Alanine Metabolism in *Bacillus subtilis*

Karzan R. Sidiq[1,2], Man Chow[1], Zhao Zhao[1] and Richard A. Daniel[1]

[1] Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle Upon Tyne, NE2 4AH, UK

[2] College of Health and Applied Sciences, Charmo University, 46023 Chamachamal, Sulaimani, Kurdistan Region-IRAQ,

Karzan R. Sidiq , College of Health and Applied Sciences, Charmo University, 46023 Chamachamal, Sulaimani, Kurdistan Region-IRAQ, Karzan.sidiq@charmouniversity.org , Mobile: +964 (0)7503891657.

Richard A. Daniel (Corresponding author), Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle Upon Tyne, NE2 4AH, UK richard.daniel@newcastle.ac.uk , Tel: +44 (0) 191 208 3239, Fax: +44 (0) 191 222 7424.

Abstract:

Despite a long history of genetic manipulation of *Bacillus subtilis* using auxotrophic markers in genetic manipulation, the genes involved in alanine metabolism have not been characterised fully. Here we show that *B. subtilis* expresses an alanine uptake permease, YtnA (DatA), that has a major role in the assimilation of D-alanine from the environment. Since this isomer of the amino acid is not normally abundant it likely source is form the cells own cell wall probably through the action of carboxypeptidases and/or the spontaneous release of D-alanine from the teichoic acids. Also in this work we clarify the synthetic pathways acting in the biosynthesis of alanine. Genetically we show that, unlike *E. coli* where multiple enzymes have a biochemical activity that can generate alanine, in *B. subtilis* the primary synthetic enzyme for alanine is encoded by *alaT*, although a second gene, *dat,* is present that can support slow growth of an alanine auxotroph however our data suggests that this enzyme probably synthesises D-alanine. In summary this work has provided an explanation of the observation that growth of *B. subtilis* is linked with an efficient recycling system for D-alanine that is released from the cell as the cell envelope is processed to permit cell enlargement. The results also suggest that the relative abundance of D- and L-alanine that might be linked with cytosolic pool of D and L-glutamate, and so enabling tight coupling protein and cell envelope synthesis with the metabolic status of the cell.

Introduction

Bacterial cell growth requires the coordinated synthesis of all the metabolites that are necessary for the enlargement of the cell by both new cell wall and membrane synthesis together with the replication of the genome and the synthesis of cytosolic components. A major part of a Gram positive bacterium's cells metabolism is directed toward the *de novo* synthesis of cell envelope, since this is an essential arrangement of peptides and sugars polymerised together to form an exoskeleton like structure linked to the cell membrane that protects the cell from the external environment. One essential component of

the bacterial cell wall that is unique to bacteria is D-alanine. This amino acid isomer is universally used in the synthesis of the peptidoglycan and in Gram positive bacteria is also used to modify the teichoic acids, which are present as a second major component of the cell wall and membrane ((1, 2)).

Due to the structure of the Gram positive cell envelope it has always been proposed that cell growth resulted in degradation of the old cell wall material and this was shed into the culture medium. Hence the degradation products would be expected to accumulate over time unless actively re-assimilated by the bacterial cells. Pervious analyses of composition of culture media after the growth of various bacterial species has revealed that many release D-amino acids on reaching stationary phase growth, presumably as secondary metabolites or signalling molecules (3). Surprisingly the analysis of spent media form *B. subtilis* cultures showed that it was essentially devoid of D-alanine, whereas media in which *S. aureus* has been grown had significant levels of this isomeric form of alanine present (4). Since both bacterial species incorporate this amino acid in the cell envelope in similar ways this suggested that *B. subtilis* has an efficient recycling system for this amino acid.

The recent characterisation of the roles of DacA/LdcB ((5) (6)) in *B. subtilis* indicated that there is a high demand for the D-isomer of alanine for cell envelope biosynthesis which can be supplied by either cytosolic isomerisation of alanine by the action of AlrA (7) or assimilation form the environment, although this isomeric form is not normally available in nature in significant concentrations. Both alanine and glutamate are abundant in the cytoplasm of growing cells and present in both the L- and D- isomeric form and there strong evidence for the presence of mechanisms for precise control of the concentration of these amino acids and presumably their isomeric state ((8)). Surprisingly, in contrast to glutamate, the biosynthetic pathway for alanine is poorly defined in *B. subtilis* with much of our understanding of the biochemistry being reliant on comparison to *E. coli* where it has been shown that several enzymes are capable of the necessary transamination reaction to generate alanine (9). Although this may not be their major/natural roles in cellular metabolism (10). Also little is known about the uptake mechanisms

involved in the assimilation of exogenous alanine by bacteria, although many transporters are bioinformatically annotated as being potentially involved in alanine uptake, experimental evidence is limited and only one from *E. coli* has been implicated in D-alanine transporter (11).

The results presented identify *ytnA* as encoding an integral membrane protein, which is suggested to have similarity to various permeases, that is required for efficiently transport exogenous D-alanine into the cell. Consequently we have renamed this gene DatA (D-alanine transporter A) to reflect its biological role and this annotation will be used form this point on. *In vivo* characterisation of this gene suggests that although it has specificity of the D- isomer of alanine, the L-isomeric for is also transported. Through this analysis we also clarify aspects of the active synthetic pathways for both isomeric forms of alanine in *B. subtilis* in vegetative growth.

General Material and methods

Escherichia coli DH5α was used for plasmids amplification. *Bacillus subtilis* strains were mutant derivatives of *168CA* and their relevant characteristics are shown in Table 1. All the chemicals and reagents used in this work were obtained from Sigma except where stated otherwise.

Plasmid DNA was routinely extracted using Plasmid MiniPrep Kit (Qiagen), whilst the extraction of *B. subtilis* chromosomal DNA was either done using DNA wizard kit (Promega) for sequencing and PCR, or as a simple DNA extract (12) to permit strain construction. The transformation of *B. subtilis* strains done according to the method developed by Anagnostopoulos and Spizizen (13), modified by Young and Spizizen (14) and *E. coli* was as described by Hanahan,1985 (15), selecting for markers using antibiotics at the following concentrations: chloramphenicol (5 µg/ml), erythromycin (1 µg/ml), spectinomycin and ampicillin (100 µg/ml) in Nutrient Agar (Oxoid). Where required 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to a final concentration of 0.002% to provide a visual indication of β-galactosidease activity.

Bacterial strains and culture manipulation

The bacterial strains were routinely grown in LB (Lysogeny broth) and on nutrient agar with added supplements when required (0.5 mM D-alanine; 1 mM IPTG). Where bacterial strains were grown in a defined media based on Spizizen minimal medium (SMM, Anagnostopoulos and Spizizen (13), Young and Spizizen (14); 0.2 % w/v ammonium sulphate, 1.4 % dipotassium phosphate, 0.6 % potassium dihydrogen phosphate, 0.1 % sodium citrate dehydrate, 0.02 % magnesium sulphate, to which was added 5 mM magnesium sulphate heptahydrate, 0.1 mM calcium chloride dehydrate, 65 µM manganese (II) sulphate tetrahydrate) this provided a basic salts medium to which supplements were added to permit growth (where necessary glucose was added to a final concentration of 10g/l and each amino acids were present at a final concentration of 1.25 mM each except where specifically stated). However where amino acid utilisation as a nitrogen source was investigated the SMM was modified by omitting the ammonium sulphate. To simplify the description of the media we have adopted M to denote minimal with any omissions in brackets followed by the additions, G for glucose, A for amino acids or the three letter code for individual amino acids, again indicating any omitted components in brackets. Hence, MGA indicates the medium has salts, glucose and all amino acids added, in contrast M-NH3 G D-Ala indicates that the medium has the salts mix, but lacks the ammonium sulphate, Glucose and D-alanine.

For growth curve assays in LB, the bacterial strains of interest were grown in LB or defined media (supplemented with ~450 µM of D-alanine where necessary) at 30°C overnight. The overnight cultures were diluted 1:30 with pre-warmed medium and grown for about 2 hours at 37°C to obtain exponentially growing cultures. The optical density of the culture were then determined (OD_{600nm}) was read and measured volumes of the cultures were centrifuged for 3 minutes in a benchtop centrifuge (RCF 9,509). The cell pellets were then quickly suspended with pre-warmed culture medium and

dispensed into pre warmed flasks or the wells of microplates, which were already loaded with pre-warmed medium such that the final OD_{600} in each flask was ~ 0.1 and in each well of microplate was ~ 0.05, the cultures were then incubated with shaking, reading the optical density (OD_{600nm}) at regular intervals using a spectrophotometer or automatically by the plate reader (BMG Fluorostar). Where the experiment required the medium composition to be changed cultures were grown overnight in a complete medium and back diluted as described above into the same medium and incubated for 2 h. The optical density at 600 nm (OD_{600}) was the determine and measured volumes of the cultures were centrifuged at 13,300 rpm for 3 minutes in a benchtop centrifuge (Heraeus Pico17, 45° angle rotor, 24 places, RCF 17000 x g). The pellets were suspended in appropriate volumes of the new medium and distributed into culture vessels, either Flasks or sterile microplates that were pre-loaded with appropriate volume of media with relevant supplements such that the correct final optical density was obtained. The growth of the strains was then monitored as described above.

Determination of growth on solid media

For growth analysis using solid media, agar plates (1.5 % w/v) were prepared using SMM salts to which was added 5 mM magnesium sulphate, 0.1 mM calcium chloride dehydrate and 65 µM manganese (II) sulphate. Variations of this base medium were made by the addition of different supplements; in general 10 g/l glucose was added as a carbon source (Glucose minimal; GM), plates supplemented with 98 µM L-tryptophan (GMT) or 1 mM L-alanine GMala. GMTala plates were supplemented with both L-tryptophan and 1 mM D-alanine, GMaa plates contained 1.25 mM of the 19 standard amino acids omitting alanine. For plates testing for utilisation of alanine as sole carbon source, glucose was removed from SMM and replaced with 25 mM L- or D-alanine (Mala). For those testing for utilisation of alanine as sole nitrogen source, ammonium sulphate was removed from SMM salt mix and the medium supplemented with 10 g/l glucose and 25 mM alanine (GM-NH_3 ala plates). Strains were streaked on

prepared agar plates and incubated at 37°C for 24 hours (GMaa plates) or 48 hours (GMT, GM, GMala, Mala and GM-NH_3ala plates) and growth determined by visual inspection was recorded by photography.

Bacterial strain construction

Mutants with single gene deletion were obtained from the Bacillus Genetic Stock Centre (BGSC) where single gene deletions had been generated by insertional deletion using a erythromycin resistance cassette (16). Chromosomal DNA from these strains at low DNA concentrations were used to transform 168CA or 168+ to ensure isogenic strains were characterised. Where necessary the temperature-sensitive vector pDR244 (16) with a functional *cre* recombinase gene was used to loop out the erythromycin marker between the lox sites, leaving the resultant strain a marker-less deletion mutant. Repetition of this method was used to generate double or triple mutants where required. The transformed strains were checked that they had the appropriate antibiotic resistance, and the gene knockouts were confirmed by PCR. The list of constructed strains of *B. subtilis* is shown in Table 1.

To identify potential D-alanine synthesis pathways, searches of the bioinformatics databases (*Subti*Wiki, SubCyc and Metacyc) were used to identify any genes that have either putative or been functionally characterised to have roles in D-amino acid metabolism. Strains deleted for the genes identified were obtained from BGSC and are listed in Table S4.

The deletion of the *dlt* operon was generated by ligation of 3 DNA fragments generated by PCR. Firstly, oligonucleotides oKS01 and oKS02 were used to amplify a 2.0 kb DNA fragment, complimentary to the DNA sequence upstream of *dltA* gene. Then oKS03 and oKS04 were used to generate a 2.0 kb DNA fragment at the downstream of *dltD* gene. Both of these DNA fragments were then digested with *Bgl*II, and after purification, appropriate quantities of the digested DNA fragments were ligated with a chloramphenicol resistant cassette (*cat*) obtained from plasmid pSG1 as a *BamH*I fragment. The ligation product was used in the transformation

of *B. subtilis* 168CA strain. The correct knockout strain (*ΔdltABCD::cat*; KS11) was isolated as a chloramphenicol resistant colony and confirmed by PCR.

Amino acid competition assay

The 168CA and RD 180 (*ΔalrA::zeo*) strains were grown in LB medium, supplemented with 450 µM of D-alanine to which other individual amino acids (Melford Bioscience) at concentrations between 0.0098 mM – 5.0 mM were added. The growth of the strains at 37 °C was then monitored over time using microplate reader (BMG, Fluorostar). The readings obtained were then used to plot growth curves for the culture and determine if the addition of a specific amino acid had any specific significant impact on D-alanine dependent strain (RD180) and not on the wild-type strain (168CA).

