Published in final edited form as:

Mol Microbiol. 2009 May ; 72(4): 895–904. doi:10.1111/j.1365-2958.2009.06687.x.

Evidence for a dual role of PBP1 in the cell division and cell separation of *Staphylococcus aureus*

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SUMMARY

Penicillin-binding proteins (PBPs) catalyze the synthesis of cell wall peptidoglycan. PBP1 of *Staphylococcus aureus* is a high molecular weight monofunctional transpeptidase (TPase) and previous studies with a conditional mutant showed that this protein was essential for bacterial growth and survival: cells in which PBP1 was depleted stopped dividing but continued to enlarge in size, accompanied by rapid loss of viability. Also, cell walls produced under PBP1 depletion appeared to have normal composition. We describe here construction of a second PBP1 mutant in which the active site of TPase domain was inactivated. Cells in which the wild type PBP1 was replaced by the mutant protein were able to initiate and complete septa and undergo at least one or two cell divisions after which growth stopped accompanied by inhibition of cell separation, down-regulation in the transcription of the autolytic system and production of cell walls with increased proportion of monomeric and dimeric mucopeptides and decrease in mucopeptide oligomers. PBP1 seems to perform a dual role in the cell cycle of *S. aureus*: as a protein required for the initiation of septation and also as a transpeptidase that generates a critical signal for cell separation at the end of cell division.

INTRODUCTION

Peptidoglycan (PG) is a macromolecule composed of linear glycan strands cross-linked by flexible peptide bridges. This strong, yet flexible structure provides the framework of the bacterial cell wall, withstanding the high osmotic pressure and maintaining cell shape (Pogliano, 2008, Vollmer *et al.*, 2008). The integrity of the PG layer is therefore essential and its precise replication during cell division absolutely critical. Due to its unique nature, PG is the target of some of the most efficient antimicrobials available. Several of these compounds, such as β -lactams and glycopeptides, act directly on or interfere with penicillin-binding proteins (PBPs). PBPs are enzymes involved in the last stages of synthesis of PG, catalyzing the polymerization (transglycosylation) and cross-linking (transpeptidation) between glycan strands (Ghuysen, 1991, Goffin & Ghuysen, 1998). These enzymes can be divided in low molecular weight (LMW) and high molecular weight (HMW) PBPs. LMW PBPs have only a penicillin-binding domain, whereas HMW PBPs are multimodular proteins and can be further subdivided in two classes. Class A HMW PBPs are bifunctional

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enzymes, with a N-terminal domain that catalyzes the transglycosylation reaction and a C-terminal, penicillin-binding domain, which catalyzes the transpeptidation reaction. Class B HMW PBPs have a N-terminal domain that does not contain the transglycosylase-associated motifs, and a C-terminal transpeptidase (TPase) domain (Ghuysen, 1991, Goffin & Ghuysen, 1998).

The gram-positive *Staphylococcus aureus* is an important human pathogen, particularly notorious for the capacity of methicillin-resistance *S. aureus* (MRSA) strains to accumulate antibiotic resistance determinants. *S. aureus* is a round shaped bacterium that divides in three alternating perpendicular planes (Koyama *et al.*, 1977, Tzagoloff & Novick, 1977). In this organism, cell wall synthesis is believed to occur mostly, if not exclusively, at the division site, where PBPs seem to be localized (Pinho & Errington, 2003, Pinho & Errington, 2005, Pereira *et al.*, 2007). *S. aureus* has only four native PBPs (PBP1–4) (Georgopapadakou & Liu, 1980). However, MRSA strains have acquired an extra PBP (PBP2A), which has low affinity for β -lactams and is thus associated with resistance to these drugs (Hartman & Tomasz, 1984, Berger-Bachi *et al.*, 1986). In comparison to other organisms *S. aureus* has a minimal set of PBPs. Regardless, their exact role is still hard to dissect, in part because of multiple functional interactions among them. Two well-documented examples in *S. aureus* are the cooperation between the transglycosylase activity of PBP2 and the TPase activity of PBP2A in the presence of β -lactams (Pinho *et al.*, 2001a, Pinho *et al.*, 2001b), or the concerted action of the TPase activities of PBP2 and PBP4 in cell wall synthesis (Leski & Tomasz, 2005).

