Genes involved in *meso*-diaminopimelate synthesis in *Bacillus subtilis*: identification of the gene encoding aspartokinase I

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Thermosensitive mutants of *Bacillus subtilis* deficient in peptidoglycan synthesis were screened for mutations in the *meso*-diaminopimelate (LD-A₂pm) metabolic pathway. Mutations in two out of five relevant linkage groups, *lssB* and *lssD*, were shown to induce, at the restrictive temperature, a deficiency in LD-A₂pm synthesis and accumulation of UDP-MurNAc-dipeptide. Group *lssB* is heterogeneous; it encompasses mutations that confer deficiency in the deacylation of *N*-acetyl-LL-A₂pm and accumulation of this precursor. Accordingly, these mutations are assigned to the previously identified locus *dapE*. Mutations in linkage group *lssD* entail a thermosensitive aspartokinase I. Therefore, they are most likely to affect the structural gene of this enzyme, which we propose to designate *dapG*. Mutation *pyc-1476*, previously reported to affect the pyruvate carboxylase, was shown to confer a deficiency in aspartokinase I, not in the carboxylase, and to belong to the *dapG* locus. *dapG* is closely linked to *spoVF*, the putative gene of dipicolinate synthase. In conclusion, mutations affecting only two out of eight steps known to be involved in LD-A₂pm synthesis were uncovered in a large collection of thermosensitive mutants obtained by indirect selection. We propose that this surprisingly restricted distribution of the thermosensitive *dap* mutations isolated so far is due to the existence, in each step of the pathway, of isoenzymes encoded by separate genes. The biological role of different aspartokinases was investigated with mutants deficient in *dapE* and *dapG* genes. Growth characteristics of these mutants in the presence of various combinations of aspartate family amino acids allow a reassessment of a metabolic channel hypothesis, i.e. the proposed existence of multienzyme complexes, each specific for a given end product.

Introduction

Biosynthetic pathways of the aspartate family amino acids supply metabolites for two essential processes in bacteria: L-methionine, L-threonine, L-isoleucine and L-lysine are required for protein synthesis, whereas *meso*-diaminopimelate (LD-A₂pm) is an essential constituent of peptidoglycan. In spore-forming bacilli, this metabolic pathway has an even more important role since it encompasses not only the synthesis of both LD-A₂pm and dipicolinate, two essential constituents of the spore, but also that of antibiotics such as colistin in *Bacillus colistinus* (Ito *et al.*, 1969) and β -lactam antibiotics

(Higgins & Kastner, 1971; Mendelovitz & Aharonowitz, 1982). In *Bacillus subtilis*, genetic characterization of the LD-A₂pm pathway cannot be approached through isolation of auxotrophic mutants since available strains are not endowed with an active LD-A₂pm uptake system. Therefore, mutants with deficient enzymes in this pathway were searched for among stable L-forms (Ward, 1975), or conditional lethal, thermosensitive mutants (Buxton, 1978; Buxton & Ward, 1980). The latter approach resulted in the identification of only two classes of mutations; the first one was shown to affect *dapE*, a gene involved in deacylation of *N*-acetyl-LL-A₂pm, whereas the second one was mistakenly (see below) assigned to *pycA*, the structural gene of pyruvate carboxylase. Mutants blocked in LD-A₂pm synthesis offer a unique system to study *in vivo* the biological role of key enzymes involved in the pathway. Moreover, a somewhat different approach to the study of the biological role of aspartokinase I (AKI) was recently reported by Zhang & Paulus (1990). They obtained a mutant, possessing an AKI apparently no longer subject

Abbreviations: LD-A₂pm, *meso*-diaminopimelate; AKI, AKII and AKIII, aspartokinase I, II and III; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ND, nephelometric density; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramate.

Dedication: This contribution is dedicated to the late Howard J. Rogers. He will always be remembered for the enthusiasm and unfailing curiosity that kept him busy at the bench until surprised by death, and made him such a stimulating colleague.

to feedback inhibition by LD-A₂pm, likely to be affected in the AKI gene.

Previously, a large collection of thermosensitive mutants of *B. subtilis*, obtained by indirect selection, had been screened for those lysing at the restrictive temperature. Five linkage groups were identified, *lssA* to *D* and *lssF*, which encompass genes involved in peptidoglycan synthesis (Brandt & Karamata, 1987). When cultures of mutants affected in these genes are shifted to the non-permissive temperature, they undergo after a short delay a more or less massive lysis. In the present work, mutations belonging to two of the five *lss* linkage groups are shown to specifically affect the synthesis of LD-A₂pm. *lssB* is involved in *N*-acetyl-LL-A₂pm deacylation, whereas *lssD* is probably the structural gene of aspartokinase I. The existence of a mutant deficient in AKI allowed us to examine the biological role of this enzyme.

Methods

Strains. The bacterial strains used are listed in Table 1.

Media and growth conditions. LA, SA, SATT, SAT2T, TS and S media were described by Karamata & Gross (1970). MS was according to Sargent (1973), and sporulation medium according to Schaeffer *et al.* (1965). Solid media were supplemented with 1.5% (w/v) agar (Difco) or 1.2% (w/v) agar (Gibco). Unless otherwise stated, amino acids (20 µg ml⁻¹) and bases (50 µg ml⁻¹) were added when required.

Liquid cultures were aerated by bubbling, and the cell mass followed by nephelometric density (ND) measured on a Unigalvo (Corning EEL) nephelometer. For an exponentially growing culture in SA medium, a ND reading of 100 corresponds to 0.11 mg ml⁻¹ dry weight (about 6×10^7 bacteria ml⁻¹). Cultures were started by inoculating a freshly grown colony. They were incubated overnight at room temperature and further diluted while still in the exponential growth phase. For cultures in MS medium, overnight incubation was at 30 °C and the medium supplemented with 0.001% casein hydrolysate (Sargent, 1973).

Transformation. Transformation procedures and media were as described by Karamata & Gross (1970). Inheritance of the *spoVF1* marker was tested on prototrophic and thermoresistant recombinants purified by streaking on appropriately supplemented TS plates and incubation at 47 °C. The media for selection and purification of Ts⁺ recombinants contained all amino acids derived from L-aspartate (L-lysine, L-methionine, L-threonine and L-isoleucine), whose presence was shown to be necessary for a clear-cut expression of the thermosensitive phenotype by recipient *dapG* strains (Fig. 1*b* and Discussion). Purified colonies were transferred on Schaeffer sporulation medium (Schaeffer *et al.*, 1965) with or without dipicolinate (100 µg ml⁻¹). After 48 h incubation at 30 °C, spores were replicated on LA plates and screened for thermosensitivity/resistance by 16–18 h incubation at 80 °C, followed by 24–36 h incubation at 30 °C.