Synthetic lethal screen

To determine if a specific transporter was required for the uptake of D-alanine by the D-alanine auxotroph (strain RD180) the auxotroph was systematically transformed with insertional mutations in genes potentially encoding amino acid transporter proteins. Using the premise that the loss of this transporter in the *alrA* null background would be lethal and very few colonies bearing the incoming resistance marker would be obtained compared to other null mutations. Our initial screen utilised the pMUTIN knockout collection as a source of null mutations and a list of the mutations tested is shown in Table S4. Where a significant reduction in transformation efficiency was seen, the gDNA was used a second time transforming both the *alrA* null strain and the wild-type strain in parallel to confirm that it was the genetic background and not the quality of the DNA preparation causing the reduced transformation efficiency. Where reduced transformation was confirmed, the resulting colonies were then screened for the presence of expected resistance markers for the respective mutations (for *alrA,* zeocine and for the pMUTIN, erythromycin).

Stabilisation of pLOSS*alrA by deletion of datA

Plasmid pLOSS* *alrA*^{W23} was constructed by cloning a PCR generated fragment of DNA corresponding to the *alrA* gene encoded by B. subtilis W23 into the pLOSS* vector described by Claessen et al. (17), inserting it such that it was under the control of the P_{spac} promoter (using *Bam*HI and *Xba*I sites introduced by the PCR oligonucleotides alrAW23-1 and alrAW23-2). Strain RD180 (*ΔalrA::zeo*) was then transformed with a pLOSS* *alrA*^{W23} to give KS21 strain (*ΔalrA* pLOSS* *alrA*^{W23}), which exhibited plasmid instability when grown in the presence of D-alanine (see results).

Strain KS21 was then transformed with gDNA from BKE30530 (*ΔdatA::erm*) selecting for erythromycin resistance to produce strain KS23 strain (*ΔdatA ΔalrA* pLOSS* *alrA*^{W23}). These two were grown on plates of nutrient agar supplemented with X-gal and with and without D-alanine at 37 °C to determine the stability of the plasmid as indicated by the loss of β-galactosidase activity.

Complementation of datA.

The coding sequence of *datA* gene and 30 bases upstream, to include the ribosome binding site (RBS) of the gene, was inserted into plasmid pPY18 (*bla amyE3' cat* P_{spac} *lacI amyE5';*(18)) to produce plasmid pKS1 (*bla amyE3' cat* P_{spac} *datA lacI amyE5'*). Plasmid pKS6 was similarly constructed, but in this case the native ribosome binding of *datA* was modified (to a sequence corresponding to the RBS identified upstream of *ftsL*). Both pKS1 and pKS6 were then used to transform strain KS22 (*ΔdatA::erm*) such that the *amyE* locus was replaced by the inducible copy of *datA*. Correct were confirmed by determining the loss of amylase activity in the strain and by PCR to give strains, KS26 and KS41. Both of these strains were then transformed with the gDNA of RD180 strain (*ΔalrA::zeo*) selecting for zeocine resistance in the presence of 0.5 mM D-alanine and 1.0 mM IPTG to give strains KS27 and KS42.

Cross-feeding assay

In this assay the D-alanine auxotroph (*alrA*) strain (RD180) was grown as close as possible to wild type or test strains on a solid medium, ensuring that the strains did not become mixed. Through trial and error it was determine that the D-alanine prototroph (donor; $alrA^{+}$) had to be inoculated onto the plate first and incubated for 3.0 h at 37°C prior to inoculating the D-alanine auxotroph (recipient) strain in parallel to the D-alanine donor strain, the plate was then incubated for a further 23 h and the resulting growth photographed.

Culture sample processing for RP-HPLC analysis for D-alanine

For the identification of D-alanine in culture media, samples were taken from the culture at different time points and centrifuged at 3000 x g for 5.0 min and then the culture supernatant filtered through sterile syringe filter (pore size 0.2 µm, GILSON®). The resulting filtrate was then passed through spin column (Vivaspin 2.0 Hydrosart, 2000 MWCO, Generon Ltd.) before analysis. For standards, the LB medium sample was similarly processed with and without added known concentrations of D-alanine. A 150 µl of the "super" filtered culture supernatant was concentrated to approximately 50 µl using a SpeedVac concentrator (SavantSPD131DDA, Thermo Electron Corporation). To this material 150 µl of Marfey's reagent (1.0 % Nα-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide in acetone) was added and mixed. Then, 40 µl of sodium bicarbonate (1.0 M) was added and mixed prior to incubation for 1.0 h at 40 C° with shaking at 750 rpm (Thermomixer compact, Eppendorf). The mixture was then allowed to cool and 25 µl of HCl (2.2 M) added to stop the reaction and dried by SpeedVac before being suspended in 150 µl of HPLC suspension solution (90 % of 0.05M triethylamaine phosphate (pH 3.0) and 10 % of acetonitile + 0.1 % formic acid). The resulting suspension was then filtered through 0.22 µm centrifuge tube filter (cellulose acetate filter, Costar) at full speed in a micro centrifuge 1.0 min, and the filtrate use for HPLC analysis. Results shown in Fig 5 are representative for 3 independent experiments.

Samples labelled by Marfeys reagent (19) were analysed using a Perkin Elmer series 200 HPLC with a Spheri-5, RP-18 column, dimension 100 mm x 4.6 mm and particle size 5.0 µm (Perkin Elmer) and

a column guard , NewGuard RP-18, 7.0 µm, 15 x 3.2 mm (Perkin Elmer). The mobile phase was generated from 0.05 M triethylamaine phosphate (pH 3.0) (solution A) and acetonitrile + 0.1 % formic acid (solution B) as a linear gradient of solution B from 10 % to 25 % in 40 min. The flow rate was 1.0 ml/min at 35 °C and the detection wave length was 340 nm, using S200 Diode array detector (DAD). After each run, the column was washed with water (solution D) and then with 60 % methanol (solution C). The details of the run parameters are shown in (Table S3).

Determination of amino acid changes in culture media.

To identify what effect the *datA* mutation had on the assimilation/release of alanine in a growing culture, parallel cultures of the wild-type (168) and the *datA* null strain (KS22) were grown overnight at 30 ˚C in a defined minimal medium; GM Opt (M. Chow, personal communication) which was GM media supplemented with amino acids serine, glutamine, asparagine and glutamate at 0.5 mM and all others at 0.25 mM except alanine which was omitted. The cultures were then diluted 1/50 in fresh media and incubated at 37 degrees for 1 h prior to being diluted in the same medium to an optical density of 0.05 at 600 nm. At this point the cultures were divided into 3 aliquots. The first was supplemented with D-alanine at 0.5 mM, the second L-alanine (0.5 mM) and the last was left without addition of alanine. At regular intervals the culture optical density was determined and 1 ml samples were centrifuged and the culture supernatant filtered through a Microcon centrifugal filter unit (MWCO 3kDa; Sigma) and stored at -20 ˚C.

The frozen samples were than processed for HPLC analysis to determine the concentration of alanine present using pre-column OPA derivatisation as described by Henderson *et al*. (2008) (20) using an Agilent 1100 HPLC with a Poroshell 120 C18 4 µm, 4.6 x 150 mm column. The resulting HPLC traces were then analysed with reference to a T0 sample and known standard concentrations of alanine to permit the identification of the peak corresponding to alanine in the trace and to allow its concentration

to be determined. The results were then plotted against time and the values obtained for a typical experiment are shown in figure 5.

Results/Discussion:

Media dependent growth characteristics of D-alanine dependent strain of *Bacillus subtilis*.

To determine if there is a D-alanine specific uptake system or if, as has been suggested that amino acid uptake systems are degenerate in their specificity (21), we firstly characterised the growth of a strain lacking the conditionally essential alanine racemase, *alrA* (RD180) in a LB growth medium, reproducing earlier experiments of Ferrari *et al.* (22). In the absence of added D-ala the strain fails to grow in the medium and no to poor growth was observed in the presence of less than 200 μM D-alanine, but when supplemented with D-ala up to 0.5 mM the strain exhibits concentration dependent growth. Above 0.5 mM D-alanine the strain grows maximally irrespective of the D-alanine concentration (Fig 1A and B). Showing that the strain is able to take up this amino acid efficiently enough to support growth, but the resulting growth rate is significantly lower that the wild-type strain (168CA) even at the highest concentration (Fig 1B), suggesting that the uptake system is not sufficient to supply the demands of normal cell wall synthesis. From these growth curves it was determined that 450 μM D-ala was sufficient for the strain to grow optimally. We then used a fixed D-alanine concentration (450 μM) and supplemented the culture medium (LB) with different concentrations of other amino acids to determine if the presence of an excess of one particular amino acids would interfere specifically with the growth of the *alrA* null strain and not the wild type strain 168. On testing all 20 amino acids it was found that only the addition of excess L-alanine resulted in any significant deviation in the growth of the *alrA* null strain but not for 168 (Fig. 1C). None of the other amino acids tested had any significant effect on the growth of the strains, and gave essentially identical growth curves to those that are shown in Fig. 1C for glycine

and proline, apart from cysteine which at elevated concentrations was toxic to both strains (data not shown).

As the abundance of amino acids is not well defined in LB we used published analyses of media components to estimate the free L-alanine concentration of LB medium to be approximately 4 mM (23). Using this value the perturbation of growth presumably caused by L-alanine occurred when there was approximately 15 fold more L-alanine compared to the D-isomer. To confirm this observation the analysis was repeated using a defined medium (GM) where all amino acids except L-ala were present at 1 mM and D-ala was absent or present at 1 or 0.5 mM and the resulting growth of the *alrA* null strain and the wild-type monitored over time (Fig 2A and B). As seen previously growth of *alrA* required D-alanine, with 0.5 mM permitting growth comparable to that see for the wild type strain in the same medium (Fig 2, A and B). Here the slower growth of the alrA null strain compared to the wild type was not seem, presumably because the "maximal" growth rate in this medium does not exceed the rate at which D-alanine can be taken up by the auxotroph.

Using the same growth conditions, with D-alanine present at 1mM, the culture medium was supplemented with L-alanine at 5, 10, 20 and 40 mM and the growth of the *alrA* null strain compared with that seen for the wild type (Fig 2 D and E). As seen previously, when the L-alanine concentration in the medium was greater than 10 times that of D-ala the *alrA* null strains growth was perturbed, and where the L-alanine was present at high concentrations (40 fold excess) the *alrA* strain failed to grow.

These results suggest two possibilities, either the presence of L-alanine alters metabolism in such that D-alanine is metabolised efficiently into pyruvate and so is not available for cell wall synthesis as suggested by (24). Alternatively the transport of exogenous alanine is unable to differentiate between the isomeric states of alanine and consequently when the extracellular ration of L- to D-alanine exceeds a certain level, D-alanine uptake is insufficient to permit cell envelope synthesis (peptidoglycan and teichoic acid synthesis). Thus a reduced growth rate or cell lysis is observed depending on how rapidly

the culture is growing when the limitation occurs. The initial experiments in liquid media support both options, although the relative concentrations of L- to D-alanine would seem to more strongly favour the idea that transport competition is the cause of the phenotype. In contrast to these results growth on equivalent minimal solid medium was found to show that the *alrA* null strain was able to grow slowly in the absence of D-alanine, also reported previously by ((22, 24)) (Fig 3). This indicates that under these conditions an alternate pathway for the synthesis of D-ala is occurring. Interestingly this growth is specifically inhibited if L-ala is present in the medium, suggesting that the observed phenotype could be also caused by repression of the minor D-alanine synthetic pathway.

An alternate D-alanine synthetic pathway.

To try and determine the genes involved in this second synthetic pathways for D-alanine we firstly eliminated the possibility that the sporulation racemase, *alrB* (25), might be expressed under these conditions by introducing the *alrA* disruption cassette into a strain already deleted for *alrB* (Fig. 3B). We also introduced the alrA null mutation into strains deleted for known non-essential racemase like genes (*yitF* (26), yrpC (27) and *racX* (28)). All of these double mutants were able to grow slowly on GM plates in the absence of D-alanine, comparable to that seen for the *alrA* single mutant (Fig. 3A). Thus, the background D-alanine synthesis that was observed was likely to be the result of the activity of another gene. We then tested other genes known or inferred to function in alanine metabolism, *yugH (alaT)*, *dat* (29) and *ald* (30), to see if they were required in a strain lacking *alrA*.

The first of these genes, *yugH*, is annotated as being similar to a PLP-dependent aspartate aminotransferase, but has also been suggested to be involved in L-alanine biosynthesis and named *alaT* (A.L. Sonenshein; unpublished; see later). Construction of a double mutant strain bearing both <u>*alrA*</u> and *alaT* was found to be difficult to create on any rich or minimal medium suggesting that these two mutations were synthetically lethal. Later analysis revealed that the *yugH* null mutant was auxotrophic

for L-alanine and further characterisation of *yugH* is described later in this paper. In contrast null mutations for both *dat* and *ald* were easily introduced into the *alrA* background provided exogenous D-alanine was present. These double mutants were then tested for the ability to grow on minimal media in the absence of D-alanine. Strain KS79, deleted for both *alrA* and *ald*, was able to grow, but a strain lacking both *alrA* and *dat* (strain KS78) was not able to propagate even with prolonged incubation (Fig 3B). Further analysis of the growth of this strain showed that it was absolutely dependent upon exogenous D-alanine under all growth conditions we have tested so far, it was also evident that this double mutant was more sensitive to the relative concentrations of D- and L-alanine in liquid culture (Figure 2, panels C, F and I).