The primary amino acid structure of *S. aureus* PBP1 has a high degree of similarity to the sequences of several class B HMW division-specific PBPs both in Gram-positive and Gram-negative organisms (Wada & Watanabe, 1998). The predicted tri-dimensional structure of PBP1 is reminiscent of its orthologs in Gram-positive bacteria, such as PBP2X of *Streptococcus pneumoniae* (Pares *et al.*, 1996). PBP1 domain architecture is organized in a N-terminal penicillin-binding protein dimerisation domain, a TPase domain and two PASTA (penicillin-binding protein and serine/threonine kinase associated) domains in its C-terminal region (UniProt entry number Q5HGQ0).

The monofunctional TPase PBP1, and the bifunctional PBP2 are the essential PBPs in *S. aureus*, but only PBP1 remains so in the presence of PBP2A (Wada & Watanabe, 1998, Pinho *et al.*, 2001b, Pereira *et al.*, 2007). Depletion studies showed that PBP1 is required for the formation of the division septum (Pereira *et al.*, 2007); in its absence, cells are not able to divide and start rapidly to lose viability. However, in the same study, we showed that under conditions of sufficient PBP1 depletion to impair cell growth, the composition of peptidoglycan did not seem to be significantly altered (Pereira *et al.*, 2007). This, together with the essentiality of PBP1 even in the presence of PBP2A, which can replace the essential TPase function of PBP2 (Pinho *et al.*, 2001b), led to the proposal that the essential function of PBP1 in *S. aureus* is specifically related to cell division in a manner that is largely independent of its enzymatic activity (Pereira *et al.*, 2007). To explore this idea further we analyzed the expression of a *pbpA* allele coding for a PBP1 variant with a non-functional TPase domain. This allele was created by a single amino acid substitution of the catalytic serine in the TPase domain by an alanine. Our results are consistent with the notion that the essential function of PBP1 in cell division is largely independent of a functional TPase domain. However, an intact TPase domain is essential for the proper coupling of cell wall synthesis and cell division to the activity of the autolytic system in *S. aureus*.

RESULTS

A functional PBP1 TPase domain is essential for growth

In a previous study, we have used the *pbpA* conditional mutant COL*Spac*P1, derived from the MRSA strain COL, to show that PBP1 has an essential role in the cell division of *S. aureus* (Pereira et al., 2007). However, this strategy did not discriminate between the specific need for a functional TPase domain and a more structural role in recruiting other proteins to the division site, as described for PBP3 and PBP2B, PBP1 orthologs in *Escherichia coli* and *Bacillus subtilis*, respectively (Daniel et al., 2000, Vollmer et al., 2008). To overcome this limitation, we have now created a point mutant in which the catalytic serine residue at position 314 of the 744-residues long protein (Wada & Watanabe, 1998), was replaced by an alanine (S314A). The wild type and the S314A alleles of *pbpA* were cloned into the *S. aureus* replicative vector pSK5632 (Grkovic et al., 2003), and the resulting plasmids transferred in parallel to COL*Spac*P1, in which expression of the chromosomal copy of *pbpA* is controlled by the IPTG-inducible P_{spac} promoter. The strains thus generated, bearing the multicopy wt or S314A episomal alleles of *pbpA*, were termed COLPBP1WT and COLPBP1TPase⁻, respectively [for detailed information see Supplementary material Materials and Methods and Fig. S1)].

In order to determine the phenotypes associated with the production of PBP1^{S314A}, the expression of *pbpA* gene from the chromosome was turned off by growing the conditional mutant in the absence of the IPTG inducer. A wild type copy of *pbpA*, also expressed from the same plasmid, was used as a control.

We compared growth and viability of the conditional mutant COL*Spac*P1 and the TPase point mutant COLPBP1TPase⁻ during cultivation in the absence of IPTG, repressing the expression of the *pbpA* chromosomal copy (Pereira et al., 2007; see above).

As reported before, COL*Spac*P1 required four to five generations in the absence of inducer before depletion of PBP1 produced distinct phenotypes – including a complete halt in growth (Fig. 1A) (Pereira et al., 2007).

