The *gtaB* Marker in *Bacillus subtilis* 168 Is Associated with a Deficiency in UDPglucose Pyrophosphorylase

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Fifty-six mutants of *Bacillus subtilis* 168 were selected for resistance to bacteriophages $\phi 29$ or $\phi 25$. The mutations were all linked to previously described teichoic acid markers *gtaA*, *gtaB* or *gtaC*, for the first and last of which, the gene products have previously been identified. Each linkage group was shown to have two distinct phenotypes with respect to phage resistance and cell-wall galactosamine content. Recombination indexes of 0.35, 0.13 and 0.41 for groups A, B and C respectively were consistent with the presence of two average-sized genes in groups A and C. Correlation between genetic and phenotypic differences supported this conclusion and led to the designation of two new markers, *gtaD* and *gtaE*. Two- and three-factor transformation crosses suggested the order *hisA-gtaB-gtaD-gtaA-tag-1* and *gtaC-gtaE-argC*. Assays for UDPglucose pyrophosphorylase and phosphoglucomutase activities in soluble extracts of representative mutants revealed that, in contrast to previous findings, the former activity was virtually undetectable in all nine group B mutants examined, suggesting that *gtaB* is the structural gene of this enzyme. Our results allow us to account for discrepancies with respect to previous reports. The thermosensitive mutation previously designated *rodC1* was shown to be 90% cotransformable with *tag-1*. In view of their extremely similar phenotypes the former mutation was renamed *tag-3*, and the likely order obtained was *gtaA-tag-3-tag-1*. This suggests that many mutations associated with deformation of cell shape in *B. subtilis* are located in the region where teichoic acid genes map.

INTRODUCTION

The genetics of teichoic acid synthesis has received little attention since the identification of markers involved in glucosylation of poly(glycerolphosphate) [poly(groP)], the major wall teichoic acid in *Bacillus subtilis* 168 (Young, 1967; Yasbin *et al.*, 1976), and *tag-1*, a thermosensitive marker associated with a decreased cell-wall content of this polymer at the restrictive temperature (Boylan *et al.*, 1972). Recently, we have obtained interstrain *B. subtilis* 168/W23 hybrids by replacement of teichoic acid genes in strain 168 by the homologous region from strain W23 (Karamata *et al.*, 1987). The exchanged region, large enough to encompass 20 or more average-sized genes, contained most if not all of the teichoic acid genes in this organism, including previously identified *gtaA*, *gtaB* and *tag-1* markers.

Evidence has been obtained in favour of *tag-1* being involved in the synthesis of poly(groP) (Boylan *et al.*, 1972; Karamata *et al.*, 1987). Characterization of three *B. subtilis* *gta* markers, associated with resistance to bacteriophage $\phi 29$, led to the probable identification of the gene products for two of them (Young, 1967): *gtaA* codes for the membrane-associated UDPglucose

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Abbreviations: GlcNAc, *N*-acetylglucosamine; GalN, galactosamine; G1-P, glucose 1-phosphate; G1,6-DP, glucose 1,6-diphosphate; UDPG, UDPglucose; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PP_i, pyrophosphate; TEA, triethanolamine; PGM, phosphoglucomutase (EC 5.4.2.2); UDPGPPase, UDPglucose pyrophosphorylase (EC 2.7.7.9), poly(groP), poly(glycerolphosphate).

polyglycerol teichoic acid glucosyltransferase (EC 2.4.1), and *gtaC* for phosphoglucomutase (α -D-glucose 1,6-diphosphate: α -D-glucose 1-phosphate phosphotransferase, EC 5.4.2.2; PGM). The third activity required for glucosylation, UDPglucose pyrophosphorylase (UTP:glucose 1-phosphate uridylyltransferase, EC 2.7.7.9; UDPGPPase) was found to be normal in all three mutant classes, leaving the structural gene of the latter enzyme to be identified. In addition, the function associated with the *gtaB* marker, in which activities of all three enzymes were unaltered, remained unknown.

In the present work, as in a previous study (Young, 1967), resistance to ϕ 29 and ϕ 25 has been exploited to obtain a large number of mutants, partly to seek answers to the above questions and also to look for new markers affecting the synthesis of teichoic acids, a class of polymers whose role(s) remains largely unknown (Baddiley, 1970; Archibald, 1974). We report genetic analyses, chemical analyses of cell walls and assays of the UDPGPPase and PGM activities in the crude soluble fraction of selected classes of mutants.

METHODS

Strains. The *B. subtilis* 168 strains listed in Table 1 were used. The bacteriophages employed were ϕ 29, ϕ 25 (laboratory stocks), and defective bacteriophages PBSY and PBSZ harboured by *B. subtilis* strains S31 and W23 respectively. Phage stocks were prepared as previously described (Mauël & Karamata, 1984; Karamata *et al.*, 1987).

Media. LA, TS plates and SAT, as well as media used for transformation and transduction experiments, were as previously described (Karamata & Gross, 1970; Pooley & Karamata, 1984; Karamata *et al.*, 1987). Where necessary, amino acids (20 to 60 μ g ml⁻¹ final concentration), uracil and adenine (100 μ g ml⁻¹) were added. The citrate medium (Young, 1967) used to characterize mutant markers was modified as follows: supplementary MgSO₄ was decreased from 5 to 4 mM and the pH was adjusted to 7.0 with 1 M-HCl to prevent precipitation of magnesium phosphate; the addition of a trace salts solution (Schlaeppli *et al.*, 1982) led to an increased growth rate. Amino acids were added to a final concentration of 40 or 60 μ g ml⁻¹.

Enzymes and biochemicals. Bakers' yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49), PGM, UDPGPPase, uridine diphosphoglucose (UDPG), glucose 1,6-diphosphate (G1,6-DP), sodium pyrophosphate (PP_i), glucose 1-phosphate (G1-P), NADP and NADPH were all obtained from Sigma.

Mutagenesis and selection of phage-resistant mutants. A seed culture of strain L5027 (or L5028), prepared in SAT medium by inoculation from a fresh LA plate, was aerated overnight in a tube at 22 °C. Cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described by Karamata & Gross (1970). In a typical experiment survival was 75% and the frequency of auxotrophs was 4%. After incubation overnight at room temperature, appropriate dilutions of the culture and ϕ 29 (or ϕ 25), at an m.o.i. of 100, were spread onto LA plates and incubated for 24 h at 37 °C. Surviving colonies, representing about 0.1% of the inoculum, were picked at random and tested for resistance to ϕ 29 (or ϕ 25) as described by Karamata *et al.* (1987). Out of 100 colonies examined all were resistant to the phage used.

