A Heat-sensitive Lysis Mutant of *Bacillus subtilis* 168 with a Low Activity of Pyruvate Carboxylase

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A mutant of *Bacillus subtilis* which grew in complex medium at 30 °C but lysed at 45 °C has been isolated. It could only grow on minimal medium at 45 °C with added aspartate (20 µg ml⁻¹) but lysed if lysine (20 µg ml⁻¹) was also present. The requirement for aspartate was due to a low activity of pyruvate carboxylase; the site of the mutation (*pyc*) was linked (16% cotransducible using phage PBS1) to the *pyrD* locus, and the order of markers deduced was: *pyrD*–*cysC*–*pyc*. This defect appeared to lead to decreased synthesis of *meso*-diaminopimelic acid (*meso*A₂pm), an amino acid unique to peptidoglycan and its precursors. At the restrictive temperature the mutant accumulated uridine-5'-diphosphate *N*-acetylmuramyl-L-alanyl-D-glutamate, since *meso*A₂pm is the next amino acid to be added to the growing peptide chain of peptidoglycan. This resulted in an inhibition of peptidoglycan synthesis, determined as a reduced incorporation of *N*-acetyl[¹⁴C]glucosamine. Peptidoglycan synthesis was not decreased if the mutant was grown in media containing aspartate but lacking lysine.

The sensitivity to lysine may arise because (i) at 45 °C the mutant was starved for aspartate and hence *meso*A₂pm even when aspartate was present, since aspartate utilization, as estimated by the incorporation of [³H]aspartate into trichloroacetic acid precipitable material, was relatively inefficient; and (ii) this diminished level of *meso*A₂pm synthesis from aspartate was further curtailed since lysine inhibits one of the aspartokinases in *B. subtilis*. Thus, addition of lysine allowed protein synthesis and hence autolysin production to proceed whilst peptidoglycan synthesis remained inhibited.

When autolysis was blocked, either indirectly by stopping protein synthesis through starvation of aspartate and lysine, or directly by introducing a *lyt* mutation, then shifting the mutant to 45 °C did not result in lysis but growth still ceased.

INTRODUCTION

To study the control of the biosynthesis of the major structural bacterial wall polymer, peptidoglycan, heat-sensitive mutants defective in its synthesis have been isolated in *Escherichia coli* (e.g. Matsuzawa *et al.*, 1969; Lugtenberg, de Haas-Menger & Ruyters, 1971) and in *Staphylococcus aureus* (Chatterjee & Young, 1972; Good & Tipper, 1972). These mutants lysed at the non-permissive temperature since peptidoglycan, which resists the force produced by the osmotic pressure of the bacterium across the cytoplasmic membrane, was no longer made. In a number of these mutants specific defects in the enzymes involved in peptidoglycan synthesis have been identified.

In this communication I describe a heat-sensitive lysis mutant of *Bacillus subtilis* which lysed, not because it had a defect in peptidoglycan synthesis itself, but because it appeared to have a decreased level of *meso*-diaminopimelic acid (*meso*A₂pm) synthesis owing to a lowered activity of pyruvate carboxylase.

Table 1. *Bacterial strains used*

Strain	Genotype	Origin or reference
168	<i>trpC2</i>	Buxton (1976)
168 <i>thy</i>	<i>trpC2 thyA thyB</i>	Buxton (1976)
RB1476	<i>trpC2 thyA thyB pyc</i>	EMS-induced mutant of 168
M22	<i>purA16 leuA8 metB5 ilvA1</i>	Buxton (1976)
RB1950	<i>purA16 metB5 ilvA1 pyc</i>	DNA .RB1476 × M22 → Leu ⁺
MB50	<i>pyrD1* cysC7</i>	P. J. Piggot
FJ3	<i>metC3 lyt-1</i>	J. E. Fein
RB1117	<i>purA16 metB5 pyc lyt-1</i>	DNA .FJ3 × RB1950 → IlvA ⁺

* *pyrD1* is the *ura-1* marker (Potvin, Kelleher & Gooder, 1975) which was mapped relative to *cysC* and *furA* by Young (1975).

METHODS

Bacterial strains. The derivatives of *B. subtilis* 168 are shown in Table 1.

