A conditional-lethal mutant of *Bacillus subtilis* 168 with a thermosensitive glycerol-3-phosphate cytidyltransferase, an enzyme specific for the synthesis of the major wall teichoic acid

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A biochemical analysis was undertaken of thermosensitive mutants of *Bacillus subtilis* 168 harbouring mutations in several *tag* genes, involved in the synthesis of the major wall teichoic acid, poly(glycerol phosphate), poly(groP). Incorporation of a pulse of [2-³H]glycerol into whole cells, following shift to the restrictive growth temperature, was used to assess synthesis of this polymer and to seek evidence of accumulation of a specific precursor. The rate of incorporation into poly(groP) was strongly decreased in all mutants; glycerol uptake was diminished by 80% or more for a strain harbouring mutation *tagB1* (formerly *tag-1*) and one bearing *tagD11* (formerly *tag-11*). The pool of CDP-glycerol (CDP-gro), a specific precursor of poly(groP), was increased, relative to the wild-type, for all mutations except *tagD11*, where the pool of CDP-gro was reduced. Cytoplasmic extracts, assayed at the permissive temperature for glycerol-3-phosphate cytidyltransferase (gro-PCT), the enzyme synthesizing CDP-gro, revealed wild-type activities for all mutations except *tagD11*. Gro-PCT activity in the latter strain was 100-fold lower and, unlike that in all other mutant strains, highly thermolabile. This thermosensitivity suggests that *tagD* encodes gro-PCT. The identification, in a gene encoding a poly(groP)-specific enzyme, of a mutation conferring a thermosensitive growth phenotype renders explicit the conclusion that synthesis of this teichoic acid is essential for the growth of *B. subtilis*.

Introduction

Initial genetic and biochemical characterization of a collection of conditional-lethal thermosensitive mutants of *Bacillus subtilis* revealed major defects in their cell shape and cell wall teichoic acid content (Briehl *et al.*, 1989). The relevant mutations were mapped at around 310° on the chromosome in at least two genes involved in synthesis of poly(glycerolphosphate), poly(groP) (Briehl *et al.*, 1989; Honeyman & Stewart, 1989; Mauël *et al.*, 1989). The conclusion that several genes specifically concerned with the synthesis of poly(groP) are located in this region was inferred from the analysis of *B. subtilis* 168/W23 strains hybrid for teichoic acids (Karamata *et al.*, 1987). Previously, a mutation, *tagB1* (formerly *tag-1*), that mapped in the same region and showed a similar phenotype (Boylan *et al.*, 1972), was considered to affect the synthesis of poly(groP), although no specific enzyme

deficiency was identified (see below). Subsequent studies (Rogers *et al.*, 1974) with *tagB1* challenged the view that teichoic acid synthesis was the mutational target and proposed that the apparent decrease in wall teichoic acid was due to a major derepression of peptidoglycan synthesis. Although the same authors later showed that a significant 50–75% inhibition in the absolute rate of teichoic acid synthesis did indeed follow transfer of this mutant to the restrictive temperature (Rogers & Taylor, 1978), no conclusion was made concerning the nature of the function encoded by *tagB*. Furthermore, a mutation in the *rodB* locus, located at 247°, associated with a cell morphology similar to that of *tagB1* and other linked mutations (Briehl *et al.*, 1989; Karamata *et al.*, 1972), was shown not to affect teichoic acid synthesis (Rogers *et al.*, 1971). Thus, the major gap in the evidence linking *tag* genes to the synthesis of poly(groP) (Karamata *et al.*, 1987; Briehl *et al.*, 1989; Mauël *et al.*, 1989) is the failure, so far, to identify, among *tag* gene products, a single teichoic acid specific enzyme. Indeed, the activities of three teichoic acid specific enzymes, the glycerol-3-phosphate cytidyltransferase (gro-PCT), CDP-glycerol poly(glycerol phosphate) transferase and UDP-glucose

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Abbreviations: BSA, bovine serum albumin; α -groP, α -glycerol 3-phosphate; gro-PCT, glycerol-3-phosphate cytidyltransferase; poly(groP), poly(glycerol phosphate); ND, nephelometric density; PG, peptidoglycan.

poly(glycerol phosphate)- α -glucosyltransferase were all apparently normal in a strain bearing *tagB1* (Boylan *et al.*, 1972). Equally, in several mutants of *Staphylococcus aureus* lacking the ribitol teichoic acid of the cell wall (Shaw *et al.*, 1970), and exhibiting a sevenfold accumulation of a specific soluble precursor, CDP-ribitol, no specific enzymic deficiencies were identified.