COLPBP1TPase⁻ incubated under the same conditions also stopped growing after approximately 7 hours (corresponding to eight to nine generations of the control strain COLPBP1WT) (Fig. 1C).

A more detailed comparison of the response of the conditional mutant and the TPase mutant to cultivation in the IPTG-free medium showed additional differences as well. After about 2.5 hours of incubation without IPTG the viable titer of the conditional mutant COL*Spac*P1 showed a rapid and extensive decline in viable titer and resumption of growth of the bacteria – both in terms of optical density and viable counts – only began about 3 hours after readdition of the inducer to the growth medium (Fig. 1A and B). In contrast, in the TPase mutant (COLPBP1TPase⁻) the halt in the increase of optical density was not accompanied by a significant loss of viable titer and readdition of the inducer to the medium caused a more prompt resumption in the increase of both the optical density and the viable titer of the culture (Fig. 1C and D).

Importantly, we found that in the absence of IPTG, and hence under conditions where wild type PBP1 was not produced, PBP1^{S314A} accumulated to wild type levels, but did not detectably bind radiolabeled penicillin, a structural analog of the terminal D-alanyl-D-alanine of peptidoglycan stem peptides, the natural TPase substrate (Fig. 2). Thus, we can infer that the TPase activity of PBP1^{S314A} was successfully inactivated and that no gross alterations to its normal folding have been imposed by the S314A substitution.

Taken together these observations clearly indicate that the TPase activity of PBP1 is essential for the normal growth of *S. aureus*. The observations also suggest that TPase activity may not be essential for maintaining viability of non-growing cultures.

PBP1 has transpeptidase activity

In a previous study, we were unable to detect any significant alterations in the composition of peptidoglycan synthesized in the presence of a limiting amount of PBP1 (Pereira et al., 2007). However, the results described above show that a non-functional PBP1 TPase domain impaired growth, which could ultimately result from structural defects at the level of the cell wall. To test the impact of the S314A mutation on cell wall synthesis, the composition of peptidoglycan isolated from COLPBP1TPase⁻ cells was analyzed by reversed-phase high-performance liquid chromatography (HPLC). When compared with the wild type control, the HPLC elution profile of the mutant strain revealed a two-fold increase in peaks 5 and 11, representing respectively monomeric and dimeric mucopeptides (Fig. 3), as well as a ten percent decrease in the representation of oligomeric, highly cross-linked mucopeptides (peaks 18 to 23), consisting of six or more cross-linked mucopeptides in the peptidoglycan (de Jonge *et al.*, 1992). These observations indicate that PBP1 does act as a TPase in the cell wall synthesis of *S. aureus*.

The S314A form of PBP1 localizes at sites of cell division

Labeling of newly synthesized cell wall with a fluorescein conjugate of vancomycin suggested that synthesis in *S. aureus* is confined to the division site, where both PBP1 and PBP2 have been localized (Pinho & Errington, 2003, Pinho & Errington, 2005, Pereira et al., 2007). Moreover, the localization of some PBPs appears to be substrate-driven and therefore require an active catalytic site (Morlot *et al.*, 2004, Pinho & Errington, 2005, Costa *et al.*, 2008), which prompted us to test whether the S314A substitution in the TPase domain of PBP1 affected its localization to the division site. For this, immunofluorescence microscopy was used to examine the subcellular localization of PBP1^{S314A} in comparison with the wild type protein. PBP1 localized at the division septum in the two parental strains COL and COL^{spac}P1 grown in the presence of IPTG (Fig. 4A). As expected, the fluorescence signal was lost when COL^{spac}P1 was depleted of PBP1. However, the fluorescence signal remained at the division site in both COLPBP1WT and COLPBP1TPase⁻ grown in the absence of IPTG (Fig. 4A). This indicates that the subcellular localization of PBP1 is largely independent of a functional TPase domain and by inference, essentially independent of its TPase activity.

