

# The membrane anchor of penicillin-binding protein PBP2a from *Streptococcus pneumoniae* influences peptidoglycan chain length

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## Keywords

cell wall; glycosyltransferase;  
pneumococcus; transglycosylase;  
transpeptidase

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(Received 14 September 2011, revised 6 December 2011, accepted 30 March 2012)

doi:10.1111/j.1742-4658.2012.08592.x

The pneumococcus is an important Gram-positive pathogen, which shows increasing resistance to antibiotics, including  $\beta$ -lactams that target peptidoglycan assembly. Understanding cell-wall synthesis, at the molecular and cellular level, is essential for the prospect of combating drug resistance. As a first step towards reconstituting pneumococcal cell-wall assembly *in vitro*, we present the characterization of the glycosyltransferase activity of penicillin-binding protein (PBP)2a from *Streptococcus pneumoniae*. Recombinant full-length membrane-anchored PBP2a was purified by ion-exchange chromatography. The glycosyltransferase activity of this enzyme was found to differ from that of a truncated periplasmic form. The full-length protein with its cytoplasmic and transmembrane segment synthesizes longer glycan chains than the shorter form. The transpeptidase active site was functional, as shown by its reactivity towards bacillitracin and the catalysis of the hydrolysis of a thiol-ester substrate analogue. However, PBP2a did not cross-link the peptide stems of glycan chains *in vitro*. The absence of transpeptidase activity indicates that an essential component is missing from the *in vitro* system.

## Structured digital abstract

- [PBP2a](#) and [PBP2a bind](#) by [cross-linking study](#) ([View interaction](#))

## Introduction

Penicillin-binding proteins (PBPs) are enzymes that assemble the peptidoglycan on the external face of the plasma membrane of bacteria [1]. The inhibition of PBPs by  $\beta$ -lactams (such as penicillin) leads to growth arrest or cell death. Nowadays, strains of several important pathogens such as *Staphylococcus aureus*, *Enterococcus faecium*, *Neisseria meningitidis* or *Streptococcus*

*pneumoniae* resist  $\beta$ -lactams by expressing PBPs that react poorly with the drugs [2]. These 'low-affinity' PBPs remain physiologically efficient in building the cell wall, despite the fact that  $\beta$ -lactams, for which they have lost their reactivity, are structural mimics of the natural substrate. To solve this paradox, we need now to study the reactions of peptidoglycan assembly.

## Abbreviations

CMC, critical micellar concentration; DDM, *n*-dodecyl- $\beta$ -D-maltopyranoside; EGS, ethylene glycol *bis*(succinic acid N-hydroxysuccinimide ester); GT, glycosyltransferase or transglycosylase; MurNAc, N-acetylmuramic acid; PBP, penicillin-binding protein; TP, transpeptidase;  $\beta$ -OG, *n*-octyl- $\beta$ -D-glucopyranoside.

It is our long-term objective to reconstitute *in vitro* the formation of the cell wall of the pneumococcus. As an important step towards this aim, we report here a characterization of full-length PBP2a from *S. pneumoniae*, following a previous study of a truncated form [3,4].

Peptidoglycan is the major component of the bacterial cell wall, which maintains cell shape, resists intracellular pressure and acts as a platform for anchored macromolecules such as proteins and polysaccharides [5–7]. This macromolecular network is made of glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid (MurNAc) that are cross-linked by short peptides attached to the MurNAc [7]. The peptidoglycan is polymerized from a precursor (lipid II) that is synthesized in the cytoplasm and flipped to the outer face of the plasma membrane [8,9]. PBPs catalyze the polymerization of glycan chains (transglycosylation) and the peptidic cross-linking of glycans strands (transpeptidation).

PBPs have been divided into three classes [1]. Class A PBPs are bifunctional with both glycosyltransferase (GT) and transpeptidase (TP) activity. Class B PBPs have only one identified catalytic domain with potential TP activity. Class C PBPs have hydrolytic activities and participate in peptidoglycan maturation and cell separation. The domain with hydrolytic or TP activity is the target of  $\beta$ -lactams. The enzymatic GT activity of the class A PBPs constitutes a promising alternative target. Moenomycin is the principal antibiotic directed against GT activity. However, because of poor pharmacokinetic properties and oral bioavailability, moenomycin is not used in human clinical therapy [10].

In recent years, important advances have been made in both structural and functional studies of class A PBPs. The crystal structure of several PBP GTases are now available: the extracellular domain of PBP2 from *Staphylococcus aureus* [11,12], the GT module of PBP1a from *Aquifex aeolicus* [13,14] and the full-length PBP1b from *Escherichia coli* [15]. In parallel, important advances have been made in the synthesis of lipid II [16–20], enabling characterization of the GT activity of a number of proteins. To date, more than 10 bacterial GT proteins have been purified and characterized including the periplasmic form of PBP2a and PBP1a from *S. pneumoniae* [1,21]. In particular, the direction of glycan chain elongation has been elucidated, involving successive attacks of the growing chain (donor) at the reducing end of the lipid II (acceptor) [22,23]. Also, different enzymes produce chains with different length distribution [24]. Screening methods for the identification of GTase inhibitors have been proposed [25–28].

