Incorporation of [2-³H]glycerol into cell surface components of *Bacillus subtilis* 168 and thermosensitive mutants affected in wall teichoic acid synthesis: effect of tunicamycin

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A method is described for measuring the synthesis of poly(glycerol phosphate) [poly(groP)], the major wall teichoic acid (WTA), lipoteichoic acid (LTA) and phospholipid (P-lipid), through fractionation of [2-³H]glycerol ([2-³H]gro)-labelled *Bacillus subtilis* cells. When cultures of certain temperature-sensitive mutants defective in one of several *tag* genes, encoding enzymes involved in WTA synthesis, were transferred to the restrictive temperature, the synthesis of WTA underwent a specific, immediate, block, while that of LTA or P-lipid proceeded unimpeded. These results, in addition to confirming the role of *tag* genes, demonstrated, reciprocally, the specificity of the fractionation procedure used to distinguish label in WTA from that in LTA or P-lipid. Results of analysis of other, less severely affected, *tag*-deficient mutants, as well as of another genetically unrelated mutant developing comparable morphological phenotypes in non-permissive conditions, are discussed in relation to a possible mechanism generating the latter phenotype. Fractionation of *B. subtilis* 168 cells labelled either with [2-³H]gro or with [1-¹⁴C]*N*-acetylglucosamine, to which tunicamycin was added at 0.5 µg ml⁻¹ (the MIC) revealed a specific and marked inhibition of poly(groP) as well as of poly(3-*O*-β-D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate), the minor WTA. However, for 60 min at least, the syntheses of PG, LTA and P-lipid were barely affected.

Keywords: poly(glycerol phosphate), tunicamycin, teichoic acid, thermosensitive *tag* mutants, phospholipid

INTRODUCTION

Among temperature-sensitive (Ts) mutants of *Bacillus subtilis* 168, a subset develops, at the non-permissive temperature, misshapen cells with characteristic rounded, swollen forms, frequently accompanied by marked, localized thickening of the cell wall (Boylan *et al.*, 1972; Rogers *et al.*, 1970; Briehl *et al.*, 1989; Pollack & Neuhaus, 1994). The relevant genes include (i) a homologue of the *Escherichia coli* *rodA*, that, together

with PBP2, is concerned with peptidoglycan (PG) synthesis during the cylindrical extension (elongation) of the cell (Henriques *et al.*, 1998), (ii) *rodB*, a homologue of the *E. coli* *mreD* (Levin *et al.*, 1992; Varley & Stewart, 1992), which, together with the adjacent *mreC*, is concerned with *E. coli* cell-shape maintenance, and (iii) genes involved in the synthesis of poly(glycerol phosphate) [poly(groP)], the principal *B. subtilis* wall teichoic acid (WTA) (Boylan *et al.*, 1972; Mauël *et al.*, 1991; Pooley *et al.*, 1991, 1992; Lazarevic & Karamata, 1995). The complex cell-shape changes developed after several hours at non-permissive temperatures, following the expression of the phenotype caused by mutation in any of the essential *tag* genes (Mauël *et al.*, 1989, 1991), have been attributed to one initial event: the arrest of cell-surface elongation (Pooley *et al.*, 1993). This explanation is consistent with results

Abbreviations: GlcNAc, *N*-acetylglucosamine; [2-³H]gro, [2-³H]glycerol; LTA, lipoteichoic acid; ND; nephelometric density; PG, peptidoglycan; P-lipid, phospholipid; poly(GlcGalNAc 1-P), poly(3-*O*-β-D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate); poly(groP), poly(glycerol phosphate); Ts, temperature sensitive; TUN, tunicamycin; WTA, wall teichoic acid.

obtained in studies with other *B. subtilis* genes which affect the cell morphology. However, in the case of *rodA*, an additional inhibitory effect on the rate of initiation of septa was evoked (Henriques *et al.*, 1998), whereas for *rodB*, the main deficiency was considered to be that of initiation of septum formation (Varley & Stewart, 1992).

While most of these genes are known to be concerned with the synthesis of cell-surface components, knowledge of the interdependences of syntheses of the distinct surface components is limited to that of WTA and covalently linked PG (Mauck & Glaser, 1972). Notably, there is an almost total ignorance of the impact of interference with WTA on the synthesis of other cell-surface components, such as lipoteichoic acid (LTA) or phospholipid (P-lipid). It has been shown that blocking P-lipid synthesis in *E. coli* leads to a block in PG as well as of lipopolysaccharide synthesis (Ehlert & Hölte, 1996). To adequately address these questions, a reliable method for the measurement of the kinetics of synthesis of relevant surface components in growing cells is needed; none has been reported so far. One such method is described here. Its use, in conjunction with some of the aforementioned mutants, has answered some of the questions raised above.