A functional PBP1 TPase domain is not essential for septation, but is essential for cell separation

When analyzed by phase contrast microscopy, COLPBP1TPase⁻ cells showed an increase in size and appeared to fail to separate following division. These observations were confirmed by transmission electron microscopy of thin sections of COLPBP1WT and COLPBP1TPase⁻ (Fig. 4B). The majority of the cells in the population producing the PBP1^{S314A} protein were found in clusters of at least two or four cells (Fig. 4B and C), confirming an inhibition of cell separation. In addition, the cells that were not found in clusters were on average 1.7 times bigger than those from the control sample (Fig. 4D), which suggests a block in cell division. Surprisingly, around 80 % of the COLPBP1TPase⁻ cells showed a completed septum. Moreover, 40 % of these initiated formation of a second septum perpendicular to the first and 20 % were able to complete it (Fig. 4B). Also, these cells appear to have thickened septa. This is in sharp contrast with the behavior of the *pbpA* conditional mutant previously described (Pereira et al., 2007), which was essentially unable to form division septa when depleted of PBP1 (only about 15 % of the cells showed complete septa). The results suggest that the synthesis of cell wall during cell division can

take place in the absence of a functional PBP1 TPase domain and presumably, in a manner that seems to be largely independent of its enzymatic activity.

Loss of a functional PBP1 TPase domain results in strong repression of the autolytic system

The clustering of COLPBP1TPase⁻ cells described above suggested an inhibition of cell separation, and hence reduced activity of the autolytic system. To test this, rates of autolysis were assessed following exposure of COL^{spac}P1 and COLPBP1TPase⁻ cells to the detergent Triton X-100. COLPBP1TPase⁻ showed a decreased autolytic rate, but the same effect was not observed in COL^{spac}P1, when both strains were grown in the absence of IPTG (Fig. 5). This is in agreement with the morphological analysis of the strains, which revealed that in contrast with COLPBP1TPase⁻, cells depleted of PBP1 did not show signs of clustering (Fig. 3 and reference Pereira et al., 2007).

Perturbations of cell wall synthesis imposed by the limited production of PBP2 were shown before to repress the *S. aureus* autolytic system (Antignac *et al.*, 2007). Since a similar situation could occur in the case of PBP1, we have tested if the decreased rates of autolysis observed in COLPBP1TPase⁻ also reflected the expression and activity of the autolytic enzymes.

First, the *in vitro* susceptibility of cell walls to autolysins from different sources was tested (Supplementary material Fig. S2). Cell walls purified from both COLPBP1TPase⁻ or COLPBP1WT showed similar levels of susceptibility, suggesting that repression of the autolytic system in COLPBP1TPase⁻ is inflicted mostly, if not exclusively, at the transcriptional level. In order to confirm this, we used northern blotting to assess the transcription level of several genes coding for autolytic enzymes, including the genes for Atl and Sle1, the major autolysins involved in the separation of daughter cells following division (Sugai *et al.*, 1995, Yamada *et al.*, 1996, Ramadurai & Jayaswal, 1997, Kajimura *et al.*, 2005), and the genes coding for LytM and SA0620. The results in figure 6 show that the expression of all autolysin genes tested was diminished in COLPBP1TPase⁻ relative to COLPBP1WT, consistent with a repressed autolytic system.

When IPTG was added to cultures of COLPBP1TPase⁻, restoring production of wild type PBP1 from the chromosomal copy, expression of all tested autolytic genes was reestablished (Fig. 6). Moreover, after five generations in the presence of inducer, the rate of autolysis of the strain was also restored (Supplementary material Fig. S3A). Cell morphology and cell separation phenotypes also showed signs of normalization, but only after ten generations in the presence of inducer (Supplementary material Fig. S3B and S3C). This delay between the recovery of the activity of the autolytic system and the cell morphology and separation phenotypes may be explained by the constitutive expression of the episomal S314A *pbpA* allele: the continued production of the TPase⁻ PBP1 may be competing with the wild type protein. As cell division seems to continue to occur for some time after inhibition of cell separation, it is also possible that peptidoglycan hydrolases involved in cell separation relocate to future division sites and therefore do not hydrolyze cell wall in the old septa. This would also be consistent with the thickening of septa.

