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Changes in Wall Teichoic Acid during the Rod-Sphere Transition of *Bacillus subtilis* 168

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Wall teichoic acid (WTA) is essential for the growth of *Bacillus subtilis* 168. To clarify the function of this polymer, the WTAs of strains 168, 104 *rodB1*, and 113 *tagF1*(*rodC1*) grown at 32 and 42°C were characterized. At the restrictive temperature, the *rodB1* and *tagF1*(*rodC1*) mutants undergo a rod-to-sphere transition that is correlated with changes in the WTA content of the cell wall. The amount of WTA decreased 33% in strain 104 *rodB1* and 84% in strain 113 *tagF1*(*rodC1*) when they were grown at the restrictive temperature. The extent of α-D-glucosylation (0.84) was not affected by growth at the higher temperature in these strains. The degree of D-alanylation decreased from 0.22 to 0.10 in the *rodB1* mutant but remained constant (0.12) in the *tagF1*(*rodC1*) mutant at both temperatures. Under these conditions, the degree of D-alanylation in the parent strain decreased from 0.27 to 0.21. The chain lengths of WTA in strains 168 and 104 *rodB1* grown at both temperatures were approximately 53 residues, with a range of 45 to 60. In contrast, although the chain length of WTA from the *tagF1*(*rodC1*) mutant at 32°C was similar to that of strains 168 and 104 *rodB1*, it was approximately eight residues at the restrictive temperature. The results suggested that the *rodB1* mutant is partially deficient in completed poly(glycerophosphate) chains. The precise biochemical defect in this mutant remains to be determined. The results for strain 113 *tagF1*(*rodC1*) are consistent with the temperature-sensitive defect in the CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase (H. M. Pooley, F.-X. Abellan, and D. Karamata, J. Bacteriol. 174:646–649, 1992).

Wall teichoic acid plays an essential role in the growth and viability of *Bacillus subtilis* 168. Rod mutants of this organism which have temperature-sensitive defects in the assembly of this surface polymer undergo a rod-to-sphere transition at the restrictive temperature. To clarify the function of this polymer in surface assembly, it was our goal to characterize the teichoic acid in two of these mutants, 104 *rodB1* and 113 *tagF1*(*rodC1*), under restrictive and nonrestrictive growth conditions.

Rod mutants were first isolated from *B. subtilis* 168 by Rogers et al. (50) and Boylan and Mendelson (6). Three groups of mutations, *rodA*, *rodB*, and *rodC*, were defined by genetic mapping (29). The *tagF1*(*rodC1*) mutation maps at about 310° (7, 29, 42). This map position is within the poly(GroP) biosynthesis cluster (*tag* genes) (25, 39). The gene cluster is organized in two divergently transcribed operons, *tagABC* and *tagDEF* (38). The genes for D-glucosylation, previously designated *gtaA* and *gtaB*, are also located in this region (47). The *gtaA*(*tagE*) gene encodes the UDP-glucose: poly(GroP) glucosyl-transferase (26, 47, 59), while the *gtaB* gene encodes the UDP-glucose pyrophosphorylase (39, 47). The *tagF*(*rodC*) gene codes for the CDP-glycerol:poly(GroP) glycerophosphotransferase (CGPTase) (45). In contrast, the *rodB1* mutation maps at about 245° (29, 35, 48, 63). The *divIVB* region is the locus of the *rodB1* mutation and contains two gene clusters, *mreBCD* and *minCD*, that are part of a single transcription unit in *B. subtilis*. The *rodB1* mutation is allelic to the *mreD* gene in this organism (63). Insertional mutagenesis or inactivation of the *tagF*(*rodC*) and *tagB*(*rodA*) genes has not been successful (25, 39); thus, it was concluded that wall teichoic acid is essential for the growth and viability of *B. subtilis* 168. Insertional inactivation of the *mreBCD* genes also appears to be lethal in *B. subtilis* (63).

The major wall teichoic acid of *B. subtilis* 168 is D-alanyl-[α-D-glucosylated poly(GroP)] (5, 15, 55, 69). The degree of D-glucosylation is generally 0.80 but has been shown to depend on the age of the cells and the phosphate concentration in the growth medium (8, 22, 33, 52, 58, 61). A chain length of 53 glycerol phosphate residues was established from the molecular weight and the phosphorus content (16). The degree of D-alanylation ranges from 0.02 to 0.1 (8, 15, 49, 52). The linkage group between α-D-glucosylated poly(GroP) and peptidoglycan in many *Bacillus* species is $(GroP)_2$-ManNAcβ(1→4)GlcNAc (2, 24, 30, 56). *B. subtilis* 168 also contains a second teichoic acid, poly(3-*O*-β-Glc-GalNAc 1-P) (17, 57). Depending on growth conditions, this teichoic acid may contribute approximately 10% of the total wall phosphate (4). Because the experiments presented in this report reflect only the major wall teichoic acid, D-alanyl-[α-D-glucosylated poly-(GroP)], the abbreviation WTA will designate this teichoic acid.

