Genetic and biochemical characterization of *Bacillus subtilis* 168 mutants specifically blocked in the synthesis of the teichoic acid poly(3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate): *gneA*, a new locus, is associated with UDP-*N*-acetylglucosamine 4-epimerase activity

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The resistance spectrum to bacteriophage ϕ 3T of different *Bacillus subtilis* 168/W23 strains hybrid for wall teichoic acids suggested that poly(3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate), a so-called minor teichoic acid of strain 168, forms part of the receptor for this phage, and a serologically related group of phages. A representative sample of 25 mutants specifically resistant to ϕ 3T, obtained from a mutagenized culture by direct selection, were all found to have a greatly reduced galactosamine content. Relevant mutations in these strains were shown by PBS1 transduction and transformation to belong to two linkage groups; a minority, associated with an atypical colony morphology, were localized between *sacA* and *purA*, whereas the majority mapped between *gtaB* and *tagB1* (formerly *tag-1*), a region containing all known genes involved in the synthesis of the major wall teichoic acid, poly(glycerol phosphate). The former mutations mapped in a new locus, *gneA*, characterized by a deficiency in UDP-*N*-acetylglucosamine 4-epimerase, while the latter ones, as well as the previously identified *pha-3* (Estrela *et al.*, 1986, *Journal of General Microbiology* 132, 411–415), map in a locus named *gga*. They are likely to affect membrane-bound enzymes involved in the synthesis of the galactosamine-containing teichoic acid. A possible biological role of this polymer is discussed.

Introduction

Cell walls of many Gram-positive bacteria contain relatively large amounts of anionic polymers (Baddiley, 1970; Archibald, 1974). In *Bacillus subtilis* 168, several such polymers have been identified (Burger & Glaser, 1964; Shibaev *et al.*, 1973); the phosphate-rich poly(glycerol phosphate) [poly(groP)], glucosylated poly(groP) and poly(3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate) [poly(Glc-GalNAc 1-P)], present under conditions of saturating phosphate, are replaced by teichuronic acid (Janczura *et al.*, 1961; Ward, 1981)

Abbreviations: DMAB, *p*-dimethylaminobenzaldehyde; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; MMC, mitomycin C; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ND, nephelometric density; ϕ 3T^R, ϕ 3T resistant; ϕ 3T^S, ϕ 3T sensitive; poly(Glc-GalNAc 1-P), poly(3-*O*- β -D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate); poly(groP), poly(glycerol phosphate).

under phosphate-limiting growth conditions. Genetic analysis of conditional lethal mutants deficient in poly(groP) synthesis (Boylan *et al.*, 1972; Karamata *et al.*, 1972; Pooley *et al.*, 1987; Briehl *et al.*, 1989; Mauël *et al.*, 1989), and in particular the more recent nucleotide sequencing studies (Honeyman & Stewart, 1989; Mauël *et al.*, 1991) have led to the identification of six genes likely to be specifically involved in the synthesis of glucosylated poly(groP). Putative enzyme activities for the encoded products of two of these genes have been proposed (Young, 1967; Pooley *et al.*, 1991). In comparison, genetic information on the galactosamine (GalN) containing polymer is limited. No mutants specifically blocked in its synthesis have been identified. Of two markers known to affect its synthesis, neither *gtaB*, the putative structural gene for UDP-glucose pyrophosphorylase (Pooley *et al.*, 1987), nor *gtaC*, which affects the activity of phosphoglucumutase (Young, 1967), is exclusively involved in the synthesis of this polymer. Both

mutations lead to a diminished pool of a precursor, UDP-glucose.

Presence or absence of GalN in walls of different *B. subtilis* 168/W23 hybrids, obtained by exchange of the *tagB1* (formerly *tag-1*) marker (Karamata *et al.*, 1987), and known to have undergone variable deletions of the neighbouring genes of the 168 chromosome (Young *et al.*, 1989), led to the conclusion that all or part of the genetic determinants for a GalN-containing polymer were located between the *gtaA* and *gtaB* loci (Karamata *et al.*, 1987). The localization to this region of the chromosome of a new locus, *pha-3* (Estrela *et al.*, 1986), associated with a specific resistance to ϕ 3T and closely related group III phages (Dean *et al.*, 1978), but distinct from previously identified markers, *gtaA*, *gtaB*, *gtaD*, raised the possibility that the poly(Glc-GalNAc 1-P) may play a role in the adsorption of these phages. Mutations in *gtaC* and *gtaB* confer resistance to ϕ 3T and serologically related phages, whereas *gtaA* mutations allow infection (Estrela *et al.*, 1986). While mutations in all the *gta* markers prevent glucosylation of the major cell wall teichoic acid, poly(groP), those in *gtaB* and *gtaC* are associated, unlike *gtaA*, with greatly reduced amounts of *N*-acetylgalactosamine (GalNAc) in cell walls (Young, 1967; Pooley *et al.*, 1987).

