Mutants of *Bacillus subtilis* 168 Thermosensitive for Growth and Wall Teichoic Acid Synthesis

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(Received 12 September 1988; revised 22 December 1988; accepted 18 January 1989)

A protocol designed to isolate mutants with thermosensitive (Ts) synthesis of the bacteriophage φ29 receptor, which includes the major wall teichoic acid in *Bacillus subtilis* 168, yielded a significant enrichment for Ts growth mutants among colonies surviving φ29 treatment. Nine mutants, Ts for both φ29 susceptibility and cell growth, harboured mutations which were located in the *tag* locus by PBS1 transduction and recombination index with the *tag-1* marker. Physical mapping revealed that they were distributed on a segment of more than 4 kb. Chemical analysis of cell walls showed a marked reduction in phosphate relative to diaminopimelic acid content of all mutants at the non-permissive temperature. Differences between mutants were correlated with the distribution of *tag* mutations on the genetic map. We conclude (i) that the newly identified markers affect several genes involved in poly(glycerol phosphate) synthesis, and (ii) that on phosphate-rich media, cell growth cannot occur without the synthesis of the latter polymer.

INTRODUCTION

The function of the anionic polymers covalently attached to the peptidoglycan (PG) of many Gram-positive organisms remains unknown. It is important to know whether their synthesis is essential for cell growth since this could provide a potential target for antibiotics (Pooley & Karamata, 1988). Recently, both indirect and direct evidence obtained from *Bacillus subtilis* was interpreted in favour of an essential role for teichoic acid (Karamata *et al.*, 1987; Pooley *et al.*, 1987; Baddiley, 1988). This conclusion implies that it should be possible to isolate conditional lethal mutants affected in teichoic acid synthesis at the non-permissive temperature. A search for such mutants is described in this paper.

Many phage-resistant mutants of *B. subtilis* affected in teichoic acid synthesis have been described (Young, 1967; Pooley *et al.*, 1987). In particular, the major wall teichoic acid in *B. subtilis* 168, glucosylated poly(glycerol phosphate) [poly(groP)] has been identified as the receptor for bacteriophage φ29. Characterization of the functions affected in a large collection of φ29 mutants has revealed the existence of five loci, *gtaA* to *E*, all involved in the glucosylation of poly(groP) (Pooley *et al.*, 1987). None of these mutants were reported as having a thermosensitive (Ts) growth phenotype. Since genes for polymerization must represent a significant target for mutagenesis, the absence of φ29 mutants blocked in the synthesis of the

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Abbreviations: poly(groP), poly(glycerol phosphate); poly(rboP), poly(ribitol phosphate); MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; PG, peptidoglycan; DAP, diaminopimelic acid; ND, nephelometric density; RI, recombination index; φ29, φ29 sensitive; φ29*, φ29 resistant; Ts, thermosensitive; Ts*, thermoressistant.

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main chain of poly(groP) or in the PG-linkage unit is consistent with their having a lethal phenotype. Indeed, several Ts conditional lethal mutants deficient in cell wall phosphate are known (Boylan & Mendelson, 1969; Shifflett et al., 1977), and the relevant tag marker is tightly linked to gtaA (Boylan et al., 1972). Nevertheless, the authors of one study (Rogers et al., 1974) obtained results suggesting that the primary block in one such mutant may not be in the synthesis of teichoic acid.

In the present study, by exploiting resistance to phage φ29, we sought to enrich for mutants having Ts synthesis of the wall receptor and a Ts phenotype for growth. The successful outcome provides new evidence that synthesis of poly(groP) plays an essential role in cell growth. Genetic and physiological characterization of the mutants obtained will be useful in the identification of the enzyme steps undergoing mutational blockage, and in the search for the role of anionic polymers in the cell economy.