Characterization of mutant markers. When strains are grown on a minimal medium in the absence or in the presence of glucose or galactose, in each case a growth response occurs which is characteristic for each of the markers *gtaA*, *gtaB* and *gtaC* (Young, 1967). The use of a multiple auxotroph L5027 (or L5028) as parent strain for this study led to a marked reduction in the maximum cell density obtained on this minimal medium, relative to the 168 *trpC2* strain (Young, 1967), which rendered interpretation of the different growth responses more difficult. Nevertheless, the test retained some utility in differentiating mutations belonging to various *gta* markers.

Genetic exchange. Methods for transformation, PBS1-mediated transduction and measurement of the recombination index have been described (Karamata & Gross, 1970; Pooley & Karamata, 1984).

Cell wall analysis. The procedures for cell wall preparation, phosphate analysis, hydrolysis and gas chromatography have been described elsewhere (Karamata *et al.*, 1987).

Estimation of cell-wall galactosamine (GalN) by selective extraction from whole cells labelled with *N*-acetyl[1-¹⁴C]glucosamine ([¹⁴C]GlcNAc). The method of Pavlik & Rogers (1973) was modified to give a radioassay, and whole cells rather than isolated walls were used. [Givan *et al.* (1982) also used whole cells to measure cell-wall GalN chemically]. An overnight culture grown in SAT medium containing unlabelled GlcNAc (50 or 100 μ M) at 22 °C was diluted to OD₅₄₀ 0.006 in the same medium, grown for five generations at 37 °C and diluted 20-fold into the same medium to which [¹⁴C]GlcNAc [final concentration 0.025–0.125 μ Ci ml⁻¹ (0.925–4.625 kBq ml⁻¹), in different experiments] was added. After five generations of labelling, cells (5 ml) were collected on a membrane filter, exposed to trichloroacetic acid (5%, w/v) at 4 °C for about 1 min and washed several times with water. Cells, resuspended in 5 ml 0.1 M-sodium citrate buffer, pH 4.0, were incubated at 100 °C for 30 min and again collected on a filter to obtain soluble and insoluble fractions. Sample preparation and counting were performed essentially as previously described by Pooley (1976). This method exploits the specific labelling of cell wall hexosamines

Table 1. *B. subtilis* strains

Strain	Genotype	Reference or construction*
M22	<i>purA16 leuA8 metB5 ilvA1</i>	Karamata & Gross (1970)
L1440	Prototroph	Pooley & Karamata (1984)
L5027	<i>hisA1 argC4 leuA8 ilvA1</i>	Pooley & Karamata (1984)
L5028	<i>hisA1 argC4 metC3 pyrA</i>	Pooley & Karamata (1984)
172ts200-B	<i>leuA8 metB5 tag-1</i>	Karamata <i>et al.</i> (1972)
Rod113	<i>leuA8 tag-3 (rodC1)†</i>	Karamata <i>et al.</i> (1972)
BC7	<i>hisA1 cysB3 trpC2 gtaC51</i>	Young <i>et al.</i> (1969)
BC9	<i>argC4 gtaA12</i>	Young <i>et al.</i> (1976)
QB510	<i>hisA1 gtaB290</i>	G. Rapoport
RUB810	<i>lys-3 metB10 gtaB20</i>	Yasbin <i>et al.</i> (1976)
L6118	<i>hisA1 argC4 leuA8 ilvA1 gtaE151</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6119	<i>hisA1 argC4 leuA8 ilvA1 gtaE152</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6125	<i>hisA1 argC4 leuA8 ilvA1 gtaE158</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6145	<i>hisA1 argC4 leuA8 ilvA1 gtaB114</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6147	<i>hisA1 argC4 leuA8 ilvA1 gtaB116</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6162	<i>hisA1 argC4 leuA8 ilvA1 gtaD2</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6165	<i>hisA1 argC4 leuA8 ilvA1 gtaD1</i>	MNNG mutagenesis of L5027, ϕ 29 ^r
L6170	<i>hisA1 argC4 metC3 pyrA gtaE180</i>	MNNG mutagenesis of L5028, ϕ 25 ^r
L6179	<i>hisA1 argC4 metC3 pyrA gtaC189</i>	MNNG mutagenesis of L5028, ϕ 25 ^r
L6188	<i>hisA1 argC4 metC3 pyrA gtaC198</i>	MNNG mutagenesis of L5028, ϕ 25 ^r
L6254	<i>hisA1 argC4 metC3 pyrA gtaB123</i>	MNNG mutagenesis of L5028, ϕ 25 ^r
L6199	<i>hisA1 argC4 metC3 pyrA gtaB⁹100</i>	MNNG mutagenesis of L5028, ϕ 25 ^r
L6220	<i>hisA1 argC4 leuA8 ilvA1 gtaB⁹101</i>	MNNG mutagenesis of L5027, ϕ 25 ^r
L6221	<i>hisA1 argC4 leuA8 ilvA1 gtaB⁹102</i>	MNNG mutagenesis of L5027, ϕ 25 ^r
L6222	<i>hisA1 argC4 leuA8 ilvA1 gtaB⁹103</i>	MNNG mutagenesis of L5027, ϕ 25 ^r
L6200	<i>hisA1 argC4 ilvA1 gtaB⁹100</i>	Congression of L6199 → L5027
L6225	<i>hisA1 argC4 metC3 gtaB⁹101</i>	Congression of L6220 → L5028
L6228	<i>hisA1 argC4 metC3 gtaB⁹102</i>	Congression of L6221 → L5028
L6238	<i>hisA1 argC4 gtaB⁹102</i>	Congression of L6228 → L5027
L6240	<i>purA16 leuA8 ilvA1 gtaB116</i>	Congression of L6147 → M22
L6256	<i>hisA1 argC4 metC3 gtaD2</i>	Congression of L6162 → L5028
L6331	<i>purA16 leuA8 ilvA1 gtaB515</i>	Pooley & Karamata (1984)
L6333	<i>purA16 ilvA1 metB5 gtaA12</i>	Karamata <i>et al.</i> (1987)
L6334	<i>purA16 leuA8 ilvA1 gtaD1</i>	Congression of L6165 → M22
L6440	<i>hisA1 argC4 leuA8 tag-1</i>	Pooley & Karamata (1984)
L6457	<i>purA16 leuA8 ilvA1 tag-3</i>	Congression of Rod113 → M22
L6463	<i>purA16 metB5 tag-1 gtaA12‡</i>	Congression of 172ts200-B → L6333
L6464	<i>hisA1 argC4 metC3 tag-1</i>	Congression of L6440 → L5028
L6473	<i>hisA1 metC3 tag-1 gtaB515</i>	Congression of L6331 → L6464
L6475	<i>hisA1 leuA8 tag-1 gtaD1§</i>	Congression of L6334 → L6440

* MNNG mutagenesis was followed by direct selection for resistance to the bacteriophage indicated. Transformation crosses are denoted by an arrow pointing from donor to recipient; markers were transferred by congression with saturating concentrations of DNA (1–5 μ g ml⁻¹).

† Previous designation.