While the molecular genetics of the rod mutants have been extensively investigated, the WTA assembled in these mutants at the permissive and restrictive temperatures have not been well characterized. When the *rodA*(*tagB1*)(*tag-1*) mutant was grown at the restrictive temperature, WTA phosphorus was reduced by 80% (7, 49). This change correlated with a decrease in both glycerol and glucose (7, 51, 52). Briehl et al. (9) demonstrated that a *B. subtilis* strain containing the *tag-3* mutation, which is equivalent to the *tagF1*(*rodC1*) mutation (47), also has decreased levels of WTA phosphorus at the restrictive temperature. In contrast to the *tagB1*(*rodA*) and *tagF1*(*rodC1*) mutants, no changes in WTA phosphorus were observed for the *rodB1* mutant (49, 51). On the basis of the genetic and biochemical results, the *tagB*(*rodA*) and *tag-*

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F(*rodC*) genes were differentiated from the *rodB* gene. To clarify further the role of WTA in these mutants, we isolated and characterized this cell surface polymer from *B. subtilis* 168, 104 *rodB1*, and 113 *tagF1*(*rodC1*) grown at the permissive and restrictive temperatures. The analyses and the inferred structures provide insights into the role of WTA in the growth of *B. subtilis* 168.

MATERIALS AND METHODS

Growth of organisms. *B. subtilis* 168, 104 *rodB1*, and 113 *tagF1*(*rodC1*), obtained from George C. Stewart, were grown overnight at 32°C in a liquid medium containing 2% (wt/vol) tryptone, 1% (wt/vol) yeast extract, 2% (wt/vol) glucose, 40 mM KH_2PO_4 (pH 6.8), and salts (1.6 mM $MgSO_4$, 60 μM $MnSO_4$, and 9 μM $FeSO_4$). The glucose and salts were autoclaved separately, and 10 drops of concentrated HCl was added to 100 ml of the 100× salts solution to prevent precipitation. The cultures were diluted into fresh medium to an optical density at 540 nm of 0.1 (Bausch & Lomb Spectronic 20) and grown at either 32 or 42°C to an optical density of 0.9 (3.2 generations). The cells were collected and washed with 5 mM piperazine acetate buffer (pH 6.4), frozen in liquid nitrogen, and stored at −20°C.

Preparation of cell walls. For the preparation of cell walls, the procedure of Van Etten and Freer (62) was used to rupture the cells. Approximately 5 g of cells was suspended in 10 ml of 50 mM piperazine acetate buffer (pH 6.0) at 4°C and vortexed at maximum speed for 2-min intervals (six times) with 15 g of glass beads at 4°C (100 to 110 μm; B. Braun Melsungen AG), using a Vortex-Genie (Scientific Industries, Inc.). Greater than 99% breakage was achieved. After each interval, the cells and walls were centrifuged at 4,000 × *g* for 10 min at 4°C, and the supernatant fraction was removed. An equal volume of buffer was added each time. After the last interval, the walls were isolated and washed five times with water. To minimize autolysin action, the isolated walls were immediately extracted with 10% sodium dodecyl sulfate (SDS) at 100°C for 1 h and then subjected to repeated washings to remove the SDS. Optical microscopy of the wall samples did not reveal the action of autolytic activity. This procedure removed lipoteichoic acid, protein, and poly(Glc-GalNAc 1-P), the minor wall teichoic acid of *B. subtilis*. The purified walls were lyophilized and dried over phosphorus pentoxide in a vacuum desiccator.

Extraction of WTA. Two methods for WTA extraction were compared. In the first, 200 mg of purified cell wall was extracted with 4 ml of 5% trichloroacetic acid (TCA) for 5 h at 60°C. In the second, 200 mg of walls was extracted with 4 ml of 0.1 N NaOH for 5 h at 60°C. The extracts containing WTA were stored at 4°C until used for the analyses of phosphorus, glycerol, glucose, and D-alanine. These procedures extracted more than 95% of the WTA, as measured by the release of either phosphorus or glucose. In the first method, the D-alanine esters are stable while the poly(GroP) undergoes partial hydrolysis, whereas in the second, the D-alanine esters are labile and the α-D-glucosylated poly(GroP) does not appear to be hydrolyzed (3, 13, 27, 32). For quantifying D-alanine, the TCA extracts were adjusted to pH 8.5 with NaOH to hydrolyze the D-alanine esters.

Determination of WTA composition. Glucose was determined by the anthrone method of Pons et al. (44). Phosphorus was determined by the method of Chen et al. (11) as modified by Ames and Dubin (1). D-Alanine was determined either by a modification of the procedure of Diven et al. (14) or by the procedure of Graßl and Supp (23). In the modified procedure of Diven et al. (14), 0.5 ml of sample was incubated with 1 ml of 0.2 M Tris-HCl (pH 8.5) and 5 mg of D-amino acid oxidase (crude; Worthington Biochemical Corp.) at 37°C for 45 min. The reaction was stopped with 0.5 ml of 30% TCA, and the color was developed by adding 0.5 ml of 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl). After 5 min, 1 ml of 2.5 M NaOH was added and the A_{525} was determined.

