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# ABC transporters required for export of wall teichoic acids do not discriminate between different main chain polymers

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## Abstract

The cell envelopes of Gram-positive bacteria comprise two major constituents, peptidoglycan and teichoic acids. Wall teichoic acids (WTAs) are anionic glycophosphate polymers that play important roles in bacterial cell growth, division, and pathogenesis. They are synthesized intracellularly and exported by an ABC transporter to the cell surface, where they are covalently attached to peptidoglycan. We address here the substrate specificity of WTA transporters by substituting the *Bacillus subtilis* homologue, TagGH$^{Bs}$, with the *Staphylococcus aureus* homologue, TarGH$^{Sa}$. These transporters export structurally different substrates in their indigenous organisms, but we show that TarGH$^{Sa}$ can substitute for the *B. subtilis* transporter. Hence, substrate specificity does not depend on the WTA main chain polymer structure, but may be determined by the conserved diphospholipid-linked disaccharide portion of the WTA precursor. We also show that the complemented *B. subtilis* strain becomes susceptible to a *S. aureus*-specific antibiotic, demonstrating that the *S. aureus* WTA transporter is the sole target of this compound.

Bacteria are surrounded by complex cell envelopes that mediate interactions with the external milieu, act as filters to allow the passage of selected molecules into and out of the cells, and form a protective layer that stabilizes the plasma membrane against high internal osmotic pressure fluctuations (1). The most conserved component of the cell envelope in bacteria is peptidoglycan (PG), a crosslinked mesh of glycan chains connected through peptide bridges. Since it is conserved, essential, and unique to bacteria, PG is a major target for clinically used antibiotics, such as penicillin and vancomycin. However, multi-resistant pathogenic strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) pose a major problem to the public, making it crucial to explore other possible cell wall targets.

The PG layers of Gram-positive organisms are densely functionalized with anionic polymers called wall teichoic acids (WTAs) (2). These polymers, which comprise as much as 50% of the cell wall mass, are typically composed of linear sugar phosphate repeats, usually glycerol or ribitol phosphates, which are tailored with D-alanyl esters and hexoses (2,3). WTA precursors are synthesized on an undecaprenyl phosphate carrier lipid (UndP) on the inner surface of the cytoplasmic membrane and then exported through a two-component ATP-binding cassette (ABC) transporter to the cell surface where they are covalently attached to PG (4) (Fig. 1).

WTAs play important roles in determining cell morphology in *B. subtilis* and are critical for cell division in *S. aureus* (5–9). They are speculated to scaffold components of the PG biosynthetic machinery as a mechanism for regulating cell envelope biosynthesis (8,9).

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Supporting Information
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WTAs are not essential for survival *in vitro* (10–12); however, *S. aureus* strains lacking WTAs are severely impaired in cell division and unable to colonize host tissue and establish infections (8,12–14). The importance of WTAs in bacterial physiology and host infection make WTA biosynthesis a target for novel antibiotics.

The WTA biosynthetic pathway shares an unusual genetic feature with several other UndP-dependent pathways: many of the downstream genes are essential except in strains having mutations that prevent flux into the pathway (10,15). The lethality of downstream null mutations may be due to accumulated toxic intermediates and/or inhibition of PG biosynthesis because of sequestration of the UndP carrier (11). It was predicted that small molecules that inhibit these downstream enzymes would have antibacterial activity, and we confirmed this through the discovery of a WTA-active antibiotic in a cell-based high throughput screen that exploited the conditional essentiality of the downstream genes (15). The compound discovered was subsequently optimized for potency to produce a second generation antibiotic named targocil (16).

Targocil has a minimal inhibitory concentration (MIC) below 1 μM against all *S. aureus* strains examined, including MRSA, and studies in *S. aureus* have identified its target as TarGH, the essential two-component ABC transporter that exports lipid-linked WTA precursors to the cell surface (Fig. 1)(4,15). Many Gram-positive organisms make WTAs and contain TarGH orthologs, but targocil is completely specific for *S. aureus*. It was unknown whether resistance in other organisms was due to intrinsically resistant TarGH transporters, the presence of unidentified, non-homologous transporters, or other mechanisms. We address this question here using a heterologous complementation approach in which the *S. aureus* transporter is expressed in *B. subtilis* PY79. This approach provides crucial insights into how WTA transporters select their substrates.