METHODS

Bacterial strains, bacteriophages and plasmids. B. subtilis 168 derivatives used are listed in Table 1. Strains were maintained as spore preparations on Schaeffer’s agar and, in some cases, as suspensions in water (Yoshikawa, 1965). Escherichia coli LE392 (metB ksdR galK trpR lacT) was used for plasmid preparations. Phages employed were φ25 and φ29 (laboratory stocks); preparation and titrating of phage stocks were as described previously (Karamata et al., 1987). Plasmid pAH101 (Ap'Cm'), and subclones pAH120 (Tc'Cm') and pAH102 (Ap'), were a generous gift from G. R. Stewart (University of Kansas, USA). pAH101 was isolated by insertion of Tn917 in the vicinity of the tag-3 (rodCI) marker. The 11 kb of B. subtilis DNA flanking the latter marker were cloned in E. coli by Honeyman & Stewart (1988) after integrating plasmid pTV21A2 (Youngman et al., 1984) into the transposon.

Media and growth measurement. L and TS plates, SAT and L broth, and media employed for transformation and transduction have been described previously (Karamata & Gross, 1970; Pooley & Karamata, 1984; Karamata et al., 1987). TBAB contained 33 g Tryptose blood agar base (Difco) per litre. PAB contained, per litre, 1.5 g Bacto-beef extract (Difco), 5 g Bacto-peptone (Difco), and 3-5 g NaCl. Solid media were prepared with 1.2% (w/v) agar (Gibco). Selection plates and liquid media contained, where appropriate, 20 μg amino acids ml⁻¹, and 100 μg bases, adenine or uracil ml⁻¹.

Growth was monitored by optical density (OD₆₆₀) or by nephelometric density (ND); ND 100 corresponds to a dry weight of 0-11 mg ml⁻¹ (Brandt & Karamata, 1987).

Enrichment for tag markers among mutants surviving φ29 treatment following mutagenesis. A seed culture of strain L5009 grown for 16 h in SAT at 22 °C was diluted to ND 4, and incubated for 5 doubling times at 30 °C. In mid-exponential phase (ND 144), 20 ml was transferred to a tube containing 1.5 mg MNNG (final concentration, 75 μg ml⁻¹) and, after vigorous mixing, incubated for 45 min. Survival after mutagenesis was 1.4%. A 2 ml sample of surviving cells, filtered, washed and resuspended in PAB (100 ml), was incubated for 20 h at 22 °C to allow for segregation and phenotypic lag. The temperature was then gradually (over a 20 min period) increased to 37 °C, an intermediate temperature, to allow partial expression of Ts mutations. After about three doubling times (90 min) φ29 was added at a multiplicity of infection of over 20 and incubation continued for 1 h, by which time lysis had begun. After removal of unadsorbed phage by filtration and washing, survivors, resuspended in PAB, were spread onto L plates, incubated at 30 °C for 16 h and screened for Ts growth by replica plating onto L plates at 48 °C. All colonies showing reduced growth were kept for further analysis. All mutants were obtained from one mutagenesis experiment (see below).

Phage resistance. Tests were performed essentially as described previously (Karamata et al., 1987) by spotting phage stocks on cells freshly streaked onto L plates, and incubating at 30 or 37 °C.

Genetic analysis. Methods for PBS1 transduction, transformation, and measurement of recombination index (RI) were as described by Karamata & Gross (1970). Transformation of strains carrying Ts markers was performed at 30 or 32 °C according to a modified procedure (Karamata et al., 1987).

Transformation of Ts mutants with plasmid DNA. Seed cultures (10 ml) of E. coli, grown for 7-8 h at 33 °C in LB, supplemented with appropriate antibiotics (chloramphenicol, 2.5 μg ml⁻¹ or ampicillin, 10 μg ml⁻¹), were used to inoculate 200 ml cultures which were harvested after 14 h at 33 °C. Standard methods were used for phenol extraction of plasmid DNA (Rodriguez & Tate, 1983).