We report here a preliminary biochemical analysis of mutants bearing mutations in different *tag* genes and the identification of the gene which encodes gro-PCT, an enzyme specifically concerned with the synthesis of poly(groP).

Methods

Bacteria. The strains of *B. subtilis* 168 used are listed in Table 1. Apart from *tagB1* (formerly *tag-1*) (Boylan & Mendelson, 1969) and *tagF1* (formerly *rodC1*) (Karamata *et al.*, 1972), all *tag* mutations were identified among a collection of mutants with a conditional lethal thermosensitive phenotype (Briehl *et al.*, 1989).

Media. TS plates and liquid medium SAT, containing glucose and supplemented where necessary with 100 μ g adenine ml⁻¹, have been described (Karamata & Gross, 1970; Briehl *et al.*, 1989). Growth was followed by nephelometric density (ND). A ND of 1 corresponds to 1.34 μ g bacterial dry weight ml⁻¹.

Chemicals. [2-³H]Glycerol and L-[α -¹⁴C]glycerol phosphate were obtained from Amersham, cytidine 5'-mono (CMP), -di (CDP) and -tri (CTP) phosphates from Fluka (Switzerland), cytidine 5'-diphosphogly-

cerol and L- α -glycerol 3-phosphate (α -groP) from Sigma, activated charcoal from Merck, and Superose 12 from Pharmacia.

Extraction of soluble precursors, including CDP-glycerol, from cultures of thermosensitive mutants pulse-labelled with [2-³H]glycerol following shift to a restrictive temperature. [2-³H]Glycerol was employed to specifically label glycerol-containing precursors and macromolecules since metabolic conversion of glycerol to dihydroxyacetone phosphate leads to loss of the label from the C-2 position to the water pool (Daniels, 1969), so that ensuing intermediary metabolites derived from glycerol are unlabelled.

Exponential-phase seed cultures, grown for 16 h at 22 °C in SAT supplemented with 200 μ M-glycerol, were diluted into the same medium to a ND of 10 and incubated with aeration at 30 °C, the permissive temperature. At a ND of 100, to diminish the growth-inhibitory effects of a sudden shift, the temperature was progressively raised to 47 °C over a 12–14 min interval. Immediately upon reaching 47 °C, a 4–5-fold dilution was made (to a ND of 40) into the same medium supplemented with 0.4 μ Ci ml⁻¹ of [2-³H]glycerol [specific activity 2 Ci mol⁻¹, 74 GBq mol⁻¹]. After a 5 min pulse, duplicate 5 ml samples were filtered, washed with water and resuspended in 1 ml water. The suspension was placed in a boiling water bath for 40 min to extract the pool of soluble glycerol-labelled precursors, which was separated from the insoluble cell fraction by filtration. After a 20 min treatment of the filter with 1 ml 0.1 M-NaOH at 100 °C, the total radioactivity incorporated was determined by scintillation counting in 10 ml of Optifluor (Packard).

The concentration of nucleotide-containing precursors in the extract was estimated according to Strominger (1962). Nucleotide-containing precursors were then separated by selective adsorption onto activated charcoal added in amounts equivalent to 20 mg per μ mol of nucleotide. After gentle shaking for 20 min, at room temperature, the charcoal was pelleted and washed twice with 1 ml 100 mM-ammonium acetate. Adsorbed nucleotides were eluted with 50% (v/v) ethanol in water/

Table 1. *Bacillus subtilis* strains

Strain	Genotype	Reference or construction
L5025	<i>purA16 leuA8 ilvA1 lys-31</i>	Brandt & Karamata (1987)
L5087	<i>hisA1 argC4 metC3</i>	Briehl <i>et al.</i> (1989)
L6601	<i>hisA1 argC4 metC3 tagD11*†‡</i>	Briehl <i>et al.</i> (1989)
L6456	<i>purA16 hisA1 argC4 metC3 tagF1†‡</i>	Briehl <i>et al.</i> (1989)
L6476	<i>hisA1 argC4 metC3 tagB1†</i>	Briehl <i>et al.</i> (1989)
L6477	<i>hisA1 argC4 metC3 tagF1†</i>	Briehl <i>et al.</i> (1989)
L6604	<i>hisA1 argC4 metC3 tagF14†</i>	Briehl <i>et al.</i> (1989)
L6605	<i>hisA1 argC4 metC3 tagF15</i>	Briehl <i>et al.</i> (1989)
L6651	<i>leuA8 ilvA1 lys-31 tagD11†</i>	Congression of L6601 $\rightarrow$ L5025
L6653	<i>leuA8 ilvA1 lys-31 tagB1</i>	Congression of L6476 $\rightarrow$ L5025
L6654	<i>leuA8 ilvA1 lys-31 tagF1†</i>	Congression of L6477 $\rightarrow$ L5025
L2100	<i>leuA8 ilvA1 lys-31</i>	Brandt & Karamata (1987)
L2129	<i>leuA8 ilvA1 lys-31 lssA29</i>	Brandt & Karamata (1987)
L2123	<i>leuA8 ilvA1 lys-31 lssB23</i>	Brandt & Karamata (1987)

