sinR, lutR,* lactate	lactate utilisation
yvbY/lutC			unknown but for utilisation of lactate
yvfW/lutB			lactate oxidase for lactate utilisation
maeN	*maeN/yufR*	*malR*	Na^+/malate symporter
putC/ycgN	*putB*	*sigA, putR, spo0A, codY*	1-pyrroline-5-carboxylate dehydrogenase for proline utilisation
rapA	*rapA*	*sigA, spo0A, comA, codY, ccpA*	response regulator aspartate phosphatase for control of sporulation initiation
refZ/yttP	*refZ*	*ccpA, spo0A, phoP*	regulator of FtsZ, relocalisation of FtsZ ring, placement of sporulation septum
sapB	*sapB*		

sndC/yhaA	*sndC, yhfA*		sulphur compound N-deacetylase
vpr	*vpr*	*codY, phoP, lexA, dnaA*	minor extracellular serine protease
ycgA	*ycgA*	*codY*	
ycgS	*ycgS*		
yczJ	*yczJ*		
ydeK	*ydeK*		
yebD	*yebD*		
yhdJ	*yhdJ*		
yhdX	*yhdX*		
yjcK	*yjcL*		
yozE	*yozE*		
yubD	*yubD*	*yubD* or tRNA-ala	
yueE	*yueE*		
yuiB	*yuiA*		
yvdA	*yvdA*		
yxeL/snaB	*yxeK*	*sigA, cymR*	for utilisation and detoxification of S-(2-succino)cysteine
yxeN			for uptake and utilisation of S-(2-succino)cysteine
yxeO			for uptake and utilisation of S-(2-succino)cysteine
yxeK			for utilisation and detoxification of S-(2-succino)cysteine

Regulations and functions were listed according to Subtiwiki (Nicolas et al., 2012). Unknown functions or regulation of certain genes remained blank.

Table 5.3: Up-regulated genes recognised as response to specific amino acid starvation using approach 2.

	PP2 VS WP2		PP1 VS WP1		LL2 VS WL2		LL1 VS WL1	
	s1	s2* using WP2 from s1	s1	s2	s1	s2	s1* using WL1 from s2	s2
appD	11.35	3.41	1.27	0.66	32.04	17.41	0.20	1.23
yrzI	8.24	3.18	2.17	1.44	8.62	9.90	2.08	1.04
appF	7.67	2.16	1.31	0.61	30.76	10.87	0.38	1.15
yufN;nupN	6.93	5.71	1.65	0.60	21.82	41.73	0.63	0.97
ctrA;pyrG	6.71	5.01	0.63	0.31	35.05	53.81	0.17	2.13
dctP	6.56	3.96	0.83	0.38	54.18	39.66	0.82	1.35
ydfC	6.25	17.94	1.74	0.80	16.11	7.58	1.03	4.10
iolR	6.25	2.01	1.01	0.93	5.90	5.29	1.98	0.66
vpr	5.43	2.37	1.12	0.87	29.52	10.98	0.33	0.93
ybgJ;glsA	5.29	3.88	0.64	0.31	10.31	54.77	12.88	1.54
yyaO	5.24	2.40	1.70	0.52	6.73	6.37	2.78	0.95
yhzC	5.23	7.36	1.35	0.54	19.66	48.58	1.36	0.79
mmsA;iolA	5.20	1.86	1.99	1.05	5.86	6.16	1.36	1.08

Genes up-regulated in both auxotrophs relative to the wild type strain grown in limited amount of the amino acid that is essential for growth (LL2 VS WL2 and PP2 VS WP2). The genes also up-regulated by 5-fold or more in both auxotrophs relative to the wild type strain grown in excess of the amino acid required for growth were excluded (LL1 VS WL1 and PP1 VS WP1). Comparisons marked with * were excluded from data interpretation.

Table 5.4: Genes that were up-regulated in both comparison PP2 VS WP2 and LL2 VS WL2.

Gene name	Operon	Regulation	Function
appD	*appD*	*tnrA, codY, scoC*	oligopeptide ABC transporter
appF			oligopeptide ABC transporter
ctrA/pyrG	*rpoE, pyrG*	*sigA*	CTP synthase (NH3, glutamine) for pyrimidine biosynthesis
dctP	*dctP/dctS*	*sigA, ccpA, fur*	C4-dicarboxylate transport protein for uptake of succinate, fumurate, malate and oxaloacetate
iolR	*iolR*	*sigA, iolR*	transcriptional repressor for regulation of myo-inositol catabolism
iolA/ysdA	*iolA*	*sigA, iolR, ccpA*	methylmalonate-semialdehyde dehydrogenase (acylating)
vpr	*vpr*	*codY, phoP, lexA, dnaA*	minor extracellular serine protease
glsA/ybgJ	*glsA*	*sigA, glnL* (induced by presence of glutamine)	glutaminase glutamine degradation
ydfC	*ydfE?*		
yhzC	*yhzC*		
yrzI	*yrzI*	*abrB*	
yufN/nupN	*nupN*	*sigA, codY*	lipoprotein, part of guanosine transporter
yyaO	*yyaO*		

Regulations and functions were listed according to Subtiwiki (Nicolas *et al.*, 2012). Unknown functions or regulation of certain genes remained blank.

When using approach 2, only genes up-regulated in both LL2 VS WL2 and PP2 VS WP2 but not their control comparisons (LL1 VS WL1 and PP1 VS WP1) were recognised as common genes up-regulated in response to specific amino acid starvation. Unfortunately, there was no biological replicate set for this phenylalanine auxotroph comparison pair (PP2 VS WP2) due to shipment delay so only one set of data can be used (Table 5.3). Only around one third of the number of genes (13) were selected compared to that of using approach 1 (Table 5.1 and 5.3). Among these 13 genes, 9 of them were metabolism related and 3 of them encode transporters. Two of them were *appD* and *appF* which were in same operon and they encode oligopeptide transporters (Table 5.4). *dctP* encodes protein of C4-dicarboxylate transporter whilst *nupN* encodes a component which was a part of guanosine transporter. Apart from *nupO*, there were two more genes in the list, *pyrG* and *nupN*, encode proteins for acquisition of pyrine and purine (Table 5.3). For the rest of genes in the list, there are *vpr* encoding a protease and *iolR* and *iolA* related to the utilisation of inositol. In terms of transcriptional regulation, the 13 genes were of 12 different operons and there were half of the operons that were suggested to be regulated by global transcriptional factors like CcpA, CodY, ScoC and/or AbrB (Table 5.4).

Other control comparisons were done as well by comparing the transcriptome profile of the wild type strain grown in limited phenylalanine compared to that of grown in limiting lysine (WP2 VS WL2). Another control comparison was comparing the transcriptome of the wild type strain grown in excess phenylalanine and in excess lysine (WP1 VS WL1) as well as a control comparison of the transcriptome of the wild type strain grown in low concentration of phenylalanine and in low concentration of lysine (WP2 VS WL2). But no significantly changed genes were found under the current selection criteria as others.

Despite the different aspects of selecting genes that were significantly changed transcriptionally in response to specific amino acid starvation, there were genes shown up-regulated in both approaches. There were three genes shown up-regulated in both approaches, *dctP*, *glsA* and *vpr* (Table 5.2 and Table 5.4) but the suggested transcription regulation of each of them was different in that no common regulators were found (Table 5.1 and 5.2). Yet many genes from all four tables in general were suggested to be under the regulation of the global transcriptional factors, *e.g.* CodY and/or CcpA, either directly or indirectly.

5.2.2 Validation of the transcriptional data using a lacZ reporter system

Apart from the possible candidates provided in the previous section, Eymann *et al*. suggested a pool of genes that were up-regulated in response to the addition of DL-norvaline into the medium, which mimicked the starvation of isoleucine and leucine inside the cell (Eymann *et al.*, 2002). Candidates of genes, which were up-regulated in response to supposedly an isoleucine and leucine starvation due to stringent response, were selected from the mentioned study (Eymann *et al.*, 2002). The genes included *vpr*, *yvyD* and *gspA* in which the protein for mediating ribosome dimerisation (ribosome hibernation promoting factor) encoded by *yvyD* was said to be the most abundant protein induced in its category. Notably, *vpr* was also a possible candidate suggested from our RNA sequencing analysis (Section 5.2.1). *gspA* was supposedly sigma B dependent, which should be independent of stringent response (Antelmann *et al.*, 1995; Eymann *et al.*, 2002). On the other hand, *tnrA* was also included as one of the possible candidates because it was suggested to be expressed in condition with good nitrogen source and was once shown up-regulated by ammonium starvation (Tam *et al.*, 2006). Promotor regions of these selected genes were independently inserted into vector RD62, upstream of *lacZ* gene, creating a reporter vector which would be transformed into wild type strain for a blue/white colony screen. The strains carrying different reporter vectors would be grown in different medium condition and the strength of expression of the corresponding genes would be reflected in the blueness of colonies. The level of up-regulation of the selected genes in the RNA sequencing in response to specific amino acid starvation was assessed. Genes *yvyD* and *vpr* were of more significant up-regulation in the auxotrophs grown in limited amino acid that the auxotroph required for growth compared to the other two selected genes from literature regardless of the approaches used (Table 5.5 and 5.6). *tnrA* was of no significant up-regulation in all tested comparisons of the set conditions.

It was expected that the regulation region of a gene was usually mediated through their own promotor binding position and RBS binding position which are supposedly mostly located upstream of the starting codon of a gene. Hence in order to test the promotor activity of the selected genes, the DNA fragments covering the upstream of the selected gene(s) (*gspA*, *yvyD*, *vpr* and *tnrA*) were independently fused to *lacZ* which encodes β-galactosidase in vector RD62 (Section 2.4 – 2.9 , 2.11 – 2.12). The insertion of regulatory region of *gspA*, *tnrA*, *vpr* and *yvyD* inserted into vector pRD62,

a promotor probe plasmid, resulted in pMC1 (P_{gspA}-*GspA-RBS-lacZ cat*), pMC2 (P_{tnrA}-*TnrA-RBS-lacZ cat*), pMC3 (P_{vpr}-*Vpr-RBS-lacZ cat*) and pMC4 (P_{yvyD}-*YvyD-RBS-lacZ cat*) respectively and transformed into *B. subtilis* wild type strain (168+), resulting in strain MC21, MC22, MC23 and MC24 respectively (Daniel, Williams and Errington, 1996).

Table 5.5: Fold change of selection of genes in response to specific amino acid starvations using approach 1.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
yvyD	3.04	5.93	5.12	6.58	1.70	1.28	3.86	1.29
tnrA	1.64	1.76	1.39	0.99	0.37	0.90	2.06	1.46
vpr	56.57	16.26	21.41	7.17	0.63	1.38	4.43	2.62
gspA	1.38	2.52	4.02	5.36	0.86	0.93	2.97	0.97

The changes of selected genes in both auxotrophs grown in limited amount of the amino acid that is essential for growth relative to that of grown in excess. Comparisons marked with * were excluded from data interpretation.

Table 5.6: Fold change of selection of genes in response to specific amino acid starvations using approach 2.

	PP2 VS WP2		PP1 VS WP1		LL2 VS WL2		LL1 VS WL1	
	s1	s2* using WP2 from s1	s1	s2	s1	s2	s1* using WL1 from s2	s2
yvyD	2.13	2.36	1.61	0.46	2.48	3.85	1.38	0.83
tnrA	1.18	0.87	1.75	1.28	2.12	1.59	0.47	0.82
vpr	5.43	2.37	1.12	0.87	29.52	10.98	0.33	0.93
gspA	1.60	2.80	1.18	0.51	1.94	2.52	1.21	0.93

The changes of selected genes in both auxotrophs grown in limited amount of the amino acid that is essential for growth relative the wild type strain grown in same medium. Comparisons marked with * were excluded from data interpretation.

To identify genes to be up-regulated in response to amino acid starvation when the *B. subtilis* strains were grown on plates, all of the reporters strains (MC21 – MC 24) were then grown in the same medium containing glucose, solution P and X-gal with and without amino acids mixture in SMM buffer (GMaaP-X-gal and GMP-X-gal

respectively) at 37°C for 48 hours (Section 2.1). They would be screened for blue/white colonies where a bluer colony indicates a more significant upregulation of gene(s) selected within a period of incubation time (Section 2.18). When the strains were grown in the absence of amino acid mix, all reporter strains grew as blue colonies (Figure 5.3B and D). The strain with the promotor region of *yvyD* in the plasmid (MC24) produced darkest blue colonies (top left quadrant in Figure 5.3B) and the colonies were bluer than those of grown in the medium with amino acid mix (GMaaP-X-gal) as the blue pigment seemed to have leaked out of the colonies into the agar (top left quadrant in Figure 5.3A). Similarly, the strain with the regulatory region of *vpr* (MC23) was slightly bluer colonies in medium without amino acid mix (GMP-X-gal) (top left quadrant in Figure 5.3D) than those of grown with the mix (medium GMaaP-X-gal) (top left quadrant in Figure 5.3C). This indicated that the two genes were up-regulated in response to amino acid starvation on plates. However, the wild type reporter strain of *yvyD* (MC24) and *vpr* (MC23) presented as blue colonies even when the amino acid mixture was supplied after two days of 37°C incubation (top left quadrant in Figure 5.3A and C). The reporter strain of *yvyD* (MC24) had shown slightly bluer colonies compared to that of *vpr* (left of Figure 5.3A and C). Together these indicated that *yvyD* and *vpr* were up-regulated during growth on the defined rich medium plate (Figure 5.3A). This expression was less significant compared to that obtained when the strains were grown in the absence of amino acid mix. Interestingly, the reporter construct with the regulatory region of *gspA* (MC21) produced significantly bluer colonies when the strain was grown in the absence of the amino acid mix (top right quadrant in Figure 5.3B) compared to that of growth with the amino acid mix present (GMaaP-X-gal) (top right quadrant of Figure 5.3A). This suggested a significant up-regulation of the gene when the strain was grown in the absence of amino acids. In contrast, strain MC22 with the regulatory region of *tnrA* in the reporter system, had pale blue colonies when they were grown in the medium without amino acid mix (GMP-X-gal) (top right quadrant in Figure 5.3D) whereas no blue colour was present when grown in the presence of amino acid mix (top right quadrant in Figure 5.3C). This indicated that the gene was also up-regulated when the strain was grown in the medium without amino acid mix (GMP-X-gal) compared to that of grown in the medium with the mix (medium GMaaP-X-gal). But the up-regulation of this gene was less significant compared the other genes tested in the given conditions.

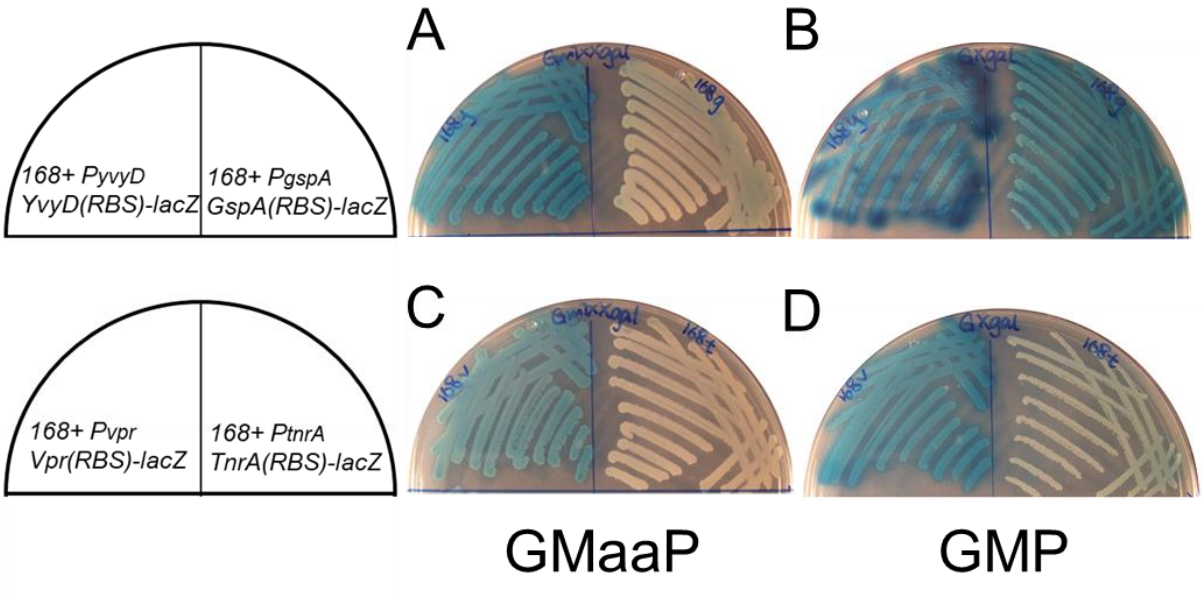


Figure 5.3: Characterisation of the expression of selected stringent response related genes. The reporter strains (MC21 – 24) were grown in either with or without amino acid mix at 37°C for 48 hours. (A) Strain MC21 (P_{gspA}-*GspA-RBS-lacZ cat*) and MC24 (P_{yvyD}-*YvyD-RBS-lacZ cat*) grown on the defined rich medium (medium GMaaP-Xgal) plates. (B) Strain MC21 (P_{gspA}-*GspA-RBS-lacZ cat*) and MC24 (P_{yvyD}-*YvyD-RBS-lacZ cat*) grown on minimal medium (medium GMP-Xgal) plates. (C) Strain MC22 (P_{tnrA}-*TnrA-RBS-lacZ cat*) and MC23 (P_{vpr}-*Vpr-RBS-lacZ cat*) grown on defined rich medium (medium GMaaP-Xgal) plates. (D) Strain MC22 (P_{tnrA}-*TnrA-RBS-lacZ cat*) and MC23 (P_{vpr}-*Vpr-RBS-lacZ cat*) grown in minimal medium (medium GMP-Xgal).

Reporter strains of *yvyD* and *gspA* had shown the largest colour difference when they were grown in the two different conditions. This suggested that the up-regulation of *yvyD* and *gspA* was more significant than that of *vpr* or *tnrA* when the strains were grown on minimal medium plates, potentially a stronger response of these genes towards amino acid starvation hence better candidates for developing reporter systems for specific amino acid transporters.

5.2.3 Characterisation of yvyD reporter system for amino acid transporter screening

To test the functionality of the transcription changes of *yvyD* in a reporter system for amino acid limitation, we exploited the previously characterised *ΔalaT::erm* (MC1) and *ΔalaT ΔalaP::erm* (MC8) strains as a positive control model for an amino acid auxotroph with its identified major transporter, pMC4 (P_{yvyD}-*YvyD(RBS)-lacZ cat*) was transformed into the two strains, MC1 and MC8. The resultant strains, MC29 (*ΔalaT::erm* P_{yvyD}-*YvyD(RBS)-lacZ cat*) and MC30 (*ΔalaT ΔalaP::erm* P_{yvyD}-*YvyD(RBS)-lacZ cat*) were then compared with the wild type strain with the same

reporter construction MC24 (P_{yvyD}-*YvyD(RBS)-lacZ cat*) grown in two different medium conditions at 37°C for 48 hours (Section 2.18). Similar to Section 5.2.1, one medium condition was adding excess amino acid needed for growth, in this case 1.25 mM L-ala and the other condition was adding sub-optimal concentration of the amino acid (0.5 mM L-ala) in the base medium GMaaP which contains glucose, amino acid mix (except alanine) and solution P in SMM buffer (Section 2.1).

In the alanine limited condition, the double mutant reporter strain (MC30) had shown a more significant blue colour and with poorer growth compared to the auxotroph and wild type reporter strains (MC29 and MC24) (top left quadrant in Figure 5.4B). MC29 and MC24 seemed to share similar blue colour colonies (bottom and top right quadrant in Figure 5.4B). This suggested that *yvyD* could be up-regulated even in the wild type background when the reporter strain was grown in the alanine limiting condition which agreed with the result from previous section. Apparently the auxotroph background did not increase much of the cellular demand of alanine, hence similar up-regulation *yvyD* as the wild type reporter strain. Only the deletion of both the main alanine transporter and the main alanine biosynthetic pathway of the reporter strain had suggested a significant higher cellular demand of alanine.

When the double mutant reporter strain (MC30) was grown in excess alanine condition, it showed significantly paler blue colonies with normal growth compared to that of grown in lower concentration of this amino acid (top left quadrant of Figure 5.4A). Similarly, the other two reporter strains (MC29 and MC24) also showed pale blue colonies with normal growth when the strains were grown in the presence of excess alanine (top right and bottom quadrant in Figure 5.4A). Nevertheless, all of these reporter strains (MC24, MC29 and MC30) showed paler blue colonies with normal growth when it was grown in excess alanine condition (Figure 5.4A). This indicated that the reduced amount of alanine resulted in the up-regulation of gene *yvyD*. But *yvyD* could be up-regulated regardless of the tested conditions or the background of reporter strain (cellular demand of alanine). As the growth of the two strains (MC29 and MC24) seemed to be similar in both conditions, this might imply the reporter system of *yvyD* provided a more sensitive approach of screening for major amino acid transporter(s) (top right and bottom in Figure 5.4A and B).

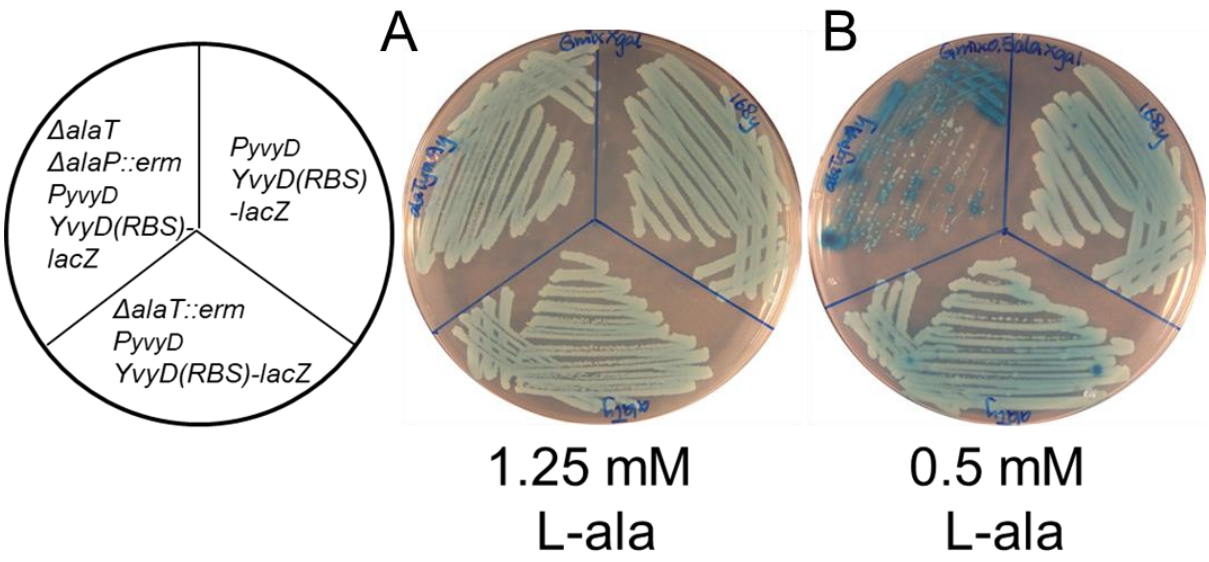


Figure 5.4: Characterisation of the *yvyD* reporter system on positive control model of amino acid transporter screening on plate. Reporter strains carrying pMC4 (P_{yvyD}-*YvyD(RBS)-lacZ cat*) in different background were grown in two different conditions to test on the sensitivity of the *yvyD* reporter system for screening amino acid transporter on plate. Strains MC29 (*ΔalaT::erm* P_{yvyD}-*YvyD(RBS)-lacZ cat*), MC30 (*ΔalaT ΔalaP::erm* P_{yvyD}-*YvyD(RBS)-lacZ cat*) and MC24 (P_{yvyD}-*YvyD(RBS)-lacZ cat*) were grown on GMaaP-Xgal plate added with either (A) 1.25 mM L-ala or (B) 0.5 mM L-ala at 37°C for 48 hours.

Together these have shown that the up-regulation of *yvyD* could be utilized as a reporter system for our specific amino acid transporter screening on plate. But the screening could only be done when the sub-optimal concentration of amino acid for the auxotroph was known. The significant up-regulation of *yvyD* only appeared when there was lower alanine in abundance in the medium. When the concentration of alanine in the medium was high enough, the cellular metabolism is presumably able to compensate for the loss of the major enzyme for synthesizing alanine through extracellular assimilation.

5.2.4 Specificity of tRNA synthetases in response to specific amino acid starvation

In the global transcriptomic study, lysine or phenylalanine specific up-regulated genes were filtered during the search of a common set of genes for specific amino acid starvation. Nevertheless, up-regulation of genes specific to the amino acid of the auxotroph depending on for growth were identified using approach 1 (**Table 8.5** and 8.4). In response to lysine specific starvation, *lysC* and *yvsH* were significantly up-regulated in the lysine auxotroph but not in the wild type strain grown in limited lysine compared to that of grown in excess lysine (**Table 8.5**). These two genes were both

suggested to be under L-box riboswitch regulation in which its specificity to lysine was recognized (Rodionov *et al.*, 2003; Wilson-Mitchell, Grundy and Henkin, 2012). For the phenylalanine pair, two genes recognized to be regulated by phenylalanine specifically by T-box riboswitch, *pheS* and *pheT* were shown up-regulated when the auxotroph and wild type strain were grown in limited phenylalanine compared to that of grown in excess (**Table 8.7**). These suggested the sensitivity of cells towards outer environment and that was demonstrated on the transcriptional level. To explore the possibility of utilizing these characteristics in the development of a transporter screening reporter, *tyrS*, which was the most studied T-box riboswitch candidate in *B. subtilis*, was selected to expand the study.