Ts mutant derivatives of L5028 (L650i series, i = 1-14) were transformed in one of the following two ways: (i) by direct selection of Ts⁺ recombinants on TBAB plates, at 48 °C, after incubation with 1-5 μg plasmid DNA ml⁻¹ (employed for L6601, L6602, L6476, L6605, L6607 and L6477), or (ii) by indirect selection (used for mutants where the background of revertants was high or the Ts phenotype too leaky, i.e. strains L6604, L6606, L6613 and L6614, as shown in Table 3). In the latter case, crosses were performed with a 1:1 mixture of plasmid and chromosomal DNA from the original mutant carrying the homologous ts marker (L650i series). arg⁺ recombinants were selected at 30 °C. Ts⁺ recombinants among Arg⁺ colonies, obtained by congression, were identified on TBAB replica plates at 48 °C.
Table 1. *Bacillus subtilis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>M22</td>
<td>purA16 leuA8 metB5 iicA1</td>
<td>Karamata &amp; Gross (1970)</td>
</tr>
<tr>
<td>L5028</td>
<td>hisA1 argC4 metC3 pyrA</td>
<td>Pooley &amp; Karamata (1984)</td>
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<tr>
<td>GSY403</td>
<td>pheA1 trpC2</td>
<td>C. Anagnostopoulos</td>
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<tr>
<td>QB935</td>
<td>aroD120 lys-1 trpC2</td>
<td>Dedonner et al. (1977)</td>
</tr>
<tr>
<td>BD21</td>
<td>str-1 cry-1 mic-1 hisA35*</td>
<td>D. Dubnau et al. (1967)</td>
</tr>
<tr>
<td>L6009</td>
<td>purA16 leuA8 metB5 hisA35</td>
<td>MNNG mutagenesis of BD21</td>
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<tr>
<td>L5087</td>
<td>hisA1 argC4 metC3</td>
<td>MNNG mutagenesis of L5009</td>
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<tr>
<td>L1440</td>
<td>Prototroph</td>
<td>MNNG mutagenesis of L5028</td>
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<tr>
<td>L6440</td>
<td>hisA1 argC4 leuA8 tag-1 φ29*</td>
<td>Same cross as for L6457 (Pooley et al., 1987)</td>
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<tr>
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<td>purA16 leuA8 ilcA1 tag-3 (rodC16) φ29†</td>
<td>MNNG mutagenesis of L5009†</td>
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<td>hisA1 argC4 metC3 tag-1 φ29*</td>
<td>MNNG mutagenesis of L5009</td>
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<td>L6477</td>
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<tr>
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<td>MNNG mutagenesis of L5009§</td>
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<td>MNNG mutagenesis of L5009†</td>
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<td>MNNG mutagenesis of L5009†</td>
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<td>MNNG mutagenesis of L5009†</td>
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<td>MNNG mutagenesis of L5009†</td>
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<td>L6614</td>
<td>hisA1 argC4 metC3 tag-20 φ29†</td>
<td>MNNG mutagenesis of L5009†</td>
</tr>
</tbody>
</table>

* A recombination index of less than 0.1 was obtained in a transformation cross with a *hisA1* recipient.
† Previous designation (Karamata et al., 1972).
‡ Resistant to φ29 at 37 °C on solid medium, but sensitive to this phage at 30 °C. No segregation of Ts and φ29" phenotypes was observed during transfer of the marker responsible for Ts phenotype.
§ Resistant to φ29 at 30 °C as well as 37 °C. This resistance phenotype is due to a closely linked mutation (probably *gtaD* or *gtaA*) shown by segregation of Ts and resistance phenotypes during crossing out of the marker conferring the former character.
|| See Methods.

Cell wall analysis. Cultures were grown in SAT (250 ml), and cells harvested in the late exponential phase (ND 200). Cell wall preparation and treatment at 100 °C with sodium dodecyl sulphate (2%, w/v) were essentially as described by Fein & Rogers (1976).

Freeze-dried cell walls were assayed for phosphate and diaminopimelic acid (DAP) according to Ames & Dubin (1960) and Work (1957), respectively.

