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Mutational Analysis of Class A and Class B Penicillin-Binding Proteins in *Streptococcus gordonii*▽

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High-molecular-weight (HMW) penicillin-binding proteins (PBPs) are divided into class A and class B PBPs, which are bifunctional transpeptidases/transglycosylases and monofunctional transpeptidases, respectively. We determined the sequences for the HMW PBP genes of *Streptococcus gordonii*, a gingivo-dental commensal related to *Streptococcus pneumoniae*. Five HMW PBPs were identified, including three class A (PBPs 1A, 1B, and 2A) and two class B (PBPs 2B and 2X) PBPs, by homology with those of *S. pneumoniae* and by radiolabeling with [^{3}H]penicillin. Single and double deletions of each of them were achieved by allelic replacement. All could be deleted, except for PBP 2X, which was essential. Morphological alterations occurred after deletion of PBP 1A (lozenge shape), PBP 2A (separation defect and chaining), and PBP 2B (aberrant septation and premature lysis) but not PBP 1B. The muropeptide cross-link patterns remained similar in all strains, indicating that cross-linkage for one missing PBP could be replaced by others. However, PBP 1A mutants presented shorter glycan chains (by 30%) and a relative decrease (25%) in one monomer stem peptide. Growth rate and viability under aeration, hyperosmolarity, and penicillin exposure were affected primarily in PBP 2B-deleted mutants. In contrast, chain-forming PBP 2A-deleted mutants withstood better aeration, probably because they formed clusters that impaired oxygen diffusion. Double deletion could be generated with any PBP combination and resulted in more-altered mutants. Thus, single deletion of four of the five HMW genes had a detectable effect on the bacterial morphology and/or physiology, and only PBP 1B seemed redundant a priori.

Penicillin-binding proteins (PBPs) catalyze the two very last steps of peptidoglycan biosynthesis, namely, transglycosylation and transpeptidation. They are divided into three groups: (i) high-molecular-weight (HMW) class A PBPs, (ii) HMW class B PBPs, and (iii) low-molecular-weight (LMW) PBPs (17). Class A PBPs are bifunctional enzymes that catalyze transglycosylation as well as transpeptidation in vitro (29). They can be individually deleted in a number of bacteria, but the presence of at least one of them is essential (10, 26, 32), probably to ensure a minimal amount of transglycosylation. Class B PBPs possess a transpeptidase activity. Although some class B PBPs can be deleted, all studied bacteria contain at least one such PBP that cannot be inactivated, indicating its essential nature. In *Streptococcus pneumoniae*, which contains two class B PBPs, none of them could be deleted (27). Finally, LMW PBPs act mainly as D,D-carboxypeptidase. They are implicated in the degree of cross-linking and the maturation of the cell wall but are not essential for peptidoglycan biosynthesis (17).

HMW PBPs are ubiquitous among gram-negative and gram-positive bacteria. Each organism possesses several different PBPs, which constitute a specific set of paralog and/or ortholog genes (18). However, their individual implications in cell wall assembly may differ among various organisms, and it is not always known which enzyme is dispensable or not for bacterial growth.

Here, we attempted to assess the functions of the five HMW PBPs of *Streptococcus gordonii*. This organism is a pioneer colonizer of the oral cavity (15, 23, 24). Although it is only an occasional pathogen (14, 22), it is phylogenetically related to *S. pneumoniae*. Its PBPs were identified by homology with those of *S. pneumoniae* as well as by radiolabeling with [^{3}H]penicillin. In order to explore their individual functions and their potential redundancy or essentiality, PBP-deleted mutants of the five HMW PBPs were generated and characterized by imaging, phenotypic experiments, and biochemical analyses.

(These data were presented at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., November 2004 [abstract C1-1309].)

MATERIALS AND METHODS

Microorganisms, growth conditions, and reagents. *Streptococcus gordonii* Challis (35) was used as the model organism. Bacteria were grown at 37°C either in brain heart infusion broth (BHI; Oxoid Ltd., Hampshire, England) without aeration or on Columbia agar (Oxoid) supplemented with 3% blood. Growth of the cultures was followed both by determination of optical density at a wavelength of 620 nm (OD_{620}), using a spectrophotometer (Novaspec II; Amersham Biosciences, Piscataway, N.J.), and by determination of viable counts on agar plates. Whenever appropriate, erythromycin was added to the media at a final concentration of 5 μg/ml. For determination of growth rate, antibiotic was omitted. Bacterial stocks were stored at −80°C in broth supplemented with 10% (vol/vol) glycerol.

DNA preparation and genetic strategies. Molecular techniques were performed using standard methods (38) or by following instructions provided with commercially available kits and reagents. Genomic DNA was extracted using a QIAGEN DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany). PCR primers were purchased from Microsynth (Microsynth GmbH, Balgach, Switzerland) and are listed in Table 1. PCR amplification was carried out with a Px2 thermal cycler (Thermo Hybaid, Ashford, Middlesex, United Kingdom). Genetic transformation of *S. gordonii* was performed as previously described, with about 1 μg of linear recombinant DNA (35).