* Formerly *tag-11* (Briehl *et al.*, 1989). Mutations *tag-11* and *tag-12* have probably arisen from the same clone, not being separated in transformation crosses (Briehl *et al.*, 1989). [2-³H]Glycerol incorporation into soluble and macromolecular fractions associated with *tag-12* (not shown) were indistinguishable from those shown here for *tagD11*.

† Distinctive colony morphology on TS plates at 30 °C; smaller, smoother and more raised colonies than those of the wild-type.

‡ The assignment of *tag* mutations to genes labelled *tagA* to *F* comes from Mauël *et al.* (1991). *tagF1* was formerly *rodC1* (Karamata *et al.*, 1972), *tag-3* (Pooley *et al.*, 1987), or *tagB3* (Mauël *et al.*, 1989). *tagB1* was formerly *tag-1* (Boylan *et al.*, 1972), *rodA* (Rogers *et al.*, 1974), or *tagA1* (Mauël *et al.*, 1989).

0.05 M-NH₄OH (1:1, v/v) by one of the following methods: (i) two 40 min treatments at room temperature with gentle shaking (Strominger, 1962), or (ii) a 60 min incubation at 70 °C (Glaser, 1965). The recovery, of approximately 80%, was slightly better with the second method. Eluted radioactivity was determined in the same way as that on the filters.

Paper chromatography. Presence of CDP-[2-³H]glycerol in the hot-water extract of cells pulse labelled with [2-³H]glycerol was determined according to Paladini & Leloir (1952) by descending chromatography on Whatman no. 3MM paper using solvents (A) ethanol/1 M-ammonium acetate, pH 7.8 (7.5:3, v/v) and (B) ethanol/1 M-ammonium acetate, pH 3.8 (7.5:3, v/v). Presence of sugar phosphates was established by the method of Bandursky & Axelrod (1952) and their position compared with those of authentic standards. Adequate separation of CDP-glycerol, UDP-*N*-acetylglucosamine, α -groP, glycerol, CMP, CDP and CTP standards, was obtained and *R_F* values agreed with previous results (Glaser, 1965; Glaser & Loewy, 1979). Scintillation counting revealed that 90% of the radioactivity in the nucleotide-containing fraction (see above, and Results) of the hot-water extract co-chromatographed with a CDP-glycerol standard.

Transformations. These were performed as previously described (Karamata & Gross, 1970).

Preparation of a crude cytosol fraction for assay of gro-PCT. Following several control experiments, the method adopted for crude cytosol preparation was that described by Glaser & Loewy (1979) modified as follows: the washing step, involving resuspension in buffer and resedimentation of the harvested cell pellet, was replaced by a simple rinse, with medium, prior to resuspension in buffer for cell breakage. This modification was accompanied by a 70% increase in final specific activity of the gro-PCT. A seed culture grown at 22 °C for 16 h in SAT supplemented with 200 μ M-glycerol was diluted to ND 10 in the same medium and incubated at 30 °C. At ND 100, the culture (200 ml) was harvested by centrifugation at 8000 r.p.m. (Sorvall rotor GSA). The pellet, after being rinsed (Pooley *et al.*, 1987) with the same medium, was resuspended in 3.5 ml TEM buffer (50 mM-Tris/HCl pH 7.5, 10 mM-MgCl₂, 1 mM-EDTA). Cells were broken by three passages through a French press. Centrifugation at 18000 r.p.m. for 15 min yielded a pellet of walls, membranes and unbroken cells. The supernatant containing the soluble fraction of the cell was either assayed for gro-PCT immediately, or small aliquots were frozen rapidly and stored at -70 °C. No significant loss of gro-PCT activity in frozen extracts (thawed once only) was noticed during a three month period. The protein content of the cytosol was assayed by the method of Bradford (1976).

