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A link in transcription between the native *pbpB* and the acquired *mecA* gene in a strain of *Staphylococcus aureus*

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Conditional mutants of *pbpB* with an IPTG-inducible promoter were used to compare the effects of interrupted transcription of this gene in a meticillin-sensitive (MSSA) and a meticillin-resistant (MRSA) strain of *Staphylococcus aureus*. After 3 h growth following the removal of IPTG, multiplication of the MSSA strain stopped abruptly, cells began to lyse, and membrane preparations showed greatly decreased quantities of penicillin-binding protein (PBP) 2. In contrast, the MRSA strain continued to grow for at least 20 h in the IPTG-free medium, but with gradually increasing doubling times, which eventually reached 180 min. The peptidoglycan produced during this period of extremely slow growth showed only minor alterations, but cells with abnormal morphology accumulated in the culture, the abundance of *mecA* transcript gradually declined, and the cellular amounts of PBP2A were significantly decreased. Adding back the IPTG inducer caused rapid resumption in the transcription of *pbpB*, followed by an increase in the transcription of *mecA*. No changes were detected in the transcription of *pbpA*, *C* and *D*, the determinant of 16S rRNA or the housekeeping gene *pta*. Promoter fusion experiments suggested that the transcription of the resistance gene *mecA* may respond to some regulatory signal generated in the bacteria during changes in the transcription of *pbpB*.

INTRODUCTION

Meticillin (formerly methicillin)-resistant *Staphylococcus aureus* (MRSA) strains carry the acquired resistance determinant *mecA* encoding the low-affinity penicillin-binding protein (PBP) 2A. The primary function of PBP2A is to take over the cross-linking of cell wall building blocks from the native PBPs when these become inactivated by β-lactam antibiotics (De Jonge & Tomasz, 1993). Recent experiments have indicated that the resistance protein PBP2A may also function in contexts other than drug resistance, and in co-operation with one of the native PBPs, namely PBP2.

The first evidence for this cooperation was the finding that inhibition of transcription of *pbpB*, which is the structural gene of PBP2, was lethal in a meticillin-sensitive *S. aureus* (MSSA), but not in the MRSA strain COL, which expresses PBP2A in a constitutive manner (Pinho *et al.*, 2001b). PBP2 is a bifunctional cell wall synthetic enzyme containing both transglycosylase (TGase) and transpeptidase (TPase) domains (Murakami *et al.*, 1994), and genetic experiments have demonstrated that the essential function of this protein, successfully replaced by PBP2A, is that of the TPase domain (Pinho *et al.*, 2001b). Cooperative functioning of PBP2 and PBP2A has been further documented by the observation that growth of MRSA in the presence of high concentrations of antibiotic requires not only PBP2A, but the functioning of the TGase domain of the native PBP2 as well (Pinho *et al.*, 2001a). Additional evidence for cooperative functioning has come from the recent observation that the presence of PBP2A is able to prevent dislocation of PBP2 from the cell wall growth zone, a phenomenon that occurs in MSSA strains exposed to β-lactam antibiotics (Pinho & Errington, 2005).

The purpose of the studies described here was to examine in more detail these intriguing and unusual phenomena, which involve cooperative functioning between two proteins: a cell wall synthetic enzyme native to *S. aureus*, and an antibiotic-resistance factor acquired by *S. aureus* from an extra-species source.

In a conditional mutant, we followed the effects of turning off and turning on the transcription of *pbpB* on the growth, oxacillin resistance, cell structure and cell wall composition and gene expression profiles, and also the production of PBPs 2 (in strain RN4220$_{spac::pbpB}$) and 2A in the MRSA

Abbreviations: C_T, threshold cycle; MRSA, meticillin-resistant *Staphylococcus aureus*; MSSA, meticillin-sensitive *S. aureus*; PAP, population analysis profile; PBP, penicillin-binding protein; Q-PCR, quantitative real-time PCR; TGase, transglycosylase; TPase, transpeptidase.

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strain $COL_{spac::pbpB}$. Extended incubation of the bacteria in the absence of *pbpB* transcription resulted in a gradual decline in the abundance of the *mecA* transcript, and a drastic reduction in the cellular amounts of PBP2A. The phenomenon appeared to be specific, and reversible by readdition of IPTG to the growth medium.

These surprising findings identify yet another cooperative phenomenon between *pbpB* and *mecA* that seems to occur at the level of gene expression. Changes in the transcription of *pbpB* seem to bring about parallel changes in transcription of the resistance gene *mecA* – apparently in response to some *trans*-acting factor that is produced in the cells as a function of the level of transcription of the native *pbpB*.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* strains were grown in tryptic soy broth (TSB) with aeration at 37 °C, or on tryptic soy agar (TSA) plates at 37 °C. *Escherichia coli* strains were grown in Luria–Bertani broth (LB) with aeration at 37 °C. For selection and maintenance of *S. aureus* and *E. coli* transformants, media were supplemented, as required, with 10 mg erythromycin l^{-1}, 10 mg chloramphenicol l^{-1} and 100 mg ampicillin l^{-1}, all obtained from Sigma.

Growth of *pbpB* conditional mutants in the presence and absence of the IPTG inducer. Cultures of *pbpB* conditional mutants of the sensitive MSSA strain $RN4220_{spac::pbpB}$ and the MRSA strain $COL_{spac::pbpB}$ were grown overnight with aeration in TSB supplemented with 100 μM IPTG (Sigma). The following morning, the turbid stationary-phase overnight cultures were diluted to an OD_{620} of 0·05 in fresh TSB containing 100 μM IPTG, and they were incubated with aeration at 37 °C in order to produce exponentially growing cultures. The effects of turning off the transcription of *pbpB* were determined when these exponentially cultures had reached an OD_{620} of 0·4.