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TABLE 1. List of the primers used for PCR amplification[a]

Primer	Forward sequence	Backward sequence	Size (bp)
Deletion construction			
*pbp1A*_H1	5′-CCTTAGCCGTGGATTAGCTG	5′-GGCGCGCCAGGGGCCTTGCTCACATAGT	590
*pbp1A*_H2	5′-GGCCGGCCCTTCCGAATCAAGTTCATCCTC	5′-CGTGTGGTTTGGTTCTGATG	582
*pbp1B*_H1	5′-AACCCAGCCTTCTACGGTTT	5′-GGCGCGCCCCAGCCACTTCCAAATTTTC	598
*pbp1B*_H2	5′-GGCCGGCCAGAGAGCGAGACTAGAACGAA	5′-AAGATACGCAGCCCATCAAC	512
*pbp2A*_H1	5′-CCCTCAGGTTTGTTGACGAT	5′-GGCGCGCCGGATCGAGACAGACGTGGTT	556
*pbp2A*_H2	5′-GGCCGGCCAAGGCGGCAATGATTTTTA	5′-TGGAACTTCAGGCGGACTAC	535
*pbp2B*_H1	5′-TTTGGGGCAGGTCTTAGATG	5′-GGCGCGCCATAAGCCAGACGGCCAATC	573
*pbp2B*_H2	5′-GGCCGGCCTTGTAGCCTATGCCCCAAG	5′-TACATGGAGGTTGCCTCTCC	617
*pbp2X*_H1	5′-AATCAGTCGCAAGCCAATTT	5′-GGCGCGCCTTTCCCCACTTGTTTTCGAT	507
*pbp2X*_H2	5′-GGCCGGCCCCAAAGAGAACGCATCAACC	5′-GAAAGCCAAAAACGTTCAGC	577
erm-PA	5′-GGCGCGCCCCGGGCCCAAAATTTGTTTGAT		860
erm-PB		5′-GGCCGGCCAGTCGGCAGCGACTCATAGAAT	
*spectino*_K7	5′-GGCGCGCCAAAAATTTAGAAGCCAATGAAATC	5′-GGCCGGCCCGCGCTTACCAATTAGAATGA	1,000
Deletion control			
*pbp1A*_ctr	5′-GCCATTTCATGCCATTTTCT	5′-TCGCCAATTCGACTTCTCTT	3,733[b]
*pbp1B*_ctr	5′-GTATTTTCCAACGTCACGTAGGAA	5′-TGTCGGTAAAGTTTGAAATTGGAA	3,755[b]
*pbp2A*_ctr	5′-TCCAGCAGGCGGTTGAGTAT	5′-GTGGTGACTTTGCCATGGC	3,364[b]
*pbp2B*_ctr	5′-ACTTTCAACGTTTGGCTCGT	5′-TTTCGATTGCACGAATCAAG	3,264[b]
*pbp2X*_ctr	5′-GCCAAGCAGATTTTTCAAGC	5′-AGCTGCCAGCATACCTCCTA	3,639[b]

[a] Engineered restriction sites are underlined. AscI, GGCGCGCC; FseI, GGCCGGCC.
[b] The size of the native fragment that does not present the integration of the PCR construct.

Identification of PBP genes in *S. gordonii*. The DNA sequences of genes encoding putative PBP 1A, PBP 1B, PBP 2A, PBP 2B, and PBP 2X of *S. gordonii* were determined by homology searches of its nonannotated sequence, available at http://www.tigr.org. BLAST searches were performed using the corresponding amino acid sequences from *S. pneumoniae*. The pneumococcal PBP sequences had the following accession numbers on the NCBI website (http://www.ncbi.nlm.nih.gov): NP_357923 (PBP 1A), NP_359500.1 (PBP 1B), NP_359415.1 (PBP 2A), NP_359110.1 (PBP 2B), and NP_357898.1 (PBP 2X). PBP sequences and their genomic environments were precisely determined with the NCBI ORFinder and Lasergene (DNASTAR Inc.) programs.

Construction of PBP gene deletions. Mutant strains were constructed using the PCR ligation mutagenesis method (28). A large internal fragment of each PBP (≥87% of the total protein) was replaced in frame by an erythromycin resistance cassette derived from the streptococcal suicide vector pJDC9 (5). In brief, the *erm* gene was amplified with the primers described in Table 1 (the forward and reverse primers are referred to as *erm*-PA and *erm*-PB, respectively), which contained the AscI and FseI restriction sites in positions 5′ and 3′. The 5′ end of the *erm* gene was ligated with an upstream fragment of the target PBP (between 500 and 600 bp) (Table 1) encompassing the ATG. Likewise, the 3′ end of *erm* was ligated with a downstream fragment of the target PBP encompassing the stop codon (Table 1). A specific amplicon was constructed for each of the five HMW PBPs. The end product was a linear PCR fragment composed of two homology segments flanking the resistance cassette, which was integrated by transformation into the parental chromosome via double crossover. Double mutants were constructed as described above, with competent bacteria deriving from the single PBP-deleted mutants. The spectinomycin resistance gene, amplified from the cloning vector pFW5 (34) with the primers listed in Table 1, was used as the second resistance cassette. Deleted mutants were selected for spectinomycin and/or erythromycin resistance. Three isolated transformants of each mutant were purified by streaking twice on fresh selective plates. The deletion of PBPs was controlled by PCR with primers external to the constructed fragment (Table 1) and by [^{3}H]penicillin radiolabeling as described below. Attempts to generate triple mutants were made using the kanamycin or chloramphenicol resistance gene, both amplified from plasmid pDONR201 (Invitrogen), as the third resistance marker.

