Biosynthesis of the Unique Wall Teichoic Acid of Staphylococcus aureus Lineage ST395

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ABSTRACT The major clonal lineages of the human pathogen Staphylococcus aureus produce cell wall-anchored anionic polyribitol-phosphate (RboP) wall teichoic acids (WTA) substituted with D-Alanine and N-acetyl-D-glucosamine. The phylogenetically isolated S. aureus ST395 lineage has recently been found to produce a unique poly-glycerol-phosphate (GroP) WTA glycosylated with N-acetyl-D-galactosamine (GalNAc). ST395 clones bear putative WTA biosynthesis genes on a novel genetic element probably acquired from coagulase-negative staphylococci (CoNS). We elucidated the ST395 WTA biosynthesis pathway and identified three novel WTA biosynthetic genes, including those encoding an α-O-GalNAc transferase TagN, a nucleotide sugar epimerase TagV probably required for generation of the activated sugar donor substrate for TagN, and an unusually short GroP WTA polymerase TagF. By using a panel of mutants derived from ST395, the GalNAc residues carried by GroP WTA were found to be required for infection by the ST395-specific bacteriophage Φ187 and to play a crucial role in horizontal gene transfer of S. aureus pathogenicity islands (SaPIs). Notably, ectopic expression of ST395 WTA biosynthesis genes rendered normal S. aureus susceptible to Φ187 and enabled Φ187-mediated SaPI transfer from ST395 to regular S. aureus. We provide evidence that exchange of WTA genes and their combination in variable, mosaic-like gene clusters have shaped the evolution of staphylococci and their capacities to undergo horizontal gene transfer events.

IMPORTANCE The structural highly diverse wall teichoic acids (WTA) are cell wall-anchored glycopolymers produced by most Gram-positive bacteria. While most of the dominant Staphylococcus aureus lineages produce poly-ribitol-phosphate WTA, the recently described ST395 lineage produces a distinct poly-glycerol-phosphate WTA type resembling the WTA backbone of coagulase-negative staphylococci (CoNS). Here, we analyzed the ST395 WTA biosynthesis pathway and found new types of WTA biosynthesis genes along with an evolutionary link between ST395 and CoNS, from which the ST395 WTA genes probably originate. The elucidation of ST395 WTA biosynthesis will help to understand how Gram-positive bacteria produce highly variable WTA types and elucidate functional consequences of WTA variation.

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Here, we identified a unique S. aureus WTA α-O-GalNAc glycosyltransferase TagN in the ST395 lineage and show that α-O-GalNAc-modified WTA is the adsorption receptor for the ST395-specific bacteriophage Φ187. We also describe that a closely related TagN homolog found in Staphylococcus carnosus TM300 (TagN-Se) can replace TagN, revealing an evolutionary connection between ST395 and CoNS. Finally, based on ectopic expression of ST395 WTA biosynthesis genes in classical RboP WTA-producing S. aureus, we identify the three enzymes, TagN, TagV, and TagF, required for GroP-GalNAc WTA biosynthesis in ST395 and provide insights into mechanisms enabling or interfering with Φ187-mediated exchange of pathogenicity islands (SaPIs) between ST395 and classical S. aureus.

RESULTS
The S. aureus ST395 lineage bears unique WTA biosynthesis genes. We have recently sequenced the genome of ST395 isolate PS187 (7) and analyzed the genome for genes potentially involved in WTA biosynthesis. Similar to classical S. aureus strains, the ST395 prototype PS187 encodes the well-studied tagO gene and the tagAHGBXD gene cluster for WTA linkage unit biosynthesis and WTA translocation (Fig. 1B), which is in agreement with the notion that most Gram-positive bacteria have the same WTA linkage unit despite different polymer composition (5, 28). PS187 has been found to bear a novel genetic element with several transposon-related sequences replacing the tar cluster for poly-RboP WTA synthesis and glycosylation found in all sequenced non-ST395 S. aureus isolates (Fig. 1B).

