An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci

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Edited by John J. Mekalanos, Harvard Medical School, Boston, MA, and approved July 17, 2001 (received for review May 24, 2001)

The blanket resistance of methicillin-resistant *Staphylococcus aureus* to all β-lactam antibiotics—which had such a devastating impact on chemotherapy of staphylococcal infections—is related to the properties of the key component of this resistance mechanism: the "acquired" penicillin-binding protein (PBP)-2A, which has unusual low affinity for all β-lactam antibiotics. Until now, the accepted model of resistance implied that in the presence of β-lactam antibiotics in the surrounding medium, PBP2A must take over the biosynthesis of staphylococcal cell wall from the four native staphylococcal PBPs because the latter become rapidly acylated and inactivated at even low concentrations of the antibiotic. However, recent observations indicate that this model requires revision. Inactivation of the transglycosylase domain, but not the transpeptidase domain, of PBP2 of *S. aureus* prevents expression of β-lactam resistance, despite the presence of the low-affinity PBP2A. The observations suggest that cell-wall synthesis in the presence of β-lactam antibiotics requires the cooperative functioning of the transglycosylase domain of the native staphylococcal PBP2 and the transpeptidase domain of the PBP2A, a protein imported by *S. aureus* from an extra species source.

Since their first appearance in clinical specimen in the early 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) strains spread globally, and by the mid-1980s they emerged as the most important nosocomial pathogens worldwide. The central genetic component of the resistance mechanism in these bacteria is *mecA,* which—embedded in a larger block of "foreign" DNA—is not native to *S. aureus*, but was imported from an as yet unidentified extraspecies source (1, 2). The *mecA* gene encodes for a 78-kDa penicillin-binding protein (PBP) 2A, which has unusually low affinity for all β-lactam antibiotics—a property that translates to the virtually complete resistance of MRSA strains to the entire β-lactam family of antimicrobial agents. Based on homologies with other PBPs, PBP2A was suggested to have both transpeptidase (TPase) and transglycosylase (TGase) activity (3), although neither of these activities was proven by biochemical assays using the purified protein. An attachment TPase activity attributed to PBP2A in methicillin-resistant *Staphylococcus epidermidis* has been demonstrated (4). More recent data (5) groups PBP2A with high molecular weight class-B PBPs, a family of proteins composed of a TPase domain and a non-penicillin-binding domain of unknown function.

PBPs are involved in the assembly of the bacterial cell-wall peptidoglycan. The strategy of β-lactam resistance in MRSA involves the addition of the new, acquired PBP2A to the complement of the four native staphylococcal PBPs. In our current model of methicillin resistance, the low-affinity PBP2A is assumed to take over the cell wall biosynthetic functions of normal PBPs in the presence of β-lactam antibiotics, which rapidly acylate (and inactivate) each of the four native staphylococcal PBPs at concentrations that are far below the minimum needed to inhibit the growth of most MRSA strains (6, 7).

In view of this, it was very surprising to find that transposon inactivation of the structural gene of the *S. aureus* PBP2 in the highly methicillin-resistant strain COL caused a massive—several hundred fold—reduction in the methicillin minimum inhibitory concentration (MIC) value, from 800 to 12 μg/ml, despite the fact that the transposon mutant produced normal amounts of PBP2A (8). Although this finding clearly demonstrated that an intact PBP2 was essential for optimal expression of methicillin resistance, the mechanism involved was perplexing because titration of the antibiotic binding capacity of PBP2 in membrane preparations clearly showed that this protein is fully acylated in the presence of a few micrograms of β-lactam antibiotics (9), i.e., several orders of magnitude below the MIC value of strain COL.

Based on amino acid sequence alignment with *Escherichia coli* PBP1A, as well as other bifunctional PBPs, the staphylococcal PBP2 belongs to high molecular weight class-A PBPs, a family of bifunctional proteins with a N-terminal TGase domain and a C-terminal TPase domain (5, 10). In an attempt to elucidate the role of PBP2 in the expression of methicillin resistance, we selectively inactivated each of the two domains of this protein by point mutations in key amino acids and determined the impact on antibiotic resistance in the background of the highly and homogeneously resistant MRSA strain COL.