Preparation of membrane-enriched protein fractions and detection of radiolabeled PBPs. Bacteria from a 1-liter culture in the late exponential phase of growth (OD_{620}, ca. 0.7) were rapidly chilled, spun down at 4°C (5,000 rpm, 15 min), and washed in a minimal volume of phosphate-buffered saline. The cells were mixed with lysing matrix B glass beads in 2-ml tubes (Qbiogene, Carlsbad, CA). They were mechanically broken with a BIO 101 FastPrep apparatus (Qbiogene) at an oscillation speed of 6.5 m/s for three runs of 45 s each. Large debris and nonlysed cells were removed by low-speed centrifugation (2,000 rpm, 15 min). The supernatant containing the membranes and soluble cytoplasmic components was subjected to two successive centrifugations (12,000 rpm, 45 min). Membrane pellets were resuspended in a small volume of phosphate-buffered saline and frozen at −20°C. Protein concentration was determined by the Bradford microassay as described by the supplier (Bio-Rad Laboratories, Richmond, California).

Membrane proteins were labeled with [^{3}H]benzylpenicillin (specific activity = 56.7 mCi/mg; Amersham Biosciences, Buckinghamshire, United Kingdom) by incubating 8-μl aliquots of membranes with 1 μCi of the radioactive penicillin for 15 min at 37°C. A 100-fold excess of unlabeled penicillin was added, and membranes were dissolved with 0.5% (final concentration) of the detergent sarkosyl. Samples were resuspended in 1/2 volume of lysis buffer (2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 10% glycerol, and 0.1% bromophenol blue in 50 mM Tris-HCl buffer [pH 6.8]) and heated at 100°C for 3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 6% gels as described previously (43). The gels were first stained with Coomassie brilliant blue to visualize all membrane proteins. They were then processed for fluorography by soaking for 30 min in 100% glycerol and for 30 min in 1 M sodium salicylate (38). PBPs were revealed using preflashed Hyperfilm MP (Amersham Biosciences) after 4 to 8 days of exposure at −80°C.

Transmission electron microscopy. Bacteria were grown in BHI until an OD_{620} of about 0.5. Aliquots (10 ml) were harvested and processed according to the method of Ryter et al. (37) with the following modifications. Cells were aligned by filtration on a 0.45-μm filter and embedded in 3% Noble agar. The agar blocks were cut in 1-mm^3 cubes, which were immediately fixed with osmium. Dehydration and embedding were performed according to the methods of Schlaeppi et al. (39). After polymerization, blocks were cut with a diamond knife on a Reichert-Jung ultramicrotome. Ultrathin sections were poststained with uranyl acetate and lead citrate (25, 36). Sections were observed with a Philips EM 201 electron microscope at 80 kV. Pictures were taken through a MegaView III digital camera driven by the analySIS program from Soft Imaging Systems Gmbh.

Biochemical analyses of the cell wall. Cell walls were prepared as described previously (9) with the following modifications. After SDS treatment and several washings, the cells were mixed with lysing matrix B glass beads in 2-ml tubes (Qbiogene). They were mechanically broken with a BIO 101 FastPrep apparatus (Qbiogene) at an oscillation speed of 6.5 m/s for 3 runs of 45 s each. The collected broken walls were pelleted at 15,000 rpm for 20 min, resuspended in 0.1 M Tris (pH 7.5) containing 0.05% sodium azide, and treated with DNase I,

TABLE 2. High-molecular-weight PBPs of *S. gordonii*

Name	Identity (%) with *S. pneumoniae* homolog	Molecular mass (kDa)
1A	65	78.76
1B	64	92.29
2A	65	80.86
2B	67	75.23
2X	59	82.96

RNase, and trypsin to remove nucleic acids and proteins. Once treated with hydrofluoric acid (49%), the peptidoglycan was digested at 37°C overnight in 25 mM phosphate buffer (pH 6.5) containing 10 mM $MgCl_2$ and 500 units of mutanolysin/mg. The samples were boiled to inactivate mutanolysin and centrifuged to pellet undigested cell walls. The supernatant containing soluble muropeptide fragments was mixed with an equal volume of 0.5 M borate buffer (pH 9) and reduced with sodium borohydride. The pH of the solution was adjusted to 2 with 20% orthophosphoric acid. Samples were stored at −20°C.