*S. aureus* makes WTA precursors consisting of a poly-ribitolphosphate chain connected through a linkage unit containing a disaccharide linked to undecapranyl pyrophosphate (17,18). The ribitolphosphate subunits carry α- or β-O-N-acetyl glucosamine modifications (Fig. 1c, d) (19). The WTA precursors of *B. subtilis* PY79 consist of a glycerolphosphate polymer tailored with α-O-glucose residues, but the linkage unit is identical to that in *S. aureus* (Fig. 1a, b). Therefore, the polymeric portions of the native substrates of the WTA transporters $TarGH^{Sa}$ and $TagGH^{Bs}$ are structurally different.

The WTA transporters consist of an ATPase portion ($TarH^{Sa}$ or $TagH^{Bs}$) and a transmembrane portion ($TarG^{Sa}$ or $TagG^{Bs}$). They belong to a family of polymer-exporting ABC transporters predicted to function as dimers containing two ATPase domains and two transmembrane domains (20,21). The transmembrane portions are moderately conserved between *S. aureus* and *B. subtilis* (36% identity, 57% similarity) (Fig. 2a). Both proteins are predicted to have six transmembrane spanning regions with the N- and C-termini inside (Fig. 2). The ATPase component of the *B. subtilis* transporter, $TarH^{Bs}$, aligns with high similarity (73%) to the first 264 amino acids of the *S. aureus* ATPase, but contains an additional 263 residue C-terminal extension that has no significant similarity to any known protein (Supplementary Fig. S1a). A single membrane-spanning region is predicted immediately after the homologous region, which would place a large C-terminal domain outside the cytoplasm (Supplementary Fig. S1b, c). Hence, both the structures of the transporters and the WTA precursors differ significantly between the two organisms.

*B. subtilis tagGH*, like its *S. aureus* counterpart, is essential for viability (4). Therefore, to determine whether $TarGH^{Sa}$ could substitute for $TagGH^{Bs}$, we first constructed a *B. subtilis* strain with $tarGH^{Sa}$ under the control of an IPTG-inducible promoter (strain KS001, Fig. 3a), and we then deleted the endogenous $tagGH^{Bs}$ genes (strain KS002, Fig. 3a). The *B.*

*subtilis* transporter could easily be deleted in the presence of IPTG, showing that TarGH$^{Sa}$ can complement the loss of TagGH$^{Bs}$. Many uniform colonies were obtained in several transformations with selection for deletion mutants, indicating that KS002 is unlikely to require additional suppressor mutations for viability. Strain KS002 was dependent on IPTG for growth (Fig. 3b, Supplementary Fig. S2), demonstrating that expression of TarGH$^{Sa}$ is essential when TagGH$^{Bs}$ is deleted. WTAs extracted from the wild-type, KS001 and KS002 strains were indistinguishable as judged by PAGE analysis of the polymers and LC/MS analysis of chemically degraded subunits (Fig. 3d, Supplementary Fig. S3). Thus, the *S. aureus* transporter is capable of transporting the polyglycerolphosphate WTA precursors produced by *B. subtilis*. Since the native substrates for the *S. aureus* transporter have poly-ribitolphosphate main chains, we conclude that substrate recognition by the ABC transporter is not dependent on the chemical structure of the main chain polymer.

Examination of KS002 by light microscopy showed that depletion of TarGH$^{Sa}$ leads to swelling and rounding of the cells (Fig. 3c), a phenotype consistent with the loss of WTAs (10). Compared to the wild-type, KS002 cells grown in the presence of IPTG had minor defects. They were slightly curved and the cell poles were wider than usual, appearing almost forked (Fig. 3c, arrows). These defects suggest imperfect cell division. KS001 cells grown in the presence of IPTG were identical to wild-type cells (Fig. 3c), indicating that the defects in KS002 are not due to over-expression of TarGH$^{Sa}$. Since IPTG-induced expression of the *B. subtilis* WTA transporter TagGH$^{Bs}$ from the same locus (strain KS005) resulted in full restoration of both growth rate and morphology (Supplementary Fig. S4), the minor defects in KS002 expressing only the *S. aureus* transporter are due either to less efficient WTA export by the exogenous transporter or possibly the absence of the C-terminal domain of the *B. subtilis* transporter.