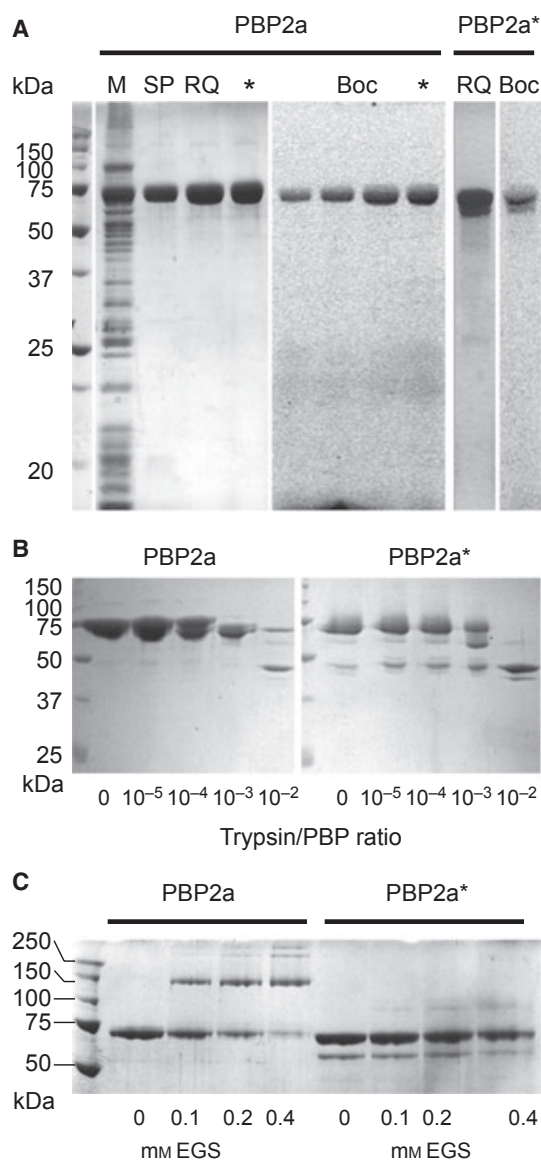
In this work with pneumococcal PBP2a, we found that the transmembrane segment influences the length of the polymerized glycan chains. We could not detect TP activity, despite the fact that the purified protein reacted with a  $\beta$ -lactam, indicating that the TP active site was functional.

## Results and Discussion

### Purification of full-length and truncated periplasmic PBP2a

First, full-length PBP2a was expressed in *E. coli* with various N-terminal affinity tags (poly-histidine, calmodulin-binding peptide or Strep-tag). Isolated membranes were solubilized in Triton X-100 and the tagged proteins were purified on the corresponding affinity resins. None of the tagged PBP2a displayed GT activity (data not shown).

We hypothesized that the tags or the linker (formed mostly by a TEV protease cleavage site) could interfere with the activity. We therefore attempted to purify untagged full-length PBP2a by ion-exchange chromatography. We applied successively cation- and anion-exchange chromatography, and to our surprise, PBP2a bound to both types of resin (SP-Sepharose and Resource Q) at nearly the same pH, pH 8 for the cation-exchange and pH 7.5 for the anion-exchange chromatography. In both cases, PBP2a was eluted with  $\sim 300$  mM of NaCl. This unexpected behavior allowed purification to near homogeneity in two steps (Fig. 1A). The yield was  $\sim 0.5$  mg protein·L culture<sup>-1</sup>. The overall predicted isoelectric point (*pI*) of PBP2a is 6.0, with a net negative charge of -8 at pH 7.5–8. The retention of PBP2a on a strong cation-exchange medium is thus unexpected. However, a closer examination of the sequence reveals that the cytoplasmic region (residues 1–56) has a *pI* of 10.3 and a net positive charge of +7 at pH 8, whereas the extracellular region (residues 78–731) has a *pI* of 5.3 and a net negative charge of -16 at pH 7.5. This charge distribution is expected from the ‘positive inside’ rule that defines the transmembrane orientation of membrane proteins [29]. We suggest that the two parts of the solubilized proteins are isolated from each other by the detergent micelle around the transmembrane segment, and that this particular arrangement may allow binding to the cation-exchanger by the cytoplasmic part, and to the anion-exchanger by the periplasmic domains. It would be of interest to test whether this behavior is general for bitopic membrane proteins that have a similar charge distribution.



**Fig. 1.** Characterization of PBP2a and PBP2a\*. (A) Coomassie Brilliant Blue- and bocillin-stained SDS/PAGE analysis of recombinant PBP2a and PBP2a\*, following SP-Sepharose (SP) or Resource Q (RQ) chromatography. M is for solubilized *E. coli* membranes prior to chromatography. Boc is for bocillin. The Boc lanes were imaged under UV illumination from the same gels prior to Coomassie Brilliant Blue staining. The asterisk signals that 25% dimethylsulfoxide was present during the reaction with bocillin. (B) Partial tryptic proteolysis of PBP2a and PBP2a\* analyzed by Coomassie Brilliant Blue-stained SDS/PAGE. (C) Cross-linking *in vitro* of PBP2a and PBP2a\* with varying amounts of EGS, analyzed by Coomassie Brilliant Blue-stained SDS/PAGE.

The periplasmic region of *S. pneumoniae* PBP2a (PBP2a\*) was prepared as described previously [4], with an additional anion-exchange chromatography following cleavage of the glutathione *S*-transferase tag

(Fig. 1A). This last step eliminated minor contaminants and allowed to exchange the detergent from Chaps to Triton X-100. The yield was  $\sim 2$  mg protein  $\cdot$  L culture $^{-1}$ .

N-Terminal sequencing yielded the expected sequence for both PBP2a and PBP2a\*, and electrospray MS confirmed the integrity of both proteins (PBP2a:  $80\,797 \pm 2$  Da measured vs  $80\,799$  expected; PBP2a\*:  $73\,810 \pm 2$  measured vs  $73\,812$  expected). The TP domains of the full-length and truncated variants were functional for the reaction with the fluorescent penicillin bocillin (Fig. 1A).