To determine glycerol, 0.4 ml of the extract containing WTA was mixed with 0.6 ml of 48% HF and hydrolyzed for 48 h at 4°C. This procedure gave the same results as that described by Lang et al. (34). The acid was removed by placing the tubes in a polycarbonate desiccator in vacuo containing NaOH pellets. The α-D-glucosyl-glycerol in the samples was hydrolyzed in 4 M HCl at 110°C for 5 h (21). After removal of the HCl, the glycerol was analyzed by the procedure of Wieland (66), which utilizes glycerol 3-phosphate dehydrogenase. To determine hexosamines, the NaOH-extracted walls were hydrolyzed in 6 N HCl at 100°C for 4 h. After removal of the HCl, total hexosamines were measured by the method of Johnson (28). Galactosamine (GalN) was determined by the method of Wagner (65), in which the molar extinction coefficient for GalN is 10 times that of mannosamine (ManN) and glucosamine (GlcN), whereas ManN was determined by a modification of the method of Ludowieg and Benmaman (36, 37) in which the molar extinction coefficient for ManN is 65 times that of GlcN and 8 times that of GalN. In this modification, a 1-ml sample containing 25 to 500 nmol of hexosamine was mixed with 0.6 ml of 4% (wt/vol) acetylacetone in 1.5 M Na_2CO_3 and allowed to stand in an ice bath for 3 h. The solution was then mixed with 2 ml of *p*-dimethylaminobenzaldehyde (3.2 g in 30 ml of HCl and 180 ml of 2-propanol) and heated for 20 min at 45°C. After cooling, the A_{530} was determined.

PAGE of WTA. The WTA in the NaOH extracts were subjected to polyacrylamide gel electrophoresis (PAGE) to visualize the distribution of polymer chain lengths. This analysis, similar to that used for lipoteichoic acid by Pollack et al. (43), also allows a comparison of the distributions in various samples of WTA. Slab gels (1.5-mm thickness), containing 15% (wt/vol) acrylamide (29:1 acrylamide/bisacrylamide; Bio-Rad Laboratories), were prepared in a 0.25 M Tris-borate buffer (pH 8.2) (67). Samples were electrophoresed (32 V/cm) in this buffer until the phenol red tracking dye was 1 cm from the bottom of the gel. WTA was visualized by the alcian blue-silver stain of Min and Cowman (40) as described by Wolters et al. (67).

The calibration of the electrophoretogram relied on two analyses: (i) compositional analysis of WTA for glycerol (phosphorus) and ManN and (ii) electrophoretic separation of partially degraded WTA. Since ManN is a constituent of the linkage group (2, 30), the molar ratio of glycerol (P) to ManN of the linkage group provides a measure of the average chain length of WTA. The average length in *B. subtilis* 168 was determined to be 53, a length that is identical to that established by Doyle et al. (16) from the molecular weight and the phosphorus content. Thus, the average chain length of WTA from the parent was set at 53 residues. Electrophoresis of partially degraded WTA prepared by extraction with 5% TCA for 5 h at 60°C provided a counting ladder. This ladder was used to calibrate the relative chain lengths. Lengths generally less than 10 residues are not visualized with the alcian blue stain procedure (40, 67). Thus, this procedure was not satisfactory for visualizing WTA of short chain length.

Scanning electron microscopy (SEM). *B. subtilis* 168, 104 *rodB1*, and 113 *tagF1*(*rodC1*) were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer for 2 h and dehydrated with a graded series of acetone concentrations. The

