JOURNAL OF BACTERIOLOGY, Dec. 2006, p. 8313–8316 Vol. 188, No. 23
0021-9193/06/$08.00+0 doi:10.1128/JB.01336-06

Wall Teichoic Acid Polymers Are Dispensable for Cell Viability in *Bacillus subtilis*▽

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Received 22 August 2006/Accepted 20 September 2006

An extensive literature has established that the synthesis of wall teichoic acid in *Bacillus subtilis* is essential for cell viability. Paradoxically, we have recently shown that wall teichoic acid biogenesis is dispensable in *Staphylococcus aureus* (M. A. D'Elia, M. P. Pereira, Y. S. Chung, W. Zhao, A. Chau, T. J. Kenney, M. C. Sulavik, T. A. Black, and E. D. Brown, J. Bacteriol. 188:4183–4189, 2006). A complex pattern of teichoic acid gene dispensability was seen in *S. aureus* where the first gene (*tarO*) was dispensable and later acting genes showed an indispensable phenotype. Here we show, for the first time, that wall teichoic acid synthesis is also dispensable in *B. subtilis* and that a similar gene dispensability pattern is seen where later acting enzymes display an essential phenotype, while the gene *tagO*, whose product catalyzes the first step in the pathway, could be deleted to yield viable mutants devoid of teichoic acid in the cell wall.

The bacterial cell wall is a complex meshwork of carbohydrates and amino acids linked as a rigid structure termed peptidoglycan, which is responsible for a variety of cellular functions, including growth, division, maintenance of shape, and protection from osmotic stress (10). In gram-positive organisms, in addition to this dense layer of peptidoglycan, the cell wall contains an equal amount of a highly charged anionic polymer of polyol phosphate, called wall teichoic acid. Although variability exists among the polymers from various organisms, these polymers have been found in all gram-positive bacteria studied. Remarkably, despite its discovery nearly 50 years ago, the cellular function of wall teichoic acid remains speculative. Nevertheless, a significant body of literature using the model organism *Bacillus subtilis* has identified a requirement for teichoic acid polymers in cell viability (3).

Beginning with temperature-sensitive mutants and more recently with the creation of deletion strains that were conditionally complemented using a tightly regulated promoter, nearly every gene responsible for wall teichoic acid biosynthesis has been shown to be required for viability in *B. subtilis* (2, 4, 6, 7, 15). In contrast, we recently demonstrated that wall teichoic acid was dispensable in *Staphylococcus aureus* (8). Paradoxically, that work indicated that the first step in polymer synthesis was dispensable, while the later steps were not (8). This apparent contradiction was resolved with the finding that a lesion in the first step of the biochemical pathway (TarO) suppressed the lethal phenotype associated with mutations in the later steps. Here, we have reevaluated the dispensability of teichoic acid biosynthesis genes in *B. subtilis*, with particular attention to the dispensability of the first biosynthetic step encoded in *tagO* (orthologue of *tarO*).

The *tagO* gene was the subject of a relatively recent dispensability study of *B. subtilis* where the failure to create insertional mutants led to the conclusion that disruption of *tagO* was lethal to the cell (16). In the work reported here, we employed a precise deletion strategy using double recombination of a PCR product targeting *tagO*. The PCR product contained a central erythromycin cassette flanked by 1,000-bp regions 5′ and 3′ of *tagO*. To our surprise, we were able to successfully create a strain with a deletion in *tagO* (EB1451) that was viable but slow growing (Table 1 shows the strains and plasmids used in this study). The failure in the previous study (16) to isolate mutants in *tagO* by insertional inactivation may stem from the slow growth and altered colony morphology of this mutant. These colonies were significantly smaller and smoother than colonies of wild-type *B. subtilis* but could be repeatedly subcultured onto fresh medium (data not shown). Additionally, transformation (11) of chromosomal DNA from the deletion strain back into the wild-type background (EB6) occurred at a frequency within twofold that obtained by an unlinked, dispensable marker (data not shown) and gave rise to colonies with growth rates and morphology identical to those of the donor strain, arguing against the existence of a secondary site mutation leading to viability.