Cultures were centrifuged, bacterial pellets were washed twice with TSB (to remove IPTG), and the bacteria were resuspended in fresh pre-warmed TSB without IPTG to an initial OD_{620} of 0·05 (time point 1). Both the $RN4220_{spac::pbpB}$ and $COL_{spac::pbpB}$ cultures were then incubated in the IPTG-free medium at 37 °C with aeration. Both cultures grew with close-to-normal doubling times for about 2 h, and, by hour 3, the OD_{620} increase of the MSSA culture ($RN4220_{spac::pbpB}$) had come to an abrupt stop (time point 2), while the MRSA culture ($COL_{spac::pbpB}$) continued to grow with gradually decreasing generation times for a total of 20 h after the removal of IPTG from the medium. In order to prevent the $COL_{spac::pbpB}$ culture from entering stationary phase during this extended period, the culture was periodically diluted to an OD_{620} of 0·05 in fresh TSB without IPTG each time the culture density had reached an OD_{620} of 0·4. Samples were removed from the $COL_{spac::pbpB}$ culture after the 11th hour (time point 3) and 20th hour (time point 4) of incubation in the IPTG-free medium. A portion of this culture was diluted to an OD_{620} of 0·05 in fresh TSB containing a full supplement of IPTG (100 μM), and growth of the culture was monitored. After a lag of about 2 h, the culture of $COL_{spac::pbpB}$ supplemented with IPTG began to grow at an increasing rate, eventually reaching a virtually normal doubling time. Samples were removed from this culture at time point 5, which corresponded to a total of 26·5 h of incubation from the beginning of the experiment. Determination of OD_{620} was carried out in a Spectrophotometer Ultraspec III (Pharmacia).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype/features	Source or reference
S. aureus		
RN4220	Mc^s, restriction negative	R. Novick†
$RN4220_{spac::pbpB}$	RN4220 with P_{spac}–*pbpB* fusion in the chromosome transformed with pMGPII, Em^r Cm^r	Pinho *et al.* (2001b)
COL	Homogeneous Mc^r, Em^s MIC 400 μg ml^{-1}*	Rockefeller University collection
$COL_{spac::pbpB}$	COL with P_{spac}–*pbpB* fusion in the chromosome transformed with pMGPII, Em^r Cm^r	Pinho *et al.* (2001b)
$COL_{spac::pbpB}$ + pLC4	$COL_{spac::pbpB}$/pLC4, negative control strain for assay of promoter activity (without plasmid pMGPII)	This study
$COL_{spac::pbpB}$ + pSG3	$COL_{spac::pbpB}$/pLC4 : : P_{pta} (without plasmid pMGPII)	This study
$COL_{spac::pbpB}$ + pLCSW-5	$COL_{spac::pbpB}$/pLC4 : : P_{mecA} (without plasmid pMGPII)	This study
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* ϕ80Δ*lacZ*ΔM15	Bethesda Research Laboratories
Plasmids		
pMGPII	*S. aureus* replicative plasmid containing *lacI* gene, Ap^r Cm^r	Pinho *et al.* (2001b)
pLC4	Promoterless *xylE* gene, Ap^r Cm^r	Ray *et al.* (1985)
pSG3	pLC4 containing the 443 bp fragment with the promoter region upstream from the *pta* gene of COL strain	This study
pLCSW-5	pLC4 containing the 545 bp fragment with the promoter region upstream from the *mecA* gene of COL strain	Wu *et al.* (2001)

*Oxacillin MIC.

†New York University School of Medicine.

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Population analysis profiles (PAPs). Antibiotic susceptibility of $COL_{spac::pbpB}$ was determined by PAPs, as described previously (Tomasz *et al.*, 1991), on agar plates containing various concentrations (mg l^{-1}) of oxacillin (Sigma), and supplemented with 0, 50 or 500 μM IPTG. Colonies were counted after incubation of the plates at 37 °C for 48 h.

Luciferin–luciferase assay. Cultures of *pbpB* conditional mutants of the sensitive MSSA strain $RN4220_{spac::pbpB}$ and the MRSA strain $COL_{spac::pbpB}$, grown overnight with aeration in TSB supplemented with 100 μM IPTG, were diluted to an OD_{620} of 0·05 in fresh TSB containing 100 μM IPTG, and incubated with aeration at 37 °C until they reached an OD_{620} of 0·4. The cultures were centrifuged, and traces of IPTG were removed, as described above. The bacteria were then resuspended in fresh pre-warmed TSB without IPTG to an initial OD_{620} of 0·05, and the amounts of ATP released by the bacteria were determined indirectly by the luciferin–luciferase assay, which was adapted from O'Neill *et al.* (2004). The ATP levels (expressed as relative luminescence units) produced by each strain were normalized to the OD_{620}.

DNA methods. DNA manipulations were performed by standard methods. Restriction enzymes were used as recommended by the manufacturer (New England Biolabs). Routine PCR amplification was performed with *Tth* DNA polymerase (HT Biotechnology). Wizard *Plus* Minipreps and Midipreps (Promega) systems were used for plasmid extraction. PCR and digestion products were purified with Wizard PCR Preps and Wizard DNA Clean-up systems (Promega). Ligation reactions were performed with T4 ligase (New England Biolabs). DNA sequencing was done at the Rockefeller University Protein/DNA Technology Center using the BigDye terminator cycle sequencing method, with either a 3700 DNA analyser for capillary electrophoresis, or ABI Prism 377 DNA sequencers for slab gel electrophoresis.

RNA preparation, Northern blotting, and analysis of the relative mRNA abundance. After extraction of RNA (Sobral *et al.*, 2003), 5 μg of each RNA sample was analysed by electrophoresis in a 1·2 % (w/v) agarose gel containing 0·66 M formaldehyde and MOPS (Sigma). The RNA was blotted onto Hybond-N^+ membranes (Amersham) with a turbo blotter alkaline transfer system (Schleicher & Schuell) with 20 × SSC. The PCR-amplified DNA probes were labelled with [α-^{32}P]dCTP (Amersham) by using a Ready to Go labelling kit (Amersham), and hybridized under high-stringency conditions. The blots were subsequently washed and autoradiographed. In order to compare the relative abundance of *mecA*, *pbpC* and 16S rRNA transcripts produced by $COL_{spac::pbpB}$ grown in the presence or absence of IPTG, a semi-quantitative method was introduced that allowed normalization of the Northern signals to an internal control (Matsuzaki *et al.*, 2001; Schelert *et al.*, 2004). For this purpose we used the value obtained for the Northern signal of the housekeeping gene *pta*, which encodes the phosphatase acetyl transferase, and which is constitutively expressed in *S. aureus* (Enright *et al.*, 2000). The intensities of the *mecA*, *pbpC* and 16S rRNA Northern blot signals were divided by that of the *pta* transcript in the same preparation.