Materials and Methods

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* strains were grown on tryptic soy broth (TSB, Difco) with aeration, at 37°C, except where indicated. *E. coli* strains were grown in Luria–Bertani broth (Difco) with aeration at 37°C.

DNA Methods. Routine DNA manipulations were performed by using standard methods (13, 14). Restriction enzymes were purchased from New England Biolabs. Plasmid DNA was purified by using Wizard Plus SV Minipreps or Midipreps DNA Purification Systems (Promega). Except where noted, PCR was done with the GeneAmp PCR reagent kit with AmpliTaq polymerase (Perkin–Elmer) using 20 pmol of each primer under the following conditions: 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 3 min; and one final extension step of 72°C for 5 min. PCR products were cleaned by using Wizard PCR Preps DNA Purification System (Promega). DNA sequencing was done at The Rockefeller University Protein/DNA Technology Center with the BigDye terminator-cycle sequencing method and either the 3700 DNA Analyzer for

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; PBP, penicillin-binding protein; TPase, transpeptidase; TGase, transglycosylase; PAPs, population analysis profiles; MIC, minimum inhibitory concentration.

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 www.pnas.org/cgi/doi/10.1073/pnas.191260798

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
S. aureus RN4220	MSSA strain restriction−	R. Novick
S. aureus COL	Homogeneous MRSA (MIC 1,600 μg/ml)	RU collection
COLpSPTM4–5	COL with Ser-398→Gly mutation in functional copy of *pbp2*	This study
COLpSPTM4–10	COL with Ser-398→Gly mutation in nonfunctional copy of *pbp2*	This study
COLTG42	COL with Glu-114→Gln mutation in functional copy of *pbp2*	This study
COLTG2–1	COL with Glu-114→Gln mutation in nonfunctional copy of *pbp2*	This study
pSP64E	Integrational vector for *S. aureus*	Ref. 11
pSPT181c	Thermosensitive shuttle vector *E. coli*/*S. aureus*	Ref. 12
pPBP2M1	pSP64E/2.1 kb encoding amino acids 54 to C-terminal of *pbp2*	This study
pPBP2M4	pPBP2M1 with Ser-398→Gly mutation	This study
pPBP2M5	pPBP2M1 with second *Hpa*I site of insert removed	This study
pPBP2M6	pPBP2M1 with Glu-114→Gln mutation	This study
pSPTM4	pSPT181c/2.1-kb insert from pPBP2M4	This study

R. Novick is located at Skirball Institute, New York; MSSA, methicillin-susceptible *S. aureus*; RU, The Rockefeller University.

capillary electrophoresis or the Applied Biosystems Prism 377 DNA Sequencers for slab-gel electrophoresis.

Mutagenesis of TPase Domain Using an Integrational Plasmid. A fragment of *pbp2* gene starting at codon 53, and therefore not including the membrane anchor, was amplified by PCR using PfuTurbo DNA polymerase (Stratagene) and primers PBP2Mut1 (5′-aatcccgggtgtttgcttattatgcttgg-3′) and PBP2P7B (5′-gcggatcctcccaccataaaagatgaag-3′). The following conditions were used: 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 2 min 30 s; and one final extension step of 72°C for 5 min. The PCR product was digested with *Ava*I and *Bam*HI and cloned into the integrational plasmid pSP64E, giving rise to plasmid pPBP2M1, which was sequenced. Mutation of the active serine (Ser_{398}→Gly) and introduction of a *Kpn*I restriction site was done by using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and primer PBP2Mut6 (5′-gatcctcaccctactggt-**ggtacc**ttaaaacctttcttagcg-3′) and its reverse, PBP2Mut7. The QuikChange Site-Directed Mutagenesis kit was used for the introduction of all mutations. The plasmid containing the mutation was sequenced and named pPBP2M4. Plasmid pPBP2M4 was introduced into RN4220 by electroporation essentially as described (15). Chromosomal DNA from the transformants was purified by using the Wizard Genomic DNA Purification System (Promega), except that cells were lysed with lysostaphin (300 μg/ml) in the presence of RNase (300 μg/ml) for 30 min at 37°C. Chromosomal DNA was used as PCR template to amplify the functional copy of *pbp2*, by using primers PrfA1 (5′-atgtcaactatcctaagcgg-3′) and pSP64–1 (5′-cgcaacgcaattaatgtgag-3′), and the nonfunctional copy, by using primers Tn551L2 (5′-caatcactctcggacaatac-3′) and PBP2P5 (5′-gaatcgtaggtggatttctt-3′). The PCR products were digested with *Kpn*I and the restriction products were run on an agarose gel to detect which transformants have the mutation in the functional or the nonfunctional copy. As a control, a silent mutation that removes an *Hpa*I site located 37 nt upstream of the codon 398 was also introduced in pPBP2M1 by site-directed mutagenesis using primer PBP2Mut8 (5′-gatttcaaagacgtcgt**c**aacagaaaccaagcaac-3′) and its reverse, PBP2Mut9. The plasmid was sequenced, named pPBP2M5, and electroporated into RN4220. Analysis of the transformants was done as described above, but the PCR products were digested with *Hpa*I.