The LaChrom high-pressure liquid chromatography (HPLC) system (Merck KGaA, Darmstadt, Germany/Hitachi Instruments, Inc., Separation Systems Group, San Jose, CA) consisted of an L-7100 pump and an L-7400 UV detector. Soluble muropeptide samples were applied to a 250- by 4.6-mm reversed-phase column (ODS-hypersil; Thermo Electron Corporation). The column was eluted at a flow rate of 0.5 ml/min with a linear gradient of 5% (vol/vol) methanol in 100 mM NaH_2PO_4 (pH 2.5) to 30% (vol/vol) methanol in 100 mM NaH_2PO_4 (pH 2.8) over 160 min. The column temperature was 34°C. Muropeptides were detected at an absorbance of 210 nm.

For analysis of glycan chain length, teichoic acid-free peptidoglycan was first reduced in sodium borohydride for 1 h at room temperature. It was then hydrolyzed with 6 N HCl for 18 h. Following the incubation, the hydrolysate was dried in a vacuum, derivatized with ortho-phthaldialdehyde, and separated by HPLC using a fluorescent detector (6, 7, 42). The amount of reduced amino sugars (expressed by the area under the peak) divided by the total amount of amino sugars and multiplied by 100 gave the percentage of free termini in the glycan chain (2). The higher the percentage of free termini was, the shorter the glycan chain length was. Each strain was analyzed in triplicate.

Exposure of the parent and mutants to various stress conditions. Generation times were determined in the mid-logarithmic phase of growth, either under standard conditions or under stress conditions, including oxygenation (by constantly rotating the cultures at 220 rpm) and hyperosmosis (in BHI supplemented with 15% mannitol). The MICs for penicillin were determined by a previously described broth macrodilution method (1) with a final inoculum of ca. 10^6 CFU/ml. The MIC was defined as the lowest antibiotic concentration that inhibited visible bacterial growth after 24 h of incubation at 37°C. Time kill curves were determined by adding either penicillin G at a concentration of 2 μg/ml (125 times the MIC) or Triton X-100 at a final concentration of 0.012% to bacterial cultures in the early exponential phase of growth (OD_{620} of 0.15). Colonies were counted after 24 h of incubation at 37°C.

RESULTS

Identification of the *S. gordonii* PBPs. The sequences of putative PBP genes of *S. gordonii* were identified by comparison of its as yet incomplete and nonannotated genome (http://www.tigr.org) with the sequences of the five HMW PBP genes of *S. pneumoniae* (see accession numbers in Materials and Methods). This revealed five potential open reading frames (Table 2), which carried a transpeptidase homologous domain, as experimentally confirmed by protein radiolabeling with [^{3}H]benzylpenicillin followed by separation on 6% SDS-PAGE gels and fluorography (Fig. 1, lane 1). An additional sixth band of ca. 45 kDa was also visible on 10% gels, corresponding to the LMW PBP 3 (data not shown). For the sake of coherence, the *S. gordonii* PBPs were not named according to their molecular weights but according to their pneumococcal homologues. A diagram depicting the main domains of these proteins is presented in Fig. 2.

Generation of PBP-deleted mutants. Mutants were generated by substituting at least 87% of the internal part of each PBP with the *erm* resistance cassette (see Materials and Methods). Transformants were controlled by PCR and by assessing the loss of specific PBP bands on SDS-PAGE gels (Fig. 1, lanes 2 to 5). Individual deletion of PBP 1A, PBP 1B, PBP 2A, and PBP 2B resulted in the disappearance of a specific band on the gel. In the case of PBP 1A, the disappearance of the main band was accompanied by the loss of two weaker bands at the bottom of the gel. These weaker bands are likely to be degradation products of PBP 1A, due to the presence of several successive serines at the C-terminal part of the protein, which are potentially random coil structures that are susceptible to enzymatic digestion (16). Finally, despite several attempts to delete PBP 2X, no deletion in this gene could be obtained.

Morphology. Because PBP deletion could lead to morphological alterations (4), each mutant was examined by optical as well as by electron microscopy. When examined by phase contrast microscopy, PBP 1A mutants formed slightly shorter chains; PBP 2A mutants grew in very long chains, with an average of about 40 bacteria; and PBP 2B mutants presented aberrant and lysed forms (Fig. 3, left panel). Quantitative fluorescence-activated cell sorting (FACS) analysis (FACSCalibur; Becton-Dickinson) with forward- and side-scattered light, indicating the sizes and granularities of the bacteria, confirmed this qualitative evaluation of cell shape (Fig. 3, right panel). Indeed, the PBP 2B pattern reveals a particularly heterogeneous population, while the PBP 1A and PBP 2A patterns show an overall decrease and increase in particle size, respectively. On the other hand, no differences could be detected between the wild type and the PBP 1B mutant.