The exploitation of this method has provided a clearer picture of the effect on *B. subtilis* of tunicamycin (TUN), an antibiotic known to affect the synthesis of both PG and WTA (Ward *et al.*, 1980). *In vitro* studies (Ward *et al.*, 1980) with membrane preparations revealed that, in the initial binding reaction of soluble precursor to lipid carrier, there was a fivefold greater sensitivity to TUN of the WTA-specific step as compared to the PG-specific one. However, data were inadequate to demonstrate that at the MIC, WTA was indeed the effective target *in vivo*. The cell fractionation method reported here for [2-³H]glycerol ([2-³H]gro)-labelled cell populations has been used to address the question of the *in vivo* target of TUN at the MIC for growth.

METHODS

Strains. *B. subtilis* strains are listed in Table 1.

Media. Luria agar (L medium) was used for maintaining viable cell populations. SATrpgro is SATr medium (Karamata & Gross, 1970) supplemented with 2.5 mM MgCl₂ and 20 µg tryptophan ml⁻¹, and, as specified, either 2.5 or 6 mM glycerol. When necessary, 100 µg adenine ml⁻¹ was added. For experiments involving labelling with *N*-acetyl[1-¹⁴C]glucosamine ([1-¹⁴C]GlcNAc), *N*-acetylglucosamine (100 µM, GlcNAc) was added. TMS, the hypertonic buffer, contained 50 mM Tris, pH 8.0, 10 mM MgCl₂, 33% sucrose and, unless otherwise stated, 120 µg lysozyme ml⁻¹. Protoplast lysis buffer contained Tris (50 mM, pH 8.0), MgSO₄ (5 mM) and DNase (80 µg ml⁻¹). TUN (Sigma) was used at a final concentration of 0.5 µg ml⁻¹.

Growth conditions. The protocol was devised for the study of Ts mutants, the phenotype of which is generally well expressed when exponentially growing cells are shifted to the non-permissive temperature. The increase was effected progress-

ively over 10–13 min to attenuate the heat shock (Pooley *et al.*, 1991). Growth was expressed as nephelometric density (ND), measured with a Corning EEL nephelometer. An ND of 100 corresponds to approximately 10⁸ cells ml⁻¹ and a bacterial dry weight of 1.34 µg ml⁻¹. Appropriately supplemented SATrpgro medium was inoculated from a 1–3-d-old L plate and brought into exponential phase by overnight incubation at room temperature. This seed culture was diluted with the same medium to an ND of 4, incubated at 30 °C until ND 40, then progressively shifted to 47 °C and further incubated.

Radioactive labelling of cell surface components. LTA, P-lipids and poly(groP), the major WTA (Sargent, 1973; Fischer *et al.*, 1983; Koch *et al.*, 1984), were labelled during growth at 30 and 47 °C in SATrpgro (6 mM glycerol) supplemented with 1.5 µCi (5.55 × 10⁴ Bq) ml⁻¹ of [2-³H]gro. Samples (1 ml, generally), removed at regular intervals, were filtered, and the cells washed four times with prewarmed SATrpgro containing 20 mM glycerol, and processed (see below).

To label the PG as well as poly(3-*O*-β-D-glucopyranosyl-*N*-acetylgalactosamine 1-phosphate) [poly(GlcGalNAc 1-P)], the minor WTA, cultures were grown in SATrpgro medium supplemented with 100 µM GlcNAc and 0.2 µCi (7.4 × 10³ Bq) ml⁻¹ of [1-¹⁴C]GlcNAc. To follow incorporation into PG, samples, withdrawn at regular intervals, were filtered, and cells were washed with SATrpgro containing 100 µM GlcNAc before being processed (see Results). Radioactive GlcNAc incorporation into poly(GlcGalNAc 1-P) was determined on separate samples according to Pavlik & Rogers (1973) as follows. Cell samples (2–5 ml) were collected on a membrane filter, washed well with water, and resuspended in 1 ml potassium phosphate buffer (0.1 M, pH 7.0). The soluble hexosamine nucleotide pool was extracted by a 5 min incubation in a boiling water bath. Cells were spun down and resuspended in 2 ml 0.1 M sodium citrate buffer pH 4.0. Extraction of poly(GlcGalNAc 1-P) was achieved by a 30 min incubation in a boiling water bath, and the soluble radioactivity separated by centrifugation from the bulk of the sedimentable radioactivity, corresponding to PG (Pavlik & Rogers, 1973).