Mutagenesis of TPase Domain Using a Thermosensitive Plasmid. The insert from pPBP2M4 was excised by using *Ava*I and cloned into the thermosensitive shuttle vector pSPT181c, giving rise to plasmid pSPTM4. This plasmid was electroporated into RN4220 at 30°C and selected for with 20 μg/ml of chloramphenicol. Plasmid pSPTM4 was subsequently introduced into COL by transduction using the phage 80α (16) at 43°C and 20 μg/ml of chloramphenicol or 37°C and 20 μg/ml of chloramphenicol plus 12 μg/ml of methicillin. Transductants were analyzed as described for pPBP2M4, except that the functional copy was amplified by using primers prfA1 and pSPT181cr (5′-ccaattgctttattgacgttg-3′) and the nonfunctional copy by using primers PBP2P6 (5′-gcaaacatgttgatatttcactg-3′) and pSPT181d (5′-caggaaacagctatgacatg-3′).

Mutagenesis of the TGase Domain. Mutation Glu-114→Gln and a silent *Nru*I restriction site were introduced in plasmid pPBP2M1 using primer PBP2Mut12 (5′-gaaagacgcagtact**c**gc**g**act**c**aaga-caatcgtttctacg-3′) and its reverse, PBP2Mut13. The plasmid was sequenced, named pPBP2M6, and electroporated into RN4220. Transformants were screened for increased moenomycin susceptibility by using tryptic soy agar plates (Difco) containing 0, 0.12, 0.25, 0.5, and 1 μg/ml of moenomycin. Several mutants that either grew or did not grow in 0.25 μg/ml of moenomycin were then analyzed as described for pPBP2M4, except that PCR products were digested with *Nru*I. One mutant with mutation Glu-114→Gln in the functional copy of *pbp2* and one mutant with the mutation in the nonfunctional copy were used as donors in a transduction to COL, giving rise to COLTG42 and COLTG2-1, respectively.

Determination of Methicillin Resistance. Population analysis profiles (PAPs) were done by plating 10-μl samples of 10^0 to 10^{-5} dilutions of an overnight culture on plates containing increasing concentrations of methicillin, as described (17). For the TGase mutants, PAPs were done in the presence of 10 μg/ml of erythromycin. For synergy assay, PAPs were done in the presence of 0.5 μg/ml of moenomycin or 200 μg/ml of cefotaxime.

Cell-Wall Analysis of the TGase Mutants. Cells were grown in tryptic soy broth (TSB) with 10 μg/ml of erythromycin until OD_{620} has reached 0.3. The isolation of cell-wall peptidoglycan and the analysis of the family of enzymatically released muropeptides, by reverse-phase HPLC, were carried out essentially as described (18). The purification of glycan strands and analysis by HPLC was done by a previously described procedure (19). The HPLC profile of the glycan strands was adjusted by subtracting the baseline.