Extraction and analysis of peptidoglycan nucleotide precursors. Cultures (200 ml) in SATT medium supplemented with *N*-acetylglucosamine (GlcNAc) (75 µM) were maintained in the exponential growth phase at 30 °C for at least five generations. They were shifted to 45 °C at an ND between 70 and 100. To obtain a short transition period

between permissive and restrictive temperature (about 1 min), flasks containing the cultures were briefly dipped into a waterbath at 70 °C and then transferred to 45 °C. After 13 min further incubation, bacteria were harvested on a Whatman filter (GF/F, diameter 45 mm), washed for less than 2 min with ice-cold water, and extracted from the filter into 10% (w/v) trichloroacetic acid (TCA) at room temperature. After ether extraction of TCA, the aqueous extract was neutralized with NaOH and concentrated by evaporation under vacuum. *N*-Acetyl-D-[1-¹⁴C]glucosamine (0.12 µCi ml⁻¹; 4.44 kBq ml⁻¹) was added to the culture 12 min before the temperature shift. Under the growth conditions used here, labelling of the hexosamine-containing soluble precursors can be considered as similar to continuous labelling, since (i) the global intracellular amount of precursors is equivalent to the cell wall mass synthesized during about only 0.1 generation time (unpublished observations) and (ii) presence of 75 µM-GlcNAc in the growth medium almost completely inhibits endogenous GlcNAc synthesis (Bates & Pasternak, 1965). Indeed, our experiments (results not presented) show that the specific activity in extracted nucleotide pools amounts to at least 80% of that of [¹⁴C]GlcNAc present in the medium.

The nucleotide precursors in the extracts were first partially purified on a Sephadex G25 column (1.5 × 90 cm) (Mengin-Lecreux *et al.*, 1982). Elution with deionized water was at a flow rate of 0.2–0.5 ml min⁻¹. Hexosamine-containing nucleotides, excluded from the gel by a charge effect, were recovered immediately after the void volume in two peaks (a rather wide one followed by a small one), which were pooled and concentrated by evaporation under vacuum. These pools were adjusted to 10 mM-HCl (5 ml) and chromatographed according to Good & Tipper (1972) on a Dowex-1 × 2 (200–400 mesh) column (0.6 × 5.5 cm), equilibrated with 10 mM-HCl. They were eluted with a 100 ml linear gradient of NaCl (0–0.6 M in 10 mM-HCl) at a rate of 22 ml h⁻¹. Nucleotide precursor peaks were detected by UV absorption and radioactivity. The identity of UDP was confirmed by its UV absorption spectrum. Fractions corresponding to each main peak were pooled and desalted on a Sephadex G25 column. UDP-MurNAc-tripeptide was well separated in the first elution peak, which was followed by the UDP-MurNAc-pentapeptide peak, and by a rather wide peak containing UDP-GlcNAc, UDP-MurNAc and UDP-MurNAc-L-alanine. UDP-MurNAc-dipeptide, the last precursor eluted, was poorly separated from the preceding one. These nucleotide preparations were hydrolysed in 4.16 M-HCl under vacuum in nitrogen-flushed ampoules (8 h at 105 °C). Under these conditions, the peptide moiety of the precursors is fully hydrolysed without excessive degradation of hexosamines, allowing simultaneous estimation of amino acids and hexosamines on a Beckman Multichrome B amino acid analyser using ninhydrin detection. Hydrolysis of GlcNAc and MurNAc standards gave glucosamine and muramic acid yields of 90% and 87%, respectively; these figures were used to correct values obtained for our samples.

Analysis of LD-A₂pm soluble precursor pools. Culture conditions, labelling by L-[¹⁴C]aspartate, and precursor extraction (from one-litre cultures) were as described for preparation of the substrate for the *N*-acetyl-LL-A₂pm deacylase assay (see below). Precursor-containing solutions were lyophilized. Adequate samples in H₂O were loaded on Whatman 3MM paper and chromatographed, according to Ruhland *et al.* (1955). The chromatograms were analysed by ninhydrin staining in acid conditions. The spots of LD-A₂pm, LL-A₂pm and *N*-acetyl-LL-A₂pm were identified on a chromatogram obtained with the standard compounds. For a quantitative estimation of the labelled compounds, the spots were cut out, eluted with 1 ml water, and the radioactivity was measured by liquid scintillation. To ascertain the identity of the *N*-acetyl-LL-A₂pm, the spot was cut out, eluted with water and hydrolysed (6 M-HCl, 105 °C, 2 h); the hydrolysis product was chromatographed again and identified as LL-A₂pm.

Table 1. Strains of *B. subtilis* 168 and *B. licheniformis*

Strain	Genotype or phenotype	Source, reference or construction
<i>B. subtilis</i>		
168	<i>trpC2</i>	BGSC*; Balassa <i>et al.</i> (1979); <i>spoVF1</i> was formerly designated <i>dap-1</i> (Piggot & Hoch, 1985)
1S83	<i>pheA1 spoVF1 trpC2</i>	Dubnau <i>et al.</i> (1967)
BD96	<i>furA1</i>	Strain PB5033 of Gianni & Galizzi (1986)†
JH879	<i>pyrA5 metC3 pycA19</i>	C. Anagnostopoulos, INRA, Jouy-en-Josas, France
GSY403	<i>pheA3‡ trpC2</i>	Karamata & Gross (1970)
E10	<i>trpC2 thyA1 thyB1 gtaC xtl-2</i>	Mauël & Karamata (1984)
L1440	Prototroph	Brandt & Karamata (1987)
L5025	<i>purA16 leuA8 ilvA1 lys-31</i>	Brandt & Karamata (1987)
L3890	<i>purA16 metC3 ilvA1</i>	
L2010	<i>trpC2 thyA1 thyB1 gtaC2 xtl-2 dapE10</i>	Brandt & Karamata (1987); <i>dapE10</i> was previously designated <i>lssB10</i>
L2012	<i>trpC2 thyA1 thyB1 gtaC2 xtl-2 lssB12</i>	Brandt & Karamata (1987)
L2023	<i>trpC2 thyA1 thyB1 gtaC2 xtl-2 dapE23</i>	Brandt & Karamata (1987); <i>dapE23</i> was previously designated <i>lssB23</i>
L2028	<i>trpC2 thyA1 thyB1 gtaC2 xtl-2 dapG28</i>	Brandt & Karamata (1987); <i>dapG28</i> was previously designated <i>lssD28</i>
L2031	<i>trpC2 thyA1 thyB1 gtaC2 xtl-2 lssB31</i>	Brandt & Karamata (1987)
L2100	<i>leuA8 ilvA1 lys-31</i>	Brandt & Karamata (1987)
L2123	<i>leuA8 ilvA1 lys-31 dapE23</i>	Brandt & Karamata (1987); <i>dapE23</i> was previously designated <i>lssB23</i>
L2127	<i>leuA8 ilvA1 lys-31 dapG27</i>	Brandt & Karamata (1987); <i>dapG27</i> was previously designated <i>lssD27</i>
L2223	<i>leuA8 ilvA1 dapE23</i>	L3890 → L2123§
L2227	<i>leuA8 ilvA1 dapG27</i>	L3890 → L2127§
L2128	<i>leuA8 ilvA1 lys-31 dapG28</i>	L2028 → L5025¶
L2328	<i>leuA8 furA1 dapG28</i>	BD96 → L2128¶
L2327	<i>pheA3 ilvA1 lys-31 dapG27</i>	GSY403 → L2127¶
MS320	<i>trpC2 thyA1 thyB1 dapE320</i>	Buxton & Ward (1980)
RB1950	<i>purA16 metB5 ilvA1 dapG1476</i>	Buxton (1978); <i>dapG1476</i> was previously designated <i>pyc</i>
<i>B. licheniformis</i>		
NCTC 6346	His ⁻	Ward (1974)