DISCUSSION

In a previous study we used a conditional mutant of PBP1 to test the essential function of this protein in staphylococcal cell division. Cells with depleted PBP1 stopped dividing but continued to grow in size, produce cell walls but also began to rapidly lose viability (Pereira *et al.*, 2007). Further, the cell wall peptidoglycan produced under conditions of PBP1 depletion sufficient to impair cell growth, showed only minor – if any – abnormalities in

composition (Pereira et al., 2007). This suggested that PBP1 was not a major TPase in *S. aureus*, and that its essential function in cell division may be independent of its TPase activity.

Although supported by the experimental evidence, this conclusion was surprising. PBP1 is a HMW class B PBP (Goffin & Ghuysen, 1998, Wada & Watanabe, 1998), localized at the cell division septum (Pereira et al., 2007), which is the main site of cell wall synthesis in *S. aureus* (Pinho & Errington, 2003), and orthologs of the *S. aureus* PBP1, such as the PBP3 of *E. coli* and *Caulobacter crescentus* and PBP2B of *B. subtilis*, were shown to be involved in peptidoglycan synthesis during cell division (Spratt, 1975, Botta & Park, 1981, Wientjes & Nanninga, 1991, Daniel et al., 2000, Costa et al., 2008).

The paradox of the essential nature of PBP1 and its disproportional small impact on the structure of the peptidoglycan, raised the question of exactly how was PBP1 contributing to viability and prompted the present investigation, in which a mutant form of PBP1 bearing a non-functional TPase domain (PBP1^{S314A}) was constructed and tested in the background of the conditional mutant in which production of the wild type PBP1 was turned off.

Similarly to the wild type protein, PBP1^{S314A} also localized to sites of cell division. After a residual growth – to deplete copies of the wild type PBP1 – the replication of such a TPase[−] mutant (COLPBP1TPase[−]) continued for a time period after which growth came to a complete halt. The composition of cell walls produced during this period was abnormal showing significant decrease in peptidoglycan crosslinking.

These findings provide a striking direct experimental evidence that the catalytic (transpeptidase) domain of PBP1 performs an essential function for the bacteria. Nevertheless, the extensive changes in peptidoglycan composition observed in the TPase mutant were at variance with the relatively minor changes reported earlier in experiments with the conditional mutant. In order to resolve this apparent contradiction, the phenotypes of the two kinds of PBP1 mutants were carefully compared.

In contrast to the findings with the conditional mutant, inactivation of the TPase activity in PBP1^{S314A} did not cause loss of cell viability. Also in contrast with the conditional mutant, most cells expressing PBP1^{S314A} were able to complete septation and a significant fraction of the bacteria also proceeded to at least a second round of cell division, after which growth came to a halt. Also unlike cells of the conditional mutant which continued to enlarge to abnormal size after depletion of PBP1, the size of most of the TPase[−] cells remained normal. The most striking and distinct phenotype of the TPase mutant was the accumulation of doublets and “tetrads” of normal size cells that were apparently unable to separate from one another. It is also possible that “octets” of cocci, (indicating the completion of a third round of cell division) were also present, but could not be distinguished from tetrads by transmission electron microscopy.

The differences between the PBP1 conditional mutant and the mutant carrying the PBP1^{S314A} allele suggest that this penicillin-binding protein performs a dual role in *S. aureus*. The first one of these roles seems to be in the formation of septa, which can occur in cells carrying a PBP1 without a functioning TPase domain. Only the presence of PBP1 protein, but not of its TPase activity, seems to be required in this process. Thus, in contrast to homologous division-specific PBPs of other microorganisms (Spratt, 1975, Botta & Park, 1981, Wientjes & Nanninga, 1991, Daniel et al., 2000, Costa et al., 2008), cell wall synthesis during cell division in *S. aureus* may not require the TPase activity of PBP1.

The accumulation of pairs and tetrads of cells observed in cultures of the COLPBP1TPase[−] mutant suggests that the second essential role of PBP1 – its TPase activity – may be timed to

occur towards the end of the septal growth and is linked – as a prerequisite – to the mechanism that catalyzes separation of daughter cells at the end of cell division.

Because of the particular mode of cell wall synthesis and cell division in staphylococci, which divide in three alternating perpendicular planes (Koyama et al., 1977, Tzagoloff & Novick, 1977), separation of daughter cells appears to be essential for continued cell divisions and growth (Giesbrecht *et al.*, 1998, Kajimura *et al.*, 2005). During cell division, cells double their mass while cell wall is synthesized at the septum (Pinho & Errington, 2003). Only once the septum is completed can daughter cells begin to separate and initiate a new round of cell division, which occurs in a plane perpendicular to the previous.