Assay of gro-PCT in cytosolic extracts. The reaction

was measured by a radioassay in the direction of CDP-glycerol formation as described by Glaser & Loewy (1979). The CDP-[¹⁴C]gro formed from L-[¹⁴C]glycerol phosphate was determined in the nucleotide-containing fraction eluted from active charcoal. For reasons of economy, the L-[¹⁴C]groP was used at a less than saturating concentration, approximately one-fourth of the apparent *K_m* of 6.2×10^{-4} M (Glaser, 1965). Under the assay conditions used, the measured rate of accumulation of CDP-[¹⁴C]gro was proportional to the quantity of protein present in the crude cytosol fraction of the wild-type up to at least 500 μ g. In most cases 10-fold lower amounts of enzyme were assayed. To obtain measurable amounts of CDP-gro formation with enzyme deficient strains, 10- to 20-fold higher concentrations of extract or of substrates, or of both simultaneously, were employed. Initially, following Glaser & Loewy (1979), the reaction mixture contained 10 μ mol Tris/HCl (pH 7.5), 2 μ mol MgCl₂,

0.2 μ mol EDTA, 0.25 μ mol CTP and 0.1 μ mol L-[¹⁴C]glycerol 3-phosphate (1 μ Ci mmol⁻¹) in a final volume of 700 μ l. After finding that 1% (w/v) bovine serum albumin (BSA) efficiently stabilized the gro-PCT activity at temperatures above 30 °C (see Results), it was routinely added to the reaction mixture during assay at all temperatures employed.

The enzyme-containing cytosol, after being thawed on ice, was prewarmed to the assay temperature just prior to addition to all remaining components of the reaction mixture. After 10 min incubation, in the majority of cases, the reaction was stopped by addition of 700 μ l trichloroacetic acid (2%, w/v) in acetate buffer (pH 4.2; 0.15 M). After 30 min on ice, precipitated proteins were removed by centrifugation. The supernatant contains the CDP-[¹⁴C]glycerol formed, which was separated from [¹⁴C]groP by adsorption to, and elution from, active charcoal as described above (method ii). In the course of the experiments described below, the specific activity of gro-PCT in extracts of the wild type strain, measured under standard conditions, fell from around 30 units, initially, to a stable figure of 7 units. One unit is defined as the activity yielding 1 nmol CDP-glycerol min⁻¹ (mg protein in the extract)⁻¹. Several control experiments were performed, varying the method of extract preparation. The results did not allow identification of the factor(s) responsible for the decrease in activity. Nevertheless, comparison with previous results revealed that the lowest figure obtained here of 33 nmol min⁻¹ (mg protein)⁻¹, measured in the presence of 10-fold higher concentrations of α -groP and CTP than those in the standard assay, is close to that reported (Anderson *et al.*, 1973) for a partially purified preparation of gro-PCT. When assayed under the standard (non-saturating) conditions employed here, the final figure of 7 units is about one-half the value (between 14 and 19 units) reported by Glaser & Loewy (1979) for a partially purified enzyme preparation.

Apparent molecular size determination of gro-PCT. Exclusion from a molecular sieve was employed. A 200 μ l aliquot, containing 470 μ g cytosol protein of the strain carrying the *tag*⁺ allele, was introduced onto a column (1.0 $\times$ 30 cm, 23.5 ml) of Superose 12 equilibrated with the same buffer (TEM, see above) as used for elution. Fractions (500 μ l) collected were assayed for gro-PCT and activity was found in a single peak spread over four fractions with an apparent molecular mass of about 30.5 kDa based on the exclusion volumes of molecular size standards, BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

Results

Incorporation of [2-³H]glycerol into the soluble pool and macromolecular fractions of B. subtilis 168 strains bearing mutations in different tag genes

Eleven *B. subtilis* 168 thermosensitive mutants bearing linked mutations in at least two different *tag* genes (Briehl *et al.*, 1989; Honeyman & Stewart, 1989; Mauël *et al.*, 1989), were examined, under restrictive conditions, for incorporation of [2-³H]glycerol into macromolecular fractions and accumulation of CDP-glycerol, a specific precursor of poly(groP). To attenuate the effects of heat shock, exponentially growing cultures were brought up to the restrictive temperature, 47 °C, over a 12–14 min transition period. Immediately upon reaching 47 °C, cultures were diluted and pulse-labelled with [2-³H]glycerol. Aliquots were filtered and the cells, after washing,

Table 2. Incorporation of [2-³H]glycerol into the insoluble fraction and accumulation of CDP-[2-³H]glycerol in the soluble fraction of *Bacillus subtilis* 168 isogenic mutant strains bearing *tag* or *lss* markers during a 5 min pulse at 47 °C, the non-permissive temperature