‡ This strain, like another one carrying *tag-1* and *gtaA12* markers, is resistant to ϕ 25 at 30 °C and 37 °C.

§ This strain, like several transformants with the same genotype obtained in another cross, does not grow on plates at 37 °C. At 30 °C it forms small colonies whose morphology resembles that of *gtaB*- and *gtaC*-containing strains (see Results). The resistance to ϕ 29 is not expressed on LA.

during growth in the presence of [¹⁴C]GlcNAc (Pooley, 1976). Confirmation of the specific extraction of labelled GalN is obtained by the insignificant (1%) release of radioactivity from *gtaB* and *gtaE* strains (Table 5), whose walls lack GalN (Young, 1967, and data not shown).

Preparation of cell extracts for the assay of UDPGPPase and PGM activities. Cultures (250 ml) were grown at 37 °C in SAT medium supplemented with MgSO₄ (5 mM). Cells in the late exponential phase (2 × 10⁸ ml⁻¹; 0.3 mg dry wt ml⁻¹) were harvested by pouring onto ice and centrifuging. In some control experiments cells were washed in various media or buffers (see below) and sedimented by centrifugation. The pellet was rinsed several times with water (0 °C). Thereafter, pellets rinsed or washed (by resuspension and resedimentation) were

suspended in buffer (2.5 ml). Suspensions were sonicated for a total of 40 s (5 s pulses followed by 45 s cooling periods) in a tube surrounded by an ice-bath. Cell debris and unbroken cells were removed by centrifugation (20 min at 20000 g) and the supernatant was immediately assayed for UDPGPPase and PGM activities. Protein concentration in the soluble fraction, assayed with the Bio-Rad reagent (Bradford, 1976), was in the range 7–20 mg ml⁻¹.

In other experiments following previously described protocols, the washing medium used (at 0 °C) was water, Spizizen's salts (as in Maino & Young, 1974), SAT medium (with various concentrations of glucose), or one of the following buffers: Tris/HCl (pH 7.0, 50 mM) as in Young (1967); Tris/HCl (pH 8.0, 50 mM) to which MgSO₄ (2.5 mM) and EDTA (0.5 mM) had been added (Forsberg *et al.*, 1973); Tricine (pH 8.0, 100 mM) as in Edmundson & Ashworth (1972); potassium phosphate (pH 6.5, 10 mM) containing EDTA (0.2 mM) (Maino & Young, 1974) or triethanolamine (TEA) (pH 8.0, 100 mM) containing various concentrations of Mg²⁺ and EDTA (see below). Sigma triethanolamine buffer (product no. 665-5), as supplied with unspecified concentrations of Mg²⁺ and EDTA, was also used.

UDPGPPase assay. The enzyme was assayed in the direction of G1-P formation, beginning with UDPG and PP_i. Activity was measured by coupling three reactions (Kalckar, 1955) leading to the formation of NADPH, measured by its absorbance at 340 nm. One unit of activity represents the conversion of 1 nmol UDPG to G1-P or an increase in A_{340} of 0.0063 min⁻¹ at 25 °C.

The reaction mixture used in most cases was as follows: MgSO₄ (2.5 µmol), EDTA (2.2 µmol), NADP (0.5 µmol), glucose-6-phosphate dehydrogenase (0.14 units), PGM (0.05 units), UDPG (0.9 µmol), G1,6-DP (2 or 14 nmol), PP_i (1.1 µmol), cell extract and TEA buffer (pH 8.0, 100 µmol) in a total volume of 1 ml. Stock solutions of UDPG, G1-P and G1,6-DP in TEA were either prepared freshly for each series of assays or, in a few cases, stored frozen.

UDPGPPase activity in crude extracts was stable for at least 1 h at 0 °C, and was proportional to total protein concentration up to about 100 units per ml of the reaction mixture. According to the activity present, between 50 and 600 µl of extract was assayed, such that the A_{340} increase was less than 0.6 absorbance units min⁻¹. All components of the reaction mixture were essential for the maximum rate of NADPH production.

Optimal results for the reaction were obtained when all components except UDPG, G1,6-DP and PP_i were incubated for 10 min at 25 °C, followed by the addition of UDPG and G1,6-DP; 5 min later, the reaction was initiated by the addition of PP_i. A short lag was followed by a linear increase in A_{340} , lasting between 30 s and 1 min, after which the rate of A_{340} increase slowed, to be followed by a phase of decrease in A_{340} . A second addition of PP_i provoked a reaction similar to the initial one. Subsequent addition of PP_i, particularly if the quantity was doubled or tripled, gave, after a considerable lag, a linear reaction, usually slower than the initial one, which often lasted up to 10 min.

Initial difficulties in obtaining UDPGPPase activity in extracts led to examination of the importance of various factors affecting its activity. In view of the instability of the enzyme (Young, 1967; Yasbin *et al.*, 1976) particular attention was given to the methods of cell handling prior to breakage. When harvested cells were washed by resuspension in water, Spizizen's salts, or any of the above-mentioned buffers, the UDPGPPase activity was found to be either zero or extremely low, in contrast to previous reports. However, washing cells with SAT or the same medium with a decreased glucose content (0.02%) yielded extracts with activities about half the values obtained with the technique finally adopted, i.e. omission of the cell washing step. Partial retention of activity when cells were washed with complete medium suggested that activity might be lost when cell metabolism was halted, in the absence of glucose, for example. The delay between harvesting and breaking cells was an important factor – we found that the longer the delay, the lower the final activity obtained. Omitting the washing step allowed a significant shortening of this delay, which was around 25 min in the method finally adopted. Removal of contaminating medium from the cell pellet by rinsing was, however, found to be essential, because of an interfering reaction. Indeed, cell extracts obtained without rinsing, or by deliberate addition of traces of medium, led to rapid increase of A_{340} in the absence of UDPG, PP_i and G1,6-DP. Medium components essential for the latter reaction were glucose and phosphate, suggesting the presence of phosphokinase. We found that contaminating medium was decreased to tolerable levels by rinsing the pellet several times with water, the technique which gave highest values for UDPGPPase activity.

The choice of buffer in which cells were resuspended for sonication was also important: the use of Tris/HCl pH 7.0 (Young, 1967), Tricine pH 8.0 (Edmundson & Ashworth, 1972), or phosphate, pH 6.5 (Maino & Young, 1974) led to low or zero activities. TEA alone (pH 8.0, 100 mM), or with MgSO₄ (2.5 mM) and EDTA (2.2 mM) (the buffer finally adopted), gave the highest activities consistently.

The choice of buffer used in the assay mixture had less influence on the final result, and Tris pH 8.5 and TEA gave comparable values. However, the relative concentrations of Mg²⁺ and EDTA were found to be extremely important. With Mg²⁺ at 2.5 or 5 mM and EDTA at 0.5 mM, UDPGPPase activity was barely detectable or absent. With Mg²⁺ at 2.5 or 5 mM and EDTA at 2.2 or 4.5 mM, respectively, UDPGPPase was readily detectable. The Sigma TEA buffer, as supplied with Mg²⁺ and EDTA of unspecified concentrations, when used in the assay mixture gave results comparable to those obtained in the last two conditions, and was employed in some cases.