Fractionation of [2-³H]gro-labelled cells. [2-³H]Gro-labelled cells, harvested on membrane filters and washed, were resuspended, by vigorous mixing, in 500 µl of TMS, the hypertonic buffer, containing 60 µg lysozyme. To ensure maximal solubilization of the cell-wall fraction, the suspension was generally incubated for up to 90 min at 37 °C. Total radioactivity was determined on a 50 µl aliquot. Protoplasts were sedimented at 15000 *g* for 15 min, and the radioactivity of the supernatant, containing the solubilized cell-wall fraction, was determined on a 400 µl aliquot, to which 1 ml water and 9 ml of a scintillation cocktail (Optifluor, Packard) were added. Following the removal of the supernatant, the protoplast pellet was washed by resuspension in 500 µl TMS, and resedimented. To extract the other, membrane-associated glycerol-containing cell components, i.e. LTA and P-lipid, the pellet, after being resuspended in 25 µl TMS with a toothpick, was mixed with 600 µl 0.2 M acetate buffer, pH 3.8, and 600 µl 80% aqueous phenol, and incubated with agitation for 60 min at 50 °C (modified from Fischer *et al.*, 1983; Koch *et al.*, 1984). After cooling on ice, the aqueous and the phenol layers, containing LTA and P-lipid, respectively, were separated by a brief centrifugation, and the radioactivity present in each phase was measured.

Table 1. *B. subtilis* strains

Strain	Genotype or phenotype	Reference or source
168	<i>trpC2</i>	
L1440	Prototroph	Mauël & Karamata (1984)
L5047	<i>purA16 pheA3 hisA35 metB5 trpC2</i>	Mauël <i>et al.</i> (1989)
L5087	<i>hisA1 argC4 metC3</i>	Briehl <i>et al.</i> (1989)
L6477	<i>hisA1 argC4 metC3 tagF1</i> (Ts)	Briehl <i>et al.</i> (1989)
L6476	<i>hisA1 argC4 metC3 tagB1</i> (Ts)	Briehl <i>et al.</i> (1989)
L6601	<i>hisA1 argC4 metC3 tagD1</i> (Ts)	Briehl <i>et al.</i> (1989)
L6604	<i>hisA1 argC4 metC3 tagF14</i> (Ts)	Briehl <i>et al.</i> (1989)
L6563	<i>hisA35 leuA8 metB5 yvyH1</i> (Ts)*	This work
104	<i>hisA1 argC4 leuA8 rodB1</i> (Ts)	Rogers <i>et al.</i> (1971)
L6565	<i>purA16 leuA8 metB5 Ts25</i> †	This work

* Ts mutation transferred by transformation from a strain present in the previously described collection (Briehl *et al.*, 1989) into strain L5047, and localized in the *yvyH* gene (Kunst *et al.*, 1997). The latter, previously designated *orfX*, is located close to the *tagA* to *H* region (Soldo *et al.*, 1993).

† Ts mutation in a strain present in the previously described collection (Briehl *et al.*, 1989), and shown to be unlinked by PBS1 transduction to the *tagA* to *H* gene cluster. Although at non-permissive growth temperature the strain develops a cell morphology comparable to that conferred by mutations in several *tag* genes, its cell wall phosphate content is indistinguishable (not presented) from that of the parent strain.

RESULTS

Optimization of the procedure for fractionating [2-³H]gro-labelled cells

To characterize the synthesis of three *B. subtilis* 168 cell surface components, i.e. WTA, LTA and P-lipids, we employed radioactive glycerol incorporation followed by cell fractionation aimed at separating these components from each other. The bulk of the radioactivity incorporated entered the poly(groP), the major WTA of strain 168 (Table 2). It was solubilized by treating washed intact cells with lysozyme in a hypertonic buffer. The protoplasts obtained were subjected to a two-phase phenol/water treatment which resulted in the extraction of LTA and P-lipid. Experiments designed to establish the critical conditions for optimizing fractionation are reported below.

Limiting cross-contamination of glycerol-containing fractions

Separation, by centrifugation, of the solubilized WTA from the LTA-linked and P-lipid-containing protoplasts was found to lead to good recoveries of all relevant labelled components, while limiting cross-contamination such as that occurring with a previously reported procedure (Sargent, 1973) in which the author's intention was to selectively precipitate, by means of trichloroacetic acid (TCA), the LTA and P-lipid fractions in a whole-cell lysate. In this procedure, a non-specific co-precipitation of high-molecular-mass cell-wall components present in cell lysates, obtained by lysozyme digestion in the absence of an osmotic support, was most likely responsible for the inadequate sep-

aration of P-lipid. It has long been known that treating cells with TCA leads to release of WTA to the soluble fraction (Archibald, 1974). In the procedure described (Sargent, 1973), the TCA supernatant was thought to contain poly(groP), assumed to be entirely TCA soluble under the conditions employed, while being devoid of (precipitated) LTA and P-lipids. However, in preliminary control experiments, we found that the proportion of the total cell-associated radioactivity that was TCA precipitable varied considerably and, notably, was dependent upon cell density, increasing from 33 to 71 % of the total glycerol label for cell densities of ND 250 and 1500, respectively. In view of these results, physical separation of protoplasts from cell-wall-associated label was preferred to TCA precipitation of whole-cell lysates, most likely involving, at higher molecular concentrations, co-precipitation of WTA and other macromolecules.