As resistance to ϕ 3T is correlated with the absence of the GalNAc-containing polymer, we verified the resistance spectrum to ϕ 3T of a selection of 168/W23 strains hybrid for wall teichoic acids (Karamata *et al.*, 1987) known to differ in their GalN content, and then proceeded to isolate a representative collection of ϕ 3T^R mutants blocked in the synthesis of the GalN-containing polymer. We report here the genetic analysis and biochemical characterization of these mutants.

Methods

Strains. The *B. subtilis* strains used are listed in Table 1. Bacteriophages ϕ 25, ϕ 29 and SPO1 were from laboratory stocks; ϕ 3Tc, ρ 11c, ϕ 105c and SPO2c were spontaneous clear plaque mutants. Stocks of the above phages and SPR were prepared on strain YB886 as previously described (Estrela *et al.*, 1986).

Media. M broth, M agar and M soft-agar (Yehle & Doi, 1967) supplemented as described by Yasbin *et al.* (1973) were used to assay phage adsorption and infection. L medium, TS plates and SAT medium as well as media for transformation and transduction were as previously described (Karamata & Gross, 1970; Pooley & Karamata, 1984; Karamata *et al.*, 1987). Minimal medium was supplemented with amino acids (20 μ g ml⁻¹) and bases (100 μ g ml⁻¹) when necessary. *sacA*⁺ and *ctrA*⁺ transductants were selected on a modified Spizizen's medium (Dedonder *et al.*, 1977) containing 0.1% sucrose (*sacA*⁺) in place of glucose or 0.1% glutamate in place of ammonium (*ctrA*⁺).

Biochemicals. UDP-*N*-acetylgalactosamine and mitomycin C (MMC) were purchased from Sigma, *N*-acetyl[1-¹⁴C]glucosamine from Amersham, and *p*-dimethylaminobenzaldehyde (DMAB) from Merck.

Phage assay. Phage infection and determination of efficiency of infection were as described by Estrela *et al.* (1986). Suitable dilutions of phage lysates were allowed to adsorb to 10⁸ cells for 15 min, at room temperature, before plating in top agar.

Mutagenesis and selection of phage-resistant mutants. Strain SL1020 was grown on SAT medium and cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described by Karamata & Gross (1970). After addition of the mutagen, the culture was divided into five tubes, and incubated for 30 min at 37 °C. Survival, measured as viable counts, was 17% and frequency of auxotrophs was 10%. The mutagenized cultures were diluted 20-fold into L medium, and further incubated overnight at room temperature to allow expression. ϕ 3T was added to appropriate dilutions of the five sub-cultures, at a multiplicity of infection of 10–100. After 15 min at room temperature, to allow adsorption, 0.1 ml samples were plated in top agar overlays and incubated overnight at 30 °C. Plates with well-distributed surviving colonies (representing from 1 to 4.8% of the inoculum from different sub-cultures) were chosen and, from certain plates, all colonies were picked and streaked. The spectrum of resistance to phages ϕ 3T, ϕ 25, ϕ 29 and ϕ 105 of about 90 colonies was examined as previously described (Estrela *et al.*, 1986). Approximately 7% of all isolates were found to be lysogenic for ϕ 3T (see below). The overall frequency of mutants specifically resistant to ϕ 3T in different sub-cultures varied from 8×10^{-4} to 2×10^{-3} . These values are more than 100-fold higher than the frequency, 5×10^{-6} , of spontaneous ϕ 3T-resistant mutants.