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† Genotype *pyr metC3 pycA* of strain PB5033, constructed by J. Hoch, was reported by Gianni & Galizzi (1986). Precise designation of the *pycA* mutation is from J. Hoch (personal communication).

‡ *pheA1* and *pheA3* are different mutations (C. Anagnostopoulos, personal communication), but both were called in the past *pheA1*. λ *phe*⁺ (Mauël *et al.*, 1989) corrects *pheA3*, but, according to the restriction map of Ferrari *et al.* (1982), it should not correct *pheA1* (P. Hauser, personal communication).

§ Lys⁺ recombinant was obtained by transformation with non-saturating concentration of DNA. The arrow points from donor to recipient.

¶ Construction by conjugation with saturating concentrations of DNA. The arrow points from donor to recipient.

Apartokinase (AK; EC 2.7.2.4) assay. Cell extracts were obtained essentially according to Ward (1975). One-litre cultures were grown in MS medium supplemented with required amino acids (40 μ g ml⁻¹) and bases (100 μ g ml⁻¹), as well as with 0.001% casein hydrolysate. Even in the absence of any specific amino acid requirement, MS medium was supplemented with 20 μ g ml⁻¹ each of L-lysine, L-methionine, L-threonine and L-isoleucine. After at least two generations at 30 °C, cells were harvested by centrifugation and the pellet resuspended in 25 ml 20 mM-potassium phosphate, pH 7.5. Cells were recentrifuged, resuspended in 3 ml of the same buffer and sonicated 16 × 10 s. The sonicate was mixed with 9 ml of a solution of saturated ammonium sulphate and 0.1 mM-EDTA at pH 7.7. After 1 h, the solution was centrifuged (20 min at 39100 g), the pellet resuspended in a solution (50 mM-diethanolamine.HCl, 1 mM- β -mercaptoethanol, 1 mM-EDTA) at pH 8.5 and dialysed for 4 h at 4 °C against the same solution. The extract was stored below -20 °C and its protein content determined by the Lowry method.

AK activity was measured according to Paulus & Gray (1967). Formation of β -L-aspartyl phosphate, subsequently converted into β -L-

aspartyl hydroxamate, was followed by colorimetry: 1 μ mol of β -L-aspartyl phosphate ml⁻¹ is equivalent to an A_{520} of 0.4. The reaction mixture contained: 11.1 mM-ATP, 11.1 mM-creatine phosphate, 2.47 U ml⁻¹ creatine kinase (EC 2.7.3.2), 11.1 mM-MgCl₂, 11.1 mM-L-aspartate, and 0.88 mM-NH₂OH.KCl, pH 8. The reaction was initiated by enzyme addition. For initial experiments, the total volume of the reaction mixture was 0.45 ml, with 0.5 mg protein of the enzyme extract, and the reaction was stopped after 30 min by addition of 0.25 ml 50% TCA and 0.1 ml 2 M-FeCl₃. For the following experiments, the volume of the reaction mixture was 0.21 ml with 0.42 mg protein of the enzyme extract, and the reaction was terminated by addition of TCA and FeCl₃ as before. Background was measured on an incubation mixture where the substrate, L-aspartate, was added after TCA and FeCl₃. The activity associated with AKI was obtained as the difference of activities measured with and without 2.22 mM-LD- A_2pm , a specific inhibitor of AKI (Rosner & Paulus, 1971; Zhang *et al.*, 1990).

N-Acetyl-L-L- A_2pm deacylase (EC 3.5.1.47) assay. Cell extracts were obtained from cultures grown in SAT2T. After five generations at

30 °C, cells were harvested by centrifugation at a ND of 90 (0.1 mg dry weight ml⁻¹). The pellet was resuspended in 4 ml buffer (150 mM-Tris, 0.75 mM-MnCl₂, 0.5 mM-pyridoxal phosphate, pH 7.5) and sonicated 24 × 5 s with 25 s intervals between successive sonications. After removal of debris (39100 g, 20 min, at 4 °C), the supernatant was immediately used as a crude extract. The protein content of the latter was determined by the biuret method (Layne, 1957).

The enzyme substrate was obtained from mutant MS320, which accumulates *N*-acetyl-LL-A₂pm at 45 °C (Buxton & Ward, 1980), grown in five one-litre batches of appropriately supplemented MS medium. After incubation at 30 °C to a ND of 80 (0.085 mg dry weight ml⁻¹), the culture was shifted to 45 °C, and 8 min after transfer L-[¹⁴C]aspartate (1 nCi ml⁻¹) was added to the culture, which was centrifuged 52 min later (5 min at 13700 g). Cells harvested by centrifugation were resuspended in 100 ml H₂O, heated for 5 min at 100 °C, and recentrifuged for 5 min at 4 °C. The supernatant was kept and the pellet re-extracted in the same way. Pooled supernatants were partly purified according to Ward (1975), i.e. they were chromatographed on a Dowex 50 (H⁺) column (1.5 × 10 cm) equilibrated with 0.01 M-HCl. Elution (60 ml h⁻¹) with a 400 ml linear gradient of HCl (0.01–2 M) yielded three radioactive peaks. Presence of *N*-acetyl-LL-A₂pm in the major peak (71% of the radioactivity loaded on the column) was confirmed by paper chromatography after hydrolysis (6 M-HCl, 2 h at 105 °C) and removal of HCl under vacuum; the ninhydrin-staining and radioactivity-containing spot was indistinguishable from that containing authentic LL-A₂pm (Gilvarg, 1959). The specific activity of *N*-acetyl-LL-A₂pm was estimated by colorimetric assay of diaminopimelate after acid hydrolysis (Work, 1971).