Because a deletion in *tagO* is expected to disrupt the first step of wall teichoic acid biosynthesis, we reasoned that the deletion strain should be devoid of any wall teichoic acid. Using previously established protocols, the cell walls from both the wild type and the deletion strain were isolated, and the phosphate content was analyzed (4). Compared to the wild type, the cell wall phosphate content was decreased by nearly 95% in the *tagO* null mutant (EB1451) (2.01 ± 0.04 μg phosphate/mg cell wall versus 0.14 ± 0.02 μg phosphate/mg cell wall). These data support the absence of teichoic acid in the cell wall and indicate that the activity of TagO was not bypassed by an alternative biosynthetic mechanism.

Further characterization of the *tagO* deletion strain was performed through the investigation of the growth kinetics by

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▽ Published ahead of print on 29 September 2006.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
B. subtilis		
EB6	*hisA1 argC4 metC3*	L5087 (2)
EB240	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagD cat86 tagD*::Spec^r	2
EB633	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagB cat86 tagB*::Spec^r	4
EB669	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagF cat86 tagF*::Spec^r	4
EB892	EB6 transformed with pRB*tagBgfp*	5
EB1451	*hisA1 argC4 metC3 tagO*::Erm^r	This study
EB1453	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagB cat86 tagB*::Spec^r *tagO*::Erm^r	This study
EB1559	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagD cat86 tagD*::Spec^r *tagO*::Erm^r	This study
EB1560	*hisA1 argC4 metC3 amyE*::*xylR* P_{xylA} *tagF cat86 tagF*::Spec^R *tagO*::Erm^r	This study
Plasmids		
pMUTIN4	IPTG[a]-inducible integration vector, source of Erm^r cassette	17
pUS19	pUC19 derivative containing Spec^r cassette	1

[a] IPTG, isopropyl-β-D-thiogalactopyranoside.

comparison to the wild type in Luria-Bertani broth (Fig. 1A). It is clear that the failure to synthesize teichoic acid had a drastic effect on the growth of *B. subtilis*. The lag phase of the mutant strain was considerably longer than that of the wild type and was coupled with a decreased growth rate. The growth kinetics were also examined with the addition of 20 mM $MgCl_2$ in the medium. Previous reports have demonstrated that Mg^{2+} supplement in the medium has a positive effect on the growth of certain morphology mutants (9, 13). The most dramatic effect was observed with an *mreB* mutant whose viability was dependent on the addition of Mg^{2+}. Although the addition of $MgCl_2$ does not restore growth of the *tagO* deletion mutant to wild-type levels, supplementation resulted in a shorter lag phase and increased growth rate (doubling time of 1.4 ± 0.1 h for the supplemented cultures versus 2.1 ± 0.1 h for the nonsupplemented cultures). Although the effect of Mg^{2+} on the enhancement of growth is not well understood, several explanations have been suggested. Most proposals have implied some impact on peptidoglycan structure or the stabilization of cell wall-enzyme complexes that are relevant to cell wall remodelling or synthesis (9). Furthermore, given the potential role for teichoic acid polymers in binding Mg^{2+} ions (12), supplementation of this ion might compensate for the loss of teichoic acid polymers in the cell wall.

Light microscopy and transmission electron microscopy in the presence and absence of $MgCl_2$ are shown in Fig. 1B and 2, respectively. Light microscopy of the *tagO* mutant revealed a loss of the rod shape and swelling of the cell volume in addition to cell aggregation. These phenotypes were not alleviated by the addition of $MgCl_2$. Interestingly, these characteristics were previously evident in micrographs of a TagO-depleted strain that were published by Soldo et al. (16). Transmission electron microscopy in the work reported here revealed aberrant septation and nonuniform thickening of the peptidoglycan layer, hallmarks associated with a loss of teichoic acid in *B. subtilis* (2). From these findings, it is clear that the loss of teichoic acid

