Observation of the time-course for peptidoglycan lipid intermediate II polymerization by *Staphylococcus aureus* monofunctional transglycosylase

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The polymerization of lipid intermediate II by the transglycosylase activity of penicillin-binding proteins (PBPs) represents an important target for antibacterial action, but limited methods are available for quantitative assay of this reaction, or screening potential inhibitors. A new labelling method for lipid II polymerization products using Sanger’s reagent (fluoro-2,4-dinitrobenzene), followed by gel permeation HPLC analysis, has permitted the observation of intermediate polymerization products for *Staphylococcus aureus* monofunctional transglycosylase MGT. Peak formation is inhibited by 6 μM ramoplanin or enduracidin. Characterization by mass spectrometry indicates the formation of tetrasaccharide and octasaccharide intermediates, but not a hexasaccharide intermediate, suggesting a dimerization of a lipid-linked tetrasaccharide.

Numerical modelling of the time-course data supports a kinetic model involving addition to lipid-linked tetrasaccharide of either lipid II or lipid-linked tetrasaccharide. Observation of free octasaccharide suggests that hydrolysis of the undecaprenyl diphosphate lipid carrier occurs at this stage in peptidoglycan transglycosylation.

INTRODUCTION

The polymerization and cross-linking of peptidoglycan precursor lipid intermediate II (undecaprenyl-PP-MurNAc(3’-lactyl-l-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala)-GlcNAc) to form mature peptidoglycan cell wall, catalysed by transglycosylase and transpeptidase activities of the penicillin-binding proteins (PBPs), has for many years been a highly attractive target for antibacterial drug discovery (Sauvage et al., 2008). Lipid intermediate II is formed enzymically by reaction of UDPMurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala with lipid carrier undecaprenyl phosphate, catalysed by translocase MraY, followed by transfer of GlcNAc to the 4’-OH of muramic acid, catalysed by glycosyltransferase MurG (see Fig. 1) (Bouhss et al., 2008). Transglycosylation is inhibited by the vancomycin group of glycopeptide antibiotics, and by moenomycin, whilst transpeptidation is inhibited by the penicillin class of β-lactam antibiotics (Sauvage et al., 2008). In spite of the great interest in these classes of antibacterial agents, detailed enzymological studies of these enzymes have been limited, due to the practical difficulties of isolating the lipid II substrate, purifying the PBP transmembrane enzymes in active form, and the lack of quantitative assay methods for transglycosylation and transpeptidation.

PBPs are classified into class A bifunctional transglycosylase/transpeptidase PBPs, and class B monofunctional transpeptidases (Sauvage et al., 2008). In addition, a monofunctional transglycosylase enzyme, MGT, which is sequence-related to the class A PBP transglycosylase domains, has been characterized in *Staphylococcus aureus* (Wang et al., 2001; Terrak & Nguyen-Diste`che, 2006; Heaslet et al., 2009; Huang et al., 2012), and a second monofunctional transglycosylase SqtA has also been identified in *S. aureus* (Reed et al., 2011). Crystal structures of the transglycosylase domains of *S. aureus* PBP2 (Lovering et al., 2007) and *Aquifex aeolicus* PBP1a (Yuan et al., 2007) were first published in 2007, followed by structures of *Escherichia coli* PBP1b (Sung et al., 2009) and the *S. aureus* MGT (Heaslet et al., 2009). The transglycosylase fold contains a small ‘jaw’ region close to the membrane bilayer, and a larger α-helical head region. The active site contains catalytic residues Glu-114 and Glu-171, which participate in an acid–base glycosyl transfer mechanism (Lovering et al., 2012). Co-crystallization of S.
**Fig. 1.** Illustration of transglycosylation and transpeptidation reactions, and the labelling strategy used in this work.

