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

Michael A. D’Elia, Kathryn E. Millar, Terry J. Beveridge, and Eric D. Brown

Antimicrobial Research Centre and Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, L8N 3Z5, Canada, and Department of Molecular and Cellular Biology, College of Biological Science, University of Guelph, Guelph, ON, N1G 2W1, Canada

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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 insertion 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 growing (Table 1 shows the strains and plasmids used in this study). 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 viablility.

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
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 MgCl₂ in the medium. Previous reports have demonstrated that Mg²⁺ 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 Mg²⁺. Although the addition of MgCl₂ 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 Mg²⁺ 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 Mg²⁺ 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 MgCl₂ 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 MgCl₂. 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

![Figure 1](https://example.com/figure1.png)

**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 MgCl₂. Cultures were inoculated at a starting optical density at 600 nm (OD₆₀₀) 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 MgCl₂. Bar, 5 μm.
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<sup>r</sup>) and one copy of *tagB*, *tagD*, or *tagF* (marked with Spec<sup>r</sup>) (*tagBDF*) that contained a complementing copy of *tagBDF* at *amyE* (marked with Chl<sup>r</sup>). 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).

**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<sub>2</sub> 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<sup>r</sup>) and one copy of *tagB*, *tagD*, or *tagF* (marked with Spec<sup>r</sup>) (*tagBDF*) that contained a complementing copy of *tagBDF* at *amyE* (marked with Chl<sup>r</sup>). 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<sup>r</sup>, all of the clones selected were also Erm<sup>r</sup> and/or Chl<sup>r</sup>. Erm, erythromycin; Chl, chloramphenicol.
were unable to generate any clones that were solely Spec\(^c\), 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 echoed in larger scale screens performed for to isolate clones that were Spec\(^r\) and Erm\(^r\) yet Chls indicates beyond, are indispensable for viability. Furthermore, the ability linked from the other two, indicating that the first enzyme of the teichoic acid biosynthesis pathway is dispensable, yet the remaining enzymes, at least tagO, were unable to generate any clones that were solely Spec\(^c\), 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\(^c\). To confirm that Spec\(^c\) could be unlinked from Erm\(^r\) and/or Chl\(^r\), a similar resistance profile of a double mutant (e.g., ΔtagO ΔtagB). In agreement with the peculiar dispensability pattern seen in these organisms may be a mechanism 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.

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### REFERENCES