**RESULTS**

Search for mutants characterized by Ts synthesis of the φ29 receptor

To isolate the predicted class of mutants, i.e. Ts for growth and φ29 plaque formation, a MNNG-mutagenized population was shifted to 37 °C, an intermediate temperature, and three generations later infected with φ29. Following the onset of lysis, survivors were plated, and colonies which developed at 30 °C were examined for Ts growth and resistance to phages φ29 and φ25 (Table 2). Although the proportion of auxotrophs was not significantly altered by the
Table 2. Enrichment for mutants with Ts growth and φ29 susceptibility among colonies surviving φ29 treatment at 37 °C

Following expression for 20 h at 22 °C, a MNNG-mutagenized population of *B. subtilis* 168 (strain L5009) was gradually increased (20 min) to 37 °C and, after three doubling times, infected by phage φ29 at a multiplicity of infection of over 20. One hour later, survivors were filtered, washed, plated onto L medium, incubated at 30 °C and screened for Ts colonies by replica-plating at 48 °C. Representative samples of Ts and Ts+ populations were examined for phage sensitivity by spotting phage onto freshly streaked cells and incubating at 30 or 37 °C.

<table>
<thead>
<tr>
<th>Growth phenotype</th>
<th>Phage sensitivity at 30 °C*</th>
<th>Ts φ29 susceptibility among class A isolates†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Class</td>
<td>φ29</td>
</tr>
<tr>
<td>Ts</td>
<td>44</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Ts+</td>
<td>78</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
</tbody>
</table>

* + and − indicate sensitivity and resistance respectively.
† Mutants sensitive to φ29 at 30 °C and resistant at 37 °C. Two mutants (L6511 L6512) harbouring *issG* markers (see text), did not grow sufficiently at 37 °C to allow a meaningful test.

Enrichment procedure, that of mutants Ts for growth at 47 °C increased at least tenfold, i.e. from less than 1%, the figure normally obtained for MNNG-mutagenized populations being 0.1–0.2% (Karamata & Gross, 1970), to 5% (15 out of 306). Several phage resistance phenotypes were identified (Table 2). The spectrum of phage resistance within a representative sample of Ts+ colonies was identical to that previously described (Pooley et al., 1987), i.e. over 80% of phage-resistant colonies were resistant to both φ29 and φ25 – corresponding to *gtaB*, *C* or *E* markers (class C) – whereas the remaining ones were sensitive to φ25 – corresponding to *gtaA* and *D* markers (class B). The presence of colonies with wild-type phage sensitivity at 30 °C (class A) suggests that a small proportion of wild-type cells may have survived the selection for resistance to φ29. In comparison, mutants Ts for growth exhibited a different phage resistance spectrum; a relatively high proportion of them were sensitive to φ29 and to φ25 at 30 °C (class A). Of 15 such mutants, 13 grew sufficiently at 37 °C to allow phage sensitivity to be measured; nine of these were phage-resistant at 37 °C. Therefore, in this class, Ts φ29 susceptibility and Ts growth are correlated in many cases. In conclusion, the enrichment procedure yielded mutants of the predicted phenotype, which are candidates for having a conditional defect in φ29 susceptibility, i.e. in teichoic acid synthesis.