The novel element was named S. aureus GroP WTA island (SaGroWI) because it turned out to be responsible for biosynthesis and glycosylation of GroP WTA (see below). Two of the proteins encoded by SaGroWI were related to previously characterized WTA biosynthetic enzymes over their entire length. One of these (TagF) was 24.06% and 24.1% identical to the GroP WTA polymerases of Bacillus subtilis 168 and of Staphylococcus epidermidis, respectively, but lacked an N-terminal portion of ca. 330 amino acids (see Fig. S1 in the supplemental material). The strongest similarity was found for the equally short product of a B. subtilis W23 gene (66% similarity), which has in vitro Gro polymerase activity but an uncertain role in WTA biosynthesis (29) (see Fig. S1). Nevertheless, the tagF homolog was the only putative GroP polymerase gene in the PS187 genome. SaGroWI encoded also a second homolog of TagD known to generate the CDP-glycerol substrate for the polymerase. However, the SaGroWI TagD was 26 amino acids shorter than that encoded in the GroP WTA polymerases of Bacillus subtilis 168 and of Staphylococcus epidermidis, respectively. SaGroWI TagD was 26 amino acids shorter than that encoded in the GroP WTA polymerases of Bacillus subtilis 168 and of Staphylococcus epidermidis, respectively. SaGroWI TagD was 26 amino acids shorter than that encoded in the GroP WTA polymerases of Bacillus subtilis 168 and of Staphylococcus epidermidis, respectively. SaGroWI TagD was 26 amino acids shorter than that encoded in the GroP WTA polymerases of Bacillus subtilis 168 and of Staphylococcus epidermidis, respectively.

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A third SaGroWI-encoded protein, TagN, has a domain related to the TagB superfamily at its N terminus and a C-terminal (WabH-like) glycosyltransferase domain previously not implicated in WTA biosynthesis. Because PS187 GroP WTA is glycosylated with α-linked GalNAc, it was tempting to speculate that TagN might be the GroP WTA GalNAc transferase. In line with this notion, all ST395 clones seem to contain tagN (7), and tagN homologs were also found in the genomes of GroP WTA-
producing CoNS, such as *S. carnosus* and *Staphylococcus simulans*, and even in *Bacillus cereus* and *Bacillus subtilis* strain BEST 195 with an unusual set of WTA genes (Fig. 2; see also Fig. S3 in the supplemental material). Upstream of *tagN*, SaGroWI contains a potentially cotranscribed gene whose product shared similarity with NAD-dependent nucleotide sugar epimerases (Fig. 1B and 2). The protein was named TagV and was assumed to generate UDP-GalNAc, the putative donor substrate for GroP WTA glycosylation with GalNAc.

*tagV* homologues were found in genomes of the GroP WTA-producing CoNS species *S. epidermidis*, *Staphylococcus pseudintermedius*, and *Staphylococcus lugdunensis* (Fig. 2). Thus, SaGroWI contains candidate genes for all steps required for GroP WTA polymer biosynthesis and its glycosylation.

**TagN is required for GalNAc modification of GroP WTA in *S. aureus* PS187.**

While inactivation of genes for WTA backbone synthesis is usually lethal because of the accumulation of lipid carrier-linked WTA precursors (30), the recently discovered genes for WTA glycosylation are dispensable for bacterial viability (17). In order to assess if TagN is indeed responsible for GroP WTA glycosylation, we set out to inactivate the entire gene in the genome of PS187, but none of our numerous attempts were successful, suggesting that TagN may have an essential role in GroP WTA biosynthesis. However, we easily succeeded with deletion of the C-terminal glycosyltransferase domain of *tagN*. WTA preparations from the resulting mutant GN1 and its parental strain contained similar amounts of phosphate, indicating that WTA amounts were the same in the two strains (Fig. 3A). When the samples were applied to polyacrylamide gel electrophoresis (PAGE), the WTA of GN1 migrated much faster than that of the parental strain (Fig. 3B). This pattern resembled the different electrophoretic mobilities of WTA from *RhoP* WTA-producing *S. aureus* RN4220 and its GlcNAc-deficient mutant (14, 19), suggesting that GN1 may indeed lack the WTA GalNAc residues. When GN1 was complemented with a plasmid-encoded copy of *tagN*, WTA of the resulting strain c-GN1 migrated like that of the wild type (Fig. 3B).