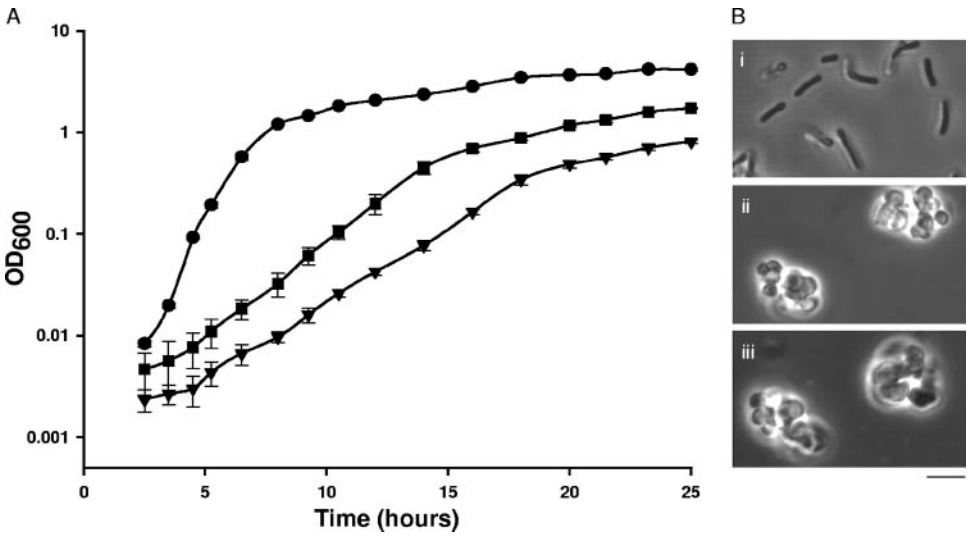

FIG. 1. Growth of *tagO* deletion mutant. (A) Growth analysis was performed in LB for EB6, i.e., the wild-type *B. subtilis* (●), and the *tagO* deletion strain (EB1451) grown in the presence (■) and absence (▼) of $MgCl_2$. Cultures were inoculated at a starting optical density at 600 nm (OD_{600}) of 0.001, and absorbance measurements were taken every 1 or 2 h. (B) Phase-contrast microscopy was performed on stationary-phase cultures of the (i) wild-type strain and the *tagO* deletion strain grown in the (ii) presence and (iii) absence of $MgCl_2$. Bar, 5 μm.

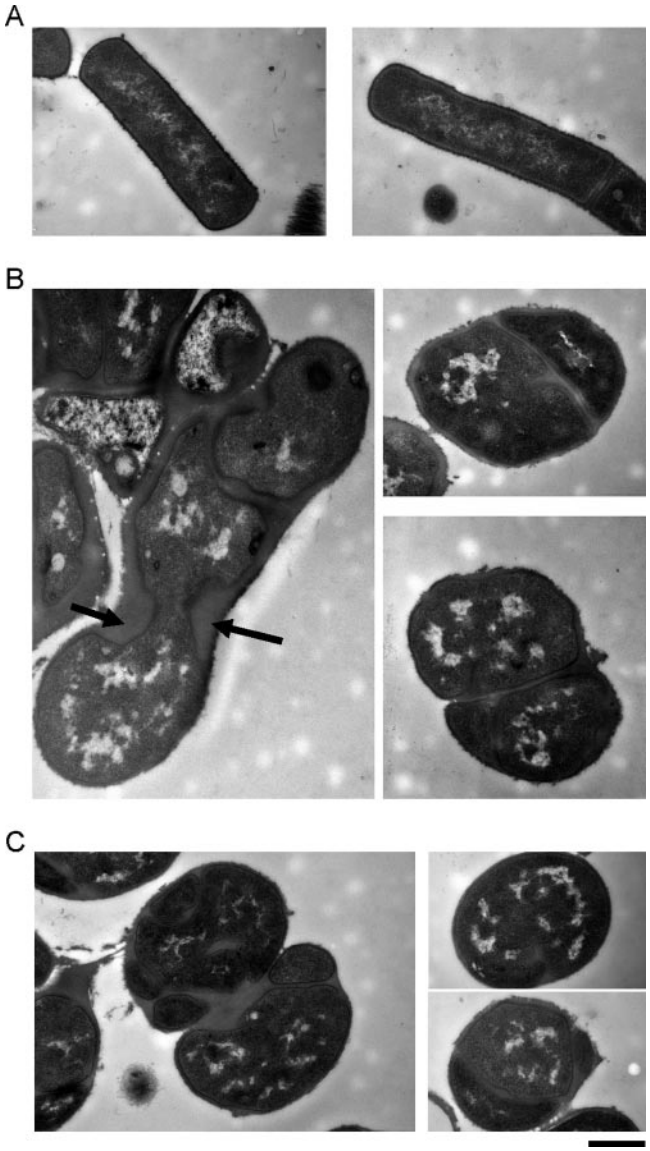

FIG. 2. Ultrastructure of *B. subtilis* lacking wall teichoic acid. Strains of *B. subtilis* 168 were harvested at late log phase of growth and conventionally embedded in thin sections for examination with transmission electron microscopy as described previously (14). The (A) wild-type strain (EB6) along with the *tagO* deletion mutant (EB1451) in the (B) absence and (C) presence of $MgCl_2$ are depicted. Arrows highlight areas of thickened cell wall. Bar, 500 nm.