Genetic analysis of Ts mutants which survived φ29 treatment

Recent (Karamata et al., 1987) and previous (Young et al., 1969; Boylan et al., 1972) evidence that teichoic acid genes are clustered near the *hisA* locus prompted a screening, by PBS1 transduction, for linkage to *hisA* of the mutations responsible for the Ts φ29 susceptibility. However, in view of (i) the nature of the mutagen used, MNNG, which is known to induce clusters of linked mutations affecting neighbouring genes (Guerola et al., 1971), and (ii) the proximity of *tag* and *gta* genes, Ts growth mutants resistant to φ29 at both 30 and 37 °C (classes B and C) were also examined. Indeed, the presence of a *gta* marker could have masked an as yet undiscovered locus involved in the synthesis of the φ29 receptor. Thus, the 44 Ts growth mutants isolated here (Table 2) were treated with PBS1 produced on a wild-type donor, L1440, and at least 100 *his*+ transductant colonies were screened for Ts+ by replica plating for those growing at 47 °C. Among 39 mutants able to be transduced, 12 showed linkage to *hisA*, with co-transduction frequencies ranging between 50% and 80% (not presented). When the results were examined in relation to the previously determined phage resistance phenotype at 30 °C (Table 2), a strikingly non-random distribution was observed with respect to *his*-linked Ts markers. Of the 15 mutants...
of class A, seven exhibited a Ts φ29 susceptibility and carried a hisA-linked Ts growth mutation. Two were Ts φ29 susceptible but their Ts growth phenotype was due to a mutation not linked to hisA: these were not studied further. The two mutants that did not grow sufficiently at 37 °C for a test of phage sensitivity harboured Ts growth markers linked to hisA and shown below to map in lssG. The four remaining mutants were φ29 sensitive at 37 °C and had a Ts growth defect that was not linked to hisA. Thus, nine of the twelve his-linked Ts growth markers were found among class A. The three remaining his-linked mutants with a Ts growth phenotype were found within class B (φ25, φ29) and were shown (see below) to harbour two mutations: one conferring Ts growth as well as Ts susceptibility to φ29, and a second, probably gtaA or gtaD, conferring φ29 resistance at all temperatures. None of the Ts growth mutants belonging to class C, showing resistance to both phages, harboured a Ts growth marker linked to hisA. These were probably double mutants containing a gta marker, and an unlinked one, affecting some unrelated essential function, associated with the Ts phenotype.

Inspection by phase-contrast microscopy of the 12 mutants harbouring his-linked mutations (see above) revealed that after a shift to 47 °C, cells of 10 strains – all those with Ts φ29 susceptibility – presented a coccal morphology, characteristic of the previously described mutants affected in the tag locus (Cole et al., 1970; Rogers et al., 1970; Shifflett et al., 1977). The latter include rodC, recently remapped (Pooley et al., 1987). The remaining two strains, L6511 and L6512, retained a rod shape at 47 °C. These observations prompted a screening for linkage to the tag-1 locus by determining the recombination index (RI) between newly identified mutations and the reference tag-1 marker. Transformation crosses were performed with donor DNA prepared from all strains carrying newly identified mutations linked to hisA, i.e. L6501 to L6507 and L6501 to L6508 and L6511 to L6514, and an auxotrophic recipient L6476 harbouring the reference tag-1 marker. A saturating DNA concentration was used to ensure congression. At least 600 Met+ recombinants were scored for Ts+, and for a second, reference marker (arg+). It appeared that the 10 mutants exhibiting the coccal cell morphology at 47 °C were more or less closely linked to tag-1 (RI between 0-17 and 0-41, data not shown) and they were thus designated as tag markers. The mutants L6511 and L6512, which remain rod-like at 47 °C (see above), showed no linkage to tag-1 (RI of 1). The higher co-transduction index with hisA, and the phenotype of these mutants, recalled the properties of a previously identified marker, lssG20 (Brandt & Karamata, 1987). Transformation crosses with the latter marker yielded RI values of 0-1 or less (not presented), strongly suggesting the presence of mutations in the same gene. The relevant markers were designated lssG11 and lssG12, and were not studied further.

Mutants L6505 and L6507, belonging to the group of three class B Ts mutants which exhibit the Tag-like morphology, harbour two mutations: a tag mutation conferring Ts φ29 susceptibility, and a closely linked φ29 resistance mutation most likely affecting either the gtaA or the gtaD locus (Pooley et al., 1987). This was revealed in one case by congression during the construction of strain L6605 (Table 1) and for L6507 during a direct selection for Ts+ recombinants with wild-type donor DNA; 10% retained the gta marker.

Between six and ten Ts+ revertant colonies were isolated at 47 °C from the confluent growth zone on L medium of several mutants – L6601 (tag-1), L6602 (tag-12), L6603 (tag-13), L6613 (tag-19), L6456 (tag-3) – and from L6605, a group B mutant, in which the tag-15 marker had segregated from a second closely linked gta marker present in the original isolate L6505. L6607 served as control in view of the presence of both tag and gta markers. Of 42 Ts+ revertants of mutants L6601, L6602, L6603, L6605, L6613 and L6456, 40 had become sensitive to φ29 at 37 °C whereas 18 Ts+ revertants of the control double mutant L6607 all retained the parental resistance to this phage at 37 °C. The simultaneous loss of both Ts phenotypes among Ts+ revertants of these mutants shows that mutation in a single locus is responsible for both phenotypes.