To test whether the lack of this C-terminal domain was responsible for the phenotype of strain KS002, a strain with a truncated version of TagH$^{Bs}$ in the native locus was constructed (KS003), leaving only the N-terminal part of the protein from amino acid 1 to 275. Strain KS003 was indistinguishable from the wild-type in cell morphology and growth rate (Supplementary Fig. S5), indicating that the extracellular domain of TagH is not required for function of the ABC transporter, consistent with previous results based on transposon mutagenesis (4). Therefore, the absence of this domain does not lead to the phenotypic changes seen when TarGH$^{Sa}$ is the sole transporter.

Having established that the *S. aureus* transporter complemented the loss of *B. subtilis* TagGH, albeit imperfectly, we examined whether targocil, a *S. aureus*-specific antibiotic with a triazoloquinazoline core (Fig. 4a) would have antibiotic activity against KS002. Wild-type *B. subtilis* PY79 is resistant to targocil at concentrations exceeding 200 μM (Table S2; 15), and expression of TarGH$^{Sa}$ in a wild-type background (strain KS001) had no effect on resistance (Fig. 4b, c; Supplementary Table S2). However, the minimal inhibitory concentration (MIC) of targocil against KS002 was 1.25 μM, which is comparable to its MICs against *S. aureus*. No other compound tested (kanamycin, penicillin G, tunicamycin, carbenicillin, erythromycin) showed a changed MIC in KS002 compared with the wild-type strain, demonstrating that targocil's activity against this strain was due solely to inhibition of the complementing *S. aureus* transporter (Supplementary Table S2). Consistent with this interpretation, targocil-treated KS002 cells were morphologically similar to KS002 cells following depletion of TarGH$^{Sa}$. Upon treatment with targocil, KS002 cells progressively rounded up along their cylindrical axis (Fig. 4c). In contrast, the wild-type and KS001 strains were unaffected by growth in the presence of the inhibitor (Fig. 4c).

Targocil resistant mutants of strain KS002 were selected on plates containing 5 μM targocil and 1 mM IPTG, required for TarGH$^{Sa}$ expression. TarG was sequenced from 18 colonies

with stable mutations that conferred resistance to targocil. In all cases, *tarG*$^{Sa}$ was found to contain a point mutation at one of several sites (Fig. 2, yellow stars), implying that mutation of the target is the major mechanism of resistance in *B. subtilis*. In *S. aureus*, in addition to *tarG* point mutations, null mutations in *tarO* or *tarA* that result in avirulent strains are found. The different resistant populations in the two organisms probably reflect the more severe growth defects following deletion of the WTA pathway in the rod-shaped *B. subtilis*. The most frequent TarG mutations were M87T (6x) and F89L (6x) and these mutations were also common when targocil resistance was selected in *S. aureus* (Fig. 2, black stars) (15). The mutated residues are predicted to be located in the middle of the second transmembrane domain of TarG (TM2), and from these and other, less common point mutations conferring targocil resistance (Fig. 2b) we speculate that the binding site for the inhibitor might be located in a tertiary structure formed by predicted transmembrane helices TM1, 2, 4 and 5.

## Conclusions

The export of large, highly polar bacterial polymers by ABC transporters is a remarkable but poorly understood process. Almost nothing is known about the mechanism of polymer transport and little is understood about substrate specificity (20). Here we have shown that the WTA transporters from *B. subtilis* and *S. aureus* can be exchanged without loss of function, even though they normally transport different substrates, *i.e.*, poly-glycerol and poly-ribitol WTA precursors tailored with different sugars. Therefore, the structure of the polymer itself is not critical for substrate recognition. Since both WTA precursors contain the same disaccharide linkage unit bound to UndP, we speculate that this diphospholipid-anchored disaccharide unit is recognized by the transport machinery. If so, WTA transporters may function as linkage unit “flippases” with main chain export occurring as a result. Alternatively, initiation of transport from the non-reducing end could occur if general chemical characteristics (*e.g.*, repeating phosphates) rather than specific structural features of the main chain polymer are involved in recognition. It has been previously suggested that polymer synthesis and transport are coupled through protein-protein interactions between the biosynthetic enzymes and the transporter, but successful heterologous complementation of *B. subtilis* with the *S. aureus* transporter suggests that such interactions are not essential for WTA export.