Limited proteolysis of PBP2a and PBP2a\* with trypsin produced a common major protein fragment with an apparent molecular mass of 44 kDa (Fig. 1B). N-Terminal sequencing showed that this fragment starts at residue 264 and thus comprises the TP domain. This main trypsin-resistant domain had been identified previously in PBP2a\* [4]. Mass spectrometry of this fragment had revealed that it consisted of residues 264–678 [4]. The shorter minor tryptic fragment, which also appears with both PBP2a and PBP2a\*, had been found in the previous study of PBP2a\* to encompass residues 301–678 [4]. The GT domain in full-length PBP2a is therefore susceptible to tryptic digestion, as in the truncated form. However, greater amounts of trypsin seem to be required to digest full-length PBP2a, indicating that the presence of the transmembrane and cytoplasmic regions may stabilize the GT domain to some extent, at least in the presence of detergent.

The oligomeric state of PBP2a was probed by chemical cross-linking followed by SDS/PAGE. Following incubation with the cross-linking agent [ethylene glycol *bis*(succinic acid) *N*-hydroxysuccinimide ester; EGS], additional bands were observed in the case of PBP2a, but not with PBP2a\* (Fig. 1C). The apparent mass of the main species is consistent with the presence of a dimer ( $\sim 150$  kDa). The presence of species with higher masses hints at the presence of higher orders oligomers or aggregates. The absence of cross-linking of PBP2a\* by EGS (Fig. 1C) suggests that the self-association of PBP2a may depend on its transmembrane and/or cytoplasmic regions.

Size-exclusion chromatography in the presence of 0.02% Triton X-100 did not show the presence of aggregates with either PBP2a or PBP2a\* (not shown). The presence of Triton X-100, which absorbs at 280 nm, precluded the monitoring by UV absorbance; instead, elution fractions were analyzed by SDS/PAGE. Both the full-length and truncated forms were eluted with comparable volumes of solution, corresponding to an apparent molecular mass of globular

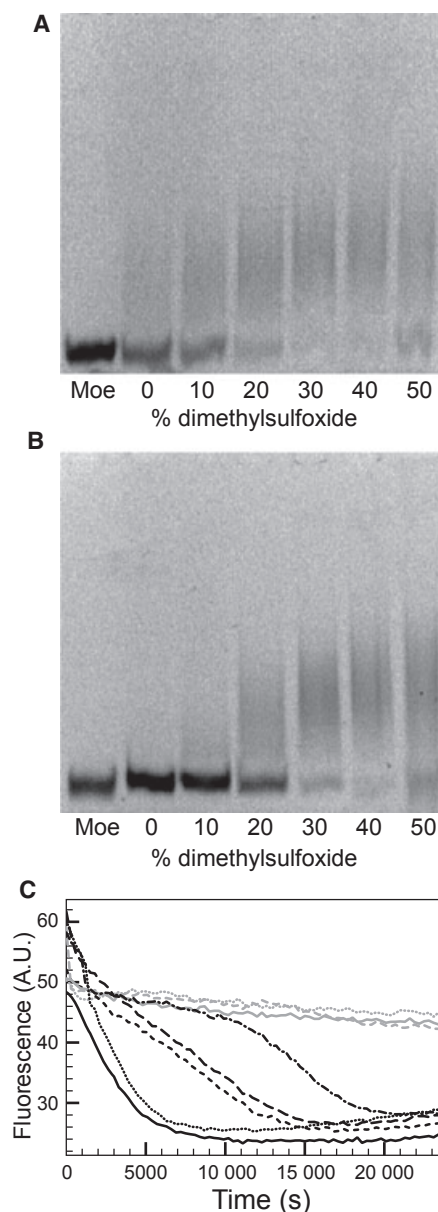
proteins comprised between 500 and 300 kDa. It is unclear why both proteins behave similarly in size-exclusion chromatography and differently in the cross-linking reaction. The presence of large micelles of detergent may mask size differences of the proteins.

The dimerization of PBP1b from *E. coli* has been observed by SPR with a dissociation constant  $K_D$  of  $\sim 100$  nm [30]. The dimerization was found to stimulate the GT activity of *E. coli* PBP1b and to be necessary for its TP activity [30]. The oligomerization of class A PBPs has also been observed *in vivo* [31,32].

### Optimization of GT reaction conditions

Reaction conditions are critical for PBP GT *in vitro* activity, for example, the nature of the divalent cation or the detergent and dimethylsulfoxide concentration [28,33,34]. Full-length PBP2a was incubated with labeled fluorescent lipid II in the presence of different concentrations of dimethylsulfoxide and Triton X-100 detergent. The detergent was either added to a concentration about fourfold its critical micellar concentration (CMC) in water, or only brought to the reaction mix with the purified protein, resulting in a concentration about fivefold below the CMC. The reaction products were analyzed by Tris/Tricine SDS/PAGE [35] (Fig. 2A,B). The lipid II migrates near the front and appears as dark thick bands at the bottom of the gel. The polymerized glycan chains appear as a smear above the lipid II band (see below for a more complete description of the assay). In this small 5-cm-long gel system, discrete chain lengths were not resolved. The reaction was optimal with 30–40% dimethylsulfoxide. The reaction started to be less efficient with 50% dimethylsulfoxide, as seen by the amount of unprocessed lipid II remaining after 40 min of reaction. The inhibitory effect of dimethylsulfoxide at high concentration may be due to denaturation of the enzyme.