Parent and mutant cells were further examined by electron microscopy (Fig. 4). PBP 1B mutants did not display any difference from the wild type (Fig. 4A). On the other hand, PBP 1A mutants presented a lozenge instead of ovoid shape (Fig. 4B). The mega-chaining PBP 2A mutants had the same overall morphology as the parent, but cells remained attached by a

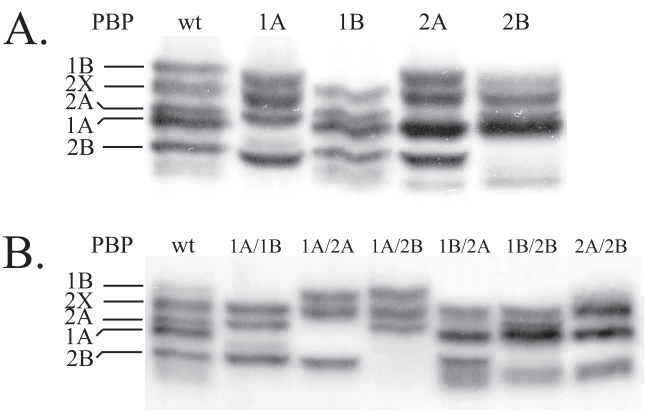

FIG. 1. Patterns of radiolabeled PBPs from the parent and PBP-deleted mutants. PBPs were visualized on fluorograms after labeling membrane proteins with [^{3}H]benzylpenicillin and separation on 6% SDS-PAGE gels. The profiles of single PBP mutants (A) and double PBP mutants (B) are presented. The parent (wt) and the deleted PBP mutants are indicated at the tops of the lanes. Positions of PBPs are listed on the left of the fluorograms.

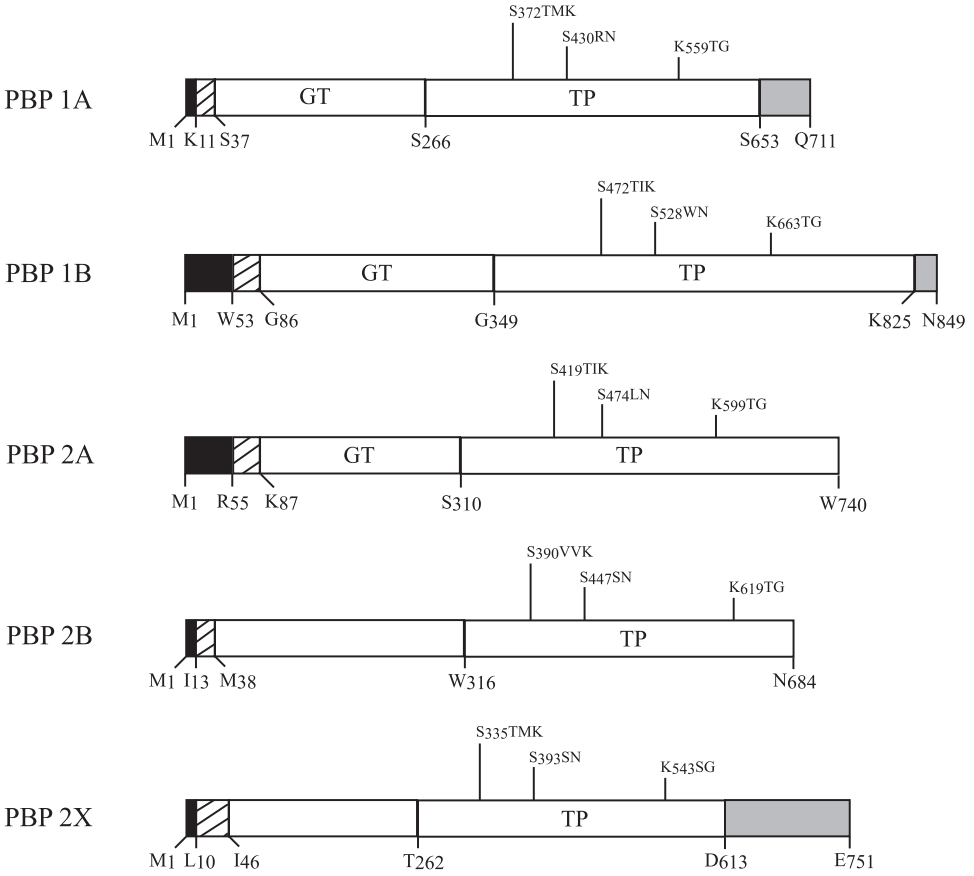

FIG. 2. Diagrams of *S. gordonii* PBPs. Domains were determined in silico by homology with previously characterized PBPs in *S. pneumoniae* (11–13, 19, 31, 33). GT and TP stand for transglycosylase and transpeptidase domains, respectively. Annotations above the transpeptidase domains indicate their conserved motifs. Conserved motifs of the transglycosylase domains are not presented but are identical to those of *S. pneumoniae*. Annotations below the diagrams define the limits of the various protein domains. Solid, hatched, and shaded boxes represent the N-terminal cytoplasmic regions, the membrane anchors, and the C-terminal extensions, respectively.

small fragment of uncleaved peptidoglycan (Fig. 4C). Finally, the PBP 2B mutant displayed the most dramatic morphological alterations, especially at the level of septation (Fig. 4D to F). Septa were often aberrant and desynchronized, with dissymmetrical and abortive forms on each side of the bacteria (Fig. 4D to E). In addition, ghosts appeared much more frequently than in the wild type (Fig. 4F). Yet, a significant subpopulation of bacteria remained intact enough to ensure the growth and the survival of these deleted mutants.