Since the presence of TarGH$^{Sa}$ as the only WTA transporter confers susceptibility against targocil to *B. subtilis*, this organism’s intrinsic resistance is not due to general differences in uptake, mechanism of action, or the presence of an unknown transporter, but solely to a resistant TagGH transporter. Since the MICs of targocil against *S. aureus* and KS002 are comparable, the affinity and the mechanism of action of the inhibitor are likely the same in the two organisms. Since targocil blocks the *S. aureus* transporter in *B. subtilis* but does not inhibit the *B. subtilis* transporter, we conclude that the interaction of targocil with *S. aureus* TarG is highly specific, explaining its narrow spectrum.

Nonetheless, there is potential to develop WTA transport inhibitors with an expanded spectrum since our experiments show that blocking WTA transport is lethal not only in *S. aureus*, but also in *B. subtilis*. The transmembrane component for WTA transport is promiscuous with respect to substrate structure and tolerant of many changes in amino acid sequence since different mutations confer resistance without affecting transport. However, it may be possible to identify inhibitors with an expanded spectrum by targeting other regions of the transporter that are involved in more conserved recognition events. These regions include the ATP binding site of the ATPase domain and possibly the WTA linkage unit recognition region (22). We are working towards the reconstitution of a WTA transporter to enable the identification of the structural features that determine how WTA substrates are selected for export, and to lay the groundwork for studies to elucidate how these machines

export such large polymers and yet can be inhibited with exquisite specificity by a small molecule.

## METHODS

### Strain construction

Lists of strains, plasmids and oligonucleotides are given in Table S1.

Since in *S. aureus tarG* and *tarH* are oriented in opposite directions and are each transcribed from their own promoter in the intergenic region, the genes were amplified separately and an optimal ribosome binding site was added upstream of each. *tarG* and *tarH* were amplified from *S. aureus* Newman using primer pairs 1+2 and 3+4 respectively. The two PCR products were digested with XbaI, ligated, and the ligation product was used as a template for a PCR using primers 1+4. This product was cloned into pDR111 using SalI and SphI. The plasmid insert was validated by sequencing and then transformed into *B. subtilis*, where it integrated into the *amyE* locus, giving strain KS001.

Strain KS002 was constructed by deleting $tagGH^{Bs}$ from the chromosome of KS001. For this, approximately 1000 bp up- and down-stream of *tarGH* were amplified using primers 5+6 and 7+8 respectively, and both PCR products were ligated to the *cat* resistance cassette from pKM074 using BamHI and EagI. The ligation product was used as a template for a PCR using primers 5+8 and the product was directly transformed into *B. subtilis* selecting for chloramphenicol resistance. Deletion of $tagGH^{Bs}$ was confirmed by PCR.

Transformation of *B. subtilis* was done according to the method of Anagnostopoulos and Spizizen (23) as modified by Jenkinson (24).

### Culture Conditions

Bacterial cultures were grown at 37°C under shaking conditions in LB medium or on Nutrient Agar (NA) plates. If required, antibiotics were added: chloramphenicol 5 μg $m^{-1}$, spectinomycin 100 μg $ml^{-1}$, erythromycin 1 μg $ml^{-1}$. 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce expression from the $P_{hyperspank}$ promoter as indicated.

### Raising of targocil resistant mutants

Dilutions of exponentially growing cultures of strain KS002 were plated on NA plates containing 5 μM targocil and 1 mM IPTG. After incubation for 24 h at 37°C colonies were picked and streaked on NA plates containing IPTG. Single colonies were then streaked on NA with 5 μM targocil and 1 mM IPTG. From all strains chromosomal DNA was isolated and *tarG* was amplified and sequenced.