S. aureus MGT with lipid II has highlighted several residues involved in lipid II binding, including Arg-103 and Arg-117, which bind the pyrophosphoryl group of the lipid II substrate (Huang et al., 2012). Assay methods for lipid II polymerization were restricted for many years to radiochemical assay of 14C-labelled lipid II, followed by paper chromatography, and counting of the baseline spot corresponding to labelled polymer (Terrak et al., 1999). This method has been refined by lysozyme or muramidase digestion of the polymeric product (Bertsche et al., 2003), giving values of $K_m$ 2.0 μM, $k_{cat}$ 0.008–0.01 s$^{-1}$ for truncated *S. aureus* MGT (Liu & Wong, 2006), and $K_m$ 9.6 μM, $k_{cat}$ 0.4 s$^{-1}$ for full-length *S. aureus* MGT (Huang et al., 2012). A fluorescence-based assay has been reported, using dansyl-labelled lipid II, followed by muramidase digestion (Schwartz et al., 2002). The above methods can be used to measure kinetic constants for transglycosylase enzymes, but they generate mixtures of polymerized products. Gel-based methods for visualization of the polymerization products have also been reported, which show different extents of polymerization by different transglycosylase enzymes (Barrett et al., 2007; Wang et al., 2008; Offant et al., 2010). In order to aid the discovery of novel small molecule transglycosylase inhibitors, there is still a need for a convenient quantitative transglycosylase assay. We have previously published fluorescence labelling methods for lipid-linked intermediates in peptidoglycan assembly, via replacement of D-Ala for D-Cys (Schouten et al., 2006; Vinatier et al., 2009). In this report we describe a new labelling method, yielding a UV-active chromophore, which we have used to study the time-course of the early stages of lipid II polymerization by *S. aureus* MGT, and which could be used as a basis for inhibitor screening.

**METHODS**

**Materials.** Lipid intermediate II was prepared by enzymic conversion of UDPMurNAc-pentapeptide (obtained from BaCWAN Cell Wall Synthesis Facility, University of Warwick) to lipid II using *Micrococcus flauus* membranes, as previously described (Breukink et al., 2003; Lloyd et al., 2008), and was characterized by electrospray mass spectrometry (observed $m/z$ 1874.7, calculated 1874.0 for (M–H)$^-$; see Supporting Information S1, available in the online Supplementary Material).

**Purification of *S. aureus* MGT.** The *S. aureus* MGT gene was overexpressed in *E. coli* BL21 (pRosetta) in a pET46 inducible expression vector, containing a T7 promoter and inserting an N-terminal His$_6$ tag. The genetic construct encoded an N-terminally truncated protein lacking the N-terminal 67 amino acid residues, as previously published (Terrak & Nguyen-Diste`che, 2006). After induction with 1 mM IPTG, harvesting of the cells by centrifugation, and sonication, an intense band at 24 kDa was revealed in the membrane fraction by SDS-PAGE, corresponding to the desired protein (24.3 kDa). Membranes were solubilized in 50 mM sodium phosphate buffer (pH 7.6) containing 1% (w/v) n-dodecyl-$\beta$-maltopyranoside (DDM), 500 mM NaCl, and 20% (w/v) glycerol.
(25 ml), followed by centrifugation at 50 000 g (30 min). Solubilized protein was purified via a Co(talon) affinity column, eluting with 50 mM sodium phosphate buffer (pH 7.6) containing 500 mM NaCl and 20% (w/v) glycerol in the absence of detergent, supplemented with 0, 15 or 500 mM imidazole. MGT eluting in the 500 mM imidazole fraction. Fractions containing MGT were concentrated, and further purified by gel filtration on a Superose 12 column, eluting with 20 mM HEPES buffer (pH 7.6) containing 500 mM NaCl and 10% (w/v) glycerol, which yielded MGT essentially free of protein contaminants (see Supporting Information S2 and S3).

**Labelling with fluoro-2,4-dinitrobenzene (Sanger’s reagent).** Samples of lipid II or MGT reaction products were adjusted to pH 9.0 by addition of 1 M Bicine buffer, and were labelled by addition of 1-fluoro-2,4-dinitrobenzene (5 µl of 25 mM solution in methanol added to 100 µl sample), and incubated overnight at room temperature. Labelled samples were then used directly for HPLC analysis.