Construction of promoter fusions. A DNA fragment of 443 bp, encompassing the region upstream of the *pta* gene from the MRSA strain COL, was amplified by PCR with *Pfu* Turbo DNA polymerase (Stratagen), and primers Pta*Bam*HI (5′-CGGGATCCGCTTGATCACCAGATTTTG-3′) and Pta*Hin*dIII (5′CGTAAGCTTCGTTCGTCCTCTCCTTCA-3′). The primers were engineered to carry restriction sites (underlined). The following PCR conditions were used: 94 °C for 4 min, 40 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min. The purified PCR product was subsequently cloned into plasmid pLC4 to generate the recombinant plasmid pSG3. The recombinant plasmid was then introduced into *S. aureus* RN4220 electrocompetent cells by electroporation with Gene Pulser apparatus (Bio-Rad), as described by Kraemer & Iandolo (1990), and finally transduced into strain $COL_{spac::pbpB}$ by using phage 80α, as previously described (Oshida & Tomasz, 1992), except that 100 μM IPTG was added to the medium for selecting $COL_{spac::pbpB}$ + pSG3 transductants.

Enzyme assays. Catechol 2,3-dioxygenase activity was used to measure the activity of *mecA* and *pta* promoters using the assay of Sheehan *et al.* (1992), except for the lysis of bacteria, which was done by using glass beads and FastPrep 120 (Bio 101 Savant) in 100 mM phosphate buffer (pH 7·5) containing 10 % (v/v) acetone. The reaction mixture, consisting of 100 mM potassium phosphate buffer (pH 7·5), 0·2 mM catechol, and 200 μg crude extract, was incubated at room temperature for 20 min, with A_{375} readings taken at 2 min intervals in an Ultraspec III spectrophotometer (Pharmacia). Enzymic assays were done in triplicate using crude extracts prepared on three different days. One milliunit corresponds to the formation at room temperature of 1 nmol 2-hydroxymuconic semialdehyde min^{-1}. Specific activity is reported in milliunits per milligram of total protein. Protein concentrations were measured by using the Modified Lowry Protein Assay (Pierce), with bovine serum albumin as a standard. $COL_{spac::pbpB}$ harbouring the plasmid pLC4 was used as the negative control.

Quantitative real-time PCR (Q-PCR). The levels of *pbpA*, *pbpB*, *pbpC*, *pbpD* and *mecA* mRNA transcripts in strains COL and $COL_{spac::pbpB}$ grown under different conditions were determined by Q-PCR. Total RNA extracts were used as templates for cDNA synthesis by random priming. To remove genomic DNA contamination, RNA samples were treated with the RNase-free DNase I set (Qiagen), and further purified with the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. RT-PCR was performed by using the GeneAmp RNA PCR kit (Applied Biosystems) in a 100 μl reaction mixture under the following conditions: 10 min at 25 °C, 60 min at 48 °C, and 5 min at 94 °C. To quantify cDNA generated by reverse transcription from target RNA, real-time PCR with SYBR Green I was performed by using SYBR Green PCR master mix in the ABI Prism 7900 Sequence Detection System (Applied Biosystems). The 25 μl reaction mixture contained 1 × i*Taq* SYBR Green PCR supermix with ROX (Bio-Rad), forward and reverse primers (each at a concentration of 100 nM), and 5 μl template (reverse transcription product). The primers were designed by Primer Express software (Applied Biosystems) and are listed in Table 2. The thermal conditions were: 2 min at 60 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. Three different RNA samples of the same culture and the various controls were processed in duplicate. Fluorescence was measured at the end of the annealing-extension phase of each cycle. A threshold value for the fluorescence of all samples was set manually. The reaction cycle at which the PCR product exceeds this fluorescence threshold was identified as the threshold cycle (C_T). The C_T was then converted to relative quantity of mRNA by using a standard curve. The standard curve was generated, via the Q-PCR program conditions, using serial twofold dilutions (2 ng to 66 pg) of genomic DNA. Relative gene expression was expressed as a ratio of target gene (*mecA*, *pbpA*, *pbpB*, *pbpC* and *pbpD*) concentration to housekeeping gene (*pta*) concentration. To verify the specificity of the PCR amplification products, melting curve analyses were performed using the following thermal cycling profile: 95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s, with 2 % increment.

Membrane purification. Membrane proteins were prepared as previously described (Sieradzki *et al.*, 1999). Protein concentrations were determined using the Modified Lowry Protein Assay (Pierce), with the bovine serum albumin as a standard.

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Table 2. Primers used for Q-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
pta	AGAAGCAATCATTGATGGCGA	ACCTGGCGCTTTTTTCTCAG
mecA	AACATTGATCGCAACGTTCAAT	TGGTCTTTCTGCATTCCTGGA
pbpA	TTTTAGCATACAGTCAGCGACCA	TCCAGGCTCGTATGTGTTTTGA
pbpB	CAGAAACCAAGCAACAGATCCTC	TTCAATGGCAGGTCCATACG
pbpC	ACATTCTTGCGCTTGCCG	TACAGAAGATCCAACCGCAAATT
pbpD	AGGTTTGTCGGCTGCATACG	ACGCTGGATTCCACTTAGTATCG

Western blotting analysis. For detection of PBP2A in the membrane protein fraction, 60 μg of each membrane protein preparation was resolved on an 8 % (w/v) acrylamide/0·06 % (w/v) bisacrylamide gel at a constant current of 20 mA, and transferred to a nitrocellulose membrane by Western blotting, as previously described (Wu *et al.*, 2001). Incubation with a monoclonal antibody against PBP2A of an MRSA strain (Eli Lilly & Co.) was carried out with the ECL Western blot analysis system (Amersham) (Wu *et al.*, 2001).

Penicillin-binding assays. Membrane proteins (150 μg protein per sample) were labelled with benzyl[^{14}C]penicillin potassium ($5{\cdot}846 \times 10^{12}$ Bq mg^{-1}; GE Healthcare), at a final concentration of 20 mg l^{-1} in a 20 μl volume, for 10 min at 30 °C. Addition of an excess of unlabelled benzylpenicillin [1000 mg l^{-1} in 10 % (v/v) SDS] was used to stop the reaction. An equal volume of sample buffer [125 mM Tris/HCl, pH 6·8, 4 % (v/v) SDS, 2 % (w/v) glycerol, 100 mg bromophenol blue l^{-1}, and 1·43 M 2-mercaptoethanol] was added to the samples, which were boiled for 5 min at 95 °C. Protein separation on SDS-PAGE was carried out on an 8 % (w/v) acrylamide/0·06 % (w/v) bisacrylamide gel, at a constant current of 20 mA. The gel was exposed to a tritium storage phosphor screen (GE Healthcare) for 2 weeks, and the screen was scanned with a typhoon scanner (GE Healthcare).