### Microscopy

For microscopy, overnight cultures were diluted into fresh medium with or without 1 mM inducer as indicated. Samples were taken at the time points indicated and placed on cover slips with agarose pads. Images were acquired with a Hamamatsu digital camera model ORCA-ER connected to a Nikon Eclipse TE2000-U microscope with X-cite 120 illumination system. Image manipulation was limited to changing brightness and contrast using ImageJ (25).

### WTA extraction and analysis

WTAs were extracted, degraded and analyzed by LC/MS as described previously (17).

### Determination of Antibiotic Sensitivity

Antibiotic sensitivity was tested by diluting an overnight culture into fresh medium containing IPTG to an $OD_{600}$ of 0.001. Antibiotics were added in dilution steps 1:2 and the cultures were incubated overnight whilst shaking at 30°C. Lack of visible growth was interpreted as sensitivity against the respective antibiotic concentration.

For the plate diffusion assay overnight cultures were plated on NA containing IPTG, then filter disks soaked with antibiotic were placed on top. The plates were incubated over night at 37°C and zones of clearing around the filter disks were interpreted as sensitivity against the antibiotic.

### Bioinformatic predictions

Sequences for $TarG^{Sa}$ and $TagG^{Bs}$ were obtained from the Genolist webserver (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList)(26). Alignments were done using the ClustalW webserver (27) and the appearance was modified using Boxshade (Institute Pasteur, France; http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList). The topology model is based on predictions by the TMHMM and the SOSUI webservers.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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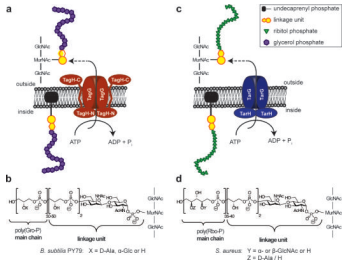

**Figure 1. Schematic of the WTA exporters of *B. subtilis* PY79 and *S. aureus***

a) Model of WTA precursor export by TagGH from *B. subtilis* PY79. b) Structure of WTA from *B. subtilis* PY79 showing the linkage unit, its connection to N-acetyl muramic acid (MurNAc) of peptidoglycan and the poly-glycerol phosphate [poly(GroP)] main chain. c) Schematic view of the WTA exporter TarGH from *S. aureus*. d) Structure of linkage unit and poly-ribitol phosphate [poly(RboP)] main chain from *S. aureus*.

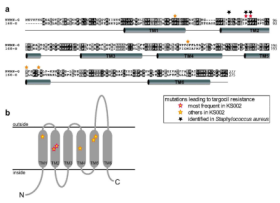


**Figure 2. Alignment of *B. subtilis* and *S. aureus* TagG/TarG**

(a) Alignment of *S. aureus* TarG (NWMN-G) and *B. subtilis* TagG (168-G). Conserved amino acids are shown with a black background, similar amino acids with a grey background. Barrels indicate predicted transmembrane helices. b) Predicted topology of $TarG^{Sa}$ with stars marking the sites of point mutations leading to targocil resistance in *B. subtilis* KS002.