As mentioned previously, the specific codon, in the leading region between position +99 to +101 within the regulatory region of *tyrS*, was responsible for the amino acid specificity of *tyrS* responding to starvation of tyrosine tRNA inside the cell (Henkin, Glass and Grundy, 1992; Grundy *et al.*, 1997). To test if this specificity would contribute to the specificity of the specific amino acid transporters screening on plate, regulatory region of *tyrS* was fused into vector pRD62 resulting in pMC5 (P_{tyrS}-*TyrS(RBS)-lacZ cat*). The vector pMC5 was then transformed into *B. subtilis* wild type strain, creating MC31. Then as suggested by Grundy *et al*., the specifier region of *tyrS* was modified by site-directed mutagenesis in order to change the target of the specifier region of gene *tyrS* from tyrosine tRNA to other amino acids including alanine and leucine tRNA (Grundy and Henkin, 1993). In other words, the specifier codon (GTA) of *tyrS* was changed to ‘GAG’ specifying leucine, ‘TGC’ and ‘GGC’ for alanine and ‘TAA’, a stop codon as a negative control, creating vectors pMC9, pMC7, pMC8 and pMC6 respectively (Section 2.4 – 2.9 , 2.11 – 2.13). These vectors were independently transformed into *B. subtilis* wild type strain, resulting in MC35, MC33, MC34 and MC32 respectively. These strains were then grown on the defined rich medium (GMaaP) and in the defined rich medium with either lower tyrosine (GMaaP_tyr-l) or alanine (GMaaP_ala) concentrations. The strains were also grown in a condition where higher concentration of tyrosine was provided into the defined rich medium (GMaaP_tyr-h) incubated at 37°C for 48 hours (Section 2.18). Also, the strains were grown in minimal medium plate (GMP) in parallel. X-gal was added to all the plates to allow screening for differences in expression according to the degrees by which blue color was developed.

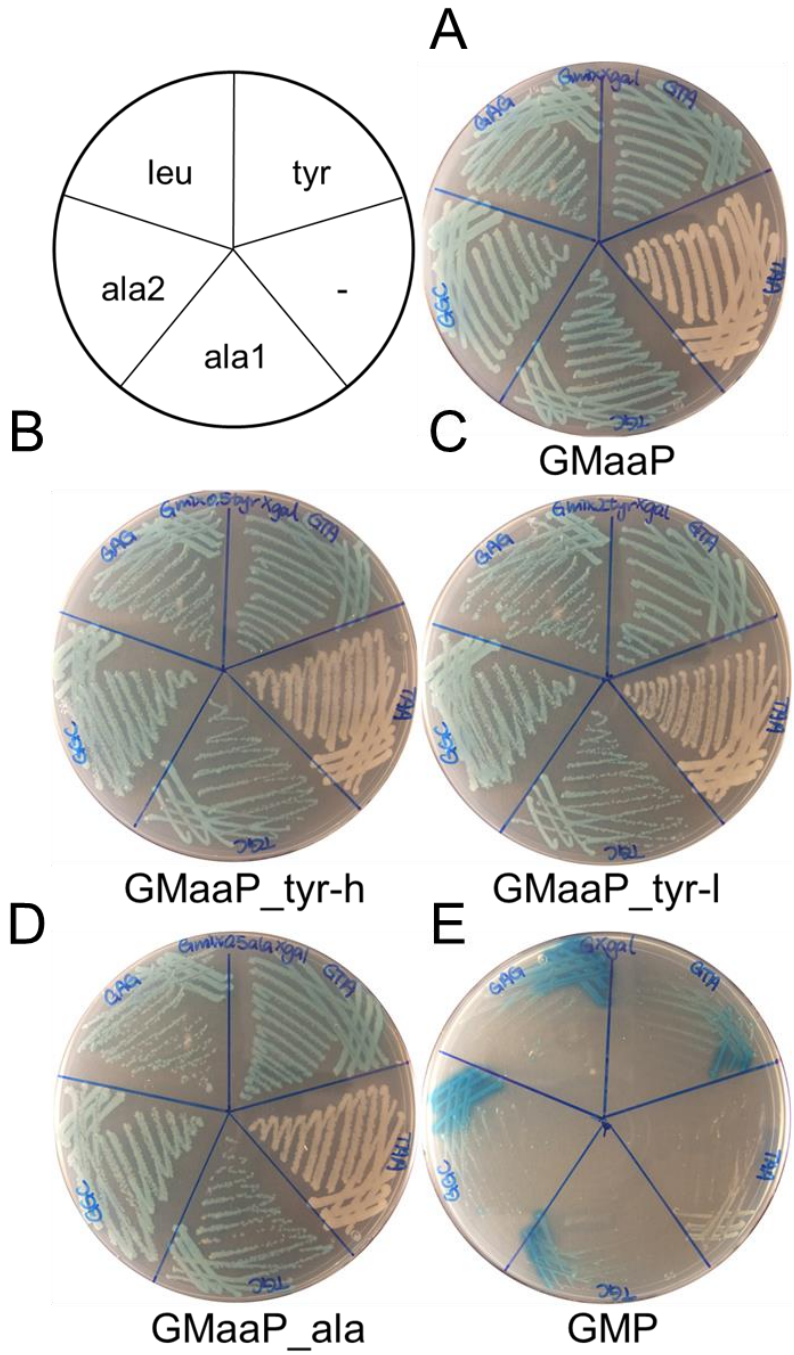


Figure 5.5: Characterisation of the reporter systems controlled by specifier codon of riboswitch controlling transcription of *tyrS* targeting tRNA of different amino acids on plate. Reporter systems with the specifier codon of *tyrS* targeting tRNA of different amino acids including alanine and leucine were transformed into the wild type strain background. Strain targeting a stop codon in the wild type strain background was also included as negative control. The strains were grown on plates containing different variations of medium at 37°C for 48 hours. tyr = MC31 targeting tyrosine tRNA; - = MC32 targeting stop codon; ala1 = MC33 targeting alanine tRNA; ala2 = MC34 targeting alanine tRNA; leu = MC35 targeting leucine tRNA. GMaaP = with all amino acids at 1.25 mM; GMaa_tyr-h = with all amino acids at 1.25 mM except tyrosine at 2 mM; GMaa_tyr-l = with all amino acids at 1.25 mM except tyrosine at 0.5 mM; GMaa_ala = with all amino acids at 1.25 mM except alanine at 0.5 mM; GMP = with no amino acid mixture.

When MC31 (P_{tyrS}-*TyrS(RBS)-lacZ cat*) was grown in the defined medium containing 1.25 mM tyrosine, the colonies turned pale blue after 48 hours of incubation at 37°C (GTA section in Figure 5.5A). There was no significant difference in growth or colour when the strain was grown in higher (2 mM) or lower (0.5 mM) concentration of tyrosine in the medium (GMaaP_tyr-h and GMaaP_tyr-l) (GTA in Figure 5.5B and C). This suggested that the reporter of *tyrS* was not sensitive to the different concentrations of tyrosine in the medium. Also, this might be due to the lower cellular demand of tyrosine as this amino acid has been shown remained abundant in the medium during exponential growth in Section 3.2.2). The strain was grown in lower alanine concentration (0.5 mM) in which alanine was shown efficiently assimilated in the same previous analysis in Section 4.2.7. But the strain obtained similar pale blue color colonies with similar growth as that of in other conditions mentioned (GTA section in Figure 5.5D). This suggested that the cellular demand of tyrosine could be similar for the wild type reporter strain grown in lower alanine concentration (0.5 mM) condition. Only when the strain was grown in minimal medium (medium GMP), the growth was poor and was showing more significant blue colored colonies (GTA section in Figure 5.5E).

When the specifier codon of *tyrS* was changed to alanine tRNAs (MC33 and MC34) and grown under alanine limiting condition (GMaaP_ala) (TGC and GGC section in Figure 5.5D), the two strains were of pale blue and were of similar colour to each other. There was no observable colour difference when the reporter strains were grown in defined rich medium, which contained higher concentration of alanine (GMaaP) (TGC and GGC in Figure 5.5A). This suggested that the reporter targeting alanine tRNA was not sensitive to the abundance of alanine, which agreed to that of when the specifier codon has not been changed. The pale blue colonies were similar to that of the reporter strain targeting tyrosine tRNA (MC31) in all tested conditions except minimal medium (GMP) (GTA, TGC, GGC in Figure 5.5A – D). Similar to the original reporter strain with specifier codon for tyrosine tRNA, although the strains exhibited poor growth and were grown into more significant blue colonies and shared similar shades of blue (GTA, TGC, GGC in Figure 5.5E). Interestingly, one of the reporter strains (MC34) seemed to show slightly more significant blue colonies than MC33 even they were supposed to target the tRNA of the same amino acid (TGC and GGC section in Figure 5.5E). This could be due to the relatively more abundant variances of uncharged alanine tRNA with anticodon GGC rather than TGC.

When the specifier codon of *tyrS* altered for leucine tRNA (MC35), the colonies were of similar pale blue colour in all tested conditions except minimal medium (GMP). This was expected as the concentration of leucine in those conditions was the same (1.25 mM). This reporter strain had shown slightly more significant blue colonies than the other reporter strains when it was grown in the absence of amino acid mix (GMP) (Figure 5.5E). This suggested that there could be a more significant starvation of leucine compared to tyrosine or alanine. The reporter strain as the negative control (with the specifier codon targeting a stop codon) remained as white colonies in all tested conditions, which was expected ('-' in Figure 5.5A - E).

To conclude, the reporter strains grown in different variations of defined rich medium were grown to similar shades of blue colonies (Figure 5.5A – D). This suggested that the upregulation of tRNA synthetase was likely to be caused by a more universal factor to all involved strains. It was possible the rate of protein synthesis decreased during the later stage of growth on plate, leaving the tRNAs of different amino acids uncharged. But the observation could also be due to the prototrophic nature of the wild type strain in that it can generate the amino acids by itself with no constraints.

5.2.5 Testing of the reporter system using T-box switch from tyrS on positive control model of transporter screening

To test if this *tyrS* reporter system could be applied in the specific amino acid transporter screen on plate, the reporter system of *tyrS* targeting uncharged tyrosine tRNA (pMC5) was transformed into *ΔalaT::erm* (MC1), *ΔalaP::erm* (MC7) and *ΔalaT ΔalaP::erm* (MC8), creating MC36, MC51 and MC52 respectively (Section 2.4 – 2.9 , 2.11 – 2.13). The resultant strains (MC36, MC51 and MC52) were then grown in parallel with the wild type reporter strain (MC31) in two medium conditions on plate at 37°C for 24 hours (Section 2.18). One condition was the defined rich medium (GMaaP) where excess amount of alanine (1.25 mM) was present whereas the other condition was medium with reduced concentration of alanine (0.5 mM) (GMaaP_ala). All plates were added X-gal for blue/white colonies screening.

The reporter strains MC40, MC51 and MC52 had shown blue colonies at a similar level whilst the wild type reporter strain (MC31) has shown white colonies when they were grown in excess concentration of alanine (1.25 mM) (GMaaP) (Figure 5.6A). However, strain MC52 (*ΔalaT ΔalaP* P_{tyrS}*-TyrS(RBS)-lacZ cat*) had impaired growth and exhibited significant blue colonies when only 0.5 mM alanine was present in the medium with other amino acids (GMaaP_ala) (top left quadrant in Figure 5.6B). The

growth of strain MC40 (*ΔalaT* P_{tyrS}*-TyrS(RBS)-lacZ cat*) and strain MC51 (*ΔalaP* P_{tyrS}*-TyrS(RBS)-lacZ cat*) was of similar growth but strain MC40 had shown a slightly bluer colonies when the two strains were grown in the presence of limited alanine (bottom quadrants in Figure 5.6B). The reporter in the wild type background (MC31) remained white (top right quadrant in Figure 5.6B). Together the results suggested that this reporter system with T-box switch regulation of *tyrS* was applicable in specific amino acid transporter screening on plate in our defined rich medium. The sensitivity of this reporter system seemed to be higher than the *yvyD* reporter in Section 5.2.3. But the medium condition and the incubation time for strains have to be better controlled in order to obtain an indicative phenotype. This matched with the suggestions from last section where reporter systems of *yvyD* were characterized (Section 5.2.3).

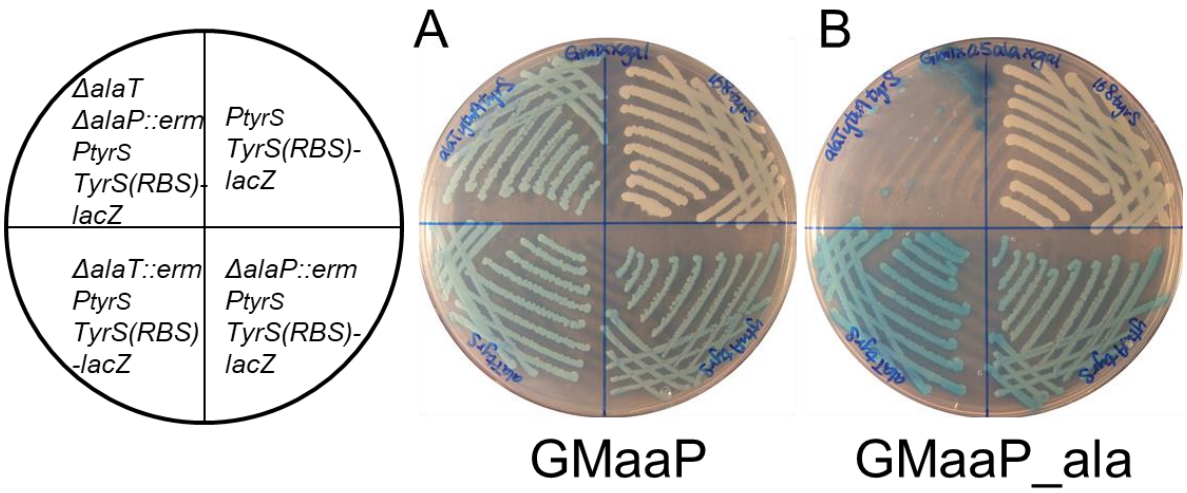


Figure 5.6: Characterisation of the reporter system of specifier codon of *tyrS* targeting tRNA on positive control model of amino acid transporter screening on plate. Reporter strains carrying pMC5 (P_{tyrS}*-TyrS(RBS)-lacZ cat*) in different background were grown in two different conditions to test on the sensitivity of the *tyrS* reporter system for screening amino acid transporter on plate. Strains MC31 (P_{tyrS}*-TyrS(RBS)-lacZ cat*), MC36 (*ΔalaT* P_{tyrS}*-TyrS(RBS)-lacZ cat*) and MC50 (*ΔalaP* P_{tyrS}*-TyrS(RBS)-lacZ cat*) and MC52 (*ΔalaT ΔalaP::erm* P_{tyrS}*-TyrS(RBS)-lacZ cat*) were grown on GMaaP plate added with either (A) 1.25 mM L-ala or (B) 0.5 mM L-ala at 37°C for 24 hours.

5.2.6 Improvement of reporter system with specifier codon targeting the amino acid

The T-box switch regulation was shown applicable in the reporter system. To test if a matched target of tRNA of an amino acid by the specifier codon might improve sensitivity of the reporter system in the screening of the specific transporter of that amino acid, the reporter system with the specifier codon targeting either tyrosine or

alanine as well as the negative control (pMC5 – 8) were separately transformed into *ΔalaT::erm* (MC1) and *ΔalaT ΔalaP::erm* (MC8), creating strains MC36 – MC39 and MC52 – MC55 respectively. The strains with different reporter systems were grown until they reached exponential phase in which the cells were collected and then washed and grown on plates of different variations of the defined rich medium (GMaaP, GMaaP_ala and GMaaP_tyr-l) at 37°C for 24 hours (Section 2.1 , 2.4 – 2.9 , 2.11 – 2.13 , 2.18). For the defined rich medium (GMaaP), there was 1.25 mM of amino acids mix present but with a reduced concentration of tyrosine (0.5 mM) in (GMaaP_tyr-l) and another variation containing 0.5 mM alanine (GMaaP_ala).

When the wild type reporter strains were grown in the defined rich medium (GMaaP), the strains MC31, MC33 and MC34, all exhibited blue color, except the negative control (MC32) (first row in Figure 5.6A). Both MC31 and MC33 displayed a darker blue spot with a ring of lighter blue whilst those of MC34 were of a lighter blue inner spot with an even lighter ring (first row in Figure 5.7A). These suggested that the up-regulation of this *tyrS*, either specifying alanine or tyrosine, was more significant for the cells grown within the inner spot, possibly due to nutrient starvation among the inner colonies. It also suggested that specifier codon targeting the anticodon TGC of alanine tRNA might be more abundant or a more dominant one responsible for the binding as it showed bluer colonies than that of targeting anticodon GGC. This was supported by a similar observation obtained when strain MC33 and MC34 were grown in defined rich medium with less alanine or tyrosine (MC33 and MC34 in Figure 5.7B and C).

With respect to response towards specific amino acid starvation, the reporter system of the specifier codon targeting anticodon GGC of alanine tRNA in either wild type (MC34), alanine auxotrophic (MC39) or double mutant background (MC55) was shown to develop bluer colour only when it was grown in the presence of lower concentration of alanine (0.5 mM) (GMaaP-ala) (MC34, MC39, MC55 in Figure 5.7A - C). And when the reporter was in the double mutant background (MC55), the strain grew with a very significant blue colour only in the alanine-limiting condition (GMaaP-ala) and it showed the most significantly blue colonies compared to MC34 and MC39 (MC55 in Figure 5.7B). When these strains (MC34, MC39, MC55) were grown in tyrosine-limiting condition (GMaaP_tyr-l), they showed similar degree of blue colour as that of grown in the defined rich medium (GMaaP) regardless of the background of the reporter strains (MC34, MC39 and MC55 in Figure 5.7A and C). However, no

significant difference observed for reporter system with the specifier codon targeting anticodon TGC in either wild type or alanine auxotrophic background (MC33 and MC38) (MC33 and MC38 in Figure 5.7A - C). But strain MC54, carrying reporter system targeting anticodon TGC in the double mutant background, was shown to develop a significant blue colour similar to that of MC55 when they were grown in alanine-limiting condition (GMaaP_ala) (MC54 in Figure 5.7B). All three of these reporter strains (MC33, MC38 and MC54) did not show significant difference when they were grown in lower concentration of tyrosine compared to that obtained on defined rich medium (GMaaP). Together these suggested that the reporter system targeting anticodon GGC (pMC8) might be more sensitive to the concentration of alanine in medium. This matched with the suggestion from Section 5.2.5 where reporter strain targeting GGC (MC55) was shown have a slightly more significant blue colour compared to MC54 targeting TGC when they were grown in minimal medium without amino acid mix (GMP). For the reporter system targeting tyrosine tRNA (pMC5), it had shown slightly more significant blue coloour when it was in the double mutant background (MC52) compared with that of in wild type or alanine auxotroph background (MC31 and MC36) when they was grown in lower concentration of alanine (GMaaP_ala) (MC52 in Figure 5.7B). The strain had also shown a darker blue colour when it was grown with less alanine (medium GMaaP_ala) compared to that obtained in the other two conditions with a higher concentration of alanine (medium GMaaP and medium GMaaP_tyr-l) (MC52 in Figure 5.7A – C). This matched with the result from last section where the reporter system was shown applicable in our designed specific amino acid transporter screening on plate. Reporter strains acting as the negative control (reporter system targeting stop codon TAA) (MC32, MC37 and MC53) remained white colonies under most conditions tested except when it was in the double mutant background (MC53) and grown in alanine-limited condition (GMaaP_ala) (MC53 in Figure 5.7B). This suggested the possibility of tRNA mismatches when demand of certain amino acids increased in the cells.

Since all strains except the three negative control reporter strains (MC32, MC37 and MC53) have turned into blue colonies in all tested conditions. This suggested the up-regulation of the reporter systems was likely caused by some common factors in bacterial colony formation on plate, which was similar to that suggested for reporter systems using *yvyD* (Section 5.2.3). This could be related to the shortage of amino

acids, possibly a competition of amino acids for cellular metabolism or protein synthesis. The use of reporter system of ribo-switch of *tyrS* could be useful in the designed specific amino acid transporter screening. With the specifier codon targeting matched tRNA of the amino acid with the amino acid transporter screening of interest, the sensitivity of the reporter system might be increased. Nevertheless, concentration of the amino acid of interest in the defined rich medium would need to be fine-tuned for the transporter screening.

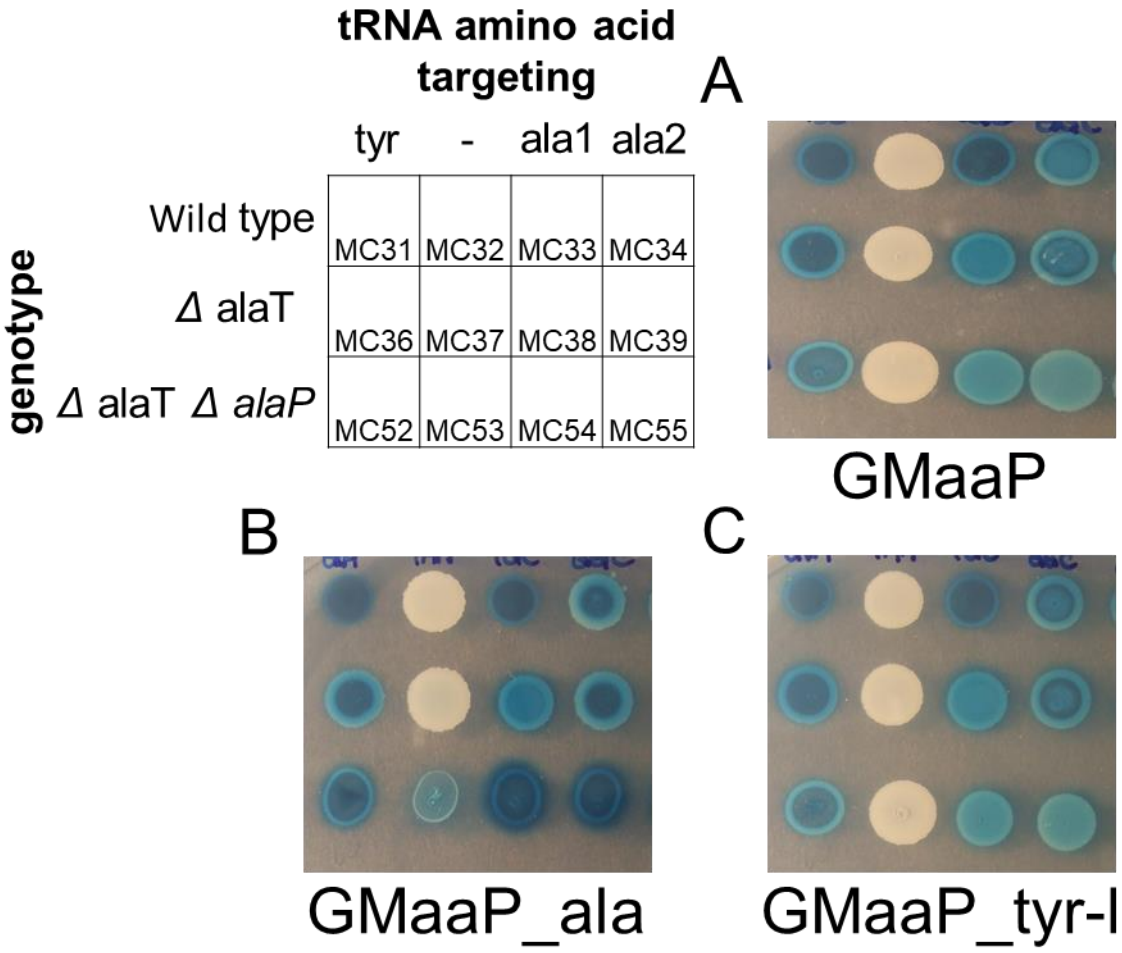


Figure 5.7: Characterisation of the reporter system targeting tRNA of two different amino acids on positive control model of amino acid transporter screening on plate. Reporter systems of the specifier codon targeting either tyrosine or alanine tRNAs in wild type, alanine auxotrophic (*ΔalaT::erm*) and double mutant (*ΔalaT ΔalaP::erm*) background were grown in variation of defined rich medium at 37°C for 24 hours. Strains were grown in either (A) defined rich medium, the medium with lower concentration of (B) alanine (0.5 mM) or (C) tyrosine (0.5 mM). (A) GMaaP= with all amino acids at 1.25 mM. GMaaP_ala = with all amino acids at 1.25 mM except alanine at 0.5 mM. GMaaP_tyr-l = with all amino acids at 1.25 mM except tyrosine at 0.5 mM. Schematic diagram showing plan of reporter strains on plates A – C.

5.2.7 Use of transcriptomic data to screen for possible specific transporters

One of the other uses of the transcriptome analysis in Section 5.2.1 was to identify potential transporters specific to an amino acid. The assumption was that when the auxotroph of an amino acid was grown in limited amount of the amino acid it needs for growth, transporter for the needed amino acid would be up-regulated.

With reference to the transcriptome data, approach 1 was used to filter up-regulated genes specific to lysine starvation on the lysine auxotroph, *ΔlysA::erm*, by comparing its transcriptome when it was grown in limited lysine to that of in excess (LL2 VS LL1) (Figure 5.2, Section 5.2.1). And its control, the comparison made for the wild type strain grown in the same condition, was WL2 VS WL1. All of these genes up-regulated specifically to lysine starvation were not up-regulated in the comparisons for the phenylalanine auxotroph (PP2 VS PP1) and its corresponding control (WP2 VS WP1). 36 genes were found up-regulated by 5-fold or more when the auxotroph was grown in limited lysine compared to that of grown in excess (LL2 VS LL1). But these genes in the wild type strain (WL2 VS WL1) were of less than 5-fold difference when the strain was grown in two different concentrations of lysine (Table 8.1). 4 out of these 36 genes encoded transporters in which one of them, *tcyN*, encodes cystine ABC transporter (Table 5.7). Two out of the four genes (*ydeG* and *yfmI*) were categorised as encoding transporters whereas the remaining one (*ycxC*) was categorised as membrane proteins (Table 5.7). *yvsH* was up-regulated by 5-fold or more when the strains (lysine auxotroph and wild type strain) were grown in limited lysine compared to that of grown in excess (**Table 8.5**). In other words, it was up-regulated in the comparison for lysine auxotroph pair (LL2 VS LL1) and the wild type control pair (WL2 VS WL1) (Table 5.7). *lysC*, a gene recognised as the positive control for lysine starvation due to its regulation under L-box riboswitch, was shown up-regulated in both LL2 VS LL1 and WL2 VS WL1 comparisons, indicating its up-regulation was shown in both lysine auxotroph and the wild type strain when they were grown in limited lysine compared to that of grown in excess (**Table 8.5**) (Kochhar and Paulus, 1996; Grundy, Lehman and Henkin, 2003; Wilson-Mitchell, Grundy and Henkin, 2012). And when approach 2 was used to filter possible lysine transporters, the transcriptome of auxotroph was compared to that of the wild type strain when they were grown in medium with sub-optimal concentration of lysine (LL2 VS WL2) with the control comparison grown in excess lysine (LL1 VS WL1). All of these genes were not shown up-regulated in comparisons of phenylalanine

auxotroph including PP2 VS WP2 and PP1 VS WP1. 79 genes were of higher expression in the auxotroph compared to that of wild type strain grown in limited lysine (**Table 8.8**) (LL2 VS WL2). 15 out of 79 encode transporters of various metabolites (Table 5.7) but one of them (*yrdR*) encodes poorly characterised transporter (Table 5.7). There were 3 genes that were of higher expression in lysine auxotroph relative to the wild type strain when they were grown in limited and excess lysine (**Table 8.9**) (LL2 VS WL2 and LL1 VS WL1) but none of them were categorised as membrane proteins according to Subtiwiki (Nicolas *et al.*, 2012). Interestingly, three genes (*yxeM*, *yczI* and *yxkD*) were found up-regulated through both approaches and one of them (*yxeK*) encodes putative S-(2-succino)cysteine transporter (Table 5.7). The other two were poorly characterised. Together, there were 23 genes recognised as possible candidates encoding lysine specific transporter.