Lysogenic assay. ϕ 3T-resistant (ϕ 3T^R) colonies were tested for ϕ 3T lysogeny by assaying supernatants of overnight shaken cultures grown in M broth at 37 °C for spontaneously liberated phage. ϕ 3T^R resistant colonies which did not liberate phage were further tested for lysogeny by MMC induction. Overnight cultures in M broth were diluted to a nephelometric density (ND) of 5 in fresh prewarmed M broth and incubated at 37 °C with shaking until they reached a ND of 40. MMC was added to a final concentration of 1 μ g ml⁻¹, and incubation continued for another 15 min. Cultures were centrifuged and cells, resuspended in fresh pre-warmed M broth, were incubated at 37 °C with shaking for at least 3 h or until lysis was visible. Supernatants were assayed for the presence of phage.

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

Estimation of cell wall GalN by selective extraction from whole cells labelled with [1-¹⁴C]GlcNAc. The method was as described by Pooley *et al.* (1987). The labelling medium contained [1-¹⁴C]GlcNAc at a final concentration of 0.025 μ Ci ml⁻¹ (0.92 kBq ml⁻¹).

Preparation of cell-free extracts. Cell-free extracts for the assay of UDP-GlcNAc 4-epimerase were prepared according to Glaser (1959). They were obtained from 500 ml cultures grown at 37 °C in NY broth (Glaser, 1959) with aeration to a ND of 120. Cells were harvested by centrifugation, washed in 0.05 M-Tris, 0.01 M-MgCl₂, 0.001 M-EDTA pH 7.5 at 4 °C, resuspended in 3 ml of the same buffer and maintained at 0 °C. Bacteria were ruptured by sonication and cell debris removed by centrifugation. The supernatant was stored at –70 °C before assay.

Total protein determinations. Total protein in cell-free extracts was measured by the Bio-Rad protein assay using the microassay procedure (Bradford, 1976) with bovine serum albumin as standard.

Assay for UDP-GlcNAc 4-epimerase (EC 5.1.3.7). The assay, performed as described by Glaser (1959), uses the conversion of GalNAc to GlcNAc, which is accompanied by a three-fold increased colour yield when the hexosamine content is measured by the Morgan-Elson colour reaction as modified by Oguchi & Oguchi (1979). Enzyme reaction mixtures contained 10 mM-glycine, 1 mM-MgCl₂, 0.1 mM-

Table 1. *Bacillus subtilis* strains

Strain	Genotype/phenotype	Origin/reference
SL1020	<i>metB5 leuA8 thr-5 su³⁺ attSPβ</i>	P. Piggot, Temple Univ., Philadelphia, USA
YB886	<i>metB5 trpC2 xin-1 attSPβ</i>	Yasbin <i>et al.</i> (1980)
CU1448	<i>hisA1 thr-5 metB5 attSPβ</i>	S. A. Zahler, Cornell Univ., Ithaca, USA
M22	<i>purA16 leuA8 metB5 ilvA1</i>	Karamata & Gross 1970
GSY403	<i>pheA3 trpC2</i>	C. Anagnostopoulos, INRA, Jouy-en-Josas, France
W23	Sm ^r	BD1037 in Dubnau <i>et al.</i> 1967
L5009	<i>purA16 leuA8 metB5 hisA35</i>	Briehl <i>et al.</i> , 1989
L5047	<i>purA16 hisA35 pheA3 metB5 trpC2</i>	Congression GSY403→L5009
L5048	<i>purA16 pheA3 hisA35 metB5</i>	Congression GSY403→L5009
L5054	<i>purA16 hisA1 pheA3 trpC2 gtaB515</i>	Congression L6303→L5047
QB944	<i>purA16 cysA1 trpC2</i>	Dedonder <i>et al.</i> (1977)
QB123	<i>sacA321 ctrA1 trpC2</i>	Dedonder <i>et al.</i> (1977)
L6440	<i>hisA1 argC4 leuA8 tagB1</i>	Pooley & Karamata (1984)
LAI000	<i>pheA3 leuA8 hisA1 attSPβ</i>	Congression L5048→SL1020
IGCg113	<i>metB10 trpC2 gga-1 (pha-3)*</i>	Estrela <i>et al.</i> (1986)
IGCg311	<i>thr-5 hisA1 xin-1 attSPβ</i>	Collection Instituto Gulbenkian de Ciência
IGCg314	<i>trpC2 hisA1 xin-1 attSPβ</i>	Congression IGCg311→YB886
<i>W23/168 hybrid strains</i>		
L5705	<i>metB5 tar Δ(tag)[†]</i>	Same cross as L5706; Karamata <i>et al.</i> (1987)
L5763	<i>hisA1 argC4 tar Δ(tag)</i>	Same cross as L5764
L5709	<i>metB5 tar Δ(tag)</i>	Same cross as L5706
L5710	<i>metB5 tar Δ(tag)</i>	Same cross as L5706
L5706	<i>metB5 tar Δ(tag)</i>	Karamata <i>et al.</i> (1987)
L5764	<i>hisA1 argC4 tar Δ(tag)</i>	Karamata <i>et al.</i> (1987)
<i>ϕ3T specifically resistant strains</i>		
LAI002	<i>metB5 leuA8 thr-5 attSPβ gga-2</i>	MNNG mutagenesis of SL1020, ϕ 3T ^R , ϕ 29 ^S ‡
to		
LAI020	<i>metB5 leuA8 thr-5 attSPβ gga-20</i>	
LAI021	<i>metB5 leuA8 thr-5 attSPβ gneA1§</i>	MNNG mutagenesis of SL1020, ϕ 3T ^R , ϕ 29 ^S ‡
to		
LAI026	<i>metB5 leuA8 thr-5 attSPβ gneA6§</i>	
LAI101	<i>pheA3 leuA8 attSPβ gga-1</i>	Congression IGCg113→LAI000
LAI120	<i>pheA3 leuA8 attSPβ gga-20</i>	Congression LAI020→LAI000
LAI121	<i>pheA3 leuA8 attSPβ gneA1§</i>	Congression LAI021→LAI000
LAI122	<i>pheA3 leuA8 attSPβ gneA2</i>	Congression LAI022→LAI000
LAI123	<i>pheA3 leuA8 attSPβ gneA3</i>	Congression LAI023→LAI000
LAI124	<i>pheA3 leuA8 attSPβ gneA4</i>	Congression LAI024→LAI000
LAI130	<i>xin-1 attSPβ gga-20</i>	Congression LAI020→IGCg314
LAI131	<i>hisA1 xin-1 attSPβ gneA1</i>	Congression LAI021→IGCg314