Interestingly, at a high concentration of Triton X-100, dimethylsulfoxide was required to observe an activity of PBP2a, whereas at a low detergent concentration, the reaction could occur to some extent in the absence of dimethylsulfoxide. Thus, dimethylsulfoxide appears to rescue the GT activity in the presence of detergent concentrations that are normally inhibitory, as observed previously with PBP1a from *Thermotoga maritima* and PBP1b from *E. coli* in decyl-poly (ethylene glycol) [28,34]. This effect of dimethylsulfoxide has been attributed to a greater solubility of the lipidic substrate and to faster exchange between detergent micelles [34]. Note that the *in vitro* activity of the N-acetylglucosaminyl transferase MurG to convert



**Fig. 2.** Effect of dimethylsulfoxide and detergent on the GT activity of PBP2a. (A,B) Effect of the concentration of dimethylsulfoxide on the GT reaction in the presence of 0.002 and 0.04% Triton X-100, respectively. Reactions using dansylated lipid II at 30 °C were stopped after 40 min, analyzed by Tris–Tricine SDS/PAGE and imaged under UV light. The lane noted with Moe was with 0.5 mM moenomycin and no dimethylsulfoxide. (C) Comparison of the effect of different detergents on the reaction followed by the decrease of fluorescence, using dansylated lipid II as substrate. See Offant *et al.* and Schwartz *et al.* for a description of the assay [28,34]. All reactions contained 0.02% Triton X-100. Added detergents were: none (black solid line), 1% Chaps (black dotted line), 0.02% DDM (black short dashes), 0.02% Triton X-100 (black long dashes), 0.25% Cymal-5 (black dot-and-dashed line), 0.08% Cymal-4 (gray solid line) and 1%  $\beta$ -OG (gray dashes). The gray dotted line is without enzyme. Cymal-3 was omitted for clarity.

lipid I into lipid II, the activity was highest with 35% dimethylsulfoxide in the presence of 0.1% deoxycholate [36].

To assess the effect of the nature of the detergent on the GT activity, we used a continuous fluorescence assay in a 96-well format [28,34]. In this assay, a modified lipid II is used, which has a fluorescent dansyl probe attached to the stem peptide. When glycan chains are polymerized, the probe is incorporated in the growing chains. However, a muramidase is present in the reaction mix that hydrolyzes the chains into disaccharide pentapeptides, which still harbor the fluorescent probe. Thus, the dansyl probe, which is initially bound to lipid II, is found in molecules devoid of lipid moiety as the reaction proceeds. This change of environment of the dansyl probe is accompanied by a decrease of fluorescence.

Detergents were tested at about twice their CMC in water. We observed no GT activity in the presence of *n*-octyl- $\beta$ -D-glucopyranoside ( $\beta$ -OG), Cymal-3 and Cymal-4 (Fig. 2C). PBP2a was active in the presence of Chaps, *n*-dodecyl- $\beta$ -D-maltopyranoside (DDM) or Triton X-100, and less so in Cymal-5. The highest activity was found with Chaps, Triton X-100 and DDM. In all cases, we used detergent concentrations above their CMC in water. The shortest detergents ( $\beta$ -OG, Cymal-3 and Cymal-4) were not suitable. Because short detergents have high CMCs, it is possible that the inhibitory effect results from the decreased probability that enzyme-containing micelles encounter substrate-containing micelles, as proposed previously [34]. Considering these results, partially presented in Fig. 2, we chose for further work to use the intermediate dimethylsulfoxide concentration of 25% and 0.02 or 0.04% of Triton X-100 as detergent.

### GT enzymatic activity

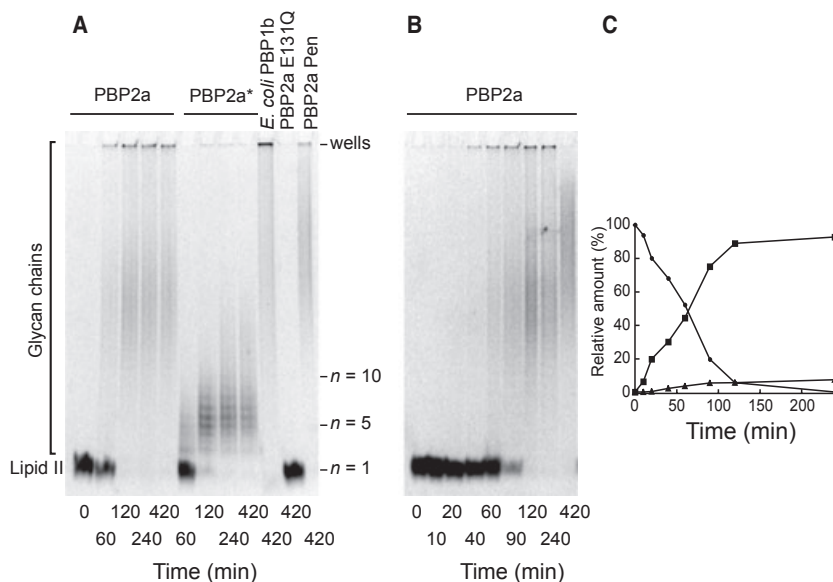
We aimed to determine the rate of transglycosylation catalyzed by PBP2a, PBP2a\* and *E. coli* PBP1b, using the 96-well microplate assay. Because of substrate limitation, we were not able to measure enough rates to obtain accurate values of the enzymatic parameters. However, by measuring the initial rates of lipid II consumption at two concentrations of lipid II, we could estimate that the  $k_{\text{cat}}$  for both PBP2a and PBP2a\* is  $> 5 \times 10^{-4} \text{ s}^{-1}$ . PBP2a\* was somewhat more efficient. The specific activity measured with 10  $\mu\text{M}$  dansylated lipid II was 0.38 nmol lipid II  $\cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$  for PBP2a, and slightly faster at 0.5 nmol lipid II  $\cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$  for PBP2a\*. For comparison, we measured a  $k_{\text{cat}}$  of  $(3.0 \pm 0.5) 10^{-2} \text{ s}^{-1}$  for *E. coli* PBP1b. The specific activity of *E. coli* PBP1b

was 22.5 nmol lipid II  $\cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$ . These findings are comparable to published values [37].