Similarly, the two approaches were used to filter up-regulated genes in response to specific phenylalanine starvation (Figure 5.2, Section 5.2.1). Using approach 1 on the phenylalanine auxotroph, *ΔpheA::erm*, the auxotroph grown in limited phenylalanine was compared to that of grown in excess of this amino acid (PP2 VS PP1). And the wild type strain grown in the same conditions (WP2 VS WP1) acted as the control. The genes were not up-regulated in comparisons of lysine auxotroph which are LL2 VS LL1 and WL2 VS WL1. For phenylalanine specific starvation, there were 110 genes up-regulated by 5-fold or more when the auxotroph was grown in limited phenylalanine compared to that of grown in excess (PP2 VS PP1) but not in the control comparison (WP2 VS WP1) (**Table 8.6**). However, only 9 of them were recognised as transporters of different metabolites with another 6 genes categorised as membrane proteins on Subtiwiki (Nicolas *et al.*, 2012) (Table 5.8). There were 14 genes that were up-regulated by 5-fold or more in the comparison for phenylalanine auxotroph pair (PP2 VS PP1) and the wild type control pair (WP2 VS WP1) in which three of them encode transporters of peptides, glutamine and a multidrug transporter and one encodes an unknown transporter (**Table 8.7**). *pheS* and *pheT*, which could act as the positive control for phenylalanine starvation as they were regulated under T-box riboswitch, were also shown up-regulated in both phenylalanine auxotroph and the wild type strain when they were grown in limited phenylalanine compared to that of grown in excess (**Table 8.7**) (Brakhage, Wozny and Putzer, 1990). Using approach 2 on the phenylalanine auxotroph, *Δ pheA::erm*, comparison of PP2 VS

WP2 has also been made with its control comparison PP1 VS WP1 (Figure 5.2, Section 5.2.1). For specific phenylalanine starvation of phenylalanine auxotroph grown in limited phenylalanine relative to the wild type strain (PP2 VS WP2), 51 genes were of higher expression in the auxotroph compared to that of wild type strain grown in limited phenylalanine (PP2 VS WP2) (Table 8.10). 13 of the 51 genes encode transporters but 2 of them encode poorly characterised transporters (Table 5.8). No genes were filtered as higher expression in phenylalanine auxotroph relative to the wild type strain when they were grown in limited and excess phenylalanine (PP2 VS WP2 and PP1 VS WP1). Interestingly, 6 genes were shown up-regulated when using either of the approaches. These genes encode transporters of various carbon sources and one of them encodes cystine transporter with another one poorly characterised (Table 5.8). Altogether there were 38 genes recognised as possible candidates encoding phenylalanine specific transporter.

Table 5.7: Possible gene candidates for transporters screening of lysine.

gene name	operon	function	comparisons pairs
tcyN	*snaA*	cystine ABC transporter (ATP-binding protein)	Approach 1: LL2 VS LL1
ydeG			
yfmI	*yfmI*		
ycxC	*ycxC*		
yvsH	*yvsH*	putative lysine transporter	Approach 1: LL2 VS LL1 and WL2 VS WL1
appC	*appD*	oligopeptide ABC transporter	Approach 2: LL2 VS WL2
appB		oligopeptide ABC transporter (permease)	
cysP	*cysH*	sulphate transport via proton symport	
gabP	*gabP*	gamma-amino butyric acid permease for utilisation of the acid	
glnH	*glnQ*	glutamine ABC transporter	
glnQ		glutamine ABC transporter (ATP-binding protein)	

glnT	*glsA*	glutamine transporter (proton symport) for glutamine uptake	
maeN	*maeN*	Na^+/malate symporter	
nupP	*nupN*	ABC transporter of guanosine	
nupQ		ABC transporter of guanosine	
ssuC	*ssuB*	aliphatic sulphonate ABC transporter (permease) for sulphonate uptake	
yhjB	*yhjC*		
yxeN	*yxeK*	for uptake and utilisation of S-(2-succino)cysteine	
yxeO		for uptake and utilisation of S-(2-succino)cysteine	
yrdR	*yrdR*	unknown protein	
yxeM	*yxeK*	putative S-(2-succino)cysteine ABC transporter (binding protein)	Both approach 1 and 2
yczI	*yczI*		
yxkD	*yxkD*		

Genes that were up-regulated by 5-fold in the concerned comparisons in column and encode transporters. The rightmost column indicates the comparisons pairs that the genes were shown up-regulated. Unknown functions or regulation of certain genes remained blank.

Table 5.8: Possible gene candidates for transporters screening of phenylalanine.

gene name	operon	function	comparisons pairs
cimH	*cimH*	transporter for citrate	Approach 1: PP2 VS PP1
cstA	*cstA*	putative pyruvate transporter; carbon starvation-induced protein	
frlN	*frlB*	amino sugar ABC transporter for uptake of sugar amines	
glpT	*glpT*	glycerol-3-phosphate permease for uptake	
gutP	*gutB*	glucitol permease for its uptake	
amyD	*msmE; msmR*	ABC transporter permease for probably melibiose uptake	

narK	*narK*	nitrite extrusion protein for nitrate respiration	
ptsH	*ptsH; ptsG*	histidine-containing phosphocarrier protein HPr of the PTS	
tcyJ	*snaA*	cystine ABC transporter (binding protein)	
ybxG	*ybxG*		
yjnA	*yjnA*		
yvfS	*yvfR*	ABC transporter	
ywbA	*ywbA*		
yebE	*yebE*		
yxjN	*yxjN*		
dppC	*dppA*	dipeptide ABC transporter	Approach 1: PP2 VS PP1 and WP2 VS WP1
glnP	*glnQ*	glutamine ABC transporter (membrane protein)	
yvdS	*yvdT*	subunit of unidentified multidrug transporter	
yhjN	*yhjN*		
citM	*citM*	Mg2+ citrate transporter	Approach 2: PP2 VS WP2
glnM	*glnQ*	glutamine ABC transporter (membrane protein)	
iolF	*iolA*	D-chiro-inositol transport protein	
lnrL	*lnrL*	ABC transporter for resistance to linearmycin	
pucI	*pucI*	allantoin permease for purine utilisation	
rhiF	*rhiL*	rhamnose oligosaccharide ABC transporter	
rhiG		rhamnose oligosaccharide ABC transporter for uptake of rhamnose oligosaccharides	
tcyL	*snaA*	cytine ABC transporter (permease) for cystine uptake	

ssuB	*ssuB*	aliphatic sulphonate ABC transporter (binding protein)	
lplA			
ytcQ	*ytcP*	polygalacturonan and rhamnogalacturonan ABC transporter	
gmuB	*gmuB*	glucomannan-specific lichenan-specific PTS, EIIB component	
ywnC	*ywnC*		
araP	*araA*	for uptake of alpha-1-5-arabinooligosaccharides	Both approach 1 and 2
citH	*citH*	secondary transporter of divalent metal ions/citrate complex	
manP	*manP*	mannose-specfific PTS, EIIBCA	
ssuA	*ssuB*	aliphatic sulphonate ABC transporter (binding lipoprotein)	
tcyM	*snaA*	cytine ABC transporter (permease) for cystine uptake	
yrhP	*yrhP*		

Genes that were up-regulated by 5-fold in the concerned comparisons in column and encode transporters. The rightmost column indicates the comparisons pairs that the genes were shown up-regulated. Unknown functions or regulation of certain genes remained blank.

5.3 Discussion

In this chapter, in order to better understand the transcriptional aspect of amino acid assimilation in *B. subtilis*, the transcriptional response towards specific amino acid starvation in the defined medium has been investigated. The use of low number of replicates due to budget limits could severely limit the predictive power of the RNA sequencing analysis. Fortunately, the up-regulated genes selected to be used in the reporter systems indirectly given certain credits to the sequencing analysis because those genes were shown up-regulated in RNA sequencing analysis as well in response to specific amino acid starvation. But a more quantitative comparison of the fold changes of selected genes (qPCR) in response to the set condition in experiment would be preferred. Nevertheless, the global transcriptome study with the validation of the identified genes using reporter system has also emphasized the

complexity of the transcriptome in order to respond to extreme conditions outside of the cells in order to maintain growth.

5.3.1 Global transcriptomic analysis on specific amino acid starvations

We first explored the transcriptome of two starvation conditions using amino acids that were probably least utilised for metabolism and so would provide an indication of the response to limited protein synthesis rather than changes in metabolism (phenylalanine and lysine). From this we thought we would identify common genes or regulators responsible for the change in transcriptome using RNA sequencing (Section 5.2.1) that were specific to the response to a single amino acid being limited rather than global alterations. However, due to the lack of replicates involved, some genes might be missed from the selection. Also, in order to avoid stress responses, the samples were collected at the time point slightly earlier than the point where the auxotroph detectably deviated in its growth compared to the wild type strain when grown in limited amino acid that the auxotroph relied on survival (Section 5.2.1).

To identify a transcriptional response to specific amino acid starvation, two approaches were taken to compare the transcriptome profile of the bacteria grown in different conditions. The first approach aimed to select genes which were up-regulated in response to starvation in the auxotrophs when they were grown in sub-optimal concentration of the amino acids the auxotrophs needed for growth compared to that of grown in excess. The second one aimed to differentiate the transcriptional profile of the auxotroph relative to the wild type when they were grown in same medium where the auxotrophs were exposed to limited nutritional condition (Section 5.2.1). The first one focused on the transcriptional regulation of the auxotroph grown in different conditions whilst the second one focused on the difference of transcriptome between the auxotroph and wild type strain.

When using approach 1, whilst 65 % of the up-regulated genes (28 out of 42) were encoding proteins of unknown functions, one fourth of the up-regulated genes (12 out of 42) were encoding proteins related to biosynthesis or acquisition of amino acids (Table 5.2). Within this category, cysteine transporter was shown up-regulated in both lysine and phenylalanine auxotrophs when they were grown in limited lysine and phenylalanine respectively. This could be a result of an increased demand of sulphur for growth. The upregulation of *sndC*, which encodes a sulphur compound, could be suggesting a higher demand of cysteine or sulphur metabolism related metabolites by the cells in the starvation condition as this compound can produce cysteine

(Niehaus *et al.*, 2018). Concomitantly, the *yxeK* operon with genes encoding proteins for utilisation of S-(2-succino)cysteine was also up-regulated which matched with the suggestion above. On the other hand, the operon *glsA* which included two genes encoding proteins for glutamine degradation and uptake was also up-regulated. This may suggest a higher demand of glutamine which might be caused by the increase in the demand of cellular activities upregulation of glutamine inside the cells as it has been suggested to be easily metabolised for growth and it was the preferred nitrogen source of *B. subtilis*. Hence the up-regulation of the glutamine transporter might imply the cells struggling to maintain exponential growth (Section 5.2.1). The upregulation of *dctP* was also observed in which the transcriptional expression of these two genes was suggested to be determined by the sensor kinases, DctST system respectively (Fabret, Feher and Hoch, 1999; Asai *et al.*, 2000; Satomura *et al.*, 2005). The expression of these genes was suggested to be positively regulated by the sensor kinase signalling systems in response to the fumarate and succinate level in the medium. Since fumarate and succinate were not added into the initial media, possibility of overflow metabolites during glycolytic growth was suggested (Meyer *et al.*, 2014). And the upregulation of detected might be a sign of glucose exhaustion in which the cells reutilize the secreted metabolites (Kleijn *et al.*, 2010; Meyer *et al.*, 2014). But further analysis for tracking extracellular metabolites is necessary. *dctP* was also reported to be CcpA dependent in that the gene was shown upregulated when the global regulator derepressed the gene, supposedly when carbon source becoming limiting (Asai *et al.*, 2000; Fujita, 2009). Both of these possibilities could suggest that glucose exhaustion has occurred in the culture in general and this could potentially complicate the analysis. Also, genes encoding peptide transporter and extracellular proteases, *dppB*, *vpr* and *aprE* were also up-regulated (Table 5.1). Transcriptional expression of these three genes were indicated to be under the control of CodY, one of the global nitrogen metabolism regulator in that its repression on its target genes were shown usually relieved during the transition of growth from a medium of rich amino acids to a media of limited nutrients (Molle *et al.*, 2003; Belitsky and Sonenshein, 2008, 2013; Brinsmade *et al.*, 2014). *vpr* and *dppB* were also reported in other global transcriptomic analyses when *B. subtilis* was facing general amino acid or ammonium starvation (Eymann *et al.*, 2002; Tam *et al.*, 2006). From the physiological point of view, extracellular proteases were expected to degrade proteins and the cleaved peptides would be recycled for growth, probably through peptide transporters (Barbieri et al., 2016). The functions of the proteins encoded by

aprE, *vpr* (extracellular proteases) and *dppB*, a peptide transporter, were expected to be useful for this process. Together, the list of genes selected using approach 1 seemed logical from the physiological standpoint for a *B. subtilis* strain facing general amino acid starvation, were not very informative about any specific response.

Using the alternate approach to analysing the data (approach 2), only 13 genes were identified as being more significantly expressed in the auxotrophs relative to the wild type strain when they were grown in sub-optimal concentration of the specific amino acid (Table 5.3). This suggested the difference in response to starvation on the transcriptional level between auxotroph and the wild type might be similar. As amino acids were limited, protein synthesis would be expected to eventually stop hence the growth. This seemed to agree with the growth difference observed in last chapter. When the alanine auxotroph was grown in limited alanine, they had a shorter exponential growth (Section 4.2.8). Together, the response to the starvation between auxotroph and the wild type strain would be similar except that the auxotroph might starve earlier, reflected in a shorter exponential growth. The small difference in transcriptome might also suggest a sequential change on the transcriptional level in response to the starvation in both strains and the specific changes we were interested in may have been missed (due to the high cut off value used in data analysis) or are hidden by a global response. Using approach 2 coupled with approach 1, a more complete picture of transcriptome to understand the physiological state and development of the cells might be determined, but more replicate of the data are required to ensure some features are reproducible and determine the level of noise in the system. This is perhaps indicated by the fact that the *yxeK* operon was not identified using approach 2 but seen in approach 1.

About one third of the up-regulated genes were encoding transporters of peptides, carbon and nucleotides (Table 5.4). One of the genes encoding extracellular protease, *vpr*, was up-regulated as well. Also, three out of five of these genes were previously suggested to be directly or indirectly under the regulation of CodY (Section 5.2.1). Or, the upregulation of *nupN* operon could be the response to the drop of GTP in cells as the action of CodY is accompanied by intracellular GTP (Handke, Shivers and Sonenshein, 2008). Together, these demonstrated a higher demand of nutrient uptake in the auxotroph relative to the wild type strain in response to the starvation. This also suggested that the CodY was responsible controlling nutrient uptake in response to the set amino acid starvation to adapt to conditions of nutrient limitation

(Ratnayake-Lecamwasam *et al.*, 2001). Quite a number of genes/operons which were shown to be upregulated in the RNA sequencing analysis were suggested to be under the control of CodY directly or through cascades of reactions responding to the starvation including ScoC and AbrB (Kallio *et al.*, 1991; Belitsky *et al.*, 2015; Barbieri *et al.*, 2016). But further experiments are necessary to confirm whether the genes were up-regulated by CodY in the set condition or through other factors in the experiment. Similar to the findings using approach 1, genes encoding extracellular protease and peptide transporter, *vpr* and *appD*, were upregulated in which they were expected for recycling of material to maintain growth. The upregulation of *vpr* and *appD* was also reported in other global transcriptomic analyses when *B. subtilis* was facing amino acid starvation (Eymann *et al.*, 2002; Tam *et al.*, 2006). These observations seemed to fit with the physiological implication of our designed specific amino acid starvation, but are not useful in determining the specific uptake mechanisms.

Both lists of genes identified by using approach 1 and 2 suggested that the cells were preparing or experiencing a change in cell growth where genes encoding extracellular proteases, uptake system of some peptides, amino acids and sugars being upregulated. Up-regulated genes were also nitrogen source related or sometimes carbon sources related which seemed to be highly relevant to amino acid starvation in cells. Although lysine or phenylalanine specific up-regulated genes were filtered, lysine or phenylalanine specific up-regulation of genes were shown in the corresponding auxotrophs and the wild type strain (**Table 8.5** and **Table 8.7**). The up-regulation of these genes further supported the relevance of the data to the starvation and that also demonstrated the specificity and sensitivity of the cells response towards the designed starvation condition. Together, these observations seemed to be logical and with physiological implications of the set specific amino acid starvation. By combining both lists of up-regulated genes from the two approaches, three genes were shown commonly upregulated at least 5-fold in response to the set specific amino acid starvation in the defined medium. This common set of “stress” genes were *dctP*, *glsA* and *vpr* and each with their own regulation hence no universal transcriptional factor could be identified to be response for the response observed in the study. Instead, the genes were more likely regulated by interdependent regulators.

Regardless of the approach used, genes up-regulated were quite often under CodY regulation hence CodY could possibly play a more dominant role in the response towards amino acid starvation (Section 5.2.1). However, the CodY regulon was reported to cover nearly 200 genes of different functions including sporulation at early stage or for adapting to a transient state of growth in general (Sonenshein, 2005). The number of genes possibly under the control of CodY in the transcriptome analysis was significantly smaller than that of a CodY regulon. This could suggest the specific amino acid starvation induced a relatively mild reaction from CodY as the binding efficiency of CodY on DNA fragments could vary (Molle *et al.*, 2003). Alternatively, this suggested that the bacterial growth could be progressively developing by sequential changes to adapt its metabolism to the changing growth condition. This also matched with the suggested similarities of the response in wild type and the auxotroph using approach 2. A time course of transcriptome analysis would be useful to track the changes of genes in response to the specific amino acid starvation.

5.3.2 Stringent response genes in response to amino acid starvation

Three stringent response related genes, particularly *yvyD*, which were shown significantly up-regulated in response to amino acid starvation induced by a 10-minute treatment of antagonist of certain amino acids in the transcriptome study were selected to test for their response in our designated conditions for specific amino acid transporter screening on plate (Eymann *et al.*, 2002) (Section 5.2.1). The up-regulation of *vpr*, *yvyD*, *gspA* was also shown in the transcriptome analysis on lysine and phenylalanine specific starvation, which increased our confidence in the selection although *tnrA* was shown with little differences in all comparisons tried (Section 5.2.2). On the other hand, *vpr* has been shown up-regulated in response to starvation of certain amino acids in two different studies, suggesting this gene to be a more promising candidate for amino acid transporter system on plate (Eymann et al., 2002, Section 5.2.2). However the up-regulation of this gene in our set condition was not significant as expected.

When the strains were grown in our conditions on plate, all of these three genes were shown up-regulated when the wild type reporter strain was grown in minimal medium (GMP) on plate (Section 5.2.2). Although *yvyD* was shown most significantly up-regulated with the darkest blue colonies with the up-regulation of *vpr* and *gspA* on plate also significant as suggested by the blue coloured colonies (Section 5.2.2).

Together these probably reflect the difference between the previous study looking at immediate effects of the stringent response and our study where more long term expression effect will be present. Here, the important point is that the plan for the transporter identification was to screen colonies on plates in which the up-regulation of genes could be triggered by the specified amino acid starvation. This was to permit higher throughput and so allow the analysis of multiple mutants in an efficient way. However, it would seem that other cellular activities during colony formation of the bacterial cells on plate may present a problem. The appearance of blue colonies in these reporter strains in the wild type background when they were grown in defined rich medium (GMaaP), suggested that the up-regulation of these genes could be triggered by the same factor(s) during its growth in either condition but to different degree. Or the up-regulation of the genes in two conditions could be caused by different factors. In other words, the up-regulation of these genes might not be solely a stringent response towards starvation of amino acids on plate.

However, having exponentially growing *B. subtilis* cells been described as heterogenous in various aspects, the transcriptome of bacterial colonies on the agar plate was expected to be difficult to predict (Gonzalez-Pastor, Hobbs and Losick, 2003; Kearns and Losick, 2005). The physiological conditions (oxygen level and nutrient level) of bacterial cells within a colony were also expected to vary depending on their positions on the plate hence the transcriptome of each cell as well as colony remained poorly understood. Therefore, it remained difficult to identify which other factor(s) might have caused the up-regulation of these genes.

Nevertheless, one of the ways to better understand the metabolic status of the cells was to identify common regulators of the two selected genes as better reporter systems for the transporter screening, *yvyD* and *gspA*. Apart from stringent response regulation on the genes suggested, the other common regulation acting on the two genes was performed by sigma B and the gene encoding this sigma factor, *sigB*, was suggested to be under the regulation of CcpA the global transcriptional regulator of carbon metabolism (Choi and Saier, 2010). Hence it implied that the cells were likely in glucose exhaustion which triggered the activity of CcpA on *sigB*. Also, amino acid starvation was shown unable to induce sigma B response but glucose or phosphate limitation (Maul *et al.*, 1995; Voelker *et al.*, 1995; Zhang and Haldenwang, 2003). Hence, the blue colonies could be a result of glucose exhaustion of the strains grown in the medium. And the limiting amino acid might have induced additional workload

on the cells causing earlier glucose exhaustion in the double mutant than the other strains. Or the strains decided to enter stationary phase due to the absence of amino acid for survival despite the presence of glucose in medium. Other possibilities that the two genes were up-regulated by sigma B could be a response to oxygen and/or phosphate limitation (Wendrich and Marahiel, 1997; Zhang and Haldenwang, 2003). For the more significant blue observed in colonies of *yvyD* reporter system compared to that of *gspA* reporter system, it could be attributed by the additional regulation of sigma H on *yvyD* whilst *gspA* was found solely of sigma B regulon (Antelmann *et al.*, 1995; Wendrich and Marahiel, 1997; Drzewiecki *et al.*, 1998). Also, *yvyD* was suggested to have a CodY binding site upstream of the starting codon and *sigH*, encoding sigma H was reported to be regulated by CodY (Strauch, 1995; Drzewiecki *et al.*, 1998; Belitsky and Sonenshein, 2013). This seemed to match with the results from Section 5.2.1 suggesting the involvement of CodY in the up-regulation of genes in response to specific amino acid starvation and matched with the expected physiological status of cells in response to amino acid starvation. But with the possible involvement of sigma B attributing to the blue colour of the colonies, the cells were more likely facing general amino acid starvation rather than a specific one.

5.3.3 Ribo-switch tyrS response in amino acid starvation

To improve the specificity of the response to the amino acid that the bacterial cells were starved for, an alternative approach of reporter system was to utilise the ribo-switch anti-termination mechanism upstream of *tyrS* where the specifier codon targeting tRNA of the cognate amino acid. The change of specifier codon was also shown sufficient to change the specificity of the induction of the gene (*tyrS*) from one amino acid to another, including leucine and phenylalanine (Henkin, Glass and Grundy, 1992; Grundy and Henkin, 1993; Grundy *et al.*, 1997, 2000; Rollins, Grundy and Henkin, 1997). From the global transcriptome study in Section 5.2.1, the genes encoding phenylalanine tRNA synthetase which were also regulated by T-box riboswitch as *tyrS*, *pheS* and *pheT*, were shown up-regulated in both wild type and phenylalanine auxotrophic background when the strains were grown in limited phenylalanine compared to that of grown in excess phenylalanine (Table 5.9). However, they were not considered as significantly up-regulated genes when the phenylalanine auxotroph was compared to the wild type strain using another approach (Table 5.10). Together these suggested that the starvation response could be observed even in the presence of excess nutrients. The limitation of amino acid

for growth was acting similar level of starvation stress on the strains in different background.

Whilst the specificity of the T-box was evident in the global transcriptome analysis (Section 5.2.1), the specifier codon directing reporter systems have been suggested to give only slightly higher specificity given a suitable condition and strain background (Section 5.2.6). Despite the different efficiency in assimilation of lysine, alanine and tyrosine observed in Section 3.2.3, the blue colonies of *tyrS* reporter systems targeting alanine, leucine and tyrosine in wild type strain background grown in different abundance of alanine or tyrosine were comparable, suggesting the starvation stress of the three amino acids was similar. Also, apparently a tyrosine specific starvation could be induced earlier when the strain was introduced an alanine auxotroph background or an alanine transporter defect (Section 5.2.5). This demonstrated the starvation of a specific amino acid could concomitantly trigger the starvation of another amino acid in the cells. Together, the performance of the *tyrS* reporter system on strains grown on plates matched with that of the *yvyD* reporter system, which likely a reflection of a more general amino acid starvation inside the cells despite the proven specificity towards cognate amino acid of the tyrosine tRNA synthetase (Henkin, Glass and Grundy, 1992; Grundy and Henkin, 1993). Nevertheless, the observation seemed logical as a shorter exponential growth was observed in the alanine auxotroph compared to wild type strain in Section 4.2.8. The tRNAs level was suggested to decrease in the amino acid auxotrophs encountering starvation and the decrease was not only that of the specific one essential for the growth of the auxotroph but others as well (Greenwich *et al.*, 2019). In *E. coli*, stability of tRNA might decrease in response to amino acid starvation and so led to the reduction in tRNA charging, and hence translation would be reduced, resulting in a decelerated growth was thus expected (Svenningsen *et al.*, 2017).

Table 5.9: Transcription expression of different tRNA synthetases which were regulated by T-box in response to leucine and phenylalanine specific amino acid starvation.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
leuS	0.68	0.57	0.60	0.82	1.73	0.77	0.68	1.03
pheS	0.87	0.45	8.57	9.30	1.09	0.96	6.38	13.01
pheT	0.60	0.50	10.68	11.48	1.04	0.87	6.63	5.41
tyrS	0.23	0.14	0.20	0.24	1.13	0.53	0.33	0.45

Table showed the fold change of each gene specified on the left compared in two different conditions. *pheS* and *pheT* were shown significantly up-regulated (5 fold in change) in both phenylalanine and wild type background when they were grown in the phenylalanine limiting medium compared to that of in excess phenylalanine medium (indicated in red box). Other genes encoding other non-cognate amino acid tRNA synthetase were not up-regulated.