* Estrela *et al.* (1986); mutation *pha-3* was renamed *gga-1*.

† *tar* represents the genetic determinants (Boylan *et al.*, 1972) for the synthesis of the poly(ribitol phosphate) present in the cell wall.

‡ R and S, resistant and sensitive, respectively to bacteriophages. See Results.

§ Mutations in the *gneA* locus are associated with an atypical colony morphology (see text).

EDTA, 0.1 mM-UDP-GalNAc and 200 μ l of suitable dilutions of the cell-free extract in a final volume of 0.5 ml at pH 9. After 10 min, the reaction was stopped by addition of enough HCl to bring the pH to 2.0, and the reaction mixture was incubated for 20 min at 100 °C. Reaction mixtures were neutralized with NaOH and total hexosamine was measured. GlcNAc and GalNAc standards were run simultaneously. Freshly prepared 1.5% (v/v) acetic anhydride in acetone (0.05 ml) was added to the neutralized reaction mixture. After 5 min at room temperature, 0.15 ml of a 0.7 M-potassium tetraborate solution was added. Tubes were covered with glass marbles and heated in boiling water for 3 min. After cooling (15–23 °C), 0.3 ml DMAB reagent, prepared as described by Reissig *et al.* (1955), was added without shaking, followed by addition of 2.7 ml glacial acetic acid. Incubation at 37 °C for 20 min was followed by a 5 min cooling period in a water bath (15–23 °C). Absorbance at 585 nm was measured without delay.

Results

Resistance to phages ϕ 3T and p11 of W23/168 strains hybrid for wall teichoic acids

We sought further evidence for the proposed role (see Introduction) of the GalN-containing teichoic acid in the adsorption of phage ϕ 3T, and related phages, by examining the adsorption and infection behaviour of these phages on W23/168 strains hybrid for cell wall teichoic acids (Karamata *et al.*, 1987). These strains, the walls of which all contain poly(ribitol phosphate), instead of poly(groP), were divided into two classes with

Table 2. Differential plating efficiencies of *B. subtilis* phages on 168/W23 strains hybrid for wall teichoic acids

Plating efficiencies are mean values of at least two different experiments, and are given as the ratio of the number of plaque-forming units (p.f.u.) obtained by plating on each strain to p.f.u. obtained by plating on the parental strain. Phages ϕ 29 and SPO2c were used as controls; ϕ 29 has an absolute requirement for glucosylated poly(groP) for adsorption, and SPO2c infection is unaffected by mutants devoid of this glucosylated teichoic acid. Cell walls of all 168/W23 hybrid strains, like W23, contain poly(ribitol phosphate) and lack poly(groP).