Consequences of incomplete cell-wall digestion

To determine adequate conditions for lysozyme digestion, [2-³H]gro- or [1-¹⁴C]GlcNAc-labelled exponentially growing cells were collected by filtration, washed, resuspended in the TMS lysis buffer and incubated at 37 °C. Samples, removed at regular time intervals, were centrifuged for 15 min at 15000 *g* and the radioactivity of the protoplast-free supernatant measured. After 45 min in the presence of lysozyme, inspection by phase-contrast microscopy revealed that over 95 % of cells of the parent strain had been converted into protoplasts, whereas up to 90 min of lysozyme treatment was required for the maximum solubilization of the cell-wall-associated label, i.e. its complete release

Table 2. [2-³H]Gro label in different cell fractions of strains bearing the *tag*⁺ allele after various fractionation procedures

Cultures were grown in the presence of [2-³H]gro (see Methods) for four generations at 30 °C prior to transfer to 47 °C. Unless indicated otherwise, samples, taken after various times at 47 °C, were fractionated, according to one or other of the procedures described in the text, by treating the washed cells with lysozyme in a hypertonic buffer yielding a soluble fraction containing poly(groP). The LTA and P-lipid fractions were obtained by extraction with two-phase aqueous phenol either directly from the protoplast pellet or following the recovery of the membrane pellet after a protoplast lysis step.

Strain	Fraction of total label*			
	Poly(groP)	P-lipid	LTA	Cytosol
L1440*	0.73 ± 0.02	0.18 ± 0.025	0.075 ± 0.025	¶
L6565 Ts*	0.73 ± 0.04	0.18 ± 0.025	0.10 ± 0.03	¶
168 <i>trpC2</i> *	0.74 ± 0.02	0.16 ± 0.015	0.093 ± 0.006	¶
168 <i>trpC2</i> †	0.705 ± 0.035	0.12 ± 0.025	0.085 ± 0.035	0.105 ± 0.01
168 <i>trpC2</i> ‡	0.69 ± 0.02	0.12 ± 0.003	0.077 ± 0.01	0.12 ± 0.01
L5087‡	0.84 ± 0.014	0.15 ± 0.014		0.014
L5087§	0.85 ± 0.017	0.074 ± 0.013	0.074 ± 0.006	¶

* Values (fraction of total incorporated radioactivity) represent the mean of four samples withdrawn at 0, 20 or 40 min (two samples) at 47 °C. Intact protoplasts were fractionated with Bligh–Dyer P-lipid extraction (see Results), followed by LTA extraction. For the time 0 sample, total radioactivity incorporated represents 62, 15 and 81 × 10³ d.p.m. for strains L1440, 168 *trpC2* and L6565, respectively.

† Results of two separate experiments are shown: means of duplicate samples taken 50 min after shift to 47 °C. P-lipids and LTA were extracted in a single step with phenol/acetate buffer from membrane preparations obtained by centrifugation of lysed protoplasts.

‡ Incubation with lysozyme was prolonged, to 60 min, with respect to experiments described in the preceding two footnotes (* and †). Total radioactivity in the membrane pellet, obtained from lysed protoplasts, is shown in the absence of further fractionation into P-lipid and LTA. Mean values for four cell samples are shown.

§ Incubation with lysozyme, prolonged, to 60 or to 90 min, with respect to experiments described in the first two footnotes (* and †). Label in LTA and P-lipid was obtained by direct extraction of pelleted intact protoplasts with phenol/acetate buffer. Mean values for five cell samples are shown.

|| Radioactivity determined by counting, in 4 ml Scintillator (Optifluor), a total of 460–500 µl of the lysozyme-solubilized fraction, obtained by centrifuging protoplasts, without dilution by added water. Subsequently, it was found that dilution in water increased the counts by up to 33 %.

¶ Not applicable. Direct extraction of P-lipids and LTA from intact protoplasts.

to the supernatant fraction (Fig. 1). Digestion for even longer periods was required in the case of certain *tag* (Ts) mutants after their transfer to 47 °C, the non-permissive temperature, and for cell walls made in the presence of TUN. The solubilization of the cell-wall fraction of [2-³H]gro-labelled cells followed a similar time course (data not presented) to that of [1-¹⁴C]-GlcNAc-labelled cells (Fig. 1a, b).