Strain*	Macromolecular (insoluble) fraction		Soluble fraction glycerol equivalents [nmol (mg cell dry wt) ⁻¹]					
	Glycerol† [nmol (mg cell dry wt) ⁻¹]	(R)‡	Total 100 °C water extract	(R)‡	Non-nucleotide	(R)‡	CDP-gro§ [nmol (mg cell dry wt) ⁻¹]	(R)‡
L6653 <i>tagB1</i>	42	(0.21)	14	(4.0)	3.3	(1.9)	10	(12)
L6651 <i>tagD11</i>	37	(0.18)	2	(0.6)	1.4	(0.8)	0.13	(0.16)
L6654 <i>tagF1</i>	94	(0.47)	8	(2.3)	2.5	(1.5)	4.6	(5.8)
L2129 <i>lssA29</i>	78	(0.39)	9	(2.6)	1.9	(1.1)	3.8	(4.8)
L2123 <i>lssB23</i>	84	(0.42)	12	(3.1)	4.5	(2.6)	3.4	(4.3)
L2100 <i>tag</i> ⁺ , <i>lss</i> ⁺	199	(1)	3.5	(1)	1.7	(1)	0.8	(1)

* Relevant mutations only shown.

† Calculated from the specific activity (2 Ci mol⁻¹) of [2-³H]glycerol in the medium. It is assumed that all cellular glycerol is obtained from exogenous glycerol without dilution. If, as appears unlikely, endogenous synthesis were to occur, there is no reason to expect differences between a set of isogenic strains (see Table 1).

‡ (R) represents the ratio of incorporation into the mutant compared with that into the wild-type strain.

§ Radioactivity adsorbed to charcoal is essentially accounted for by CDP-glycerol (Methods and Results).

|| Markers involved in the synthesis of peptidoglycan (Brandt & Karamata, 1987).

and resuspension in water, were placed in a boiling water bath. The distribution of label between the filtrate, containing the soluble precursors, and the insoluble fraction was determined. Comparison of strains bearing representative mutations (Table 2) revealed that, relative to the *tag*⁺ allele, *tagF1* and *tagB1* (formerly *tag-1*) mutations conferred a three- to fourfold increase in the [2-³H]glycerol in the soluble fraction, which points to a substantial increase in the CDP-glycerol pool, following transfer to restrictive conditions, where synthesis of wall teichoic acid is inhibited (Briehl *et al.*, 1989). The remaining mutation, *tagD11* (*tag-11*), was associated with a soluble pool virtually indistinguishable from that of the wild type, even though showing an inhibition of teichoic acid synthesis comparable to that of the other *tag* mutations. The phenotype of *tagD11*-bearing strains, clearly distinct from that conferred by *tagB1*, is consistent with the genetic evidence that the relevant mutations map in different genes (Mauël *et al.*, 1991).

To assess if the increase in [³H]glycerol-containing pools is specific to *tagB* and *tagF* markers, incorporation of [2-³H]glycerol into four thermosensitive mutants bearing different *lss* markers (Brandt & Karamata, 1987) was examined. The latter strains were shown to be affected in different steps of the synthesis of soluble precursors of peptidoglycan (PG) (Brandt & Karamata, 1987; Roten *et al.*, 1991). It appeared (Table 2, and data not presented) that all the *lss* loci were characterized by significantly increased soluble pools and a reduced incorporation of [2-³H]glycerol into the macromolecular fraction which were comparable to those associated with

tagF markers (see below). This result is consistent with a reduction of glycerol incorporation into poly(groP), which is probably the consequence of a prior inhibition of PG synthesis. Indeed, synthesis of both polymers occurs coordinately in *B. subtilis*, and teichoic acid chains are attached to those of PG made simultaneously (Mauck & Glaser, 1972).

The rate of incorporation of label into the insoluble fraction revealed some striking differences between *tag*-bearing mutants (Table 2). While significant reductions, relative to the wild-type, were associated with all three markers, that for *tagF1* was 50%, whereas those for the remaining markers were more pronounced, i.e. nearly 75% for *tagB1* and 80% for *tagD11*. [2-³H]Glycerol labels essentially three fractions in *B. subtilis* 168 (Sargent, 1973; H. M. Pooley, unpublished results); 75% is accounted for by poly(groP), and the remainder is distributed, in roughly equivalent amounts, in lipid and lipoteichoic acid. Thus, the reduction in glycerol incorporation associated with all *tag* mutations examined is consistent with the markedly reduced phosphate contents of isolated cell walls (Briehl *et al.*, 1989) and suggests a selective slowing of wall teichoic acid synthesis. Eight more strains (Briehl *et al.*, 1989), each carrying a different mutation in the *tagF* locus, behaved similarly to that bearing *tagF1* (data not presented). However, mutations *tagF14*, *F16*, *F18* or *F19*, associated with a leaky phenotype (Briehl *et al.*, 1989), were characterized by a less pronounced decrease in macromolecular synthesis, as well as by smaller increases in the soluble pool.