WTA preparations from GN1 were further purified by ion-exchange chromatography and analyzed by 1H nuclear magnetic resonance (1H-NMR) and heteronuclear 13C,1H single quantum correlation (HSQC) spectroscopy. Two characteristic signals of \(^\text{\textalpha}\)-GalNAc (anomeric proton signal of \(^\text{\textalpha}\)-GalN at \(\delta\) 5.1 and NAc at \(\delta\) 2.1) were present in the spectra of the wild type and the *tagN*-complemented mutant c-GN1 but absent in mutant GN1 (Fig. 3C; see also Fig. S4A and Table S1 in the supplemental material), demonstrating that GN1 WTA lacks GalNAc and requires *tagN* for WTA glycosylation.

To analyze if the TagN homologs of CoNS have similar functions as TagN, mutant GN1 was complemented with a *tagN*-like gene from *S. carnosus* (*tagN-Sc*). The resulting strain produced
WTA that exhibited wild-type electrophoretic migration behavior (Fig. 3B) and contained GalNAc, as shown by NMR (Fig. 3C) and HSQC (see Fig. S4B and Table S1 in the supplemental material). Taken together, these data strongly suggest that tagN and its close homolog from S. carnosus encode GroP WTA/\(\alpha\)-O-GalNAc trans-

WTA GalNAc modification is required for phage \(\Phi 187\) infection of S. aureus ST395 and facilitates the HGT of SaPIs. Most phages infecting RboP WTA-producing S. aureus, including transducing phages such as \(\Phi 11\), require GlcNAc-modified WTA for efficient adsorption and infection (17–19). The ST395 lineage-specific \(\Phi 187\) also requires WTA for adsorption and infection (7), but it has remained unknown if \(\Phi 187\) has a preference for WTA with GalNAc residues. Disruption of tagN in GN1 resulted in resistance to \(\Phi 187\) and in drastically reduced adsorption of \(\Phi 187\), while complementation of GN1 with tagN restored both phage

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**FIG 3** Impact of two novel WTA glycosyltransferases, TagN and TagN-Sc, on electrophoretic mobility and composition of PS187 WTA. (A) Cell wall phosphate determination upon tagN disruption in the S. aureus PS187 panel compared to PS187 wild type (w.t.), PS187 \(\Delta\)tagO strain lacking WTA, and its complemented strain (c-\(\Delta\)tagO). Statistically significant differences compared to the wild type calculated by the unpaired two-tailed Student t test are indicated: ns, not significant, \(P > 0.05\); ***, \(P < 0.001\). (B) WTA PAGE analysis of WTA preparations. Samples were resolved in polyacrylamide gels and visualized with alcian blue/silver staining. One representative experiment is shown. (C) \(^1\)H NMR spectrum of purified WTAs. Arrows indicate characteristic signals for GalNAc at \(\delta\) 5.1 (anomeric proton signal) and \(\delta\) 2.1 and for methyl group of NAc at \(\delta\) 2.1. Wild type (w.t.), tagN mutant (GN1), tagN-complemented GN1 mutant (c-GN1), and tagN-Sc-complemented GN1 mutant (c-GN1 [tagN-Sc]) are indicated.
susceptibility and adsorption (Fig. 4A and B; see also Fig. S5A in the supplemental material), indicating that Φ187 is specific for host bacteria with GalNAc-modified WTA. Moreover, complementation of GN1 with the S. carnosus tagN homolog tagN-Sc restored Φ187 susceptibility, thereby confirming that TagN and TagN-Sc have similar functions (Fig. 4A and B). Of note, expression of only the C-terminal TagN glycosyltransferase domain along with an appropriate ribosomal binding site in GN1 did not restore Φ187 susceptibility, indicating that both the tagB-like and the glycosyltransferase domains of TagN are required for WTA glycosylation (Fig. 4B).

The broad-host-range phage ΦK from the myovirus family has previously been shown to use the backbone of RboP-type WTA as a receptor for infection (18, 19). More recently, we have found that this phage is also virulent to strain PS187 (7). As shown in Fig. 4B, ΦK was unable to form plaques on the WTA-deficient ΔtagO mutant but could form plaques on GN1, albeit with reduced infection efficiency (and reduced adsorption; see Fig. S5B in the supplemental material) compared to the parental strain. This finding indicates that ΦK infection depends on the main WTA chain, while GalNAc residues on WTA are not essential but increase the plaquing efficiency (Fig. 4B).