Fractionation of the protoplast pellet of samples having incompletely digested cell walls (after 45 min lysozyme treatment) allowed determination of the ultimate localization of any residual cell wall that had not been released to the protoplast supernatant. Although protoplast formation was practically complete after 45 min, a significant proportion of ³H label, up to or even more than 10 % of the radioactivity in cell samples taken at higher cell densities (Table 2, footnote †), was found in the cytoplasmic fraction, obtained after spinning down

the membrane fraction of lysed protoplasts. When lysozyme action was allowed to continue for 75, or often for 90 min, the label in the cytosol fraction (Table 2, footnote ‡) was found to be little more than 1 %. These results, together with those reported above (Fig. 1a), suggested that the label appearing in the cytosol fraction after shorter times of lysozyme exposure represented incompletely solubilized wall fragments that co-sedimented with the protoplasts.

Selective extraction of LTA and of P-lipid

Following cell-wall digestion, the extraction procedure for P-lipid and LTA from [2-³H]gro-labelled protoplasts, on which we finally settled (see Methods), was chosen after examining two additional methods of processing the washed protoplast pellet. Initially, (i) the Bligh–Dyer methanol/chloroform extraction of P-lipids from intact protoplasts (Rogers & Taylor, 1978),

Fig. 1. Kinetics of radioactivity solubilization during protoplast formation from [¹⁴C]GlcNAc-labelled cells (a) of *B. subtilis tagB1*, *tagD1* and *tag*⁺ strains, and (b) of *B. subtilis tag*⁺ labelled in the presence or the absence of TUN. (a) Time course of [1-¹⁴C]GlcNAc-labelled PG solubilization by lysozyme treatment of cells in a hypertonic buffer. Cultures grown at 30 °C in the presence of [1-¹⁴C]GlcNAc were transferred to 47 °C at an ND of 60. Samples were harvested 40 min after the temperature shift. After washing and resuspension in a lysozyme-containing buffer, 50 µl aliquots, removed at regular time intervals, were spun down, and sedimentable and non-sedimentable radioactivity determined. □, L5087 *tag*⁺; ◇, L6476 *tagB1*; ○, L6601 *tagD1*. (b) Effect of 1 µg TUN ml⁻¹ on the time course of lysozyme-induced release of PG from cultures of *B. subtilis* 168. The culture labelled with [1-¹⁴C]GlcNAc at 30 °C was split in two parts, to one of which TUN was added at a final concentration of 1 µg ml⁻¹. After approximately one mass doubling, samples were withdrawn and harvested cells treated as described in Methods. ■, Culture with TUN; □, culture without TUN.

followed by biphasic aqueous phenol treatment to remove LTA, was used (Table 2), and subsequently, (ii) an osmotic-shock-mediated lysis of protoplasts followed by the two-phase aqueous phenol extraction of LTA and P-lipids from cytoplasmic membranes obtained by centrifugation (Table 2). Compared with the procedure finally adopted, both these methods were cumbersome, involved more manipulations and led to less accurate, more disperse measures. Nonetheless, they gave results substantially in agreement with those obtained with the simpler method finally adopted.

Synthesis of WTA, LTA and P-lipid in *B. subtilis* 168 and in mutants deficient in poly(groP) synthesis

To assess the reliability of the [2-³H]gro-labelling method, we determined the pattern of WTA synthesis, while simultaneously examining the kinetics of LTA and P-lipid synthesis, in several *tag* (Ts)-bearing mutants affected in one of the following enzymes: TagD, the glycerol-3-phosphate cytidyltransferase; TagF, the CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase; TagB, the putative CDP-glycerol *N*-acetylmannosamine glycerophosphotransferase, the enzyme adding two or more glycerol units from CDP-glycerol to the *N*-acetylmannosamine residue of the PG-WTA linkage unit (Mauël *et al.*, 1994); or YvyH, the protein encoded by the previously characterized gene *orfX* (Soldo *et al.*, 1993). All but the last named were previously shown, after 2.5 h incubation at the restrictive temperature, to be deficient in WTA synthesis (Briehl *et al.*, 1989).

Following the protocol devised, exponentially growing cells of the 168 wild-type strain and Ts mutants were continuously labelled with [2-³H]gro and shifted to 47 °C. Samples, withdrawn at different times, were processed and the radioactivity corresponding to WTA, LTA and P-lipid fractions was measured. It appeared that during growth at 30 °C [2-³H]gro incorporation into all fractions paralleled overall mass increase (Fig. 2a). Following the temperature shift of the strain bearing the *tag*⁺ allele, the rate of incorporation into all fractions increased, as did the growth rate, although the relative incorporation into P-lipids and LTA was somewhat slower. At 47 °C, the *tagF1*-bearing strain was characterized by a rapid slow-down of [2-³H]gro incorporation into the WTA, an observation in full agreement with *in vitro* assays revealing an extremely Ts poly(groP) polymerase activity (Pooley *et al.*, 1992) exhibited by this mutant. However, incorporation into P-lipid and LTA continued upon the shift (Fig. 2b), reaching, 20 min later, a rate parallel to that of mass increase. The two strains carrying mutated *tagB* (Fig. 2c) or *tagD* (Fig. 2d) alleles yielded results very comparable to those obtained with the *tagF1*-bearing strain (Fig. 2b). Transfer to 47 °C provoked an immediate and strong reduction of the rate of isotope incorporation into poly(groP), whereas incorporation into P-lipid- and LTA-containing fractions continued, albeit at a slightly reduced rate relative to that of the wild-type strain.