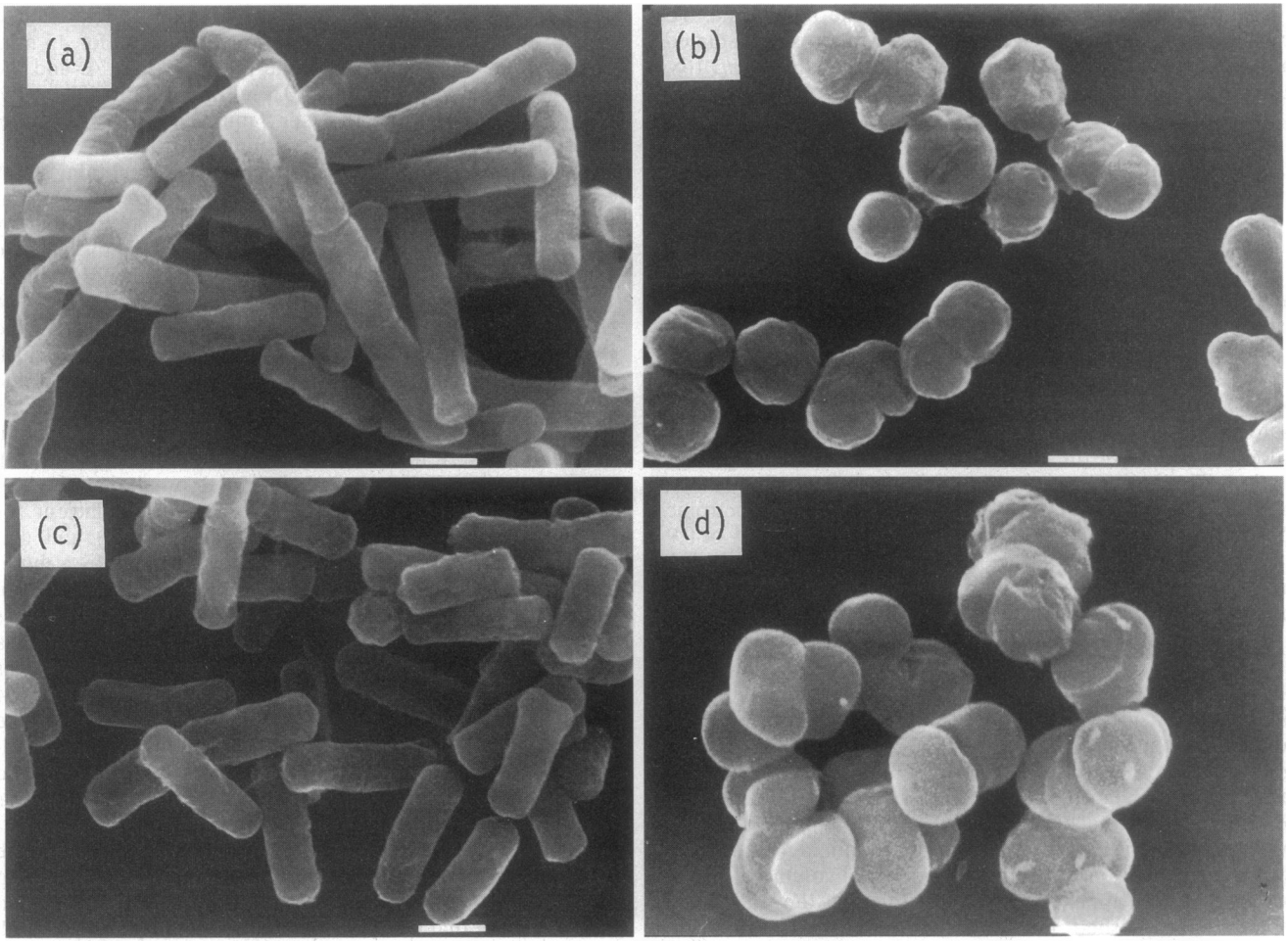

FIG. 1. Morphology of Rod mutants. Strain 104 *rodB1* (a and b) and strain 113 *tagF1*(*rodC1*) (c and d) grown at 32 and 42°C, respectively, for 3 h were examined by SEM as described in Materials and Methods. Bar = 1 μm.

cells were placed on a glass slide and critical point dried. The dried cells were sputter coated with 80-Å (8-nm) gold palladium (SEM Coating Unit E5100; Polaron Instruments). Cells were observed with a JEOL scanning electron microscope (JSM-35 CF).

RESULTS

Growth and SEM of *B. subtilis* Rod mutants. For the investigation of strains 104 *rodB1* and 113 *tagF1*(*rodC1*), growth at 32°C was used to obtain rod-shaped cells and growth at 42°C was used to obtain spherical cells. The higher temperature was found to be the minimum at which all cells are spherical (53, 64). The lower temperature was chosen as an average between 30 and 35°C, temperatures used by previous workers (12, 29, 54, 64) to grow rod-shaped cells.

B. subtilis 168 grew as rods which were 1.9 ± 0.2 and 2.1 ± 0.5 μm in length at 32 and 42°C, respectively. Strains 104 *rodB1* and 113 *tagF1*(*rodC1*) grown at 32°C were rods of 1.7 ± 0.3 and 1.6 ± 0.3 μm, respectively (Fig. 1a and c). When grown at 42°C, both the *rodB1* and *tagF1*(*rodC1*) mutants continued to divide until the interseptal (polar) distance was approximately 0.9 μm. These cells rounded and eventually separated resulting in the sphere-like morphology (Fig. 1b and d). SEM observations of the *rodB1* and *tagF1*(*rodC1*) mutants justified the use of 32 and 42°C to demonstrate the rod-sphere transition. When strain 104 *rodB1* was grown at an intermediate temperature (37°C), bifurcation and aberrant cell organization were observed (Fig. 2).

Compositional analyses of wall extracts containing WTA. A detailed chemical analysis of WTA synthesized at 32 and 42°C was undertaken to clarify those features which correlate with the rod-sphere transition. WTA was isolated from the SDS-extracted cell walls with either 0.1 N NaOH or 5% TCA at 60°C for 5 h. Both methods of extraction removed approximately 50% of the dry weight from walls of rod-shaped cells of strains 168, 104 *rodB1*, and 113 *tagF1*(*rodC1*). From the spherical cells of strains 104 and 113, 37 and 10%, respectively, of the wall were extracted by these procedures (data not shown). The compositional analyses are summarized in Table 1. Significant differences were found between the amounts of each component in the extracts containing WTA of rod-shaped cells grown at 32°C and spherical-shaped cells grown at 42°C. The extracts from strain 168 grown at 32°C had phosphorus and glycerol contents of 1.38 and 1.23 μmol/mg, respectively. At 42°C, these amounts decreased approximately 10%. The extracts from the *rodB1* mutant grown at 32°C had phosphorus and glycerol contents of 1.28 and 1.20 μmol/mg, respectively. When this strain was grown at 42°C, the phosphorus and glycerol contents were 0.85 and 0.82 μmol/mg, respectively. The extracts from the *tagF1*(*rodC1*) mutant grown at 32°C had