Cell wall analysis. Cell walls were isolated; the peptidoglycan was purified, and hydrolysed with M1 muramidase, and the resulting muropeptides were reduced with borohydride and separated by reverse-phase HPLC, as previously described (De Jonge *et al.*, 1992).

Electron microscopy. Aliquots (1 ml) of bacterial cultures were harvested by low-speed centrifugation, and fixed with 1 ml 2·5 % (v/v) glutaraldehyde. Electron microscopy was done at the Electron Microscopy Service of The Rockefeller University.

Reproducibility. All experiments, including growth curves, Northern blots and PAPs, were repeated at least five to seven times, and HPLC profiles were obtained at five different time points in order to assure reproducibility. Details about the reproducibility of Q-PCR and promoter fusion experiments are described below. Determination of protein profiles and the radioactive PBP binding assay (Fig. 2), ATP release and Western blotting were only performed once. Electron micrographs were selected after scanning numerous fields at low-power magnification to make sure that photos presented at high-power magnification represented 'typical' structures.

RESULTS

Inhibition of *pbpB* transcription in an MSSA strain: effect on growth, cell structure and cellular amounts of PBP2

MSSA strain RN4220$_{spac::pbpB}$ was resuspended in growth medium free of IPTG (time point 1, Fig. 1), and transcription of *pbpB* and rate of growth (OD_{620} increase) were followed over time. Transcription of *pbpB* was turned off within 10 min after the removal of the inducer, as indicated by Northern blotting (data not shown). On the other hand, the culture continued to grow for the first 2 h after the removal of IPTG from the medium, with a doubling time virtually the same as that in the presence of IPTG (55 versus 49 min). After about 3 h growth (time point 2), corresponding to approximately 2·5 generation times, the OD_{620} increase of strain RN4220$_{spac::pbpB}$ came to an abrupt halt. Electron microscopic observation showed the accumulation of empty cells, suggesting lysis, and extending the incubation time by a further 4 h resulted in the loss of about 50 % of the viable titre of the culture (data not shown). Testing the growth medium with the luciferin–luciferase assay indicated extensive release of ATP during incubation in the IPTG-free medium (see Fig. 1a–c).

Total protein extracts were prepared from RN4220$_{spac::pbpB}$ cultures grown in the presence of 100 μM IPTG, and in IPTG-free medium, in order to compare the number of protein bands, and also for testing PBP patterns using [^{14}C]penicillin. Fig. 2 shows the Coomassie-blue-stained gel, and the PBP pattern obtained by the radioactive-penicillin-binding assay. A diminished amount of the Coomassie-stained material was evident in the position corresponding to that of PBP2 in the stained gel, and PBP2 was no longer detectable in the penicillin-binding assay. Large amounts of PBP4 were observed for the RN4220$_{spac::pbpB}$ mutant grown in the absence of the inducer, suggesting that PBP2, which has previously been proposed to cooperate functionally with PBP4 in the cross-linking of peptidoglycan (Leski & Tomasz, 2005), may exert some kind of positive control in the translation of this PBP.

Inhibition of *pbpB* transcription in an MRSA strain: effect on growth, cell structure and cellular amounts of PBP2A

The design of this experiment was exactly the same as that for RN4220$_{spac::pbpB}$. MRSA strain COL$_{spac::pbpB}$ was resuspended in growth medium free of IPTG, and the transcription of *pbpB* and rate of growth (OD_{620} increase) were followed over time. Similar to RN4220$_{spac::pbpB}$, in COL$_{spac::pbpB}$, transcription of *pbpB* was turned off 10 min after the removal of the inducer (data not shown), but the culture continued to grow for 20 h. During the first 3 h, the doubling time of the culture was virtually the same as that in

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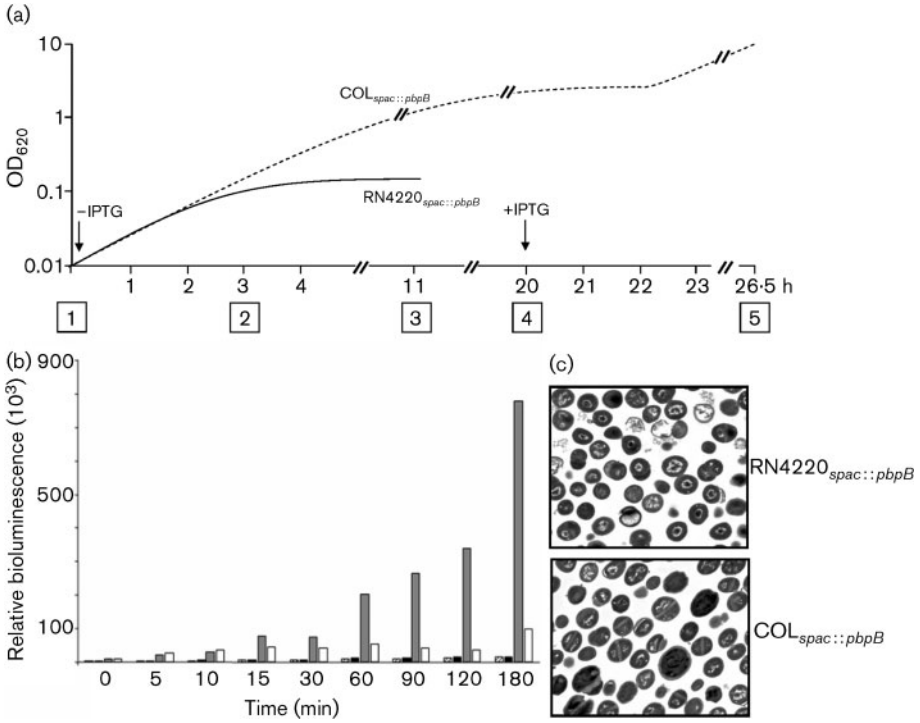