**HPLC-based methods for studying S. aureus MGT activity.** Samples of lipid II (5–20 nmol) were applied to a microcentrifuge tube, and chloroform/methanol removed using a stream of nitrogen gas. The dried lipid was then solubilized in 20 mM Tris buffer (pH 7.5) containing 0.25% (w/v) DDM, 150 mM NaCl and 0.2 mg purified MGT in a total volume of 200 µl. Enzyme assays were incubated at 20 °C for 5–60 min, and protein removed by passage through a 10 kDa Amicon Ultra 0.5 ml centrifugal filter (Millipore), prior to labelling with Sanger’s reagent. Aliquots (15 µl) of labelled samples were diluted with water/methanol (1 : 1, 100 µl) and injected onto a BioSep3000 (Phenomenex) size exclusion column. The absorbance of the eluate was monitored at 400 nm.

Method A (solvent-based method) used an isocratic gradient of water/methanol (1 : 1), eluting at 0.3 ml min⁻¹, for 70 min. In method B (detergent-based method), the size exclusion column was eluted with 10 mM HEPES buffer (pH 7.6) containing 150 mM NaCl and 0.06% (w/v) Triton X-100 detergent, at 0.3 ml min⁻¹, for 60 min. Control assays in which (a) MGT was pre-treated with EDTA (5 mM), (b) MGT was boiled for 10 min prior to assay, and (c) enzyme products were treated with lysozyme (0.2 mg ml⁻¹; Sigma-Aldrich) each resulted in >70% reduction in intensity of the observed product peak. Enzyme inhibition assays contained ramoplanin (gift of BioSearch Italia) or enduracidin (Sigma-Aldrich) at 6 µM final concentration.

**Numerical modelling methods.** Three kinetic models were devised for the transglycosylase-catalysed reaction, shown in Fig. 2, scheme 1. Rate constants were assigned to each step, and rate equations were composed using ordinary differential equations in each case (see Supporting Information S4–S6). Each kinetic model was expressed in the Matlab SimBiology package, using the graphical interface package. Initial conditions were entered with known experimental conditions, and initial estimates for each rate constant k₁–k₅ were entered. The following assumptions were made, in order to minimize the number of variables for numerical modelling (more complex models were found not to be convergent): (1) binding of each equivalent of substrate occurs with the same association/dissociation rate constants, (2) each glycosyltransfer reaction occurs with similar rate constant, (3) each glycosyltransfer reaction is irreversible. Parameter sensitivity was tested for rate constants k₁–k₅, and output concentration for each species was found to be more sensitive to rate constants k₁, k₃ and k₅. Fitting of models to experimental data used nonlinear regression to minimize the weighted sum of squares of the data residuals, iteratively modifying the rate constants k₁–k₅ in each cycle, to minimize the weighted sum of squares (see Supporting Information S7). Parameter estimates from data fitting of each model are shown in Table 1.

**RESULTS**

**Labelling of lipid II with UV-active chromophore**

Lipid intermediate II was prepared by enzymic conversion of UDPMurNAc-pentapeptide to lipid II by M. flavus membranes (Breukink et al., 2003; Lloyd et al., 2008). Purified lipid II was characterized by negative ion electrospray mass spectrometry, showing the expected [M—H]− peak at m/z 1874.7 (calculated 1874, see Supporting Information S1). Analysis of lipid II by HPLC is problematic, since lipid II contains no UV–vis chromophore; however, it was found that treatment of lipid II with 2 equivalents of Sanger’s reagent (fluoro-2,4-dinitrobenzene) in 10 mM sodium bicarbonate buffer at pH 8.0–9.0 gave a sharp peak by reverse phase HPLC. The labelled lipid II was characterized by negative ion electrospray mass spectrometry, giving m/z 2040.6 (calculated [M—H]− 2040.1). Since this labelling method is selective for amine nucleophiles, Sanger’s reagent was examined as a method for observation of the products of lipid II polymerization, as illustrated in Fig. 1.

**Polymerization of lipid II with S. aureus monofunctional transglycosylase, MGT**

*S. aureus* MGT was expressed as an N-His fusion protein, truncated by removal of amino acid residues 1–67 encoding a transmembrane α-helix, as previously published (Terrak & Nguyen-Diste`che, 2006). Analysis of cell extract by SDS-PAGE indicated that the expressed protein was insoluble, but the protein was successfully solubilized using 50 mM sodium phosphate buffer (pH 7.6) containing 500 mM NaCl, 20% (w/v) glycerol, and 1% (w/v) DDM. Active enzyme was purified by co-affinity chromatography, followed by Superose 12 gel filtration chromatography. Analysis by SDS-PAGE showed a 24 kDa band essentially free of protein contaminants (see Supporting Information S2 and S3).