As more than 80% of mutants isolated here with Ts φ29 susceptibility contained markers mapping in the tag locus, this phenotype offers a highly specific means of obtaining conditional lethal mutants affected in teichoic acid synthesis. Although several previously described tag markers were not isolated by this selection method, all that were examined share a temperature-dependent φ29 resistance phenotype (Shifflett et al., 1977).
Assignment of tag markers to the physical map

Localization of most of the newly isolated tag markers was achieved with plasmid pAH101 and its derivatives pAH120 and pAH102. Plasmid pAH101 contains 11 kb of B. subtilis chromosomal DNA and was identified as correcting the tag-3 (rodC) marker which was recently shown (Pooley et al., 1987) to be closely linked to tag-1, gtaA and gtaD markers. Assessment of the ability of pAH101 and its subclones to transform Ts mutations showing linkage to tag-1 led to identification of three groups (Fig. 1).

Markers tag-11, tag-12 and tag-1, which apparently had no alleles on pAH101, were assigned to group I. The latter has since been shown to be on a fragment separated by over 7 kb from tag-3 (Mauël et al., 1989). The remaining tag mutations, all corrected by pAH101, were divided into two groups: group II markers, tag-15, tag-17 and tag-20, were corrected by pAH120 but not by pAH102, whereas group III, which includes tag-3, tag-13, tag-14, tag-16 and tag-19, was located on a 2 kb fragment common to both pAH120 and pAH102. Determination of recombination indexes by pairwise two-factor transformation crosses (Briehl, 1987) of several representatives of each group provided the order: group I (tag-11, tag-12, tag-1), group II (tag-15, tag-17, tag-20), group III (tag-13, tag-14, tag-16, tag-18, tag-3). Comparison of the restriction map of B. subtilis DNA cloned in pAH101 with that of a larger region flanking tag-3, cloned by Mauël et al. (1989), allowed orientation of this segment with respect to hisA, i.e. group I, group II, group III, hisA. Inspection of Fig. 1 reveals that groups II and III lie within a fragment of about 4 kb. Nevertheless, tag markers are distributed over an appreciably larger segment since tag-3 and tag-1 (group I) are separated by over 7 kb (Mauël et al., 1989). This is consistent with the mutations identified here affecting several essential genes whose products are involved in φ29 susceptibility.

Interestingly, group II contained exclusively mutations derived from three original mutants sensitive to φ25, but resistant to φ29 at 30 °C (Table 2, class B) which, in two instances, were shown (see above) to be due to the simultaneous presence of tag and neighbouring gta mutations. Thus, the tag gene(s) in group II is (are) probably very close to gtaA and D loci.
Teichoic acid mutants of *B. subtilis*

Table 3. Cell wall content of phosphate and DAP for cells grown at permissive (30 °C) and restrictive (47 °C) temperatures

Cultures (250 ml in SAT) grown at 30 °C were shifted to 47 °C at an OD<sub>640</sub> of 0.01 by gradually increasing the bath temperature over a 15 min period. For all cultures, cells were harvested at an OD<sub>640</sub> of 0.2, which was reached by different mutants 2-3.5 h after the shift. Cell wall preparation and chemical assays were according to standard procedures. Phosphate and DAP contents are given in μmol (mg cell wall dry weight)<sup>-1</sup>. Controls grown at 30 °C were also harvested at OD<sub>640</sub> 0.2.

<table>
<thead>
<tr>
<th>Linkage group</th>
<th>Strain</th>
<th>Marker</th>
<th>Phosphate</th>
<th>DAP</th>
<th>R*</th>
<th>R/R&lt;sub&gt;0&lt;/sub&gt;</th>
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<th>DAP</th>
<th>R*</th>
<th>R/R&lt;sub&gt;0&lt;/sub&gt;</th>
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* Ratio of phosphate concentration to that of DAP. R<sub>0</sub>, ratio for wild-type strain carrying the tag<sup>+</sup> allele.