The mechanism of cell separation in *S. aureus* is known to be catalyzed by the autolytic system and interruption of cell separation was shown to produce accumulation of doublets and tetrads of cells (Giesbrecht et al., 1998, Kajimura et al., 2005). The phenotype of the TPase[−] mutant suggests that the transpeptidase activity of PBP1 is linked to such autolytic events.

Clustering of cells – similar to the phenomenon described in this communication – was also reported in *S. aureus* in which transcription of PBP2 – the second major *S. aureus* TPase – was blocked (Gardete *et al.*, 2006). Inhibition of autolysis and rapid decline in the cellular amounts and transcription of most autolytic enzymes has also been described recently in *S. aureus* treated with inhibitors of cell wall synthesis or by inhibition in the transcription of the major staphylococcal transpeptidase PBP2 (Antignac *et al.*, 2007).

We propose – as a working hypothesis – that the alterations in the composition of peptidoglycan caused by the absence of a functional TPase domain in PBP1 generate a signal that is sensed, directly or indirectly, and processed by a signal transduction pathway that controls the expression of autolytic genes, resulting in the inhibition of cell separation. Consistent with this hypothesis, we found that the halt of growth of the TPase[−] mutant was accompanied by a striking decline in the activity of the autolytic system and a reduction in the transcription of most of the *S. aureus* autolytic enzymes (see Fig 5 and Fig 6). The existence of complex regulatory circuits coupling the autolytic system to the synthesis of cell walls has been described recently (Dubrac *et al.*, 2007, Dubrac *et al.*, 2008).

It is interesting to consider the transpeptidase activity of PBP1 as part of a potential checkpoint-type mechanism that controls progression through the cell cycle in *S. aureus*. This regulatory circuit would function at the end of each round of cell division to monitor proper cell wall synthesis during cell division. Defects in the cell wall composition, caused in this case by the lack of PBP1 TPase activity, would trigger repression of the autolytic system. Such a control mechanism would ensure that a cell could only proceed to the next division upon successful completion of cell wall synthesis in the previous round.

Another point that merits discussion is that the localization of PBP1 does not seem to follow the substrate-driven mechanism that has been proposed for several other proteins, including PBP2 of *S. aureus* (Pinho & Errington, 2005), PBP2X of *S. pneumoniae* (Morlot et al., 2004) and more recently, PBP3 of *C. crescentus* (Costa et al., 2008). It is possible that the two PASTA domains present in the C-terminal region of PBP1 may be involved in the recruitment of this protein to the division site as suggested for PBP2X from *S. pneumoniae* (Jones & Dyson, 2006). These domains are hypothesized to recognize and interact with the stem peptides of uncrosslinked peptidoglycan (Yeats *et al.*, 2002, Jones & Dyson, 2006). Alternatively, PBP1 may follow a localization pathway similar to PBP3 of *E. coli*, whose primary septal targeting determinants are found on its 26 residues-long N-terminal transmembrane anchor (Weiss *et al.*, 1999, Piette *et al.*, 2004, Wissel & Weiss, 2004, Wissel

et al., 2005). It will be interesting to test whether the localization mechanism of PBP1 in *S. aureus* is somehow related to its function in coupling cell division to cell separation.

EXPERIMENTAL PROCEDURES

Strains and plasmids

Strains and plasmids used in this study are listed in SI Table 1. The construction of *S. aureus* strain COL Δ pacP1 has been described before (Pereira et al., 2007). The construction of strains COLPBP1WT and COLPBP1TPase⁻ is described in Supplementary material Materials and Methods. The MRSA strain COL, which constitutively produces PBP2A, was chosen as a parental strain in this study in order to further explore the previously demonstrated finding that this resistance-associated protein can not replace the essential function(s) of the *S. aureus* PBP1 (Pereira et al., 2007).