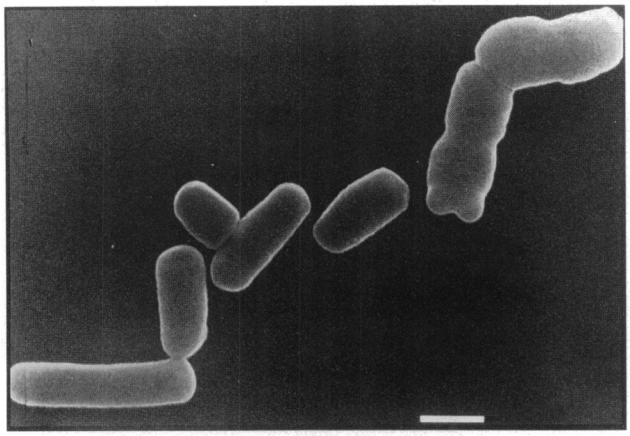

FIG. 2. Aberrant morphology of strain 104 *rodB1* 104 grown at 37°C. Bar = 1 μm.

values of 1.18 and 0.92 μmol/mg for phosphorus and glycerol, respectively. Growth of this mutant at 42°C yielded extracts with phosphorus and glycerol values of 0.19 and 0.16 μmol/mg, respectively. Thus, the amount of WTA, as measured by the average changes in phosphorus and glycerol contents, decreased 33% in the *rodB1* mutant and 84% in the *tagF1*(*rodC1*) mutant grown at 42°C.

Two additional components of WTA, D-glucose and D-alanine, were examined to determine whether they reflect the changes in phosphorus and glycerol. Growth of strain 168 at 42°C resulted in a 12% decrease of glucose compared with growth at 32°C. When strains 104 *rodB1* and 113 *tagF1*(*rodC1*) were grown at 42 instead of 32°C, the glucose content decreased from 0.94 to 0.61 μmol/mg and from 0.92 to 0.13 μmol/mg, respectively. Thus, the percentage decreases in glucose of 35% in the *rodB1* mutant and of 86% in the *tagF1*(*rodC1*) mutant corresponded to the changes in phosphorus and glycerol noted above. The ratios of glucose to glycerol (or to phosphorus) indicate that the WTA from both the wild-type and mutant cells grown at either temperature are approximately 80% D-glucosylated (Table 2).

The changes in D-alanine content, however, did not correspond with the changes in phosphorus and glycerol. For example, in strain 168, the D-alanine ester content decreased 30% when growth occurred at the elevated temperature. The D-alanine ester content of RodB1 WTA decreased 69% from 0.26 to 0.08 μmol/mg when growth occurred at the restrictive temperature (Table 1). Phosphorus and glycerol in this strain decreased 34 and 32%, respectively. The degree of D-alanylation, D-alanine/glycerol, of this WTA at 42°C decreased from 0.22 to 0.10 (Table 2). The D-alanine ester content of the TagF1(RodC1) WTA decreased 80% from 0.10 at 32°C to 0.02 μmol/mg at 42°C. On the other hand, the degree of D-alanylation remained at approximately 0.11 (Table 2). Therefore, the degree of D-alanylation in the *tagF1*(*rodC1*) mutant, unlike that of the *rodB1* mutant and strain 168, does not vary during the transition from rod to sphere.

From the D-alanine ester content of strain 113 *tagF1*(*rodC1*) at 32°C, we speculated that this temperature might not have been appropriate for demonstrating rod morphology. The D-alanine content of the WTA in this strain grown at 32°C was different from that of *B. subtilis* 168 (0.10 versus 0.33 μmol/mg) and more comparable to that of the *rodB1* mutant grown at 42°C (0.10 versus 0.08 μmol/mg). In addition, the ratio of D-alanine to glycerol in the WTA of the rod-shaped *tagF1*(*rodC1*) mutant (0.11) at 32°C was comparable to that of the spherical-shaped *rodB1* mutant at 42°C (Table 2). In contrast, strain 168 at 32 and 42°C and the *rodB1* mutant at 32°C had ratios of D-alanine to glycerol of 0.27, 0.21, and 0.22, respectively. To test our speculation, strain 113 *tagF1*(*rodC1*) was grown at 25°C and the WTA was analyzed. The results showed phosphorus and glucose contents of 1.40 ± 0.14 and 1.21 ± 0.05 μmol/mg, respectively, with a D-alanine content of 0.04 ± 0.02 μmol/mg (data not shown). Thus, growth of the *tagF1*(*rodC1*) mutant at 25°C did not yield a D-alanine content similar to that of strain 168, whereas the phosphorus and glucose contents were similar.

It was suggested that growth of strain 104 *rodB1* at a temperature higher than 42°C might result in a greater decrease in the amount of WTA. Our analyses of this strain at 48°C showed no significant differences in phosphorus and glucose contents from those observed at 42°C. In contrast, the D-alanine content of the *rodB1* mutant grown at 48°C was less than 0.01 μmol/mg (data not shown). Thus, it would appear that the synthesis of poly(GroP) in the *rodB1* mutant does not exhibit the pronounced temperature sensitivity that is evident in the *tagF1*(*rodC1*) mutant.

