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The *N*-Acetylmannosamine Transferase Catalyzes the First Committed Step of Teichoic Acid Assembly in *Bacillus subtilis* and *Staphylococcus aureus*▽§

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There have been considerable strides made in the characterization of the dispensability of teichoic acid biosynthesis genes in recent years. A notable omission thus far has been an early gene in teichoic acid synthesis encoding the *N*-acetylmannosamine transferase (*tagA* in *Bacillus subtilis*; *tarA* in *Staphylococcus aureus*), which adds *N*-acetylmannosamine to complete the synthesis of undecaprenol pyrophosphate-linked disaccharide. Here, we show that the *N*-acetylmannosamine transferases are dispensable for growth in vitro, making this biosynthetic enzyme the last dispensable gene in the pathway, suggesting that *tagA* (or *tarA*) encodes the first committed step in wall teichoic acid synthesis.

The cell wall of gram-positive bacteria is composed of not only peptidoglycan, but also a significant proportion of the polyol phosphate polymer known as teichoic acid. Wall teichoic acid has long been held as an essential component of the cell wall architecture (2–5, 19). However, recently, our group has demonstrated a complex pattern of dispensability for wall teichoic acid biosynthetic genes of both *Bacillus subtilis* and *Staphylococcus aureus* (9, 10).

The synthesis of wall teichoic acid polymers occurs through the sequential action of several enzymes (14, 17). The action of no less than seven enzymes is thought to synthesize the completed polymer on the cytoplasmic face of the cell membrane for export to the outside of the cell. Once outside, the completed polymer is covalently attached to the C-6 of the *N*-acetylmuramic acid of peptidoglycan through the action of an uncharacterized transferase. The best-characterized wall teichoic acid biosynthetic machinery is that for polymers composed of glycerol phosphate and ribitol phosphate. In the last several years, biochemical experiments have characterized the activities of nearly all of the enzymes responsible for the synthesis of both glycerol phosphate and ribitol phosphate polymers (6, 11, 18).

Work on the essential nature of wall teichoic acid dates back many years to the discovery and characterization of temperature-sensitive *B. subtilis tag* mutants for poly(glycerol phosphate) synthesis by D. Karamata's lab (4, 5, 19). That work and follow-up studies by our research group (2, 3, 20) showed convincingly that genetic lesions in several wall teichoic acid biosynthetic steps led to cell death in vitro. Recently, however, we uncovered some remarkable complexity in the dispensability pattern of wall teichoic acid synthetic genes. Working with both *B. subtilis* and *S. aureus*, we showed that viable deletions could be generated in the first gene of the pathway, encoding the *N*-acetylglucosamine-1-phosphate transferase (*tagO* in *B. subtilis*; *tarO* in *S. aureus*), while deletions could not be made for late-acting genes, including those encoding the glycerol phosphate primase (*tagB* in *B. subtilis*; *tarB* in *S. aureus*) and downstream enzymes. This apparent paradox was resolved when it was discovered that all of the indispensable genes became dispensable in a *tagO* (or *tarO*) deletion background and suggested that lesions in late steps of wall teichoic acid synthesis lead to a premature termination of the pathway, causing a buildup of toxic intermediates or the sequestration of a common and vital precursor molecule (i.e., undecaprenol phosphate).

While extensive investigations have charted the complex genetics of wall teichoic acid synthesis in both *B. subtilis* 168 (2–5, 9, 15, 16, 19, 21) and *S. aureus* (10, 23), no experiments have so far been reported to characterize the dispensability phenotype of the *N*-acetylmannosamine transferase encoded by *tagA* (*B. subtilis*) and *tarA* (*S. aureus*). Indeed, *tagA* from *B. subtilis* was recently shown to catalyze the addition of *N*-acetylmannosamine to complete the synthesis of undecaprenol pyrophosphate-linked disaccharide, a core component of the "linkage unit" of wall teichoic acid (6, 11, 25). This places TagA (TarA) as an enzyme catalyzing the second step in wall teichoic biosynthesis after TagO (TarO), the *N*-acetylglucosamine-1-phosphate transferase. Given the dispensable phenotype of *tagO* (*tarO*) and the capacity of this deletion for suppression of downstream, essential, late-acting genes, we were motivated to explore the dispensability phenotype of this as-yet-unexplored step of wall teichoic acid synthesis. Here, we analyzed the dispensability of the *N*-acetylmannosamine transferase genes of both *B. subtilis* and *S. aureus* (*tagA* and *tarA*, respectively) for growth in vitro.