Full-length PBP2a and PBP2a\* showed comparable specific activities for the consumption of lipid II, indicating that the membrane anchor does not have an important effect on this aspect of the enzymatic activity. The catalytic constant was 10<sup>4</sup>-fold higher than reported in a previous study of PBP2a\* [3], but still 40–60-fold lower than that of *E. coli* PBP1b. The dimerization of *E. coli* PBP1b was found to promote GT activity [30]. If this finding applies to PBP2a, one factor limiting the activity of PBP2a *in vitro* might be a concentration that is below its dissociation constant.

The Tris–Tricine SDS/PAGE assay developed by Barrett *et al.* [35] allows the visualization of the synthesized glycan chains length, using radio-labeled lipid II as precursor. In this study, we used a fluorescently labeled lipid II, with a dansyl group attached on the third position of the substrate pentapeptide (lysine amine). This kind of modification should not affect the kinetics parameters of the GT reaction [18,38]. However, because the lysine amine that bears the dansyl probe is normally the acceptor group of the transpeptidation, no TP activity can occur with this precursor. Gels were imaged on a UV transilluminator. Lipid II migrates near the front of the electrophoresis, chains of disaccharide units migrate more slowly as they are longer. For short chains, individual bands were resolved with this 20 cm gel system, up to  $\sim 10$  disaccharide units. Longer chains were not resolved and merged in a smear. Some fluorescent material, likely constituted of longer chains remained in the wells. Using this assay, we examined the distribution of products of full-length PBP2a and PBP2a\*. We found that remaining free lipid II could not be detected after  $\sim 2$  h of reaction (Fig. 3). The lack of resolution of glycan chains of high molecular mass prevented precise determination of the chain length. However, by comparison with chains assembled by *E. coli* PBP1b, known to synthesize chains of 30–50 units [24,28,30], we estimated that PBP2a produced chains of 20–30 units. By contrast, PBP2a\* produced much smaller chains of five to six disaccharides. These results indicate that full-length PBP2a from *S. pneumoniae* is more processive than its truncated form PBP2a\*. Because both forms of the enzyme use up lipid II at similar rates, the different chain lengths that are produced suggest that PBP2a\* starts assembling chains more easily, but also releases glycan chains more readily than the full-length proteins. This phenomenon could be due to the transmembrane and/or cytoplasmic region directly, or indirectly through the absence of dimerization of PBP2a\*. Interestingly, the absence





**Fig. 3.** GT activity using dansylated lipid II, monitored by Tris–Tricine SDS/PAGE and imaged under UV light. (A) Comparison of the chain length distribution resulting from the activity of PBP2a and PBP2a\*. As a positive control, a reaction catalyzed by *E. coli* PBP1b was included. As negative control, a PBP2a variant with the active site residue replaced E131Q was used. The last lane demonstrates the absence of effect of penicillin (Pen) on the GT activity. Reaction times are given below the lanes. (B) Kinetics of the polymerization of glycan chains by PBP2a, as in (A) with more time points. (C) The fluorescence in the lipid II band (●), the glycan chains smear (■) and the well (▲) were quantified and represented on the graph.

of the transmembrane segment of *E. coli* PBP1b was found to decrease fivefold the affinity for the drug moenomycin [25]. Examination of the structure of *E. coli* PBP1b, the sole structure of a full-length class A PBP, led Sung *et al.* to propose that removal of the transmembrane segment would impact on the binding of lipid II and moenomycin [15]. Alternatively, an indirect effect of the detergent micelle surrounding the hydrophobic transmembrane segment could change the dynamics of the enzyme–substrate–product interactions.

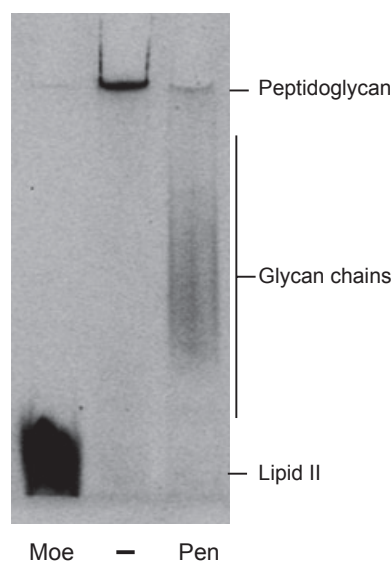
A former comparative study of several PBPs from different organisms showed that PBP2a from *Enterococcus faecalis* and PBP2 from *Staphylococcus aureus* produced chains of only 15 units. PBP1a from *E. coli* made longer chains than PBP1b from the same bacteria. The glycan chain length was not dependent on the enzyme-to-substrate ratio [24].