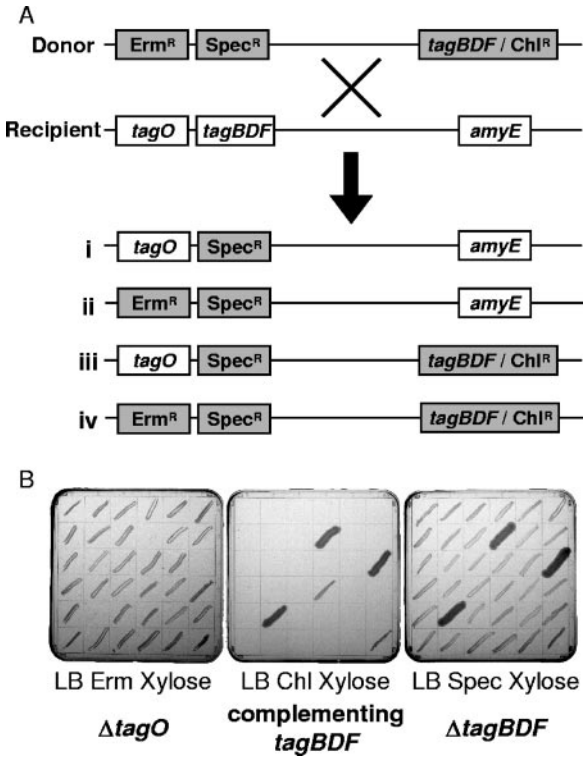

FIG. 3. Testing *tag* gene dispensability in *B. subtilis*. (A) To address the dispensability of *tagB*, *tagD and tagF* donor strains were created containing deletions of *tagO* (marked with Erm^r) and one copy of *tagB*, *tagD*, or *tagF* (marked with Spec^r) (*tagBDF*) that contained a complementing copy of *tagBDF* at *amyE* (marked with Chl^r). Transformation into a recipient (wild-type) strain and selection on spectinomycin (Spec) (150 μg/ml) and xylose (2%) could allow for four possible outcomes (i to iv). (B) The outcome of this selection procedure performed to test the dispensability of *tagD* is depicted. In addition to showing Spec^r, all of the clones selected were also Erm^r and/or Chl^r. Erm, erythromycin; Chl, chloramphenicol.

polymers has a dramatic effect on the cellular morphology of *B. subtilis*.

Given the surprising dispensability pattern associated with teichoic acid biosynthesis genes in *S. aureus*, where the first step was dispensable and remaining steps had an essential phenotype (8), we were interested, in this work, to reevaluate the dispensability of several late-acting teichoic acid genes (*tagB*, *tagD*, and *tagF*) in *B. subtilis*. The low transformability of *B. subtilis* makes it difficult to differentiate between a failed transformation and a lethal event; therefore, we endeavored to examine the dispensability of these late-acting genes by congression analysis (transformation of chromosomal DNA into the recipient strain and analysis of resistance markers transferred). Strains were generated that contained a deletion of *tagO* (marked with Erm^r, *ermAB*); a deletion of the late-acting gene *tagB*, *tagD*, or *tagF* [marked with Spec^r, AAD(9)]; and a complementing copy of the late-acting gene at *amyE* (marked with Chl^r, *cat86*). Each strain was produced by transforming chromosomal DNA from the *tagO* deletion strain (EB1451) into the complemented deletion strains of *tagB* (EB633), *tagD* (EB240), and *tagF* (EB669), giving rise to strains EB1453, EB1559, and EB1560.

Chromosomal DNA from each of the strains constructed (EB1453, EB1559, and EB1560) was transformed into a wild-type background (EB6) and growth selected on LB medium containing spectinomycin (150 μg/ml) and xylose (2%). After 2 days, 100 colonies from each transformation were examined for erythromycin and/or chloramphenicol resistance. Figure 3A provides a schematic of the experimental methodology and possible outcomes expected. Figure 3B shows the outcome of a typical experiment where 36 clones were chosen from the transformation of chromosomal DNA of strain EB1559 into strain EB6. Here, 31 clones were Spec^r Erm^r, 3 clones were Spec^r Chl^r, and 2 clones were Spec^r Erm^r Chl^r. Notably, we