These results have interesting parallels with *Listeria monocytogenes* where the aminotransferase Dat has been shown to function in the synthesis of D-alanine when the supposed essential racemase was deleted (31). However, the mutant strains of *L. monocytogenes* described do not seem exhibit any sensitivity to L-alanine, as is seen for *B. subtilis*. This suggests that either the expression or the biochemical activity of Dat is significantly different in these related bacterial species. This possibility is further supported by the recent characterisation of a similar enzyme in *Mycobacterium smegmatis* (32) where it is found that a Dat homologue is able to correct both an alanine racemase and the glutamate racemase null mutation, suggesting that in this bacterial species the enzymatic activity is bidirectional.

Identification of the D-alanine uptake system

To determine if a gene encoding a D-ala specific transporter exists, a simple synthetic lethal screen was employed, using the *alrA* null mutant and systematically introducing null mutations for genes predicted to encode proteins involved in transport of solutes across the membrane, with the expectation that the loss of D-ala uptake would be inviable in combination with the *alrA* null mutation. In an initial screen

using a collection of 43 transporter like gene knockouts in the form of pMUTIN integrations (33) were introduced into the *alrA* null background and the transformants selected for on nutrient agar with 1 mg/ml erythromycin and supplemented with 0.5 mM D-alanine. Most of those tested were found to transform the strain efficiently and the resulting colonies were found to have both of the expected antibiotic resistance markers and were dependent upon exogenous D-alanine. However 3 knockout mutations, corresponding to *yfkT, ydgF and datA*, were found to give rise to very few colonies on selective plates and all of which were found to have lost the *alrA* null mutation, suggesting that the loss of these functions in combination with *alrA* were inviable.

To determine if these mutations were specifically perturbing the uptake of D-alanine a second screen was developed using a strain where the *alrA* null mutation complemented by a heterologous alanine racemase (*alrA* from *B. subtilis* W23 including its own promoter) cloned into the unstable plasmid, pLOSS* (17) . This resulted in a strain (KS21) that was chromosomally deleted for *alrA*, but was able to grow in the absence of D-ala by virtue plasmid encoded racemase and when grown on normal media lacking D-alanine. The presence of the plasmid (pLOSS* $alrA^{W23}$) was also indicated by the fact that is encodes β-galactosidase, and so strains bearing the plasmid give rise to blue colonies (Fig 4A). However, if the media was supplemented with D-alanine the strain was able to grow with or without the plasmid and so both blue and white colonies would be generated (Fig.4 B and C). Thus if a mutation that interfered with D-ala uptake in this strain, it would result in the plasmid becoming essential for viability and hence the strain would always carry the plasmid even in the presence of excess exogenous D-ala.

The 3 mutations initially identified as potentially being synthetically lethal with *alrA* were then introduced into the modified host strain (KS21) and strains with the null alleles were easily obtained in all cases. However, of the 3 mutations only the deletion of *datA,* resulted in the pLOSS*$alrA^{W23}$ plasmid being stabilised when grown in the presence of D-alanine (Fig. 4, A, B and C). The other 2 null mutations generated strains that easily lost the plasmid, resulting in a high proportion of white colonies in the

absence of spectinomycin (the resistance marker encoded on pLOSS*$alrA^{W23}$), provided that D-ala was present in the culture medium. This indicated that these strains were able to assimilate D-ala sufficiently to allow growth without the need for the complementing copy of *alrA* on the plasmid. Thus these genetic screens indicated that the loss of *datA* resulted in a strain that was not able to exploit D-alanine in the culture medium to the extent necessary to support growth in the absence of the alanine racemase, AlrA.

Cross-feeding assay

If DatA is involved in the assimilation of exogenous D-ala, it would be expected that a strain lacking this transporter would not be able to re-assimilate D-ala released from the cell wall during growth. To test this hypothesis we initially used a simple bioassay using the *alrA* null mutant as a reporter strain for the presence of D-alanine. Previous publications had suggested that *B. subtilis* does not release D-alanine into the culture medium during growth (4) on the basis that it was not detectable in culture supernatants. Thus the growth of a wild-type Bacillus strain was not expected to release D-ala and so would not support the growth of a D-ala auxotroph. Thus a linear streak of the wild type strain (168CA) on a nutrient agar plate with a parallel streak of the *alrA* null mutant placed as close as possible would only result in the growth of the wild type strain (Fig. 4D). In contrast using the same inoculation technique using a *datA* null strain in place of 168 resulted in clear growth of the *alrA* null strain (Fig. 4D), indicating that the growth of *datA* null strain results in the release of D-ala into the surrounding medium which can be utilised by other strain (the *alrA* null strain). This suggested that D-alanine was diffusing from the *datA* null strain in sufficient concentrations to support the growth of the D-alanine auxotroph (Strain RD180; *alrA* null). This released D-alanine presumably originated from either the maturation of the peptidoglycan, through the action of DacA and LdcB (6) or could be the result of spontaneous cleavage of D-ala from the teichoic acids resent in the cell envelope. Using the same cross-feeding assay,

but with a strain deleted for both *datA* and the *dltABCD* operon (strain KS36) as the feeding strain where D-alanine is not present on the teichoic acid (1), a similar but less dramatic result was obtained (Fig. 4E), suggesting that a large proportion of released D-alanine originates from the teichoic acids under these conditions.

These results are consistent with the idea that *datA* is required for D-ala uptake and that the most likely sources of exogenous D-alanine in vegetative growth are from the teichoic acids and the maturation of the cell wall. They also suggest that the growing bacteria normally efficiently recover the released D-alanine, presumably for reuse in future cell wall synthesis.

As the bioassay indicated that D-alanine was being released by the *datA* null strain when grown on agar plates it was expected that in liquid growth the released D-ala should be detectable at the chemical level. For this parallel cultures of 168CA and *datA* null strain (KS22) were grown in LB medium and the culture supernatant was treated with Marfeys reagent prior to HPLC analysis (19) to permit separation and quantitation of optical isomers of alanine. Using a dilutions series of D-ala in sterile LB medium it was possible to identify the peak corresponding to D-ala on the HPLC trace (Fig S1) and generate a standard curve for the quantitative determination of D-ala in the sample. Using this standard curve it was possible to determine that D-ala is present in the culture medium of *B. subtilis* during exponential growth (Fig S2A and B). As expected, for the wild type strain the concentration was low and reduced to undetectable levels at the end of exponential growth. In contrast the *datA* null strain exhibited elevated levels of D-ala throughout exponential growth (Fig. S2B). A Similar result was obtained for cultures supernatants of the *dltABCD* null strains (Fig. S2C), although at the released D-alanine was less significant, as was expected here D-alanine would not be present on the teichoic acids. This was consistent with the cross feeding results, but since the analytical method has multiple steps the absolute values cannot be considered to be reliable.

To resolve the question of the role of DatA in alanine uptake/release the wild-type and the *datA* null strain were grown in a defined minimal media supplemented with either 0.5 mM L-alanine, D-alanine or in the absence of any alanine. The growth of the culture was then monitored and samples taken a regular intervals to allow the concentration of free alanine in the culture medium to be determined and the results obtained were then plotted against time (Fig 5 shows results obtained for a characteristic experiment). When the strains were grown in the absence of alanine the HPLC analysis clearly showed the accumulation of a peak corresponding to alanine appearing after about 90 min of growth. For the wild-type strain this peak was relatively small, corresponding to about 5-7 μM and was seen to disappear toward the end of exponential growth (Fig. 5A). In dramatic contrast a significantly stronger alanine peak was obtained for the *datA* null strain staring at about the same time, but rapidly increasing to over 100 mM and remaining at this level. Where alanine was present in the culture medium it was clear that the wild-type strain efficiently assimilated this amino acid in both isomeric forms over the duration of the experiment (Fig 5B), reducing its concentration to undetectable levels between 120 and 150 min of incubation, whereas the *datA* null strain was unable to efficiently utilise either isomeric form of alanine. Interestingly, it was reproducibly observed that the wild-type strain more efficiently utilised the D-isomeric form of alanine. In contrast the poor uptake of alanine exhibited by the *datA* mutant was more evident for L-alanine than for the D-isomer.

From the chemical analysis and the cross feeding, it is clear that alanine is released as *B. subtilis* grows, and strains lacking DatA do not seem as efficient as the wild type strain in the re-assimilation of this amino acid. In both cases it was repeatedly evident that the concentration of alanine reduced toward the end of exponential growth, suggesting that there may be other uptake systems acting to assimilate alanine in later phases of growth. However, it would seem that these are not sufficient to support vegetative growth as indicated by the synthetic lethality of *datA* and *alrA* mutations.

Genetic context and expression of *datA*

Looking at the genome in combination with transcriptional data (34) it suggested that *datA* is the last gene in an operon with transcription passing from between SAM, *metK*, passing through *asnB* before reaching *datA.* To confirm this constructed a large set of oligonucleotides that would permit the amplification of various regions of the genome surrounding *datA* (Fig S3A). The functionality of these primer pairs we then confirmed using genomic DNA extracted from 168CA as a template (Fig S3B) and then these oligonucleotide combinations were then used to amplify cDNA generated from total RNA extracted from exponentially growing 168CA (Fig S3C). The resulting DNA fragments were then resolved on an agarose gel to provide an indication of the size of the possible RNA transcripts in this region of the genome. This analysis supports the previous microarray data (34) suggesting that *datA* is certainly co-transcribed with *asnB* and *metK*. As such it was possible that the insertional deletion of *datA* could have unforeseen effects on *asnB* and *metK*, both of which have been shown to be required for normal growth ((35) and (36) respectively). To eliminate the possibility that the phenotype for the *datA* null mutation in combination with *alrA* was the result of a polar effect of the inserted antibiotic resistance cassette used to make the deletion, we cloned a copy of *datA,* using its own ribosome binding site (RBS), into pPY18 so as to place it under the control of the P_{spac} promoter and permit it to be inserted into *amyE* locus. This construction (pKS1) was then transformed into strain KS27 (*alrA* null mutant) and the resulting strain checked for the correct insertion of the ectopically expressed copy of *datA*. This strain was then transformed with the *datA* null mutation using chromosomal DNA from strain BKE30530. However none of the resulting colonies carried the correct markers, looking at the native RBS of *datA* a simple explanation for this result was that there was poor translation of the ectopic copy of *datA*. To eliminate this possibility the inducible construct was reconstructed, this time changing the RBS to one closer to the consensus (in this case we took a segment of sequence upstream of the cell division gene *ftsL* corresponding to the RBS sequence and extending to the initiation codon) to give plasmid pKS6. A

strain carrying this construction combined with *datA* deletion (KS41) was found to be easily transformed with the *alrA* null mutation in the presence of the inducer (0.1 mM IPTG; Fig S3A). Thus, enhancing the translation of the *datA* transcript was sufficient to complement the chromosomal null mutation, showing that the observed phenotype was caused by the deletion of *datA* and not due to a polar effect on other genes and that efficient expression of native *datA* seems to be coupled with the translation of the upstream genes.

Characterisation of an L-Alanine auxotroph

Alanine metabolism in *B. subtilis* has not been clearly defined although various genes have been identified as potentially acting in the synthesis or degradation of this amino acid. However much of this work relates to the analysis of sporulation and/or germination defects (37) or biochemical characterisation of enzymes extracted out of total lysates of *B. subtilis* or other related species (38-40). To our surprise, as mentioned earlier we found that the *alaT* null mutant alone exhibited a significant growth defect on minimal media that was corrected by the presence of D- or L-alanine in the growth medium, implicating it as having a major role in the normal biosynthesis of alanine. However, with prolonged incubation this null mutant was able to generate visible biomass in the absence of alanine (Fig. 6A; top right quadrant), suggesting the existence of another biosynthetic pathway. Three possibilities existed, firstly other transaminases are encoded in the genome that are able to catalyse the necessary conversion of precursors into alanine, as has been determined for *E. coli* ((41)). Secondly, as the growth of the strain was very slow, it was possible that the alternate D-ala synthetic pathway in combination with the racemase was acting to generate sufficient L-ala to permit limited growth (Fig. 6A). To test this latter possibility we constructed a strain deleted for both *alaT* and *dat* (strain MC4) and on inoculating on glucose minimal plates with and without alanine this strain was found to be unable to grow in the absence of L-ala, suggesting that has *dat* a minor role in L-alanine synthesis. Interestingly we

could not show that Dat had any role in the utilisation of D-or L-alanine as a carbon or ammonium source as had previously been suggested (42), but this role was specific to the alanine dehydrogenase encoded by *ald* (Fig. S4) as had previously been suggested (43).

Is DatA a transporter for both D and L-alanine?