N-acetyl-LL-A₂pm deacylase was assayed by colorimetry (Sundhara-das & Gilvarg, 1967). The assay mixture (32.5 µl), buffered by Tris/HCl (28.5 mM, pH 7.5), contained 325 nmol *N*-acetyl-LL-A₂pm and crude enzyme extract (200–300 µg protein). After 20 min incubation at 37 °C, the reaction was stopped by addition of 17.5 µl of 6 M-HCl; 100 µl ninhydrin (5%, w/v, in methyl cellosolve) was then added and the tube was incubated at 100 °C for 5 min. After cooling under running water, 1.1 ml propanol was added and the A₄₂₀ recorded. Background was estimated on a mixture to which HCl was added before the enzyme extract. In these conditions, an A₄₂₀ increase of 0.005 corresponds to the conversion of 1 nmol *N*-acetyl-LL-A₂pm into LL-A₂pm.

Pyruvate carboxylase (EC 6.4.1.1) assay. Crude extracts, prepared like those for the AK assay (see above), were obtained from cells harvested in the late exponential growth phase (ND about 200). Pyruvate carboxylase activity was measured by transfer of radioactivity from labelled CO₂ (Diesterhaft & Freese, 1973). Labelled oxaloacetate, produced by pyruvate carboxylase, was converted into L-aspartate through coupling with aspartate aminotransferase (Fisher & Magasanik, 1984). The reaction medium contained: 25 mM-Tris pH 7.5, 10 mM-MgCl₂, 5 mM-NaH¹⁴CO₃ (0.1 µCi ml⁻¹; 3.7 kBq ml⁻¹), 5 mM-sodium pyruvate, 0.12 mM-acetyl-CoA, 5 mM-ATP, 10 mM-sodium glutamate, 20 mM-pyridoxal phosphate, and 2 U ml⁻¹ aspartate aminotransferase (EC 2.6.1.1). The reaction was initiated by addition of the enzyme extract and, 30 min later, stopped by addition of 2 M-HCl (half the volume of the reaction mixture). Samples (100 µl) were dried for 3 h at 80 °C, resuspended in 1 ml H₂O and their radioactivity measured by liquid scintillation counting. Volumes of the reaction mixtures were either 200 or 80 µl, with the addition of enzyme extracts containing 200 or 50 µg protein, respectively.

Control experiments revealed that absence of acetyl-CoA totally abolished the observed enzyme activity, in agreement with Diesterhaft & Freese (1973). To confirm that pyruvate carboxylase activity was actually measured, we have shown that radioactivity incorporation was also totally abolished when the substrate, pyruvate, was omitted, or when the enzyme extract (50 µg of protein) was incubated for 5 min with 16.7 µg avidin just before the assay.

Results

Preliminary screening of *lss* mutants

To identify mutants deficient in LD-A₂pm synthesis, we screened our collection of thermosensitive lysis mutants, *lss*(Ts), deficient in peptidoglycan synthesis at the non-permissive temperature. Shifting the latter strains from 30 °C to 45 °C results in a limited nephelometric density increase followed, at around 20 min, by a more or less massive lysis (Brandt & Karamata, 1987). We exploited the well-known phenomenon that lysis induced by interference with peptidoglycan synthesis can be suppressed by simultaneous inhibition of protein synthesis (Buxton, 1978; Kirby & Burnell, 1954; Meynell & Meynell, 1970). It is generally accepted that mutants affected in the synthesis of LD-A₂pm are intrinsically also deficient in the formation of L-lysine or, depending on the exact site of the enzymic block (Fig. 2), of L-lysine and other amino acids belonging to the aspartate family. Therefore, the thermosensitive lysis phenotype of mutants deficient in LD-A₂pm synthesis should not be expressed unless the growth medium contains all amino acids whose synthesis has been affected by their *dap* mutations. Supplementation with LD-A₂pm is ineffective since *B. subtilis* cells lack an active LD-A₂pm uptake system (Ward, 1975; Buxton, 1978).

Cultures of *lys*⁺ derivatives harbouring *lss* mutations, grown in minimal media with or without L-lysine, were shifted from 30 °C to the non-permissive temperature, and their nephelometric density was followed. Only thermosensitive lysis strains belonging to linkage groups *lssB* and *lssD* were affected by the medium composition (Figs. 1*a*, *b*), revealing a deficiency in LD-A₂pm synthesis. However, strains belonging to linkage group *lssB* behaved differently from those belonging to group *lssD* when the growth medium was supplemented with various combinations of aspartate family amino acids. Lysis of L2223 (*lssB23*) was abolished in absence of L-lysine (Fig. 1*a*); supplementation with L-methionine and L-threonine as well as L-lysine did not influence the lysis kinetics observed in presence of L-lysine alone. Surprisingly, in the absence of L-lysine, growth of strain L2227 (*lssD27*) continued at 45 °C, but more slowly than that of the parent strain (Fig. 1*b*). Although supplementation of the medium with L-lysine stopped growth, the cultures did not show a clear lysis. The latter appeared only in medium supplemented with L-lysine as well as L-threonine and L-methionine. These observations suggest that *lssB* and *lssD* loci concern enzymes located down- and upstream from L-aspartate-β-semialdehyde, respectively (Fig. 2).

Absence of L-lysine almost completely abolished the lysis of mutant L2223 (*lssB23*) at 45 °C (Fig. 1*a*). This can

Fig. 1. Growth and lysis kinetics of mutants L2223 (*lssB23*) and L2227 (*lssD27*) at the non-permissive temperature in the presence of various combinations of aspartate family amino acids. Cultures, grown exponentially for four to five generations at 30 °C in appropriately supplemented MS medium, were shifted to 45 °C and the nephelometric density was followed. The time of shift is indicated by an arrow. Media were always supplemented by 40 $\mu\text{g ml}^{-1}$ of L-leucine and L-isoleucine, which are required by all strains used. (a) Strain L2223 (*lssB23*) in MS medium with (○) or without (●) 20 $\mu\text{g L-lysine ml}^{-1}$. (b) Strain L2227 (*lssD27*) in MS medium without L-lysine (■), with 20 $\mu\text{g L-lysine ml}^{-1}$ (●), or with 20 $\mu\text{g ml}^{-1}$ each of L-lysine, L-methionine and L-threonine (○). (c) strain L2227 (*lssD27*) in MS medium supplemented with 20 $\mu\text{g L-lysine ml}^{-1}$ with (○) or without (●) 20 $\mu\text{g ml}^{-1}$ each of L-methionine and L-threonine, and in MS medium supplemented with 20 $\mu\text{g L-lysine ml}^{-1}$ and 200 $\mu\text{g ml}^{-1}$ of a mixture of D- and L-aspartate with (□) or without (■) 20 $\mu\text{g ml}^{-1}$ each of L-methionine and L-threonine.

Table 2. Pools of peptidoglycan nucleotide precursors in mutant and wild-type strains at non-permissive temperature

Labelled nucleotide precursors were extracted from cultures grown at 30 °C and shifted to 45 °C, as described in Methods. Following a preliminary purification step on Sephadex G25, and chromatography on a small Dowex-1 $\times$ 2 column, the nucleotide precursor content in major elution peaks was determined after analysis of amino acids and amino sugars. Minor peaks were quantified by their amount of radioactivity.