Fig. 1. Growth, cell morphology and ATP release in *pbpB* conditional mutants of *S. aureus*. (a) Schematic representation of the growth curves of *S. aureus* strains $COL_{spac::pbpB}$ (with *mecA*) and $RN4220_{spac::pbpB}$ (without *mecA*) grown in the presence and absence of IPTG. Cultures of these strains grown with an optimal concentration of IPTG (100 μM), and the appropriate antibiotics, were centrifuged, washed twice, and resuspended in fresh TSB without IPTG (time point 1). Growth of the mutant cultures was monitored as OD_{620} in the absence of IPTG until either the mutant cells stopped growing, or the growth rate had slowed down considerably (time points 2 and 4, respectively). At time point 4, IPTG was added back to the medium, and growth was followed until the MRSA mutant culture reached a normal generation time (time point 5). (b) ATP production in the conditional mutant strains $RN4220_{spac::pbpB}$ and $COL_{spac::pbpB}$ grown in the presence and absence of 100 μM IPTG at different time points, measured by using the luciferin–luciferase assay. The experiment was performed once. The total ATP content was expressed as relative luminescence units, and normalized to the OD_{620}: $COL_{spac::pbpB}$ grown in the absence of IPTG (hatched bars), $COL_{spac::pbpB}$ grown with 100 μM IPTG (black bars), $RN4220_{spac::pbpB}$ grown in the absence of IPTG (grey bars), and $RN4220_{spac::pbpB}$ grown with 100 μM IPTG (white bars). (c) Impaired cell division of $RN4220_{spac::pbpB}$ mutant cells lacking transcription of the *pbpB* gene. Electron micrographs of $RN4220_{spac::pbpB}$ and $COL_{spac::pbpB}$ grown for 3 h in the absence of IPTG (time point 2) are shown.

the presence of IPTG (51 versus 48 min), but from the third hour on, the doubling time began to gradually increase, to reach – between the 11th and the 20th hour of incubation – a value as long as 180 min (see Fig. 1). A portion of this extensively 'starved' culture was resuspended in medium containing an optimal concentration of IPTG. Within 30 min after readdition of IPTG, the *pbpB* transcription signal reappeared (data not shown), and, after a lag of about 2 h, the culture began to grow with a doubling time approaching that of the original culture.

Samples were removed from $COL_{spac::pbpB}$ grown in the absence of IPTG at several times during the experiment: at the beginning of cultivation (time point 1), after 20 h incubation in the IPTG-free medium (time point 4), and at a time after the readdition of IPTG to the medium when the culture of $COL_{spac::pbpB}$ had resumed normal growth (time point 5). Electron microscopic thin sections began to show a few cells with extensive morphological abnormalities by the end of the 3-h period of growth in the absence of IPTG (data not shown).

The release of ATP into the growth medium was relatively small compared with the extensive release of ATP observed in samples from $RN4220_{spac::pbpB}$ starved for the same length of time (see Fig. 1b). Cells with abnormal morphology became predominant in the cultures of $COL_{spac::pbpB}$ grown for 20 h in the absence of IPTG (see Fig. 3b). The abnormal morphology included a block in cell separation, enlarged and abnormally placed septa, and amorphous cell-wall-like material accumulating at the cell surface. These abnormal structures gradually decreased in number as the bacteria resumed multiplication in the IPTG-containing medium (see Fig. 3b).

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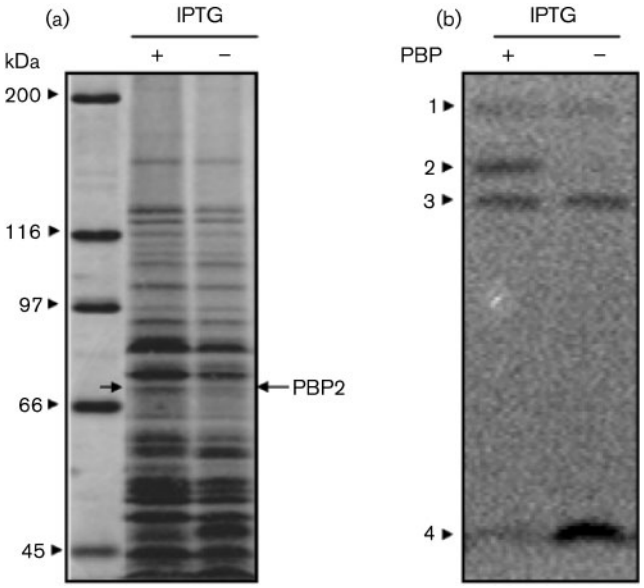

Fig. 2. Production of PBP2 in RN4220$_{spac::pbpB}$ grown in the presence and absence of IPTG. Membrane proteins extracted from RN4220$_{spac::pbpB}$ grown in medium supplemented with 100 μM (+IPTG), or incubated in the absence of the inducer for 3 h (−IPTG), were run in an SDS-PAGE gel, and stained with Coomassie blue (a). (b) The same membrane preparations were assayed for PBP patterns using [^{14}C]penicillin.