Lipid II (7.5 nmol) was treated with purified *S. aureus* MGT (50 µM), and the products were derivatized using Sanger’s reagent. After precipitation of protein, the derivatized products were analysed by size exclusion chromatography, using a Phenomenex Biosep S3000 column. Two elution methods were used. Method A, involving isocratic elution with water/methanol (1 : 1), resulted in a new high molecular mass envelope at retention time 18–21 min, not found in enzyme-only or substrate-only controls (see Fig. 3a), followed by two peaks which were also found in substrate-only controls. A time-course experiment using this method, shown in Fig. 3b, revealed that the envelope of products could be resolved into three species: peak 1 at 18.0 min with a high molecular mass was formed predominantly at longer reaction times (60 min), whereas peaks 2 (18.4 min) and 3 (18.8 min) were formed at shorter reaction times, with peak 3 formed after 5 min, and mixtures of peaks 2 and 3 formed at 10, 20 and 30 min incubation time (see Fig. 3). Incubation of MGT with 5 mM EDTA prior to assay was found to decrease the
formation of peaks 1–3 by 70–80 %, presumably due to chelation of the Mg$^{2+}$ cofactor required for transglycosylation. Attempts to observe polymerization products using purified *Staphylococcus pneumoniae* PBP1A, PBP1B, or *S. aureus* PBP2 gave no observable peaks via this method (data not shown), using a range of substrate and enzyme concentrations, suggesting that they are more highly processive enzymes for lipid II polymerization; therefore, we focused on characterization of the *S. aureus* MGT reaction.

Peaks from method A were collected and analysed by mass spectrometry, and data were obtained by MALDI mass spectrometry at $m/z$ 5408±2 and 4453±2 (for peaks 1 and 2), and $m/z$ 2204±1 for peak 3. The peak at $m/z$ 5408 matches the calculated MNa adduct ($m/z$ 5407) for undecaprenyl-PP-(MurNAc-GlcNAc)$_4$, suggesting that this species is a lipid-linked octasaccharide. The species at $m/z$ 4453 does not match undecaprenyl-PP-(MurNAc-GlcNAc)$_3$ (calculated MH$^+$ 4270, MNa$^+$ 4292), but may correspond to free octasaccharide (MurNAc-GlcNAc)$_4$ in which a fragmentation of −43 (removal of N-acetyl group) has occurred (calculated MNa−COCH$_3$ 4455), a fragmentation which is preceded by GlcNAc sugars (Lee et al., 2005). Similarly, the species at $m/z$ 2205 does not match undecaprenyl-PP-(MurNAc-GlcNAc)$_2$ (calculated MH$^+$ 3155, MNa$^+$ 3177), but may correspond to free tetrasaccharide (MurNAc-GlcNAc)$_2$ with the same fragmentation (calculated M−COCH$_3$ 2204).

Observation of polymerization products by method A was found to be condition-dependent: although consistent results were obtained for 2–3 months; upon replacement of the HPLC column, the same peaks were not observed, which we hypothesize is due to the limited solubility of the polymerization products in the buffer. Therefore, method B was developed, in which 10 mM HEPES buffer (pH 7.6) was used, containing 150 mM NaCl and 0.06 % (w/v) Triton X-100 detergent. Using this method, a high molecular mass product envelope was observed at retention time 11–13 min, whose size was dependent upon the amount of lipid II and MGT (see Fig. 4). Samples from an incubation of MGT with 7.5 nmol lipid II were removed at 5, 30, 60 and 90 min, and analysed by method B. As shown in Fig. 5, both the size and the shape of the product envelope changed, with a shift to shorter retention time at longer incubation times. The 90 min sample was, in addition, treated with lysozyme, resulting in a disappearance of the product envelope.