Our initial phenotypic characterisation of the *datA* null mutant clearly indicated that it had an important role in the uptake of D-ala. However, in the initial analysis of the *alrA* null strain it was observed that that excess L-ala in the culture medium interfered with the strain ability to grow, implying that DatA could function as an alanine uptake system and not be specific for the D-isomer. This possibility was further indicated by the fact that both D- and L-alanine assimilation was perturbed in the *datA* null strain (Fig. 5B). But as the strains used were capable of alanine biosynthesis, the uptake of Alanine was not necessary for their ability to grow, and so may not represent the uptake capability of an alanine auxotroph. However as AlaT is the major biosynthetic enzyme in the alanine pathway, it was now possible to determine if DatA was required for L-alanine uptake. Using a genetic approach we constructed a strain deleted for both *alaT* and *datA,* and then tried to propagate the strain in on GM media supplemented with alanine. Initial results suggested that DatA was not required for L-alanine uptake as the double mutant appeared to grow normally on minimal media supplemented with 1 mM L-alanine (Fig.6B, upper plates). However, on plates where L-alanine was provided at a lower concentration (0.4 mM) the double mutant failed to show any growth (Fig 6B, lower plates). To confirm these observations in a more quantitative way we analysed the growth of the strain in liquid culture using a range of alanine concentrations (Fig 6C). From the growth curves generated it was clear that normal growth of an alanine auxotroph require the presence of at least 0.8 mM alanine in the culture medium, whereas a strain deleted for both *alaT* and *datA* required 1 mM or more to exhibit the same growth profile.

Analysis of the growth of the *alaT* null strains in liquid culture confirmed that requirement of DatA for the efficient uptake of L-ala in media with less the 1 mM free alanine (Fig 6C). Similar results were also obtained when D-alanine was used to feed the strain. Surprisingly D-ala at concentrations over 1 mM were able to support the growth of an *alaT* alanine auxotroph even when *datA* was deleted, suggesting that both D and L-alanine were able to be taken up by some other transport system when present exogenously in sufficient concentration.

Summary:

From our current understanding of cell envelope biosynthesis and maturation in *B. subtilis*, D-alanine plays an essential role in peptidoglycan precursors by acting as the substrate for PBP mediated crosslinking. The formation of each crosslink in the peptidoglycan will result in the release of a D-alanine residue combined with the fact PG peptide side chains that do not take part in cross links are also matured soon after synthesis, with both of the terminal D-alanine residues being cleaved off sequentially (5, 6). Combined with the synthesis of the PG, D-alanine is also released from the teichoic acids by spontaneous hydrolysis (44) consequently there should be an accumulation of free D-alanine residues in the immediate vicinity of a growing cell. Our initial analysis sought to identify how D-alanine was re-assimilated form the extracellular medium such that D-ala is essentially undetectable in culture medium post exponential growth (4). Exploiting the expected synthetic lethality expected for a D-alanine auxotroph that lost the ability to assimilate exogenous D-alanine identified the gene *datA* as a potential permease necessary for efficient D-alanine uptake. Characterisation of the phenotype of the D-alanine auxotroph shows that the uptake of exogenous D-alanine is not sufficient to support high growth rates seen for a wild-type strain even in the presence of excess D-alanine (Fig. 1) indicating that the uptake is the rate limiting step. Consequently we propose to change the name of *datA* to **D**-**a**lanine **t**ransporter **A**, *datA*, to reflect its apparent function in the re-use of the D-alanine that is released during

the normal processes of cell wall synthesis and maturation. From the phenotypic analysis presented in this work, the logical role of DatA would seem to be the re-assimilation of D-alanine released from the cell wall as the bacterium grows. This role would be consistent with growth in a nutrient limited environment, interestingly this recycling function seems to be absent in *S. aureus*, although potential homologues of DatA can be tentatively identified by similarity. However, since the similarity is low (below 48 % identity) they may have other roles and as yet no functional characterisation has been done for these genes in this organism.

Unfortunately attempts to biochemically characterise DatA were not successful as we were unable to purify the protein. Thus we employed phenotypic and genetic methods to try and determine the specificity of the transporter. To make this possible we defined the late biosynthetic steps of L-alanine synthesis in *B. subtilis.* In contrast to *E. coli* where a functionally redundant set of 3 transaminases (AvtA, YfbQ and SerC; (41)) have been shown to participate in alanine synthesis, alanine seems to be the product of only one major transaminase, AlaT. Deletion of which results in a strain that grows significantly slower in minimal media than the wild-type, suggesting that although this was the major biosynthetic enzyme for L-alanine synthesis a second synthetic pathways existed. However, the availability of an alanine auxotroph then permitted us to show that DatA also acts in the uptake exogenous of L-alanine, but only when present at concentrations below 1 mM, this could potentially indicate that the affinity of the transporter of L-alanine is lower than for the D- isomer, but since this is an *in vivo* assay other factors may also be influencing the growth of the strain that we cannot take into account. Further analysis of the phenotype of the L-alanine auxotroph (*alaT*) showed that the slow growth when deprived of exogenous alanine was surprisingly dependent upon the transaminase that we found acting in D-alanine synthesis, Dat. Previous analysis of other enzymes acting in alanine metabolism is rather limited with two enzymes being identified in the literature from various Bacillus species. The first being an alanine dehydrogenase (Ald (43)) has previous been identified as functioning

in the use of alanine as a carbon source in sporulation and a D-alanine transaminase (Dat (45)) proposed to function in the metabolic conversion of D-alanine into D-glutamate (46), perhaps in stationary phase growth or sporulation. However, transcriptional micro-array data and proteomics (42) seems to suggest that both of these enzymes are maintained as a relatively constant abundance in cells, inferring more of a central role in cellular metabolism. In our analysis found that Ald is required for alanine to function as a carbon source, with the suggestion that this enzyme acts on only the L-isomer and Dat is implicated in the synthesis of both D- and L-alanine, either directly or through the action of the racemase. Interestingly the activity of Dat is however inefficient in *B. subtilis* and is unable to fully correct the auxotrophy caused by the loss of AlaT (the main L-alanine synthase) or AlrA (the alanine racemase for D-alanine production). It was also evident that exogenous L-alanine is toxic to a strain lacking the alanine racemase, suggesting that the activity of Dat is modulated in some way by the availability of the L-isomer of alanine. Putting these results together indicates that alanine biosynthesis is potentially integrated the glutamate biosynthetic pathway via Dat in such a way as to permit the generation D-alanine which in turn can be converted into the L-isomeric form for protein. This integration of pathways seems to be supported by the characterisation of other bacterial species where it has been shown that Listera possess an equivalent Dat enzyme which seems to be fully capable of supplying the cells D-alanine requirements in the absence of a functional alanine racemase (31) and the mutant strains do not exhibit L-alanine sensitivity. This idea is supported by the characterisation of an enzyme comparable to Dat in *Mycobacterium smegmatis* shows that it has different properties and can act to supply the cell with D-glutamate in the absence of the cognate racemase, MurI, as well as function to provide D-alanine in a strain deleted for the alanine racemase (32). Thus it would seem this set of enzymes are well conserved in a diverse range of bacteria, but most obviously in Firmicutes and close relatives. However their activity is modulated in different ways, presumably reflecting different metabolic priorities. This then raises the question of how the activity of these enzymes are modulated in the cytosol to ensure

that the necessary precursors are available and that the metabolic pathways do not undergo futile cycles of interconversion. As such our work is now focused on the subcellular localisation of these enzymes in combination with determining biochemical activity and substrate preference.

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Figure legends:

Figure 1

Panel A - The growth characteristics of of D-alanine auxotroph (alrA) strain. The growth curves of wild-type (168CA) and ΔalrA::zeo (RD180) strains in LB medium, supplemented with different concentrations of D-alanine (0.2-1 mM) at 37 °C. The growth curves were monitored by plate reader and shown in Log10 of optical density (OD600). The plotted line shows the mean values for 3 biological replicates with error bars indicating the standard deviation between replicates.

Panel B - The growth rates of the same strains measured at three time point intervals (60-80, 80-100 and 100-120 min) of panel (A). The error bars represent the standard deviation of mean of growth rate in two independent experiments.

Panels C and D - Growth curves showing the inhibitory effect of increasing concentrations of L-alanine, glycine or L- proline on the growth of the D-alanine dependent strain (RD180) compared to the wild type strain (168CA). Both strains were grown at 37 ˚C in LB medium with fixed concentration of D-alanine and supplemented with different concentrations of L-alanine (C) or 5 mM Glycine or L-Proline (D) at 37 C˚.

Figure 2

Inhibitory effect of L-alanine on the growth of a D-alanine auxotroph in a defined media. The growth of wild-type (168CA), *ΔalrA* (RD180) and *ΔalrA Δdat* (KS78) in GMaa with 1 or 0.5 mM of D-alanine or no alanine were shown in panels **A**, **B** and **C** respectively. Strains were grown in GMaa with 1 mM D-alanine supplemented with different concentrations of L-alanine (D-F), or in GMaa with L-alanine but without D-alanine (**G-I**). Growth determine in terms of optical density (600 nm) in plate reader with shaking at 37 ˚C. Numbers in the legend represents different molarities (mM) of either D-/L-alanine (for A-C): D-alanine; for D-I: L-alanine) in the media. '-' indicates no alanine was added in the media.

Figure 3

Identification of the second D-alanine synthetic enzyme.

Panel A - The D-alanine auxotroph strains *ΔalrA* (RD180), *ΔalrA ΔyitF* (KS35), *ΔalrA ΔyrpC* (KS34), and *ΔalrA ΔracX* (KS33) were found to be able to grow well on Minimal medium (MG) in the absence of D-alanine, (plate was incubated at 37 °C for 36 hours).

Panel B - Growth of strains *ΔalrA ΔalrB* (KS32), *ΔalrA* (RD180) and *ΔalrA Δdat* (KS78) on MM with and without supplementation of 0.5 mM D- or L-alanine, at 37 °C for 48 hours. As a positive control the strains were also inoculated on nutrient agar (NA) supplemented with D-alanine (0.5 mM)

Figure 4

Genetic confirmation of the role of *datA* in D-alanine uptake. A D-alanine dependent strain with the sole functional copy of *alrA* encoded on a unstable plasmid was constructed, KS21 (*ΔalrA + pLOSS* Ω alrA*) and compared to a strain also lacking *datA*, KS23 (*ΔalrA ΔdatA + pLOSS* Ω alrA*) when grown on different media. **Panel A** shows the strains grown on nutrient agar (NA) plus X-gal. In contrast **panels B** and **C** show the same strains on NA + X-gal supplemented with 450 µM of D-alanine and X-gal and 900 µM of D-alanine respectively. All three plates were photographed after 16 h incubation at 37 °C. The

blue colonies indicate that the *pLOSS* Ω alrA* plasmid is conserved in the cells, whereas the cells produced white colonies after losing the plasmid. (D & E) In this cross-feeding assay, the 168CA, KS11 (*ΔdltA-D*), KS22 (*ΔdatA*) and KSS36 (*ΔdltA-D ΔdatA*) strains (D-alanine donors) and RD180 (*ΔalrA::zeo*) and KS12 (*ΔalrA ΔdltA-D*) strains (D-alanine recipients) were used in this assay. The D-alanine donor strains were firstly streaked on nutrient agar plates and incubated for 3 h at 37 °C. Later, the D-alanine recipient strains were streaked closely parallel to the donor strains and kept incubating for a further 23 h. The total incubation time was 26 h.

Figure 5

Determination the concentration of alanine present in the culture medium during growth of wild-type *B. subtilis* and a *datA* null strain by OPA derivatisation and HPLC. The line graphs show growth curves expressed as the optical density of the cultures at 600 nm (○ symbols for the wild type and ● for the *datA* null strain and the column plots in panel **A** indicate the concentration of free alanine (μM). Panel B presents the growth curves for the wild-type strain and the *datA* null strain when grown the presence of exogenous alanine (0.5 mM), for simplicity the growth curves shown are for the cultures supplemented with D-alanine as those supplemented with L-alanine were essentially identical. On the same plot the alanine concentration in the respective cultures was determined over time with triangles for L-alanine (△ for the wild-type culture and ▲ for the *datA* mutant) and inverted triangles for D-alanine (▽ for the wild-type and ▼ *datA* mutant).

Figure 6

DatA transports both isoforms of alanine. **Panel A** shows how the growth of Δ*alaT* (MC2) and Δ*alaT* Δ*dat* (MC4) on GM plates is altered by the presence or absence of 1mM L-alanine. The plates were incubated at 37°C for 48 hours. **Panel B** Shows how the growth of wild-type (168+), Δ*alaT* (MC2) and Δ*alaT* Δ*datA* (MC8) on GMaa plates with either D-/L-alanine at higher (1 mM) and lower (0.4 mM)

concentrations. The plates were incubated at 37°C for 24 hours**. Panel C** shows typical growth curves obtained for the wild-type strain (168), the *alaT* null strain (MC2) and the double mutant *alaT datA* (MC4, identical results were obtained for strain MC8) grown at 37 °C in a plate reader in the liquid GMaa supplemented with a range of concentrations of D-/L-alanine from 0.4 mM to 1 mM (C).