But the phenotype was different from the expectation derived from the global transcriptome. This indicated that the specific response reflected in the transcriptome study could not be directly applied to the reporter system when the strains were grown on plates. The difference might be due to the limitation of nutrients for growth on plate with longer incubation compared to that of performed in flask. The cells grown by that stage were starved not only for alanine but other amino acids as well in general when the strains were grown on plate and a decelerated protein synthesis and cellular metabolism was deduced. But an auxotrophic background might be a severe enough condition to differentiate the starvation of amino acids for protein synthesis from that of a wild type strain when the strains were grown on plate (Section 5.2.6). The specificity suggested of the *tyrS* reporter system was only demonstrated when the double mutant, with auxotrophic background and transporter defect, was grown in limited amino acid it required for survival. The reporter system targeting alanine was showing observable but only slightly bluer colonies compared to that of targeting tyrosine when they were grown in the right condition and strain background (Section 5.2.6). Together these emphasized the importance of a right

medium condition and a right background of the strain of interest to demonstrate the specificity of the reporter system. This also resembled the suggestion from Section 4.2.8 where evidence of AlaP as an alanine transporter was only observed given a range of alanine concentrations.

Whilst extreme conditions allowed the demonstration of the specificity of the reporter system, the negative control reporter system grown in same condition suggested possible mismatches of specifier codon with unknown uncharged tRNA (Section 5.2.6). Furthermore, it suggested possible mismatches in the other reporter systems in the same double mutant (*ΔalaT ΔalaP*) background grown in same condition. Hence the more significant blue colonies relative to the auxotrophs (MC36, MC38 and MC39) formed by MC52, MC54 or MC55 could be partly due to mismatches of specifier codon with uncharged cognate tRNA. If only limited amino acids were available, the proportion of uncharged tRNA should increase, raising the probability of lowering ppGpp concentration which would then lead to a suppression in growth (Tollerson and Ibba, 2020).

Table 5.10: Transcription expression of different tRNA synthetases which were regulated by T-box in response to leucine and phenylalanine specific amino acid starvation.

	PP2 VS WP2		PP1 VS WP1		LL2 VS WL2		LL1 VS WL1	
	s1	s2* using WP2 from s1	s1	s2	s1	s2	s1* using WL1 from s2	s2
leuS	0.86	0.70	0.97	0.87	0.59	0.80	1.50	1.09
pheS	1.27	0.94	0.95	1.32	0.59	0.54	0.75	1.16
pheT	1.69	1.74	1.05	0.82	0.66	0.59	1.14	1.03
tyrS	0.66	0.56	1.09	1.04	0.26	0.29	1.28	1.10

Table showed the fold change of each gene specified on the left compared in two different conditions. *pheS* and *pheT* were shown significantly up-regulated (5 fold in change) in both phenylalanine and wild type background when they were grown in the phenylalanine limiting medium compared to that of in excess phenylalanine medium (indicated in red box). Other genes encoding other non-cognate amino acid tRNA synthetase were not up-regulated.

Two types of alanine tRNA models were suggested in *B. subtilis* hence two reporter systems targeting the specifier codon of these two types of alanine tRNA (pMC7 and pMC8) were compared in this study (Chan and Lowe, 2009, 2016). The major difference of the two types of tRNA was the presence of the 3' tail which could be served as another binding position with the side bulge of the antiterminator to enhance the structural binding with uncharged tRNA (Grundy, Rollins and Henkin, 1994). Whilst pMC7 was targeting alanine tRNA with the 3' tail, pMC8 was targeting the other one without the tail (Chan and Lowe, 2009, 2016). But the reporter system (pMC8) targeting alanine tRNA without the tail seemed to give a more significant response to the starvation condition created on plate regardless of the presence of amino acid mixture (Section 5.2.4 and 5.2.6). Nevertheless, the 3' tail was suggested not essential for the binding of tRNA with the antiterminator (Green and Vold, 1988). On the other hand, some might suggest the antiterminator to be recognizing the overall tertiary structure of the tRNA for binding (Grundy *et al.*, 2000). Hence it was possible that the alanine tRNA without the 3' tail at the acceptor end might be more structurally fit into the pocket of the antiterminator. If this was true, it could imply the importance of a fit tertiary structure over the 3' tail at the accept end for better binding. However, this question was not further explored. AlaS was identified as the alanine tRNA synthetase in *B. subtilis* but not much about it has been explored. Assuming *B. subtilis* with only one alanine synthetase for charging alanine tRNA, it was possible that the difference in alanine tRNAs to affect translation efficiency. Serine starvation was once suggested leading to ribosomes preferentially pausing on some of the serine codons but not the others during translation (Subramaniam *et al.*, 2013). For the observed difference in response of the two types of alanine tRNA, it could reflect their difference in structure and stability which mentioned earlier, and abundance. This might suggest biased codon preference for alanine in *B. subtilis* or a preference in ribosome pausing during translation (Greenwich *et al.*, 2019). But this was not further explored in this project.

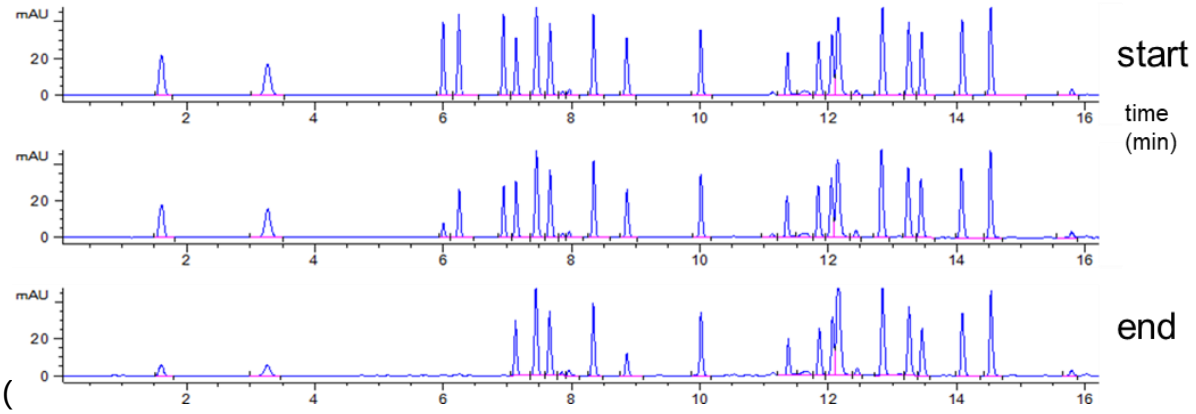
5.3.4 Using RNA sequencing filtering specific amino acid transporters

In the global transcriptome study, *lysC* was shown up-regulated in both the wild type strain and the lysine auxotroph under the regulation of L-box riboswitch (Section 5.2.7). On the other hand, *pheS* was shown up-regulated in both the wild type strain and the phenylalanine auxotroph under the regulation of T-box riboswitch (Section 5.2.7). The up-regulation of both genes suggested lysine and phenylalanine specific

starvation respectively and hence the wild type strain was suggested to have the ability to sense the concentration difference in medium as the auxotroph and reacted as the auxotroph on the transcriptional level (Section 5.2.7). The observation might also help to explain the absence of assimilation difference between auxotrophs and the wild type strain observed in Section 3.2.4 as the assimilation of the amino acid might have been increased in both strain at similar level. Still this might not be a universal phenotype for all amino acid auxotrophs.

With the indication of specific amino acid starvation presented, the transcriptomic analysis could also be utilised to filter more promising genes encoding specific amino acid transporter(s). Using the two approaches introduced in Section 5.2.1, 23 genes were selected to be more promising candidates encoding lysine specific transporter, including *yvsH*, a putative lysine transporter regulated by L-box as *lysC* the positive control (Table 5.7, Section 5.2.7) (Hahne *et al.*, 2008; Gao *et al.*, 2009). However, experimental evidence of *yvsH* as a lysine transporter is lacking in *B. subtilis*. With the established reporter system, it can be transformed into lysine auxotroph, the wild type strain and a double mutant of *lysC* and *yvsH*. Similar to the positive control in the reporter system development (Section 5.2.3 and 5.2.5), growing the three strains with the reporter system in limited lysine on plate could be screened for blue/white colony. If *yvsH* encodes a lysine specific transporter, both the poor growth and bluer colonies of the double mutant compared to the other two should appear in the presence of limited lysine. With gene encoding transporter of interest deleted in an amino acid auxotroph, the reporter system can help increase effectiveness in determining the specificity of the transporter of interest towards the corresponding amino acid that the auxotroph required to grow. In the case of phenylalanine starvation, *pheS* was shown up-regulated but no putative phenylalanine transporter was recognized in the filtering step although 38 genes encoding different types of transporters were selected (Table 5.8). Instead, there were genes encoding transporters of different carbon sources up-regulated including citrate and pyruvate (Table 5.8). The genes encoding proteins of unknown function and categorized as membrane proteins were also included in the list for screening. This filtering step using transcriptomic sequencing data would help to reduce the scale of the screening assay, lysine and phenylalanine transporter identification in this case. However, screening of transporters for both auxotrophs was not further explored.

Through the selection of up-regulated genes specific to lysine starvation as well as phenylalanine starvation, there were more genes shown up-regulated in response to phenylalanine starvation compared to the lysine starvation



(

Figure **8.1: Chromatogram of amino acids content in defined medium of culture taken during time course experiment for quantitative analysis.**

Table 8.1: Relative abundance of amino acids of *168+* growing in Gaa medium at different time points.

	Relative level of amino acid (percentage to T0 at T150)	Relative level of amino acid (percentage to T0 at T250)	Relative level of amino acid (percentage to T0 at T400)
Asparagine	19.4	0.0	N.D
Serine	60.4	12.3	N.D
Glutamine	62.6	20.7	N.D
Aspartate	80.5	53.6	25.0
Glutamate	90.0	62.5	33.7
Alanine	84.7	57.5	39.5
Isoleucine	92.4	81.5	73.7
Leucine	92.9	85.3	81.7
Lysine	89.8	86.5	86.6
Valine	95.9	90.8	88.2
Threonine	94.5	90.1	88.6
Arginine	95.2	91.4	90.8
Phenylalanine	96.2	94.2	95.2
Tyrosine	96.3	94.7	96.9
Glycine	98.5	98.3	99.5
Tryptophan	95.1	98.0	99.9

The relative abundance of each amino acid at each time point (T150, T250 and T400 from left to right) was calculated in percentage relative to their initial concentration correspondingly and the numbers were corrected to 1 decimal place. Data shown is from single experiment. The experiment was repeated and similar trend of assimilation profile was obtained. N.D = amino acid concentration was below detectable range.

Table 8.2: Depletion rates of amino acids from defined medium of growing *168+*

	Gaa	Maa	GMaa
Asparagine	-25.1	-19.3	-22.6
Glutamine	-42.7	-21.7	-20.9
Serine	-18.5	-23.6	-24.2
Aspartate	-17.6	-17.7	-27.3
Alanine	-11.3	1.8	0.5
Glutamate	-17.7	-27.4	-31.7
Leucine	-8.2	-7.5	-5.0
Isoleucine	-8.6	-7.7	-1.7
Lysine	-7.1	-7.7	-1.3
Valine	-6.8	-11.8	4.9
Phenylalanine	-3.3	-3.4	1.9
Arginine	-4.1	-3.8	1.7
Tyrosine	-0.4	-0.4	7.3
Tryptophan	-0.4	-0.6	3.0
Threonine	-5.4	-2.7	-2.4
Glycine	0.4	0.0	6.2

The depletion rates (% decrease per OD600) were calculated as the percentage change of the relative abundance of amino acids per change of optical density. Column Gaa represents the relative abundance of amino acids recorded when the strain was grown in Gaa whilst column Maa represents those when the strain was grown in Maa. Column GMaa shows the relative abundance of amino acids when the strain was grown in GMaa. The order of amino acids follows that of Table 3.2 for easier reference. Data shown is from single experiment. The experiment was repeated and similar trend of assimilation profile was obtained.

Table 8.3: Tables of assimilation efficiency of the amino acid specified for different auxotrophs and the wild type strain.

A Serine

Time (min)	Wild type strain	Auxotroph (*ΔserA*)
0-160	-28.7	-34.9
0-100	-37.1	-55.3
100-160	-26.5	-28.7

B Alanine

Time (min)	Wild type strain	Auxotroph (*ΔalaT*)
0-160	-13.7	-16.9
0-100	-10.2	-14.5
100-160	-14.6	-17.7

C Leucine

Time (min)	Wild type strain	Auxotroph (*ΔleuA*)
0-160	-14.1	-9.5
0-100	-20.8	-4.3
100-160	-12.3	-11

D Tryptophan

Time (min)	Wild type strain	Auxotroph (*ΔtrpC2*)
0-160	-2.9	-1.8
0-100	2.8	8.6
100-160	-4.4	-5.1

The strains were grown in defined medium (GaaP) containing equal concentrations of 20 amino acids. Each table shows the assimilation efficiency rate calculated as percentage change per OD600 of the amino acid that the auxotroph required for growth for the auxotroph compared to the wild type strain during exponential growth over three time points. The first row of each table shows the overall rate during exponential growth. The second row shows the first 100 minute of the assimilation efficiency of the specified amino acid whilst the third row shows the following 60 minute of assimilation efficiency of the amino acid. The more negative the value in each table, the higher assimilation efficiency of the specified amino acid. A: comparison of assimilation efficiency of serine between serine auxotroph (MC16) and wild type strain. B: comparison of assimilation efficiency of alanine between leucine auxotroph (MC2) and wild type strain. C: comparison of assimilation efficiency of leucine between leucine auxotroph (MC17) and wild type strain. D: comparison of assimilation efficiency of tryptophan between tryptophan auxotroph (*168CA*) and wild type strain. The experiment has been repeated once and the data above come from a single experiment

Table 8.4 – 8.7). The difference in the number may suggest a more significant response from the phenylalanine auxotroph to the starvation of the amino acid it required for growth compared to the lysine auxotroph. This may relate to the observed difference in growth where the wild type strain demonstrated a slightly slower growth when it was in limited phenylalanine compared to that of in excess (Figure 5.1B). The difference was not observed for the wild type strain grown in two different lysine concentrations. phenylalanine auxotroph. The wild type strain might have metabolic pressure but more in-depth studies are necessary to confirm and further explore.

5.3.5 Proposed transcriptional response to specific amino acid starvation

The involvement of different regulation mechanisms in response to the starvation was observed in the global transcriptome study, including riboswitches, stringent response, CodY and its related global regulators (Section 5.2.1). In the validation of up-regulated genes in the reporter system, participation of sigma B for general stress response was suggested. Together, a model of hypothetic sequential order of transcriptional regulation in response to specific amino acid starvation is proposed.

When specific amino acid is starved, riboswitch including T-box or L-box which is based on the abundance of uncharged tRNA of *B. subtilis* in response to starvation of specific amino acid depending on the starvation of which amino acid the cell encountered (Section 5.2.1 and 5.2.6). As the cells to be triggered by amino acid starvation for longer period, the proportion of uncharged tRNA increased over charged tRNA and the stringent response might be triggered (Section 5.2.1) (Wendrich and Marahiel, 1997). Concomitantly, the drop of GTP and branched-chain amino acid in cytosol during amino acid starvation would trigger the activity of CodY on different CodY-binding DNA fragment. There are also other CodY-controlled regulators, *e.g.*, ScoC, that were suggested responsible for many of the up-regulated genes in response to amino acid starvation in addition to CodY (Section 5.2.1). The interaction of CodY and ScoC allowed fine adjustment of gene expression level (Belitsky *et al.*, 2015; Barbieri *et al.*, 2016). This flexibility and fine-tune of the transcriptional expression of genes resembled that of the suggested flexibility in enzyme kinetic activities in alanine metabolism (Section 4.3.1). These regulatory responses could be regarded as the early response of cells towards the said starvation as they were observed in the transcriptome analysis.

As the duration of starvation increased, starvation of other nutrients would be triggered and the cells would then experience a general amino acid starvation as well as glucose exhaustion in which the glucose limitation would likely trigger the activity of sigma B (Voelker *et al.*, 1995; Kroos and Yu, 2000). The participation of sigma B was suggested only when the strains were grown on plates (Section 5.2.2) hence it was likely sequentially later than the other mentioned. This also matched with the metabolic study where sigma B activity was shown activated when nutrients become limiting (Meyer *et al.*, 2014). As more cells responded and together brought about a drop in ATP, GTP companied by a decelerated growth which could be seen often as

a phenotype when the cells were not able to obtain the amino acid it needed for growth in this project (Section 4.2.8, 5.2.3 and 5.2.5).

The up-regulation of *lysC* and *pheS* suggested lysine and phenylalanine specific starvation respectively in the wild type strain as the corresponding auxotroph (Section 5.2.7). Thus, the wild type strain was suggested the ability to sense the concentration difference in medium as the auxotroph and reacted as the auxotroph on the transcriptional level (Section 5.2.7). Therefore, the sequential regulation of genes in the wild type strain was expected to be likely similar to the auxotrophs but possibly responding later than the auxotrophs. The size of difference remains unclear though.

Chapter 6 Final discussion and future work

Previous studies on amino acid assimilation or the identification of specific amino acid transporters were commonly demonstrated using bacteria strains grown in essentially minimum medium condition to obtain a clear indication of the assimilation (Section 1.2). The choice of minimal medium over complex medium was also to provide a cleaner environment to avoid complications of the components interfering with uptake of the amino acid of interest in the study. However, the transcriptome studies revealed a significant difference of *B. subtilis* grown in the two different conditions (Nicolas *et al.*, 2012). This raised the questions about the specificity as well as the gene expression level of the transporters identified if the bacterial cells were grown in two completely different conditions (minimal and rich media condition).

6.1.1 Final discussion

This project began by establishing the protocol for quantifying amino acid content in medium was first developed (Section 3.2.2). A rich medium supporting an active growth of *B. subtilis* with all 20 standard amino acids provided was also developed, offering the possibility of determining if the transporters are specific for their substrate or are redundant in their transport capability (Section 3.2.1). Amino acid assimilation profile of *B. subtilis* in a defined rich medium during exponential growth was established. The assimilation efficiency of the amino acids seemed to be dependent on the correlation of each related to the central metabolism involving glycolysis and TCA cycle (Section 3.2.2). This matched with the nature of *B. subtilis* in metabolizing rapidly usable resources for growth, reflected the high cost of maintaining efficient growth and agreed to the proposed higher cost effectiveness of uptake if available relative to the biosynthesis of amino acids (Akashi and Gojobori, 2002). As an extension of this work, it is clear that the methods used have the potential to allow analysis of overflow metabolites observed from the growth of *B. subtilis* in the presence of alternate carbon sources. An initial analysis showed that malate resulted in amino acids apparently being released into the medium during rapid growth could be re-utilised and the extracellular medium acted as a storage pool for *B. subtilis* for use at a later stage of growth (Meyer *et al.*, 2014). The cells seemed in general to prefer assimilation over biosynthesis of a metabolite. Efficient assimilation of serine, glutamine and other amino acids from group 1 have also been observed in *E. coli* in which the importance of these amino acids towards glycolytic flux has been emphasized (Selvarasu *et al.*, 2009).

The difference in the relative abundance of alanine in medium with glucose, malate or with both attracted attention for a more focused study on its metabolism (Section 3.2.4). Alanine biosynthetic and utilization pathways were demonstrated *in vivo* with Dat being shown involved as a minor alanine biosynthetic pathway for both isoforms of alanine (Section 4.3.1). Although the regulation of Dat remains to be explored, the activity of Dat was shown possibly mediated by L-alanine (Section 4.3.1). With the characterized alanine racemase, AlrA, together they could form a dynamic alanine metabolism in fine-tuning the abundance of both isoforms of alanine, D- and L-alanine, inside the cells (Sidiq *et al.*, 2021). From this dynamic nature of alanine metabolism, it seems logical that insignificant difference of amino acid assimilation profile of auxotrophs compared to the wild type strain was observed (Section 3.2.4). The dynamic metabolism of different amino acids, particularly those of efficiently assimilated ones, could be important in fine-tuning the abundance of metabolites to support the central metabolism for growth.

The understanding of alanine metabolism allowed better characterization of the newly identified alanine transporter by our colleague. With the confirmation of the specificity of AlaP, the alanine transporter, other alanine transporters were also suggested (Section 4.2.8 and 4.2.9). With the long existing debate on the degeneracy of transporters, the characterization of AlaP seemed to suggest a balance between degeneracy and specificity of transporters in *B. subtilis* in which the level of specificity was likely to be regulated depending on the medium conditions. The transporters efficiency and hence the capacity of the assimilation of one amino acid might be adjusted as well where necessary. On the other hand, the role of regulation involved in response to amino acid starvation was of interest as the other transporters were suggested to be expressed in later stage of growth or with lower specificity to alanine (Section 4.2.9).

A dynamic network of metabolism and a flexible capacity of transporting an amino acid in *B. subtilis* for maintaining intracellular pool of amino acids for growth was suggested by the results of chapter 3 and 4. This raised the question of the cells by what means they control the metabolism and nutrient uptake and whether there was the existence of any related regulations in *B. subtills* in response to starvation of amino acids. The transcriptome analysis and the reporter systems together suggested the participation of various global regulators including CodY and sigma B as well as stringent response (Section 5.2.1 – 5.2.4). These suggested how the

bacterial cells reacting to the amino acid starvation in a multi-disciplinary approach and all of these would eventually bring to an arrest of growth that resembled the observed shorter exponential growth of alanine auxotroph with the alanine transporter defect (Section 4.2.8). The importance of GTP as a sensor to amino acid starvation was also highlighted as it was suggested to be involved in different mentioned transcription regulation mechanisms (Section 5.3.2).

6.1.2 Proposal of specific amino acid transporters screening

The initial plan for specific amino acid transporter screening was a live/dead colony screening for specific amino acid starvation and it was used first to screen for a D-alanine transporter (Sidiq *et al.*, 2021). However, this seemed not applicable for screening transporter(s) specific to L-amino acid (Section 5.1).

On the whole, this project provided a better understanding of the assimilation of amino acids in *B. subtilis* which is helpful in optimising the screening plan for specific amino acid transporters. Few criteria to an ideal screening plan are outlined. The first one was to identify a 'clean' auxotroph for transporters screening. The absence of an expected different phenotype between the reporter strain of the wild type background and the auxotrophic one was observed (Section 5.2.3 and 5.2.5). This may be due to the presence of Dat where low level of alanine might be produced (Section 4.2.2). The second one was the medium condition. As there was observable difference in colonies when the reporter strains in different background were grown in low concentration of alanine but not in high one (Section 4.2.8, 5.2.3 and 5.2.5). This demonstrated the importance of setting the screening in a right condition in order to screen for the expected phenotype. There was complication of the transcriptional profile of bacterial colonies on plate but an on-plate screening was easier to handle in large numbers than cellular assay. The use of reporter system for the screening would allow the appearance of blue colonies which might serve as an indicator of successful transformation in the screening in order to avoid false positive candidates that could be brought from technical issues like transformation efficiency. Although the reporter systems of *yvyD* or *tyrS* were suggested to be a response to a more general starvation in chapter 5, they could still be used in the screening of specific amino acid starvation condition on plate as demonstrated in Section 5.2.3 and 5.2.6. And it was expected that other genes in both sigma B and sigma H regulon like *yvyD* could also be potential candidates. Alternatively, *tyrS* reporter system could be engineered to target another amino acid to improve specificity of the reporter system

in starvation of an amino acid. To reduce the scale of the screening assay, the RNA sequencing result in a similar setting as Section 5.2.1can be done in advance to select more promising gene candidates for screening as in Section 5.2.7.

6.1.3 Summary

This project emphasised the better understanding of cellular metabolism including focused study of the metabolism of the metabolites of interest as well as the, transcriptional regulation of cells on mediating cellular metabolism to feed cellular demand is necessary for amino acid transporters screening. The understanding of the uptake and specificity of transporters would now be mechanistically possible with the help of the availability of rich defined medium which can be manipulated for specificity assays. The specificity of transporter can then be matched with structural studies to determine how the substrate binds and being taken into the cell.

Not only the transporter screening and transporters characterisation, the study of cellular metabolism is also beneficial for optimising growth medium for amino acid or protein production in industry. With the understanding of the uptake capacity of the strain of interest, growth medium for the strain can be optimised, permitting clean synthesis of bioproducts in which the medium will be exhausted of nutrients and the wanted product could be more easily isolated. Current bottleneck of antibiotics development is in the uptake of a created inhibitor into cells. The understanding of the structural characteristics and specificity of identified uptake systems in rich medium condition could be useful in modifying the bacterial inhibitors of interest to be recognised by the uptake system hence killing bacterial cells.

With the help of advanced technology development in ‘omics’, the molecular level study of the cellular metabolism could be applied into a systemic biology tool to calculate the optimum growth of a bacterial cell and the essential components for it to maintain such growth. A flux could be determined by the carbon source and the kinetics of involved reactions corresponding to different direction of flux in the main central metabolism might help to refine the estimated energy cost of nutrients to maintain growth of the strain of interest. To obtain a more complete picture of metabolism, the expression level was ideally measured when the strain was grown in a variation of conditions. Therefore, the flux calculation has to be companied by a database of transcriptional expression level of enzymes in each reaction in different medium condition. All of these require the combination of concrete experimental

evidence on the molecular level in large scale. This project can serve as an initial blueprint of what needs to be considered in such physiological studies.

Chapter 7 References

Akashi, H. and Gojobori, T. (2002) 'Metabolic efficiency and amino acid composition in the proteomes of *Escherichia coli* and *Bacillus subtilis*', *Proceedings of the National Academy of Sciences*, 99(6), pp. 3695–3700. Available at: https://doi.org/10.1073/PNAS.062526999.

Amir, A. *et al.* (2014) 'Bending forces plastically deform growing bacterial cell walls.', *Proceedings of the National Academy of Sciences of the United States of America*, 111(16), pp. 5778–83. Available at: https://doi.org/10.1073/pnas.1317497111.

Anagnostopoulos, C. and Spizizen, J. (1961) 'Requirements for transformation in *Bacillus subtilis*.', *Journal of bacteriology*, 81(5), pp. 741–6.