Table 2. *Distribution of $\phi 29^r$ mutants into linkage groups*

$\phi 29^r$ mutants were distributed into linkage groups by PBS1 co-transduction with reference markers to which previously isolated *gta* mutations were shown to be linked (Young *et al.*, 1969). The homogeneity of each group was assessed by growth on different carbon sources in liquid media (Young, 1967), and by their distinctive colony morphology.

Linkage group	Reference mutation	Colony morphology	No. of mutations (%)	Highest recombination index*
<i>gtaA</i>	<i>gtaA12</i>	Rough (wild-type)	10 (18)	0.45
<i>gtaB</i>	<i>gtaB515</i>	Smooth†	16 (29)	0.13
<i>gtaC</i>	<i>gtaE151‡</i>	Smooth†	30 (53)	0.41
Total examined:				56 (100)

* To determine the maximum distance between *gta* markers within each group, mutations with highest and lowest PBS1 co-transfer indexes with the reference marker were chosen, and their recombination indexes determined by transformation. Five to ten crosses were performed in each case. Allele numbers giving the highest recombination indexes to the reference mutations were *gtaD2*, *gtaB*103* and *gtaC189* respectively.

† Such colonies were small, shiny, raised and round.

‡ The *gtaC* linkage group contains two genes (see Results); the second is designated *gtaE*.

PGM assay. The activity was measured in the direction of glucose 6-phosphate formation, starting from G1-P. Coupling with glucose 6-phosphate dehydrogenase activity leads to formation of NADPH, measured as A_{340} . One unit was defined as for UDPGPPase (see above). Crude extracts were prepared as for the UDPGPPase assay and the PGM activity was determined by two methods, based on those of (a) Forsberg *et al.* (1973) and (b) Maino & Young (1974). In the method usually used (a), the reaction mixture was: glucose-6-phosphate dehydrogenase (0.05 units) G1-P (1 μ mol), G1,6-DP (2 nmol), NADP (1 μ mol), $MgSO_4$ (2.5 μ mol) EDTA (2.2 μ mol) and TEA (pH 8.0, 100 μ mol) in a total volume of 1 ml. The reaction was initiated by addition of G1-P to the remaining components prewarmed to 25 °C. Method (b), used occasionally, was as follows. Between 50 and 600 μ l cell extract in TEA buffer (pH 8.0, 100 mM) containing $MgSO_4$ (2.5 mM) and EDTA (2.2 mM) was incubated for 15 min at 25 °C. Then, glucose-6-phosphate dehydrogenase (0.05 units) G1,6-DP (17 nmol), NADP (1 μ mol) and the above-mentioned buffer, prewarmed to 25 °C, were added to give a total volume of 0.99 ml. The reaction was initiated by the addition of G1-P (1 μ mol). With both methods PGM activity was comparable irrespective of the buffer used in cell washing or sonication. With method (b) it was confirmed that preincubating the extracts for 15 min at 25 °C, prior to substrate addition, increased the measured activity several fold.

During the assays of both UDPGPPase and PGM, the NADPH formed is degraded by an NADPH oxidase present in crude extracts (Young, 1967; Yasbin *et al.*, 1976). This activity was measured by the decrease in A_{340} following addition of NADPH to crude extracts prepared for the assay of UDPGPPase.

RESULTS

Preliminary characterization of B. subtilis mutants resistant to phage $\phi 29$

In a search for new genetic determinants involved in teichoic acid synthesis, a collection of mutants resistant to bacteriophage $\phi 29$ ($\phi 29^r$) were obtained by direct selection from a mutagenized population of *B. subtilis* 168. Their relatedness to previously isolated and mapped $\phi 29^r$ mutations, *gtaA*, *gtaB* and *gtaC*, all involved in glucosylation of poly(groP), was first ascertained by PBS1 transduction of 56 mutants chosen at random (Table 2). Thirty mutations were linked to *argC*, to which the *gtaC* marker has been shown previously to be linked (Young *et al.*, 1969). The remaining 26 mutations were about 50 % cotransducible with *hisA*, i.e. they mapped in the region containing *gtaA* and *gtaB* markers. Distinction between mutants belonging to the latter groups was tentatively achieved on the basis of their growth characteristics in liquid media on appropriate carbon sources (see Methods). It appeared that 10 strains were of the *gtaA* type whereas 16 were *gtaB*-like (data not presented). The colony morphology on TS medium provided a useful means of distinguishing *gtaA* strains from other classes: all 10 putative *gtaA* strains formed rough colonies, indistinguishable from the $\phi 29$ -sensitive parent, whereas *gtaB* and *gtaC* strains grew as small, raised, more or less shiny and

Table 3. *Phage resistance pattern of gta mutants*

Linkage group	Sensitivity/resistance* to phage:				No.
	$\phi 29$	$\phi 25$	PBSY	PBSZ	
A	—	+	—	+	7†
D	—	+	+	+	2
B	—	—	—	—	5‡
B ^o	—	—	+	+	3§
C	—	—	—	+	2§
E	—	—	—	—	10
Total examined:					29

* + and — indicate sensitivity and resistance respectively.

† Phage resistance pattern identical to that of the reference mutation *gtaA12* (Young, 1967).

‡ Phage resistance pattern identical to that of the reference mutation *gtaB290* (Young, 1967).

§ These strains were obtained by selection for $\phi 25$ resistance. *gtaB^o* mutations include *gtaB^o101*, *gtaB^o102* and *gtaB^o103*. *gtaC* mutations are *gtaC189* and *gtaC198*.

|| The phage resistance pattern of strain BC7 (*gtaC51*) was identical to this class.

round colonies. Thus, at this stage, all $\phi 29^r$ mutants identified here appeared, both genetically and phenotypically, to be related to previously described *gta* mutants (Young, 1967; Yasbin *et al.*, 1976).

To estimate the size of linkage groups A, B and C we determined, for each of them, recombination indexes between mutations which gave the smallest and the highest co-transfer indexes in PBS1 transduction experiments (see above). The highest recombination indexes, obtained by transformation, were 0.35, 0.13 and 0.41 for linkage groups A, B and C respectively (Table 2). Since a recombination index of 0.2 corresponds to an average-sized gene, coding for a 40 kDa protein (Bodmer & Ganesan, 1964; Henner & Hoch, 1980), group B probably consists of only one gene, whereas groups A and C could well contain more than one gene. It is not surprising that the latter marker, associated with PGM deficiency (Young, 1967), should contain two genes, at least. Indeed, SDS-PAGE analysis of *B. subtilis* preparations of PGM, an important enzyme involved in glycolysis as well as in the synthesis of many polysaccharides, revealed a complex pattern of polypeptide bands (Maino & Young, 1974). In *Neurospora*, two genes are required for formation of the PGM enzyme complex (Mishra & Tatum, 1970).