We have recently reported on WTA structure-dependent and Φ187-mediated transfer of mobile genetic elements (MGEs) such as SaPIs from S. aureus ST395 to CoNS and even to certain Listeria monocytogenes serotypes (7), which led to the conclusion that SaPI particles adopt the receptor requirements of cognate helper phages. Interestingly, although GN1 lacks GalNAc modification, SaPI transfer to GN1 via Φ187 occurred albeit at very low efficiencies (Fig. 5). These data suggest that Φ187-derived SaPI particles require GalNAc modification for efficient adsorption, although...
FIG 5  The absence of a suitable Φ187 receptor prevents Φ187-mediated import of SaPIs to classical *S. aureus*. Phage Φ187-mediated transfer of SaPIbov1 or SaPI187β to the *S. aureus* PS187 strain panel, including GN1 lacking WTA GalNAc modification and *S. aureus* RN4220 wild type and RN4220-H. SaPI donor strains were VW1 (SaPIbov1) and VW7 (SaPI187β). Values represent the ratio of transduction units (TRU; transductants/ml phage lysate) to PFU (plaques/ml phage lysate on *S. aureus* PS187 wt.) and are given as means (n = 3 experiments) ± SD. No TRU were observed in controls lacking phages or SaPI particles. PS187 and RN4220 wild type (w.t.), *tagN* mutant (GN1), *tagN*-complemented GN1 mutant (c-GN1 [*tagN*]) and RN4220-H expressing ST395 WTA are indicated.

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characterized GroP WTA polymerases (35, 36) by the lack of a large N-terminal domain whose sequence varies largely between species and whose function is unknown (37). However, we provide evidence that even the short TagF is functional, which is in agreement with recent biochemical and structural data demonstrating that the C-terminal domain of S. epidermidis TagF is responsible for binding of the donor substrate CDP-glycerol and for polymerase activity (37). Our finding is also in agreement with the recently described in vitro GroP WTA polymerase activity of the short B. subtilis W23 TarF (29).

We describe a new type of WTA glycosyltransferase, TagN, which is unrelated to two recently identified WTA glycosyltransferases, TarM and TarS. The C-terminal domain of TagN, related to the GT1 family of glycosyltransferases, was required for GalNAc modification of PS187 WTA. It remains unclear why TagN encloses also an N-terminal domain that is weakly homologous to the TagB protein superfamily. The fact that we could not delete this part of the tagN gene suggests that it may have an additional role in WTA biosynthesis. Since several Staphylococcus and Bacillus genomes contain TagN homologs, the PS187 TagN appears like a prototype of a common GroP WTA biosynthetic module (see Fig. 2). Indeed, one of these homologs from S. carnosus TM300 (TagN-Sc) has an identical function.

The exact origin of WTA genes found on the SaGroWI element and when and how they may have jumped from CoNS to ST395 during evolution remains unknown. The fact that these genes are separated by transposase-related DNA fragments suggests that they have been combined by rather recent recombination events into a mosaic-like structure. WTA genes have been found on other MGEs, such as a copy of a WTA translocase gene on a multiresistance plasmid pSK1 (38), providing further evidence for the mobility of WTA biosynthetic genes. Of note, there is an evolutionary link between ST395 clones and CoNS since all three genes have their closest counterparts in different CoNS and at least some CoNS share the tagV-tagN cluster composition with ST395 clone PS187 (see Fig. 2). Although many CoNS produce GroP WTA and some of them even bear tagV and tagN homologs, neither S. epidermidis, S. lugdunensis, Staphylococcus warneri, nor Staphylococcus hominis has been found to glycosylate their WTA with GalNAc (6, 39). This discrepancy might result from strain-dependent differences in the presence or expression of the tagN homologs. Along this line, environmental conditions in the ecological niche might influence WTA gene expression levels and WTA glycosylation processes. However, while S. epidermidis and S. lugdunensis bear tagV and tagN next to each other but in opposite directions (Fig. 2), only S. pseudintermedius ED99 seems to share the operon-like organization found in PS187 (Fig. 2). Nevertheless, S. simulans has previously been found to glycosylate its GroP WTA with both GlcNAc and GalNAc (6), although tagN is not directly flanked by a tagV homolog in this species. Possibly, other UDP-4-glucose epimerases located at distant genome loci can replace tagV. In most of the CoNS species and even in B. subtilis strain BEST195, tagN and tagV are colocalized with other WTA biosynthesis genes in a cluster, which is a common feature of most WTA biosynthesis genes in Gram-positive bacteria. In contrast, tagN or