Media and chemicals. L-broth, Spizizen's minimal medium and MS minimal medium have all been described previously (Buxton, 1976). CH/S/C was a casein acid-hydrolysate medium (Janczura, Perkins & Rogers, 1961). Difco Bacto Casamino acids [present at 1.0% (w/v) in medium CH/S/C] contained 0.5% (w/w) aspartic acid and 7.0% (w/w) lysine (H. Saunders, personal communication). Wall medium was 0.1 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.3, containing: MgSO₄, 2 mM; glucose, 0.5% (w/v); chloramphenicol (Parke-Davis, Hounslow, Middlesex), 50 µg ml⁻¹; *N*-acetylglucosamine, 0.15 mM; L-arginine, 0.2% (w/v); and DL-alanine, 0.02% (w/v). Schaeffer's sporulation agar was as described by Schaeffer, Millet & Aubert (1965).

Radioactively labelled compounds were obtained from The Radiochemical Centre, Amersham.

Mutant isolation. The isolation of the mutant strain RB1476 from the parent strain 168*thy*, after mutagenesis with ethyl methanesulphonate (EMS), has been described (Buxton, 1976). Briefly, heat-sensitive mutants were screened in L-broth for those which showed visible lysis after a period (3 h or overnight) at the non-permissive temperature, 45 °C. The mutation giving rise to this phenotype was transferred to strain M22 (*leuA*) by DNA-mediated transformation using a saturating concentration of DNA (congression) and selecting for Leu⁺ recombinants. The experiments described in this paper were performed on one such recombinant (strain RB1950), its parent (M22) and a *Lyt*⁺ recombinant (RB1117, see below).

Enzyme assays. Sonic extracts were prepared from cells grown in L-broth. Glutamate-oxaloacetate transaminase [L-aspartate: 2-oxoglutarate aminotransferase; EC 2.6.1.1] was assayed by measuring the formation of oxaloacetate spectrophotometrically (Gunsalus & Stamer, 1955); pyruvate carboxylase [pyruvate: carbon-dioxide ligase (ADP-forming); EC 6.4.1.1] was assayed by coupling the reaction to malate dehydrogenase (Sigma) and following the rate of NADH oxidation spectrophotometrically (Seubert & Weiker, 1969). The enzyme assays were performed using a Unicam SP800 recording spectrophotometer. Like pyruvate carboxylase from *Pseudomonas citronellolis*, but unlike the enzyme from liver, yeast or *Arthrobacter globiformis* (see Kornberg, 1966), the *B. subtilis* enzyme did not appear to require acetyl-CoA for its activity (unpublished data). Protein was determined by the method of Lowry *et al.* (1951).

Estimation of peptidoglycan synthesis. Bacteria were grown overnight with aeration at 30 °C in CH/S/C medium containing: *N*-acetylglucosamine, 0.5 mM; MgSO₄, 3.3 mM; isoleucine, leucine and methionine, each at 20 µg ml⁻¹; and adenine, 40 µg ml⁻¹. The cultures were then diluted into similar medium, with added glucose (0.4%, w/v), and grown with aeration at 30 °C until an absorbance of approximately 0.8 at 600 nm was reached. An absorbance of 1.0 was equivalent to 300 µg dry weight ml⁻¹. Cultures (30 ml) were harvested by centrifugation at 35 °C and resuspended in wall medium (20 ml) pre-warmed to 30 °C; samples (10 ml) were then incubated at 30 °C or 45 °C for 10 min. *N*-Acetyl-D-[1-¹⁴C]glucosamine (0.2 µCi, 58 mCi mmol⁻¹) was added to each 10 ml culture and samples (0.5 ml) were taken at intervals into 0.5 ml ice-cold 10% (w/v) trichloroacetic acid (TCA). After storage on ice for at least 15 min, the samples were heated at 80 °C for 15 min to remove teichoic acid, and then filtered under vacuum through glass-fibre filters (Whatman GF/C). The filters were washed successively with cold 5% (w/v) TCA, ethanol and ether, dried and immersed in 2 ml scintillation fluid [toluene containing 2,5-diphenyloxazole (0.4%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%, w/v)] and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer. Pooley (1976) has shown that the measurement of *N*-acetyl[¹⁴C]-glucosamine incorporation in this manner is a valid estimation of peptidoglycan synthesis.