Analysis of CDP-glycerol and non-nucleotide glycerol-containing pools in *tag* mutants

To separate nucleotides from other soluble precursors, extracts obtained in boiling water (see above) were treated with activated charcoal. Nucleotide precursors were processed by paper chromatography for identification of CDP-glycerol and quantification of radioactivity in isolated spots (see Methods). It appeared that CDP-glycerol represents about 30% of the total soluble label for the wild-type. For strains carrying *tagB* or *tagF* mutations, the amount of CDP-glycerol was 6- to 12-fold higher than in the wild-type. Following expression of the phenotype associated with *tagF* and *tagB* mutations, the measured amounts of CDP-glycerol were between 4.6 and 10 nmol (mg cell dry weight)⁻¹, as against 0.8 for the *tag*⁺ allele. These figures are comparable to those reported (Shaw *et al.*, 1970) for the major soluble precursor, CDP-ribitol, in teichoic acid deficient mutants of *Staphylococcus aureus* and their parent strain: 3 and 0.4 nmol (mg cell dry weight)⁻¹, respectively. Increased pools of CDP-glycerol were found in all strains (Briehl *et al.*, 1989) carrying *tagF* markers (data not presented). This accumulation of a specific poly(groP) teichoic acid precursor, CDP-glycerol, is consistent with a block in wall teichoic acid synthesis. *tagD11* contrasts strikingly with other *tag* mutations, since it confers a considerably reduced CDP-glycerol pool, barely 16% of that of the *tag*⁺-bearing strain. This failure to accumulate CDP-glycerol under restrictive conditions, where its incorporation into the wall is strongly inhibited (Briehl *et al.*, 1989) argues in favour of a block in CDP-glycerol formation. The function affected by *tagD11* is not primarily concerned with glycerol uptake or glycerol kinase activity since, under restrictive conditions, glycerol incorporation into the macromolecular fraction continues with linear kinetics, albeit at a reduced rate, for a generation at least (data not presented).

Interestingly, label in the non-nucleotide fraction of the soluble pool (Table 2), of which α -[2-³H]groP, the immediate precursor of CDP-glycerol, forms about half (not presented), was also apparently somewhat increased in strains bearing *tagB1* and *tagF* markers (Table 2). The *tagD11* mutation was again exceptional in not being associated with any apparent accumulation of α -groP (see Discussion).

gro-PCT activity in crude cytosol fractions of strains bearing *tag* mutations

The enzyme gro-PCT, responsible for synthesis of CDP-glycerol from α -groP and CTP, appeared to be the prime candidate for the deficient activity in strain L6601 (*tagD11*). The enzyme activity was assayed at 25 °C on

Table 3. *gro-PCT* activity in the crude cytosol fraction of *B. subtilis* 168 strains bearing mutations in different *tag* genes: thermosensitivity of *gro-PCT* activity in a *tagD*-bearing mutant

Strain*	gro-PCT† [nmol min ⁻¹ (μg protein) ⁻¹]		
	25 °C	42 °C	<i>R</i> _{42/25} ‡
L5087 <i>tag</i> ⁺	6.4 ± 0.2	5.2 ± 0.2	0.8
L6456 <i>tagF1</i>	2.5 ± 0.1	2.3 ± 0.2	0.9
L6604 <i>tagF14</i>	5.6 ± 0.2	5.1 ± 0.1	0.9
L6605 <i>tagF15</i>	3.4 ± 0.3	3.1 ± 0.1	0.9
L6476 <i>tagB1</i>	3.8 ± 0.1	2.6 ± 0.2	0.7
L6601 <i>tagD11</i>	0.08 ± 0.02	< 0.01	< 0.1

* Relevant mutations only shown.

† Specific activity was measured under standard conditions (see Methods) at the temperature indicated, with 50 μg protein per assay, except for *tagD11*, where 1 mg of extract protein was used. Results shown are mean values, with standard deviations, of a minimum of two separate assays; for *tagD11* and *tag*⁺ alleles, four separate cytosol preparations were assayed.

‡ The ratio of activity measured at 42 °C compared with that at 25 °C.