Some studies have reported a delay in the onset of the reaction catalyzed by *E. coli* PBP1b, which has been attributed either to a slow initial coupling step or to a slow rearrangement to the active conformation [34,39]. This lag phase has not been observed with PBP2a from *S. pneumoniae* (Fig. 3C). Note that in Fig. 3A,B, the images were overexposed to better visualize the glycan chains. This caused most of the lipid II bands to be saturated and to not reflect their actual amount. For the quantification shown in Fig. 3C, a

shorter exposure was used, which revealed the concomitant disappearance of lipid II with the appearance of the glycan chains. The short exposure image is not shown as the glycan chains are then barely visible, although the amount of fluorescence integrated over the length of the gel remains constant.

#### Absence of TP activity *in vitro*

The TP assay was based on the SDS/PAGE assay, but using a mix of fluorescent (for detection) and nonfluorescent lipid II (for cross-linking). Pure labeled lipid II cannot be used to investigate the TP activity, because it cannot serve as an acceptor in the reaction. Indeed, the fluorescent probe is attached to the third residue-free amine that participates in the reaction. By using 95% of unlabeled and 5% fluorescent lipid II, we hoped to allow transpeptidation to occur, while incorporating enough labeled material to visualize the products after SDS/PAGE. The experimental setup was validated using *E. coli* PBP1b, which is known to display both GT and TP activities *in vitro* [30,40]. In the absence of  $\beta$ -lactam, using a mixture of lysine-dansylated (5%, 10  $\mu$ M) and *meso*-diaminopimelate-containing lipid II (95%, 200  $\mu$ M), *E. coli* PBP1b (0.5  $\mu$ M) generated in 2 h a fluorescent product of high molecular mass that was retained on the top of the



**Fig. 4.** Assembly of peptidoglycan *in vitro* by *E. coli* PBP1b monitored by Tris–Tricine SDS/PAGE. Reactions were carried out with a mixture of dansylated lipid II (5%) and unlabeled *meso*-diaminopimelate-containing lipid II, without and with moenomycin (Moe) or penicillin G (Pen). Cross-linked peptidoglycan does not enter the gel system and remains trapped at the level of the loading well.

polyacrylamide gel (Fig. 4). We used *meso*-diaminopimelate-containing lipid II, the natural precursor of *E. coli* peptidoglycan, because PBP1b does not catalyze transpeptidation with lysine-containing precursors (W. Vollmer, unpublished). In the presence of penicillin, *E. coli* PBP1b produced glycan chains of the expected size, demonstrating that the large polymer obtained in the absence of penicillin resulted from transpeptidation. Under the same experimental conditions, using lysine-containing lipid II, PBP2a did not display transpeptidase activity, even with 10 times higher concentration of enzyme after 5 h.

Despite the absence of detectable transpeptidase activity of PBP2a and PBP2a\*, both variants reacted with bocillin (Fig. 1A), and both catalyzed the hydrolysis of S2d, a thiol-ester analogue of the stem peptide. Hydrolysis of thiol-ester analogues has been used traditionally as a proxy for the TP activity in the absence of physiologically relevant substrate [41]. Values given in Table 1 show that the activity of the pneumococcal PBPs is on par with that of *E. coli* PBP1b with respect to S2d hydrolysis. This observation raises the question of the absence of transpeptidase activity.

One trivial explanation would have been that the DMSO required for optimal GT activity is inhibiting the TP activity. However, we showed that reaction with bocillin is not hampered by 25% DMSO (Fig. 1A, \* lanes). Conversely, because we found that

**Table 1.** S2d hydrolysis activity. The specific activity measured with 2 mM S2d.

Protein	Specific activity (nmol·min <sup>-1</sup> ·mg enzyme <sup>-1</sup> )
PBP2x	1058
PBP2x*	1884
PBP2a	403
PBP2a*	964
<i>E. coli</i> PBP1b	432

PBP2a exhibits some residual GT activity in the absence of DMSO at low a concentration of Triton X-100, we repeated the assay under these conditions (no DMSO, 0.002% Triton X-100) but did not observe TP activity (not shown).

To our knowledge, no transpeptidation catalyzed by purified PBPs from Gram-positive organisms has ever been reported. Early experiments with enzymes from *Bacillus* species showed that although GT activity was observed, no transpeptidation occurred with PBPs from *Bacillus subtilis*, and only minor amount with PBPs from *Bacillus stearothermophilus* [42]. It is possible that the assembly of a thick peptidoglycan depends on a more controlled activity of the PBPs interacting with activating factors, and that isolated pneumococcal PBPs are inactive. Activators of peptidoglycan assembly are known in some Gram-negative species [43,44].

An alternative hypothesis is that the lipid II used in our study is not the proper substrate for PBP2a or for pneumococcal PBPs in general. The peptidoglycan of pneumococcus contains a significant fraction of ‘branched’ muro-peptides. In those, a Ser–Ala or Ala–Ala dipeptide is added onto the side chain of the third residue lysine. Cross-linking by transpeptidation then occurs through the free amine of the Ala. The presence of these branched peptides is necessary for the expression of  $\beta$ -lactam resistance [45]. It is conceivable that the TP domain of PBP2a is specialized for branched peptides. Also, the lysine-containing lipid II used in our study contains a glutamate as second residue of the stem peptide [16]. In most Gram-positive bacteria, the second residue is an iso-glutamine that results from the amidation of the glutamate by the recently identified essential amidotransferase MurT/GatD [46,47]. Depletion of the MutT/GatD amido transferase impairs growth and increases the susceptibility of *Staphylococcus aureus* to  $\beta$ -lactams. These observations suggested that one or more staphylococcal PBPs may require or prefer amidated lipid II [46,47]. This modification occurs at the stage of the lipid II, as shown in early studies with particulate extracts from *Staphylococcus aureus* [48]. Finally, the correct substrate for

PBP2a-catalyzed transpeptidation, at least as acceptor, might be the pre-existing peptidoglycan itself.