Teichoic acid and peptidoglycan contents of the cell wall fraction of mutants with Ts φ29 susceptibility, incubated at permissive and restrictive temperatures

Synthesis of poly(groP) and of its linkage unit requires several genes. Previous work (Karamata *et al.*, 1987) strongly suggested that they are all clustered near the tag-1 marker. Our observations on the newly identified markers, i.e. (i) their distribution over a DNA segment of more than 4 kb and (ii) the Tag-like morphology they confer at 47 °C, provide new support for this conclusion, and raise the possibility that under restrictive conditions all such mutations may, like tag-1, specifically affect poly(groP) synthesis.

To test this prediction, cell walls were prepared by standard procedures from cultures of strains grown at both the restrictive and the permissive temperatures. At 30 °C, they were harvested in the mid-exponential phase (OD<sub>640</sub> 0.2). After shifting to 47 °C, cells were incubated until their mass increase was at least 20-fold to ensure that any remaining wall formed at the permissive temperature was diluted at least 20-fold (see Methods) even in the absence of cell wall turnover. The ratio of teichoic acid to PG in wall preparations was determined by measurement of specific components, i.e. phosphate and DAP, respectively (Table 3). At the permissive temperature, all the mutants had phosphate/DAP ratios comparable to that of the wild-type, but at the restrictive temperature they showed, without exception, like tag-1-bearing strains (Boylan *et al.*, 1972), a marked and specific reduction in the content of phosphate. At 30 °C the DAP contents of all the mutants were close to that for the wild-type, whereas at 47 °C, they were markedly (between 50% and 85%) higher than that in the wild-type. This increased value is to be expected for cell walls with little teichoic acid, of which PG must represent by far the greater part.

A strong inhibition of teichoic acid incorporation into the cell wall is the dominant overall feature. However, this inhibition was far from uniform, as phosphate/DAP values were between 3% and 42% of that of the wild-type, suggesting an overall reduction in cell wall teichoic acid of at least 60%, and in some cases of more than 95%. An inhibition of 80% or more was found for 8 of the 12 mutants examined. It appears that incorporation of phosphate into the cell wall at the restrictive temperature could continue in most if not all of the mutants. Although the phosphate/DAP values for some mutants (notably L6602 and L6476) of barely 3% appear...
compatible with a complete block in teichoic acid incorporation, a low rate of phosphate incorporation into the cell wall, equivalent to 3-4% of that of the wild-type, cannot be excluded. Inspection of data (Table 3) for strains grouped according to the preliminary physical mapping (Fig. 1) reveals that phosphate/DAP values are not distributed randomly. Markers belonging to group I are all characterized by very low phosphate/DAP ratios; three of the four lowest values (below 0.07) are found in this group. Group II markers also form a homogeneous group, with phosphate/DAP values ranging from 0.14 to 0.2, i.e. on average fourfold higher than those of the first group. Group III, comprising half the markers, is heterogeneous; several mutants have ratios close to 0.4 – an order of magnitude higher than those characterizing group I – whereas the remainder includes markers with much lower values (0.06-0.07), overlapping with those of the first group. Although these correlations do not permit conclusions to be drawn as to the nature of the block affecting teichoic acid synthesis in different mutants, they strongly encourage belief that different defects are likely to be revealed by biochemical and enzymic assays.