Isolation and characterization of nucleotide-bound peptidoglycan precursors. After 90 min in wall medium as described above, the culture (7.0 ml) was centrifuged at 4 °C, and the bacteria were resuspended in

Fig. 1. Effect of a temperature shift on the growth of *B. subtilis* strains M22 (wild-type), RB1950 (*pyc*) and RB1117 (*pyc lyt*). Bacteria were grown with aeration (bubbling) in MS medium containing Casamino acids at 30 °C and shifted to 45 °C at the time indicated by the arrows. Growth was monitored using an EEL nephelometer (see Buxton, 1976). A scattered light reading of 100 corresponds to a viable count of 6×10^8 bacteria ml^{-1} .

0.1 vol. distilled water. This suspension was heated at 100 °C for 5 min, centrifuged and the supernatant liquid was retained. The pellet was washed again with 0.5 ml water, centrifuged, and the supernatants were pooled, freeze-dried and taken up in 0.1 ml water. This solution was chromatographed on Whatman no. 1 paper for approximately 3 d. Descending chromatography was carried out in the solvent system: isobutyric acid/0.5-M- NH_3 (5:3, v/v), and nucleotides were located by their absorption of ultraviolet light. Peptidoglycan precursors were identified by reference to the position of known precursors (see Ward, 1975) and by amino acid analysis.

Amino acid analysis and the determination of the bound *N*-acetylhexosamine content of nucleotide precursors was determined as described by Ward (1975). Radioactivity on paper was determined by autoradiography and counted directly in a Packard Tri-Carb liquid-scintillation spectrometer (see Ward, 1975).

Incorporation of aspartate and lysine. Bacteria were grown in MS medium containing aspartate and lysine (each at $20 \mu\text{g ml}^{-1}$) at 35 °C until an absorbance at 600 nm of 0.2 to 0.3 was reached. L-[G- ^3H]Aspartic acid (1.1 Ci mmol^{-1} , 25 μCi) and DL-[4,5(n)- ^3H]lysine monohydrochloride (10 Ci mmol^{-1} , 25 μCi) were added to separate 10 ml cultures, and samples (0.5 ml) were taken at intervals into 0.5 ml ice-cold 10% (w/v) TCA. After storage on ice, the samples were filtered, treated, and counted as described above.

Genetical methods. Transduction using phage PBS1 and DNA-mediated transformation have been described previously (Buxton, 1976).

The *pyc lyt* recombinant strain RB1117 was isolated by transforming strain RB1950 with a saturating amount of DNA isolated from the autolytic enzyme-deficient mutant FJ3 (Fein & Rogers, 1976) and selecting for H^+V^- recombinants. Amongst these, *lyt* recombinants were identified by their tendency to form long chains of bacteria as seen under the phase contrast microscope. Their Lyt^- phenotype was confirmed by testing for lysis of whole bacteria at pH 8.5 at 45 °C (unpublished data).

RESULTS

Growth characteristics of mutant strain RB1950

A culture of the mutant strain RB1950, when shifted from 30 °C to 45 °C in MS medium containing Casamino acids (1%, w/v), began to lyse after 60 min (Fig. 1). At 30 °C, M22 had a doubling time of 32 min, whereas that for RB1950 was 76 min. Strain RB1950 grew at 45 °C on Spizizen's minimal medium with glucose as carbon source if aspartate ($20 \mu\text{g ml}^{-1}$) was added, but not if an equal amount of lysine was also added, or if lysine and threonine ($20 \mu\text{g ml}^{-1}$) were also added. It did, however, grow on medium containing aspartate ($200 \mu\text{g ml}^{-1}$) and lysine ($20 \mu\text{g ml}^{-1}$). The mutant also grew on minimal glucose medium at 45 °C if malate ($40 \mu\text{g ml}^{-1}$) or glutamate ($20 \mu\text{g ml}^{-1}$) were added, although not if 2-oxoglutarate ($40 \mu\text{g ml}^{-1}$) or succinate ($20 \mu\text{g ml}^{-1}$) were substituted (this may have been because the latter compounds have difficulty in entering the bacterium; 2-oxoglutarate is also somewhat unstable). No other amino acid addition (including A_2pm and D-alanine)

Fig. 2. Pathways leading to the synthesis of L-lysine. Not all intermediate steps are shown. The enzymes numbered are: 1, pyruvate carboxylase (EC 6.4.1.1); 2, glutamate-oxaloacetate transaminase (EC 2.6.1.1); 3, aspartokinase (EC 2.7.2.4); 4, aspartate- β -semialdehyde dehydrogenase (EC 1.2.1.11); 5, dihydrodipicolinate synthase (EC 4.2.1.52); 6, dihydrodipicolinate reductase (EC 1.3.1.aa); 7, diaminopimelate decarboxylase (EC 4.1.1.20).

allowed growth at 45 °C. At 30 °C, growth occurred on minimal glucose agar except if lysine (20 $\mu\text{g ml}^{-1}$) alone was added, although again protection was afforded by an equal amount of aspartate.