Anderson, A.J. *et al.* (1978) 'Cell wall assembly in *Bacillus subtilis*: location of wall material incorporated during pulsed release of phosphate limitation, its accessibility to bacteriophages and concanavalin A, and its susceptibility to turnover.', *Journal of bacteriology*, 136(3), pp. 886–99.

Antelmann, H. *et al.* (1995) 'A gene at 333 degrees on the *Bacillus subtilis* chromosome encodes the newly identified sigma B-dependent general stress protein GspA.', *Journal of bacteriology*, 177(12), pp. 3540–5.

Armstrong, J.J., Baddiley, J. and Buchanan, J.G. (1959) 'Teichoic Acids from Bacterial Walls: Structure of Teichoic Acid from the walls of *Bacillus subtilis*', *Nature*, 184(4682), pp. 248–249. Available at: https://doi.org/10.1038/184248a0.

Asai, K. *et al.* (2000) 'Regulation of the transport system for C4-dicarboxylic acids in *Bacillus subtilis*', *Microbiology*, 146(2), pp. 263–271. Available at: https://doi.org/10.1099/00221287-146-2-263.

Ashiuchi, M. *et al.* (1998) 'Properties of Glutamate Racemase from *Bacillus subtilis* IFO 3336 Producing Poly-γ-Glutamate', *The Journal of Biochemistry*, 123(6), pp. 1156–1163. Available at: https://doi.org/10.1093/OXFORDJOURNALS.JBCHEM.A022055.

Ashiuchi, M., Soda, K. and Misono, H. (1999) 'Characterization of yrpC Gene Product of *Bacillus subtilis* IFO 3336 as Glutamate Racemase Isozyme', *Bioscience, Biotechnology, and Biochemistry*, 63(5), pp. 792–798. Available at: https://doi.org/10.1271/BBB.63.792.

Atkinson, M.R. *et al.* (1990) ‘Regulation of histidine and proline degradation enzymes by amino acid availability in *Bacillus subtilis*.’, *Journal of bacteriology*, 172(9), pp. 4758–65.

Atkinson, M.R., Wray, L. V. and Fisher, S.H. (1990) ‘Regulation of histidine and proline degradation enzymes by amino acid availability in *Bacillus subtilis*’, *Journal of Bacteriology*, 172(9), pp. 4758–4765. Available at: https://doi.org/10.1128/jb.172.9.4758-4765.1990.

Atrih, A. *et al.* (1999) ‘Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation.’, *Journal of bacteriology*, 181(13), pp. 3956–66.

Ausubel, M.F. *et al.* (2003) *Current protocols in molecular biology*, *John Wiley and Sons, Inc.* Available at: https://www.worldcat.org/title/current-protocols-in-molecular-biology/oclc/936946914?referer=di&ht=edition (Accessed: 7 October 2021).

Babitzke, P. and Gollnick, P. (2001) ‘Posttranscription initiation control of tryptophan metabolism in *Bacillus subtilis* by the trp RNA-binding attenuation protein (TRAP), anti-TRAP, and RNA structure’, *Journal of Bacteriology*, 183(20), pp. 5795–5802. Available at: https://doi.org/10.1128/JB.183.20.5795-5802.2001.

Barbieri, G. *et al.* (2016) ‘Interplay of CodY and ScoC in the Regulation of Major Extracellular Protease Genes of *Bacillus subtilis*.’, *Journal of bacteriology*, 198(6), pp. 907–20. Available at: https://doi.org/10.1128/JB.00894-15.

Beeby, M. *et al.* (2013) ‘Architecture and assembly of the Gram-positive cell wall’, *Molecular Microbiology*, 88(4), pp. 664–672. Available at: https://doi.org/10.1111/MMI.12203.

Belitsky, B.R. (2002) ‘Biosynthesis of amino acids of the Gglutamate and aspartate families, alanine, and polyamines’, in *Bacillus subtilis and Its Closest Relatives*. American Society of Microbiology, pp. 203–231.

Belitsky, B.R. *et al.* (2015) ‘Interactive regulation by the *B acillus subtilis* global regulators CodY and ScoC’, *Molecular Microbiology*, 97(4), pp. 698–716. Available at: https://doi.org/10.1111/mmi.13056.

Belitsky, B.R. (2015) ‘Role of branched-chain amino acid transport in *Bacillus subtilis* CodY activity’, *Journal of Bacteriology*, 197(8), pp. 1330–1338. Available at: https://doi.org/10.1128/JB.02563-14.

Belitsky, B.R., Brinsmade, S.R. and Sonenshein, A.L. (2015) ‘Intermediate Levels of *Bacillus subtilis* CodY Activity Are Required for Derepression of the Branched-Chain Amino Acid Permease, BraB’, *PLOS Genetics*. Edited by L. Søgaard-Andersen, 11(10), p. e1005600. Available at: https://doi.org/10.1371/journal.pgen.1005600.

Belitsky, B.R. and Sonenshein, A.L. (1998) ‘Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes’, *Journal of Bacteriology*, 180(23), pp. 6298–6305. Available at: https://doi.org/10.1128/.180.23.6298-6305.1998.

Belitsky, B.R. and Sonenshein, A.L. (2008) ‘Genetic and Biochemical Analysis of CodY-Binding Sites in *Bacillus subtilis*’, *Journal of Bacteriology*, 190(4), pp. 1224–1236. Available at: https://doi.org/10.1128/JB.01780-07.

Belitsky, B.R. and Sonenshein, A.L. (2013) ‘Genome-wide identification of *Bacillus subtilis* CodY-binding sites at single-nucleotide resolution’, *Proceedings of the National Academy of Sciences*, 110(17), pp. 7026–7031. Available at: https://doi.org/10.1073/pnas.1300428110.

Berberich, R. *et al.* (1968) ‘D-amino acids as inducers of L-alanine dehydrogenase in *Bacillus subtilis*’, *J. Biol. Chem.*, 243(5), pp. 1006–1011.

Bergara, F. *et al.* (2003) ‘CodY is a nutritional repressor of flagellar gene expression in *Bacillus subtilis*’, *Journal of Bacteriology*, 185(10), p. 3118. Available at: https://doi.org/10.1128/JB.185.10.3118-3126.2003.

Bernlohr, R.W. (1967) ‘Changes in amino acid permeation during sporulation.’, *Journal of bacteriology*, 93(3), pp. 1031–1044. Available at: https://doi.org/10.1128/jb.93.3.1031-1044.1967.

Beveridge, T.J. and Murray, R.G.E. (1979) ‘How thick is the *Bacillus subtilis* cell wall?’, *Current Microbiology*, 2(1), pp. 1–4. Available at: https://doi.org/10.1007/BF02601723.

Birdsell, D.C., Doyle, R.J. and Morgenstern, M. (1975) ‘Organization of teichoic acid in the cell wall of *Bacillus subtilis*.’, *J. Bacteriol.*, 121(2), pp. 726–734.

Blencke, H. *et al.* (2006) ‘Regulation of citB expression in *Bacillus subtilis*: integration of multiple metabolic signals in the citrate pool and by the general nitrogen regulatory system’, *Archives of microbiology*, 185(2), pp. 136–146. Available at: https://doi.org/10.1007/S00203-005-0078-0.

Blencke, H.-M. *et al.* (2003) ‘Transcriptional profiling of gene expression in response to glucose in *Bacillus subtilis*: regulation of the central metabolic pathways.’, *Metabolic engineering*, 5(2), pp. 133–49.

Borkowski, O. *et al.* (2016) ‘Translation elicits a growth rate-dependent, genome-wide, differential protein production in *Bacillus subtilis*’, *Mol Syst Biol*, 12, p. 870. Available at: https://doi.org/10.15252/msb.20156608.

Boylan, R.J. *et al.* (1972) ‘Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid.’, *Journal of Bacteriology*, 110(1), pp. 281–90.

Brakhage, A., Wozny, M. and Putzer, H. (1990) ‘Structure and nucleotide sequence of the *Bacillus subtilis* phenylalanyl-tRNA synthetase genes’, *Biochimie*, 72(10), pp. 725–734. Available at: https://doi.org/10.1016/0300-9084(90)90157-C.

Brans, A. *et al.* (2004) ‘New integrative method to generate *Bacillus subtilis* recombinant strains free of selection markers’, *Applied and environmental microbiology*, 70(12), pp. 7241–7250. Available at: https://doi.org/10.1128/AEM.70.12.7241-7250.2004.

Brantl, S. and Licht, A. (2010) ‘Characterisation of *Bacillus subtilis* transcriptional regulators involved in metabolic processes.’, *Current protein & peptide science*, 11(4), pp. 274–91.

Brinsmade, S.R. *et al.* (2010) ‘Regulation of CodY Activity through Modulation of Intracellular Branched-Chain Amino Acid Pools’, *Journal of Bacteriology*, 192(24), pp. 6357–6368. Available at: https://doi.org/10.1128/JB.00937-10.

Brinsmade, S.R. *et al.* (2014) ‘Hierarchical expression of genes controlled by the *Bacillus subtilis* global regulatory protein CodY’, *Proceedings of the National Academy of Sciences*, 111(22), pp. 8227–8232. Available at: https://doi.org/10.1073/pnas.1321308111.

Burguière, P. *et al.* (2004) ‘Three different systems participate in L-cystine uptake in *Bacillus subtilis*.’, *Journal of bacteriology*, 186(15), pp. 4875–84. Available at: https://doi.org/10.1128/JB.186.15.4875-4884.2004.

Calogero, S. *et al.* (1994) ‘RocR, a novel regulatory protein controlling arginine utilization in *Bacillus subtilis*, belongs to the NtrC/NifA family of transcriptional activators.’, *Journal of bacteriology*, 176(5), pp. 1234–41.

Cashel, M. *et al.* (1996) ‘The stringent response in: *Escherichia coli* and *Salmonella* Cellular and Molecular Biology’, *Cellular and Molecular Biology*, 2, pp. 1458–1496.

Cava, F. *et al.* (2011) ‘Distinct pathways for modification of the bacterial cell wall by non-canonical D -amino acids’, *The EMBO Journal*, 30(16), pp. 3442–3453. Available at: https://doi.org/10.1038/emboj.2011.246.

Champney, W.S. and Jensen, R.A. (1970) ‘Metabolic influences on tyrosine excretion in *Bacillus subtilis*.’, *Journal of bacteriology*, 104(1), pp. 351–9.

Chan, P. and Lowe, T. (2009) ‘GtRNAdb: a database of transfer RNA genes detected in genomic sequence’, *Nucleic acids research*, 37(Database issue), pp. D93-97. Available at: https://doi.org/10.1093/NAR/GKN787.

Chan, P.P. and Lowe, T.M. (2016) ‘GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes’, *Nucleic Acids Research*, 44(Database issue), p. D184. Available at: https://doi.org/10.1093/NAR/GKV1309.

Cheung, H.Y., Vitković, L. and Freese, E. (1983) ‘Rates of peptidoglycan turnover and cell growth of *Bacillus subtilis* are correlated.’, *Journal of bacteriology*, 156(3), pp. 1099–106.

Choi, S.-K. and Saier, M.H. (2010) ‘Transcriptional regulation of the rsbV promoter controlling stress responses to ethanol, carbon limitation, and phosphorous limitation in *Bacillus subtilis’*, *International Journal of Microbiology*, 2010. Available at: https://doi.org/10.1155/2010/263410.

Chopin, A., Biaudet, V. and Ehrlich, S. (1998) ‘Analysis of the *Bacillus subtilis* genome sequence reveals nine new T-box leaders’, *Molecular microbiology*, 29(2), pp. 662–664. Available at: https://doi.org/10.1046/J.1365-2958.1998.00912.X.

Chubukov, V. *et al.* (2013) ‘Transcriptional regulation is insufficient to explain substrate-induced flux changes in *Bacillus subtilis*.’, *Molecular systems biology*, 9, p. 709. Available at: https://doi.org/10.1038/msb.2013.66.

Clark, V.L. and Young, F.E. (1974) ‘Active transport of D-alanine and related amino acids by whole cells of *Bacillus subtilis*.’, *Journal of bacteriology*, 120(3), pp. 1085–92.

Cohen, G. and Monod, J. (1957) ‘Bacterial permeases’, *Am Soc Microbiol*, 21, pp. 169–194.

Czaplewski, L.G. *et al.* (1992) ‘Purification and initial characterization of AhrC: the regulator of arginine metabolism genes in *Bacillus subtilis*’, *Molecular Microbiology*, 6(2), pp. 267–275. Available at: https://doi.org/10.1111/j.1365-2958.1992.tb02008.x.

Daniel, R. and Errington, J. (1993) ‘Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in *Bacillus subtilis*’, *Journal of Molecular Biology*, 232(2), pp. 468–483. Available at: https://doi.org/10.1006/JMBI.1993.1403.

Daniel, R.A., Williams, A.M. and Errington, J. (1996) ‘A complex four-gene operon containing essential cell division gene pbpB in *Bacillus subtilis*.’, *Journal of bacteriology*, 178(8), pp. 2343–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8636036 (Accessed: 29 November 2016).

Darmon, E. *et al.* (2002) ‘A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*.’, *Journal of bacteriology*, 184(20), pp. 5661–71.

Dijkstra, A.J. and Keck, W. (1996) ‘Peptidoglycan as a barrier to transenvelope transport.’, *Journal of bacteriology*, 178(19), pp. 5555–62.

Doan, T. *et al.* (2003) ‘The *Bacillus subtilis* ywkA gene encodes a malic enzyme and its transcription is activated by the YufL/YufM two-component system in response to malate’, *Microbiology*, 149(9), pp. 2331–2343. Available at: https://doi.org/10.1099/mic.0.26256-0.

Drzewiecki, K. *et al.* (1998) ‘The yvyD gene of *Bacillus subtilis* is under dual control of sigmaB and sigmaH.’, *Journal of bacteriology*, 180(24), pp. 6674–80.

Dul, M.J. and Young, F.E. (1973) ‘Genetic mapping of a mutant defective in D,L-alanine racemase in *Bacillus subtilis* 168.’, *Journal of bacteriology*, 115(3), pp. 1212–4.

Eymann, C. *et al.* (2002) ‘Bacillus subtilis functional genomics: global characterization of the stringent response by proteome and transcriptome analysis.’, *Journal of bacteriology*, 184(9), pp. 2500–20. Available at: https://doi.org/10.1128/jb.184.9.2500-2520.2002.

Fabret, C., Feher, V.A. and Hoch, J.A. (1999) ‘Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world.’, *Journal of bacteriology*, 181(7), pp. 1975–83.

Ferrari, E., Henner, D.J. and Yang, M.Y. (1985) ‘Isolation of an Alanine Racemase Gene from Bacillus subtilis and its Use for Plasmid Maintenance in *B. subtilis’*, *Nature Biotechnology*, 3(11), pp. 1003–1007. Available at: https://doi.org/10.1038/nbt1185-1003.

Ferson, A.E., Lewis V. Wray, J. and Fisher, S.H. (1996) ‘Expression of the *Bacillus subtilis* gabP gene is regulated independently in response to nitrogen and amino acid availability’, *Molecular Microbiology*, 22(4), pp. 693–701. Available at: https://doi.org/10.1046/J.1365-2958.1996.D01-1720.X.

Fischer, E. and Sauer, U. (2005) ‘Large-scale in vivo flux analysis shows rigidity and suboptimal performance of *Bacillus subtilis* metabolism’, *Nature Genetics*, 37(6), pp. 636–640. Available at: https://doi.org/10.1038/ng1555.

Fisher, S.H. (1999) ‘Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference!’, *Molecular Microbiology*, 32(2), pp. 223–232. Available at: https://doi.org/10.1046/j.1365-2958.1999.01333.x.

Fisher, S.H., Rohrer, K. and Ferson, A.E. (1996) ‘Role of CodY in regulation of the *Bacillus subtilis* hut operon.’, *Journal of bacteriology*, 178(13), pp. 3779–84.

Fisher, S.H. and Sonenshein, A.L. (1991) ‘Control of Carbon and Nitrogen Metabolism in *Bacillus Subtilis’*, *Annual Review of Microbiology*, 45(1), pp. 107–135. Available at: https://doi.org/10.1146/annurev.mi.45.100191.000543.

Foster, S.J. and Popham, D.L. (2002) *Bacillus subtilis and Its Closest Relatives*. Edited by A.L. Sonenshein, J.A. Hoch, and R. Losick. American Society of Microbiology. Available at: https://doi.org/10.1128/9781555817992.

Fotheringham, I.G., Bledig, S.A. and Taylor, P.P. (1998) 'Characterization of the genes encoding D-amino acid transaminase and glutamate racemase, two D-glutamate biosynthetic enzymes of *Bacillus sphaericus* ATCC 10208', *Journal of Bacteriology*, 180(16), pp. 4319–4323. Available at: https://doi.org/10.1128/jb.180.16.4319-4323.1998.

Freese, E. and Oosterwyk, J. (1963) 'The Induction of Alanine Dehydrogenase', *Biochemistry*, 2(6), pp. 1212–1216. Available at: https://doi.org/10.1021/BI00906A006.

Freese, E., Park, S.W. and Cashel, M. (1964) 'The developmental significance of alanine dehydrogenase in *Bacillus subtilis'*, *Proceedings of the National Academy of Sciences of the United States of America*, 51(6), pp. 1164–72.

Fujita, Y. (2009) 'Carbon Catabolite Control of the Metabolic Network in *Bacillus subtilis*', *Bioscience, Biotechnology, and Biochemistry*, 73(2), pp. 245–259. Available at: https://doi.org/10.1271/bbb.80479.

Gagnon, Y. *et al.* (1994) 'Clustering and co-transcription of the *Bacillus subtilis* genes encoding the aminoacyl-tRNA synthetases specific for glutamate and for cysteine and the first enzyme for cysteine biosynthesis.', *The Journal of biological chemistry*, 269(10), pp. 7473–82.

Gao, X. *et al.* (2009) 'Structure and mechanism of an amino acid antiporter', *Science (New York, N.Y.)*, 324(5934), pp. 1565–1568. Available at: https://doi.org/10.1126/SCIENCE.1173654.

Gardan, R., Rapoport, G. and Débarbouillé, M. (1995) 'Expression of the rocDEF operon involved in arginine catabolism in *Bacillus subtilis'*, *Journal of Molecular Biology*, 249(5), pp. 843–856. Available at: https://doi.org/10.1006/jmbi.1995.0342.

Geiger, T. and Wolz, C. (2014) 'Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria', *International Journal of Medical Microbiology*, 304(2), pp. 150–155. Available at: https://doi.org/10.1016/j.ijmm.2013.11.013.

Glaser, L. and Lindsay, B. (1977) ‘Relation between cell wall turnover and cell growth in *Bacillus subtilis*.’, *Journal of bacteriology*, 130(2), pp. 610–9.

Gonzalez-Pastor, J., Hobbs, E. and Losick, R. (2003) ‘Cannibalism by Sporulating Bacteria’, *Science*, 301, pp. 510–513. Available at: https://doi.org/10.1126/science.1086462.

Gonzy-Tréboul, G. *et al.* (1991) ‘The glucose permease of the phosphotransferase system of *Bacillus subtilis*: evidence for IIGlc and IIIGlc domains.’, *Molecular microbiology*, 5(5), pp. 1241–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1956301 (Accessed: 30 April 2018).

Goodell, E. and Schwarz, U. (1985) ‘Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*’, *Journal of Bacteriology*, 162(1), pp. 391–397. Available at: https://doi.org/10.1128/JB.162.1.391-397.1985.

Grandoni, J.A., Zahler, S.A. and Calvo’, J.M. (1992) ‘Transcriptional regulation of the ilv-leu operon of *Bacillus subtilis*’, *Journal of Bacteriology*, 174(10), pp. 3212–3219.

Green, C.J. and Vold, B.S. (1988) ‘Structural requirements for processing of synthetic tRNAHis precursors by the catalytic RNA component of RNase P.’, *Journal of Biological Chemistry*, 263(2), pp. 652–657. Available at: https://doi.org/10.1016/S0021-9258(19)35402-X.

Greenwich, J. *et al.* (2019) ‘A decrease in serine levels during growth transition triggers biofilm formation in *bacillus subtilis*’, *Journal of Bacteriology*, 201(15). Available at: https://doi.org/10.1128/JB.00155-19.

Grimshaw, C.E. and Cleland, W.W. (1981) ‘Kinetic mechanism of *Bacillus subtilis* L-alanine dehydrogenase’, *Biochemistry*, 20(20), pp. 5650–5655. Available at: https://doi.org/10.1021/bi00523a002.

Grundy, F. *et al.* (1997) ‘Specificity of tRNA-mRNA interactions in *Bacillus subtilis* tyrS antitermination.’, *Journal of Bacteriology*, 179(8), pp. 2587–94.

Grundy, F., Lehman, S. and Henkin, T. (2003) ‘The L box regulon: lysine sensing by leader RNAs of bacterial lysine biosynthesis genes’, *Proceedings of the National Academy of Sciences of the United States of America*, 100(21), pp. 12057–12062. Available at: https://doi.org/10.1073/PNAS.2133705100.

Grundy, F.J. *et al.* (2000) 'tRNA determinants for transcription antitermination of the *Bacillus subtilis* tyrS gene.', *RNA (New York, N.Y.)*, 6(8), pp. 1131–41.

Grundy, F.J. and Henkin, T.M. (1993) 'tRNA as a positive regulator of transcription antitermination in *B. subtilis'*, *Cell*, 74(3), pp. 475–482. Available at: https://doi.org/10.1016/0092-8674(93)80049-K.

Grundy, F.J. and Henkin, T.M. (1994) 'Conservation of a Transcription Antitermination Mechanism in Aminoacyl-tRNA Synthetase and Amino Acid Biosynthesis Genes in Gram-positive Bacteria', *Journal of Molecular Biology*, 235(2), pp. 798–804. Available at: https://doi.org/10.1006/jmbi.1994.1038.

Grundy, F.J. and Henkin, T.M. (2014) 'Synthesis of serine, glycine, cysteine, and methionine', *Bacillus subtilis and Its Closest Relatives*, pp. 245–254. Available at: https://doi.org/10.1128/9781555817992.CH18.

Grundy, F.J., Rollins, S.M. and Henkin, T.M. (1994) 'Interaction between the Acceptor End of tRNA and the T Box Stimulates Antitermination in the *Bacillus subtilis* tyrS Gene: a New Role for the Discriminator Base', *Journal of Bacteriology*, 176(15), pp. 4518–4526.

Grundy, F.J., Turinsky, A.J. and Henkin, T.M. (1994) 'Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA.', *Journal of bacteriology*, 176(15), pp. 4527–33.

Gunka, K. and Commichau, F.M. (2012) 'Control of glutamate homeostasis in *Bacillus subtilis*: a complex interplay between ammonium assimilation, glutamate biosynthesis and degradation', *Molecular Microbiology*, 85(2), pp. 213–224. Available at: https://doi.org/10.1111/j.1365-2958.2012.08105.x.

Hahne, H. *et al.* (2008) 'From complementarity to comprehensiveness--targeting the membrane proteome of growing *Bacillus subtilis* by divergent approaches', *Proteomics*, 8(19), pp. 4123–4136. Available at: https://doi.org/10.1002/PMIC.200800258.

Handke, L.D., Shivers, R.P. and Sonenshein, A.L. (2008) 'Interaction of *Bacillus subtilis* CodY with GTP', *Journal of Bacteriology*, 190(3), pp. 798–806. Available at: https://doi.org/10.1128/JB.01115-07.

Hayhurst, E.J. *et al.* (2008) 'Cell wall peptidoglycan architecture in *Bacillus subtilis*', 105(38).

Hecker, M. *et al.* (1987) 'Synthesis of heat shock proteins following amino acid or oxygen limitation in *Bacillus subtilis* relA+ and relA strains', *Z Naturfosch C J Biosci.*, 42(7–8), pp. 941–947.

Heijenoort, J. Van (1994) 'Chapter 3 Biosynthesis of the bacterial peptidoglycan unit', *New Comprehensive Biochemistry*, 27(C), pp. 39–54. Available at: https://doi.org/10.1016/S0167-7306(08)60406-8.

Heijenoort, J. van (2001) 'Recent advances in the formation of the bacterial peptidoglycan monomer unit', *Natural Product Reports*, 18(5), pp. 503–519. Available at: https://doi.org/10.1039/A804532A.

Henderson, J.W. *et al.* (no date) 'Rapid, accurate, sensitive, and reproducible HPLC analysis of amino acids- Amino acid analysis using zorbax eclipse-AAA columns and the Agilent 1100 HPLC'.

Henkin, T.M., Glass, B.L. and Grundy, F.J. (1992) 'Analysis of the *Bacillus subtilis* tyrS gene: conservation of a regulatory sequence in multiple tRNA synthetase genes.', *Journal of bacteriology*, 174(4), pp. 1299–306.

Hols, P. *et al.* (1999) 'Conversion of Lactococcus lactis from homolactic to homoalanine fermentation through metabolic engineering', *Nature biotechnology*, 17(6), pp. 588–592. Available at: https://doi.org/10.1038/9902.

Höltje, J. V (1998) 'Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*', *Microbiology and molecular biology reviews : MMBR*, 62(1), pp. 181–203. Available at: https://doi.org/10.1128/MMBR.62.1.181-203.1998.

Hörl, M., Fuhrer, T. and Zamboni, N. (2021) 'Bifunctional Malic/Malolactic Enzyme Provides a Novel Mechanism for NADPH-Balancing in Bacillus subtilis', *mBio*, 12(2). Available at: https://doi.org/10.1128/MBIO.03438-20.

Hosie, A.H.F. and Poole, P.S. (2001) 'Bacterial ABC transporters of amino acids', *Research in Microbiology*, 152(3), pp. 259–270. Available at: https://doi.org/10.1016/S0923-2508(01)01197-4.

Hullo, M.-F. *et al.* (2004) 'The metNPQ operon of *Bacillus subtilis* encodes an ABC permease transporting methionine sulfoxide, D- and L-methionine', *Research in*

Microbiology, 155(2), pp. 80–86. Available at: https://doi.org/10.1016/J.RESMIC.2003.11.008.

Hyyrylainen, H.L. *et al.* (2000) 'D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*.', *The Journal of biological chemistry*, 275(35), pp. 26696–703. Available at: https://doi.org/10.1074/jbc.M003804200.

Inaoka, T. *et al.* (2002) 'Guanine Nucleotides Guanosine 5-Diphosphate 3-Diphosphate and GTP Co-operatively Regulate the Production of an Antibiotic Bacilysin in *Bacillus subtilis**'. Available at: https://doi.org/10.1074/jbc.M208722200.