crude extracts obtained from cultures grown at 30 °C (Table 3). While gro-PCT activity was readily detectable in extracts of the wild-type, as well as in those of mutants L6604 (*tagF14*) and L6605 (*tagF15*), it was practically undetectable in that of mutant L6601 (*tagD11*). This result, in agreement with the observed large increases in the CDP-glycerol pool associated with mutations *tagF1* and *tagB1*, implies that gro-PCT is not affected by mutations in the *tagF* and *tagB* genes. Assay of 20-fold higher amounts of crude extracts from strain L6601 (*tagD11*) did yield a detectable activity. However, the specific activity thus measured was 100-fold lower than that of the wild-type strain: 0.08 versus 6.4 nmol min⁻¹ (mg protein)⁻¹. Assay of amounts as high as 1 mg cytosol protein in the presence of 10-fold higher concentrations of both substrates, CTP and α -groP, yielded specific activities about five times higher: 0.43 and 33 nmol min⁻¹ mg⁻¹ for strains L6601 (*tagD11*) and L5087, (wild-type), respectively. The strongly reduced gro-PCT activity measured in cell-free extracts of mutant L6601 at 25 °C, below the permissive growth temperature, is entirely consistent with the failure to accumulate a pool of CDP-gro at the non-permissive temperature. The alternative possibility of an inhibitor of this enzyme being present in the extracts of this mutant was examined by measuring the gro-PCT activity when extracts from mutant L6601 (*tagD11*) and the *tag*⁺ strain were pooled in equal proportions. Gro-PCT activity was found to be strictly proportional to the amount of *tag*⁺ cytosol protein, arguing in favour of a defective protein

and against the presence of an inhibitor in the mutant extract.

Thermostability of the gro-PCT in extracts of strains bearing mutations in tagA, tagB and tagF genes

Assaying gro-PCT in extracts of the wild-type strain at different temperatures showed that temperatures above 30 °C strongly diminished the measured activity: at 37 °C, it was barely 30% of that at 30 °C, whereas at 42 °C, no activity could be detected. Stabilization of the activity of the wild-type enzyme at higher temperatures was attempted by addition of bovine serum albumin (BSA). In the presence of 1% (w/v) BSA the measured activity at 37 °C was identical to that at 30 °C, whereas at 42 °C, it was barely 20% lower. The growth temperature of the culture from which the extracts were prepared did not influence this result (not shown). Comparison of the gro-PCT activities (Table 3) at two assay temperatures (42 and 25 °C) expressed as the ratio $R_{42/25}$ did not reveal significant differences with respect to the wild-type ($R_{42/25} = 0.84$) for extracts of three representative strains carrying *tagF* markers – *tagF1*, *F14* and *F15*. With an $R_{42/25}$ of 0.7, *tagB1* may be associated with a slightly more pronounced temperature sensitivity. This absence of thermosensitivity was only to be expected in view of the rapid accumulation of CDP-glycerol in these strains following transfer to the non-permissive temperature. A striking contrast was offered by the *tagD11*-bearing mutant. At the non-permissive temperature, gro-PCT activities were dramatically reduced ($R_{42/25} \leq 0.1$), implying the existence of a modified, heat-sensitive protein, strongly suggesting that the mutation affects the structural gene of the gro-PCT.

Apparent molecular size determination

Passage through a Superose 12 column of the crude cytosol of the wild-type strain L5087 yielded gro-PCT activity in a single peak with an apparent molecular size of about 30.5 kDa. Comparison with the mass, 14.5 kDa, of the putative polypeptide predicted from the nucleotide sequence of the *tagD* gene (Mauël *et al.*, 1991) suggests the presence of a dimer.

Discussion

The results presented here reveal that *tagD* is most likely to be the structural gene of gro-PCT, an enzyme specific for the synthesis of poly(groP), the major cell wall anionic polymer in *B. subtilis* 168. Thus, they eliminate any remaining doubts (Rogers *et al.*, 1974; Rogers & Taylor, 1978) that the conditional lethal phenotype of

a *tagD*-bearing mutant may not be a consequence of a single primary defect specific to teichoic acid synthesis. The likelihood that other *tag* genes encode poly(groP)-specific proteins is also reinforced. The conclusion that poly(groP) synthesis is essential for growth (Karamata *et al.*, 1987; Briehl *et al.*, 1989; Mauël *et al.*, 1989) is now made explicit.

It is interesting that the existence of another *B. subtilis* mutant deficient in gro-PCT has been reported (Rosenberger, 1976; Rogers, 1979). Although, like the *tagD*-bearing mutant, it grew as a coccus and was deficient in wall phosphate, this mutant was reported not to have a conditional lethal phenotype. However, in the absence of any published data, it is difficult to evaluate this observation. Furthermore the mutant is likely to be genetically complex. In this respect it may resemble the partial revertants of other *tag*-bearing mutants (Shifflett *et al.*, 1977) which, at the restrictive temperature, grow slowly, form small colonies, and have low but significant amounts of phosphate in their walls.