**FIG 6** Ectopic expression of ST395 WTA in S. aureus RN4220 leads to production of two types of WTA. (A) Phage susceptibility assay for S. aureus RN4220 strain panel encompassing engineered RN4220 strains expressing ST395 WTA biosynthesis genes tagN, tagV plus tagN, tagF plus tagN, and tagF in combination with tagV and tagN. Ph187, Ph11, Ph00a, and ΦK lysates were spotted onto bacterial lawns and analyzed after overnight incubation for macroplaque formation. One representative experiment is shown. (B) WTA PAGE analysis of WTA preparations of PS187 wild type, RN4220 wild type, and RN4220 hybrid (RN4220-H). Samples were resolved in polyacrylamide gels and visualized with amion blue/silver staining. Arrows indicate WTA alteration in RN4220-H compared to that in its parental strain. One representative experiment is shown. (C) Heteronuclear 13C,1H single quantum correlation (HSQC) spectrum from purified wild-type S. aureus RN4220 and PS187 WTA repeating units. (D) HSQC spectrum from purified S. aureus RN4220-H WTA expressing dual WTA. Arabic numerals label the proton/carbon atoms of the residues. Spectra were recorded at 700 MHz (at 600 MHz for RN4220 w.t.) and 300 K (PS187 w.t. and RN4220 w.t.) or 330 K (RN4220-H) relative to acetone (δH 2.225, δC 31.50). 1H and 13C chemical shifts are indicated in Table S1 in the supplemental material. Arrows indicate strain origin of WTA sample for HSQC spectra.
tagV do not colocalize with other WTA biosynthetic genes in some CoNS (e.g., S. epidermidis), thereby resembling the isolation of the tarM locus in RboP-producing S. aureus (4) and suggesting that the staphylococcal WTA glycosylation genes are more variable than those for WTA backbone biosynthesis.

It remains unclear why ST395 clones produce an unusual WTA type. Early reports have suggested a canine origin of PS187-related strains, pointing to a specific niche of this lineage in dogs (40). The homology and the similar gene organization of tagV and tagN in S. pseudintermedius, which colonizes in particular dogs (41), further substantiates this hypothesis. Nevertheless, recent reports on ST395 from noses or infections of hospital patients in northern Germany and Poland (42, 43) indicate that ST395 clones are at least partly adapted to the human host. Since WTA has been found to be crucial for host-pathogen interaction (3), the discovery of tagN can help to unravel the role of WTA glycosyl residues in host-pathogen interaction and host specificity in the future.

MATERIALS AND METHODS

Bacterial strains and growth media. All bacterial strains listed in Table S2 in the supplemental material were grown in basic medium (BM) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K2HPO4, 0.1% glucose) or Luria-Bertani (LB) broth supplemented with the appropriate antibiotics at a concentration of 10 µg/ml for chloramphenicol, 3 µg/ml for tetracycline, 2.5 µg/ml for erythromycin, or 100 µg/ml for ampicillin.

Molecular genetic methods. For the construction of markerless ΔtagN or ΔtagX mutants in S. aureus PS187 by allelic replacement, the pKO1 shuttle vector was used (44). Primers are listed in Table S3 in the supplemental material. Gene deletion was performed as described previously (44).

For the construction of tagN, tagVN, and tagN-Sc complementation vectors, PS187 wild-type tagN (and the tagVN operon) and S. carnosus TM300 wild-type tagN-Scott genes were amplified via PCR from PS187 wild-type or TM300 wild-type genomic DNA (for primers, see Table S3). The resulting PCR products were purified, digested, and cloned into the Escherichia coli/S. aureus shuttle vector pBR474 (31) at the PstI/BamHI (tagN, tagVN, and tagN-Sc) sites, resulting in plasmids pBR474-tagN, pBR474-tagVN, and pBR474-tagN-Sc. For cloning of the tagN glycosyltransferase domain (for primers, see Table S3), the PCR product was purified, digested, and cloned into pBR474 at the BamHI/EcoRI site, resulting in plasmid pBR474-tagN (GTD). For ectopic expression of ST395 WTA, all required genes, tagF, tagV, and tagN, were amplified from genomic DNA (PS187 wild type) using the corresponding primers P1, P2, P3, and P4 (see Table S3). The resulting PCR products (tagF and tagVN PCR products) were purified and cloned into pBR474 using the infusion cloning technique accordingly to the manufacturer’s instructions (Clontech), resulting in plasmid pRB474-FEPiN. The plasmid was isolated from E. coli TOP10 and used to transform RN4220 resulting in the dual WTA-producing strain RN4220 hybrid (RN4220-H). For the construction of the pBR474-tagFN plasmid, infusion cloning technique was used again (primers P1, P2, P4, and P5). Plasmid pBR474-tagO has been described recently (18).