Johnson, J.W., Fisher, J.F. and Mobashery, S. (2013) 'Bacterial cell-wall recycling', *Annals of the New York Academy of Sciences*, 1277(1), p. 54. Available at: https://doi.org/10.1111/J.1749-6632.2012.06813.X.

Jourlin-Castelli, C. *et al.* (2000) 'CcpC, a novel regulator of the LysR family required for glucose repression of the citB gene in Bacillus subtilis', *Journal of Molecular Biology*, 295(4), pp. 865–878. Available at: https://doi.org/10.1006/JMBI.1999.3420.

Jung, K., Branciamore, S. and Martini, G. (2000) 'Electron spin resonance of copper(II) as a tool for the determination of asparagine concentration in *Bacillus subtilis* cultures', *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1523(1), pp. 1–5. Available at: https://doi.org/10.1016/S0304-4165(00)00112-4.

Kallio, P.T. *et al.* (1991) 'The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein', *Journal of Biological Chemistry*, 266(20), pp. 13411–13417. Available at: https://doi.org/10.1016/S0021-9258(18)98855-1.

Kearns, D.B. and Losick, R. (2005) 'Cell population heterogeneity during growth of *Bacillus subtilis'*, *Genes & Development*, 19(24), p. 3083. Available at: https://doi.org/10.1101/GAD.1373905.

Kern, T. *et al.* (2010) 'Dynamics characterization of fully hydrated bacterial cell walls by solid-state NMR: evidence for cooperative binding of metal ions.', *Journal of the American Chemical Society*, 132(31), pp. 10911–9. Available at: https://doi.org/10.1021/ja104533w.

Kim, S.H., Schneider, B.L. and Reitzer, L. (2010) ‘Genetics and regulation of the major enzymes of alanine synthesis in Escherichia coli’, *Journal of Bacteriology*, 192(20), pp. 5304–5311. Available at: https://doi.org/10.1128/JB.00738-10.

Kimura, K., Tran, L.S.P. and Itoh, Y. (2004) ‘Roles and regulation of the glutamate racemase isogenes, racE and yrpC, in *Bacillus subtilis*’, *Microbiology*, 150(9), pp. 2911–2920. Available at: https://doi.org/10.1099/mic.0.27045-0.

Kleijn, R.J. *et al.* (2010) ‘Metabolic fluxes during strong carbon catabolite repression by malate in *Bacillus subtilis*.’, *The Journal of biological chemistry*, 285(3), pp. 1587–96. Available at: https://doi.org/10.1074/jbc.M109.061747.

Klewing, A. *et al.* (2020) ‘Resistance to serine in *Bacillus subtilis* : identification of the serine transporter YbeC and of a metabolic network that links serine and threonine metabolism’, *Environmental Microbiology*, 22(9), pp. 3937–3949. Available at: https://doi.org/10.1111/1462-2920.15179.

Koch, A.L. and Doyle, R.J. (1985) ‘Inside-to-outside growth and turnover of the wall of gram-positive rods’, *Journal of Theoretical Biology*, 117(1), pp. 137–157. Available at: https://doi.org/10.1016/S0022-5193(85)80169-7.

Kochhar, S. and Paulus, H. (1996) ‘Lysine-induced premature transcription termination in the lysC operon of *Bacillus subtilis*’, *Microbiology*, 142(7), pp. 1635–1639. Available at: https://doi.org/10.1099/13500872-142-7-1635.

Koide, A. and Hoch, J.A. (1994) ‘Identification of a second oligopeptide transport system in *Bacillus subtilis* and determination of its role in sporulation’, *Molecular Microbiology*, 13(3), pp. 417–426. Available at: https://doi.org/10.1111/j.1365-2958.1994.tb00436.x.

Koide, A., Perego, M. and Hoch, J.A. (1999) ‘ScoC regulates peptide transport and sporulation initiation in *Bacillus subtilis*.’, *Journal of bacteriology*, 181(13), pp. 4114–7.

Kolodkin-Gal, I. *et al.* (2010) ‘D-amino acids trigger biofilm disassembly’, *Science (New York, N.Y.)*, 328(5978), pp. 627–9. Available at: https://doi.org/10.1126/science.1188628.

Koo, B.M. *et al.* (2017) ‘Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis’*, *Cell systems*, 4(3), p. 291. Available at: https://doi.org/10.1016/J.CELS.2016.12.013.

Krämer, R. (1994) ‘Functional principles of solute transport systems: concepts and perspectives’, *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1185(1), pp. 1–34. Available at: https://doi.org/10.1016/0005-2728(94)90189-9.

Krásný, L. *et al.* (2008) ‘The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*’, *Molecular Microbiology*, 69(1). Available at: https://doi.org/10.1111/j.1365-2958.2008.06256.x.

Kroos, L. and Yu, Y. (2000) ‘Regulation of sigma factor activity during *Bacillus subtilis* development’, *Current opinion in microbiology*, 3(6), pp. 553–560. Available at: https://doi.org/10.1016/S1369-5274(00)00140-5.

Kunst, F. *et al.* (1997) ‘The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*.’, *Nature*, 390(6657), pp. 249–56. Available at: https://doi.org/10.1038/36786.

Lachowlcz, T.M. *et al.* (1996) ‘Inhibitory action of serine on growth of bacteria of the genus Bacillus on mineral synthetic media’, *Folia Microbiol*, 41(1), pp. 21–25.

Lam, H. *et al.* (2009) ‘D-amino acids govern stationary phase cell wall remodeling in bacteria.’, *Science (New York, N.Y.)*, 325(5947), pp. 1552–5. Available at: https://doi.org/10.1126/science.1178123.

Lambt, D.H. and Bott, K.F. (1979) Inhibition of *Bacillus subtilis* growth and sporulation by threonine, *Journal of Bacteriology*.

LeDeaux, J.R., Solomon, J.M. and Grossman, A.D. (1997) ‘Analysis of non-polar deletion mutations in the genes of the spo0K (opp) operon of *Bacillus subtilis*’, *FEMS Microbiology Letters*, 153(1), pp. 63–69. Available at: https://doi.org/10.1111/J.1574-6968.1997.TB10464.X.

Lee, M. *et al.* (2004) ‘Aerobic production of alanine by *Escherichia coli* aceF ldhA mutants expressing the *Bacillus sphaericus* alaD gene’, *Applied microbiology and biotechnology*, 65(1), pp. 56–60. Available at: https://doi.org/10.1007/S00253-004-1560-3.

Lerondel, G. *et al.* (2006) ‘YtsJ has the major physiological role of the four paralogous malic enzyme isoforms in *Bacillus subtilis*.’, *Journal of bacteriology*, 188(13), pp. 4727–36. Available at: https://doi.org/10.1128/JB.00167-06.

Levdikov, V.M. *et al.* (2017) ‘Structure of the Branched-chain Amino Acid and GTP-sensing Global Regulator, CodY, from *Bacillus subtilis*’, *The Journal of biological chemistry*, 292(7), pp. 2714–2728. Available at: https://doi.org/10.1074/JBC.M116.754309.

Litzinger, S. *et al.* (2010) ‘Muropeptide rescue in *Bacillus subtilis* involves sequential hydrolysis by beta-N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase’, *Journal of bacteriology*, 192(12), pp. 3132–3143. Available at: https://doi.org/10.1128/JB.01256-09.

Liu, P., Xu, H. and Zhang, X. (2021) ‘Metabolic engineering of microorganisms for L-alanine production’, *Journal of Industrial Microbiology and Biotechnology*, 0, p. 57. Available at: https://doi.org/10.1093/JIMB/KUAB057.

Lopez, J.M., Dromerick, A. and Freese, E. (1981) ‘Response of guanosine 5’-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation.’, *Journal of bacteriology*, 146(2), pp. 605–13.

Lopez, J.M., Marks, C.L. and Freese, E. (1979) ‘The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*’, *Biochimica et Biophysica Acta (BBA) - General Subjects*, 587(2), pp. 238–252. Available at: https://doi.org/10.1016/0304-4165(79)90357-X.

Lorca, G., Winnen, B. and Saier, M.H. (2003) ‘Identification of the L-aspartate transporter in *Bacillus subtilis*.’, *Journal of Bacteriology*, 185(10), pp. 3218–22. Available at: https://doi.org/10.1128/jb.185.10.3218-3222.2003.

Mäder, U. *et al.* (2002) ‘Transcriptome and proteome analysis of *Bacillus subtilis* gene expression modulated by amino acid availability.’, *Journal of Bacteriology*, 184(15), pp. 4288–95.

Majumdar, S.K. and Bose, S.K. (1958) ‘Utilization of amino acids by *Bacillus subtilis* during growth and antibiotic production’, *BBA - Biochimica et Biophysica Acta*, 29(3), pp. 509–513. Available at: https://doi.org/10.1016/0006-3002(58)90006-4.

Marta, P.T., Ladner, R.D. and Grandoni, J.A. (1996) ‘A CUC triplet confers leucine-dependent regulation of the *Bacillus subtilis* ilv-leu operon’, *Journal of Bacteriology*, 178(7), pp. 2150–2153. Available at: https://doi.org/10.1128/JB.178.7.2150-2153.1996.

Martínez del Pozo, A. *et al.* (1989) ‘Stereospecificity of reactions catalyzed by bacterial D-amino acid transaminase.’, *The Journal of biological chemistry*, 264(30), pp. 17784–9.

Martinez-Carrion, M. and Jenkins, W.T. (1963) ‘Some properties of a D-alanine D-glutamate aminotransferase purified from Bacillus,subtilis’, *Biochemical and Biophysical Research Communications*, 12(5), pp. 365–368. Available at: https://doi.org/10.1016/0006-291X(63)90106-2.

Martinez-carrion, M. and Terry Jenkins, W. (1965) ‘D-alanine-D-glutamate transaminase’, *The Journal of Biological Chemistry*, 240(9). Available at: https://doi.org/10.1016/S0021-9258(18)97177-2.

Mauck, J., Chan, L. and Glaser, L. (1971) ‘Turnover of the cell wall of Gram-positive bacteria’, *Journal of Biological Chemistry*, 246(6), pp. 1820–1827. Available at: https://doi.org/10.1016/S0021-9258(18)62382-8.

Maul, B. *et al.* (1995) ‘σB-dependent regulation ofgsiB in response to multiple stimuli *inBacillus subtilis*’, *Molecular and General Genetics MGG 1995 248:1*, 248(1), pp. 114–120. Available at: https://doi.org/10.1007/BF02456620.

May, J.J. *et al.* (2005) ‘Inhibition of the D-alanine:D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium’s susceptibility to antibiotics that target the cell wall.’, *The FEBS journal*, 272(12), pp. 2993–3003. Available at: https://doi.org/10.1111/j.1742-4658.2005.04700.x.

Melin, L., Rutberg, L. and Von Gabain’, A. (1989) ‘Transcriptional and Posttranscriptional Control of the *Bacillus subtilis* Succinate Dehydrogenase Operon’, *JOURNAL OF BACTERIOLOGY*, 171(4), pp. 2110–2115.

Meyer, F. and Stülke, J. (2013) ‘Malate metabolism in *Bacillus subtilis*: distinct roles for three classes of malate-oxidizing enzymes’, *FEMS microbiology letters*, 339(1), pp. 17–22. Available at: https://doi.org/10.1111/1574-6968.12041.

Meyer, H. *et al.* (2014) ‘A time resolved metabolomics study: the influence of different carbon sources during growth and starvation of *Bacillus subtilis*’, *Mol. BioSyst.*, 10(7), pp. 1812–1823. Available at: https://doi.org/10.1039/C4MB00112E.

Meyer, H., Weidmann, H. and Lalk, M. (2013) ‘Methodological approaches to help unravel the intracellular metabolome of *Bacillus subtilis*.’, *Microbial cell factories*, 12, p. 69. Available at: https://doi.org/10.1186/1475-2859-12-69.

Mirel, D. *et al.* (2000) ‘Environmental regulation of *Bacillus subtilis* sigma(D)-dependent gene expression’, *Journal of bacteriology*, 182(11), pp. 3055–3062. Available at: https://doi.org/10.1128/JB.182.11.3055-3062.2000.

Mitani, T., Heinze, J.E. and Freese, E. (1977) ‘Induction of sporulation in *Bacillus subtilis* by decoyinine or hadacidin’, *Biochemical and Biophysical Research Communications*, 77(3), pp. 1118–1125. Available at: https://doi.org/10.1016/S0006-291X(77)80094-6.

Mobley, H.L. *et al.* (1984) ‘Insertion and fate of the cell wall in *Bacillus subtilis*.’, *Journal of bacteriology*, 158(1), pp. 169–79.

Moe, L.A. (2013) ‘Amino acids in the rhizosphere: from plants to microbes.’, *American Journal of Botany*, 100(9), pp. 1692–705. Available at: https://doi.org/10.3732/ajb.1300033.

Molle, V. *et al.* (2003) ‘Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis.’, *Journal of bacteriology*, 185(6), pp. 1911–22. Available at: https://doi.org/10.1128/JB.185.6.1911-1922.2003.

Moreno, M.S. *et al.* (2001) ‘Catabolite repression mediated by the CcpA protein in *Bacillus subtilis*: Novel modes of regulation revealed by whole-genome analyses’, *Molecular Microbiology*, 39(5), pp. 1366–1381. Available at: https://doi.org/10.1111/j.1365-2958.2001.02328.x.

Mortuza, R. *et al.* (2018) ‘Overexpression of a newly identified D-amino acid transaminase in *Mycobacterium smegmatis* complements glutamate racemase deletion’, *Molecular Microbiology*, 107(2), pp. 198–213. Available at: https://doi.org/10.1111/mmi.13877.

Muntel, J. *et al.* (2014) ‘Comprehensive absolute quantification of the cytosolic proteome of *Bacillus subtilis* by data independent, parallel fragmentation in liquid chromatography/mass spectrometry (LC/MS(E)).’, *Molecular & cellular proteomics : MCP*, 13(4), pp. 1008–19. Available at: https://doi.org/10.1074/mcp.M113.032631.

Nanamiya, H. *et al.* (2007) ‘Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*’, *Molecular Microbiology*, 67(2), pp. 291–304. Available at: https://doi.org/10.1111/j.1365-2958.2007.06018.x.

Natori, Y. *et al.* (2009) ‘Transcription activity of individual rrn operons in *Bacillus subtilis* mutants deficient in (p)ppGpp synthetase genes, relA, yjbM, and ywaC.’, *Journal of bacteriology*, 191(14), pp. 4555–61. Available at: https://doi.org/10.1128/JB.00263-09.

Neuhaus, F.C. and Baddiley, J. (2003) ‘A Continuum of Anionic Charge: Structures and Functions of d-Alanyl-Teichoic Acids in Gram-Positive Bacteria’, *Microbiology and Molecular Biology Reviews*, 67(4), pp. 686–723. Available at: https://doi.org/10.1128/mmbr.67.4.686-723.2003.

Nicolas, P. *et al.* (2012) ‘Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*’, *Science*, 335(6072), pp. 1103–1106. Available at: https://doi.org/10.1126/science.1206848.

Niehaus, T.D. *et al.* (2018) ‘Identification of a metabolic disposal route for the oncometabolite S-(2-succino)cysteine in *Bacillus subtilis*’, *Journal of Biological Chemistry*, 293(21), pp. 8255–8263. Available at: https://doi.org/10.1074/jbc.RA118.002925.

Nishino, T. *et al.* (1979) ‘Regulatory nucleotides involved in the Rel function of *Bacillus subtilis*.’, *Journal of Bacteriology*, 140(2), p. 671.

Ochi, K., Kandalas, J.C. and Freese, E. (1981) Initiation of *Bacillus subtilis* Sporulation by the Stringent Response to Partial Amino Acid Deprivation*, *THE JOURNAL OF BIOLOGICAL CHEMISTRY*.

Ohne, M. (1974) ‘Regulation of aconitase synthesis in *Bacillus subtilis*: induction, feedback repression, and catabolite repression.’, *Journal of Bacteriology*, 117(3), pp. 1295–1305. Available at: https://doi.org/10.1128/JB.117.3.1295-1305.1974.

Ordal, G.W. and Gibson, K.J. (1977) ‘Chemotaxis toward amino acids by *Bacillus subtilis*’, *Journal of Bacteriology*, 129(1), pp. 151–155. Available at: https://doi.org/10.1128/jb.129.1.151-155.1977.

Ordal, G.W., Villani, D.P. and Gibson, K.J. (1977) ‘Amino acid chemoreceptors of *Bacillus subtilis*’, *Journal of Bacteriology*, 129(1), pp. 156–165. Available at: https://doi.org/10.1128/jb.129.1.156-165.1977.

Oxendeb, D.L. (1972) Memebrane transport.

Park, J.T. and Uehara, T. (2008) ‘How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan).’, *Microbiology and molecular biology reviews : MMBR*, 72(2), pp. 211–27, table of contents. Available at: https://doi.org/10.1128/MMBR.00027-07.

Pelchat, M. and Lapointe, J. (1999) ‘Aminoacyl-tRNA synthetase genes of *Bacillus subtilis*: organization and regulation.’, *Biochemistry and cell biology = Biochimie et biologie cellulaire*, 77(4), pp. 343–7.

Peña-Soler, E. *et al.* (2014) ‘Structural Analysis and Mutant Growth Properties Reveal Distinctive Enzymatic and Cellular Roles for the Three Major L-Alanine Transaminases of *Escherichia coli*’, *PLoS ONE*. Edited by A.O. Hudson, 9(7), p. e102139. Available at: https://doi.org/10.1371/journal.pone.0102139.

Perego, M. *et al.* (1995) ‘Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. Identification of genes and regulation.’, *The Journal of biological chemistry*, 270(26), pp. 15598–606.

Pierce, K.J., Salifu, S.P. and Tangney, M. (2008) ‘Gene cloning and characterization of a second alanine racemase from *Bacillus subtilis* encoded by yncD’, *FEMS Microbiology Letters*, 283(1), pp. 69–74. Available at: https://doi.org/10.1111/j.1574-6968.2008.01151.x.

Pine, M. (1967) ‘Response of intracellular proteolysis to alteration of bacterial protein and the implications in metabolic regulation’, *Journal of bacteriology*, 93(5), pp. 1527–1533. Available at: https://doi.org/10.1128/JB.93.5.1527-1533.1967.

Pinhal, S. *et al.* (2019) ‘Acetate metabolism and the inhibition of bacterial growth by acetate’, *Journal of Bacteriology*, 201(13), pp. 147–166. Available at: https://doi.org/10.1128/JB.00147-19.

Ponce-de-Leon, M.M. and Pizer, L.I. (1972) ‘Serine biosynthesis and its regulation in *Bacillus subtilis*.’, *Journal of bacteriology*, 110(3), pp. 895–904.

Pooley, H.M. (1976) ‘Layered distribution, according to age, within the cell wall of bacillus subtilis.’, *Journal of bacteriology*, 125(3), pp. 1139–47.

Poolman, B. and Konings, W.N. (1993) ‘Secondary solute transport in bacteria’, *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1183(1), pp. 5–39. Available at: https://doi.org/10.1016/0005-2728(93)90003-X.

Pozo, A.M. del *et al.* (1989) ‘Activity and Spectroscopic Properties of Bacterial D-Amino Acid Transaminase after Multiple Site-Directed Mutagenesis of a Single Tryptophan Residue’, *Biochemistry*, 28(2), pp. 510–516. Available at: https://doi.org/10.1021/bi00428a015.

Putzer, H. *et al.* (1995) ‘Aminoacyl-tRNA synthetase gene regulation in *Bacillus subtilis*: induction, repression and growth-rate regulation’, *Molecular Microbiology*, 16(4), pp. 709–718. Available at: https://doi.org/10.1111/j.1365-2958.1995.tb02432.x.

Radkov, A. and Moe, M. (2014) ‘Bacterial synthesis of D-amino acids’, *Applied microbiology and biotechnology*, 98(12), pp. 5363–5374. Available at: https://doi.org/10.1007/S00253-014-5726-3.

Ratnayake-Lecamwasam, M. *et al.* (2001) ‘*Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels’, *Genes & Development*, 15(9), pp. 1093–1103. Available at: https://doi.org/10.1101/gad.874201.

Reig, N. *et al.* (2007) ‘Functional and structural characterization of the first prokaryotic member of the L-amino acid transporter (LAT) family: a model for APC transporters.’, *The Journal of biological chemistry*, 282(18), pp. 13270–81. Available at: https://doi.org/10.1074/jbc.M610695200.

Reith, J. and Mayer, C. (2011) ‘Peptidoglycan turnover and recycling in Gram-Positive bacteria’, *Applied Microbiology and Biotechnology*. Springer, pp. 1–11. Available at: https://doi.org/10.1007/s00253-011-3486-x.

Resnekov, O. *et al.* (1992) ‘Organization and regulation of the *Bacillus subtilis* odhAB operon, which encodes two of the subenzymes of the 2-oxoglutarate dehydrogenase complex’, *Molecular & general genetics : MGG*, 234(2), pp. 285–296. Available at: https://doi.org/10.1007/BF00283849.

Robbins, J.C. and Oxender, D.L. (1973) ‘Transport systems for alanine, serine, and glycine in *Escherichia coli* K-12’, *Journal of Bacteriology*, 116(1), pp. 12–18. Available at: https://doi.org/10.1128/JB.116.1.12-18.1973.

Rodionov, D.A. *et al.* (2003) ‘Regulation of lysine biosynthesis and transport genes in bacteria: yet another RNA riboswitch?’, *Nucleic acids research*, 31(23), pp. 6748–57. Available at: https://doi.org/10.1093/NAR/GKG900.

Rollins, S.M., Grundy, F.J. and Henkin, T.M. (1997) ‘Analysis of cis-acting sequence and structural elements required for antitermination of the *Bacillus subtilis* tyrS gene’, *Molecular Microbiology*, 25(2), pp. 411–421. Available at: https://doi.org/10.1046/J.1365-2958.1997.4851839.X.

Saier, M.H. (2000) ‘Families of transmembrane transporters selective for amino acids and their derivatives.’, *Microbiology (Reading, England)*, 146 (Pt 8, pp. 1775–95. Available at: https://doi.org/10.1099/00221287-146-8-1775.

Saier, M.H. *et al.* (2002) ‘Overall transport capabilities of *Bacillus subtilis*’. Available at: http://www.asmscience.org/docserver/fulltext/10.1128/9781555817992/9781555812058_Chap10.pdf?expires=1506967006&id=id&accname=esid055672&checksum=920FD7CF210EBB17D92906CA3DB30C3E (Accessed: 2 October 2017).

Salzberg, L.I. and Helmann, J.D. (2008) ‘Phenotypic and Transcriptomic Characterization of *Bacillus subtilis* Mutants with Grossly Altered Membrane Composition’, *Journal of bacteriology*, 190(23), pp. 7797–807. Available at: https://doi.org/10.1128/JB.00720-08.

Sarsero, J.P., Merino, E. and Yanofsky, C. (2000) ‘A *Bacillus subtilis* gene of previously unknown function, yhaG, is translationally regulated by tryptophan-activated TRAP and appears to be involved in tryptophan transport.’, *Journal of bacteriology*, 182(8), pp. 2329–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10735881 (Accessed: 14 September 2017).

Satomura, T. *et al.* (2005) ‘Enhancement of glutamine utilization in *Bacillus subtilis* through the GlnK-GlnL two-component regulatory system’, *Journal of Bacteriology*, 187(14), pp. 4813–4821. Available at: https://doi.org/10.1128/JB.187.14.4813-4821.2005.

Schilling, O. *et al.* (2007) ‘Transcriptional and Metabolic Responses of *Bacillus subtilis* to the Availability of Organic Acids: Transcription Regulation Is Important but Not Sufficient To Account for Metabolic Adaptation’, *Applied and Environmental Microbiology*, 73(2), pp. 499–507. Available at: https://doi.org/10.1128/AEM.02084-06.

Scott, J.R. and Barnett, T.C. (2006) ‘Surface proteins of gram-positive bacteria and how they get there.’, *Annual review of microbiology*, 60, pp. 397–423. Available at: https://doi.org/10.1146/annurev.micro.60.080805.142256.

Scott, M. *et al.* (2010) ‘Interdependence of cell growth and gene expression: Origins and consequences’, *Science*, 330(6007), pp. 1099–1102. Available at: https://doi.org/10.1126/science.1192588.

Selvarasu, S. *et al.* (2009) ‘Characterizing *Escherichia coli* DH5α growth and metabolism in a complex medium using genome-scale flux analysis’, *Biotechnology and Bioengineering*, 102(3), pp. 923–934. Available at: https://doi.org/10.1002/bit.22119.

Serror, P. and Sonenshein, A.L. (1996) ‘Interaction of CodY, a novel *Bacillus subtilis* DNA-binding protein, with the dpp promoter region.’, *Molecular microbiology*, 20(4), pp. 843–52.

Sezonov, G., Joseleau-Petit, D. and D’Ari, R. (2007) ‘*Escherichia coli* physiology in Luria-Bertani broth.’, *Journal of bacteriology*, 189(23), pp. 8746–9. Available at: https://doi.org/10.1128/JB.01368-07.

Shivers, R.P. and Sonenshein, A.L. (2004) ‘Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids’, *Molecular Microbiology*, 53(2), pp. 599–611. Available at: https://doi.org/10.1111/j.1365-2958.2004.04135.x.

Sidiq, K.R. (2016) ‘Cell Wall Metabolism in *Bacillus subtilis*’.

Sidiq, K.R. *et al.* (2021) ‘Alanine metabolism in *Bacillus subtilis*’, *Molecular Microbiology*, 115(4), pp. 739–757. Available at: https://doi.org/10.1111/MMI.14640.

Silhavy, T.J., Kahne, D. and Walker, S. (2010) ‘The bacterial cell envelope’, *Cold Spring Harbor perspectives in biology*, 2(5), p. a000414. Available at: https://doi.org/10.1101/cshperspect.a000414.

Siranosian, K.J., Ireton, K. and Grossman, A.D. (1993) ‘Alanine dehydrogenase (ald) is required for normal sporulation in *Bacillus subtilis*.’, *Journal of bacteriology*, 175(21), pp. 6789–96.

Slack, F.J. *et al.* (1991) ‘Transcriptional regulation of a *Bacillus subtilis* dipeptide transport operon.’, *Molecular microbiology*, 5(8), pp. 1915–25.

Slack, F.J. *et al.* (1995) ‘A gene required for nutritional repression of the *Bacillus subtilis* dipeptide permease operon.’, *Molecular microbiology*, 15(4), pp. 689–702.