Growth of the mutant strains

S. aureus strains were grown in tryptic soy broth (TSB, Difco Laboratories) at 37°C. To maintain aeration conditions, the volume of medium used was always 10 % of the flask volume and strains were incubated in an orbital shaker at 180 rpm. Alternatively bacteria were plated on tryptic soy agar (TSA, Difco Laboratories). Strain COL Δ pacP1 was grown as described before (Pereira et al., 2007) and unless otherwise stated, samples were collected after five generations in the absence or presence of 0.5 mM of IPTG. Strains COLPBP1WT and COLPBP1TPase⁻ were grown as follows: overnight cultures grown in the presence of IPTG (0.1 mM), were rediluted in fresh medium and grown for five generations with IPTG. This step was introduced to allow overnight cultures to leave the stationary phase of growth. After this initial step, cultures were washed with fresh TSB to remove IPTG and grown in TSB without inducer. To maintain cells in the exponential growth phase, cultures were again rediluted after five generations. Erythromycin (10 µg/ml) was always present in the growing medium. Unless otherwise stated, samples were collected after eight generations in the absence of IPTG. The doubling time of strains COL Δ pacP1 (with 0.5 mM of IPTG) and COLPBP1WT was approximately 50 minutes.

Immunoblot analysis

Membrane proteins were isolated and purified from cultures harvested at an OD₆₂₀ of 0.8 as previously described (Sieradzki *et al.*, 1999). Membrane protein preparations (10 µg/sample) were resolved on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS). Transfer to a Hybond ECL membrane (GE Healthcare) was done using a Mini-Protean 3 system (Biorad) according to the manufacture instructions. Immunoblotting was carried out according to the ECL Western blotting analysis system (GE Healthcare). The primary anti-PBP1 antibody was used over-night at 4°C at a dilution of 1:1000 and the peroxidase labeled anti-rabbit secondary antibody (GE Healthcare) was used for 1h at room temperature at a dilution of 1:50000.

Penicillin-binding assay

The same membrane protein preparations used in immunoblotting were used (150 µg/sample) to detect PBPs essentially as described before (Zhou *et al.*, 2008). Briefly, PBPs were labeled with 20 µg/ml of [¹⁴C]benzylpenicillin and resolved on a 8 % polyacrylamide gel containing SDS. PBPs were detected by fluorography using Hyblot CL autoradiography film (Denville Scientific).

Peptidoglycan composition

The purification of cell walls is described in SI Materials and Methods. The peptidoglycan fraction was extracted from purified cell walls as described before (de Jonge *et al.*, 1992). Purified peptidoglycan was digested with mutanolysin (Sigma), the resulting muropeptides were reduced with sodium borohydride (Sigma) and analyzed by reverse-phase high performance liquid chromatography (HPLC) using a Hypersil ODS column (Thermo Electron Corporation).

Immunofluorescence microscopy

Samples were grown to an OD₆₂₀ of 0.7 and prepared for immunofluorescence as described before (Pinho & Errington, 2003). Cells were incubated over-night at 4°C with the primary anti-PBP1 antibody (diluted 1:800) and 1h at room temperature with anti-rabbit immunoglobulin G-fluorescing isothiocyanate conjugate (diluted 1:500). Fluorescent images were acquired by a CoolSNAP HQ Photometrics camera coupled to a Leica DMRA2 microscope.

Transmission-electron microscopy

Samples were collected at an OD₆₂₀ of 0.8 and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.0. Cells were processed for electron microscopy according to the procedure of Ryter *et al.* (Ryter *et al.*, 1958), as modified by Tomasz *et al.* (Tomasz *et al.*, 1964).

Triton-induced autolysis

Cells were grown to an OD₆₂₀ of 0.3 and processed as described before (de Jonge *et al.*, 1991). The OD₆₂₀ of the suspensions was measured every 15 minutes for three hours and autolysis was expressed as a percentage of the initial OD.