The cumulative deficiencies in WTA synthesis previously established by chemical methods as well as by enzymic assays are strongly confirmed by these observations. The apparently unimpeded incorporation of [2-³H]gro into LTA and P-lipid at the non-permissive

Fig. 2. Incorporation of $[2\text{-}^3\text{H}]\text{gro}$ into poly(groP), LTA and P-lipids by *B. subtilis* 168 and relevant Ts mutants. Cultures of Ts mutants and parent strains grown in SATrpgro containing $[2\text{-}^3\text{H}]\text{gro}$ at 30°C were transferred to 47°C at an ND of 40. Treatment of washed cell samples with lysozyme in a hypertonic buffer allowed the solubilization of WTA. LTA and P-lipids were selectively extracted from the protoplast pellet with a two-phase phenol/aqueous buffer treatment. Culture density is shown as ND (---□---). Time 0 is the time at which the culture reached 47°C following transfer from 30°C . The radioactivity per ml of culture was determined for the following fractions: the protoplast supernatant (□) containing poly(groP); the phenolic phase containing P-lipid (◆); the aqueous acetate buffer (pH 4.7) extract containing LTA (○). The following strains were examined: L5087 *tag*⁺ (a), L6477 *tagF1* (b), L6476 *tagB1* (c), L6601 *tagD1* (d), and L6563 *yvyH1* (e).

temperature clearly points to the overall rate of synthesis of these surface components not being dependent upon continuing WTA synthesis.

Results obtained with three further strains, bearing mutations in *yvyH1* (*orfX1*) (Soldo *et al.*, 1993), or *tagF14* or *rodB1*, a mutation affecting a gene highly homologous to *mreD* of *E. coli* (Levin *et al.*, 1992;

Varley & Stewart, 1992), were for the last two barely distinguishable from those of the strain with the *tag*⁺ allele during 50 min of incorporation at the restrictive temperature (not presented). For the mutant affected in *yvyH1*, a somewhat reduced rate of incorporation for all glycerol-containing fractions was relatively more pronounced for the WTA during the initial 20 min at 47°C (Fig. 2e).

That the $[2\text{-}^3\text{H}]\text{gro}$ incorporation phenotype of the *rodB1*-bearing mutant was barely distinguishable from that of the strain with the *tag*⁺ allele is not surprising in that no WTA enzyme deficiencies were reported, neither was there any significant reduction in the phosphate content of cell walls prepared even after 2.5–3 h at 47°C , i.e. after three to four times longer growth at the restrictive temperature than in the present incorporation studies. In strains with mutant *yvyH1* and *tagF14* alleles after 3 h at 47°C , cell-wall phosphate was reduced but by only 10 and 40 % (not presented), respectively, much less than the greater than 90 % reduction observed in the case of the foregoing mutant alleles, *tagB1*, *tagD1* and *tagF1* (Briehl *et al.*, 1989).

PG synthesis in strains bearing *tag* mutations

The method, as devised, providing relatively accurate kinetics of WTA synthesis, was exploited to examine the interdependence of WTA and PG synthesis. Cultures of the wild-type and of strains bearing mutant alleles of *tagB*, *tagD* or *tagF* growing exponentially in SATrpgro medium supplemented with GlcNAc, were continuously labelled with $[1\text{-}^{14}\text{C}]\text{GlcNAc}$. Aliquots collected at regular time intervals were split into two parts and treated as follows: (i) one series of samples was subjected to lysozyme digestion in hypertonic buffer, and the radioactivity in the solubilized cell wall (principally present in PG, but including that in the minor galactosamine-containing WTA), from which protoplasts were separated by centrifugation, was measured, and (ii) the other series was treated with a sodium acetate buffer (pH 4) to extract the poly(GlcGalNAc 1-P), the minor WTA of strain 168 (Estrela *et al.*, 1991), as described in Methods. Incorporation kinetics (not presented) confirmed previous observations (Pooley *et al.*, 1993), i.e. the overall PG synthesis proceeds at rates comparable to that of the strain with the *tag*⁺ allele despite the inhibition of WTA synthesis.