Figure S1

Standard curve for D-alanine quantification in LB media. A) The RP-HPLC analysis of 2000 MWCO filtered LB medium with and without D-alanine (mM). The red arrows indicate the D-alanine peaks, which were raised around 34 min. B) A standard curve was generated from the data of figure (A) for quantitative determination of D-alanine in the growth medium.

Figure S2

RP-HPLC analysis for the detection of D-alanine in culture supernatants.

Panel A - Growth curves for cultures samples for HPLC analysis (panel B) the wild-type strain (●) 168CA), *ΔdatA* (○; KS22), *ΔdltA-D* (▼; KS11) and *ΔdltA-D datA* (△; KS36) strains in LB medium at 37 °C. The black arrows represent the time points, when the samples were taken for RP-HPLC analysis.

Panel B - RP-HPLC analysis of 2000 MWCO filtered culture supernatants of 168CA and *ΔdatA* (KS22) strains at different time points (90, 180, 390 min and O/N).

Panal C - RP-HPLC analysis of *ΔdltA-D* (KS11) and *ΔdltA-D ΔdatA* (KS36) strains at 180 min of incubation. The red arrows indicate D-alanine peaks, which appeared at around 34 min.

Figure S3

Complementation and Transcription analysis of *datA* gene.

Panel A - Growth of KS22 (*ΔdatA::erm*), RD180 (*ΔalrA::zeo*) and KS42 (*ΔdatA::erm amyE Ω (cat Pspac datA-ftsL*(RBS)) *ΔalrA::zeo*) strains on nutrient agar (NA) plates with and without 1.0 mM IPTG and 0.5 mM D-alanine. The plates were incubated at 37 °C overnight.

Panel B provides a diagrammatic representation of the chromosomal region up and downstream of the *datA* gene below which are shown numbered DNA fragments which were amplified by PCR. The numbers indicate which specific pair of oligonucleotides (sequences of which can be found in Table S2) were used to generate the DNA fragment. The images of agarose gel show products of PCR reactions (32 cycles), using gDNA (**panel C**) and cDNA (**panel D**) as templates. The lane numbers correspond to the numbered bars in panel A.

Figure S4

Genes involved in the use of alanine as sole carbon/nitrogen source. For testing alanine used as sole carbon source, *ΔalaT* (MC2) *Δdat* (MC3) and *Δald* (MC5) were grown on M plates with 25 mM D-/L-alanine. For testing alanine used as nitrogen source, these three strains were grown on GM-NH_3 plates supplemented with 25 mM D-/L-alanine. The plates were incubated at 37°C for 48 hours.

Table 1 Strains collection

***B. subtilis* strains**	**Genotype**	**Construction or Source or Reference**
168 CA	*trpC2 Bacillus subtilis*	Laboratory strains collection
168 +	Prototrophic derivative of 168 CA	R. Daniel, unpublished
W23	*Bacillus subtilis* W23	Laboratory strains collection
RD180	*trpC2* Δ*alrA::zeo*	R. Daniel, unpublished
BKE30530	*trpC2* Δ*datA::erm*	BGSC (16)
BKE 17640	*trpC2* Δ*alrB::erm*	“
BKE 34430	*trpC2* Δ*racX::erm*	“
BKE09670	*trpC2* Δ*dat::erm*	“
BKE31400	*trpC2* Δ*alaT::erm*	“
BKE31930	*trpC2* Δ*ald::erm*	“
BKE 26810	*trpC2* Δ*yrpC::erm*	“
BKE10970	*trpC2* Δ*yitF::erm*	“
KS11	*trpC2* Δ*dltABCD::cat*	deletion of *dltABCD* genes
KS21	*trpC2* Δ*alrA::zeo* p*LOSS*alrA*	p*LOSS*alrA* transformed into RD180
KS22	*trpC2* Δ*datA::erm*	168CA transformed with gDNA of BKE30530
KS23	*trpC2* Δ*datA::erm* Δ*alrA::zeo pLOSS*alrA*	KS21 transformed with gDNA of BKE30530
KS26 KS27	*trpC2* Δ*datA::erm* Δ*amyE (cat* P_{spac} *datA)* *trpC2* Δ*alrA::zeo* Δ*amyE (cat* P_{spac} *datA)*	pKS1 transformed into KS22 pKS1 transformed into RD180
KS32	*trpC2* Δ*alrA::zeo* Δ*alrB::erm*	RD180 transformed with gDNA of BKE17640
KS33	*trpC2* Δ*alrA::zeo* Δ*racX::erm*	RD180 transformed with gDNA of BKE34430
KS34	*trpC2* Δ*alrA::zeo* Δ*yrpC::erm*	RD180 transformed with gDNA of BKE 26810
KS35	*trpC2* Δ*alrA::zeo* Δ*yitF::erm*	RD180 transformed with gDNA of BKE10970
KS36	*trpC2* Δ*datA::erm* Δ*dltA-D::cat*	KS22 transformed with gDNA of KS11
KS41	*trpC2* Δ*datA::erm* Δ*amyE*(*cat* P_{spac} *ftsL*(RBS)- *datA*)	pKS6 transformed into KS22
KS42	*trpC2* Δ*datA::erm* Δ*amyE* (*cat* P_{spac}	KS41 transformed with gDNA of

	ftsL(RBS)- *datA)* Δ*alrA::zeo*	RD180
KS78	*trpC2* Δ*alrA::zeo* Δ*dat::erm*	RD180 transformed with gDNA of BKE09670
KS79	*trpC2* Δ*alrA::zeo* Δ*ald::erm*	RD180 transformed with gDNA of BKE31390
MC1	Δ*alaT::erm*	gDNA of BKE31400 transformed into 168+.
MC2	Δ*alaT*	Erythromycin marker was removed using pDR244.
MC3	Δ*dat::erm*	gDNA of BKE09670 transformed into 168+.
MC4	Δ*alaT* Δ*dat::erm*	gDNA of BKE 09670 transformed into MC2.
MC5	Δ*ald::erm*	gDNA of BKE 09670 transformed into 168+.
MC6	Δ*alaT* Δ*ald::erm*	gDNA of BKE 09670 transformed into MC2
MC7	Δ*datA::erm*	gDNA of BKE 09670 transformed into 168+.
MC8	Δ*alaT* Δ*datA::erm*	gDNA of BKE 09670 transformed into MC2.
***E. coli* strains**	**Genotype/Relevant features**	**Construction or Source or Reference**
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England BioLabs®

Table S1. Plasmid constructs used in this study.

Name plasmid	Relevant features	Construction	Source/Reference
pPY18	*bla amyE3' cat* P_{spac} *lacI amyE5'*	------	(18)
pSG1	*bla cat*	-------	(47)
pLOSS**alrA*	*bla spc* P_{spac} $alrA^{W23}$ - *PdivlA- lacZ lacI rep pLS20**	Inserstion of *alrA* gene into *pLOSS** vector	This work
pKS1	*bla amyE3' cat* P_{spac} *datA*	Insertion of *datA*	This work

	lacI amyE5'	gene and its upstream 30 bases into *Xba*I cut pPY18	
pKS6	*bla amyE3' cat* P_{spac} *datA-ftsL(RBS) lacI lacZ amyE5'*	Insertion of *datA* gene and RBS of *ftsL* gene into *Xba*I and *BglI*Icut pPY18	This work

Supplemental methods and materials

Real-time PCR assay

The wild type strain (168CA) was grown in LB media to mid-exponential growth (OD_{600} ~ 1.0) at 37 °C. Total RNA was extracted from 1.0 ml the culture, using total RNA purification plus Kit (NORGEN Biotek Corp.) according to the manufacturer's protocol. The high capacity cDNA reverse transcription kit (Applied Biosystems™) was used for making cDNA according to manufacturer's protocol. The total RNA extract was used as template and non-specific random hexamers as primer in cDNA production. Real-time PCR was then performed on normal PCR machine, using DNA as template, gene specific primers and Q5™ High-Fidelity DNA polymerase (New England BioLabs®) according to the manufacturer's instructions. The Real-time PCR products were run on agarose gel (Fig S2).

Table S2. Oligonucleotides

Name	Sequence	Restriction site	Purpose
alrAW23-1	5’-ATC**TCTAGA**CGTTAGGCATCGTTTCCCTTTGTCC-‘3	*Xba*I	Construction of p*LOSS*alrA*^{W23}
alrAW23-2	5’- ACT**GGTACC**CTATTTGCTTGTATTTTCCTGCAATAAAGGATTTC	*Bam*HI	
oKS01	5´-AAGGAACAGATGATGCGCACG-´3	---------	For deletion of *dltABCD* genes
oKS02	5´AGT**TCTAGA**TCCGCATGTGTTTGAATAGC-´3	*Xba*l	
oKS03	5´ATA**TCTAGAAGATCT**AGCGAAGGGCTTCCAGGTTGC-´3	*Xba*l and *Bgl*II	
oKS04	5´-TGCGATTTCTCCTGTTTCACCG-´3	---------	
oKS12	5´-TAGTATTCGGCACAGATCGGC-´3	---------	
oKS21	5´ATC**TCTAGA**AATGATATTTTTTTCTAGGGGAGAAGAAGC-´3	*Xba*I	For pKS1 construction
oKS22	5´TGA**TCTAGA**TCAGCTGATATTTCGTTCGCTGGC-´3	*Xba*I	
oKS25	5´ATC**TCTAGA**AAAATTAAAAGGAGGTCATCAGCCTATGCAAAAACAAAAACAAGAGCTGCACCGC-´3	*Xba*I	For pKS6 construction
oKS26	5´TGA**AGATCT**TCAGCTGATATTTCGTTCGCTGGC-´3	*BglII*	
oKS38	5´-AACGGCTCGGTGACATGTAT-´3	---------	
oKS39	5´-TGTTCAATGGCTTTGATCGT-´3	---------	
oKS40	5´-GGACTTGGACCAGCAAACAG-´3	---------	
oKS41	5´-CGGAAAAACCTCCCTTTTCT-´3	---------	
oKS42	5´-AGCGGTGCTTTCTGTGAAAT-´3	---------	
oKS43	5´-GTGCGGCTATTATTGCGATT-´3	---------	
oKS44	5´-TGTCCAGCTCTTGATGAACG-´3	---------	
oKS45	5´-CAGAGAGTGGGAAGGGCTTT-´3	---------	
oKS46	5´-GCATCACAAGAAGCGGAACT-´3	---------	
oKS47	5´-TCGCTCCGTCACTTAAGGTT-´3	---------	

oKS48	5´-ACTTGCCCCGTGAATGATTA-´3	---------	oKS38 to oKS69 used for transcription analysis of *datA* gene
oKS49	5´-TTCTCCGATCACGACATCAC-´3	---------	
oKS50	5´-CGGAGAACAGTCCAGCATTT-´3	---------	
oKS51	5´-ATCCCGATTAAGGCGTTCTT-´3	---------	
oKS52	5´-CGCCCTGCAAAAGTCATC-´3	---------	
oKS53	5´-TTGCTGGCTGTGTACTATGGA-´3	---------	
oKS54	5´-TTTCTTTTCTTCCGGCTTCA-´3	---------	
oKS55	5´-ATCCCGGGTAAAGCTGTTCT-´3	---------	
oKS56	5´-GCGGTGCAGCTCTTGTTT-´3	---------	
oKS57	5´-AGCTGAGCCATCAGCCTAAA-´3	---------	
oKS58	5´-CCGTTCTTCGTTTTCAGCTC-´3	---------	
oKS59	5´-CGCGATGTGACGAAGACTTA-´3	---------	
oKS60	5´-GCTTGTTAAAAACCCCGACA-´3	---------	
oKS61	5´-GGAGCAGCTGCGTAAAGAAG-´3	---------	
oKS62	5´-GGGCAAGTGAAATTGGAAGA-´3	---------	
oKS63	5´-ATCGGATACACACGTGCAAA-´3	---------	
oKS64	5´-GCCCCTCCGTAACAGATTC-´3	---------	
oKS65	5´-TGAAAGGCACAAAGACCAAA-´3	---------	
oKS66	5´-TCGTAAAAGGGTTTGCAATG-´3	---------	
oKS67	5´-CAGCGGTCAAATCAACTGAG-´3	---------	
oKS68	5´-AGCGGCTGTACACGAAAGTT-´3	---------	
oKS69	5´-AACGGCTGCTCAACTGTTTT-´3	---------	

- The restriction sites are shown as bold, underlined letters.