Enzyme defect of the mutant

The growth characteristics suggested there may be a block in the synthesis of aspartate (see Fig. 2). No difference in glutamate-oxaloacetate transaminase activity could be detected between strain RB1950 and its parent M22, but a significant difference was noted in the specific activity of pyruvate carboxylase. At an assay temperature of 45 °C, the specific

Table 2. Genetic cross to determine the location of the *pyc* mutation

Phage PBS1 was grown on strain RB1950 at 30 °C, and used to transduce strain MB50 (*pyrD1 cysC7*) to *PyrD*⁺ on minimal plates containing cysteine and aspartate (each at 20 µg ml⁻¹) at 30 °C. After purification by single colony isolation, the recombinants were scored as *cys*⁺ or *cys* at 30 °C on agar plates lacking cysteine, and as *pyc*⁺ or *pyc* on L-broth plates at 45 °C and on minimal plates lacking aspartate at 45 °C. There was 100 % correlation of *pyc*⁺ and *pyc* as scored on L-broth plates and on minimal plates lacking aspartate. On the latter plates *pyc* recombinants grew a little, but lysed.

Selected marker	Unselected markers		Indicated order		
	<i>pyc</i> ⁺	<i>cysC</i> ⁺ 32	<i>cysC</i> 35	Donor	<i>pyc</i> <i>cysC</i> ⁺ <i>pyrD</i> ⁺
<i>pyrD</i> ⁺	<i>pyc</i>	13	0	Recipient	<i>pyc</i> ⁺ <i>cysC</i> <i>pyrD</i>

activity of the enzyme from strain RB1950 grown at 30 °C in L-broth was 4.8 nmol NADH oxidized min⁻¹ (mg protein)⁻¹, whereas from strain M22, it was 120. At an assay temperature of 30 °C, the specific activities were 17 for RB1950 and 50 for M22. Thus strain RB1950 had 33 % of the pyruvate carboxylase activity of M22 when assayed at 30 °C and only 4 % at 45 °C. It therefore had a heat-sensitive pyruvate carboxylase, the mutation being somewhat leaky at 30 °C. This was also indicated by a requirement for very great aeration (by shaking) at 30 °C to avoid lysis, which may reflect the need for a continuous supply of CO₂ to enable the lower amount of pyruvate carboxylase to function at its maximal rate.

The inability of the mutant to grow on minimal glucose medium except when utilizable intermediates of the tricarboxylic acid cycle (or their precursors) were added, suggested that the mutant lacked the anaplerotic enzyme, pyruvate carboxylase, for growth on C₃ compounds (see Kornberg, 1966). In the Enterobacteriaceae, mutants lacking the analogous enzyme, phosphoenolpyruvate carboxylase, have similar nutritional phenotypes (Theodore & Englesberg, 1962; Amarasingham, 1959; Ashworth & Kornberg, 1963).

Genetic analysis of the mutant

Phage-mediated transduction using PBS1 showed that the site of the mutation (*pyc*) giving rise to the heat-sensitive phenotype of strain RB1950 was 16 % cotransducible with *pyrD1*. From the three-point cross shown in Table 2, the order of markers was deduced to be *pyrD1-cysC-pyc*, assuming that recombinants requiring a minimum of four exchanges between donor and recipient DNA will occur significantly less frequently than those requiring a minimum of two. [Young (1975) has mapped *pyrD* and *cysC* relative to other markers.] *pyc* was therefore very probably identical to the *aspA* locus, a mutant of which has previously been shown to be defective in pyruvate carboxylase (Hoch & Mathews, 1972). The *aspA* locus was closely linked to *recA1* (Hoch & Mathews, 1972), a position which is compatible with the mapping described above (see Lepesant-Kejzlarová *et al.*, 1975). [The symbol *pyc* is used here to avoid confusion since *aspA* in *E. coli* has been used for aspartase; see Bachmann, Low & Taylor (1976).]