**Discussion**

We have identified a class of mutants with a Ts growth phenotype, deficiency in cell wall phosphate, and Ts susceptibility to phage φ29, most probably due, for each mutant, to one and the same mutation. This phenotype provides new evidence supporting the essentiality for cell growth of the synthesis of glucosylated poly(groP) – the φ29 receptor. However, since mutants affected in the glucosylation of the latter polymer are not impeded in their growth, our data can only be interpreted in favour of an essential role in cell growth of the main polyol chain of poly(groP). Nevertheless, a previous study (Rogers et al., 1974) of a mutant harbouring the tag-1 marker reached a different conclusion. Following transfer from permissive (30 °C) to restrictive (45 °C) temperature, careful measurements of PG synthesis, both chemically, and by fractionation of cells labelled with [1-14C]GlcNAc, revealed a four- to fivefold specific increase in the ratio of PG relative to protein, or to optical density. No such increase occurred with the wild-type strain. This result was attributed to a selective derepression of PG synthesis, and led to the suggestion that the reduced content of teichoic acid in the cell wall was essentially due to a relative dilution, rather than to a specific inhibition of teichoic acid synthesis. This explanation, invoked by the authors of a subsequent study (Shifflett et al., 1977) identifying new tag-like markers, has not been put in question, although the authors of the original study modified their earlier conclusion, suggesting that some inhibition of teichoic acid synthesis accompanied derepression of PG synthesis (Rogers, 1979). In view of this, the effect of tag-1, and of many of the tag markers described here, on the relative and absolute rates of synthesis of PG and protein have been re-examined at the restrictive temperature. The results (not presented) revealed a significant increase in PG to protein ratio, confirming the previous finding with tag-1. Nevertheless, as measured by labelling with [1-14C]GlcNAc, the kinetics of PG synthesis in wild-type and mutants reveal minimal differences in absolute rates, whereas with several tag-bearing mutants, but not the wild-type, a markedly decreased rate of incorporation into protein was observed. Thus, it is essentially through decreased protein synthesis, rather than increased PG synthesis, that the observed variation of the ratio of PG to protein is obtained. Accordingly, the marked decrease in the cell wall teichoic acid content, characterizing all tag markers under restrictive conditions, can only be due, we believe, to a specific reduction in the incorporation of this polymer in the absence of any significant change in the rate of PG synthesis.

Not surprisingly, the markers in linkage group III associated with relatively high wall phosphate content under restrictive conditions, tag-14, tag-16, tag-18 and tag-19, were also accompanied by a more leaky Ts growth phenotype than was the case for remaining markers.

Mapping of tag mutations, obtained in this and previous studies, revealed that they were all clustered in a relatively small region around 310° on the B. subtilis chromosome. This observation is consistent with the analysis of 168/W23 hybrids which has shown (Karamata et al., 1987) that exchange in that region of a small 168 segment by a 'functionally' analogous segment from strain W23 resulted in synthesis of poly(ribitol phosphate) [poly(rboP)], implying
that genes responsible for poly(groP) were exchanged for those determining the synthesis of poly(rboP). Physical mapping revealed that the majority of tag markers span a 4 kb interval, and further mapping (Mauel et al., 1989) has shown that the outside tag markers are 7–8 kb apart, and contain at least two transcription units. Such a region could accommodate genes encoding most, if not all, of the enzymes involved in the synthesis of poly(groP) and the PG-linkage unit.

In conclusion, our observations reveal that all tag mutants so far isolated are Ts in the synthesis of the \( \phi 29 \) receptor and for incorporation of phosphate into the cell wall, strongly suggesting that they are deficient in poly(groP) synthesis. Although in such mutants mass increase continues for some time at high temperature they are unable to grow indefinitely, which leads us to conclude that the synthesis of poly(groP) is essential for cell growth. A related study (Mauel et al., 1989), aimed at insertional inactivation of tag genes, reached the same conclusions.

The tag mutants examined here reveal good correlation between mapping and phenotype, i.e. linkage groups I and II are characterized by a quantitatively homogeneous phosphate incorporation. Identification of enzymes encoded by different linkage groups will open the way to investigation of the role of the major anionic polymer through morphological and biochemical characterization of mutants blocked at different steps in the synthesis of poly(groP) and the PG-linkage unit.

Finally, that poly(groP) synthesis must play a role in cell morphogenesis appears incontrovertible now that 12 tag markers distributed in several linkage groups exhibit, without exception, a disturbed morphology under restrictive conditions.

We thank Professor N. H. Mendelson for his interest, Monique Evard for excellent technical assistance, and George Stewart and Alan Honeyman for the generous gift of plasmid pAH101 and subclones pAH102 and pAH120.

**REFERENCES**