Phage resistance spectrum of gta mutations

To identify possible new classes among mutants belonging to the linkage groups described above, we examined the resistance of different $\phi 29^r$ (or $\phi 25^r$) mutants to both phages as well as to defective bacteriophages PBSY and PBSZ (Table 3). Among mutants examined belonging to group A, two were sensitive to all phages but $\phi 29$, whereas the remaining seven were resistant to $\phi 29$ and PBSY, as was a strain containing the previously identified marker *gtaA12* (Young, 1967). The close linkage between mutations harboured by the first two strains (recombination index less than 0.1) and their apparent distance (recombination index 0.3) from remaining *gtaA* mutations (including *gtaA12*) suggest that they belong to a separate gene, designated *gtaD*. Screening of linkage group B revealed that five of the strains examined were resistant to all four phages whereas four were sensitive to both PBSY and PBSZ. Nevertheless, the highest recombination index of group B is too small (Table 2) to warrant assigning these phenotypes to different genes. Results for enzyme activities provide further support for considering that all *gtaB* mutations belong to the same gene (see below). However, since these differences in phage sensitivity were found to be correlated with cell-wall GalN content (see below) we have designated mutations with the latter phenotype *gtaB^o*. Finally, group C mutants also split into two classes: ten were resistant to all phages whereas two were sensitive to PBSZ. The latter markers mapped at one extreme of this relatively large linkage group and were different from all other mutants in this group in their UDPGPPase activity (see below). These differences, and

Table 4. Cell wall teichoic acid composition of *gta* mutants

Results, obtained by GLC, are given in $\mu\text{mol (mg cell wall)}^{-1}$ for all components. Phosphate was determined colorimetrically (Ames & Dubin, 1960).

Strain	Mutation	Phosphate	Glycerol	Glucose	GalN
M22		1.28	0.84	0.45	0.16
L6333	<i>gtaA12</i>	1.43	0.85	0.15	0.20
L6334	<i>gtaD1</i>	1.3	1.0	0.23	0.19
L6331	<i>gtaB515</i>	1.5	0.92	0.04	0.02
L6225	<i>gtaB⁹101</i>	1.33	0.84	0.06	0.04
L6228	<i>gtaB⁹102</i>	1.05	0.67	0.06	0.07

Table 5. GalN-containing teichoic acids in cell walls of strains carrying different *gta* markers

Cells, continuously grown with [^{14}C]GlcNAc to specifically label cell wall hexosamines, were precipitated by 5% TCA and the GalN-containing teichoic acids were extracted by heating for 30 min at 100 °C at pH 4. Results were corrected for radioactivity incorporated into protein as previously described (Pooley, 1976).

Marker	No. of strains examined	GalN*
<i>gta</i> ⁺	2	15.3 $\pm$ 1.5
<i>gtaA</i>	6	20.7 $\pm$ 1.4
<i>gtaD</i>	2	18.4 $\pm$ 1.5
<i>gtaB</i>	9†	0.9 $\pm$ 0.1
<i>gtaB⁹</i>	3	4.5 $\pm$ 0.4
<i>gtaC</i>	2‡	9.5
		3.3
<i>gtaE</i>	6§	0.9 $\pm$ 0.1

* Expressed as a percentage of the total radioactivity incorporated into cell-wall hexosamines. The values are means $\pm$ standard error; a minimum of two independent measurements were made for each strain examined.

† Includes strains RUB810 (*gtaB20*) and QB510 (*gtaB290*).

‡ Strains L6188 and L6179.

§ Strain BC7 was identical to this class.

those in cell-wall GalN content (see below), led to the mutations in the former group being assigned to a new marker, designated *gtaE*. This is in agreement with the conclusion, made above on genetic grounds, that group C consists of at least two genes.

Anionic polymer components in cell walls of *gta* mutants

The presence of different phage-resistance phenotypes among all linkage groups suggested the existence of differences in cell-wall composition. Accordingly, cell walls, isolated from several mutant classes, were hydrolysed and subjected to GLC analysis. Results, in agreement with previous findings, showed that all mutants examined were glucose deficient, although to various degrees, whereas their phosphorus and glycerol contents were comparable to those of the wild-type (Table 4). Further analyses (F. Fiedler, personal communication) have revealed that walls from both *gtaA* and *gtaD* as well as *gtaB⁹* mutants were devoid of the glucosylated form of poly(*groP*). This confirms the previous conclusion (Young, 1967) that glucosylated poly(*groP*), absent from all classes of *gta* mutants examined, forms an essential part of the $\phi 29$ receptor. The substantial amounts of glucose associated with mutations *gtaA12* and *gtaD1* may well be correlated with a wild-type content of the GalN-glucose phosphate polymer (Shibaev *et al.*, 1973; Archibald, 1980). Indeed, these mutations were associated with wild-type levels of GalN and between one-third and one-half of the glucose of a *gta*⁺ strain, in contrast to the much lower contents of these components in three *gtaB* group mutants.

Fig. 1. Probable order of *gta* and *tag* markers determined by two-factor transformation crosses. Co-transformation frequencies shown are means of two or more crosses. Saturating concentrations of DNA were used in all cases except for crosses involving *gtaB* and *tag-1* markers, i.e. when *tag*⁺ recombinants were selected, 0.03 µg DNA ml⁻¹ was used. In crosses involving markers *gtaD*, *gtaA*, *tag-3* (*rodC1*) and *tag-1*, co-transfer of unlinked reference markers was in the range 0.01–0.12. Arrows point from the selected to the co-transferred marker. Linkage between *tag-3* and *tag-1* as well as that between *gtaD* and *gtaA* corresponds to 1 minus the recombination index.

The data (Table 4) suggested differences in GalN content among group B mutants: two *gtaB*⁹ strains, showing sensitivity to phages PBSY and PBSZ, appeared to have slightly higher contents of GalN than a group B mutant resistant to these phages. However, the poor sensitivity of the hexosamine assay prompted a more accurate measurement of this component. Accordingly, cell wall GalN was measured in all mutant classes so far identified. Cultures grown at 37 °C in the presence of [¹⁴C]GlcNAc were treated to selectively extract GalN from the cell-wall fraction (Table 5). Strains containing *gtaA* or *gtaD* markers released 20% of the cell hexosamine, a figure not smaller than that of the wild-type, in agreement with the results in Table 4. Suggested differences (Table 4) in GalN content among *gtaB* mutants were clearly confirmed with a larger number of strains: whereas, in one group, GalN represented less than 1% of the wall hexosamines, three *gtaB*⁹ mutants contained significantly higher (4%) amounts of GalN. Likewise, examination of eight mutants in the *gtaC*–*gtaE* linkage group revealed that the two *gtaC* mutations, distinguished from the remaining *gtaE* mutations by phage sensitivity, were associated with a significant wall GalN content (3 and 9%), whereas this component was 1% or less in all six *gtaE* strains. These results and those presented below strongly suggest that the synthesis of one or both GalN-containing polymers can proceed in the absence of either PGM or UDPGPPase, activities essential for glycosylation of the poly(groP) teichoic acid (see below).