In conclusion, we aimed to purify and to characterize recombinant full-length and periplasmic PBP2a from *S. pneumoniae*, in order to reconstitute *in vitro* the peptidoglycan synthesis from this important Gram-positive pathogen. We found that the GT activity of PBP2a was very sensitive to the reaction conditions such as the concentration of DMSO and the detergent. Also, we observed that membrane anchor influences glycan chain length *in vitro*. The next challenge will be to demonstrate TP activities from pneumococcal PBPs, as it has never been obtained with recombinant enzymes from Gram-positive organisms.

## Experimental procedures

### Materials

Triton X-100 and Chaps were purchased from Euromedex (Souffelweyersheim, France); DDM;  $\beta$ -OG, Cymal-3, Cymal-4 and Cymal-5 were from Anatrace (Maumee, OH, USA). Moenomycin was a gift from Aventis (Frankfurt am Main, Germany). The thio-ester substrate S2d (N-benzoyl-D-alanylmercaptoacetic acid) was obtained from B. Joris (Centre d'Ingénierie des Protéines, Université de Liège, Belgium). Lipid II (with Ala-D-iGlu-Lys-D-Ala-D-Ala) was obtained from University of Warwick, UK-BaCWAN synthetic facility [49]. Lysine-dansylated lipid II was prepared as described previously [16], with the modification that the lysine was dansylated by incubation of UDP-MurNAC-pentapeptide (22 mg) with dansyl chloride (25 mg) in 20 mL of dimethyl formamide 50%, *N,N*-diisopropylethylamine 0.5%, for 3 h at room temperature.

### Plasmids for expression of pneumococcal PBP2a and PBP2x

The *pbp2a* gene was amplified from R6 *S. pneumoniae* genomic DNA by PCR with the primers GAGAATTCC ATATGAAATTAGATAAATTATTTGAG and GGTC GACGGATCCACTAGTGCTAGCTTAGCGAAATAGATTG, and introduced as a *NdeI*–*Bam*HI fragment into pET-30b, to express full-length PBP2a. The plasmid used to express the truncated PBP2a\* was that described previously [4]. The plasmid for the expression of the PBP2a–E131Q mutant was constructed using the primers GCAGAATG CTGTTATCGCGACACAGGACCGTTCCTTC and its reverse complement, with the Quickchange kit (Stratagene, Santa Clara, CA, USA).

The *pbp2x* gene was amplified using the primers GAG AATCCATATGAAGTGGACAAAAAGAGTAATC and GGTTCGACGGATCCGTCTCCTAAAGTTAATG, and

introduced as a *NdeI*–*Bam*HI fragment into a modified pET-30b vector (A. Zapun, unpublished) that allows expression of proteins with a C-terminal Strep-tag.

### Production and purification of PBPs

Full-length PBP2a or PBP2x–Strep were overexpressed in *E. coli* BL21(DE3) cells (Invitrogen, Paisley, UK). Cells were grown at 37 °C in 2 L of Luria–Bertani broth supplemented with kanamycin to  $D_{600} = 2$  and expression was induced overnight at 20 °C in the presence of 1 mM isopropyl thio- $\beta$ -D-galactoside. Cells were resuspended in 40 mL of lysis buffer (50 mM Hepes, pH 7.5, 500 mM NaCl, 10 mM MgCl<sub>2</sub>) containing one tablet of Complete protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were broken in a Microfluidizer (Microfluidics, Newton, MA, USA) at 10 000 psi. Membranes were sedimented by ultracentrifugation (150 000 g, 1 h, 4 °C). The pellet was resuspended in 10–20 mL of lysis buffer to reach  $D_{600} = 1$ , and membranes were solubilized with Triton X-100 1% for 30 min at 4 °C.

Full-length PBP2a was purified by two chromatography steps performed at 4 °C. Solubilized membranes were loaded onto a cation exchange 5 mL SP-Sepharose column (GE Healthcare, Chalfont St Giles, UK) equilibrated with 50 mM Tris pH 8.0, 10 mM MgCl<sub>2</sub>, containing 0.2% Triton X-100. Bound proteins were eluted with a 0–1 M NaCl gradient over 10 column volumes. Pooled fractions containing PBP2a were diluted 10-fold and loaded onto a 6 mL Resource Q column (GE Healthcare) equilibrated with 50 mM Hepes pH 7.5, 10 mM MgCl<sub>2</sub>, containing 0.2% Triton X-100. The PBP2a–E131Q mutant was prepared in the same manner. PBP2a\* and the membrane-bound  $\gamma$ -isoform (46–844) PBP1b from *E. coli* were prepared as described previously [4,30].

PBP2x–Strep was purified by a single affinity chromatography step. Solubilized membranes were incubated for 1 h with 0.5 mL of Strep-Tactin Superflow resin (IBA, Göttingen, Germany) equilibrated with the lysis buffer containing 0.02% DDM. After washing with 8 column volumes, the protein was eluted with the same buffer containing 2.5 mM desthiobiotin. The truncated periplasmic PBP2x\* was prepared as described previously [50].