TABLE 2. Testing the dispensability of the late-acting teichoic acid gene product

Gene tested	Donor strain	Recipient strain	No. of clones that were phenotype:			
			Spec^r Erm^r Chl^{sc}	Spec^r Erm^s Chlrd	Spec^r Erm^r Chl^{re}	Spec^r Erm^s Chl^{sf}
tagB[a]	EB1453	EB6	95	4	1	0
tagD[a]	EB1559	EB6	92	6	2	0
tagF[a]	EB1560	EB6	83	17	0	0
tagB[b]	EB1453	EB892	1	0	0	24

[a] A total of 100 colonies was examined.
[b] A total of 25 colonies was examined.
[c] Resistance profile of a double mutant (e.g., Δ*tagO* Δ*tagB*).
[d] Resistance profile of a complemented deletion strain (e.g., EB633).
[e] Resistance profile of the donor strain (e.g., EB1453).
[f] Resistance profile of a strain with a chromosomal deletion for the tested gene (e.g., Δ*tagB*).

were unable to generate any clones that were solely Spec^r, suggesting that *tagD* is indeed essential and that it is only possible to obtain a deletion of *tagD* if it is accompanied by a complementing copy or by a deletion of *tagO*. These results were echoed in larger scale screens performed for *tagB*, *tagD*, and *tagF* outlined in Table 2. In each case, the majority of clones (80 to 90%) were Spec^r Erm^r. Under no circumstances were clones generated that were exclusively Spec^r. To confirm that Spec^r could be unlinked from Erm^r and/or Chl^r, a similar congression sought to transform chromosomal DNA from strain EB1453 into EB892 (a strain containing a plasmid-borne copy of *tagB*). Here 24 of the 25 clones selected were Spec^r Erm^s Chl^s, demonstrating that the Spec^r marker could be unlinked from the other two, indicating that the *tagO* locus can be unlinked from the *tagB* locus and therefore the entire *tag* operon. Taken together, these data support the conclusion that the first enzyme of the teichoic acid biosynthesis pathway is dispensable, yet the remaining enzymes, at least *tagB* and beyond, are indispensable for viability. Furthermore, the ability to isolate clones that were Spec^r and Erm^r yet Chl^s indicates that the essential nature of *tagB*, *tagD*, and *tagF* can be suppressed by a deletion in *tagO*. These data parallel those obtained using *S. aureus* as a model, and thus, the peculiar dispensability pattern seen in these organisms may be a mechanistic feature associated with teichoic acid biosynthesis genes in gram-positive bacteria.

Here we show that despite a significant literature to the contrary, teichoic acid polymers are not essential to the viability of *B. subtilis* but nevertheless appear to play a crucial role in maintaining the shape of this organism. Through the replacement of *tagO* with an erythromycin resistance cassette in the absence of complementation, we have circumvented the ability of the organism to produce cell wall containing teichoic acid polymers, as shown by the drastic reduction in phosphate content. The creation of this mutant is in contradiction to the work by Soldo et al., who reported the inability to generate viable mutants in *tagO* through insertional inactivation (16). We attribute this discrepancy to the slow growth and altered morphology of this mutant that may have mistakenly led these authors to conclude that these mutants were not viable. Perhaps most remarkable is that, despite the dispensability of *tagO*, late-acting gene products are required for viability. This is in agreement with the peculiar dispensability pattern seen in *S. aureus* teichoic acid genes. Indeed it may reflect a mechanistic feature that is paradigmatic of the dispensability patterns of these genes in all gram-positive bacteria. As speculated in our previous work, we believe that the essentiality of the late-acting gene products may arise from the build up of toxic intermediates or from the sequestration of a crucial metabolite, such as undecaprenol phosphate, which is also required for the production of peptidoglycan.

We thank Bob Harris of the University of Guelph for his technical assistance in preparing samples for electron microscopy. Microscopy was performed in the NSERC Guelph Regional Integrated Imaging Facility (GRIIF).

This work was supported, in part, by a Canadian Institutes of Health Research (CIHR) operating grant (MOP-15496). M.A.D. holds a CIHR Canadian Graduate Scholarship. Both E.D.B. and T.J.B. hold Canada Research Chairs. Microscopy performed at the NSERC Guelph Regional Integrated Imaging Facility (GRIIF) is partially funded by an NSERC Major Facility Access grant to T.J.B.

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