Precursor	Amount of precursor [nmol (mg dry wt) $^{-1}$] in strain		
	L2100 (Ts $^{+}$)	L2123 (<i>lssB23</i>)	L2127 (<i>lssD27</i>)
UDP-GlcNAc*	0.3	0.1	0.2
UDP-MurNAc	0.5	3.1	1.2
UDP-MurNAc-monopeptide			
UDP-MurNAc-dipeptide			
UDP-MurNAc-tripeptide			
UDP-MurNAc-pentapeptide	1.1	<0.1	<0.1
UDP-MurNAc-pentapeptide	1.1	<0.1	0.2
Total	3.0	9.8	6.9

* Includes UDP-GlcNAc and UDP-GlcNAc-enolpyruvate since both substances produce glucosamine upon acid hydrolysis.

be attributed to complete inhibition of protein synthesis due to L-lysine starvation, strongly suggesting that mutation *lssB23* totally abolishes the activity of the enzyme affected. The latter statement correlates well with the absence of the UDP-MurNAc-pentapeptide pool (Table 2) and of peptidoglycan synthesis, at the non-permissive temperature (Brandt & Karamata, 1987, and not presented). However, absence of L-threonine and L-methionine had a less drastic effect on the lysis kinetics

of L2227 (*lssD27*); the decrease in nephelometric density was only slowed down (Fig. 1b), but lysis did occur even in the presence of L-lysine only. This behaviour is likely to be due to a limited protein synthesis at 45 °C, and thus to some residual activity of the impaired function; this is in full agreement with both a reduced but significant UDP-MurNAc-pentapeptide pool (Table 2) and reduced peptidoglycan synthesis (Brandt & Karamata, 1987, and not presented) at high temperature.

Fig. 2. Biosynthesis of the amino acids belonging to the L-aspartate family in *B. subtilis* (Ward, 1975). Enzymic activities are indicated as follows: 1, pyruvate carboxylase (EC 6.4.1.1); 2, aspartate aminotransferase (EC 2.6.1.1); 3, aspartokinases I, II and III (EC 2.7.2.4); 4, aspartate-semialdehyde dehydrogenase (EC 1.2.1.11); 5, dihydrodipicolinate synthase (EC 4.2.1.52); 6, dihydrodipicolinate reductase (EC 1.3.1.26); 7, tetrahydrodipicolinate acetyltransferase; 8, acetyl-diaminopimelate aminotransferase; 9, acetyl-diaminopimelate deacetylase (EC 3.5.1.47); 10, diaminopimelate epimerase (EC 5.1.1.7); 11, dipicolinate synthase; 12, the pyruvate dehydrogenase complex; 13, diaminopimelate decarboxylase (EC 4.1.1.20); 14, homoserine dehydrogenase (EC 1.1.1.3); 15, homoserine kinase (EC 2.7.1.39); 16, threonine synthase (EC 4.2.99.2). Genetic markers so far identified in *B. subtilis* are indicated in *italics*, and their positions on the chromosome are estimated in degrees essentially according to Zeigler (1990). *lysR* is a new locus identified by mapping lysine auxotrophic mutations, which are associated with a deficiency in diaminopimelate decarboxylase activity (Shevtchenko *et al.*, 1988). With the exception of reactions in L-threonine and L-methionine pathways, cosubstrates and coproducts of all enzymic steps are specified.

Analysis of peptidoglycan and LD-A₂pm soluble precursor pools

Mutants deficient in LD-A₂pm synthesis are affected in peptidoglycan formation since absence of LD-A₂pm blocks peptidoglycan precursor synthesis at the level of UDP-MurNAc-dipeptide, which accumulates in the cytoplasm (Buxton & Ward, 1980; Ward, 1975). Thus, to confirm our preliminary evidence that *lssB* and *lssD* loci are involved in LD-A₂pm synthesis, we measured the

nucleotide precursor pools of peptidoglycan in relevant strains at the non-permissive temperature, as described in Methods. Cells growing exponentially at 30 °C were shifted to 45 °C; they were collected by filtration before the onset of lysis so as to minimize the loss of soluble metabolites. After TCA extraction and a partial purification step by gel filtration, the nucleotide precursors were fractionated on a Dowex-1 × 2 ion-exchange resin. Finally, amino acid and amino sugar analyses of fractions corresponding to different peaks allowed

Table 3. *N*-Acetyl-LL-A₂pm deacylase activity in mutant and wild-type strains

Crude extracts were prepared from cultures growing exponentially in SAT2T medium at 30 °C. The reaction mixture contained 200–300 µg protein from crude extracts and was always incubated for 20 min at 37 °C. Conversion of *N*-acetyl-LL-A₂pm into LL-A₂pm was followed by colorimetry.

Strain	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	Percentage relative to L2100
L2100	1836	100
MS320 (<i>dapE320</i>)	31	1.7
L2123 (<i>lssB23</i>)	281	15.3
L2127 (<i>lssD27</i>)	1441	78.5

quantification of different precursors. As expected, mutations *lssD27* and *lssB23* conferred accumulation of considerable amounts of UDP-MurNAc-dipeptide and very low levels, or even absence, of UDP-MurNAc-pentapeptide (Table 2), which is consistent with a defect in LD-A₂pm synthesis. Furthermore, accumulation of CDP-glycerol, a soluble precursor of poly(glycerol phosphate), has also been observed in *lssD27*- and *lssB23*-bearing strains at the non-permissive temperature (Pooley *et al.*, 1991). However, since excretion of UDP-linked compounds has been reported (Szabo *et al.*, 1989), the measured pools may well be underestimates of synthesized quantities of nucleotide precursors. Control experiments (not presented) revealed that peptidoglycan nucleotide precursor pools in strain L2100, the reference Ts⁺ strain, were not significantly affected by the temperature shift.

In the experiment shown, the reference strain had a surprisingly high level of UDP-MurNAc-dipeptide, a level comparable to that of UDP-MurNAc-pentapeptide (Table 2). However, in other experiments performed on the same strain, UDP-MurNAc-pentapeptide was found to be the most abundant precursor specific for peptidoglycan synthesis (not presented). Comparable results were observed in *Escherichia coli* (Mengin-Lecreux *et al.*, 1982, 1983) and *Staphylococcus aureus* (Good & Tipper, 1972).