Results

Strategy for Mutagenesis of PBP2 Domains. A fragment of *pbp2* that does not include its promoter or its membrane anchor was cloned into the integrational plasmid pSP64E, which does not replicate

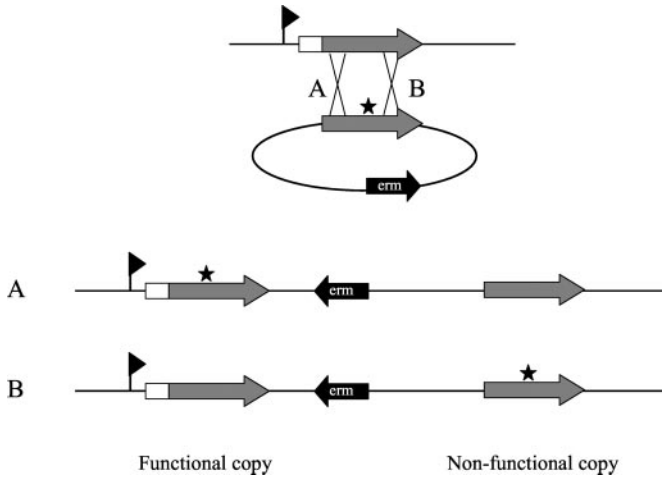

Fig. 1. Strategy for mutagenesis of PBP2 domains. A fragment of *pbp2* that does not include its promoter (indicated by flag) or its membrane anchor (□) was cloned into the integrational plasmid pSP64E, generating plasmid pPBP2M1, which was mutagenized by site-directed mutagenesis (⋆). The mutated plasmid was then introduced into the methicillin-susceptible strain RN4220 by electroporation. After insertion in the chromosome by a Campbell-type mechanism, the plasmid should generate a strain that has a functional copy of *pbp2* followed by the integrational vector and by a nonfunctional copy of *pbp2* (without its promoter and membrane anchor). If the single crossover recombination that occurs during transformation takes place before the mutated codon (*A*), the end result will be a strain with the mutation in the functional copy of *pbp2*, whereas if the recombination point is located after the mutated codon (*B*) the end result will be a strain with a native functional copy and a mutated nonfunctional copy, which should have a phenotype similar to the wild type.

in *S. aureus*, generating plasmid pPBP2M1, which was mutagenized by site-directed mutagenesis.

The mutated plasmid was then introduced into RN4220 by electroporation. After insertion in the chromosome by a Campbell-type mechanism, the mutated plasmid generates a strain that has a functional copy of *pbp2* followed by the integrational vector and by a nonfunctional copy of *pbp2* (without its promoter and membrane anchor). Depending on the recombination point of the single crossover that occurs during transformation, the end result will be a strain with the mutation either in the functional or the nonfunctional copy of *pbp2* (Fig. 1).

Mutagenesis of the TPase Domain. Based on homology with PBP1A of *E. coli*, the serine at the 398th position has been proposed to be the active serine of *S. aureus* PBP2 (10). Mutagenesis of the active-site serine residue was shown to completely remove penicillin-binding activity from a PBP (20, 21). To eliminate the TPase activity of PBP2, the mutation Ser-398→Gly was introduced in pPBP2M1 by site-directed mutagenesis, giving rise to plasmid pPBP2M4. To have an easy way of screening the mutants, a *Kpn*I site was introduced together with the mutation of the active-site serine. As a control of the technique, a silent mutation that removes an *Hpa*I site located 37 nt upstream of the codon for the active serine residue was introduced in pPBP2M1, generating plasmid pPBP2M5.

Plasmids pPBP2M4 and pPBP2M5 were first electroporated into the β-lactam-susceptible strain RN4220. As the mutations of these plasmids were located 1 kb after the 5′ end of the 2.1-kb fragment of *pbp2* cloned in pPBP2M1, we expected ≈50% of the mutants resulting from the integration of pPBP2M4 or pPBP2M5 to have the mutation in the functional copy of *pbp2*, based on the predicted recombination frequencies. After the integration of pPBP2M4, none of the 57 mutants that were analyzed had the mutation in the functional copy, whereas after the integration of pPBP2M5, this fraction was 16 of 29 analyzed mutants. This result suggests that PBP2 may perform an essential function in β-lactam-susceptible *S. aureus* (but not necessarily in the background of an MRSA strain).

Because an MRSA strain that can be directly electroporated is not available, we used the thermosensitive shuttle vector pSPT181c to overcome the problems of obtaining TPase mutants in RN4220. The insert from pPBP2M4 was transferred to pSPT181c, giving rise to plasmid pSPTM4, which was next electroporated into RN4220 at the permissive temperature and subsequently transduced to the methicillin-resistant strain COL. The plasmid insertion in the chromosome was not very stable when mutants were subjected to several cycles of growth. Therefore, after each experiment, chromosomal DNA was extracted and PCR and restriction digestions were done to confirm that the mutation was still present in the functional copy of *pbp2*. Mutants COLpSPTM4–5 and COLpSPTM4–10 had the mutation in the functional and the nonfunctional copy of *pbp2*, respectively, and were chosen for further characterization.