B. subtilis 168 also contains a second teichoic acid, poly(3-*O*-β-Glc-GalNAc 1-P) (17, 56). Depending on growth conditions, this teichoic acid may contribute approximately 10% of the total wall phosphorus (4). In the present work, the walls prepared by extraction in 10% SDS at 100°C for 1 h did not contain significant amounts of GalN; thus, poly(Glc-GalNAc 1-P) was extracted from the wall by this treatment. Therefore, the results in this report do not reflect this polymer. Pavlik and Rogers (41) also observed a difference in the extractabilities of this teichoic acid and the α-D-glucosylated poly(GroP). For example, the poly(Glc-GalNAc 1-P) was extracted with 0.1 M

TABLE 1. Analyses of cell wall extracts containing WTA of *B. subtilis* Rod mutants

Strain	Growth temp (°C)	Content (μmol/mg of cell wall)[a]				
		Phosphorus	D-Glucose	D-Alanine	Glycerol	ManN[b]
168	32	1.38 ± 0.10	1.08 ± 0.07	0.33 ± 0.06	1.23 ± 0.16	0.023 ± 0.004
	42	1.25 ± 0.06	0.94 ± 0.02	0.23 ± 0.03	1.12 ± 0.09	0.021 ± 0.002
104 *rodB1*	32	1.28 ± 0.06	0.94 ± 0.07	0.26 ± 0.04	1.20 ± 0.08	0.021 ± 0.006
	42	0.85 ± 0.07	0.61 ± 0.08	0.08 ± 0.01	0.82 ± 0.16	0.017 ± 0.006
113 *tagF1*(*rodC1*)	32	1.18 ± 0.13	0.92 ± 0.16	0.10 ± 0.03	0.92 ± 0.16	0.031 ± 0.007
	42	0.19 ± 0.04	0.13 ± 0.07	0.02 ± 0.01	0.16 ± 0.08	0.021 ± 0.008

[a] Mean ± standard deviation of three growth experiments, each analyzed with duplicate extractions. One extraction was performed with 5% TCA at 60°C for 5 h, and the other was performed with 0.1 N NaOH at 60°C for 5 h. Since these procedures extract >95% of the WTA, the values for each were averaged. In each extraction, 200 mg of SDS-purified wall from either *B. subtilis* 168, 104 *rodB1*, or 113 *tagF1*(*rodC1*) grown at either 32 or 42°C was used.

[b] Measured in the NaOH-extracted wall as described in Materials and Methods.

TABLE 2. Ratios of WTA components in Rod mutants of *B. subtilis*

Strain	Growth temp (°C)	Ratio[a]			
		Glucose/glycerol (glucose/P)	D-Alanine/glycerol (D-alanine/P)	Glycerol/P	Glycerol/ManN (P/ManN)
168	32	0.88 (0.78)	0.27 (0.24)	0.89	53 (60)
	42	0.84 (0.75)	0.21 (0.18)	0.90	53 (60)
104 *rodB1*	32	0.78 (0.73)	0.22 (0.20)	0.94	57 (61)
	42	0.74 (0.72)	0.10 (0.09)	0.96	48 (50)
113 *tagF1(rodC1)*	32	1.00 (0.78)	0.11 (0.08)	0.78	30 (38)
	42	0.81 (0.68)	0.13 (0.10)	0.84	8 (9)

[a] Calculated from the data in Table 1.

citrate or acetate buffer (pH 4.0) at 100°C, conditions that did not extract the glucosylated poly(GroP) (20, 47). This difference in solubility may be similar to that observed in the present work. In conclusion, the walls prepared by the SDS extraction procedure appear to contain only the D-alanyl-[α-D-glucosylated poly(GroP)] linked to peptidoglycan.

Length of teichoic acid. Since the WTA-peptidoglycan linkage unit in many bacilli consists of $(GroP)_2$-ManNAcβ (1→4)GlcNAc (2, 30), quantification of ManN provides a means for determining the average number of glycerol phosphate residues per WTA chain. The amount of this amino sugar ranged from 0.021 to 0.034 μmol/mg in a variety of *B. subtilis* strains (68). In the present work, the amounts of ManN in strains 168, 104 *rodB1*, and 113 *tagF1(rodC1)* decreased 9, 19, and 32%, respectively, when the growth temperature was shifted to 42°C. Because of the magnitude of the standard deviation of the ManN analyses (Table 1), the changes in RodB1 and TagF1(RodC1) ManN may not be statistically significant. The ratio of glycerol to ManN (Table 2) was used to calculate the average chain length of the WTA in these strains. This determination assumes that each potential linkage unit containing ManNAc has a poly(GroP) chain attached. Results of this analysis indicated that strains 168, 104 *rodB1*, and 113 *tagF1(rodC1)* grown at 32°C had 53, 57, and 30 residues per chain, respectively. The value for strain 168 was identical to that calculated from the apparent weight-average molecular weight (24,800) (16). At 42°C, the numbers of residues per chain from compositional analyses were 53, 48, and 8, respectively (Table 2). Thus, the chain length of WTA from the *tagF1(rodC1)* mutant grown at the elevated temperature decreased from 30 to 8 residues per linkage unit.