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TABLE 1. Allelic replacement for testing gene dispensability in *S. aureus*

Strain	No. of colonies with indicated phenotype		
	Wild type	Nonexcisant	Mutant
No complementation			
tarA mutant	65	1	34
Δ*tarA* background			
tarB mutant	75	0	15
tarF mutant	50	2	48
tarIJ mutant	88	0	12

TABLE 2. Phosphate content of cell wall isolated from *B. subtilis* and *S. aureus*

Strain	Phosphate content (μmol phosphate/mg cell wall)
Bacillus subtilis	
Wild type	1.6 ± 0.4
Δ*tarO* mutant	0.09 ± 0.02
Δ*tarA* mutant	0.10 ± 0.03
Staphylococcus aureus	
Wild type	1.2 ± 0.1
Δ*tarO* mutant	0.140 ± 0.003
Δ*tarA* mutant	0.140 ± 0.005

Gene *tarA* from *S. aureus* COL was identified as SACOL0693, using BLAST analysis. Dispensability testing of *tarA* was done in *S. aureus* strain SA178RI, using an allelic replacement system developed by us (pSAKO) and described previously (10). Using this methodology (see the supplemental material for detailed methods), we demonstrated that in a wild-type background, *S. aureus tarA* could be readily replaced with an erythromycin resistance cassette, allowing for mutant generation at a high frequency (Table 1). Thus, our data reveal that this locus is dispensable for growth in vitro. In *B. subtilis*, we were likewise able to replace the *tagA* gene with a spectinomycin resistance cassette after the generation and transformation of a PCR product containing the flanking regions of *tagA* surrounding the resistance cassette. To confirm that the deletion of *tagA* was not the result of a suppressor mutation elsewhere in the chromosome, we performed an analysis of congression to compare the efficiency of recombination of the Spec resistance determinant (replacing *tagA*) into wild-type *B. subtilis* to that of the Erm resistance determinant (replacing *tagO*). We also compared these with that of a control Chl resistance cassette at the *amy* locus. The frequencies of recombination for all of these experiments were very similar (data not shown). These findings indicated that the loss of *tagA* was not the result of a concomitant suppressor mutation. The resulting colonies (Δ*tagA*) were small, smooth, and very similar in morphology to the *tagO* mutant that we have described previously (9).

To confirm that these strains were devoid of teichoic acid polymers, the cell wall phosphate contents for *B. subtilis* (*S. aureus*) wild-type, *tagO* (*tarO*), and *tagA* (*tarA*) null strains were analyzed (Table 2). These results revealed that the cell wall phosphate content of *tagA* and *tarA* null strains were approximately 10% that of the wild type and comparable to those found in the cell walls of the *tagO* and *tarO* null strains. The generation of *tagA* (*tarA*) mutants in conjunction with a significant loss of cell wall phosphate content was consistent with the conclusion that *B. subtilis tagA* and *S. aureus tarA* mutants were devoid of wall teichoic acid. While the presence of residual phosphate in this mutant was noteworthy, it was not surprising. Previous analyses by our group and another group have revealed residual phosphate in a *tagO* mutant (9, 21). We speculate that this phosphate might originate from minor teichoic acid species or other phosphate-containing cellular components.

As stated above, our group has previously been able to demonstrate, using both *B. subtilis tagO* and *S. aureus tarO*, that these deletions were able to suppress the lethality associated with deletion of late-acting gene products (9, 10). Having succeeded in making strains of *B. subtilis* and *S. aureus* that lacked the *N*-acetylmannosamine transferase gene and wall teichoic acid, we were interested in testing for genetic interactions with the late-acting genes in the pathway. Previously, we were able to leverage the capacity of allelic replacement with pSAKO to test the dispensability of late-acting teichoic genes in the presence and absence of a *tarO* deletion (10). We reasoned that the dispensable phenotype of *tarA* should provide for a dispensable phenotype of the downstream genes *tarB*, *tarF*, and *tarIJ* just as we have seen for *tarO*. From Table 1, it is clear that in the absence of *tarA*, the otherwise essential genes *tarB*, *tarF*, and *tarIJ* become dispensable. These data demonstrate that *tarA* has the same peculiar genetic interactions previously observed with *tarO*.