Mutagenesis of the TGase Domain. It was recently reported that Glu-233 of PBP1B of *E. coli* is essential for the TGase reaction catalyzed by this protein. The Glu233→Gln PBP1B mutant protein has negligible glycan chain elongation activity (0.2% of that of the nonmutated PBP1B) and has normal penicillin-binding activity (22). Protein sequence alignment between *E. coli* PBP1B and *S. aureus* PBP2 suggests that Glu-114 of PBP2 is the essential glutamic acid residue of the PBP2 TGase domain.

Glu-114 was mutated to glutamine in pPBP2M1, giving rise to plasmid pPBP2M6, which was then electroporated into RN4220. Because only 10% of the mutants were expected to have the mutation in the functional copy (the mutation to be introduced is located 0.2 kb after the 5′ end of the 2.1-kb fragment of *pbp2* cloned in pPBP2M1), mutants were first screened for increased susceptibility to moenomycin, an antibiotic that inhibits the TGase reaction. Six of 50 analyzed mutants had a 2- to 4-fold reduction in moenomycin MIC value, and all of them were subsequently confirmed by PCR and restriction analysis to have the TGase Glu-114→Gln mutation in the functional copy of *pbp2*. Mutant COLTG42 and control COLTG2-1, which has the TGase mutation in the nonfunctional copy of *pbp2*, were chosen for further study.

Effect of Mutations in the TGase and TPase Domains of PBP2 on Methicillin Resistance. PAPs were done for the TPase mutant COLpSPTM4–5 and for the TGase mutant COLTG42 and respective control strains. The TPase mutation had no effect on methicillin resistance, but the TGase mutants showed drastic decrease in the MIC to 3 μg/ml (Fig. 2).

Similar—selective—effects were observed when moenomycin (an inhibitor of transglycosylation) or cefotaxime (a β-lactam with selective affinity for PBP2) were tested for their impact on methicillin resistance—each tested at one-quarter of their respective MIC values. The methicillin MIC of strain COL was reduced from 800 μg/ml to 1.5 μg/ml in the presence of moenomycin (0.5 μg/ml), but was not affected by cefotaxime (200 μg/ml) (Fig. 2).

The PAPs of the TGase mutants were done in the presence of 10 μg/ml of erythromycin to prevent growth of cells in case there is excision of the suicide plasmid. Yet the shape of PAPs showed that small subpopulations of cells that have retained high-level resistance to methicillin were present in the mutant cultures at frequencies of 10^{-6} to 10^{-7}. We analyzed four such colonies that grew at 200 and 400 μg/ml of methicillin, and verified that in each of the colonies the Glu-114→Gln mutation (that was initially present on the functional copy of *pbp2*) has reverted to the