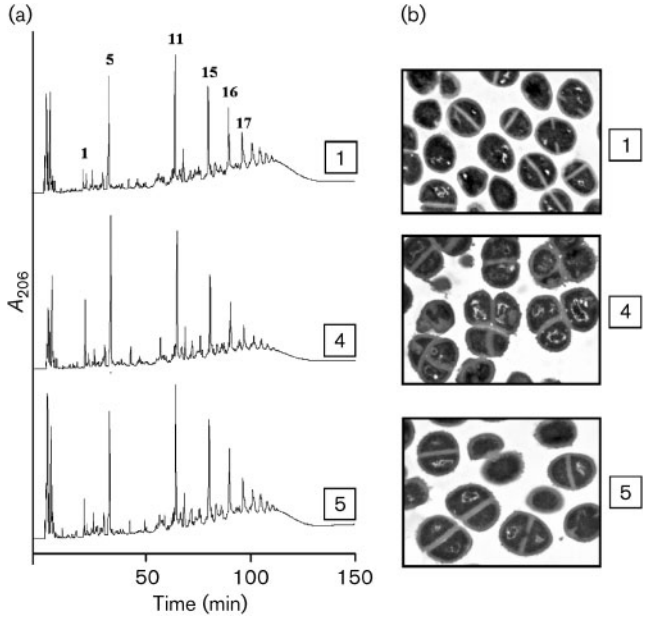

Fig. 3. (a) Effect of *pbpB* transcription on the composition of cell wall peptidoglycan. Peptidoglycan was prepared from COL$_{spac::pbpB}$ grown in the presence or absence of IPTG for different lengths of time, and muropeptide patterns were analysed by HPLC, as described in Methods. Top, COL$_{spac::pbpB}$ grown in optimal IPTG conditions (time point 1). Middle, COL$_{spac::pbpB}$ grown in the absence of IPTG for 20 h (time point 4). After 20 h growth in the absence of the inducer, IPTG was added to the growth medium of the mutant culture. Bottom, COL$_{spac::pbpB}$ reincubated with 100 μM IPTG for 6·5 h (time point 5). Numbers above the peaks in the HPLC elution profile identify the structure of the particular muropeptide, as described earlier (De Jonge *et al.*, 1992). The poorly resolved part ('hump') of the HPLC profile eluting with retention times of greater than 90 min contains highly cross-linked oligomeric components. (b) Cell morphology of COL$_{spac::pbpB}$ grown in the presence and absence of IPTG for different time periods. Shown are electron micrographs from COL$_{spac::pbpB}$ grown in an optimal IPTG concentration (time point 1), and in the absence of IPTG for 20 h (time point 4). After 20 h growth, IPTG was added to the medium, and the mutant culture was incubated for 6·5 h (time point 5).

Cell wall peptidoglycan was prepared from COL$_{spac::pbpB}$ at the beginning (time point 1), after 20 h growth in IPTG-free medium (time point 4), and from a culture that had resumed normal growth after the readdition of IPTG to the medium (time point 5). Fig. 3(a) shows the increase in the relative proportions of the monomeric muropeptides 1 and 5, and a parallel decrease in the proportion of the highly cross-linked muropeptides (elution time >90 min) in the cell walls of bacteria grown in the absence of IPTG for 20 h. These structural abnormalities had virtually disappeared from the cell wall of the culture that had resumed growth in the IPTG-containing medium.

Effect of inhibited *pbpB* transcription on the transcription of *mecA*, and on the production of PBP2A

Samples were removed from COL$_{spac::pbpB}$ grown in IPTG-free medium, and also from the same culture in which *pbpB* transcription was reinitiated (by addition of IPTG to the medium). The samples were used to test the effect on the transcription of a variety of genetic determinants (determined by Northern blotting), and also on the cellular amounts of the *mecA* gene product PBP2A (determined by Western blotting). All samples were removed from cultures at an OD_{620} of 0·4.

The extended growth in the absence of *pbpB* transcription did not alter the transcription of *pta*, 16S rRNA and *pbpC*, but removal of IPTG caused an immediate overexpression of *mgtB*, a mono-functional TGase, which showed normal levels of expression equally promptly upon renewed transcription of *pbpB*. Most interestingly and unexpectedly, growth in the IPTG-free medium caused a gradual slow decrease in the intensity of the transcriptional signal of *mecA*, which was then reversed as the culture resumed growth following readdition of IPTG to the medium (Fig. 4a). Testing the transcription of *mecA* in COL and COL+pMGPII (Pinho *et al.*, 2001b) grown in increasing concentrations of IPTG demonstrated that neither LacI, nor IPTG itself, interfered with expression of *mecA*.

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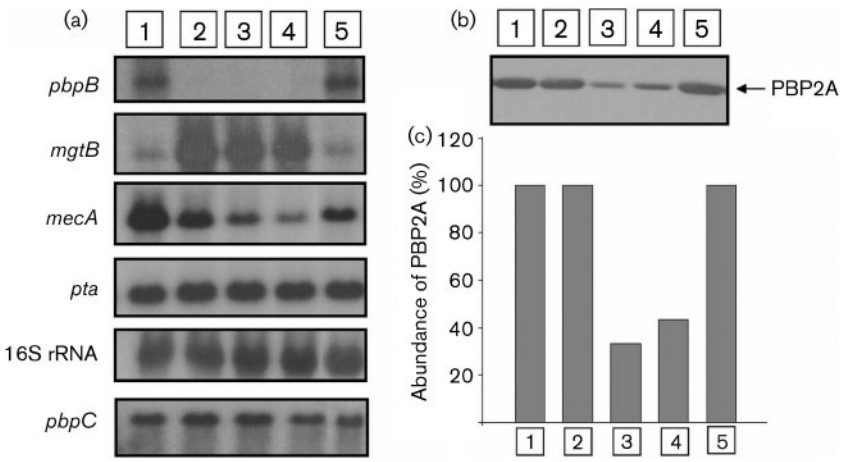

Fig. 4. Changes in transcriptional profiles and PBP2A concentration during growth of $COL_{spac::pbpB}$. (a) Transcription analysis was done for *pbpB*, *mgtB*, *mecA*, *pta*, 16S rRNA and *pbpC* by Northern blotting in cultures of the promoter-controlled *pbpB* mutant $COL_{spac::pbpB}$ grown in the absence of IPTG at different time points. $COL_{spac::pbpB}$ was grown in the presence of an optimal IPTG concentration (time point 1), and subsequently washed and resuspended in TSB. The mutant strain was incubated in IPTG-free medium for 3 h (time point 2), 11 h (time point 3) and 20 h (time point 4). After 20 h growth, IPTG was added back to medium, and the culture was grown for 6·5 h (time point 5). (b) Membrane proteins were extracted, and tested by Western blotting for the production of PBP2A at the time points indicated. (c) Relative abundance of PBP2A at the time points indicated.

Parallel samples removed at the time points 1–5 showed a decrease in the Western blot signal for PBP2A, in parallel with the decrease of transcription of *mecA*. Reappearance of a stronger Western blot signal was apparent in the samples taken from the culture at time point 5, i.e. in bacteria that had resumed growth with normal doubling time (see Fig. 4b, c).