Smith, T.J., Blackman, S.A. and Foster, S.J. (2000) ‘Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions’, *Microbiology*, 146(2), pp. 249–262. Available at: https://doi.org/10.1099/00221287-146-2-249.

Smith, T.J., Blackman, S.A. and Foster, S.J. (2009) ‘Peptidoglycan Hydrolases of *Bacillus subtilis* 168’, *https://home.liebertpub.com/mdr*, 2(1), pp. 113–118. Available at: https://doi.org/10.1089/MDR.1996.2.113.

Sojka, L. *et al.* (2011) ‘Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in *Bacillus subtilis*’, *Nucleic Acids Research*, 39(11), pp. 4598–4611. Available at: https://doi.org/10.1093/NAR/GKR032.

Sonenshein, A. (2002) ‘Developmental biology: regulation by selective gene localization’, *Current biology : CB*, 12(3). Available at: https://doi.org/10.1016/S0960-9822(02)00668-1.

Sonenshein, A.L. (2005) ‘CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria’, *Current Opinion in Microbiology*, 8(2), pp. 203–207. Available at: https://doi.org/10.1016/j.mib.2005.01.001.

Sonenshein, A.L. (2007) ‘Control of key metabolic intersections in *Bacillus subtilis*’, *Nature Reviews Microbiology*, 5(12), pp. 917–927. Available at: https://doi.org/10.1038/nrmicro1772.

Stauffer, G. (2004) ‘Regulation of serine, glycine, and one-carbon biosynthesis’, *EcoSal Plus*, 1(1). Available at: https://doi.org/10.1128/ECOSALPLUS.3.6.1.2.

Strauch, M. (1995) ‘Delineation of AbrB-binding sites on the *Bacillus subtilis* spo0H, kinB, ftsAZ, and pbpE promoters and use of a derived homology to identify a previously unsuspected binding site in the bsuB1 methylase promote’, *Journal of*

Bacteriology, 177(23), pp. 6999–7002. Available at: https://doi.org/10.1128/JB.177.23.6999-7002.1995.

Stülke, J. and Hillen, W. (2000) 'Regulation of carbon catabolism in *Bacillus* species', *Annual review of microbiology*, 54, pp. 849–880. Available at: https://doi.org/10.1146/ANNUREV.MICRO.54.1.849.

Subramaniam, A.R. *et al.* (2013) 'A serine sensor for multicellularity in a bacterium', *eLife*, 2013(2). Available at: https://doi.org/10.7554/ELIFE.01501.

Sutrinas, S.L. *et al.* (1990) 'The glucose permease of *Bacillus subtilis* is a single polypeptide chain that functions to energize the sucrose permease', *The Journal of Biological Chemistry*, 265(30), pp. 18581–18589. Available at: http://www.jbc.org/content/265/30/18581.full.pdf (Accessed: 30 April 2018).

Svenningsen, S. Lo *et al.* (2017) 'Transfer RNA is highly unstable during early amino acid starvation in *Escherichia coli*', *Nucleic Acids Research*, 45(2), p. 793. Available at: https://doi.org/10.1093/NAR/GKW1169.

Tam, L.T. *et al.* (2006) 'Global gene expression profiling of *Bacillus subtilis* in response to ammonium and tryptophan starvation as revealed by transcriptome and proteome analysis', *Journal of Molecular Microbiology and Biotechnology*, 12(1–2), pp. 121–130. Available at: https://doi.org/10.1159/000096467.

Tanaka, K., Kobayashi, K. and Ogasawara, N. (2003) 'The *Bacillus subtilis* YufLM two-component system regulates the expression of the malate transporters MaeN (YufR) and YflS, and is essential for utilization of malate in minimal medium', *Microbiology (Reading, England)*, 149(Pt 9), pp. 2317–2329. Available at: https://doi.org/10.1099/MIC.0.26257-0.

Tännler, S. *et al.* (2008) 'CcpN controls central carbon fluxes in *Bacillus subtilis*.', *Journal of bacteriology*, 190(18), pp. 6178–87. Available at: https://doi.org/10.1128/JB.00552-08.

Tempest, D.W., Meers, J.L. and Brown, C.M. (1970) 'Influence of environment on the content and composition of microbial free amino acid pools', *Journal of General Microbiology*, 64(2), pp. 171–185. Available at: https://doi.org/10.1099/00221287-64-2-171.

Thompson, R.J. *et al.* (1998) ‘Pathogenicity and immunogenicity of a *Listeria monocytogenes* strain that requires D-alanine for growth.’, *Infection and immunity*, 66(8), pp. 3552–61.

Thorne, C.B., Gomez, C.G. and Housewright, R.D. (1955) ‘Transamination of D-amino acids by *Bacillus subtilis*.’, *Journal of Bacteriology*, 69(3), pp. 357–62.

Tobisch, S. *et al.* (1999) ‘Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*’, *Journal of bacteriology*, 181(22), pp. 6996–7004. Available at: https://doi.org/10.1128/JB.181.22.6996-7004.1999.

Tojo, S. *et al.* (2008) ‘Molecular Mechanisms Underlying the Positive Stringent Response of the *Bacillus subtilis* ilv-leu Operon, Involved in the Biosynthesis of Branched-Chain Amino Acids’, *Journal of Bacteriology*, 190(18), pp. 6134–6147. Available at: https://doi.org/10.1128/JB.00606-08.

Tojo, S. *et al.* (2010) ‘Heavy Involvement of Stringent Transcription Control Depending on the Adenine or Guanine Species of the Transcription Initiation Site in Glucose and Pyruvate Metabolism in *Bacillus subtilis*’, *Journal of Bacteriology*, 192(6), pp. 1573–1585. Available at: https://doi.org/10.1128/JB.01394-09.

Tollerson, R. and Ibba, M. (2020) ‘Translational regulation of environmental adaptation in bacteria’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., pp. 10434–10445. Available at: https://doi.org/10.1074/jbc.REV120.012742.

Tolner, B. *et al.* (1995) ‘Characterization of the proton/glutamate symport protein of *Bacillus subtilis* and its functional expression in *Escherichia coli*.’, *Journal of bacteriology*, 177(10), pp. 2863–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7751298 (Accessed: 14 September 2017).

Tomoyoshi Soga, *,† *et al.* (2003) ‘Quantitative Metabolome Analysis Using Capillary Electrophoresis Mass Spectrometry’. Available at: https://doi.org/10.1021/PR034020M.

Turner, R., Hobbs, J. and Foster, S. (2016) ‘Atomic Force Microscopy Analysis of Bacterial Cell Wall Peptidoglycan Architecture’, *Methods in Molecular Biology (Clifton, N.J.)*, 1440, pp. 3–9. Available at: https://doi.org/10.1007/978-1-4939-3676-2_1.

Turner, R.D., Vollmer, W. and Foster, S.J. (2014) ‘Different walls for rods and balls: the diversity of peptidoglycan.’, *Molecular microbiology*, 91(5), pp. 862–74. Available at: https://doi.org/10.1111/mmi.12513.

U, S. *et al.* (1996) ‘Physiology and metabolic fluxes of wild-type and riboflavin-producing *Bacillus subtilis*’, *Applied and environmental microbiology*, 62(10), pp. 3687–3696. Available at: https://doi.org/10.1128/AEM.62.10.3687-3696.1996.

Villapakkam, A.C. *et al.* (2009) ‘Genetic and Biochemical Analysis of the Interaction of *Bacillus subtilis* CodY with Branched-Chain Amino Acids’, *Journal of Bacteriology*, 191(22), pp. 6865–6876. Available at: https://doi.org/10.1128/JB.00818-09.

Voelker, U. *et al.* (1995) ‘Separate mechanisms activate sigma B of *Bacillus subtilis* in response to environmental and metabolic stresses’, *Journal of bacteriology*, 177(13), pp. 3771–3780. Available at: https://doi.org/10.1128/JB.177.13.3771-3780.1995.

Vollmer, W. (2008) ‘Structural variation in the glycan strands of bacterial peptidoglycan.’, *FEMS microbiology reviews*, 32(2), pp. 287–306. Available at: https://doi.org/10.1111/j.1574-6976.2007.00088.x.

Vollmer, W., Blanot, D. and de Pedro, M. a (2008) ‘Peptidoglycan structure and architecture.’, *FEMS microbiology reviews*, 32(2), pp. 149–67. Available at: https://doi.org/10.1111/j.1574-6976.2007.00094.x.

Vollmer, W. and Höltje, J. V (2001) ‘Morphogenesis of *Escherichia coli*’, *Current Opinion in Microbiology*, 4(6), pp. 625–633. Available at: https://doi.org/10.1016/S1369-5274(01)00261-2.

Vollmer, W. and Seligman, S.J. (2010) ‘Architecture of peptidoglycan: more data and more models.’, *Trends in microbiology*, 18(2), pp. 59–66. Available at: https://doi.org/10.1016/j.tim.2009.12.004.

Walsh, C.T. (1989) ‘Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly.’, *The Journal of biological chemistry*, 264(5), pp. 2393–6.

Ward, J.B. (1973) ‘The chain length of the glycans in bacterial cell walls.’, *The Biochemical journal*, 133(2), pp. 395–8.

Ward, J.B. and Zahler, S.A. (1973) ‘Genetic studies of leucine biosynthesis in *Bacillus subtilis*.’, *Journal of bacteriology*, 116(2), pp. 719–26.

Wargel, R.J., Hadur, C.A. and Neuhaus, F.C. (1971) ‘Mechanism of D-cycloserine action: transport mutants for D-alanine, D-cycloserine, and glycine.’, *Journal of Bacteriology*, 105(3), pp. 1028–1035. Available at: https://doi.org/10.1128/JB.105.3.1028-1035.1971.

Wargel, R.J., Shadur, C.A. and Neuhaus, F.C. (1970) ‘Mechanism of D-cycloserine action: transport systems for D-alanine, D-cycloserine, L-alanine, and glycine.’, *Journal of Bacteriology*, 103(3), pp. 778–788. Available at: https://doi.org/10.1128/JB.103.3.778-788.1970.

Wecke, J., Madela, K. and Fischer, W. (1997) ‘The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*’, *Microbiology*, 143(9), pp. 2953–2960. Available at: https://doi.org/10.1099/00221287-143-9-2953.

Wecke, J., Perego, M. and Fischer, W. (1996) ‘D-alanine deprivation of *Bacillus subtilis* teichoic acids is without effect on cell growth and morphology but affects the autolytic activity’, *Microbial Drug Resistance*, 2(1), pp. 123–129. Available at: https://doi.org/10.1089/mdr.1996.2.123.

van Wely, K.H.M. *et al.* (2001) ‘Translocation of proteins across the cell envelope of Gram-positive bacteria’, *FEMS Microbiology Reviews*, 25(4), pp. 437–454. Available at: https://doi.org/10.1111/j.1574-6976.2001.tb00586.x.

Wendrich, T.M. and Marahiel, M.A. (1997) ‘Cloning and characterization of a relA/spoT homologue from *Bacillus subtilis*.’, *Molecular microbiology*, 26(1), pp. 65–79.

Wiame, J.M. and Pierard, A. (1955) ‘Occurrence of an L(+)-Alanine dehydrogenase in *Bacillus subtilis*’, *Nature*, 176(4492), pp. 1073–1075. Available at: https://doi.org/10.1038/1761073b0.

Willetts, A.J. and Turner, J.M. (1970) ‘Threonine metabolism in a strain of *Bacillus subtilis*.’, *The Biochemical journal*, 117(2), p. 438. Available at: https://doi.org/10.1042/bj1170027Pb.

Wilson-Mitchell, S.N., Grundy, F.J. and Henkin, T.M. (2012) ‘Analysis of lysine recognition and specificity of the *Bacillus subtilis* L box riboswitch’, *Nucleic Acids Research*, 40(12), pp. 5706–5717. Available at: https://doi.org/10.1093/nar/gks212.

Wray, L. V., Ferson, A.E. and Fisher, S.H. (1997) ‘Expression of the *Bacillus subtilis* ureABC operon is controlled by multiple regulatory factors including CodY, GlnR, TnrA, and Spo0H’, *Journal of Bacteriology*, 179(17), pp. 5494–5501. Available at: https://doi.org/10.1128/JB.179.17.5494-5501.1997.

Yakhnin, H. *et al.* (2004) ‘The trp RNA-binding attenuation protein of *Bacillus subtilis* regulates translation of the tryptophan transport gene trpP (yhaG) by blocking ribosome binding.’, *Journal of bacteriology*, 186(2), pp. 278–86. Available at: https://doi.org/10.1128/JB.186.2.278-286.2004.

Yamane, K., Bunai, K. and Kakeshita, H. (2004) ‘Protein traffic for secretion and related machinery of *Bacillus subtilis*.’, *Bioscience, biotechnology, and biochemistry*, 68(10), pp. 2007–23.

Yang, Y. *et al.* (2015) ‘Relation between chemotaxis and consumption of amino acids in bacteria’, *Molecular Microbiology*, 96(6), pp. 1272–1282. Available at: https://doi.org/10.1111/mmi.13006.

Yonaha, K. *et al.* (1975) ‘D-amino acid aminotransferase of *Bacillus sphaericus*. Enzymologic and spectrometric properties.’, *Journal of Biological Chemistry*, 250(17), pp. 6983–6989. Available at: https://doi.org/10.1016/S0021-9258(19)41029-6.

Yoneyama, H. *et al.* (2011) ‘Isolation of a Mutant Auxotrophic for L-Alanine and Identification of Three Major Aminotransferases That Synthesize L -Alanine in *Escherichia coli*’, *Bioscience, Biotechnology, and Biochemistry*, 75(5), pp. 930–938. Available at: https://doi.org/10.1271/bbb.100905.

Yoshida, A. and Freese, E. (1964) ‘Purification and chemical characterisation of alanine dehydrogenase of *Bacillus subtilis*.’, *Biochimica et biophysica acta*, 92, pp. 33–43.

Yoshida, A. and Freese, E. (1965) ‘Enzymic properties of alanine dehydrogenase of *Bacillus subtilis*’, *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis*, 96(2), pp. 248–262. Available at: https://doi.org/10.1016/0005-2787(65)90588-5.

Yoshimura, T. and Esak, N. (2003) ‘Amino acid racemases: functions and mechanisms’, *Journal of Bioscience and Bioengineering*, 96(2), pp. 103–109. Available at: https://doi.org/10.1016/S1389-1723(03)90111-3.

Young, F.E. and Spizizen, J. (1961) ‘Physiological and genetic factors affecting transformation of *Bacillus subtilis*.’, *Journal of bacteriology*, 81(5), pp. 823–9.

Zalieckas, J.M., Wray, L. V and Fisher, S.H. (1999) ‘Trans-acting factors affecting carbon catabolite repression of the hut operon in *Bacillus subtilis*.’, *Journal of bacteriology*, 181(9), pp. 2883–8.

Zaprasis, A. *et al.* (2015) ‘Uptake of amino acids and their metabolic conversion into the compatible solute proline confers osmoprotection to *Bacillus subtilis*.’, *Applied and Environmental Microbiology*, 81(1), pp. 250–9. Available at: https://doi.org/10.1128/AEM.02797-14.

Zhang, S. and Haldenwang, W.G. (2003) ‘RelA is a component of the nutritional stress activation pathway of the *Bacillus subtilis* transcription factor σB’, *Journal of Bacteriology*, 185(19), p. 5714. Available at: https://doi.org/10.1128/JB.185.19.5714-5721.2003.

Zhang, X. *et al.* (2007) ‘Production of L-alanine by metabolically engineered *Escherichia coli*’, *Applied Microbiology and Biotechnology*, 77(2), pp. 355–366. Available at: https://doi.org/10.1007/S00253-007-1170-Y.

Zhao, H., Roistacher, D.M. and Helmann, J.D. (2018) ‘Aspartate deficiency limits peptidoglycan synthesis and sensitizes cells to antibiotics targeting cell wall synthesis in *Bacillus subtilis*’, *Molecular Microbiology*, 109(6), pp. 826–844. Available at: https://doi.org/10.1111/mmi.14078.

Chapter 8 Appendix

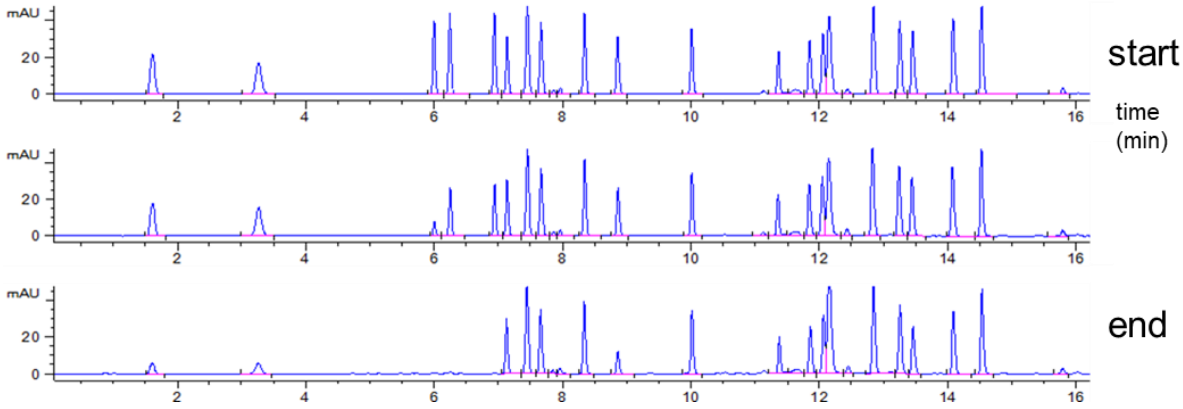


Figure 8.1: Chromatogram of amino acids content in defined medium of culture taken during time course experiment for quantitative analysis.

Table 8.1: Relative abundance of amino acids of *168+* growing in Gaa medium at different time points.

	Relative level of amino acid (percentage to T_0 at T_{150})	Relative level of amino acid (percentage to T_0 at T_{250})	Relative level of amino acid (percentage to T_0 at T_{400})
Asparagine	19.4	0.0	N.D
Serine	60.4	12.3	N.D
Glutamine	62.6	20.7	N.D
Aspartate	80.5	53.6	25.0
Glutamate	90.0	62.5	33.7
Alanine	84.7	57.5	39.5
Isoleucine	92.4	81.5	73.7
Leucine	92.9	85.3	81.7
Lysine	89.8	86.5	86.6
Valine	95.9	90.8	88.2
Threonine	94.5	90.1	88.6
Arginine	95.2	91.4	90.8
Phenylalanine	96.2	94.2	95.2
Tyrosine	96.3	94.7	96.9
Glycine	98.5	98.3	99.5
Tryptophan	95.1	98.0	99.9

The relative abundance of each amino acid at each time point (T_{150}, T_{250} and T_{400} from left to right) was calculated in percentage relative to their initial concentration correspondingly and the numbers were corrected to 1 decimal place. Data shown is from single experiment. The experiment was repeated and similar trend of

assimilation profile was obtained. N.D = amino acid concentration was below detectable range.

Table 8.2: Depletion rates of amino acids from defined medium of growing *168+*

	Gaa	Maa	GMaa
Asparagine	-25.1	-19.3	-22.6
Glutamine	-42.7	-21.7	-20.9
Serine	-18.5	-23.6	-24.2
Aspartate	-17.6	-17.7	-27.3
Alanine	-11.3	1.8	0.5
Glutamate	-17.7	-27.4	-31.7
Leucine	-8.2	-7.5	-5.0
Isoleucine	-8.6	-7.7	-1.7
Lysine	-7.1	-7.7	-1.3
Valine	-6.8	-11.8	4.9
Phenylalanine	-3.3	-3.4	1.9
Arginine	-4.1	-3.8	1.7
Tyrosine	-0.4	-0.4	7.3
Tryptophan	-0.4	-0.6	3.0
Threonine	-5.4	-2.7	-2.4
Glycine	0.4	0.0	6.2

The depletion rates (% decrease per OD600) were calculated as the percentage change of the relative abundance of amino acids per change of optical density. Column Gaa represents the relative abundance of amino acids recorded when the strain was grown in Gaa whilst column Maa represents those when the strain was grown in Maa. Column GMaa shows the relative abundance of amino acids when the strain was grown in GMaa. The order of amino acids follows that of Table 3.2 for easier reference. Data shown is from single experiment. The experiment was repeated and similar trend of assimilation profile was obtained.

Table 8.3: Tables of assimilation efficiency of the amino acid specified for different auxotrophs and the wild type strain.

A Serine

Time (min)	Wild type strain	Auxotroph (*ΔserA*)
0-160	-28.7	-34.9
0-100	-37.1	-55.3
100-160	-26.5	-28.7

B Alanine

Time (min)	Wild type strain	Auxotroph (*ΔalaT*)
0-160	-13.7	-16.9
0-100	-10.2	-14.5
100-160	-14.6	-17.7

C Leucine

Time (min)	Wild type strain	Auxotroph (*ΔleuA*)
0-160	-14.1	-9.5
0-100	-20.8	-4.3
100-160	-12.3	-11

D Tryptophan

Time (min)	Wild type strain	Auxotroph (*ΔtrpC2*)
0-160	-2.9	-1.8
0-100	2.8	8.6
100-160	-4.4	-5.1

The strains were grown in defined medium (GaaP) containing equal concentrations of 20 amino acids. Each table shows the assimilation efficiency rate calculated as percentage change per OD600 of the amino acid that the auxotroph required for growth for the auxotroph compared to the wild type strain during exponential growth over three time points. The first row of each table shows the overall rate during exponential growth. The second row shows the first 100 minute of the assimilation efficiency of the specified amino acid whilst the third row shows the following 60 minute of assimilation efficiency of the amino acid. The more negative the value in each table, the higher assimilation efficiency of the specified amino acid. A: comparison of assimilation efficiency of serine between serine auxotroph (MC16) and wild type strain. B: comparison of assimilation efficiency of alanine between leucine auxotroph (MC2) and wild type strain. C: comparison of assimilation efficiency of leucine between leucine auxotroph (MC17) and wild type strain. D: comparison of assimilation efficiency of tryptophan between tryptophan auxotroph (*168CA*) and wild type strain. The experiment has been repeated once and the data above come from a single experiment

Table 8.4: Table of upregulated genes in lysine auxotroph grown in limited lysine compared to that of grown in excess.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
abnA	5.55	9.98		31.10	2.27	3.64	1.07	0.23

ald	10.78	5.05	4.02	3.42	0.92	1.62	2.01	4.58
clsB/ywjE	17.76	5.66	8.08		1.78	1.91	5.34	0.50
csbD	5.55	5.99	5.05		0.00	0.00	0.00	0.00
degQ	5.95	9.38	6.19	3.79	0.31	1.13	4.60	3.88
epr	6.68	11.95	3.39	3.03	2.70	1.51	1.82	1.17
gcvPA	17.29	5.06	4.64	4.95	0.93	1.35	3.62	6.44
hom	11.25	6.70	5.67	3.91	1.75	1.87	3.36	5.09
hutP	9.97	8.79	7.18	3.42	1.57	1.25	1.80	0.95
spoIIIAA	14.43	6.99	27.28		0.35	0.47	2.14	1.39
tcyN	6.29	14.97	2.86	48.95	0.45	1.46	3.56	2.31
tlpB	6.44	13.16	2.75	3.39	3.09	1.17	1.08	1.33
xkdD	6.94	10.98	4.24	42.49	1.89	1.62	3.42	2.22
ybgE	10.73	7.75	4.85	9.67	1.40	1.05	2.10	2.08
ycxC	12.65	5.08	3.68	4.27	0.76	0.89	2.14	4.44
yczI	5.29	9.20	4.46	5.78	1.21	1.03	3.06	4.07
ydeG	6.32	8.81	3.23	7.24	2.12	1.31	2.46	7.34
yfmB	5.86	6.73	4.65	7.14	0.89	1.35	2.21	1.97
yfmI	6.56	5.28	8.12	3.64	1.52	1.59	3.25	5.46
yheE	6.56	6.75	10.14	3.73	0.71	1.06	2.40	2.74

yisS	10.05	5.62	4.42	9.24	2.05	2.48	2.71	9.80
yjcA	14.43	7.32	0.88	7.57	0.43	0.76	1.07	1.39
ykwB	6.64	5.60	4.00	3.87	0.69	1.59	2.19	3.00
yncC	10.37	6.13	7.38	4.66	0.79	1.07	1.74	2.16
yoaA	5.03	5.87	5.09	3.86	1.01	1.28	2.82	2.54
yobF	5.18	6.13	3.28	4.36	0.69	1.14	3.55	3.61
ypzC	5.97	5.58	5.37	1.57	1.21	1.28	2.35	4.54
yqbQ	7.21	13.98	2.69	5.67	1.14	1.21	3.92	1.91
yrdB	33.79	26.29	14.57	3.69	1.39	1.62	8.19	15.94
yukJ	6.85	6.70	6.51	3.23	1.30	1.27	3.51	2.67
yuzF	7.24	13.61	4.82	6.66	1.34	1.62	3.65	3.94
ywqN	11.88	7.77	2.36	1.68	0.69	0.53	0.97	1.30
ywqO	12.07	5.54	1.01	2.35	0.95	1.52	0.75	1.19
yxeM	9.30	8.53	5.81	4.70	1.88	1.45	4.94	2.05
yxkD	5.85	7.99	2.22	5.32	1.14	0.79	1.57	1.62
yxzF	9.43	13.98	1.68	6.58	0.38	0.40	1.07	2.77

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Table 8.5: Table of upregulated genes in lysine auxotroph and wild type strain grown in limited lysine compared to that of grown in excess.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
lysC	87.14	138.72	1.67	0.96	1.17	37.32	1.06	0.74
yvsH	26.51	11.24	3.79	8.25	0.88	13.62	0.36	0.51