### Protein characterization

The protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA) using BSA as a standard. Size-exclusion chromatography was performed on a S200 Superdex GL 5/150 column (GE Healthcare) in 50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub> and 0.02% Triton X-100. Cross-linking *in vitro* was performed with EGS in the same buffer. Full-length PBP2a or PBP2a\* (1.4  $\mu$ M) were incubated for 20 min with 0–0.4 mM of EGS (Sigma-Aldrich, St Louis, MO, USA). After reaction



quenching by the addition of 50 mM Tris (pH 8.0) for 15 min, samples were analyzed by SDS/PAGE and western blot with rabbit anti-PBP2a serum. Limited proteolysis was performed on 6 mg·mL<sup>-1</sup> of PBPs, with trypsin/PBP ratios ranging from 1 : 10<sup>5</sup> to 1 : 10<sup>2</sup> (w/w). After incubation for 30 min at 37 °C, protease activity was inhibited with 1 mM phenylmethanesulfonyl fluoride and the proteolyzed samples were analyzed by SDS/PAGE (30 µg PBP·lane<sup>-1</sup>).

### GT and TP activity assays

To determine the optimal concentration of DMSO, PBP2a (2 µM) was incubated for 40 min with lysine-dansylated lipid II (25 µM) at 30 °C, in 50 mM Hepes pH 7.5, 200 mM NaCl, 25 mM MgCl<sub>2</sub> and various concentrations of DMSO (0 to 50%), with Triton X-100 (0.002%, brought by the protein preparation, or 0.04%). The reactions were stopped after 40 min by the addition 0.5 mM moenomycin. Samples were analyzed by Tris–Tricine SDS/PAGE [35] and gels were imaged on a UV (302 nm) transilluminator using a Gel Doc system (Bio-Rad, Hercules, CA, USA).

For glycan chain length determination, conditions were as above except that the reaction mixtures contained 1 µM PBP2a, 10 µM of lysine-dansylated lipid II, 25% DMSO and 0.04% Triton X-100. After various time intervals (0–7 h), reactions were stopped with 0.5 mM moenomycin. Samples were analyzed as above, bands were quantified using IMAGEJ software.

The same Tris–Tricine SDS/PAGE assay was used to demonstrate TP activity. However, a mixture of 10 µM dansylated and 200 µM unlabeled lipid II (*meso*-diaminopimelate-containing lipid II with *E. coli* PBP1b) was used, in order to provide the free amine on the third residue that is necessary to function as acceptor in the transpeptidation reaction. The concentration of *E. coli* PBP1b was 0.5 µM, for an incubation time of 2 h at 30 °C. With PBP2a, attempts were made with protein concentrations up to 6 µM and incubation times as long as 5 h.

The continuous fluorescent assay in a 96-well format was used to compare the effect of detergents and the GT kinetics of different PBPs [28,34]. The reaction mix (50 µL) included lysine-dansylated lipid II (10 µM) in 50 mM Hepes, pH 7.5, 200 mM NaCl, 25 mM MgCl<sub>2</sub>, 25% DMSO, 0.02% Triton X-100, 0.1 mg·mL<sup>-1</sup> of N-acetylmuramidase from *Streptomyces globisporus* (Calbiochem, San Diego, CA, USA). In addition to the Triton X-100 brought with the enzyme (0.02%), added detergent concentrations were: 0.02% DDM, 1% β-OG, 0.02% Triton X-100, 1% Chaps, 3% Cymal-3, 0.8% Cymal-4, 0.25% Cymal-5, which are about twofold their CMCs. Time courses at 30 °C were initiated with the addition of PBP2a or PBP2a\* (1 µM), or *E. coli* PBP1b (0.1 µM).

For the determination of kinetic parameters, measurements were made with 5 and 10 µM lysine-dansylated lipid II, in 50 mM Hepes, pH 7.5, 200 mM NaCl, 25 mM MgCl<sub>2</sub>, 25%

DMSO, 0.04% Triton X-100, at 30 °C. The amplitude of the fluorescence decrease (fluorescence at time zero minus fluorescence at the reached plateau) was taken as representing the complete conversion of dansylated lipid II into dansylated disaccharide pentapeptide. The initial slope of the fluorescent decrease could then be interpreted as a rate of lipid II consumption.

### S2d thio-ester hydrolysis

Activity of the TP domain of PBP2a, PBP2a\*, PBP2x, periplasmic PBP2x and *E. coli* PBP1b was assayed by measuring the hydrolysis of the S2d thio-ester analogue of cell-wall stem peptides [41]. Reaction mixtures (500 µL each) contained 52 mM potassium phosphate pH 7.0, 2 mM S2d, 0.8 mM 4,4'-dithiodipyridine and 3–5 µg of PBP. The thio-group release that was coupled to 4,4'-dithiodipyridine was measured by monitoring the increase in absorption at 325 nm (molar extinction coefficient  $\epsilon = 19\,800\text{ M}^{-1}\cdot\text{cm}^{-1}$ ) for 3 min at 37 °C as described [51]. Negative controls were performed in the presence of 0.5 mM penicillin G.

### Acknowledgements

We thank Luca Signor for mass spectrometry measurements and Jean-Pierre Andrieu for N-terminal sequencing. We thank Andrea Dessen, Anne Marie Di Guilmi for their comments and suggestions, Laure Roux for technical assistance, and Marjolaine Noirclerc-Savoie, from the IBS RoBioMol platform of the Partnership for Structural Biology, for expertise with the fluorimeter. We are grateful to Bernard Joris (Centre d'Ingénierie des Protéines, Liège, Belgium) for the generous gift of S2d. This work was partly funded by the FP6 EUR-INTAFAR LSHM-CT-2004-512138 project, the ANR grant PneumoPG ANR-08-BLAN-0201, and a CNRS-Royal Society grant to WV and AZ.

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