Experiments with phages and SaPI particles. Phage susceptibility was determined using the soft-agar spot assay described previously (19). A phage panel encompassing serogroup L phage Φ187 (45), two serogroup B phages, Φ11 (45) and Φ80a (46), and the broad-host-range phage ΦK (19) was used. Phages ΦK, Φ11, and Φ80a were propagated on S. aureus RN4220 wild type or on PS187 wild type (Φ187) as described previously (7).

Phage adsorption rates were determined as described previously (18). Briefly, the multiplicity of infection (MOI) was set to 0.005 (for Φ187), 0.0001 (for ΦK), 0.1 (for Φ11), or 0.05 (for Φ80a), and adsorption was calculated by determining PFU of the unbound phage in the supernatant and subtracting it from the total number of input PFU. Adsorption efficiency was indicated relative to the adsorption on the parental wild-type strain PS187 (for Φ187 and ΦK) or RN4220 wild type (for Φ11 and Φ80a), which was set to 100%.

Efficiency of plaquing (EOP) was determined by mixing 100 µl phage suspension (150 PFU, titrated on PS187 wild type) with 100 µl of bacteria (1.8 × 108 CFU) and incubating for 10 min at room temperature. After incubation, 4 ml soft agar was added, mixed well, poured onto BM agar plates supplemented with the appropriate antibiotics, and incubated overnight at 30°C. Single PFUs were counted, and EOP was indicated relative to the EOP of the parental wild-type strain, which was set as 100%.

Phage Φ187-mediated SaPI transfer was studied as described previously (7). Briefly, the recently described S. aureus PS187 SaPIbov1 and SaPI187β SaPI donor strains VW1 and VW7 bearing a resistance marker-labeled SaPIbov1 or SaPI187β (7) (see Table S2 in the supplemental material) were infected with phage Φ187 to generate lysates containing phage Φ187-derived SaPI particles. Subsequently ~8.0 × 107 bacteria were mixed with 100 µl of WTA SaPI lysates SaPIbov1 (~1.7 × 109 PFU/ml) or SaPI187β (~1.1 × 109 PFU/ml), respectively, incubated for 15 min at 37°C, diluted, and plated on BM agar supplemented with the appropriate antibiotics. To exclude spontaneous uptake of nicked DNA, SaPI-containing lysates were also treated with DNase I in controls as described previously (7). Transductants were counted after overnight incubation at 37°C, and transduction efficiency was calculated. SaPI transfer was confirmed by molecular typing of resistance markers.

WTA extraction and purification. WTA was isolated as described previously (7). Briefly, overnight cultures were washed with ammonium acetate buffer (20 mM, pH 4.6) and mechanically disrupted either in a cell disruptor (Euler; for large-scale isolation) or in buffer together with glass beads (Sigma; acid washed, 150 to 212 µm) using a FastPrep-24 MP apparatus (Biomedicals Europe) (50% beads, 50% cell paste, 10 cycles, 6 s/m). The resulting lysates were incubated at 37°C overnight in the presence of DNase and RNase. Subsequently, sodium dodecyl sulfate (SDS) was added to a final concentration of 2% followed by ultrasonication for 15 min using a Branson Sonifier 250 apparatus (pulse duration of 0.9 s, output control of 3). Cell walls were washed several times to remove SDS. A 5% trichloroacetic acid treatment for 4 h at 65°C was used to release WTA from cell walls. Peptidoglycan debris was separated via centrifugation (10 min, 14,000 × g). Determination of inorganic phosphate as described previously (10) was used for WTA quantification. WTA crude extracts were further purified and dialyzed as described before (7).