The observed shift in retention time of the product envelope suggested a shift to higher molecular mass polymerization products. In order to analyse the observed

**Table 1.** Parameter estimates from data fitting using kinetic models 1, 2 and 3

<table>
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<th>Model</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_3$</th>
<th>$k_4$</th>
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<td>0.30</td>
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<tr>
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<td>0.001</td>
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<td>Model 3</td>
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<td>0.002</td>
<td>17.3</td>
<td>0.23</td>
<td>9.2</td>
</tr>
</tbody>
</table>

**Fig. 2.** Models 1, 2, and 3 for lipid II polymerization.
behaviour in more detail, the peak data were segmented into 10 × 0.3 min retention time segments, and the area under the curve was calculated for each segment. Analysis of segment area vs incubation time shows that (1) the mean molecular mass increases vs incubation time; (see Fig. 5a); (2) the size of each higher Mₚ segment increases vs time, with the highest Mₚ segments showing nonlinear behaviour (see Fig. 5b); (3) the size of the lower Mₚ segments reaches a steady-state value after 5–10 min (see Fig. 5c), suggesting that these species represent one or more polymerization intermediates. Pre-incubation of MGT with 5 mM EDTA prior to assay was found to decrease the formation of the product envelope by 70–80 %, with a shift to longer retention time (Supporting Information S4).

Inhibition of MGT by transglycosylase inhibitors

Incubations of MGT and lipid II (7.5 nmol) were repeated in the presence of 6 μM concentrations of ramoplanin (Hu et al., 2003) and enduracidin (Fang et al., 2006), both proposed to form complexes with lipid I or lipid II. Treatment with 6 μM ramoplanin was found to prevent high and low molecular mass product formation by >95 % after 60 min (see Fig. 6). Treatment with 6 μM enduracidin reduced product formation after 5 min by approximately 50 %; after 60 min the formation of low molecular mass product was similar to the control lacking inhibitor, whereas the formation of high molecular mass product was reduced by approximately 50 %.

Numerical modelling of three kinetic models for lipid II polymerization

The apparent absence of a hexasaccharide species in the products observed in HPLC method A raised the possibility that lipid II polymerization might occur via reaction of two lipid-linked tetrasaccharide intermediates, rather than via stepwise addition of three lipid II equivalents. In order to examine this hypothesis further, three possible kinetic models for lipid II polymerization to an octasaccharide product (shown in Fig. 2, scheme 1) were simulated via numerical modelling, and fitted to the time-course data obtained from the method B HPLC data. Model 1 involves consecutive addition of lipid II equivalents, via lipid-linked tetrasaccharide and hexasaccharide intermediates, to give lipid-linked octasaccharide product; model 2 involves reaction of lipid-linked tetrasaccharide either with two consecutive lipid II equivalents or with lipid-linked tetrasaccharide to form lipid-linked octasaccharide; and model 3 involves an obligate reaction of two lipid-linked tetrasaccharides to form lipid-linked octasaccharide.

Each model was described by differential equations governed by explicit reactions, which were based on the mass-action law (see Supporting Information S5–S7), and expressed in the Matlab SimBiology package. Initial conditions were entered with known experimental concentrations, and initial estimates were entered for the five unique rate constants: k₁ (association constant for MGT + substrate), k₂ (dissociation constant for MGT–substrate...
complex), $k_3$ (association constant of MGT–substrate with second substrate), $k_4$ (dissociation constant of MGT ternary complex), $k_5$ (turnover of MGT ternary complex via glycosyltransfer). The following assumptions were made, in order to minimize the number of variables for numerical modelling (more complex models were found not to be convergent): (1) binding of each equivalent of substrate occurs with the same association/dissociation rate constants; (2) each glycosyltransfer reaction occurs with similar rate constant; (3) each glycosyltransfer reaction is irreversible.