Since the decrease in WTA content may reflect either a decrease in chain length or a decrease in the number of chains, it was important to confirm the chain lengths determined from the ratios of glycerol to ManN. The separation of WTA by PAGE provided an independent analysis of chain length as well as the distribution of the chain lengths. The WTA from all strains (Fig. 3, lanes 1 to 6), with the exception of the *tagF1(rodC1)* mutant grown at 42°C (Fig. 3, lane 7), were relatively uniform in chain length, ranging between 45 and 60 residues of glycerol phosphate. Since there was no detectable decrease in the chain length of the RodB1 WTA at 42°C, the possibility exists that not all of the linkage units have WTA attached if the amount of ManN remains constant. In the *tagF1(rodC1)* mutant grown at 42°C, no WTA was visualized in the amount of extract used to analyze the other samples. The pronounced decrease in the amount of TagF1(RodC1) WTA at 42°C would appear to be the result of a decrease in chain length, an observation consistent with the chemically determined chain length (eight residues). Because the alcian blue stain does not generally visualize short chain lengths (<10 residues), the distribution of TagF1(RodC1) WTA (42°C) lengths could not be verified by PAGE. The profile of TagF1(RodC1) WTA at 32°C (Fig. 3) shows a distribution of 40 to 55 residues, in contrast to the 30 calculated from the ratio of glycerol to ManN. The reason for this discrepancy is not understood.

DISCUSSION

This work demonstrates that both strains 104 *rodB1* and 113 *tagF1(rodC1)* exhibit significant decreases in WTA content when grown at 42 instead of 32°C. These decreases are thought to be responsible for the shape change at the nonpermissive temperature. The WTA remaining in the *tagF1(rodC1)* (16%) and *rodB1* (67%) mutants when grown at 42°C suggested that it is not necessary to eliminate all of the polymer in order to observe the rod-sphere transition. In addition to the difference in the amounts of WTA between these strains at the nonpermissive temperature, there is also a major dissimilarity in the length of poly(GroP) between these strains. The WTA of the spherical *tagF1(rodC1)* mutant grown at 42°C was short (≈8 residues) compared with that of the *rodB1* mutant, which was the same length as that found in the wild-type (≈50 to 60 residues). It was concluded that the temperature-dependent defect in strain 113 *tagF1(rodC1)* is associated with the elongation of WTA. This conclusion is consistent with the observation of Pooley et al. (45) that the TagF1(RodC1) protein is the thermosensitive CGPTase. In contrast to the *tagF1(rodC1)* mutant, the change in WTA of the *rodB1* mutant cannot be correlated with a decrease in chain length. Rather, the defect in strain 104 *rodB1* appears to be related to a decrease in the number of poly(GroP) chains. Thus, the unique mutations expressed in strains 104 *rodB1* and 113 *tagF1(rodC1)* provide one with two novel systems for examining the role of WTA in the morphogenesis of *B. subtilis*.

Although the *rodB1* and *tagF1(rodC1)* mutants were constructed to have isogenic backgrounds (29), there would also appear to be another difference between the WTA of these two mutants and the wild type. The *rodB1* mutant grown at the permissive temperature has approximately the same degree of D-alanylation (22%) as does the wild-type strain (27%). However, at the nonpermissive temperature, only 10% of the glycerol phosphate residues in strain 104 *rodB1* are D-alanylated, compared with 21% found in the parent strain. In strain 113 *tagF1(rodC1)*, on the other hand, the degree of D-alanylation was decreased at the permissive temperature (11%) and did not decrease further at the nonpermissive temperature

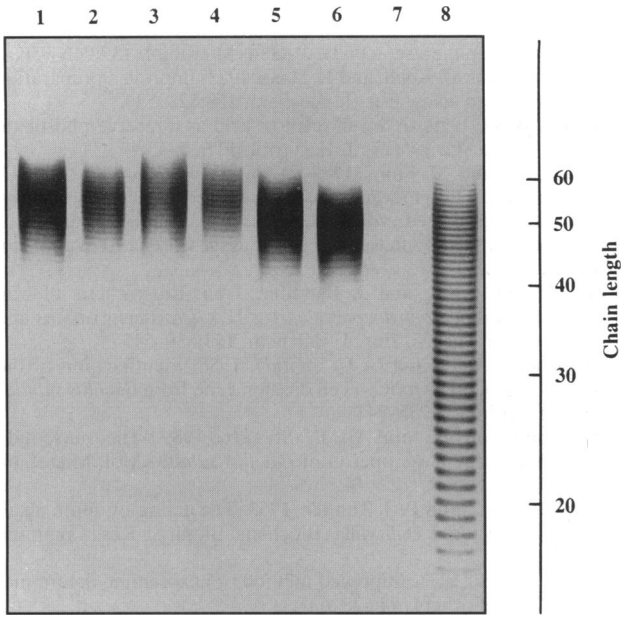

FIG. 3. PAGE profiles of WTA in *B. subtilis* 168, 104 *rodB1*, and 113 *tagF1*(*rodC1*) at 32 and 42°C. The WTA samples in lanes 1 to 7 were extracted with 0.1 M NaOH at 60°C for 5 h from *B. subtilis* 168 (lanes 1 and 2), 104 *rodB1* (lanes 3 and 4), and 113 *tagF1*(*rodC1*) (lanes 5 to 7) grown at either 25°C (lane 5), 32°C (lanes 1, 3, and 6), or 42°C (lanes 2, 4, and 7). The amount of sample per lane subjected to PAGE is the extract from 1 to 2 nmol wall phosphorus. Lane 8 is WTA extracted with 5% TCA at 60°C for 5 h from strain 168 grown at 32°C. Because of the partial hydrolysis by TCA, an extensive ladder of chain lengths is observed. In the primary electrophoretogram, 47 chain lengths were observed in the TCA extract; hence, this ladder was used for calibration. The calibration is described in detail in Materials and Methods. The bottom part of the electrophoretogram is not shown.