Tunicamycin and cell wall synthesis

Finally, we investigated the effect of TUN on PG as well as on WTA synthesis in exponentially growing cultures of *B. subtilis* 168. Addition of $0.5\ \mu\text{g TUN ml}^{-1}$ to a culture at an ND of 60, continuously labelled with $[2\text{-}^3\text{H}]\text{GlcNAc}$ at 30°C , brought the synthesis of the minor WTA (Freymond, 1995) to a halt nearly instantaneously. Although the ND increase was slightly slowed down by TUN addition (Fig. 3), $[2\text{-}^3\text{H}]\text{GlcNAc}$

Fig. 3. Effect of TUN on the incorporation of [$1\text{-}^{14}\text{C}$]GlcNAc into PG and into poly(GlcGalNAc 1-P), the minor galactosamine-containing WTA of *B. subtilis* 168. A culture of *B. subtilis* L5087 growing in SATrpgro (2.5 mM) supplemented with GlcNAc (0.1 M) and [$1\text{-}^{14}\text{C}$]GlcNAc at 30 °C was split into two parts at ND 60, corresponding to time 0. TUN at a final concentration of $0.5\text{ }\mu\text{g ml}^{-1}$ was added to one part, while the other was further incubated. At any specified time, two samples were taken and filtered. One was treated with lysozyme in a hypertonic buffer solution to solubilize the cell wall label (d.p.m. per ml culture), mainly incorporated into PG (○, ●). The second sample was resuspended in buffer (pH 7), incubated for 5 min in a boiling water bath to extract soluble label, and treated for 30 min with a sodium citrate buffer (0.1 M, pH 4.0) at 100 °C to extract radioactivity corresponding to the minor galactosamine WTA (□, ■). Growth was followed by ND (—△—, —▲—). Filled symbols correspond to the culture grown in the presence of TUN.

incorporation into PG did not significantly differ from that in the control culture for at least 60 min, indicating that, under the experimental conditions used, the overall rate of PG synthesis was not affected. A similar experiment (Fig. 4) with [$2\text{-}^3\text{H}$]gro-labelled cells revealed, as expected, that the presence of TUN did not interfere for at least 80 min with the incorporation of ^3H into the P-lipid and into LTA, which paralleled the ND increase. However, TUN addition provoked a marked reduction of poly(groP) synthesis. This finding was confirmed by a further experiment (not presented), in which inhibition of glycerol incorporation into poly(groP) became apparently complete 10 min after TUN addition. Examining cells by light microscopy 2 h after the drug addition revealed morphological changes resembling those characteristic of Ts WTA-deficient mutants, as reported previously (Takatsuki *et al.*, 1972). In conclusion, in confirmation of previous results obtained in *in vitro* studies, it would appear that *in vivo* TUN inhibits specifically the very first step of the WTA linkage unit systems, i.e. the coupling of GlcNAc to the lipid carrier. As had been predicted (Pooley & Karamata, 1994), this is a reaction common to both the minor and the major WTA (Freymond, 1995).

Fig. 4. Effect of TUN addition on the incorporation of [$2\text{-}^3\text{H}$]gro into poly(groP) and other envelope components. A culture of *B. subtilis* L5087 *tag*⁺ growing exponentially at 30 °C in SATrpgro (2.5 mM) supplemented with $1.5\text{ }\mu\text{Ci}$ ($5.55 \times 10^4\text{ Bq ml}^{-1}$) of [$2\text{-}^3\text{H}$]gro was split into two parts at ND 60, and $0.5\text{ }\mu\text{g TUN ml}^{-1}$ was added to one part. Cells, obtained by filtration of samples withdrawn at times indicated, were fractionated as described (see Fig. 2 and Methods). ND (—△—, —▲—) and radioactivity ml^{-1} of culture for cells in the absence and in the presence of TUN, respectively, are shown for poly(groP)- (□, ■), P-lipid- (◇, ◆) and LTA-containing fractions (○, ●).

DISCUSSION

A reliable, reproducible procedure for analysing [$2\text{-}^3\text{H}$]gro incorporation into three specific *B. subtilis* 168 glycerol-containing cell envelope components, i.e. WTA, LTA and P-lipids, provides a means of following their synthesis in growing cultures. Analysis, following expression of conditional-lethal mutant alleles of *tag* and other genes, provided independent confirmation, *in vivo*, of the biological function of *tag* genes, notably by revealing that, for several of them, a marked inhibition of poly(groP) synthesis occurs within a few minutes of transfer to non-permissive conditions. Thus, in several mutants, the characteristic deformations of cell morphology, clearly seen after 2–3 h, are apparently triggered by the specific inhibition of WTA synthesis that can be already blocked at the time of shift to the non-permissive temperature. The absence, during the first 50 min, of any significant effect on the rate of synthesis of P-lipids or of LTA underlines the specificity of *tag* mutations. In contrast, the expression of these mutations is accompanied by a significant but transient fall in the rate of PG synthesis (Pooley *et al.*, 1993).