**Fig. 5.** Segment analysis of product envelope data from Fig. 4, showing time-course of MGT-catalysed polymerization. (a) All segments, from high $M_r$ (left, segment 1) to low $M_r$ (right, segment 10) for each incubation time. (b) Each higher $M_r$ segment area plotted vs enzyme incubation time. (c) Each lower $M_r$ segment area plotted vs enzyme incubation time. AUC, area under curve.
Each model was used to simulate the observed experimental data for the high molecular mass species in HPLC method B, and nonlinear regression was used to minimize a weighted sum of squares of the data residuals. This fit generated revised estimates of $k_1-k_5$, and the new parameters were recycled iteratively, in order to improve the fit to the experimental data. Each model gave satisfactory least-squares fits to the experimental data (see Supporting Information S8–S10), although models 2 and 3 gave better fits to the initial sigmoidal phase of the reaction. The three models gave very different estimates for the glycosyltransfer rate constant $k_5$: 0.18 s$^{-1}$ (model 1); 0.005 s$^{-1}$ (model 2) and 9.2 s$^{-1}$ (model 3). Assuming that three glycosyltransfer turnovers are needed to produce one molecule of octasaccharide product, then the experimental data would predict a value of 0.001 s$^{-1}$ for $k_5$, and literature $k_{cat}$ values for MGT are in the range 0.008–0.01 s$^{-1}$ (Terrak & Nguyen-Diste`che, 2006; Huang et al., 2012). Therefore, model 2 provides the better fit to the experimental data.

**CONCLUSIONS**

The labelling method described in this paper provides a useful method for the direct, quantitative observation of lipid II polymerization using *S. aureus* MGT. These intermediate polymerization product peaks were only observed for *S. aureus* MGT, and not for *S. pneumoniae* PBPA1 or PB/PB2 or *S. aureus* PBP2. Due to the very low $k_{cat}$ value of *S. aureus* MGT (Terrak & Nguyen-Diste`che, 2006), in these experiments we are only observing a few turnovers of the MGT reaction, which might explain why these intermediates are observed only for this enzyme. It is known that MGT is structurally related to other published transglycosylases (Huang et al., 2012); therefore it seems likely that other transglycosylases follow a similar mechanism in the early stages of their polymerization reactions. We have demonstrated that this method can act as a read-out for transglycosylation inhibition by ramoplanin or enduracidin; therefore, this assay could in principle be used as a screening method for transglycosylase inhibitors, which remain an attractive antibacterial target (Bugg et al., 2011).

The identification of observed peaks by mass spectrometry as undecaprenyl-PP-(MurNAc-GlcNAc)$_4$, octasaccharide (MurNAc-GlcNAc)$_4$ and tetrasaccharide (MurNAc-GlcNAc)$_2$ raised the possibility that the lipid-linked hexasaccharide undecaprenyl-PP-(MurNAc-GlcNAc)$_3$ might not be an intermediate in transglycosylation for MGT, since no hexasaccharide species was observed. In order to examine this hypothesis further, kinetic models were constructed for the sequential addition of lipid II (model 1), reaction of lipid-linked tetrasaccharide either with lipid II or with a second lipid-linked tetrasaccharide (model 2), or obligate condensation of two molecules of lipid-linked tetrasaccharide (model 3), and these models were fitted numerically to the observed HPLC data. Although each model gave a satisfactory qualitative fit to the data, models 2 and 3 gave better fits to the sigmoidal early phase of the reaction, which is a noticeable feature of the observed data, and model 2 gave the better parameter estimation for the glycosyltransfer rate constant. Thus, numerical modelling supports model 2, involving reaction of lipid-linked tetrasaccharide with either lipid II or lipid-linked tetrasaccharide. Zhang et al. (2007) reported, using gel assays, that a synthetic lipid-linked tetrasaccharide (termed lipid IV) can be dimerized by *E. coli* PBPA1, but not by *E. coli* PBP1B. The ability of different bifunctional PBPs to
add either lipid II or a lipid-linked tetrasaccharide is consistent with model 2. Sigmoidal kinetic behaviour has also been observed for the polymerization of lipid II by E. coli PBP1A (Barrett et al., 2007) and E. coli PBP1B (Huang et al., 2012; Chen et al., 2003) enzymes.

The observation that free octasaccharide (MurNAc-GlcNAc)₄ is an observable product of the S. aureus MGT reaction is also of interest. It suggests that loss of the undecaprenyl diphosphate lipid tether, which must occur at some point in the polymerization process, may occur at the octasaccharide stage, and that free octasaccharide is then used as a substrate for further polymerization. Hydrolysis of the undecaprenyl diphosphate lipid anchor could occur via base-catalysed attack of water on the glycosyl phosphate centre, in place of transglycosylation.

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REFERENCES


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