The mutant had difficulty in sporulating, presumably because it was starved of A₂pm and dipicolinic acid. However, when the few spores produced on Schaeffer's sporulation agar germinated, they gave rise to heat-resistant organisms that grew at 45 °C on complex medium, and no longer required aspartate. Thus the lysis phenotype and aspartate requirement are most probably due to a single mutation.

Table 3. *Accumulation of UDP-MurNAc-dipeptide by the mutant strain RB1950*

Mutant strain RB1950 and its parent M22 were grown at 30 °C in CH/S/C (see Methods) and resuspended in wall medium. After 10 min incubation, *N*-acetyl-D-[1-¹⁴C]glucosamine was added, and after 90 min the bacteria were harvested and the nucleotide-bound precursors of peptidoglycan were isolated and identified as described in Methods.

	Strain M22		Strain RB1950	
	30 °C	45 °C	30 °C	45 °C
Radioactivity co-chromatographing with UDP-MurNAc-L-Ala-D-Glu (c.p.m.)	349	1 184	300	5 969
Total radioactivity in heat-resistant TCA-precipitable material (peptidoglycan) (c.p.m.)	19 390	49 028	18 578	12 376
Radioactivity in UDP-MurNAc-L-Ala-D-Glu as a percentage of the total radioactivity in peptidoglycan	1.8	2.4	1.6	48.2

Synthesis of peptidoglycan and accumulation of nucleotide-bound peptidoglycan precursors

Since aspartate is the precursor of *meso*A₂pm (see Fig. 2) and D-glutamate, it seemed likely that lysis at 45 °C was due to a starvation of *meso*A₂pm and D-glutamate and a consequent lowering of the rate of peptidoglycan synthesis, since D-glutamate and *meso*A₂pm are the second and third amino acids to be added to the peptide side-chain of peptidoglycan (see review by Ghuysen & Shockman, 1973). If this were so, it should be possible to detect, at 45 °C, the accumulation of peptidoglycan precursors. Cultures of strain RB1950 and its parent M22 were grown at 30 °C in CH/S/C as described in Methods, resuspended in wall medium [which lacks aspartate and contains chloramphenicol to stop protein synthesis and thus inhibit lysis whilst allowing peptidoglycan synthesis to continue (Hughes, Tanner & Stokes, 1970)], and incubated at 30 °C or 45 °C. After 10 min *N*-acetyl[¹⁴C]glucosamine was added, and after 90 min the bacteria were harvested and the nucleotide-bound precursors of peptidoglycan were isolated and identified as described in Methods. Strain M22 incorporated approximately 2.5 times more *N*-acetyl[¹⁴C]glucosamine into peptidoglycan after 90 min at 45 °C than at 30 °C. In contrast, strain RB1950 incorporated slightly less at 45 °C than at 30 °C. Thus the mutant made less peptidoglycan at 45 °C. When the experiment was performed in wall medium supplemented with aspartate (200 µg ml⁻¹), *N*-acetyl[¹⁴C]glucosamine incorporation into peptidoglycan at 45 °C by strain RB1950 was nearly as great as by strain M22.

The accumulation of peptidoglycan precursors after 90 min in wall medium is shown in Table 3. The mutant strain RB1950, unlike its parent M22, accumulated UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid, the identity of which was established by amino acid analysis (kindly performed by Dr J. B. Ward). In RB1950 at 45 °C, 48% of the total radioactivity in heat-resistant TCA-precipitable material was in UDP-MurNAc-L-Ala-D-Glu, whereas this figure was 2.4% in the parent M22 (Table 3). The mutant accumulated 3.15 µmol of UDP-MurNAc-dipeptide per 100 mg dry weight of bacteria, compared with 2.17 µmol accumulated by a stable L-form of *B. subtilis* defective in aspartate-β-semialdehyde dehydrogenase and 0.13 µmol accumulated by wild-type *B. subtilis* (Ward, 1975).

Thus the defect in pyruvate carboxylase appeared to lead to lower *meso*A₂pm synthesis, slowing peptidoglycan synthesis and resulting in the accumulation of UDP-MurNAc-L-Ala-D-Glu. The presence of aspartate allowed peptidoglycan synthesis to proceed in the mutant at nearly the same rate as in the wild type.