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Table 8.6: Table of genes upregulated in phenylalanine auxotroph grown in limited phenylalanine compared to that of grown in excess.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
yaaC	2.66	0.82	12.12	12.78	1.14	1.73	1.28	0.46
yabG	4.07	0.67	21.21	10.90	1.14	3.03	1.07	0.97
adaA	1.97	4.52	6.22	7.85	3.15	1.89	1.91	1.12
ybxG	5.54	2.60	6.97	5.44	1.22	1.08	2.09	5.77
glpT	4.99	0.64	8.59	9.13	1.14	0.93	0.36	1.11
gudD	14.43	2.11	13.13	165.33	2.50	1.94	2.85	0.46
ycbU	9.49	4.13	5.62	6.83	1.22	1.29	3.07	4.17
nasC	3.08	0.53	5.16	15.84	0.79	0.61	0.81	0.52
yczE	28.49	1.20	9.77	5.81	1.01	1.05	1.10	1.55
yclE	9.84	4.04	5.14	7.72	1.15	1.31	2.07	3.83

gerKA	4.44	2.64	5.39	11.34	1.14	2.02	2.44	1.48
phrC	2.22	3.33	6.26	5.94	1.26	2.70	2.46	1.59
ydaE	8.32	2.25	6.57	9.85	0.13	0.27	1.87	1.94
ydaL	4.99	2.27	5.84	8.93	2.40	2.02	1.12	1.21
ydaS	9.16	3.61	5.46	5.62	1.24	1.32	4.98	0.88
vmlR	3.24	5.35	7.19	8.02	0.99	1.06	1.70	1.47
gutP	3.66	1.20	6.67	14.59	0.49	0.69	1.42	1.39
yebD	6.35	30.01	15.90	6.82	1.19	1.13	4.64	5.80
yebE	2.55	5.99	7.96	6.18	1.19	0.66	1.81	0.92
yesR	5.92	0.63	5.56	33.25	1.95	0.52	0.67	0.77
yesS	9.43	1.06	5.51	47.11	0.78	0.89	1.02	0.75
yesW	8.88	2.00	6.57	66.19			1.60	0.17
yesX	3.14	8.98	10.10	16.81	1.30	0.35	1.07	0.13
yetA	2.84	0.77	9.43	15.16	3.08	2.77	0.97	0.45
yfnF	1.73	1.11	5.56	14.59	1.36	1.21	1.28	0.83
ssuA		1.66	8.08	17.55	0.57	0.61	0.15	0.35
yhbB	19.98	3.74	5.46	14.22	2.84	6.07	0.83	0.88
yhcM	1.11	2.34	5.05	7.57	0.89	1.40	2.06	1.97
yhdF	4.90	4.72	5.01	6.73	1.38	1.49	2.76	3.26
yheF	2.22	1.00	5.05	14.78	0.57	1.21	0.00	0.00
hpr	3.71	6.47	5.38	5.14	1.46	1.36	2.58	1.75
yitB	0.83	0.25	14.14	55.42	0.49	0.69	2.14	1.39
yitC	2.22	0.30	18.18	22.48	0.85	0.45	1.07	1.19
yitE	6.10	2.50	5.56	46.18		#DIV/0!	4.27	0.92

argB	25.16	3.85	7.71	21.09	1.93	0.97	3.56	1.34
manP	3.77		22.22	12.19	2.55	0.61	0.53	0.09
manA	1.66	0.67	7.58	9.61	0.57	1.82	3.20	0.38
yjgC	2.59	1.16	11.62	46.41	0.61	0.37	1.42	0.67
yjmF	7.77	1.33	9.60	11.31	0.43	0.45	2.67	0.87
uxaB	2.59	2.21	5.60	9.73	0.67	0.40	0.91	1.28
uxaA	4.84	2.27	5.67	9.66	1.48	0.96	2.14	2.29
yjnA	9.99	4.11	13.13	10.59	0.50	0.82	2.86	3.44
xtrA	14.43	2.00	16.16	7.02	1.51	0.00	1.37	2.50
xpf	17.76	0.33	32.33	10.85	1.14	0.61	1.83	8.32
ykrP	4.72	14.97	8.08	17.55	0.88	0.13	2.40	4.16
ykvT	2.59	3.49	5.56	7.62	1.14	2.43	0.00	0.00
spoIIGA	6.66	1.93	6.47	7.92	0.71	1.06	1.64	3.08
ymaG	5.55		5.05	10.62	1.14	0.00	1.07	0.46
spoVK	1.39	4.10	7.58	6.00	0.67	0.33	0.63	1.26
ynfE		3.49	6.06	8.31	0.28	0.91	0.00	0.00
yngF	3.33	2.00	5.56	5.17	0.38	0.40	0.00	0.00
yngH	2.44	1.00	5.30	40.18	0.76	0.81	0.27	0.20
yngI	5.55	0.57	5.66	8.98	4.54	6.07	0.46	0.32
yojA	2.77	1.12	7.41	9.42	1.70	0.81	1.07	1.39
yodQ		1.25	5.39	7.39		#DIV/0	1.07	0.69
yodT		0.33	9.09	37.87	0.38	0.81	1.07	0.20
mtbP	1.43	1.75	6.06	25.86			0.85	0.50
yomT	0.92	8.49	10.10	12.01	2.27	6.07	1.92	2.08

sleB	3.33	0.87	10.10	8.31	0.25	1.75	0.36	0.46
spoIIIAE	!	0.00	5.05	11.08	0.38	0.40	1.07	0.59
yrkE	1.11		8.08	6.77	0.76	0.40	4.27	2.77
yrhP	8.29	3.99	6.89	6.89	3.55	2.27	1.74	1.13
yrzI	4.47	7.75	10.66	5.11	1.08	0.82	2.81	2.31
yrrI	5.83	1.11	5.72	8.66	0.19	1.15	1.07	0.98
cstA	5.86	3.10	8.14	21.24	1.22	1.18	2.14	2.77
araP	2.54	2.00	6.47	7.81	1.23	0.81	0.40	0.18
araL	2.77	1.50	17.17	21.24	0.76	1.01	1.07	0.28
tcyJ	8.32	1.11	8.08	13.16	0.97	2.43	2.67	0.53
acsA	10.64	3.90	5.95	7.04	0.65	1.61	2.45	2.24
yteU	4.25	1.50	5.56	6.10	0.57	0.66	1.68	3.81
yteT	3.75	1.30	6.06	7.57	0.64	0.69	1.82	1.12
amyD	12.49	2.52	5.44	9.82	0.52	2.07	1.60	3.18
ytcB		2.99	8.08	27.71	1.14	0.00	3.20	2.08
ytaB	2.22	1.11	11.11	30.48	0.57	4.85	0.59	1.39
glgB	9.80	2.12	5.14	5.33	0.90	1.12	1.53	1.19
yugS	1.84	2.00	5.29	10.16	1.79	1.45	1.26	0.80
mstX		3.99	9.09	6.77	2.27	0.00	1.07	0.69
yuiA	4.62	8.86	7.45	8.80	0.61	1.37	3.34	2.07
pucR	7.07	3.60	5.05	6.77	0.78	1.17	1.49	3.28
pucE	13.32	2.75	9.09	26.32	0.85	1.21	0.80	2.08
pucD	5.77	1.60	5.51	37.21	0.83	1.38	1.17	0.74
pucB		2.66	9.09	20.32	1.14	0.30	4.27	0.69

pucA	4.16	5.32	5.05	23.09	1.14	2.43	1.60	0.59
frlN	4.44	10.26	9.09	35.10	2.43	3.47	4.51	4.79
frlB	4.92	12.12	6.96	16.63	1.31	1.76	3.97	3.81
fadM	3.70	0.67	7.07	16.86	2.27	0.00	0.36	0.13
yvgT	3.61	1.49	5.64	6.85	1.68	1.85	0.95	4.27
yvfS	4.72	1.60	5.39	12.78	3.41	1.62	1.07	1.56
yvdQ	1.85	1.34	6.47	7.85	1.65	1.43	1.07	1.39
yvyD	3.04	5.93	5.12	6.58	1.70	1.28	3.86	1.29
cotG	2.77	1.33	7.07	41.56		#DIV/0!	1.60	0.83
ureC	25.50	2.72	13.04	13.28	0.54	1.41	4.31	5.94
narG	12.78	4.43	7.30	28.53	0.52	0.88	2.64	0.55
arfM	5.87	2.58	9.26	6.59	0.63	0.96	4.13	0.29
narK	8.12	2.26	7.12	13.22	0.49	0.74	2.48	0.46
pbpG	5.95	1.75	5.23	9.35	0.59	0.81	0.65	0.60
ywfA	4.16	3.24	9.34	49.88	5.68	1.21	1.60	0.76
rocB	5.83	2.04	9.01	11.08	1.23	1.36	1.69	1.54
ywdL	3.55	2.22	6.06	7.39	1.14	1.56	1.07	1.66
ywbA	4.44	0.42	5.86	25.86	0.87	0.75	1.00	1.49
cimH	12.37	3.55	11.54	9.69	0.45	0.89	3.46	1.54
yxjN	9.20	3.83	5.65	7.19	1.25	0.94	2.24	4.92
katE	1.47	0.88	5.67	7.20	0.76	0.79	1.74	0.47
citH	27.75	1.60	14.14	33.56	2.27	4.04	0.46	0.35
hutH	13.32	3.28	5.93	26.60	0.64	0.91	3.44	1.75
yxeQ	12.69	4.03	5.25	7.88	1.17	0.89	1.63	4.24

yxeP	12.91	4.76	7.12	6.83	1.20	1.11	2.94	3.68
iolD	3.96	4.09	5.02	14.21	1.79	0.96	1.80	0.80
iolC	4.81	2.00	7.27	11.80	0.96	0.70	1.15	0.81
yycP	3.61	1.82	5.56	8.31	0.53	0.57	1.96	0.73

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Table 8.7: Table of genes upregulated in phenylalanine auxotroph and the wild type strain grown in limited phenylalanine compared to that of grown in excess.

	LL2 VS LL1		PP2 VS PP1		WL2 VS WL1		WP2 VS WP1	
	s1	s2	s1	s2	s1* using WL1 from s2	s2	s1	s2* using WP2 from s1
mutT	2.22	3.33	6.31	29.56	0.45	0.73	5.34	0.77
yhjN	8.00	2.68	9.21	6.15	0.12	1.02	14.90	26.72
xkdC	15.17	2.57	6.06	15.70	0.88	1.08	8.90	2.89
dppC	56.60	4.51	21.62	14.62	0.43	1.57	6.76	3.84
iseA	3.33	10.55	9.49	8.25	1.08	0.80	6.67	0.62
glnP	8.88	#DIV/0!	9.09	30.48	0.38	0.40	5.34	1.73
pheT	0.60	0.50	10.68	11.48	1.04	0.87	6.63	5.41
pheS	0.87	0.45	8.57	9.30	1.09	0.96	6.38	13.01
pucC	7.77	3.39	7.07	59.11	2.65	0.81	8.54	0.92
yvdS	3.51	4.66	6.31	5.77	1.14	2.55	6.94	1.29
ureB	40.51	2.40	8.18	8.53	0.49	1.23	6.33	3.91
ureA	48.61	3.58	18.27	9.54	0.51	1.40	5.84	3.76
scoA	18.87	2.33	7.58	18.24	1.14	1.82	7.47	1.62

yybH	4.81	11.72	6.38	7.34	2.13	1.21	5.04	5.11

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Table 8.8: Table of genes up-regulated in lysine auxotroph relative to the wild type strain grown in limited lysine.

	PP2 VS WP2		PP1 VS WP1		LL2 VS WL2		LL1 VS WL1	
	s1	s2* using WP2 from s1	s1	s2	s1	s2	s1* using WL1 from s2	s2
nagBB	3.13	1.55	2.08	0.98	5.10	6.63	4.30	0.94
ybgF	1.43	1.27	0.90	0.89	10.46	8.05	0.48	1.15
glnT	4.56	2.13	1.20	0.32	9.52	8.26	5.15	1.07
amhX	3.30	7.63	0.94	0.75	8.42	14.01	0.22	0.75
ycgN	2.86	1.91	0.94	0.63	6.52	8.87	0.68	1.57
ycgS	1.80	1.20	0.98	1.13	7.68	6.77	0.57	1.08
gabT	1.99	3.82	0.76	0.62	7.32	6.90	0.76	1.57
yczI	1.91	1.69	1.31	1.19	5.70	7.94	1.30	0.89
ydaF	3.78	1.09	1.50	1.03	8.28	11.46	1.77	1.24
ydaG	3.32	1.43	1.77	0.97	8.80	10.53	1.41	1.22
vmlR	4.60	3.81	1.08	0.70	5.47	5.07	1.67	1.00
gabP	1.48	1.52	0.79	1.26	7.88	6.32	0.19	1.17
yebD	4.59	1.78	1.34	1.51	9.93	18.67	1.87	0.70
sapB	2.78	1.65	0.94	1.42	16.77	14.60	0.92	0.92
ygaJ	2.27	2.63	1.57	1.31	9.29	16.34	0.94	1.23
ssuC	4.44	25.98	0.60	0.93	14.10	12.64	0.00	0.38

yhdJ	3.51	2.41	1.01	1.13	6.49	7.82	1.03	0.84
yhdX	1.06	4.01	0.52	1.57	15.61	10.65	0.32	0.87
yheE	3.11	2.44	0.73	1.79	6.13	7.15	0.67	1.12
yhjB	2.77	1.97	0.84	1.39	12.32	10.09	0.51	1.13
yhjC	4.39	2.37	1.94	0.84	16.01	14.83	1.03	2.05
sat	1.61	1.80	1.17	0.59	8.42	6.69	0.69	1.30
appB	3.91	1.76	1.07	0.60	27.20	11.00	0.20	1.10
appC	4.14	2.27	1.06	0.75	22.31	13.29	0.18	1.09
yjcI	1.55	1.15	1.17	1.02	12.26	10.72	0.32	1.26
yjcJ	1.34	1.44	1.09	0.98	14.00	10.73	0.23	1.17
yjcK	4.17	3.53	0.82	1.16	12.70	8.85	1.31	1.37
yjcL	4.77	3.45	0.79	1.50	18.42	11.57	0.28	1.37
dppA	3.33	2.11	1.12	0.65	6.48	5.14	0.36	1.33
guaD	2.17	1.79	0.68	1.69	12.81	8.56	0.47	1.13
ykoJ	1.86	2.53	1.09	1.12	6.77	7.30	1.39	0.93
ykrV	2.44	1.54	1.23	1.07	12.22	11.27	0.89	1.24
spo0E	2.93	0.78	1.64	1.51	5.53	7.38	1.32	0.87
cysH	3.50	2.81	1.83	0.52	13.30	14.25	1.04	1.09
cysP	3.32	3.25	1.09	0.52	12.17	10.17	0.38	1.28
cysC	1.61	1.62	1.16	0.48	13.58	7.66	1.00	1.54
sumT	1.30	1.54	1.24	0.99	11.10	7.42	0.81	1.02
iseA	4.07	4.03	2.86	0.30	6.22	10.39	2.01	0.79
yocR	3.23	1.78	1.27	2.50	6.06	8.15	1.74	1.01
yocS	4.22	3.32	0.86	0.86	13.14	14.61	0.15	1.26

yoyD	2.47	0.85	1.24	2.09	10.41	10.69	0.95	1.58
yodL	4.09	2.60	2.06	0.84	12.01	17.58	0.80	0.74
yozE	2.76	1.87	1.08	1.48	6.02	12.01	2.06	0.82
ilvD	3.86	2.71	1.14	0.94	20.52	18.96	0.35	1.30
ypqA	1.44	2.28	0.17	1.04	6.04	10.95	0.44	0.73
ypjB	0.89	0.49	0.93	0.79	12.46	5.40	2.21	2.46
ypzJ	3.83	0.49	1.72	1.11	7.23	5.59	1.60	0.74
yqgL	2.66	0.44	1.11	1.62	13.26	5.34	1.03	1.02
yqbQ	0.72	3.63	1.04	1.22	13.09	11.80	2.06	1.02
yrdR	2.28	3.57	0.78	1.16	13.85	7.42	1.80	0.77
yrdB	3.92	1.13	2.20	4.87	25.08	16.64	1.03	1.02
yrpB	2.07	1.69	1.35	0.97	6.60	10.76	1.61	1.07
glnQ	2.02	2.03	1.04	0.60	7.45	7.79	0.18	0.72
tlpB	3.28	1.79	1.29	0.70	7.91	11.91	3.80	1.06
yufO	4.68	5.06	0.32	0.46	22.49	22.75	0.24	0.95
yufP	4.34	4.46	1.04	0.46	18.53	16.54	0.11	1.14
yufQ	3.07	4.18	0.93	0.21	26.85	17.17	0.34	1.19
maeN	2.21	2.02	0.75	4.30	10.50	12.63	0.13	1.39
yuzF	1.66	2.27	1.25	1.34	7.19	6.82	1.33	0.81
yueE	2.38	1.72	1.21	1.61	11.60	12.91	2.65	1.27
yvdA	2.09	4.96	1.15	0.19	7.44	8.56	0.69	1.11
lytD	2.37	1.35	1.35	0.68	5.49	5.48	2.78	1.10
pgsE	0.85	2.39		0.52	11.08	5.90	2.58	1.02
ywqO	0.49	2.16	0.37	1.09	8.76	6.24	0.69	1.71

ywqN	1.38	1.92	0.57	1.48	5.73	10.55	0.33	0.71
bacC	1.56	1.29	1.07	1.08	7.97	5.13	0.35	1.09
bacB	1.99	1.71	0.98	1.43	9.30	7.53	0.40	1.26
bacA	2.78	1.35	1.62	1.22	10.34	9.98	0.68	1.41
yweA	2.76	1.91	1.18	0.78	8.27	11.77	1.39	0.93
spsG	1.87	9.59	0.85	1.19	6.38	5.62	1.16	0.90
epr	2.83	1.48	1.52	0.57	7.42	7.55	2.99	0.95
ywaA	2.17	1.88	1.35	0.93	6.14	9.38	2.01	1.24
yxzF	2.47	13.22	1.57	5.57	8.56	5.90	0.34	0.17
yxkD	1.69	2.54	1.19	0.78	9.00	8.54	1.75	0.85
hutP	3.75	2.92	0.94	0.81	6.34	5.70	1.00	0.81
yxeO	1.72	1.60	0.84	1.08	13.21	7.64	0.75	0.85
yxeN	2.01	1.59	0.93	0.45	8.06	7.16	0.80	1.49
yxeM	2.15	2.04	1.83	0.89	7.26	5.11	1.47	0.87
yxeK	2.67	2.75	0.79	0.99	7.85	6.85	1.59	0.84

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Table 8.9: Table of genes up-regulated in lysine auxotroph relative to the wild type strain grown in limited lysine as well as that of grown in excess lysine.

	PP2 VS WP2		PP1 VS WP1		LL2 VS WL2		LL1 VS WL1	
	s1	s2* using WP2 from s1	s1	s2	s1	s2	s1* using WL1 from s2	s2
cca	0.28	0.53	0.36	1.25	7.55	7.16	103.00	30.72
ypjC	1.13	1.17	1.22	3.06	7.15	12.01	5.81	5.35
ypjA	3.46	1.62	0.66	1.19	6.38	14.75	4.25	7.04

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Table 8.10: Table of genes up-regulated in phenylalanine auxotroph relative to the wild type strain grown in limited phenylalanine.

	PP2 VS WP2		PP1 VS WP1		LL2 VS WL2		LL1 VS WL1	
	s1	s2* using WP2 from s1	s1	s2	s1	s2	s1* using WL1 from s2	s2
skfA	9.58	1.22	0.63	1.25	2.79	3.73	0.72	0.66
skfB	9.34	0.87	1.06	1.10	3.17	1.36	1.23	0.99
ybxH	5.93	22.26	2.09	1.39	1.44	1.54	1.03	1.54
ybgB	6.77	3.31	1.25	0.81	5.29	2.02	1.93	1.02
ycdF	5.18	18.55	0.65	0.93	1.17	1.26	0.62	1.13
phrI	13.83	4.64	0.52	0.35	3.42	3.37	0.52	3.07
ydzR	7.90	44.53	1.04	0.20	4.03	1.12	0.26	1.02
gmuB	5.93	30.61	0.00	0.23	6.04	!	2.58	0.00
cotJC	10.86	34.32	5.22	1.39	0.81	4.21	0.00	0.68
yesP	22.71	89.06	0.00	0.40	7.05	1.69	0.34	2.05
yesQ	9.88	67.72		0.35		0.84	0.00	0.00
yesR	6.52	20.04	0.78	0.46	1.34	3.37	0.44	2.78
rhgT	6.91	37.57	6.26	0.20	3.52	0.63	0.41	0.41
yesV	15.80	83.49	1.04	0.00	9.06	2.53	0.00	0.26

yesW	8.56	66.49	2.09	0.17		6.74		
yesX	19.75	84.42	2.09	0.66	2.14	3.79	0.88	0.15
yetA	5.53	25.88	0.57	0.76	2.17	0.53	2.35	1.90
lplA	6.58	42.37	1.04	0.20	3.69	1.90	0.17	0.51
yfnH	18.76	61.23	0.00	0.93	2.62		2.06	3.07
yfmN	5.93	27.83	0.70	0.28	1.01		0.00	0.44
citM	5.05	14.74	1.04	0.46	1.55	1.36	1.39	1.33
acoB	5.68	17.16	0.70	0.00	2.01		0.00	0.23
yfiH	6.91	23.42	1.04	1.71	1.51	1.31	0.66	1.17
yfiL	11.19	28.14	0.00	0.00	2.01		3.09	2.05
yfiM	17.78	76.07	0.52	0.35	1.68	0.34	2.06	2.56
ssuB	11.85	57.52	5.22	0.56	4.03		2.06	2.05
ssuA	7.90	35.25	0.15	0.70	2.01	4.21	0.00	1.54
spoVR	8.39	26.44	1.04	1.55	3.24	3.37	0.59	0.59
yhjR	7.90	39.89		0.46		0.84	0.00	1.02
yisJ	5.93	29.22		0.00	2.52	2.53	1.03	0.51
yjzE	9.38	16.23	1.17	0.22	2.69	1.38	1.60	1.25
manP	21.73	122.46	0.52	0.93	1.90	3.37	1.29	0.00
ykrP	6.14	5.88	1.83	1.39	4.89	37.92	0.92	0.34
ykvI	5.93	22.03	1.04	2.09	2.85	10.95	3.61	5.12
yncC	5.37	2.64	1.26	1.23	13.59	4.73	1.03	0.83
yngG	15.80	50.10		1.04		1.01	0.34	0.00
yngH	20.74	80.71	1.04	0.40	5.54	3.37	1.72	2.73
yngI	9.22	33.09	0.75	1.18	2.52	1.35	2.06	14.33
yngJ	20.74	33.40	0.63	0.00	1.17	1.47	0.00	3.07
yobB	16.79	4.02	0.57	1.02	0.86	0.35	0.57	0.98
yozW	5.93	2.78	1.25	0.40	0.30	2.53	0.52	3.58
yodQ	7.90	22.26	1.57	2.09	2.01	0.84		
yodT	8.89	38.04	1.04	0.20	6.04	0.42	0.00	1.02
bcsA	6.75	7.27	0.54	2.19	0.92	2.02	1.75	1.84
spmA	8.39	8.81	3.65	1.22	4.03		0.26	1.02
ypzD	5.93	12.99	0.00	0.00				
yqjW	17.78	61.23	1.57	0.23	5.37	1.97	1.03	1.02

mmgD	7.41	35.72	2.61	0.40	2.69	0.60	0.62	0.82
mmgC	5.43	20.87		0.15	9.06	0.84	0.69	0.68
mmgA	9.88	61.23	0.00	0.00	8.06		0.00	0.00
spoIIIAA	6.67	15.31	0.52	0.00	3.27	2.36	0.08	0.16
yqfQ	7.90	54.73	0.75	0.70	1.51	0.60	0.09	0.51
yqxH	5.93	14.84		0.84		0.42	0.69	0.34
yqcA	6.91	21.34	1.04	2.78	7.05	1.26	1.03	1.02
yqbP	8.89	27.83		0.35	3.02		0.00	1.02
yqaT	5.36	3.71	0.96	1.76	3.59	5.27	1.13	0.82
yrhP	5.70	5.85	1.44	0.96	5.72	2.70	2.45	1.54
yrhH	17.78	16.70	2.87	0.38	6.49	3.93	10.67	0.65
glnM	11.85	21.34	1.04	0.00	2.01	3.37	1.03	1.54
yrzQ	5.93	12.06		0.70	1.51	1.97	0.00	1.02
yrzE	6.42	12.52	3.83	0.31	3.78	2.53	1.72	1.37
araP	10.53	28.76	0.65	0.67	1.24	1.90	0.60	0.77
araL	16.79	42.67	1.04	0.56	1.26	0.51	0.34	0.34
abnA	10.86	93.70	0.00	0.70	5.04	2.81	2.06	1.02
ytbD	7.24	15.77	0.78	0.70	1.07	0.71	1.18	0.18
tcyM	8.89	26.90		0.00	3.36	3.37	1.03	4.10
tcyL	15.80	29.69	0.00	1.11	4.03	3.79	0.00	2.05
ytmI	10.86	27.83	0.52	11.14	3.02	0.67	0.31	0.31
yteV	5.93	4.17	0.70	1.04	0.50	3.65	0.34	0.51
ytcQ	6.91	19.88	1.04	0.44	2.01	6.11	0.97	0.18
yugS	5.33	5.88	1.27	0.46	2.11	1.96	2.06	1.42
mstX	8.89	20.41	1.04	2.09	2.01		0.00	1.02
fadM	13.83	67.72	0.70	0.51	2.52		1.55	3.07
sdpI	10.54	0.94	1.48	0.93	1.70	4.21	2.84	1.15
sdpR	11.35	0.68	1.47	0.79	1.33	3.61	3.22	0.99
ganB	8.89	43.29	6.26	0.75	2.85	3.93	2.40	2.05
ywpE	13.83	38.04	1.44	0.58	0.81	0.56	1.03	0.88
pucI	5.93	26.90	1.30	0.28	1.11	0.84	1.42	1.02
ywnC	17.78	40.82	0.56	0.56	2.35	0.51	2.06	1.46
pbpG	5.11	13.81	0.63	0.89	5.40	2.11	0.54	0.98

ywfA	6.09	16.70	1.04	0.25	3.02	10.95	4.12	4.10
scoB	11.36	45.92	0.52	0.42	2.47	0.74	0.57	0.80
citH	9.22	33.71	0.30	0.35	4.20	0.67	0.34	1.71
iolF	6.42	22.26	1.96	0.41	1.59	2.41	1.72	1.71
iolC	5.08	7.62	0.80	0.52	3.27	2.15	0.65	0.75
yydF	7.79	1.56	1.30	0.29	2.16	2.16	1.93	0.76

Genes up-regulated by 5-fold or more are listed. Comparisons marked with * were excluded from data interpretation.

Chapter 9 Publication

Sidiq, K. *et al.* (2021). ‘Alanine metabolism in Bacillus subtilis.’, Molecular Microbiology. 115(4), pp.739-757.