The method described here for specifically measuring LTA synthesis offers a means of screening candidate mutants for those possibly deficient in the synthesis of this component. The current absence of LTA mutants, as well as of identified genes known to be involved in LTA synthesis, is a major handicap to the understanding of the biological role(s) of LTA, and of the regulation of its synthesis in relation to that of other surface components.

Analysis of [2-³H]gro incorporation by a strain bearing a Ts mutation in a distinct gene, *rodB*, not belonging to the *tag* gene cluster, whose expression provokes the development, under restrictive conditions, of morphological deformations comparable to those of *tag*-bearing mutants, revealed no significant reduction either in poly(groP) synthesis, or in that of the other glycerol-containing envelope components. This clear difference in response between mutations in *tag* genes and those deficient in *rodB*, a homologue of *mreD* of *E. coli*, associated with similar conditional-lethal morphological phenotypes, confirms that the so-called 'rod' cell morphology developed by certain *B. subtilis* mutants may, but need not, be the result of inhibiting synthesis of the major WTA.

The rapid shut-off of WTA synthesis in *tagF1*-, *tagB1*- and *tagD1*-bearing mutants could be sufficient to cause the block in cell elongation. However, in the case of mutants such as *tagF14* or *yvyH1*, inhibition of WTA is apparently neither rapid enough nor sufficiently pronounced to be considered as the primary cause of the defect which leads to the development of deformed swollen cells. Paradoxically, the kinetics of appearance of such deformed cells in the latter mutants are not significantly slower than in the mutants severely affected in WTA synthesis, revealing an absence of proportionality between the degree of inhibition of WTA synthesis and the extent of cell deformations.

The results can be reconciled, however, should the proteins concerned be constituents of a holoenzyme – that includes enzymes involved in the synthesis of both major cell wall components, i.e. WTA and PG – such that mutations affecting individual proteins could interfere with the normal working of the cell-wall elongation machinery, affecting protein–protein interaction within the holoenzyme, without impeding, to a great extent, the activity of the catalytic site.

Like its *E. coli* homologue, the *rodA* gene of *B. subtilis* encodes a membrane protein that plays an essential role in the control of PG synthesis, ensuring elongation of the cylindrical part of the cell's surface (Henriques *et al.*, 1998). Such a protein could be an integral part of the 'elongation' holoenzyme, although it has been suggested (Henriques *et al.*, 1998) that growth arrest could result from an inability of the swollen cells formed to initiate septation normally. However, the data provided, showing a shortening of the cells, in agreement with previous observations obtained on a *tagF* mutant (Pooley *et al.*, 1993), are consistent with a continued formation of septa, in contrast to the diminished rate of cell elongation, i.e. of the incorporation of cylindrical wall material, observed at the restrictive temperature. Since blocking cell elongation would provoke the greatest limitation of cell volume increase, we believe that the increased cell width that accompanies diminished activity of the *rodA*, *rodB* and *tag* genes reflects the tendency for the mass of cell cytoplasmic constituents to increase faster than the volume of rod-shaped cells.

In *E. coli* notably, the development of swollen cells can result from the temperature-dependent loss of activity of mutant protein(s) encoded by one of several genes, such as *glmS*, *glmM* and *glmU*, involved in GlcNAc biosynthesis, an intermediate common to both lipopolysaccharide and PG (Mengin-Lecreulx & van Heijenoort, 1996). Homologues of these genes are present in *B. subtilis*, where GlcNAc is an intermediate shared by WTA and PG. Although no *B. subtilis* mutants deficient in these genes have been examined, it will be interesting to know whether they are characterized by a conditional comparably swollen cell phenotype. Interestingly, mutants conditionally blocked downstream of GlcNAc formation, i.e. exclusively concerned in the synthesis of PG precursors, do not develop swollen cells. In *B. subtilis* (Brandt & Karamata, 1987) as well as in *E. coli* (Mengin-Lecreulx & van Heijenoort, 1996), they are all associated with cell lysis.

Experiments involving TUN at or near the MIC for growth of *B. subtilis* yielded a particularly clear and striking result in view of previous reports (Ward *et al.*, 1980). During the 50 min following TUN addition, overall mass increase, the incorporation of labelled precursors into P-lipids and LTA and, most interestingly, into PG showed virtually no change, whereas incorporation into both the minor and the major WTA was rapidly and strongly reduced. This result (i) supports the suggestion (Pooley & Karamata, 1988) that, at the MIC, WTA could be the growth-inhibitory target for TUN, and (ii) is in good agreement with results obtained *in vitro* with membrane preparations. Moreover, at a fivefold higher antibiotic concentration, the simultaneous inhibition of both WTA polymers (see above) and of PG (Ward *et al.*, 1980) is consistent with the UDP-GlcNAc translocase activity being the more sensitive target, since this enzyme is responsible for the first step in the biosynthesis of the linkage unit to PG.

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