With this work we have established that *tagA* and *tarA* are dispensable for in vitro growth in both *B. subtilis* and *S. aureus* strains, respectively. Phenotypic characterization of these mutants indicated that the strains were devoid of wall teichoic acid. Furthermore we have shown that the deletion of *tarA* in *S. aureus* is able to suppress the essential phenotypes of several late-acting wall teichoic acid synthesis genes. These findings reveal that *tagA* and *tarA* are the last dispensable genes in their respective biosynthetic pathways and suggest that the *N*-acetylmannosamine transferase commits the cell to synthesizing wall teichoic acid. This would mean that TagO (TarO) catalyzes a reversible biosynthetic step. Indeed, the reversibility of enzymes homologous and analogous to TagO (TarO) has been well established (1, 7, 13, 22). With TagO (TarO) catalyzing a reversible step, the reaction controlled by TagA (TarA) represents the first committed step in wall teichoic acid synthesis. Having committed to teichoic acid biosynthesis, the cell must complete polymer assembly to avoid the lethal consequences of blocks in the later steps of this pathway.

To further evaluate the phenotype of the deletion of the *N*-acetylmannosamine transferase gene in both *B. subtilis* and *S. aureus*, growth analysis and transmission electron microscopy were performed. The growth characteristics of the *B. subtilis tagA* and *S. aureus tarA* deletion strains with respect to those of the wild-type strains are very different (Fig. 1). Figure 1A shows the growth kinetics of the *B. subtilis tagA* null strain (EB1494) compared to those of the wild-type (EB6) and *tagO* deletion (EB1451) strains. The data reveal that the mutant is significantly impaired for growth compared to the wild-type

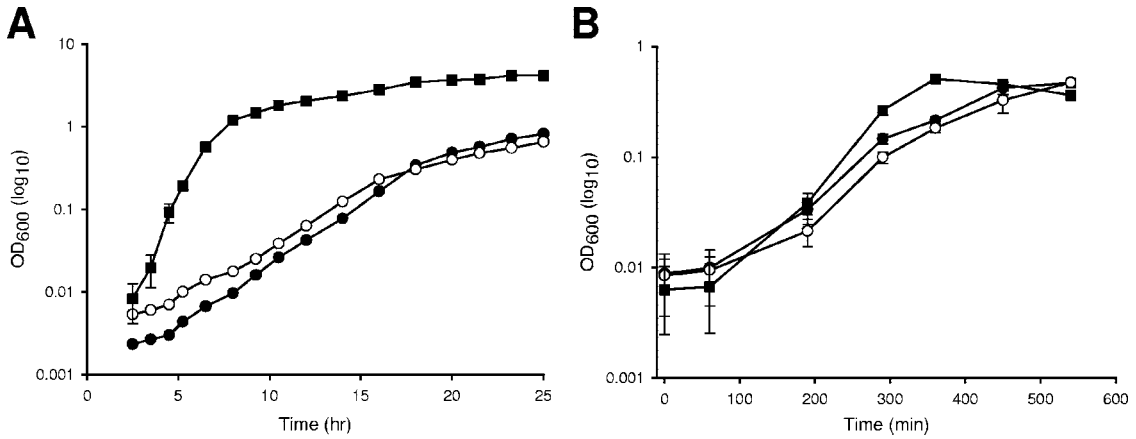

FIG. 1. Growth kinetics of *B. subtilis* and *S. aureus* deletion mutants. (A) Growth curves are depicted for the *B. subtilis tagA* deletion strain (EB1494 [○]). Growth data for the wild-type (EB6 [■]) and *tagO* deletion (EB1451 [●]) strains (9) are shown for comparison. (B) Growth curves are shown for the *S. aureus* wild-type (SA178RI [■]), *tarO* null (EBII44 [●]), and *tarA* null (EBII58 [○]) strains. All cultures were inoculated to a starting optical density value at 600 nm (OD_{600}) of 0.005, and absorbance measurements were taken every 1 to 2 h.

strain, with a growth rate comparable to that of the *tagO* mutant previously described (9). For *S. aureus*, the *tarA* deletion strain grew similarly to both the wild type and the *tarO* deletion strain (Fig. 1B).