Phage	Parent strains		Hybrids (168/W23)					
	168 M22	W23	W23-like		W23-like, ϕ 25 ^R		Mixed-type	
			L5705	L5763	L5709	L5710	L5706	L5764
GalNAc content*	++	—	—	—	—	—	++	++
ϕ 3Tc	1	0†	0	0	0	0	0.25	0.27
ρ 11c	1	0	0	0	0	0	0.61	0.52
ϕ 29	1	0	0	0	0	0	0	0
SPO2c	1	0	0	ND	0	ND	0	ND

ND, Not done.

* Relative to the wild-type GalNAc content, ++ indicates a normal level and —, a near absence of GalNAc; for values, see Karamata *et al.* (1987).

† 0 stands for $<10^{-8}$.

respect to their galactosamine content: 'W23-like', and 'mixed-type', devoid of and containing GalNAc, respectively. In agreement with the hypothesis made above (see Introduction), we found (Table 2) that only those hybrid strains which have a 168-like content of GalNAc were sensitive to ϕ 3T and ρ 11, confirming previous observations with *gta* mutations (Estrela *et al.*, 1986). Measurement of phage titres before and after adsorption to whole cells, performed as described by Estrela *et al.* (1986), revealed the failure of ϕ 3T and serologically related phages to adsorb to wild-type cells from which the GalNAc-containing polymer had been selectively extracted (see Methods). This supports the involvement of this wall teichoic acid in the adsorption of these phages.

Isolation and preliminary characterization of *B. subtilis* mutants resistant to phage ϕ 3T

A collection of 89 ϕ 3T^R mutants was isolated from a MNNG-mutagenized culture of strain SL1020, by direct selection. Those mutants which were shown not to be lysogenic for ϕ 3T (see Methods) were further analysed for their resistance pattern towards phages whose adsorption is affected by *gta* mutations, in particular towards ϕ 29, which has an absolute requirement for glucosylated poly(groP), and ϕ 25, which is able to infect *gtaA*-carrying mutants but not those bearing *gtaB* or *gtaC* mutations (Young, 1967; Pooley *et al.*, 1987).

Nineteen mutants resistant to ϕ 29 and ϕ 25 in addition to ϕ 3T were considered to harbour *gtaB*, *C*, or *E* mutations and were not further studied. The remaining mutants, all specifically resistant to phages ϕ 3T and ρ 11, were retained for further study. They were characterized by the presence of a glucosylated poly(groP), as revealed by sensitivity to ϕ 29.

Distribution of ϕ 3T^R mutants into linkage groups

Genetic analysis was focused on 25 mutants specifically resistant to ϕ 3T. They included all 14 isolates from one mutagenized sub-culture as well as representatives with all types of colony morphology originating from different sub-cultures. The spontaneous ϕ 3T^R mutant (Estrela *et al.*, 1986) containing the *pha-3* mutation localized in the *hisA1* region of the chromosome was also included in our analysis. Preliminary assignment of mutations to linkage groups was achieved by PBS1 transduction. In the initial series of strains transduced, while, as expected, several mutations were found to be linked to *hisA1*, two did not map in this region. Transducing phage stocks prepared on one of the latter, and use of the Dedonder kit of recipients (Dedonder *et al.*, 1977) revealed the presence of a mutation cotransducible with the *sacA* locus (see below). This mutation was associated with an atypical colony morphology (small, raised, round and brilliant colonies) apparently indistinguishable from that pro-

Table 3. Mapping of mutations *gneA1* and *gga-20* by two- and three-factor PBS1 transduction crosses