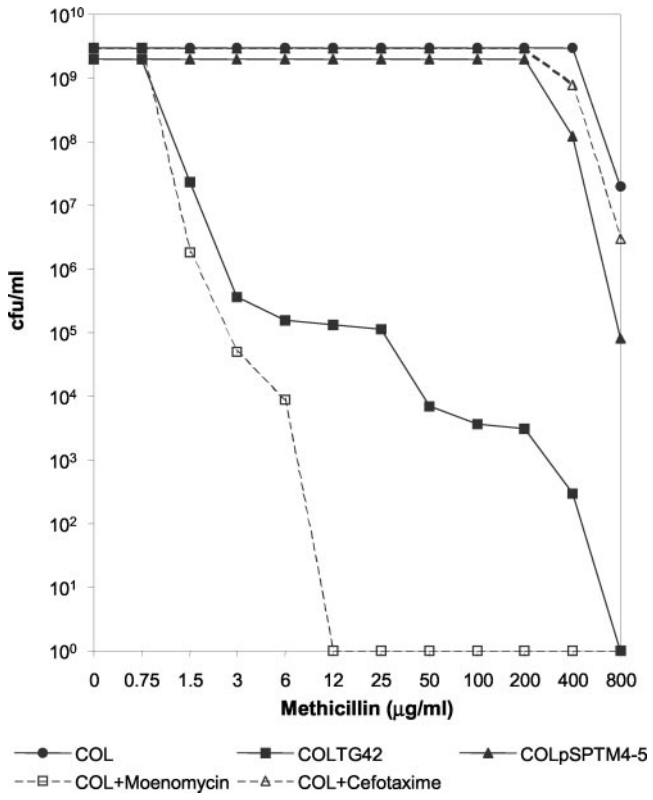

Fig. 2. Methicillin PAPs. The TPase mutation (COLpSPTM4–5) did not affect methicillin resistance, whereas the TGase mutation (COLTG42) caused more than 100-fold decrease in MIC to methicillin. The control strains COLpSPTM4–10 and COLTG2-1 had the same resistance profiles as strain COL. For the synergy assay, PAPs were done in the presence of 0.5 μg/ml moenomycin (an inhibitor of transglycosylation) or 200 μg/ml cefotaxime (a β-lactam with selective affinity for PBP2).

original wild-type sequence, thus explaining the resistance of the colonies.

Demonstration of TGase Activity in PBP2. Muropeptide composition and glycan-strand length of cell walls were analyzed in strain COLTG42, in the control strain COLTG2-1, and in the parental strain COL. There was no detectable change in muropeptide composition, but the proportion of glycan strands with shorter chain length increased significantly in the TGase mutant (Fig. 3). This finding suggests that the TGase domain of the staphylococcal PBP2 has enzymatic activity.

Discussion

Our findings identify the penicillin-insensitive TGase domain of PBP2 as the function critical for the optimal expression of methicillin resistance. Previous studies (8, 17) have shown that the structural gene of PBP2 was one of the surprisingly large number of genetic determinants ("auxiliary genes"), the normal functioning of which is essential for optimal expression of methicillin resistance (23, 24). The results described in this article provide a specific biochemical mechanism for how PBP2, an auxiliary gene, contributes to the expression of antibiotic resistance.

In the current model of bacterial peptidoglycan assembly in *E. coli*, the synthesis of the glycan strands and the crosslinking of muropeptides occur simultaneously, as a result of the action of a multienzyme complex that includes TGases and TPases (as well as murein hydrolases) (25). Our observations represent suggestive evidence for a concerted action of at least two proteins in *S. aureus* cell-wall synthesis. In this case, the cooperating molecular species involve the native PBP2 (through its TGase domain) and the acquired PBP2A—presumably "lending" its low affinity TPase domain for the synthesis of the poorly crosslinked peptidoglycan that is produced by methicillin-resistant staphylococci in the presence of β-lactam antibiotics (18, 26). This does not necessarily imply direct physical interaction between the two proteins (Fig. 4). Whether or not the TPase activity of PBP2A also functions when the bacteria grow in antibiotic-free medium remains to be determined.