In comparison with all other strains, where ^3H -labelled α -groP forms about 50% of the label in the non-nucleotide pool, the low level of ^3H label in the soluble pool of the gro-PCT-deficient mutant implies a failure to accumulate α -groP. Formation of the latter compound is not blocked in the *tagD11*-bearing mutant, however, since incorporation of [^3H]glycerol into some macromolecular fractions continues. Furthermore, the gene encoding glycerol kinase maps at 75°, together with other genes involved in glycerol metabolism (Lindgren & Rutberg, 1974). The absence of accumulation of α -groP presumably means, therefore, that glycerol uptake is subject to feedback inhibition by a relatively small increase in the α -groP pool. In the case of all other *tag* markers, presence of larger amounts of ^3H in the non-nucleotide pool is probably due to back formation of α -groP from the accumulated CDP-glycerol by an active CDP-glycerol pyrophosphatase, known to be present in this organism (Glaser, 1965).

Pulse-labelling experiments with [$2\text{-}^3\text{H}$]glycerol, immediately after transfer to 47 °C, suggest that the very low cell wall teichoic acid content of all *tag*-bearing mutants (Briehl *et al.*, 1989), observed two or more hours after transfer to 47 °C, is a consequence of a rather rapid expression of the relevant *tag* mutations. However, the increased pool of CDP-glycerol which in all but one instance accompanies this phenotype is shown here not to be limited to mutations almost certainly affecting enzymes of poly(groP) synthesis. Indeed, accumulation of this precursor is also conferred by mutations affecting different steps of the synthesis of soluble peptidoglycan precursors. This result is not surprising, since transfer of any membrane-linked poly(groP) to nascent peptidoglycan is coupled to the synthesis of the latter (Mauck &

Glaser, 1972). However, in the case of *tag* mutations, a block in teichoic acid synthesis does not appear to prevent synthesis of peptidoglycan (Rogers *et al.*, 1974; Briehl *et al.*, 1989; H. M. Pooley, unpublished observations).

The isolation of clones containing *tag* genes (Honeyman & Stewart, 1988; Mauël *et al.*, 1989) and subsequent nucleotide sequencing (Honeyman & Stewart, 1989; Mauël, *et al.*, 1991) have revealed the presence of six genes grouped in two operons, one containing *tagA*, *B* (formerly *tagA*), and *C*, and the other containing *tagD*, *E* (*gtaA*) and *F* (*rodC*) genes. So far, products of only two genes have been probably identified, namely, the UDP-glucose poly(groP)- α -glucosyltransferase likely to be encoded by *tagE* (Young, 1967; Young *et al.*, 1969; Brooks *et al.*, 1971) and gro-PCT encoded by *tagD*. Since two out of the three genes of the *tagDEF* operon encode enzymes specifically involved in the synthesis of the glucosylated poly(groP), it is most likely that the remaining gene also encodes a protein concerned with the synthesis of this polymer.

This conclusion raises the question of the striking absence from the mutant collection obtained by the rationale of Briehl *et al.* (1989) of mutations affecting the *tagABC* operon. All of the latter mutants harbour mutations in genes *tagF* or *tagD*. Indeed, up to eight mutations in this collection map in a single gene, *tagF* (Briehl *et al.*, 1989), and one in the small gene *tagD* (Mauël, *et al.*, 1991) belonging to the same operon, *tagDEF*. Based on size calculated from nucleotide sequences (Honeyman & Stewart, 1989; Mauël *et al.*, 1991), the frequency of mutations obtained (approximately one per 8 kDa of protein) is apparently not very different for these two target genes. Many mutations in the remaining locus in this operon, *tagE* (*gtaA*) have been identified (Young, 1967; Pooley *et al.*, 1987). However, they do not have a conditional lethal phenotype characteristic of mutations in the remaining genes of this operon: non-glucosylated poly(groP) is incorporated into the cell wall of *tagE*-containing mutants (Young, 1967). Interestingly, unlike most, if not all, *tagF* mutations in our collection, as well as *tagF1* (*rodC1*), obtained independently (Briehl *et al.*, 1989), the only mutation to have been mapped in the *tagABC* operon, *tagB1*, remains sensitive to $\phi 29$ at 37 °C and higher temperatures (H. M. Pooley, unpublished observations). If this property were to be shared by other mutations in this operon, their elimination during the enrichment procedure (Briehl *et al.*, 1989) could account for their absence from the above-mentioned collection. Introducing new mutations by *in vitro* mutagenesis of the relevant clones should allow this question to be answered.

Interest in components regulating bacterial morphogenesis led to the isolation, some 20 years ago (Satta *et al.*,

1969; Boylan & Mendelson, 1969; Rogers *et al.*, 1970) of conditional lethal mutants affected in cell shape, in several rod-shaped organisms. Our results imply the identification in one organism, at least, of one such component. Nevertheless, the identification of an essential function, and the knowledge of some of the complex abnormalities in the physiology, surface growth and morphology of the cell that follow a block in this function, in no way furnish a ready-made understanding of the role(s) of poly(groP) during normal growth and division. This challenge remains to be met.

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