Donor	Recipient	Selected marker	Recombinant class	No. of colonies scored	Implied order
LAI131 <i>gneA1</i>	QB944 <i>purA16, cysA1</i>	<i>pur</i> ⁺	Cys ⁺ ϕ 3T ^R	0	<i>gneA1, purA, cysA</i>
			Cys ⁺ ϕ 3T ^S	24	
			Cys ⁻ ϕ 3T ^R	8	
			Cys ⁻ ϕ 3T ^S	42	
		<i>cys</i> ⁺	Pur ⁺ ϕ 3T ^R	0	
			Pur ⁺ ϕ 3T ^S	26	
			Pur ⁻ ϕ 3T ^R	0	
			Pur ⁻ ϕ 3T ^S	49	
LAI131 <i>gneA1</i>	QB123 <i>sacA321, ctrA1</i>	<i>sac</i> ⁺	Ctr ⁺ ϕ 3T ^R	13	<i>ctrA, sacA, gneA1</i>
			Ctr ⁺ ϕ 3T ^S	36	
			Ctr ⁻ ϕ 3T ^R	32	
			Ctr ⁻ ϕ 3T ^S	19	
		<i>ctr</i> ⁺	Sac ⁺ ϕ 3T ^R	17	
			Sac ⁺ ϕ 3T ^S	33	
			Sac ⁻ ϕ 3T ^R	0	
			Sac ⁻ ϕ 3T ^S	50	
LAI130 <i>gga-20</i>	L6440 <i>hisA1, tagB1</i>	<i>his</i> ⁺	Tag ⁺ ϕ 3T ^R	37	<i>hisA, gga-20, tagB1</i>
			Tag ⁺ ϕ 3T ^S	0	
			Tag ⁻ ϕ 3T ^R	7	
			Tag ⁻ ϕ 3T ^S	53	
		<i>tag</i> ⁺	His ⁺ ϕ 3T ^R	43	
			His ⁺ ϕ 3T ^S	0	
			His ⁻ ϕ 3T ^R	53	
			His ⁻ ϕ 3T ^S	5	

voked by *gtaB* and *gtaC* markers. Inspection of the selected 26 ϕ 3T^R mutants revealed a total of six with this abnormal colony morphology. Two of the latter, strains LAI023 and LAI025, had a somewhat rougher, flatter, more irregular colony morphology, apparently intermediate between that of the remaining four and that of the wild-type. Mutations *gneA2*, -3, -4 and -6 were shown by PBS1 transduction to map in the same position on the chromosome as *gneA1* (see below). The transformational recombination index between the *gneA1* (LAI131) and *gneA5* mutations, associated with the most pronounced difference in colony morphology within this group, was 0.03. The close linkage, consistent with their being alleles, identifies a new locus designated *gneA* (for UDP-GlcNAc 4-epimerase; see below).

Mutations in the remaining 20 mutants were mapped more precisely by transformation experiments using strain L5054 (*trpC2, gtaB515*) as recipient. Double *gta*⁺ *trp*⁺ recombinants were identified among selected *trp*⁺ recombinants by loss of the characteristic colony morphology of the *gtaB* marker (see below). The correlation between *gta*⁺ and loss of this colony morphology was always confirmed by a test for ϕ 29 sensitivity. For each cross, between 20 and 24 *gta*⁺ recombinants were scored for resistance to ρ 11, indicative of the presence of a *gga* marker (for glucose galactosamine phosphate; see below). For 20 donors,

including the strain carrying the *pha-3* marker, presence of a *gga* marker was inferred from the high proportion of ρ 11 resistance among *gta*⁺ recombinants – a mean of 72% was found with extremes of 96% (*gga-8*) and 42% (*gga-14*). The mean frequency of congression of an unlinked reference marker, *pur*⁺, among *trp*⁺ recombinants was less than 9% for all but one cross. Thus, genetic analysis reveals that mutations conferring specific ϕ 3T resistance identified in the 26 mutants of our collection map in two regions of the chromosome.

Mapping of mutations *gneA1* and *gga-20*, representative of the two linkage groups

Mapping, by two- and three-factor PBS1 transduction crosses, of mutations *gneA1* and *gga-20*, representative of the linkage groups localized in the *sacA* and *gtaB* regions, provided the order *sacA, gneA, purA* and *hisA, gga, tagB*, respectively (Table 3). The results confirmed that mutations belonging to the *gga* linkage group lie in the same region as many other markers – *gtaA, gtaB, tagF1* (*rodC1*), *tagB1* (*tag-1*) – directly or indirectly involved in cell wall teichoic acid synthesis. The probable location for *gga* markers between *gtaB* and *tagB* (see above) was confirmed (unpublished results) by transformation of two representative markers *gga-1* (*pha-3*) and *gga-20* with clones from an EMBL3 λ bank (Mauël *et al.*, 1989).