However, since the defect in pyruvate carboxylase activity results in decreased ability to make oxaloacetate (and hence aspartate and A₂pm), glutamate synthesis will also be impaired because oxaloacetate (with acetyl-CoA) is also its precursor. Thus, it seems strange

Fig. 3. Possible pathway for the provision of glutamate for peptidoglycan formation.

that UDP-MurNAc-L-Ala-D-Glu was accumulated rather than UDP-MurNAc-L-Ala, unless oxaloacetate were specifically channelled to glutamate. One explanation could be that a limited supply of glutamate is formed by breakdown of arginine in the wall medium (Fig. 3), and this limited supply of glutamate is used for peptidoglycan formation rather than as a precursor of aspartate (i.e. glutamate $\rightarrow$ 2-oxoglutarate $\rightarrow$ succinate $\rightarrow$ fumarate $\rightarrow$ malate $\rightarrow$ oxaloacetate $\rightarrow$ aspartate).

Sensitivity of the mutant to lysine

The lysis of the mutant at 45 °C in complex medium may be due to its sensitivity to the lysine present in the medium, since it failed to grow on minimal medium containing both aspartate and lysine, although increasing the aspartate concentration afforded some protection. This sensitivity to lysine may be due to several effects.

1. *Bacillus subtilis* strain 168 appeared to incorporate aspartate from medium containing lysine and aspartate into TCA-precipitable material much less efficiently than lysine (Fig. 4). Similar results were obtained with strain M22, whether or not lysine was present (data not shown). This could be due either to an inefficient mechanism for uptake of aspartate or to its degradation.

2. The decreased level of *meso*A₂pm synthesis would be further curtailed when lysine was added because lysine inhibits one of the two aspartokinases of *B. subtilis* (aspartokinase II) (Hampton *et al.*, 1971; Rosner & Paulus, 1971; see Fig. 2); this would lead to a decrease in the rate of synthesis of β -aspartyl phosphate and hence of *meso*A₂pm and peptidoglycan. The action of diaminopimelate decarboxylase is not reversible; *meso*A₂pm cannot therefore be synthesized from lysine (Rosner, 1975). [*meso*A₂pm-requiring mutants of *E. coli* have also been reported to lyse in the presence of lysine (Meadow, Hoare & Work, 1957); this lysis was prevented by increasing the A₂pm concentration of the medium.]

3. When aspartokinase II was inhibited by lysine, peptidoglycan synthesis may have been slowed more rapidly by *meso*A₂pm starvation than protein synthesis by threonine starvation. (Since threonine was present in excess, starvation for this amino acid may not

Fig. 4. Incorporation of [^3H]aspartate (○) and [^3H]lysine (●) into TCA-precipitable material by *B. subtilis* strain 168. The strain was grown at 35 °C in MS medium containing aspartate and lysine as described in Methods. [^3H]Aspartate or [^3H]lysine was then added, and samples were taken at intervals and assayed for the amount of radioactivity in TCA-precipitable material. Results are expressed as a percentage of the total radioactivity in the medium.

Fig. 5. Temperature shift experiments involving amino acid starvation, with *B. subtilis* strains M22 (wild-type) and RB1950 (*pyc*). Strains M22 and RB1950, each in MS medium containing methionine, isoleucine, leucine and aspartate (20 $\mu\text{g ml}^{-1}$ each) and adenine (40 $\mu\text{g ml}^{-1}$), were grown with aeration at 30 °C to exponential phase, then harvested by centrifugation at room temperature, washed in MS medium lacking aspartate, and resuspended in MS medium lacking aspartate (□), or supplemented with aspartate (○) or lysine (■) or aspartate plus lysine (●), each at 20 $\mu\text{g ml}^{-1}$. At time 0, the cultures were shifted to 45 °C and aerated, and growth was followed using an EEL nephelometer. A scattered light reading of 100 corresponds to a viable count of 6×10^8 bacteria ml^{-1} .

have occurred. The strain used was auxotrophic for methionine and isoleucine which were present in the medium.) Protein synthesis, and hence autolysis, would then overtake peptidoglycan synthesis resulting in lysis.