Northern blot analysis

Total RNA was extracted from cultures grown to an OD₆₂₀ of 0.8 and northern blotting was performed as described before (Sobral *et al.*, 2003). 16S rRNA was used as a control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Aude Antignac for helpful discussions and technical assistance; H. Komatsuzawa and M. Sugai for the generous gift of PBP1 antibody; and R. A. Skurray for kindly providing plasmid pSK5632. Partial support for this study was provided by a grant (2 RO1A1045738-10) from the National Institute of Health, U.S. Public Health Service, to Alexander Tomasz and by contracts from Fundação para a Ciência e a Tecnologia, Portugal: POCTI/BIA-MIC/58416/2004 to Herminia de Lencastre POCTI/BCI/48647/2002 to Adriano O Henriques, and PTDC/BIA-MIC/67845/2006 to Mariana Gomes Pinho. Sandro.F.F.Pereira. was supported by grant SFRH/BD/9185/2002 from Fundação para a Ciência e a Tecnologia and by grant 79108 from Fundação Calouste Gulbenkian.

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Fig 1. Growth and viability of *S. aureus* in the absence of PBP1 transpeptidase activity
 (A) growth and (B) viability of the *pbpA* conditional mutant COLspacP1 grown in the presence and in the absence of IPTG. (C) growth and (D) viability of COLspacP1 producing the wild type PBP1 (COLPBP1WT) or PBP1^{S314A} (COLPBP1TPase⁻) from plasmid pSK5632. Solid and empty symbols represent the presence (0.5 mM) and absence of inducer, respectively. IPTG was added (arrow) when the OD of the mutant strains stopped increasing. Samples were taken every 50 min (approximate doubling time of strains) for OD measurements and for the determination of colony forming units (CFUs).

Fig. 2. Inactivation of the TPase activity of PBP1

(A) membrane proteins were analyzed by immunoblotting using an anti-PBP1 antibody. (B and C) same protein preparations were incubated with [¹⁴C]benzylpenicillin in a penicillin-binding assay. (C) shows an overexposed section of the gel in panel B to confirm the presence or absence of the PBP1 band. Membrane proteins were isolated from the parental strain COL; COL*spac*P1 grown in the presence of 0.5 mM and in the absence of IPTG; and COL*spac*P1 producing the wild type PBP1 (WT) or PBP1^{S314A} (TPase⁻) grown in the absence of IPTG.

Fig. 3. Peptidoglycan composition

Peptidoglycan synthesized in the presence of wild type and TPase⁻ PBP1, was extracted, digested with mutanolysin and analyzed by reverse-phase HPLC. Peaks 5 and 11, corresponding respectively to monomeric and dimeric mucopeptides, as well as peaks 18 to 23, corresponding of six or more cross-linked mucopeptide molecules, are identified in the chromatogram and represent the main alterations in the HPLC profile of the mutant strain.

Fig. 4. PBP1 localization and cell morphology

(A) localization of PBP1 in the septum was verified by immunofluorescence. (B) electron micrographs of *COLspacP1* cells producing wild type PBP1 (COLPBP1WT) or PBP1^{S314A} (COLPBP1TPase⁻) from plasmid pSK5632. Strains were grown in the absence of IPTG, except when indicated. (C) the number of cells that formed clusters was quantified from micrographs and expressed as a percentage of the number of cells counted. (D) the longitudinal length (from pole to pole) from every single cell within the electron microscopy field was measured to determine the average cell diameter. The total number of cells measured is shown in parenthesis.

Fig. 5. Triton-induced autolysis of whole cells

Strains were incubated with 0.01% of triton X-100 at 37°C. OD₆₂₀ was monitored every 15 minutes for 3 hours and autolysis rates were measured as a decrease of the initial OD. Strain COLspacP1 was grown with (●) or without (○) 0.5 mM IPTG. COLspacP1 producing the wild type PBP1 (COLPBP1WT, ▼) or the mutant form of PBP1^{S314A} (COLPBP1TPase⁻, ▽) from plasmid pSK5632, were grown in the absence of IPTG.

Fig. 6. Expression of autolysins

Total RNA was extracted from the PBP1 conditional mutant COL Δ pacP1 producing either the wild type PBP1 (WT) or the PBP1^{S314A} mutant form (TPase⁻) of the protein, from plasmid pSK5632. Samples were analyzed by northern blotting. RNA blots were hybridized with ³²P-labeled *atl*, *sle1*, *lytM*, and SA0620 DNA probes. The intensity of the 16S rRNA gel band was used as control. Cultures were grown in the absence of IPTG, except when indicated.