The differences shown in the growth curves were paralleled in the transmission electron micrographs shown in Fig. 2. While the *S. aureus tarA* mutant did not have any significant morphological defects, the *B. subtilis tagA* deletion mutant showed abnormalities that were very similar to those seen previously for the *tagO* deletion mutant (9). These gross morphological defects included loss of its rod shape, aberrant septation, and asymmetrical peptidoglycan architecture. Thus, the loss of wall teichoic acid had a much more profound effect on *B. subtilis* than it did on *S. aureus*. Further characterization of the *tagA* and *tarA* mutants revealed that *N*-acetylmannosamine transferase deficiency had dramatically different impacts on

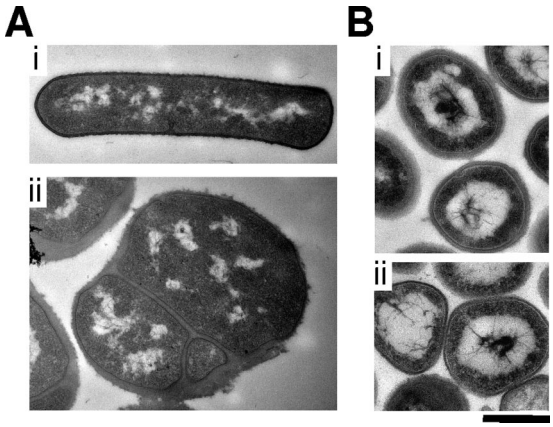

FIG. 2. Ultrastructure of *B. subtilis tagA* and *S. aureus tarA* null mutants. Bacteria were harvested at late log phase of growth and embedded in thin sections for examination with transmission electron microscopy as described in the supplemental material. Panel A shows micrographs of (i) the *B. subtilis* wild type (EB6) and (ii) the *tagA* null strain (EB1494). Panel B depicts micrographs of (i) the *S. aureus* wild-type (SA178RI) and (ii) the *S. aureus tarA* null strain (EBII58). The bar represents 500 nm.

the growth and morphology of *B. subtilis* and *S. aureus*. Deletion of *tagA* in *B. subtilis* resulted in a remarkable impact on ultrastructure including complete loss of rod shape, abberant septation and cell wall asymmetry. These observations were reminiscent of that seen for the *B. subtilis tagO* deletion described previously (9). In contrast, the *S. aureus tarA* deletion mutant had growth and ultrastructural characteristics that were not unlike those of the wild type. The gross morphological and growth defect differences between the two organisms is not understood as of yet. We predict that these differences result from the shape of the organism; in particular, there are significantly more-profound effects on *B. subtilis*, given its rod structure, while the coccoid shape associated with *S. aureus* resists such defects. Although not yet demonstrated, an interesting hypothesis may be the alternate manners in which these two organisms grow and build their cell walls (8). *S. aureus* growth occurs only at the septum, while *B. subtilis* growth occurs both at the septum and along the cell cylinder. It is interesting to speculate that teichoic acid biogenesis plays a role in only the cylinder growth or bacteria, indicating why defects are seen only with the rod-shaped *B. subtilis*.

Given the similar phenotypes of the *tagO* (*tarO*) and *tagA* (*tarA*) deletion strains, we broadened our search for phenotypes in this work to include antibiotic susceptibility. We restricted our investigations for these studies to *S. aureus* because of the robust growth of the *tarO* and *tarA* deletion strains of this organism. MIC determinations to a variety of antimicrobials are largely unchanged relative to those of the wild type. Among the 20 antibiotics tested of various chemical classes and mechanisms (see the supplemental material), *tarO* and *tarA* deletion strains showed increased susceptibility (>2-fold compared to the wild types) only to fusidic acid and phosphomycin, 8- and 16-fold reductions in MIC, respectively (data not shown). Interestingly, these two compounds are negatively charged, as are teichoic acid polymers. We posit that the increased susceptibility was due to improved delivery of these compounds to their intracellular targets. Therefore, with the exception of a potential influence of negatively charged mole-