Table S3 Pump and run parameters of RP-HPLC analysis

Step	Time (min)	Flow (ml/min)	A	B	C	D	Curve	Sample volume(µl)
0	0.5	1.00	90.0	10.0	0.0	0.0	0.0	150
1	1.5	1.00	90.0	10.0	0.0	0.0	0.0	
2	40.0	1.00	75.0	25.0	0.0	0.0	1.0	
3	25.0	1.00	60.0	40.0	0.0	0.0	1.0	
4	0.5	1.00	90.0	10.0	0.0	0.0	0.0	
5	20.0	1.00	90.0	10.0	0.0	0.0	0.0	
Total Run Time	87.00							

Table S4. Putative amino acids transport mutants

Strains	Geneotype
MGNA-C343	*trpC2* Δ*yfkT*::pMUTIN4 *(erm)*
MGNA-B989	*trpC2* Δ*ycgH*::pMUTIN4 *(erm)*
MGNA-B757	*trpC2* Δ*ywaA*::pMUTIN4 *(erm)*
MGNA-C001	*trpC2* Δ*yckA*::pMUTIN4 *(erm)*
MGNA-C394	*trpC2* Δ*yqiN*::pMUTIN4 *(erm)*
MGNA-C383	*trpC2* Δ*yqiZ*::pMUTIN4 *(erm)*
MGNA-C480	*trpC2* Δ*yqgT*::pMUTIN4 *(erm)*
MGNA-A172	*trpC2* Δ*ytmL*::pMUTIN4 *(erm)*
MGNA-B963	*trpC2* Δ*ybeC*::pMUTIN4 *(erm)*
MGNA-B783	*trpC2* Δ*hutM*::pMUTIN4 *(erm)*
MGNA-B940	*trpC2* Δ*ybgF*::pMUTIN4 *(erm)*
MGNA-C381	*trpC2* Δ*yqiX*::pMUTIN4 *(erm)*
MGNA-B939	*trpC2* Δ*ybgE*::pMUTIN4 *(erm)*
MGNA-B914	*trpC2* ΔyabM::pMUTIN4 *(erm)*
MGNA-C084	*trpC2* Δ*ydgF*::pMUTIN4 *(erm)*
MGNA-C022	*trpC2* Δ*ycsG*::pMUTIN4 *(erm)*
MGNA-A551	*trpC2* Δ*yurH*::pMUTIN4 *(erm)*
MGNA-A736	*trpC2* Δ*ykbA*::pMUTIN4 *(erm)*
MGNA-A031	*trpC2* Δ*yjoB*::pMUTIN4 *(erm)*
MGNA-A468	*trpC2* Δ*yvbW*::pMUTIN4 *(erm)*
MGNA-A012	*trpC2* Δ*ytgP*::pMUTIN4 *(erm)*
MGNA-A909	*trpC2* Δ*yecA*::pMUTIN4 *(erm)*
MGNA-A362	*trpC2* Δ*yjkB*::pMUTIN4 *(erm)*
MGNA-A311	*trpC2* Δ*yobN*::pMUTIN4 *(erm)*
MGNA-A173	*trpC2* Δ*ytmM*::pMUTIN4 *(erm)*
MGNA-A230	*trpC2* Δ*aapA*::pMUTIN4 *(erm)*
MGNA-B479	*trpC2* Δ*yhdG*::pMUTIN4 *(erm)*
MGNA-C382	*trpC2* Δ*yqiY*::pMUTIN4 *(erm)*
MGNA-A171	*trpC2* Δ*ytmK*::pMUTIN4 *(erm)*
MGNA-C231	*trpC2* Δ*yfnA*::pMUTIN4 *(erm)*
MGNA-B782	*trpC2* Δ*yxeM*::pMUTIN4 *(erm)*
MGNA-A126	*trpC2* Δ*ytnA*::pMUTIN4 *(erm)*
MGNA-A595	*trpC2* Δ*yusA*::pMUTIN4 *(erm)*
MGNA-A596	*trpC2* Δ*yusC*::pMUTIN4 *(erm)*
MGNA-A686	*trpC2* Δ*yhaG*::pMUTIN4 *(erm)*
MGNA-C257	*trpC2* Δ*yflA*::pMUTIN4 *(erm)*
MGNA-A659	*trpC2* Δ*yhcG*::pMUTIN4 *(erm)*

MGNA-A653	*trpC2* Δ*yhcH*::pMUTIN4 *(erm)*
MGNA-A655	*trpC2* Δ*yhcJ*::pMUTIN4 *(erm)*
MGNA-A834	*trpC2* Δ*yoaC*::pMUTIN4 *(erm)*
MGNA-A835	*trpC2* Δ*yoaD*::pMUTIN4 *(erm)*
MGNA-A783	*trpC2* Δ*mtnK*::pMUTIN4 *(erm)*
MGNA-A082	*trpC2* Δ*spoVG*::pMUTIN4 *(erm)*

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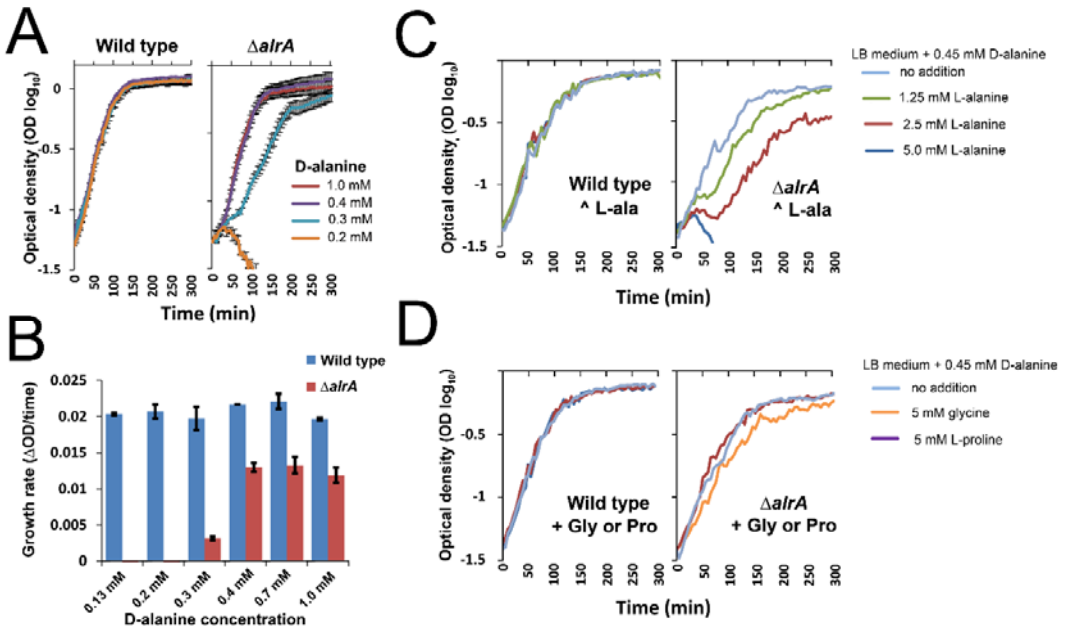
Fig. 1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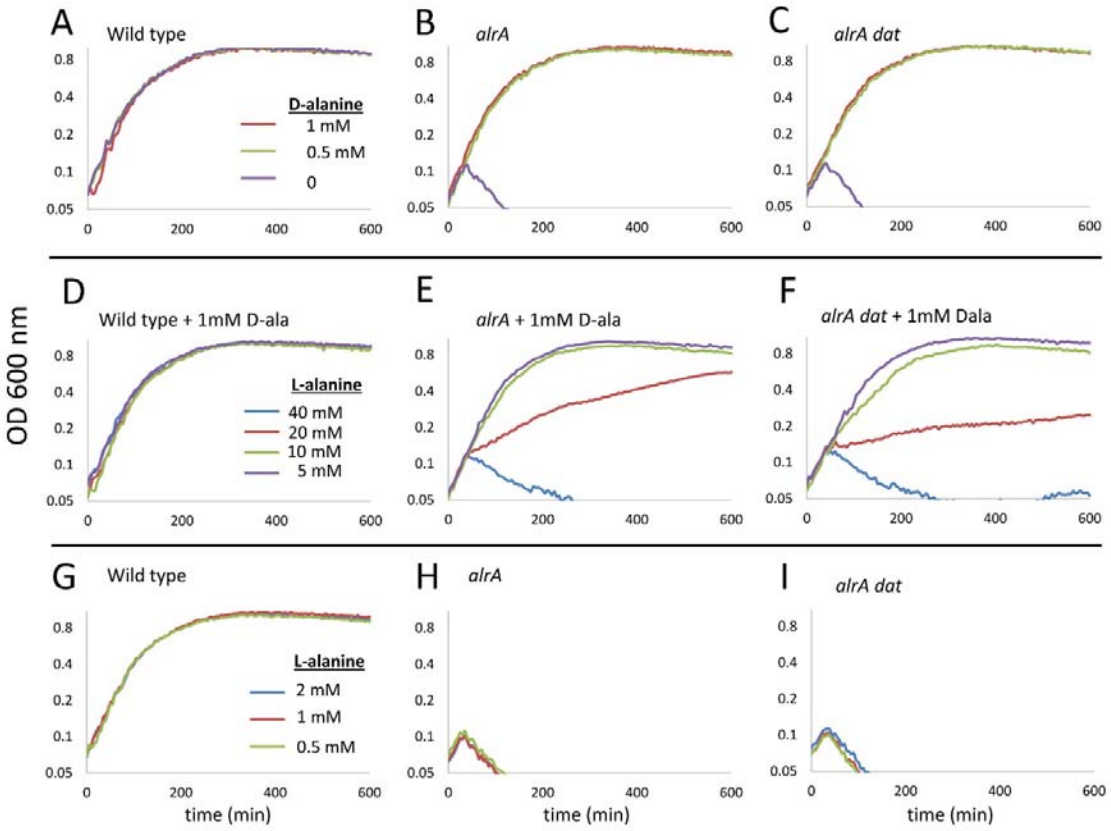
ΔalrA
Optical density (OD log10)
D-alanine
1.0 mM
0.4 mM
0.3 mM
0.2 mM
Time (min)
B
Wild type
ΔalrA
Growth rate (ΔOD/time)
0.13 mM
0.2 mM
0.3 mM
0.4 mM
0.7 mM
1.0 mM
D-alanine concentration
C
LB medium + 0.45 mM D-alanine
no addition
1.25 mM L-alanine
2.5 mM L-alanine
5.0 mM L-alanine
Wild type
^ L-ala
ΔalrA
^ L-ala
Time (min)
D
LB medium + 0.45 mM D-alanine
no addition
5 mM glycine
5 mM L-proline
Wild type
+ Gly or Pro
ΔalrA
+ Gly or Pro
Time (min)

Fig 2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D-alanine
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0.5 mM
0
B
alrA
C
alrA dat
D
Wild type + 1mM D-ala
L-alanine
40 mM
20 mM
10 mM
5 mM
E
alrA + 1mM D-ala
F
alrA dat + 1mM Dala
G
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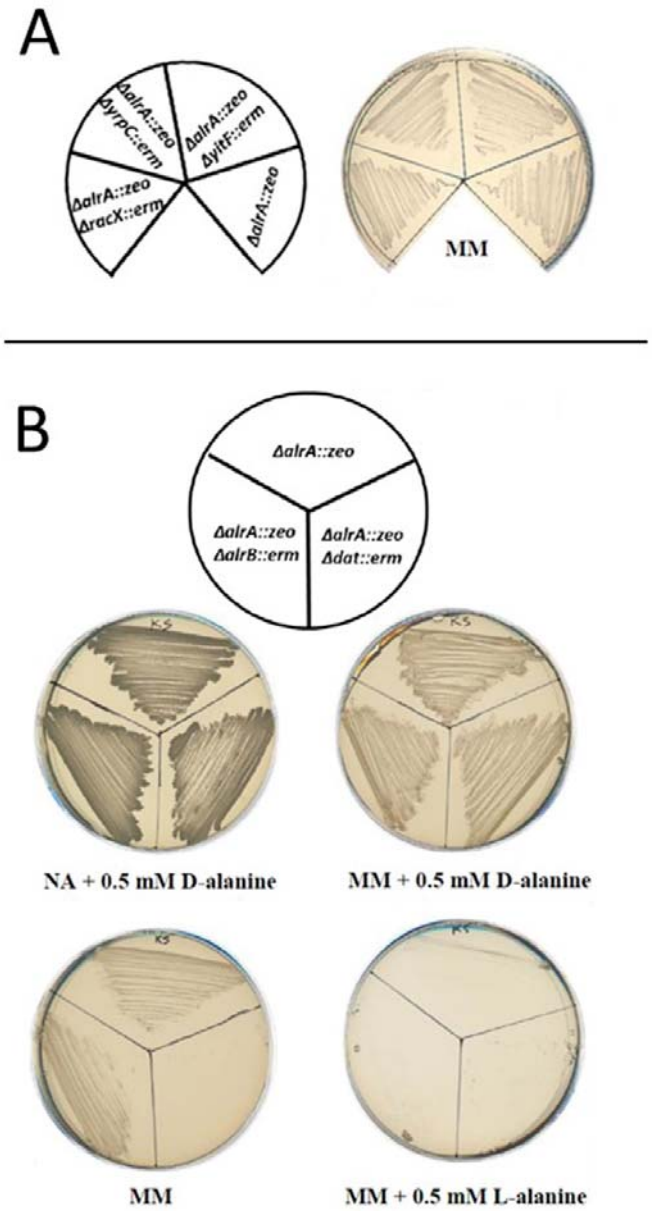
L-alanine
2 mM
1 mM
0.5 mM
H
alrA
I
alrA dat
OD 600 nm
time (min)