Table 4. Cell wall galactosamine content in representative $\phi 3T^R$ mutants

Cells labelled with [^{14}C]GlcNAc were precipitated by 5% (w/v) TCA and the GalNAc-containing polymer was selectively extracted by heating at 100 °C for 30 min at pH 4.

Linkage group	Reference mutation	No. of mutants examined	Cell wall GalNAc* (%)
<i>gneA</i>	<i>gneA1</i>	4	1.0 $\pm$ 0.15
	<i>gneA3</i>	2	4.8 $\pm$ 0
<i>gga</i>	<i>gga-1</i>	20	1.7 $\pm$ 0.5
<i>gneA</i> ⁺ <i>gga</i> ⁺ parent strain			18.3 $\pm$ 1.5

* Expressed as a percentage of total radioactivity incorporated in cell wall hexosamines. For each group indicated, values represent the mean of all mutants. They are based on at least two independent measurements for each mutant examined.

Table 5. UDP-GlcNAc 4-epimerase activity in cytoplasmic extracts of $\phi 3T^R$ mutants representative of different linkage groups

Strain	Marker	Mutation	UDP-GlcNAc 4-epimerase	
			Unit* (mg protein) ⁻¹	Activity relative to wild-type (%)
LA1121	<i>gneA</i>	<i>gneA1</i>	0.014	5
LA1122		<i>gneA2</i>	0.005	2
LA1123		<i>gneA3</i>	0.009	3
LA1124		<i>gneA4</i>	0.013	4
LA1101	<i>gga</i>	<i>gga-1</i>	0.28	97
LA1120		<i>gga-20</i>	0.26	93
LA1000	<i>gneA</i> ⁺ , <i>gga</i> ⁺		0.29	100

* One unit of enzyme is defined as the amount which catalyses the formation of 1 μ mol UDP-GlcNAc under the conditions of the standard assay.

Estimation of cell wall GalN content

Genetically characterized mutants, bearing mutations in linkage groups *gga* and *gneA*, were analysed for the GalNAc content of their walls. A selective extraction at 100 °C and pH 4 of hexosamine-labelled cells was shown to specifically remove over 95% of the cell wall GalN (Pooley *et al.*, 1987). The mutants had 4- to 18-fold less extractable cell wall GalN (hexosamine) label than the wild-type (Table 4). The average figure for 20 *gga* mutations was 1.7 $\pm$ 0.5. That for the four *gneA* markers associated with a characteristic colony morphology (see above) was 1.0 $\pm$ 0.2 while the two remaining *gneA* markers, carried by strains LA1023 and LA1025, and associated with an intermediate colony morphology (see above), were identical, with amounts of 4.8%. Results for the last-mentioned strains are consistent with a certain

leakiness in the phenotype of mutations *gneA3* and *gneA5*. These results, obtained on a representative sample of randomly isolated $\phi 3T^R$ mutants, provide a clear confirmation that specific $\phi 3T$ resistance is invariably associated with a marked reduction in the cell wall GalNAc content. Analyses of cell walls of several such mutants revealed phosphate and glycerol contents comparable to those of the parental strain SL1020 (data not presented), suggesting a normal level of poly(groP), in agreement with the $\phi 29$ sensitivity of these strains.

Assay of UDP-GlcNAc 4-epimerase activity in the soluble fraction of cell extracts

Since UDP-GalNAc is a specific precursor of poly(Glc-GalNAc 1-P), a block in the formation of this precursor

appeared to be a good candidate for a mutation that would lead to a specific inhibition of the synthesis of this polymer. UDP-GalNAc is formed from UDP-GlcNAc by the action of UDP-GlcNAc 4-epimerase, a cytoplasmic enzyme, whose presence in cell extracts of *B. subtilis* 168 was described by Glaser (1959). Assays of the activity in cell-free extracts of mutants belonging to the *gga* linkage group (Table 5) revealed a UDP-GlcNAc 4-epimerase activity identical to that of the parent strain, whereas four mutants carrying a *gneA* marker are all but devoid of epimerase activity, including one, LAI123, *gneA3*, containing significant cell wall GalN. This suggests that while the epimerase in the latter strain is inactive in the *in vitro* assay conditions, it has residual activity *in vivo*.