(13%). Thus, while the esterification of WTA with D-alanine in strain 104 would appear to correlate with the rod-sphere transition, the results with strain 113 do not correlate.

Two features of cell wall dynamics in *B. subtilis* may have introduced a slight bias into the results of this study. (i) If WTA synthesis was terminated at the shift to the nonpermissive temperature, 3.2 generations of growth at the restrictive temperature will have turned over approximately 85% of the WTA, leaving 15% of the WTA from the inoculum cells (31). Because of the complexity of the turnover process, calculation of the precise amount of WTA at 3.2 generations from turnover rates is difficult (31). (ii) The decay of CGPTase after shift-up to the restrictive temperature is not known. A combination of these features may have influenced the amounts of the WTA from cells grown at 42°C, using inocula grown at 32°C.

Rogers et al. (51) did not observe a difference in the phosphorus content between the rod and spherical cells of the *rodB* (non-temperature-sensitive, *rod-4*) mutant. This observation contrasts with the 33% decrease in WTA of the *rodB1* mutant at the nonpermissive temperature in the present study. The difference between these observations may reflect the media and growth conditions. In the experiment with the *rod-4* mutant, the cells were grown for 2 days on minimal solid medium, whereas the cells in our work were grown in an enriched liquid medium for 5 h. It was reported that the wall of this strain grown under permissive conditions contains 0.7 μmol of phosphorus per mg (51), an amount which was 45% less than that found in the present growth experiments (1.28 μmol/mg). Thus, because of the differences in the growth conditions, the results of our experiments do not appear to be comparable with those of Rogers et al. (51).

It has been demonstrated that $(GroP)_2$-ManNAc-β(1→4) GlcNAc is the linkage group between the peptidoglycan and WTA in many *Bacillus* species (2, 24, 30, 56). We also found that ManN is present in *B. subtilis* 168 and assumed that it is present in this linkage group. This assumption allowed us to determine an average poly(GroP) chain length as shown in Table 2. The wild-type strain 168 grown at either 32 or 42°C had a chain length of approximately 53 residues. This determination of average chain length from the ratio of glycerol to ManN was corroborated by visualization of the electrophoretic pattern of WTA. From this pattern, a range of 45 to 60 residues was observed, which is consistent with the glycerol-to-ManN ratio. This methodology was used to compare the WTA chain lengths of the *rodB1* and *tagF1*(*rodC1*) mutants at the permissive and nonpermissive temperatures.

No difference in the number of glycerol phosphate residues per chain was observed in strain 104 *rodB1* grown at either of the two temperatures even though a 33% decrease in WTA content was observed at the nonpermissive temperature. This decrease may result from linkage units not being substituted with WTA or fewer available linkage units in the WTA biosynthetic pathway. A more accurate assay for ManN will be required to distinguish between these possibilities. In contrast to strain 104 *rodB1*, a dramatic decrease in the chain length of WTA was observed in the *tagF1*(*rodC1*) mutant grown at 42°C. This decrease (85%) in chain length accounts for the observed reduction in the WTA content. Hence, the *tagF1*(*rodC1*) mutation affects the ability of the cell to elongate WTA at the nonpermissive temperature. Although the *rodB1* and *tagF1*(*rodC1*) mutants have similar phenotypes, it is concluded that they result from two very different mutations (45, 63).

The decrease in cell elongation and increase in cell diameter observed during the rod-sphere transition in strains 104 *rodB1* and 113 *tagF1*(*rodC1*) were similar to those observed in the *rodB1* mutant by Burdett (10). Similar changes were also observed in a mutant bearing *tagB1*(*rodA*) during a temperature shift from 30 to 45°C (58). In addition to these changes, we observed aberrant cell organization at the intermediate temperature of 37°C in the *rodB1* mutant. Spherical shape may result from an interference in deposition of peptidoglycan. Alternatively, spherical shape may be regarded as the result of a block in elongation of the cylindrical wall and unimpeded septum formation (46). Varley and Stewart (63) and Levin et al. (35) showed that the *rodB* gene is homologous with the *mreD* gene in *E. coli*, which controls septum-specific wall synthesis. It remains to be determined whether the individual defects in WTA synthesis in strains 104 *rodB1* and 113 *tagF1*(*rodC1*) have similar or different effects on cell septation and elongation.

The *tagF*(*rodC*) gene has been located at position 310° on the *B. subtilis* chromosome, a position where all other *tag* mutations have been found (38). On the other hand, the *rodB1*(*mreD*) gene is located at position 245° in a region containing genes concerned with septum formation and shape determination (35, 63). While the evidence presented here and by Pooley et al. (45) has shown that strain 113 *tagF1*(*rodC1*) is a temperature-sensitive mutant in the WTA elongation pathway, the chromosomal location of the *rodB1* mutation argues against making this assertion for strain 104 *rodB1*. Whether the decrease of WTA in the *rodB1* mutant is responsible for the rod-sphere transition or is the consequence of this transition is not known. Thus, the precise biochemical defect in the *rodB1* mutant remains to be determined.

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