Fig. 3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MM
B
ΔalrA::zeo
ΔalrA::zeo
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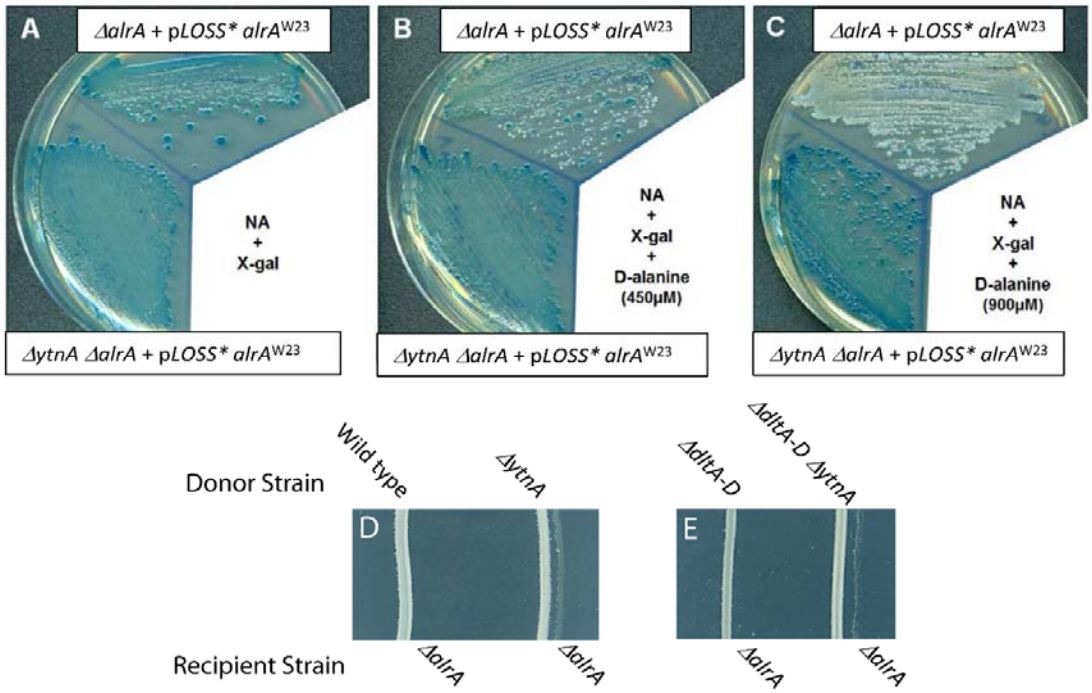
MM + 0.5 mM D-alanine
MM
MM + 0.5 mM L-alanine

Fig 4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+
X-gal
ΔytnA ΔalrA + pLOSS* alrA^W23
B
ΔalrA + pLOSS* alrA^W23
NA
+
X-gal
+
D-alanine
(450μM)
ΔytnA ΔalrA + pLOSS* alrA^W23
C
ΔalrA + pLOSS* alrA^W23
NA
+
X-gal
+
D-alanine
(900μM)
ΔytnA ΔalrA + pLOSS* alrA^W23
Donor Strain
Wild type
ΔytnA
ΔdltA-D
ΔdltA-D ΔytnA
D
E
Recipient Strain
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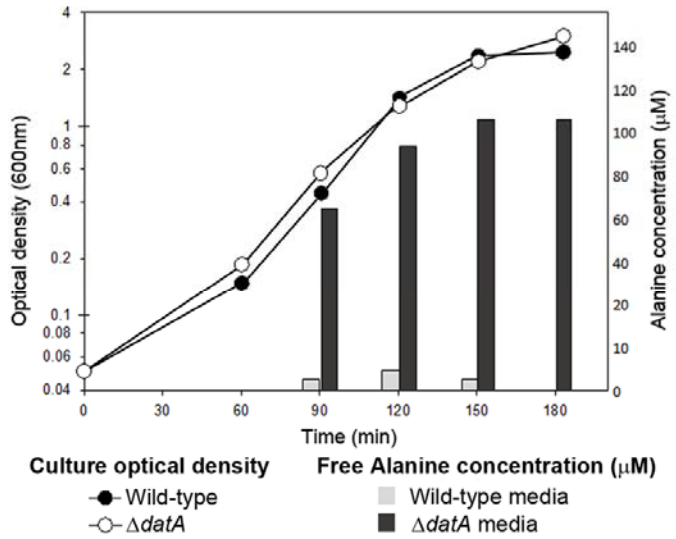
ΔalrA
ΔalrA
ΔalrA

Fig. 5

A

Detection of extracellular alanine during growth



B

Depletion of extracellular D- or L-alanine during growth

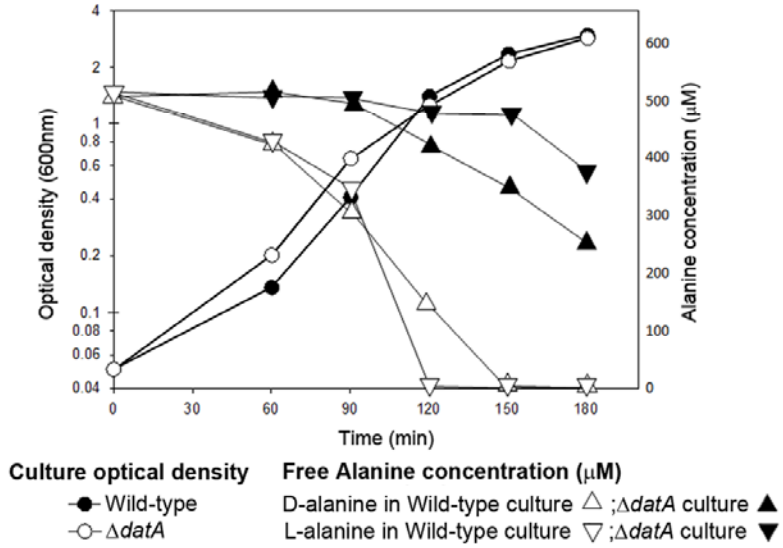


Fig 6.

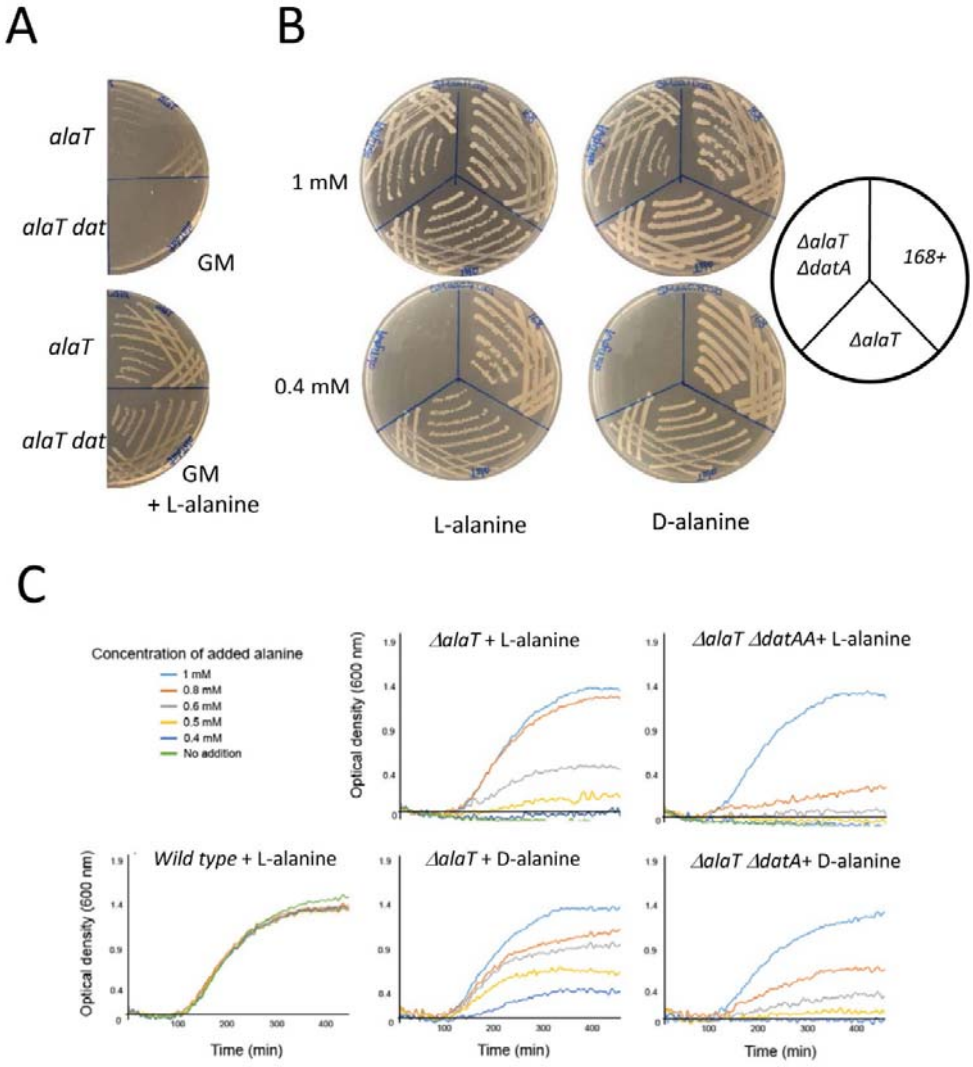

A
B
alaT
alaT dat
GM
alaT
alaT dat
GM
+ L-alanine
1 mM
0.4 mM
ΔalaT
ΔdatA
168+
ΔalaT
L-alanine
D-alanine
C
Concentration of added alanine
1 mM
0.8 mM
0.6 mM
0.5 mM
0.4 mM
No addition
ΔalaT + L-alanine
ΔalaT ΔdatAA+ L-alanine
Wild type + L-alanine
ΔalaT + D-alanine
ΔalaT ΔdatA+ D-alanine
Optical density (600 nm)
Time (min)