Discussion

Mutants specifically resistant to bacteriophage ϕ 3T allow the definition of two linkage groups, *gneA* and *gga*, involved in the synthesis of poly(Glc-GalNAc 1-P), a so called minor wall teichoic acid, that can nevertheless account for 20% of the cell's hexosamine content. Genetic and biochemical analyses of these mutants have led to the identification of a new locus, *gneA*, linked to *sacA*, which is likely to encode the UDP-GlcNAc 4-epimerase catalysing the formation of UDP-GalNAc, a precursor of at least two anionic cell wall polymers, the poly(Glc-GalNAc 1-P) (Shibaev *et al.*, 1973) and, during growth under phosphate limitation, the teichuronic acid (Ellwood & Tempest, 1972). The high density of markers involved in teichoic acid synthesis at around 310° of the *B. subtilis* chromosome is further underlined by the identification of one or several genes, designated *gga*, responsible for their ϕ 3T^R and cell wall GalN deficient phenotypes. Localization of *gga* markers between *tagB* and *gtaB* loci is in excellent agreement with genetic and cell wall analyses of different 168/W23 hybrid strains (Karamata *et al.*, 1987), as well as with hybridization studies (Young *et al.*, 1989). The *gga* loci are likely to encode membrane-linked enzymes involved in the synthesis of the poly(Glc-GalNAc 1-P). Indeed, identification of the *gneA* locus, together with *gtaB*, the putative structural gene for UDP-glucose pyrophosphorylase, and *gtaC*, essential for phosphoglucomutase activity, means that all the enzymes necessary for the formation of known soluble precursors of the GalN-containing polymer, namely UDP-glucose and UDP-GalNAc, are now associated with loci other than *gga*. The preponderance of *gga* mutations, 19 out of a total of 25, identified in a random sample of GalN deficient mutants, is consistent with a larger mutational target than that formed by the *gneA* locus.

Since this work was completed, insertional mutagenesis with clones of the *gga* region has revealed a single transcription unit of about 3 kbp which, when disrupted, is associated with a ϕ 3T^R phenotype (Mauël *et al.*, 1991). The coding capacity of this unit, equivalent to three average-size proteins of about 33 kDa, may be sufficient to accommodate several, if not all, membrane-bound enzymes required for the polymerization of poly(Glc-GalNAc 1-P) (Hayes *et al.*, 1977; Ward, 1981).

The virtual absence of GalN in the 20 *gga* mutants examined here is consistent with the presence of a single GalN-containing cell wall teichoic acid, likely to be the poly(Glc-GalNAc 1-P), previously identified in this strain (Shibaev *et al.*, 1973). The present study provides no support for the existence, in this strain and under the growth conditions used, of a second GalN-containing polymer (Duckworth *et al.*, 1972).

Although the absence of poly(Glc-GalNAc 1-P) from walls of 26 mutants specifically resistant to ϕ 3T confirms previous results (Young, 1967) suggesting the dispensability of this polymer for cell growth, its biological role remains an interesting question. When a glycerol-requiring mutant is grown in the absence of glycerol, isolated cell walls contain, as expected, reduced amounts of the major teichoic acid, poly(groP), but increased quantities of GalN (Rosenberger, 1976). Certain *tag* (Ts) mutations, which are partially expressed at 30 °C, are associated with a twofold or greater increase in wall GalN content relative to the *tag*⁺ allele (H. M. Pooley & F.-X. Abellan, unpublished). These results can be interpreted in favour of a substitution role, i.e. within certain limits, the GalN polymer may be able to compensate a deficiency of poly(groP). If this explanation were correct, it would mean that this organism has at least two substitute polymers for poly(groP), one, poly(Glc-GalNAc 1-P), for phosphate-rich conditions and the other, teichuronic acid, for growth under phosphate depletion.

The maintenance of the genetic and enzymic capacity for synthesis of three distinct anionic polymers further underlines the importance of the role(s), so far unelucidated, of cell wall anionic or polysaccharidic polymers in the economy of this and, possibly, of other organisms.

The abnormal colony morphology associated with mutations in the *gneA* locus, closely resembling that induced by mutations in the *gtaB*, *gtaC* and *gtaE* loci, was found to be a useful phenotype for genetic analysis. It is noteworthy that mutations in the above genes affect enzymes involved in the metabolic steps leading to soluble precursors of cell wall polymers whereas mutations affecting *gga* genes, likely to encode membrane-bound proteins, do not affect colony morphology. Thus, the altered morphology may be the result of a disturbance in the pools of soluble cell wall precursors provoked by a

block in one of the enzymes leading to the synthesis of one or more of them.

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