Exposure to different stresses. Since morphological alterations could be synonymous with mechanical and/or synthetic weaknesses, the growth and survival of the bacteria were tested under various conditions. Under standard conditions, the parent and all the mutants reached the same cell density (viable count of ca. 10^9 at an OD_{620} of 1.6) and had the same rate of survival in the stationary phase of growth (loss of 1 $\log_{10}$ CFU/ml after 24 h of incubation). On the other hand, while the parent and the three PBP 1A, 1B, and 2A mutants had similar growth rates (44.2 ± 1.5 min), the PBP 2B mutant grew significantly slower (55.8 ± 7.5; $P < 0.001$). Aeration also decreased the growth rate of the PBP 2B mutant (72.8 ± 2.3 min) but not of the others, while hyperosmolarity restored it (46.9 ± 2.3 min). This favorable effect might have resulted from the osmotic protection of this lysis-prone mutant. However, hyperosmosis altered the growth rate of the lozenge-shaped PBP 1A mutant (54.8 ± 0.6 min).

Bacterial lysis and killing were tested during either aeration or treatment with Triton X-100 (final concentration of 0.12%) or various penicillin concentrations (10×, 50×, and 125× MICs) applied at the beginning of the logarithmic phase of growth (OD_{620} = 0.15). No differences in spontaneous lysis and Triton-induced lysis or killing were observed. On the other hand, oxygenation inflicted a loss of viability of 5 $\log_{10}$ CFU/ml/24 h in all the tested strains, except for the PBP 2A mutant, which lost only 1 $\log_{10}$ CFU/ml/24 h.

The mutants had unaltered MICs for penicillin (0.008 μg/ml) and other beta-lactams, including amoxicillin (MIC, 0.016 μg/ml) and imipenem (MIC, 0.008 μg/ml). However, penicillin-induced lysis was clearly increased in the PBP 2B mutant, and in the PBP 2A mutant to a lesser extent, compared to that in the other strains (Fig. 5A). This correlated with a steeper time kill slope during the first 6 h of drug exposure (Fig. 5B), at least for the PBP 2B mutant. Lysis and killing trends were similar for the three concentrations of penicillin, complying with the concentration-independent bactericidal effect of beta-lactam drugs.