Fig. S1. Standard curve for D-alanine quantification in LB media

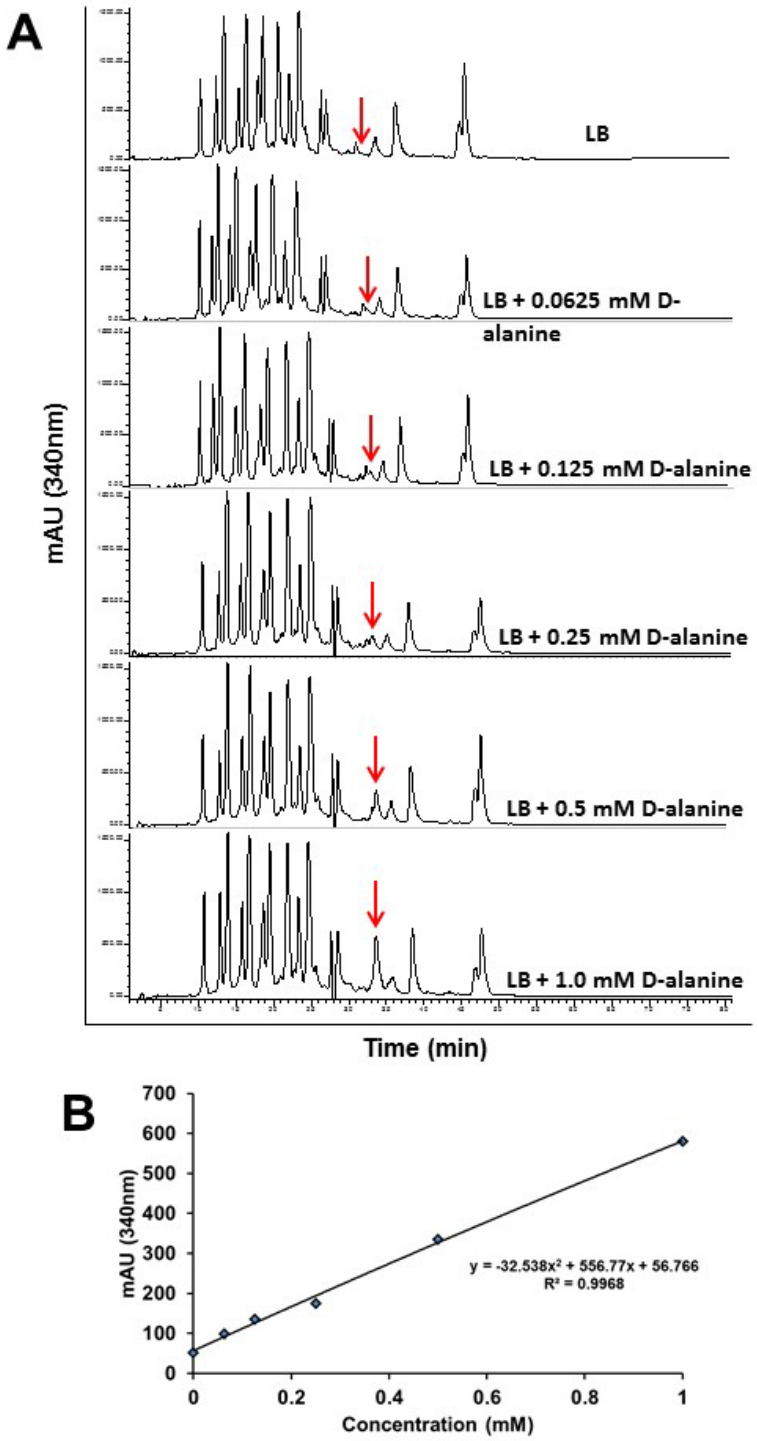


Fig S2

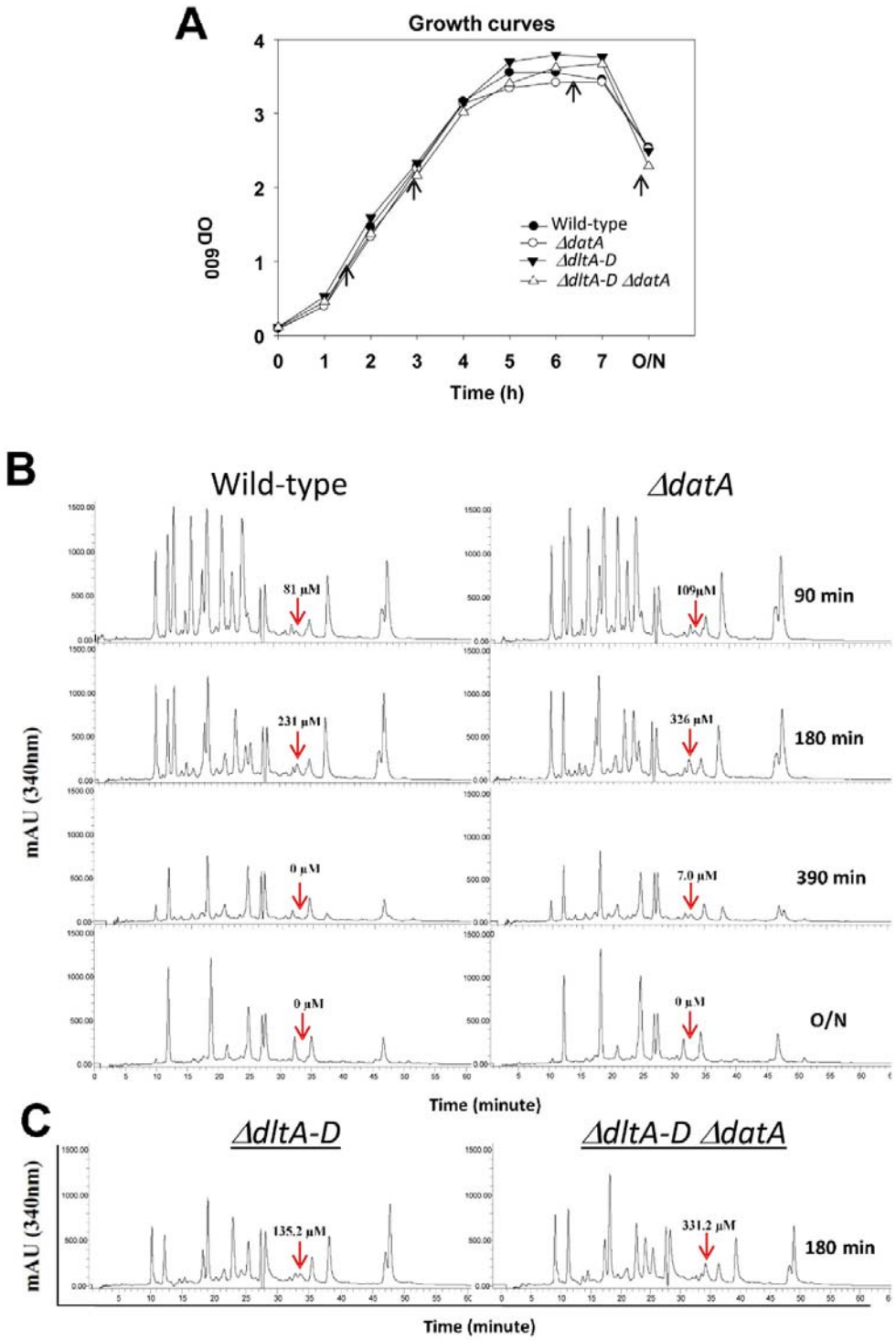
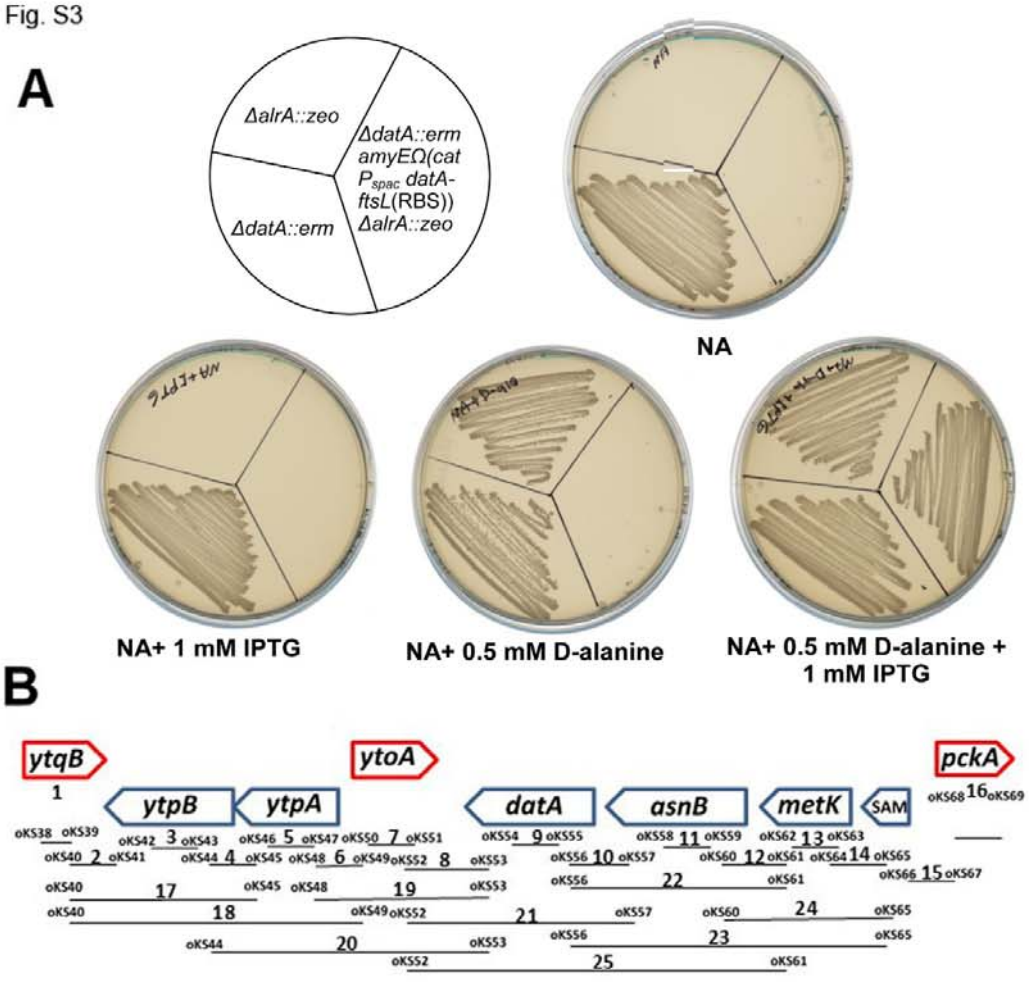

A
Growth curves
OD 600
Time (h)
Wild-type
ΔdatA
ΔdltA-D
ΔdltA-D ΔdatA
B
Wild-type
ΔdatA
mAU (340nm)
81 µM
109µM
90 min
231 µM
326 µM
180 min
0 µM
7.0 µM
390 min
0 µM
0 µM
O/N
Time (minute)
C
ΔdltA-D
ΔdltA-D ΔdatA
mAU (340nm)
135.2 µM
331.2 µM
180 min
Time (minute)

Fig. S3


A
ΔalrA::zeo
ΔdatA::erm amyEΩ(cat Pspac datA-ftsL(RBS)) ΔalrA::zeo
ΔdatA::erm
NA
NA+ 1 mM IPTG
NA+ 0.5 mM D-alanine
NA+ 0.5 mM D-alanine + 1 mM IPTG
B
ytqB
ytoA
pckA
ytpB
ytpA
datA
asnB
metK
SAM
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 M
Kb
1.5
1
0.5
0.1
5
4
3
2
1.5
1
0.5
D
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 M
Kb
1.5
1
0.5
0.1
5
4
3
2
1.5
1
0.5

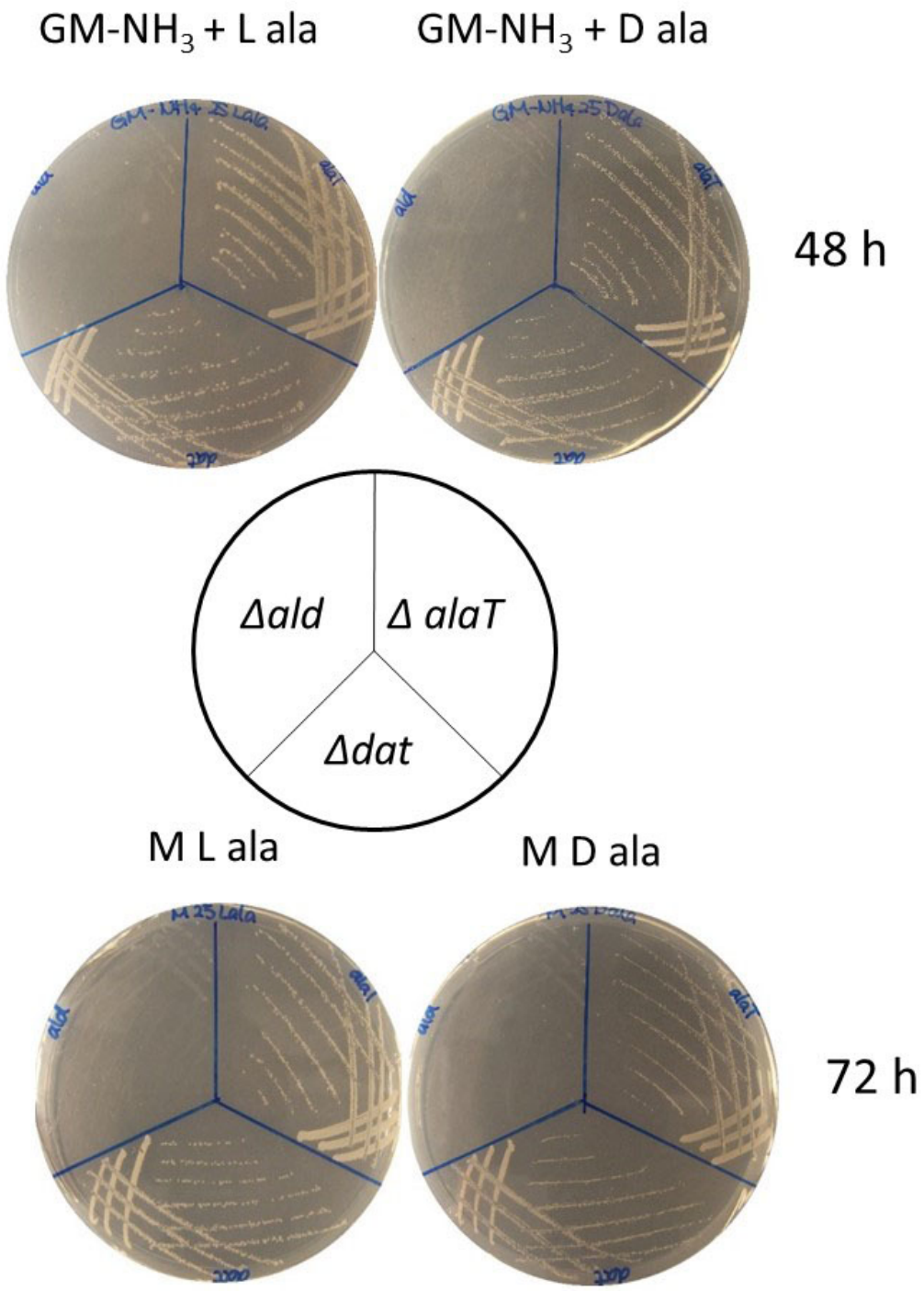



Fig S4