For mutants belonging to group *lssB*, we narrowed the range of enzymes to be assayed by examining their soluble LD-A₂pm precursor pools. Cultures growing exponentially in MS medium were shifted from 30 °C to 45 °C, and 52 min later, hot-water cell extracts were prepared. Analysis of the extracts by paper chromatography revealed that group *lssB* is heterogeneous. First, two mutations, *lssB23* and *lssB10*, conferred accumulation of *N*-acetyl-LL-A₂pm; the observed pools were 17- and 11-fold higher, respectively, than that of the wild-type strain E10. This points to a deficiency in the *N*-

acetyl-LL-A₂pm deacylation, which is in excellent agreement with the very close genetic linkage between *lssB23* and *dapE320* (Brandt & Karamata, 1987), a previously described mutation which is responsible for a deficiency of the *N*-acetyl-LL-A₂pm deacylase (Buxton & Ward, 1980). Finally, mutants bearing the remaining mutations, *lssB12* and *B31*, did not accumulate any of the ninhydrin-staining LD-A₂pm precursors, in particular neither *N*-acetyl-LL-A₂pm nor LL-A₂pm. Their chromatograms were characterized by an increased ninhydrin staining spot which may correspond to glutamate.

Assay for activities of AK and *N*-acetyl-LL-A₂pm deacylase in cell extracts

Our results suggest that group *lssB* may comprise two loci, one of which being involved in the *N*-acetyl-LL-A₂pm deacylation, whereas the *lssD* locus seems to be involved in the first stages of LD-A₂pm synthesis.

Assays for *N*-acetyl-LL-A₂pm deacylase activity (Sundharadas & Gilvarg, 1967) were performed on crude extracts obtained from exponential-phase cultures grown at 30 °C (Table 3). The very low level of deacylating activity in strain L2123 (*lssB23*), in full agreement with the analyses of precursor pools, establishes that mutation *lssB23* confers a phenotype identical to that shown by the previously characterized mutation *dapE320* (Buxton & Ward, 1980). Accordingly *lssB10* and *B23* were renamed *dapE10* and *E23*. These assays have also revealed that *lssB12*- and *B31*-bearing mutants have wild-type levels of *N*-acetyl-LL-A₂pm deacylase (not presented). Incidentally, in our experiments, extracts of the wild-type strain (L2100) yielded specific deacylase activities four times higher than that measured by Buxton & Ward (1980).

The requirement for L-lysine for full expression of the thermosensitive phenotype in mutant L2227 (*lssD27*) (Fig. 1b) suggests a deficiency of AKI activity. Indeed, AKII, the other major AK activity in *B. subtilis* 168, is known to be both inhibited and repressed by lysine (Hampton *et al.*, 1971; Graves & Switzer, 1990; Zhang *et al.*, 1990). Thus, presence of L-lysine in the growth medium would prevent AKII from substituting for the deficient AKI activity at the restrictive temperature. In addition, *lssD*, mapping at 144° (Brandt & Karamata, 1987), is located in a different region of the *B. subtilis* chromosome than *aecA*, the AKII locus, situated at 253° (Petricek *et al.*, 1989; Chen *et al.*, 1989) and more recently renamed *lysC* (Lu *et al.*, 1991). This prompted us to assay the activity of AKI. Cultures of strain L2227 (*lssD27*) and L2328 (*lssD28*), growing exponentially at 30 °C in MS medium, were harvested and AK activities were determined in ammonium sulphate precipitates of crude cell extracts (Table 4). When assayed at 37 °C, the activity of AKI was completely abolished in these

Table 4. *AK activities in cell extracts*

Extracts were prepared from cultures growing exponentially at 30 °C in minimal medium supplemented with L-lysine, L-methionine, L-threonine and L-isoleucine. When required, bases were added at 100 µg ml⁻¹. Aspartokinase activities were assayed on ammonium sulphate precipitates after suspension of the pellet and dialysis. Unless otherwise stated the reaction mixture (0.21 ml) contained 0.42 mg protein from the cell extract. Incubation time was always 30 min.

Strain	Assay temp. (°C)	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		Total	AKI*
168†	25	9.5	5.6
	37	10.9	3.8
L1440 (wild-type)	25	2.8	2.2
	37	4.6	2.9
L2227 (<i>lssD27</i>)	25	2.4	0.2
	37	2.9	0
L2227 (<i>lssD27</i>)†	25	2.7	0.7
	37	0	0
L2328 (<i>lssD28</i>)	25	0.6	0.6
	37	0	0
RB1950 (<i>pyc-1476</i>)	25	0.4	0.4
	37	0.4	0
<i>B. licheniformis</i> NCTC 6346 (His ⁻)	30	10.8	5.6

* Difference between activities obtained with and without LD-A₂pm (2.2 mM) in the reaction mixture; addition of LD-A₂pm inhibits AKI activity (Rosner & Paulus, 1971).

† The reaction mixture (0.45 ml) contained 0.5 mg cell extract protein.

partially purified enzyme extracts. *In vitro*, even at 25 °C, the activity of this enzyme was very low, strongly suggesting that *lssD* is the structural gene of AKI. Thus, *lssD* was renamed *dapG*, in line with the nomenclature used in *E. coli* (Cohen & Saint-Girons, 1987). At 25 °C, total AK activities in extracts of strains L2227, L2328, and in particular RB1950 (see below), were very low. This apparently surprising observation is easily accounted for by (i) a very low level of AKI *in vitro* (see above), and (ii) a nearly complete repression of AKII and AKIII in the media used in our experiments (see Discussion). Finally, a control experiment performed on *Bacillus licheniformis* NCTC 6346 also revealed an LD-A₂pm-sensitive AK activity in this bacterium.

Reassessment of the enzymic activity affected by the *pyc-1476* mutation

Although mutation *pyc-1476* was reported to affect pyruvate carboxylase activity (Buxton, 1978), the phenotype of strain RB1950 (*pyc-1476*) was almost indistinguishable from that of strain L2227 (*dapG27*). Indeed, (i) both strains have a lysine-dependent temperature-

sensitive lysis phenotype, (ii) they accumulate UDP-MurNAc-dipeptide, suggesting a defect in LD-A₂pm synthesis, and (iii) their thermosensitive phenotype is partially corrected by including aspartate in the growth medium (Buxton, 1978; Fig. 1c). These observations, coupled to that of very close linkage between *pyc-1476* and *dapG27* (Brandt & Karamata, 1987), suggested the need for a reassessment of enzymic activities in strain RB1950. Assays performed with crude extracts of strain RB1950 (Table 4) revealed a deficiency in AKI activity, confirming that *pyc-1476* and *dapG27* affect the same gene. Thus, we propose to designate the former marker *dapG1476*.

Pyruvate carboxylase activity in extracts of strain RB1950 exhibits the same temperature-dependent profile as the wild-type strain L1440, and thus shows no sign of thermosensitivity. The apparently reduced level of pyruvate carboxylase activity in strain RB1950 (40% of that of the wild-type) may well be due to repression by L-aspartate present in the growth medium, a repression also observed with the wild-type strain. In addition, the observation that enzyme activity in mutants RB1950 and L2227 is approximately half that of the wild-type in the same growth media, could result from an increased L-aspartate pool due to a poorly functioning mutated enzymic activity even at the permissive temperature (Buxton, 1978, and results not shown). Although we cannot account for the apparent pyruvate carboxylase deficiency in strain RB1950 reported by Buxton (1978), it is noteworthy that the assays described were performed in the absence of acetyl-CoA which, according to Diesterhaft & Freese (1973) and to our observations (see Methods), is essential for pyruvate carboxylase activity in *B. subtilis*.