Effect of *pbpB* transcription on the level of oxacillin resistance

An overnight culture of $COL_{spac::pbpB}$ was plated on three sets of agar media. In the first set, the medium contained no IPTG; in the second and third sets, the medium contained 50 and 500 μM IPTG, respectively. The agar media were then supplemented with increasing concentrations of oxacillin, and the bacterial culture was plated at different cell concentrations for the analysis of PAPs (as described in Methods). As an additional control, strain COL without the $P_{spac::pbpB}$ construct was also plated. Fig. 5(a) shows that the IPTG concentration in the agar medium had a profound effect on the shape of the PAP curves: the MIC value for the majority of the cells increased with the concentration of IPTG, which also increased the relative homogeneity of the PAP profile. It is noteworthy that the high-level and homogeneous phenotype of the parental strain COL was not attained, even at the optimal concentration of the inducer. The abundance of *pbpB* transcript produced by the conditional mutant is never as high as in strain COL due to the disruption of the *prfA–pbpB* operon, the major transcript of *pbpB* gene, after integration of the suicide vector with the IPTG-inducible P_{spac} promoter into the COL chromosome (Pinho *et al.*, 1998). Fig. 5(b) shows the degree of transcription of *pbpB* at the corresponding concentrations of IPTG, and also demonstrates the existence of only one *pbpB* transcript in the COL *pbpB* mutant.

Parallel changes in the transcription of *pbpB* and *mecA*, as determined by Q-PCR

The relative abundance of *pbpB* and *mecA* transcripts produced by $COL_{spac::pbpB}$ was determined in cultures grown for 20 h in the absence of IPTG (time point 4), and also after IPTG was added back to the medium (time point 5), using Q-PCR. The relative abundance of *pbpA*, *pbpC* and *pbpD* was also determined. The cDNA of each target gene (*mecA*, *pbpA*, *pbpB*, *pbpC* and *pbpD*) was amplified, and quantitatively estimated in duplicate, as described in Methods. The experiment was repeated three times from three independent RNA preparations, and Table 3 summarizes the data. Expression of *pbpB* was suppressed, and transcription of *mecA* was significantly lower, in $COL_{spac::pbpB}$ grown in the absence of the inducer. Transcription of *mecA* increased approximately twofold in parallel with the renewed expression of *pbpB* following the readdition of IPTG to the growth medium. Transcription of *pbpA*, *pbpC* and *pbpD* remained unaffected.

Effect of *pbpB* transcription on the *mecA* promoter

The *mecA* and *pta* promoters from *S. aureus* COL strain were cloned in pLC4 in front of the *xylE* gene to obtain pLC4::P_{mecA} and pLC4::P_{pta}; the recombinant plasmids were transformed into $COL_{spac::pbpB}$ to obtain $COL_{spac::pbpB}$+pLCSW-5, and $COL_{spac::pbpB}$+pSG3 constructs, respectively. After growth in the presence of an optimal concentration of IPTG, and in the absence of the inducer, for 11 h, crude enzyme extracts were prepared from the two constructs, and the specific catechol 2,3-deoxygenase enzyme activities were determined by a colorimetric assay. For the construct $COL_{spac::pbpB}$+pLCSW-5 this assay indicated that the activity of the *mecA* promoter of

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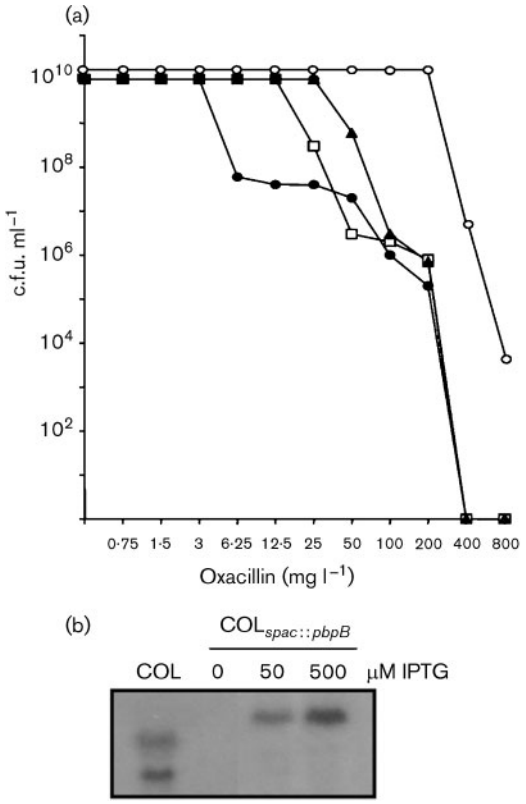

Fig. 5. Expression of β-lactam antibiotic resistance at different levels of *pbpB* transcription. (a) COL$_{spac::pbpB}$ was plated on TSA agar containing increasing concentrations of oxacillin, and supplemented with different concentrations of IPTG. Bacterial colonies were counted after 48 h incubation at 37 °C. Shown are oxacillin PAPs of strains COL (○), COL$_{spac::pbpB}$ grown without IPTG (●), COL$_{spac::pbpB}$ grown with 50 μM IPTG (□) and 500 μpM IPTG (▲). (b) Northern blotting analysis of *pbpB* transcription in COL$_{spac::pbpB}$ grown in increasing concentrations of IPTG. The parental strain COL was used as a control.

COL$_{spac::pbpB}$ grown in a medium lacking IPTG was one-third of the activity of the same promoter when the mutant strain was incubated in an optimal IPTG concentration. No significant changes were detected in the specific enzyme activity extracted from the construct COL$_{spac::pbpB}$+pSG3 grown in the absence and presence of IPTG.

DISCUSSION

In contrast to the MSSA strain, the MRSA strain COL$_{spac::pbpB}$ continued to grow in the IPTG-free medium for at least 20 h, far beyond the short 3 h time period after which the growth of the conditional mutant RN4220$_{spac::pbpB}$ had stopped, and the cells had begun to show signs of lysis. This confirms the earlier observation that PBP2A can replace the essential function of PBP2 for growth (Pinho *et al.*, 2001b). Presumably, the 'take-over' by PBP2A occurred at the time when PBP2 was no longer detectable by the penicillin-binding assay (see Fig. 2). An effective take-over by PBP2A is also supported by the analysis of the peptidoglycan isolated from COL$_{spac::pbpB}$ after 20 h growth in the IPTG-free medium, when only relatively minor abnormalities were evident (an increase in the proportion of monomeric muropeptides, and some decrease in highly cross-linked oligomers) (see Fig. 3).