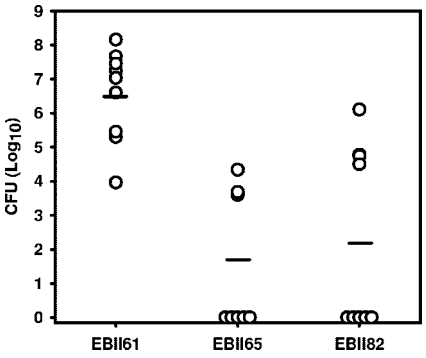

FIG. 3. Teichoic acid mutants are impaired for growth in vivo. The graph shows the CFU recovered from the homogenized kidneys of mice infected with the *S. aureus* wild-type Newman strain (EBII61) and corresponding *tarO* (EBII65) and *tarA* (EBII81) deletion mutants. In these experiments, mice were injected in the tail vein with 10^7 bacteria, and CFU were determined 5 days postinfection.

cules, the loss of teichoic acid polymers in the cell wall does not significantly alter the drug susceptibility of *S. aureus*.

Having shown that *B. subtilis tagA* and *S. aureus tarA* deletion mutants were viable and analogous in many respects to the *tagO* and *tarO* mutants characterized previously, we were interested in comparing the in vivo phenotypes of the *tarO* and *tarA* mutants. Weidenmaier et al. previously showed that the *tarO* null mutant was compromised for colonization in rat nasopharyngeal and rabbit endocarditis models (23, 24). Here, we tested the hypothesis that the *tarA* deletion would similarly impair the colonization of *S. aureus* in a mouse kidney abscess model. Figure 3 charts the colony counts recovered from mouse kidneys 5 days after infection with a wild-type *S. aureus* Newman strain (EBII61) as well as the *tarO* and *tarA* null mutants in the Newman background (EBII65 and EBII82, respectively). At the time of sacrifice, mice infected with the wild-type Newman strain (EBII61) had high bacterial cell numbers in their kidneys (average of $10^{6.5} \pm 10^{1.3}$ CFU). In stark contrast, we were unable to recover viable bacteria from most of the mice infected with either the *tarO* or *tarA* mutant strains, while some mice had low but detectable bacterial loads. The average cell number recovered from mice infected with the *tarO* (EBII65) and *tarA* (EBII82) null strains was $10^{1.7} \pm 10^{2.0}$ and $10^{2.2} \pm 10^{2.7}$ CFU, respectively. Generally, mice infected with the mutant strains were significantly more healthy than those infected with wild-type bacteria. Clinical scoring through examination of the overall fitness of the mice showed that the Δ*tarO* mutant had an average score of 0.66 ± 1, the Δ*tarA* mutant had an average score of 0 ± 0, and the wild-type strain had a significantly higher score of 3.1 ± 1.2. As a further measure of health, we observed that mice infected with mutant strains lost, on average, significantly less weight than mice infected with the wild-type Newman strain, as follows: 21 ± 6% (wild-type Newman strain), 0.6 ± 3.4% (*tarO* null strain), and 1.4 ± 4.3% (*tarA* null strain).

Here, we found that the *tarO* and *tarA* mutants were compromised similarly to the wild type in a mouse kidney abscess model of infection. It has been well established that teichoic acid polymers play a significant role in the adherence of bacteria, likely the result of the charge associated with the polymer (12, 24). The failure of the *tarA* null mutant to colonize and persist in the mouse model here provides additional support for the importance of wall teichoic acid to infection and draws further parallels with the *tarO* mutant in terms of phenotype.

In conclusion, our findings reveal that *B. subtilis tagA* and *S. aureus tarA* are dispensable in their respective biosynthetic pathways. Indeed, the encoded *N*-acetylmannosamine transferases should be considered the first committed step in wall teichoic acid polymer production. In this particular pathway, commitment to wall teichoic acid synthesis marks an obligation to complete polymer assembly and export. The consequence of failing to do so in these organisms is cell death. Thus, despite the dispensability of the polymer for in vitro growth, wall teichoic acid biosynthesis represents an exploitable target for new antibiotic development. Interestingly, results shown here and elsewhere (9, 10) predict that the suppression of lethal phenotypes associated with blocks in late steps of wall teichoic acid synthesis could be accomplished with mutations in the first steps, namely *tagO* (*tarO*) and/or *tagA* (*tarA*). Nevertheless, the requirement of wall teichoic acid for virulence in various animal models suggests that such suppressor mutations would lead to noninfectious strains. We maintain therefore that wall teichoic acid synthesis may well be an ideal target for new antibacterial drug discovery.

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