Transformation and transduction mapping in the gtaA–gtaB and the gtaC regions of the B. subtilis linkage map

Two-point transformation crosses involving relevant markers revealed the likely order *gtaB*–*gtaD*–*gtaA*–*rodC*–*tag-1* (Fig. 1). The location of *gtaD* between *gtaB* and *gtaA* was confirmed by reciprocal three-factor transformation crosses involving markers *gtaD1*, *gtaA12* and *tag-1* (Table 6). The low numbers of *gta*⁺ recombinants were due to relatively close linkage of *gtaA* and *gtaD* mutations.

The results also revealed that the *gtaA* (and *gtaD*) marker was tightly linked to both *tag-1* and *rodC1* (Fig. 1), suggesting close linkage between the latter two markers. This observation

Table 6. Mapping of *gtaD* by reciprocal three-factor transformation crosses

DNA was used at saturating concentrations (1–5 µg ml⁻¹). Recombinants at the *tag-1* locus obtained by pseudo-linkage were recognized on replica plates of selected *leu*⁺ (or *mer*⁺) recombinants at 47 °C.

Donor*	Recipient*	Selected marker	Recombinant classes among <i>tag</i> ⁺	No.	Suggested order
L1440 <i>gta</i> ⁺	L6475, <i>gtaD1 tag-1 leuA8</i>	<i>leu</i> ⁺	<i>gta</i> ⁺ (ϕ29 ^a) <i>gta</i> (ϕ29 ^a)	47 19 Total 66	<i>gtaD-gtaA-tag-1</i>
BC9, <i>gtaA12</i>	L6475, <i>gtaD1 tag-1 leuA8</i>	<i>leu</i> ⁺	<i>gta</i> ⁺ (ϕ29 ^a) <i>gta</i> (ϕ29 ^a)	0 64 Total 64	
L1440 <i>gta</i> ⁺	L6463, <i>gtaA12 tag-1 metB5</i>	<i>mer</i> ⁺	<i>gta</i> ⁺ (ϕ29 ^a) <i>gta</i> (ϕ29 ^a)	40 1 Total 41	
L6334, <i>gtaD1</i>	L6463, <i>gtaA12 tag-1 metB5</i>	<i>mer</i> ⁺	<i>gta</i> ⁺ (ϕ29 ^a) <i>gta</i> (ϕ29 ^a)	2 34 Total 36	

* Relevant markers only shown.

contrasts with a previous study (Karamata *et al.*, 1972) which reported 20% PBS1 co-transfer of *hisA* and *rodC1*, and inferred that the latter marker was widely separated from *tag-1*. A transformation cross involving *rodC1* and *tag-1* markers gave a recombination index of 0.09 (data not presented), confirming their close linkage. This result, as well as the very similar phenotype of these mutations, prompted the designation *tag-3* in place of the previous *rodC1*.

PBS1 transduction crosses involving *gtaC198*, *gtaE151* and *argC4* markers gave 42 *gta*⁺ recombinants among 684 *arg*⁺ transductants (6.1%) with a *gtaC* donor and a *gtaE* recipient. In reciprocal crosses, 0 *gta*⁺ recombinants were obtained among 630 *arg*⁺ transductants, establishing the order *gtaC-gtaE-argC*.

Assay for UDPGPPase, NADPH oxidase and PGM activities in the soluble fraction of cell extracts

Identification of mutants affected in glucosylation of poly(*groP*) with new phenotypes – distinct phage resistance spectra (Table 3) and chemical composition of the wall (Tables 4 and 5) – as well as genetic evidence suggesting that some linkage groups contained more than one gene, prompted their examination for enzyme deficiencies and, in particular, a search for mutants affected in UDPGPPase activity. However, failure to detect significant UDPGPPase activity in crude cytoplasmic extracts of sonicated cultures of the *gta*⁺ strain, when prepared as previously described (Young, 1967; Maino & Young, 1974; Forsberg *et al.*, 1973), led to a search for and identification of appropriate modifications of the assay procedure, i.e. (i) a cell washing step was replaced by rinsing the pellet of harvested cells (see Methods) and (ii) the values for UDPGPPase activity were not corrected for the destruction of NADPH, formed in the assay mixture by the NADPH oxidase activity present in crude cell extracts (Young, 1967).

Cells from representatives of all new and some previously identified *gta* markers were grown in SAT medium at 37 °C until the late exponential phase. They were harvested by centrifugation and the cell pellets, after rinsing, were broken by sonication. The soluble fraction was assayed for UDPGPPase and PGM activities (Table 7). It appeared that the UDPGPPase activities in two strains belonging to the *gtaD* group were not significantly different from that of the *gta*⁺ strain (6.6, 7.7 and 11 units mg⁻¹ protein respectively). A striking result came from the examination of the *gtaB* linkage group, where among nine strains, including one previously characterized, *gtaB290* (Young *et al.*, 1969), not one was found to have a UDPGPPase activity greater than 2% of that of the wild-type. This strongly suggested that the gene product of the

Table 7. *UDPGPPase and PGM activities in crude extracts of gta mutants*

Cell pellets, obtained from late exponential phase cultures grown on SAT at 37 °C, were rinsed with water (0 °C), but not washed, before being broken by sonication. Soluble extracts were assayed immediately after removal of cell debris. Values shown were not corrected for the destruction of NADPH by NADPH oxidase present in extracts.

Strain	Marker	Enzyme activity [units (mg protein) ⁻¹]*		
		UDPGPPase	PGM	
			Method <i>a</i>	Method <i>b</i>
M22	<i>gta</i> ⁺	11	13.6	97
L6334	<i>gtaD1</i>	7.7	10.9	
L6256	<i>gtaD2</i>	6.2	10.5	
L6331	<i>gtaB515</i>	≤ 0.1	13.1	140
L6145	<i>gtaB114</i>	≤ 0.1	9.5	
L6240	<i>gtaB116</i>	≤ 0.1	11.4	
L6254	<i>gtaB123</i>	≤ 0.1	7.3	
L6200	<i>gtaB^o100</i>	≤ 0.1	20.4	
L6220	<i>gtaB^o101</i>	0.14	9.6	
L6238	<i>gtaB^o102</i>	≤ 0.1	7.3	
L6222	<i>gtaB^o103</i>	≤ 0.1	8.3	
QB510	<i>gtaB290</i>	≤ 0.1	ND	73
L6118	<i>gtaE151</i>	0.8	< 1	
L6119	<i>gtaE152</i>	0.8	< 1	
L6125	<i>gtaE158</i>	0.5	< 1	
L6170	<i>gtaE180</i>	0.7	< 1	
L6179	<i>gtaC189</i>	12	< 1	
L6188	<i>gtaC198</i>	5.6	< 1	
BC7	<i>gtaC51</i>	6.9	< 1	

ND, Not done.