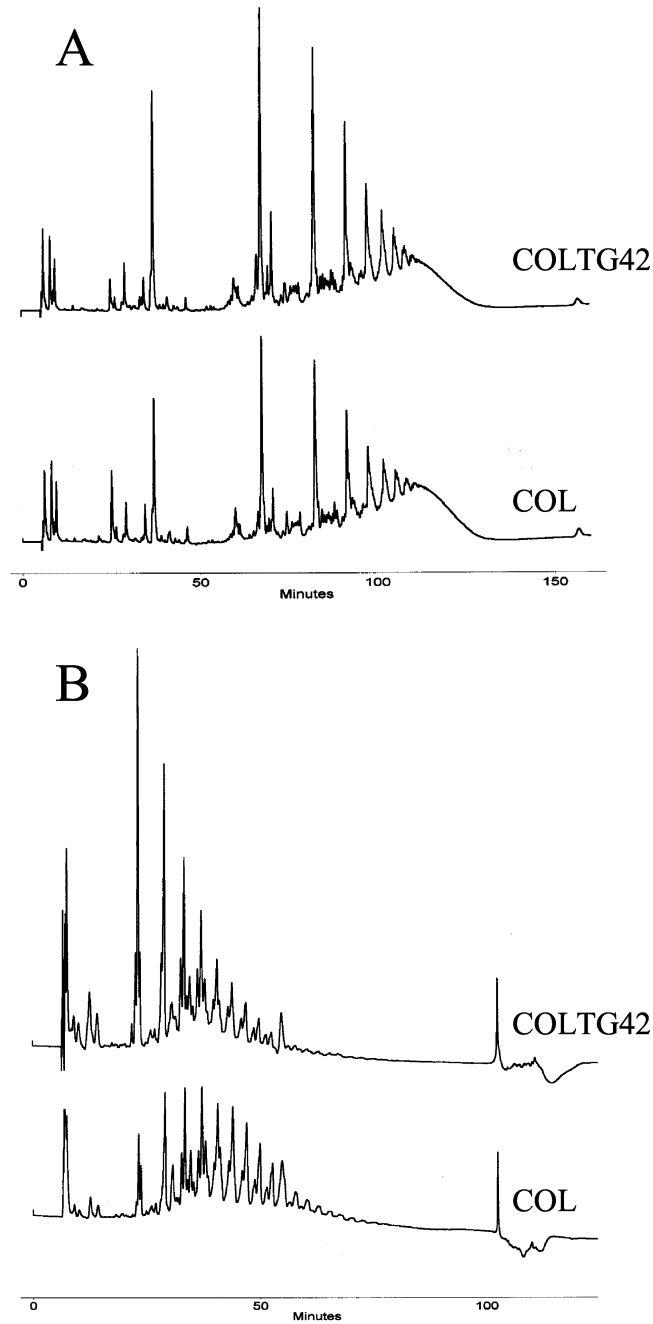

Fig. 3. HPLC cell-wall analysis of the TGase mutant COLTG42 and in the parental strain COL. Muropeptide composition (*A*) was not significantly altered, whereas the glycan profile (*B*) was found to shift in the direction of peaks with shorter retention times, corresponding to shorter glycan strands with fewer disaccharide units. Control strain COLTG2-1 had both the muropeptide and the glycan profile identical to parental strain COL.