WTA polyacrylamide gel electrophoresis (WTA PAGE). Dialyzed WTA samples were separated as described previously with minor modifications (19). Briefly, a 26% and 0.75-mm-thick resolving gel was used on which about 100 nmol phosphate was loaded per lane. Samples were electrophoresed at 4°C in a Bio-Rad Protein II Xi electrophoresis cell for 18 h using a current of 15 mA/gel to ensure that even the faster-migrating WTA species of GN1 is resolved from the wild-type WTA. The running buffer contained 0.1 M Tris base and 0.1 M Tricine at a pH of 8.2. The gel was developed using the alcian blue/silver staining method as described previously (47).

Protein sequence alignments. Multiple protein sequence alignments were performed using ClustalW (48) and online resources (49).

General and analytical chemistry methods. The amino sugar composition of isolated WTA components was determined as their amino alditois obtained after hydrolysis of the samples as described previously (7).

NMR spectroscopy. NMR experiments were carried in D2O at 300 K or 330 K in the case of RN4220-H as described previously (7). Briefly all one-dimensional (1H and 13C) and two-dimensional homonuclear (COSY, TOCSY, and ROESY) and heteronuclear (HSQC-DEPT, HMBC, and H2,H3-HMQC-TOCSY) experiments were recorded with a Bruker DRX Avance III 700-MHz spectrometer (operating frequencies of 700.75 MHz for 1H NMR, 176.2 MHz for 13C NMR, and 283.7 MHz for 31P) using standard Bruker software for all samples but RN4220, whose NMR experiments were recorded with a Bruker DRX Avance 600-MHz spectrometer (operating frequencies of 600.03 MHz for 1H NMR, 150.89 MHz for 13C NMR, 125.8 MHz for 31P NMR, 300.6 MHz for 2H NMR, and 30.02 MHz for 31P NMR).
for $^{13}$C NMR, and 242.90 for $^{31}$P using standard Bruker software. COSY, TOCSY, and ROESY were recorded using data sets (t1 by t2) of 4,096 by 512 points for PS187 (12, 16, and 16 scans were acquired for each t1 value in each case, respectively), GN1 (24, 32, and 32 scans were acquired for each t1 value in each case, respectively), c-GN1 (24, 32, and 48 scans were acquired for each t1 value in each case, respectively), c-GN1 (tagN-Sc) (4, 8, and 16 scans were acquired for each t1 value in each case, respectively), and RN4220-H (4, 8, and 8 scans were acquired for each t1 value in each case, respectively), while a data set (t1 by t2) of 2,048 by 512 was recorded for the sample RN4220 (16, 16, and 32 scans were acquired for each t1 value in each case, respectively). The TOCSY experiments were carried out in the phase-sensitive mode with mixing times of 100 ms for all samples. No 1H,31P-HMQC-TOCSY experiment was recorded using a data set of 2,048 by 512 and 32 scans (c-GN1 [tagN-Sc]), and 10 scans (RN4220-H) for each t1 value. The HMBC spectra were acquired using data sets of 4,096 by 512 points for all but RN4220 (set of 2,048 by 512) and 56 (GN1), 40 (PS187 and c-GN1), 32 (RN4220 and c-GN1 [tagN-Sc]), and 10 scans (RN4220-H) for each t1 value. The HMBC spectra were acquired using data sets of 4,096 by 512 points for all but RN4220 (set of 2,048 by 512) and 96 (RN4220), 56 (GN1), 48 (RN4220-H), 40 (PS187 and c-GN1) and 32 scans (c-GN1 [tagN-Sc]) for each t1 value. The 1H,31P-HMQC-TOCSY experiment was recorded using a data set of 2,048 by 512 points (32 scans for each t1 value for c-GN1 [tagN-Sc]), 16 for RN4220, PS187, and GN1, 12 for c-GN1, and 4 for RN4220-H, respectively, using a mixing time of 100 ms for all samples. No 1H,31P-HMQC-TOCSY experiment was recorded for the sample c-GN1 (tagN-Sc). Chemical shifts were reported relative to acetone (∆H 2.225; ∆C 31.50).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/llookup/suppl/doi:10.1128/mBio.00869-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.

Figure S2, PDF file, 0.1 MB.

Figure S3, PDF file, 0.2 MB.

Figure S4, PDF file, 0.1 MB.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

Table S3, DOCX file, 0.1 MB.

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