If protein synthesis, and hence autolysin production, were to be slowed at the same time as peptidoglycan synthesis, lysis would not be expected to occur. This is demonstrated by the results shown in Fig. 5. When the mutant was shifted to 45 °C in minimal medium lacking aspartate and lysine, it stopped growing but did not lyse; in medium containing

lysine, however, the mutant lysed, as it did somewhat later in medium containing aspartate and lysine. Raising the aspartate and lysine concentrations each to $200 \mu\text{g ml}^{-1}$ allowed growth to continue for a much longer time before lysis began (data not shown) – this probably enabled more aspartate to be utilized and peptidoglycan synthesis to proceed for a longer time. The addition of lysine to the wild-type strain M22 did not cause lysis, presumably because such bacteria were not starved for *mesoA*₂pm.

Direct inactivation of the autolytic enzymes would also be expected to inhibit lysis but the mutant would still be unable to grow even in complex medium containing aspartate because of *mesoA*₂pm starvation. In fact, an autolytic-enzyme deficient derivative of strain RB1950 (strain RB1117, *pyc 1yt*) lysed only slightly at 45 °C in Casamino acid medium (Fig. 1), although growth did stop.

DISCUSSION

The isolation of heat-sensitive lysis mutants of *B. subtilis* may be expected to provide mutants blocked in the various stages of peptidoglycan synthesis (see review by Ghuysen & Shockman, 1973). The isolation of a mutant blocked in *mesoA*₂pm synthesis does not appear to be fortuitous, since the identification of some non-conditional osmotically fragile mutants (i.e. stable L-forms) as defective in aspartate- β -semialdehyde dehydrogenase activity (Ward, 1975) seems to indicate that this type of mutant is common amongst strains selected on this basis. Furthermore, I have isolated two other heat-sensitive lysis mutants which appear to have defects in *mesoA*₂pm synthesis (unpublished data); one is sensitive to lysine at 45 °C but grows in its absence, and could have an altered aspartokinase.

The comparatively common occurrence of this type of lesion in *mesoA*₂pm synthesis amongst heat-sensitive lysis mutants may be due to the extremely autolytic nature of *B. subtilis* (Rogers & Forsberg, 1971) compared with, for example, staphylococci (Rogers, 1967). Thus even a slight reduction in the rate of peptidoglycan synthesis allows wall synthesis to be rapidly overtaken by autolysis. The common occurrence of mutants blocked in *mesoA*₂pm synthesis may merely be a reflection of the large number of enzymes and hence genes, involved in this synthesis. It is, however, also tempting to think that since *mesoA*₂pm is a unique constituent of peptidoglycan, its rate of synthesis may be the limiting factor determining the rate of peptidoglycan synthesis in bacilli. In the mutant studied in the present paper, it is also important that one of the aspartokinases is inhibited by lysine (Rosner & Paulus, 1971). The aspartokinase of *B. polymyxa*, in contrast, is only inhibited by lysine plus threonine (Paulus & Gray, 1964).

It may be possible to eliminate the class of mutants defective in *mesoA*₂pm synthesis by selecting for lysis mutants in L-broth supplemented with *mesoA*₂pm, although this compound is only taken up poorly by *B. subtilis* 168 (data not shown). The results presented in this paper, however, suggest that if lysine is omitted, mutants blocked in *mesoA*₂pm synthesis will not lyse. A non-lytic mutant (Fein & Rogers, 1976) as a parent strain could also be used to eliminate lysis mutants with only a slight reduction in the rate of peptidoglycan synthesis. Selection of secondary-site revertants of strain RB1950 which do not lyse at 45 °C may in fact be a convenient selective procedure for the isolation of such non-lytic mutants.

Al-ssum & White (1977*a*) have shown that in a *Bacillus megaterium* strain which has a biotin requirement at 37 °C, starvation of biotin resulted in abnormal morphology in the form of filaments, thin regions of wall, swollen ends and reduced ability to sporulate. The addition of aspartate restored normal morphology at 37 °C even in the absence of biotin. Since biotin is required in the growth medium for maximal pyruvate carboxylase activity (Cazzulo, Sundaram & Kornberg, 1969; Al-ssum & White, 1977*b*), it seems that a similar phenotype to that of the *pyc* mutant at 45 °C may be achieved by inhibiting pyruvate carboxylase through biotin starvation.

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