* In the majority of cases results represent the mean of a minimum of two experiments.

gtaB locus was indeed the UDPGPPase activity, as originally supposed by Young (1967). Possible reasons for the apparent contradiction, i.e. the failure (Young, 1967; Yasbin *et al.*, 1976) to detect *in vitro* such a deficiency are examined in the Discussion. Finally, assay of this activity in seven strains with the *gtaC* or *gtaE* markers, including the previously characterized mutation *gtaC51* (Young, 1967), revealed that *gtaC* strains had activities comparable to the *gta*⁺ value, while the *gtaE* strains had only 5–10% of this value.

The NADPH oxidase activity present in cell extracts (Young, 1967) degrades the NADPH formed during the reaction and thus could affect the measured UDPGPPase (see Methods). Loss of *A*₃₄₀ after addition of NADPH to cell extracts (2 mg protein), allowed measurement of this activity. The latter was found (not shown) to be (i) virtually identical for all strains shown in Table 7 and (ii) proportional to the initial concentration of added NADPH up to 0.2 μmol ml⁻¹ (*A*₃₄₀ 1.2). At the latter concentration the rate of NADPH destruction was equivalent to 10 units per mg protein of the extract, i.e. comparable to the UDPGPPase activity present in the *gta*⁺ organism. However, for smaller initial concentrations of NADPH, in the range 0.1–0.2 *A*₃₄₀ units, the measured NADPH oxidase was at least five times lower than that of NADPH formation in the UDPGPPase assay of strains not deficient in the latter activity. As the rate of destruction was concentration dependent, correction for NADPH destruction was difficult and was thus omitted. For strains deficient in UDPGPPase activity, where the increase in *A*₃₄₀ was nil or extremely low, the absence of NADPH oxidase activity, being dependent on NADPH concentration, rendered correction for NADPH destruction unnecessary.

In confirmation of previous findings, only strains belonging to the *gtaC*–*gtaE* linkage group showed significant deficiency in PGM activity (Table 7).

DISCUSSION

The attempt here to isolate new markers involved in wall teichoic acid synthesis in *B. subtilis* by exploiting, as done previously, the resistance to, principally, phage $\phi 29$ was successful to a very limited extent. Mutations in over 150 strains (Table 2 and not shown) mapped close to previously identified markers *gtaA*, *gtaB* and *gtaC*. On the one hand this is not surprising since we have shown (Karamata *et al.*, 1987) that most if not all teichoic acid genes are located in the *hisA* region. Nevertheless, genetic, chemical and phage-resistance analyses have revealed the existence of new markers with distinct phenotypes in all three linkage groups. In two of them, *gtaA* and *gtaC*, the evidence strongly suggests the presence of more than one gene.

Two closely linked mutations, clearly separated from a *gtaA* reference marker and with a distinct phage resistance spectrum, have been assigned to a new group, *gtaD*. Like *gtaA* mutants, they are characterized by a deficiency of cell-wall glucose and normal activities of PGM and UDPGPPase. We have not determined whether *gtaD* mutations affect the UDPG: poly(groP) glucosyltransferase, shown to be deficient in previously identified *gtaA* mutants (Young, 1967).

All mutations linked to the previously identified marker *gtaC51* conferred a considerably reduced PGM activity. Two of them, *gtaC189* and *gtaC198*, located at one extremity of this group, are characterized by wild-type levels of UDPGPPase, a significant cell wall GalN content and sensitivity to phage PBSZ. However, the majority of these mutants showed decreased UDPGPPase activity (about 5–10% of that of the wild-type), absence of GalN, and resistance to all phages. It is likely that the latter class maps in a regulatory gene, designated *gtaE*, which controls the level of both PGM and UDPGPPase. Mutant BC7, containing *gtaC51* (Young, 1967), is an exception since it has normal UDPGPPase activity but, like *gtaE* mutants, lacks wall GalN and is resistant to PBSZ. Genetic analysis suggests that it harbours more than one linked *gta* mutation (data not presented).

The most surprising finding concerns the probable identification of the gene product of the *gtaB* locus as UDPGPPase. This activity is strongly deficient or absent in all of the nine probably independent *gtaB* or *gtaB^o* mutants examined, including one, *gtaB290*, described previously (Young, 1967); it is 5- to 10-fold lower than that characteristic of *gtaE* mutants (see above). These results are in apparent contradiction with previous findings, where UDPGPPase activity was found to be normal in all *gtaB* mutants examined (Young, 1967; Yasbin *et al.*, 1976). We believe that the explanation could well lie in the unwarranted (see Results) application of a correction for NADPH oxidase to extracts that had little UDPGPPase activity. Indeed, when using a cell washing step, as previously described (Young, 1967; Maino & Young, 1974), we failed to obtain extracts with UDPGPPase activity (see Methods). It has been reported that use of certain buffers for extract preparation leads to inactivation of this enzyme in extracts from the slime mould *Dictyostelium discoideum* (Newell & Sussman, 1969; Edmundson & Ashworth, 1972). Introduction of a correction for NADPH oxidase activity during assay of extracts with residual UDPGPPase activity not only accounts for reported activities but is also consistent with the strikingly similar values for all strains examined in previous studies. Indeed, a virtually identical NADPH oxidase activity was obtained in all mutants examined here. Also consistent with the above interpretation are the activities reported here for the wild-type, which, compared to previous results, are broadly similar for PGM but, without any corrections are approximately threefold higher for UDPGPPase.

The specific requirement for glucosylated poly(groP) for adsorption of $\phi 29$ provides the most likely explanation for the failure to identify, among mutants resistant to this phage, more new markers affecting teichoic acid synthesis. In particular, it would account for the absence of mutants specifically blocked in the synthesis of other teichoic acids present in the cell wall of this organism (Duckworth *et al.*, 1972; Shibaev *et al.*, 1973). However, the heterogeneity among *gta* mutants with respect to GalN-containing polymers cannot be easily correlated with observed enzyme deficiencies. Understanding of this question would require a more complete knowledge of the structure and biosynthesis of GalN-containing polymers (Shibaev *et al.*, 1973). Previous results (Archibald, 1980), as well as those reported here, provide good grounds for believing that the latter polymers form part of the receptors for other bacteriophages, PBSZ for example.