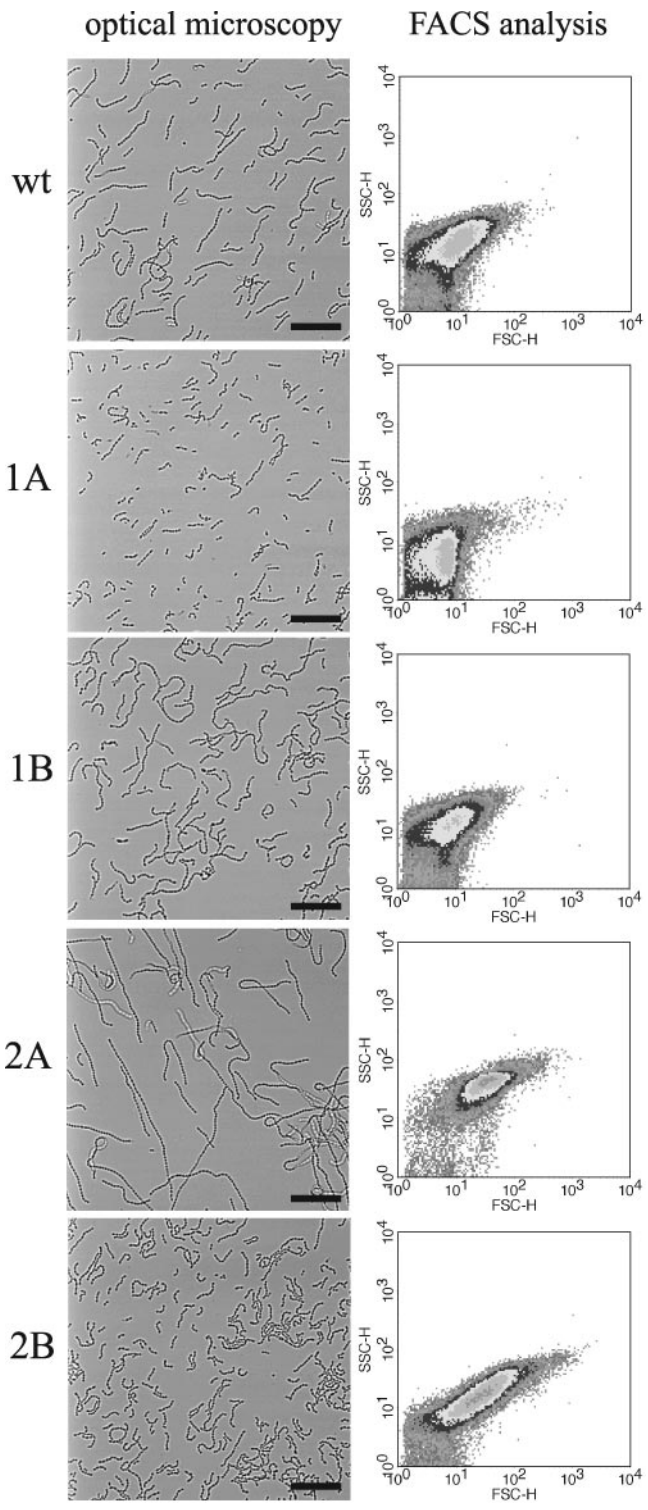

FIG. 3. Cell shape modifications detected by optical microscopy and FACS analysis. The left panel presents exponentially growing cells observed under phase contrast microscopy. Bars, 16 μm. The right panel presents bacteria from the same cultures analyzed by cell sorting. Bacteria were injected into a FACSCalibur and the results analyzed with the CELLQUEST PRO software. To compare populations, the data for each strain were plotted on a two-dimensional graph (*x* axis, forward scatter; *y* axis, side scatter). The wild type (wt) and the PBP-deleted mutants are indicated beside the panels.

Biochemical analysis. Since PBPs ensure transpeptidation and transglycosylation, we sought to determine whether deletion mutants displayed alterations in muropeptide cross-linkage or glycan chain length. The muropeptide HPLC chromatograms for the parent and the mutants presented a complex but very reproducible pattern of peaks, which were qualitatively identical for all the strains. The structures for the contents of nine major peaks were determined in a parallel study (3). One of these peaks, representing a monomer, was significantly increased by ca. 25% in the PBP 1A mutant compared to that in the other strains ($P < 0.05$; compared by analysis of variance with Tukey's multiple-comparison test over a total of four individual HPLC runs, using two wall preparations from each mutant).

The lengths of the glycan chains were indirectly evaluated by determining the percentage of unlinked-terminal muramates reducible by borohydrate, compared to the total amount of muramate in the wall. The percentages of reducible muramate (means ± standard deviations for three determinations) were 15.05 ± 2.84 in the parent, 23.96 ± 3.78 in the PBP 1A mutant, 14.16 ± 2.47 in the PBP 2A mutant, and 18.22 ± 1.56 in the PBP 2B mutant. The PBP 1B mutant was not tested because it showed no morphological alterations. The percentage of reducible muramate was clearly higher in the PBP 1A mutant (23.96 ± 3.78 versus 14 to 18% in the other strains), indicating that the average length of the glycan chains was ca. 30% shorter in this lozenge-shaped mutant.

Double mutants. Double mutants were obtained by producing a second PBP deletion in bacteria that were already lacking one of the PBPs. However, attempts to generate triple PBP-deleted mutants systematically failed. All combinations of double mutants were successfully constructed, as can be seen by protein radiolabeling with [^{3}H]benzylpenicillin (Fig. 1B). Growth rates for double-mutants were more heterogeneous than those for single mutants. PBPs 1A, 2A, and 2B mutated individually or in combination with PBP 1B had similar growth rates, suggesting the possible redundancy of PBP 1B. PBP 2A mutated with either PBP 1A or PBP 2B grew slightly slower than the corresponding individual mutants. In that case, the burden caused by each mutation seems to be additive. Finally, the simultaneous deletion of both PBP 1A and PBP 2B strongly decreased the growth rate. Morphological observations by optical microscopy revealed no differences between PBP 1A, 2A, and 2B single mutants and the corresponding double mutants with PBP 1B. The deletion of PBP 2B with either PBP 1A or PBP 2A led to additive effects. A high proportion of aberrant morphologies was detected, combined with particularly short or long chains, respectively. Finally, the mutations of both PBP 1A and PBP 2A, which are individually leading to opposite effects, engendered short chains with high numbers of enlarged cells.

DISCUSSION

This study identified the five HMW PBPs of *S. gordonii* and provided insights into their functional role. The three class A PBPs (PBPs 1A, 1B, and 2A) could be deleted individually or in tandem, indicating their nonessentiality. However, deletion of PBP 1A resulted in lozenge-shaped cells and deletion of PBP2A in increased bacterial chaining. The lozenge-shaped