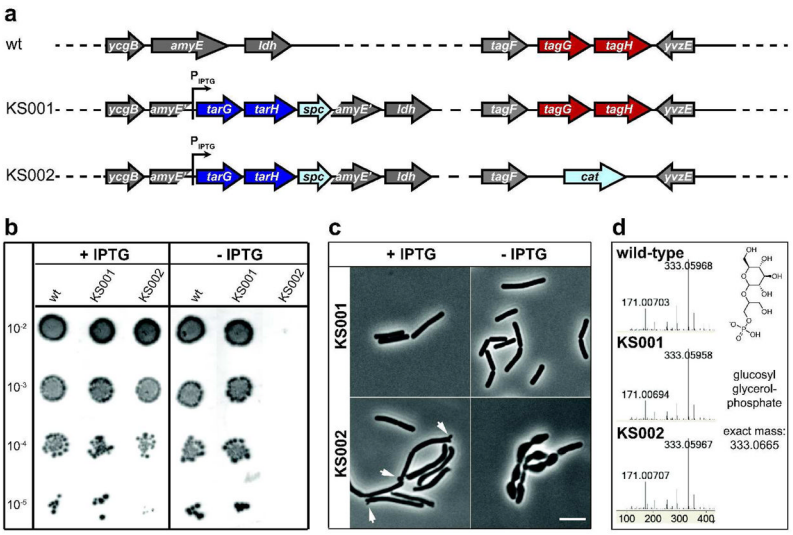


**Figure 3. *B. subtilis* is viable with TarGH$^{Sa}$ as the only WTA exporter**

a) Scheme of the genetic background of wild-type *B. subtilis* PY79 (wt) and strains KS001 and KS002. Indigo arrows indicate *S. aureus* genes, red arrows *B. subtilis* genes, light blue arrows antibiotic resistance genes, and grey arrows the flanking genes. b) Dilution series of bacterial cultures spotted on plates with (left) and without IPTG (right). c) Phase contrast microscopic images of strains KS001 and KS002 grown in LB in the presence and absence of 1mM IPTG as indicated. Scale bar 5 μm. d) Mass spectrometry results for WTA monomers of *B. subtilis* PY79 wild-type, KS001 and KS002.

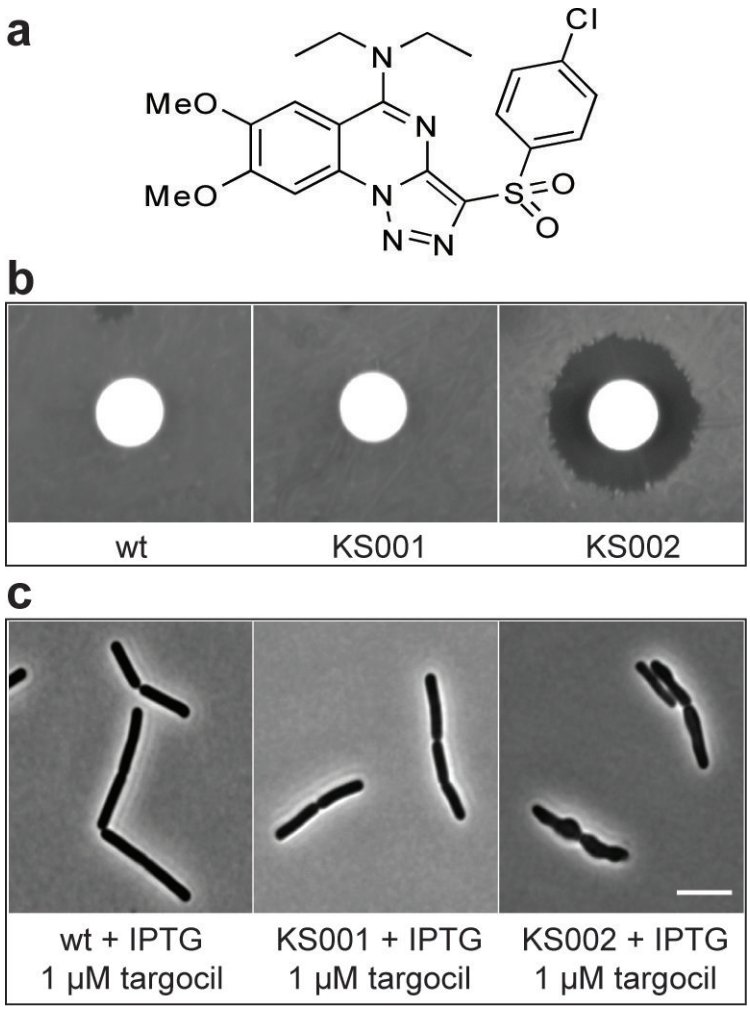


**Figure 4. TarGH$^{Sa}$ as the only WTA exporter renders *B. subtilis* sensitive to the TarG-inhibitor targocil**

a) Structure of the TarG inhibitor targocil. b) Plate diffusion assay showing degree of sensitivity of wild-type (wt), KS001, and KS002 against targocil. c) Phase contrast microscopy of wild-type, KS001 and KS002 grown in LB containing 1 mM IPTG and treated with 1 μM targocil for 2 hours. Scale bar 5 μm.