The most notable absence among mutants isolated both here and previously is that of mutations blocking synthesis of the main polyolphosphate chain, since they should be equally resistant to $\phi 29$. This absence supports previously presented evidence (Karamata *et al.*, 1987) that many such mutations are lethal, and is in favour of an essential role for this polymer in cell growth. Indeed, conditional lethal mutants genes which, when mutated, express a conditional lethal phenotype (as exemplified by *tag-1* and *tag-3* mutations) are very good candidates for being involved in the biosynthesis of the main polyolphosphate chain (Karamata *et al.*, 1987). Furthermore, the evidence obtained that *tag-1* is affected in poly(*groP*) synthesis strongly suggests that deficiencies in the synthesis of this polymer lead to disturbances in cell shape and in the orderly process of surface growth. It would appear that the majority of morphologically disturbed mutants known in *B. subtilis* harbour mutations that now map where most – if not all – teichoic acid genes have been recently mapped (Karamata *et al.*, 1987). This underlines how much remains to be learnt both of the genetic regulation of the biosynthesis and of the roles of the anionic cell wall polymers in *B. subtilis*, and in other Gram-positive organisms.

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REFERENCES

- AMES, B. N. & DUBIN, D. T. (1960). The role of polyamines in the neutralisation of bacteriophage deoxyribonucleic acid. *Journal of Biological Chemistry* **235**, 769–775.
- ARCHIBALD, A. R. (1974). The structure, biosynthesis and function of teichoic acid. *Advances in Microbial Physiology* **11**, 53–95.
- ARCHIBALD, A. R. (1980). Phage receptors in Gram-positive bacteria. In *Virus Receptors (Receptors and Recognition, Series B, vol. 7)*, pp. 7–26. Edited by L. L. Randall & L. Philipson. London: Chapman & Hall.
- BADDILEY, J. (1970). Structure, biosynthesis and function of teichoic acids. *Accounts of Chemical Research* **3**, 98–105.
- BODMER, W. F. & GANESAN, A. T. (1964). Biochemical and genetic studies of integration and recombination in *Bacillus subtilis* transformation. *Genetics* **50**, 717–738.
- BOYLAN, R. J., MENDELSON, N. H., BROOKS, D. & YOUNG, F. E. (1972). Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. *Journal of Bacteriology* **110**, 281–290.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- DUCKWORTH, M., ARCHIBALD, A. R. & BADDILEY, J. (1972). The location of *N*-acetylglucosamine in the walls of *Bacillus subtilis* 168. *Biochemical Journal* **130**, 691–696.
- EDMUNDSON, T. D. & ASHWORTH, J. (1972). 6-Phosphogluconate dehydrogenase and the assay of uridine diphosphate glucose pyrophosphorylase in the cellular slime mould *Dictyostelium discoideum*. *Biochemical Journal* **126**, 593–600.
- FORSBERG, C. W., WYRICK, P. B., WARD, J. B. & ROGERS, H. J. (1973). Effect of phosphate limitation on the morphology and wall composition of *Bacillus licheniformis* and its phosphoglucomutase-deficient mutants. *Journal of Bacteriology* **113**, 969–984.
- GIVAN, A. L., GLASSEY, R. S., GREEN, W. K., LANG, A. J. & ARCHIBALD, A. R. (1982). Relation between wall teichoic acid content of *Bacillus subtilis* and efficiency of adsorption of bacteriophages SP50 and $\phi 25$. *Archives of Microbiology* **133**, 318–322.
- HENNER, D. J. & HOCH, J. A. (1980). The *Bacillus subtilis* chromosome. *Microbiological Reviews* **44**, 57–82.
- KALCKAR, H. M. (1955). Enzymatic determination of UDPG by the specific pyrophosphorylase. *Methods in Enzymology* **2**, 676–677.
- KARAMATA, D. & GROSS, J. D. (1970). Isolation and genetic analysis of temperature sensitive mutants of *Bacillus subtilis* defective in DNA synthesis. *Molecular and General Genetics* **108**, 277–287.
- KARAMATA, D., MCCONNELL, M. & ROGERS, H. J. (1972). Mapping of *rod* mutants of *Bacillus subtilis*. *Journal of Bacteriology* **111**, 73–79.
- KARAMATA, D., POOLEY, H. M. & MONOD, M. (1987). Expression of heterologous genes for wall teichoic acid in *Bacillus subtilis* 168. *Molecular and General Genetics* **207**, 73–81.
- MAINO, V. C. & YOUNG, F. E. (1974). Regulation of glucosylation of teichoic acid. Isolation of phosphoglucomutase in *Bacillus subtilis* 168. *Journal of Biological Chemistry* **249**, 5169–5175.
- MAUËL, C. & KARAMATA, D. (1984). Prophage induction in thermosensitive DNA mutants of *Bacillus subtilis*. *Molecular and General Genetics* **194**, 451–456.
- MISHRA, N. C. & TATUM, E. L. (1970). Phosphoglucomutase mutants of *Neurospora sitophila* and their relation to morphology. *Proceedings of the National Academy of Sciences of the United States of America* **66**, 638–645.
- NEWELL, P. C. & SUSSMAN, M. (1969). Uridine diphosphate glucose pyrophosphorylase in *Dictyo-*

- stelium discoideum*. *Journal of Biological Chemistry* **244**, 2990–2995.
- PAVLIK, J. G. & ROGERS, H. J. (1973). Selective extraction of polymers from cell walls of Gram positive bacteria. *Biochemical Journal* **131**, 619–621.
- POOLEY, H. M. (1976). Turnover and spreading of old wall during surface growth of *Bacillus subtilis*. *Journal of Bacteriology* **125**, 1127–1138.
- POOLEY, H. M. & KARAMATA, D. (1984). Genetic analysis of lytic deficient and flagellaless mutants in *Bacillus subtilis*. *Journal of Bacteriology* **160**, 1123–1129.
- SCHLAEPPI, J.-M., POOLEY, H. M. & KARAMATA, D. (1982). Identification of cell wall subunits in *Bacillus subtilis* and analysis of their segregation during growth. *Journal of Bacteriology* **149**, 329–337.
- SHIBAEV, V. N., DUCKWORTH, M., ARCHIBALD, A. R. & BADDILEY, J. (1973). Structure of a polymer containing *N*-acetylgalactosamine from walls of *Bacillus subtilis* 168. *Biochemical Journal* **135**, 383–384.
- YASBIN, R. E., MAINO, V. C. & YOUNG, F. E. (1976). Bacteriophage resistance in *Bacillus subtilis* 168, W23 and interstrain transformants. *Journal of Bacteriology* **125**, 1120–1126.
- YOUNG, F. E. (1967). Requirement of glucosylated teichoic acid for adsorption of phage in *Bacillus subtilis* 168. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 2377–2384.
- YOUNG, F. E., SMITH, C. & REILLY, B. E. (1969). Chromosomal location of genes regulating resistance to bacteriophage in *Bacillus subtilis*. *Journal of Bacteriology* **98**, 1087–1097.