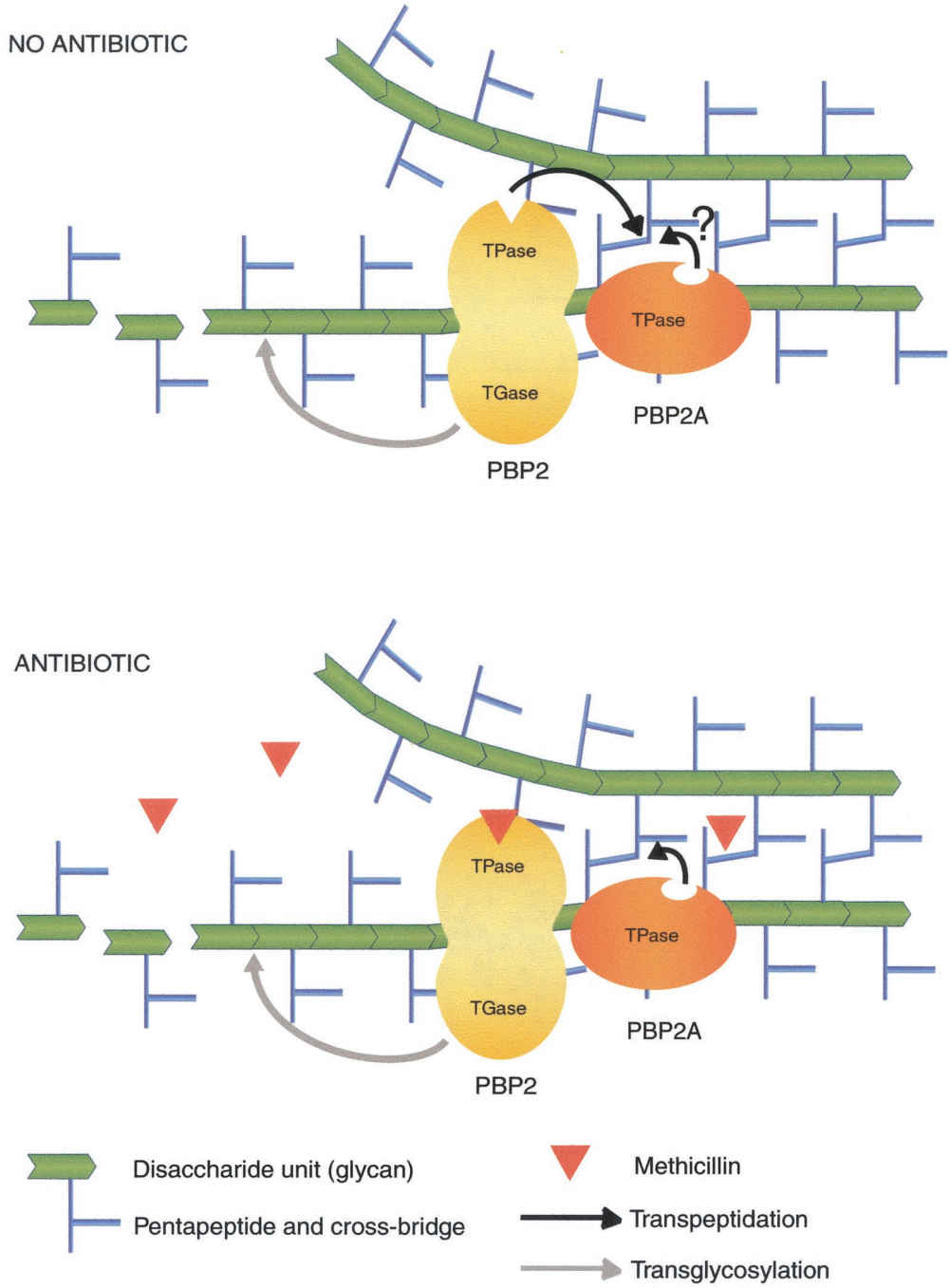

Fig. 4. Model for the cooperative functioning of the TGase domain of PBP2 and the TPase activity of PBP2A in methicillin-resistant *S. aureus*. (*Upper*) In the absence of antibiotic it is assumed that both the TPase and TGase domains of PBP2 participate in the biosynthesis of staphylococcal peptidoglycan. Whether or not the TPase activity of PBP2A (present in methicillin-resistant strains) also functions in the crosslinking of the peptidoglycan in the absence of antibiotic in the medium is not clear at the present time. (*Lower*) When antibiotic (▼) is added to the medium, the TPase domain of PBP2 is acylated and is no longer capable of performing its peptide crosslinking activity. However, the observations described in this paper demonstrate that the penicillin-insensitive TGase domain of PBP2 remains functional and cooperates with the TPase activity of the acquired PBP2A and is actually essential for cell-wall synthesis and bacterial growth in the presence of β-lactam antibiotics in the surrounding medium.

Our findings also strongly suggest an enzymatic activity of the TGase domain of the staphylococcal PBP2; until now, a putative TGase activity of *S. aureus* PBP2 was based only on sequence homologies. Park and Matsuhashi (27) were unable to confirm this activity; using an *in vitro* assay they separated the TGase activity of *S. aureus* from its PBPs and concluded that other enzymes were responsible for the TGase activity in this organism. The fact that *S. aureus* also has monofunctional glycosyltranferase(s) (28) is probably the reason the change in the glycan profile of the PBP2 TGase mutants was not more drastic than what we observed, and is also likely to explain the viability of our mutants.

Why is the TGase activity essential for the expression of methicillin resistance? Our data demonstrate that the cell wall

of TGase mutants is enriched for glycan strands of shorter disaccharide units. MRSA strains grown in the presence of β-lactam antibiotics are known to produce a peptidoglycan with greatly reduced peptide crosslinking (18, 26). It is possible that the bacteria can only survive in those conditions if the glycans are long enough to compensate for the decrease in cell-wall crosslinking. The synergy that exists between methicillin and moenomycin, an antibiotic that inhibits the transglycosylation step, is in accordance with the need for long glycan chains in the presence of β-lactams and emphasizes the usefulness of searching for new antibiotics that act at the level of the TGase reaction.

We thank Shang Wei Wu, who kindly provided plasmid pSPT181c, and Sérgio Filipe, Ivo Boneca, and Mario Ramirez for helpful discussions. These studies were supported by National Institutes of Health Grant RO1 AI45738 and by Fundaçao Para a Ciencia e Tecnologia (Portugal) Grant PRAXIS XXI/BD/9079/96.

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