Nevertheless, the gradual increase in the doubling time of COL$_{spac::pbpB}$, reaching 180 min after 11 h growth in the IPTG-free medium, indicates that the capacity of the *mecA* gene product PBP2A to fully replace the native PBP2 is not without limits. This was also supported by the accumulation in the culture of structurally abnormal cells, which showed blocked cell separation, widening of the septal areas, and appearance of amorphous cell-wall-like material at the bacterial surface (Fig. 3b). These structural features are very similar to those described in *S. aureus* with an inhibited autolytic system, which can be fully reversible (Sieradzki & Tomasz, 2006). This interpretation is also consistent with the fact that the morphological abnormalities were reversible in the COL$_{spac::pbpB}$ culture, since they became extremely rare after resumption of normal growth following the readdition of IPTG to the medium (Fig. 3b).

In order to find the reason for decreasing growth rates of COL$_{spac::pbpB}$, we tested the rate of transcription of *mecA*,

Table 3. Quantification of *mecA*, *pbpA*, *pbpB*, *pbpC* and *pbpD* transcripts by Q-PCR in COL and COL$_{spac::pbpB}$ grown in the absence of IPTG for 20 h (time point 4), and then incubated with IPTG for 6·5 h (time point 5)

Data are means ± SD for three independent experiments.

Gene	Relative abundance of mRNA transcript		
	COL	COL$_{spac::pbpB}$ (time point 4)	COL$_{spac::pbpB}$ (time point 5)
pbpA	1	0·927 ± 0·140	0·982 ± 0·126
pbpB	1	0·017 ± 0·002	0·80 ± 0·29
pbpC	1	0·876 ± 0·058	1·395 ± 0·357
pbpD	1	1·137 ± 0·185	1·280 ± 0·303
mecA	1	0·310 ± 0·076	0·659 ± 0·145

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and the cellular amounts of PBP2A, after various periods of incubation of the bacteria in IPTG-free medium. Surprisingly, both the abundance of the *mecA* transcript, and the amounts of PBP2A, showed a significant decline as the time of incubation in the IPTG-free medium progressed, suggesting that the inhibition of transcription of *pbpB* – directly or indirectly – impacts on the transcription, and also on the translation, of the resistance gene *mecA*.

Strain COL carries a *mecA* gene as part of an SCC*mec* type I cassette, which does not contain functional *mecI*/*mecR1*, and the bacterium also lacks the *blaI*/*blaR1* system (Oliveira *et al.*, 2001). In this strain, *mecA* is constitutively expressed, producing high levels of *mecA* transcript, and large amounts of PBP2A (Hackbarth & Chambers, 1993).

Our observations suggest that the transcription of this *mecA* gene lacking the dedicated controlling elements is somehow influenced by the regulatory circuitry that controls transcription of the native *pbpB*. This apparent 'regulatory' connection appears to be specific, since testing the effect of the blocked *pbpB* transcription on the transcription of a number of other genetic determinants failed to detect similar changes. These determinants included *pta*, the determinant of 16S rRNA, and *pbpC*, *A* and *D*. Most importantly, the decline in transcription of *mecA* that was observed in the PBP2-starved bacteria was reversed when the transcription of *pbpB* was reinitiated by adding the inducer back to the growth medium (Fig. 4). These parallel changes in transcription of *pbpB* and *mecA* were confirmed by Q-PCR, and by the results of promoter fusion experiments, which suggests that some factor accumulating in bacteria with an inhibited *pbpB* transcription can influence the reading of the *mecA* promoter.

An interesting consequence of the removal of IPTG from the growth medium of $COL_{spac::pbpB}$ was the overexpression of *mgtB*, a monofunctional TGase of *S. aureus*, which rapidly followed the inhibition of *pbpB* transcription. Readdition of IPTG to the medium caused an equally rapid reduction in the abundance of the *mgtB* transcript, suggesting the existence of a regulatory system that may provide an alternative TGase for the cells in which the TGase activity of PBP2 is not available. Experiments are in progress to test whether or not the TGase activity of *mgtB* becomes essential for the bacteria in which PBP2A replaces the transpeptidase activity of PBP2.

A previous study demonstrated that the TGase domain of PBP2 becomes essential for growth and cell wall synthesis when $COL_{spac::pbpB}$ is exposed to high concentration of oxacillin (Pinho *et al.*, 2001a). In view of the new observation on the overexpression of *mgtB* under the same conditions, we repeated titration of oxacillin resistance as a function of the concentrations of IPTG in the medium. Fig. 5 shows that both the resistance level of the majority of the bacteria, and the population profile of the culture, were functions of the abundance of the *pbpB* transcript, indicating that MgtB may not be able to replace efficiently the TGase function of PBP2 in the assembly of peptidoglycan. These data also suggest that the optimal expression of resistance to oxacillin may require a stoichiometric balance between the two proteins PBP2 and PBP2A, which are known to cooperate in cell wall synthesis (Pinho *et al.*, 2001a).

The observation described in this paper, namely that transcription of the resistance determinant *mecA* is under the 'control' of the determinant of an essential native *pbp*, is not only surprising, but counterintuitive as well, since survival and multiplication of *S. aureus* with an inhibited *pbpB* is dependent on the continued expression and function of the resistance gene *mecA* and its protein product. However, the physiological scenario of a *S. aureus pbpB* conditional mutant with inhibited PBP2 production is clearly an abnormal one, and the 'normal' role of *mecA* gene in *S. aureus* is to provide antibiotic resistance, not to replace the native PBP2 in the absence of antimicrobial agents in the medium.

The tuning down of transcription of *mecA*, and the decline in the cellular amounts of PBP2A, follow the halt in *pbpB* transcription only after a prolonged lag, suggesting that the link between transcription of these two genes is an indirect one. Possibly, PBP2A somehow 'senses' the lack of PBP2, the native cell wall synthesis protein with which the resistance protein is clearly able to cooperate in the functional sense (Pinho *et al.*, 2001a). We propose that the new evidence described in this paper, documenting a connection between the transcription of *pbpB* and *mecA*, may be an indication of an attempt by the *S. aureus* cell to integrate the uncontrolled transcription of *mecA* into a regulatory circuitry that allows harmonious functioning of cell wall synthetic enzymes. The mechanism by which transcription of *mecA* is downregulated in the absence of *pbpB* transcription is currently under investigation.

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