The reassignment of *pyc-1476* resolves an ambiguity in the localization of the *pycA* locus and clarifies the mapping in the *pyr* region (Buxton, 1978; Piggot & Hoch, 1985; Piggot, 1989; Zeigler, 1990). Indeed, mutation *dapG1476* (previously designated *pyc-1476*), located at 144° on the *B. subtilis* chromosome (Buxton, 1978; Brandt & Karamata, 1987), is not related to mutation *pycA19* (originally designated *aspA*), which does confer a deficiency in pyruvate carboxylase activity (Hoch & Mathews, 1972; Diesterhaft & Freese, 1973), and behaves like an aspartate requiring auxotroph at all temperatures. In addition, three-point PBS1 transduction crosses (results not presented) confirm the order *metC*, *pycA*, *pyrA* and place *pycA* at 127°, in agreement with Piggot & Hoch (1985) as well as with Mueller & Taber (1989).

Genetic linkage between *dapG* and *spoVF*

Inspection of the genetic map of *B. subtilis* suggests that the *dapG* locus maps in the same region as *dpa-1*, a

Table 5. Linkage of *spoVF* and *dapG* markers

Competent cells of strains L2327 (*dapG27*) and RB1950 (*dapG1476*) were transformed with indicated concentrations of donor DNA. Double recombinants, prototrophic and Ts⁺, obtained by pseudo-linkage, were either directly selected, or identified by screening of selected prototrophs. Cotransfer of the *spoVF* marker was determined as described in Methods.

Donor	DNA concn (ng ml ⁻¹)	Recipient	Selected marker(s)	No. of recombinants tested	Unselected marker	Percentage of co-transfer
1S83 (<i>spoVF1 pheA1</i>)	300	L2327 (<i>dapG27 pheA3 leuA8</i>)	<i>phe⁺ dap⁺⁺</i>	147	Spo ⁻	66
<i>λphe⁺</i>	25				Leu ⁺	9.5
GSY403 (<i>pheA3</i>)	300	L2327 (<i>dapG27 pheA3 leuA8</i>)	<i>phe⁺ dap⁺⁺</i>	150	Spo ⁻	0
<i>λphe⁺</i>	100				Leu ⁺	4.7
1S83 (<i>spoVF1 pur⁺</i>)	300	RB1950 (<i>dapG1476 purA16 metB5</i>)	<i>pur⁺ dap⁺⁺</i>	30	Spo ⁻	50
			<i>pur⁺</i>	300	Met ⁺	5.3
L1440	300	RB1950 (<i>dapG1476 purA16 metB5</i>)	<i>pur⁺ dap⁺⁺</i>	24	Spo ⁻	0
			<i>pur⁺</i>	300	Met ⁺	9.7

* Direct selection for inheritance of both markers at 47 °C.

† Among the selected *pur⁺* recombinants the inheritance of the Ts⁺ phenotype was assessed by streaking on the appropriate minimal medium and incubation at 47 °C (see Methods).

marker which probably belongs to *spoVF* (Piggot & Hoch, 1985) and leads to generation of thermosensitive spores. As this phenotype is reverted by supplementation of the sporulation medium with dipicolinate, it seems likely that *dpa-1* affects dipicolinate synthase (Balassa *et al.*, 1979). Since, in addition, AKI and dipicolinate synthase belong to the same metabolic pathway, we assessed the linkage of the above markers by transformation.

Competent cells of L2327 (*dapG27*) and RB1950 (*dapG1476*) were transformed with saturating concentrations of donor DNA from strain 1S83 (*spoVF1* or *dpa-1*), as well as from a reference *spo⁺* strain (Table 5). To circumvent the high background of spontaneous thermoresistant revertants, double recombinants, prototrophic and thermoresistant, were selected. When strain L2327 (*dapG27*) was the recipient, double recombinants (*Phe⁺* and *Ts⁺*) were selected directly. With strain RB1950 (*dapG1476*), selected *Pur⁺* transformants were subsequently screened for thermoresistance. Double recombinants from both crosses were finally tested for the inheritance, by co-transfer, of the *spoVF1* marker. Both *dapG27* and *dapG1476* were about 60% co-transferred with *spoVF1* (Table 5), revealing a close linkage which is compatible with the possibility of coordinate transcription during sporulation.

Discussion

In this study, we have identified mutations which affect LD-A₂pm synthesis by screening thermosensitive mutants that lyse in non-permissive conditions due to deficiencies in peptidoglycan synthesis (Brandt &

Karamata, 1987). Among five linkage groups harbouring such mutations, two, *lssB* and *lssD*, were found to be involved in LD-A₂pm synthesis.

Linkage group *lssB*, mapping at 125°, is heterogeneous. Mutations *lssB10* and *B23* confer a deficiency in *N*-acetyl-LL-A₂pm deacylation like the previously identified *dapE320* mutation (Buxton & Ward, 1980), to which they are very closely linked (Brandt & Karamata, 1987). Thus, mutations *lssB10* and *B23* certainly affect the same gene as *dapE320*. Accordingly, they were renamed *dapE10* and *E23*, respectively. However, we believe it premature to ascribe a precise function to the identified *dapE* locus, since a mutation in the *dapE* gene of *E. coli* was shown to be complemented by a clone of *B. subtilis* DNA mapping at about 204° (Shevtchenko *et al.*, 1984; Aleksieva *et al.*, 1985). The remaining group *lssB* mutations, *lssB12* and *B31*, do not confer a clear-cut phenotype; cell mass increase at 45 °C is significant and the lysis, when observed, is very limited. Since, in addition, activities of the *N*-acetyl-LL-A₂pm deacylase are normal, the function affected by these two mutations remains unclear.

Mutations in group *lssD* were shown to affect AKI activity. They are most probably located in the structural gene of this enzyme which, according to the nomenclature used in *E. coli*, was denoted *dapG*. This locus was mapped at 144° (Brandt & Karamata, 1987) and shown to encompass a previously identified mutation, *pyc-1476* (renamed *dapG1476*), which had been mistakenly assigned to the *pycA* locus (Buxton, 1978). Transformation crosses have revealed close linkage between *dapG* and *spoVF*, the putative structural gene of dipicolinate synthase. Since products of *dapG27* and *dpa-1* are part of the same metabolic pathway (Fig. 2), this linkage may

suggest a coordinate gene expression. Indeed, AKI is expressed not only during vegetative growth, but also during sporulation (Hampton *et al.*, 1971; Graves & Switzer, 1990), where it is required for the synthesis of both LD-A₂pm and dipicolinate, two essential components of the spore. Activities of AKII and AKIII are, in contrast, hardly detectable during sporulation, where amino acids required for protein synthesis are derived from protein turnover (Hampton *et al.*, 1971; Graves & Switzer, 1990).

In conclusion, we found that a very large collection of thermosensitive mutants, obtained by indirect selection (Brandt & Karamata, 1987), comprises only two classes of LD-A₂pm-deficient strains endowed with a rapid lysis phenotype, i.e. those deficient in gene *dapG* and those deficient in gene *dapE*. Interestingly, all previously isolated thermosensitive mutants (Buxton, 1978; Buxton & Ward, 1980) affected in LD-A₂pm synthesis were shown to owe their phenotype to deficiencies in the same *dapG* and *dapE* genes. These surprising observations suggest that mutations affecting nearly all of the genes involved in the eight enzymic steps required for LD-A₂pm synthesis have a very low probability of inducing a thermosensitive phenotype. Explanations based on intrinsic properties of the proteins (Karamata & Gross, 1970) cannot be ruled out. However, it is possible that mutations in structural genes of most of the enzymes involved LD-A₂pm synthesis are complemented by products of genes encoding isoenzymes. Thus, to abolish such an enzymic activity, two specific mutations affecting both genetic determinants which encode the isoenzymes would be required. Considering that one of the two mutations would have to result in thermosensitivity, their simultaneous occurrence is even more unlikely.

Availability of a mutant with a thermosensitive AKI offers the possibility to study *in vivo* the regulation of AK activities in *B. subtilis*. The three so far identified isoenzymes, AKI, II and III, participate in the first step of the biosynthesis of amino acids belonging to the aspartate family (Fig. 2). Their regulation has been studied *in vitro*. AKI appears to be rather specifically involved in the synthesis of LD-A₂pm, by which it is inhibited (Rosner & Paulus, 1971); its activity is largely independent of the amino acid composition of the growth medium (Rosner & Paulus, 1971; Graves & Switzer, 1990; Zang *et al.*, 1990). AKII is both inhibited and repressed by lysine, and induced by methionine (Graves & Switzer, 1990; Zhang *et al.*, 1990). Analysis of the sequence of its structural gene has revealed the participation of L-lysine in the mechanism of expression of that gene (Chen *et al.*, 1987; Chen & Paulus, 1988). AKIII is induced by L-lysine, and repressed by L-threonine as well as by several amino acids; in addition,

it is inhibited by the simultaneous presence of L-threonine and L-lysine (Graves & Switzer, 1990). These observations clearly imply that AKII and AKIII are essentially devoted to protein synthesis. The former appears to be more specifically responsible for the synthesis of L-lysine and the latter for that of L-homoserine or, more specifically, of L-threonine. They may well, to some extent, be interchangeable; for instance, in strain 168, AKII activity is more important than that of AKIII whereas the converse is found in the parent Marburg strain (Zhang *et al.*, 1990).

Information on the biological role of AKs obtained from physiological studies of a *dapG*-bearing mutant can be summarized as follows. In minimal medium devoid of lysine, strain L2227 (*dapG27*) sustains substantial growth at the non-permissive temperature (Fig. 1*b*); apparently, AKII and AKIII substitute for the defective AKI. However, despite their overwhelming predominance over AKI in this medium (Hampton *et al.*, 1971; Graves & Switzer, 1990; Zhang *et al.*, 1990), AKII and AKIII are unable to fully compensate the lack of AKI activity. Indeed, with the exception of a short period following the shift to non-permissive temperature, growth of L2227 (*dapG27*) is significantly impaired when compared to that of the parent strain at 45 °C (Fig. 1). Thus, it would appear that either the level, a very high one, of AKII and AKIII is still insufficient to fully compensate for absence of AKI or, for a more fundamental reason, the former enzymes are not easily interchangeable with the latter. Formation of very small colonies on minimal medium devoid of lysine, at 45 °C (not presented), provides further evidence of a very incomplete substitution of AKII and AKIII for the deficient AKI.

In the presence of lysine, shift of L2227 (*dapG27*) to the non-permissive temperature is accompanied by an abrupt halt of mass increase (Fig. 1*b*). This observation is easily accounted for by inactivation of AKI, absence of the lysine-sensitive AKII activity, and a very low level of AKIII in strain 168 (Graves & Switzer, 1990; Zhang *et al.*, 1990). Nevertheless, the residual aspartokinase activity at 45 °C, presumably that of AKIII, may sustain a limited protein synthesis as suggested by a limited and slow lysis of the culture (see Fig. 1*b* and Results). Thus, it would appear that collapse of AKI activity at the non-permissive temperature blocks peptidoglycan and protein synthesis simultaneously, strongly suggesting that at the permissive temperature these syntheses were both relying on AKI activity. In any case, a rapid lysis is observed at 45 °C only when all relevant amino acids are present in the medium allowing normal protein synthesis (Fig. 1*b*).

Finally, the inclusion of a high concentration of L-aspartate in lysine-supplemented media restores substantial growth of strain L2227 (*dapG27*) at 45 °C (Fig. 1*c*),

strongly suggesting that AK activity can be enhanced by an increased concentration of its substrate (Fig. 1c); it is not clear whether L-aspartate stimulates the activity of the thermolabile AKI or of AKIII or of both enzymes.

In summary, our *in vivo* observations are in excellent agreement with *in vitro* studies of the regulation of AK activities. They underline the important role of L-lysine in the regulation of AK activity in *B. subtilis* 168, and they fully agree with the reported constant level of AKI activity, which is independent of the growth medium composition.

Recent experiments on AKII and AKIII mutants were analysed with respect to the metabolic channel hypothesis, according to which various aspartate family amino acids, including LD-A₂pm, would each be synthesized in separate compartments (Zhang *et al.*, 1990; Zhang & Paulus, 1990). Our observations, suggesting a certain degree of interchangeability between AKI and the other AKs, would at first sight argue against independent metabolic channels. However, we would like to stress that substitution of AKI by AKII and AKIII is incomplete even in minimal medium where the latter enzymes are present at a high level. Therefore, *in vivo* studies of the AKI-deficient mutant could also be interpreted in favour of separate, but partially leaky, metabolic compartments. Indeed, if the concept of a channel were interpreted in terms of a multienzyme complex producing a specific end product, one could assume that each specific aspartokinase would bind preferentially to a given complex. For example, the LD-A₂pm channel would have the highest affinity for AKI. However, each AK isoenzyme could also bind, albeit with somewhat lower affinities, to the other complexes, assuring some of their metabolic function. This model, involving multienzyme complexes, together with partial reciprocal substitution of isoenzymes, could readily account for the apparent partially communicating precursor pools.

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