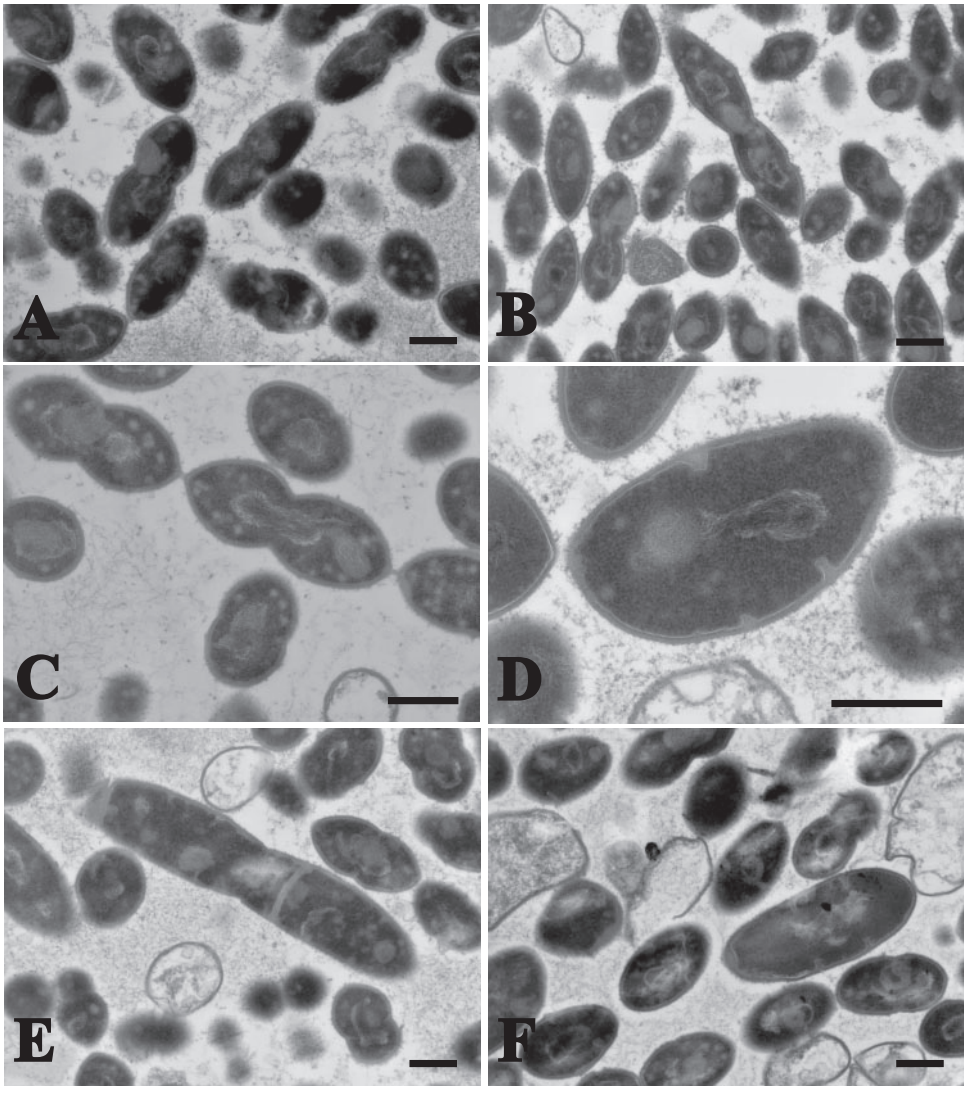

FIG. 4. Electron microscopy of individually deleted PBPs in *S. gordonii*. The parent strain (A) and the PBP 1A (B), PBP 2A (C), and PBP 2B (D to F) mutants were examined in the mid-log phase of growth in rich medium. Bars, 0.5 μm.

PBP 1A mutant grew less rapidly under hyperosmotic conditions, which may be indirect proof of a mechanical weakness in the wall leading to osmosis-induced shrinkage, and its peptidoglycan chains were ca. 30% shorter (on average) than those in the other bacteria. This might be compatible with a decreased mechanical resistance in the wall to osmosis-induced shrinkage. The chaining PBP2A mutant was more resistant to aeration-induced killing, maybe because it formed aggregates that impaired the diffusion of oxygen. Yet, it had an unaltered cell shape and displayed an unchanged muropeptide, like all the other mutants except for the PBP 1A-deleted species. Thus, although the three class A PBPs were nonessential, the deletion of at least two of them (PBP 1A and PBP 2A) had clear morphological and physiological consequences. Class A PBPs could also be deleted in the closely related *S. pneumoniae* species (26, 32). However, the morphological alterations were prominent in the double mutants.

In contrast to what was found for class A PBPs, only one of the class B PBPs, i.e., PBP 2B, could be deleted, while PBP 2X was essential. This is compatible with what was found for *Streptococcus thermophilus*, where the homologue of PBP 2B could also be successfully inactivated (41), but somewhat different from what was found for *S. pneumoniae*, in which both PBP 2B and 2X are essential (27). Together, these results highlight the unique essentiality of PBP 2X, which appeared also vital in other bacterial species, including *Bacillus subtilis* and *Escherichia coli* (8, 40).

Although PBP 2B could be inactivated, its deletion resulted in the most dramatic phenotypes. Electron microscopy revealed profound anomalies in septal formation, which is compatible with its physical localization at the equatorial plate, as reported for *S. pneumoniae* (30). There were many asymmetrical and abnormal cells during normal growth, an observation that was also made with the PBP 2B-inactivated *S. thermophilus* mutant (41). In addition, the *S. gordonii* mutant was more susceptible to penicillin-induced lysis (as expressed by the loss

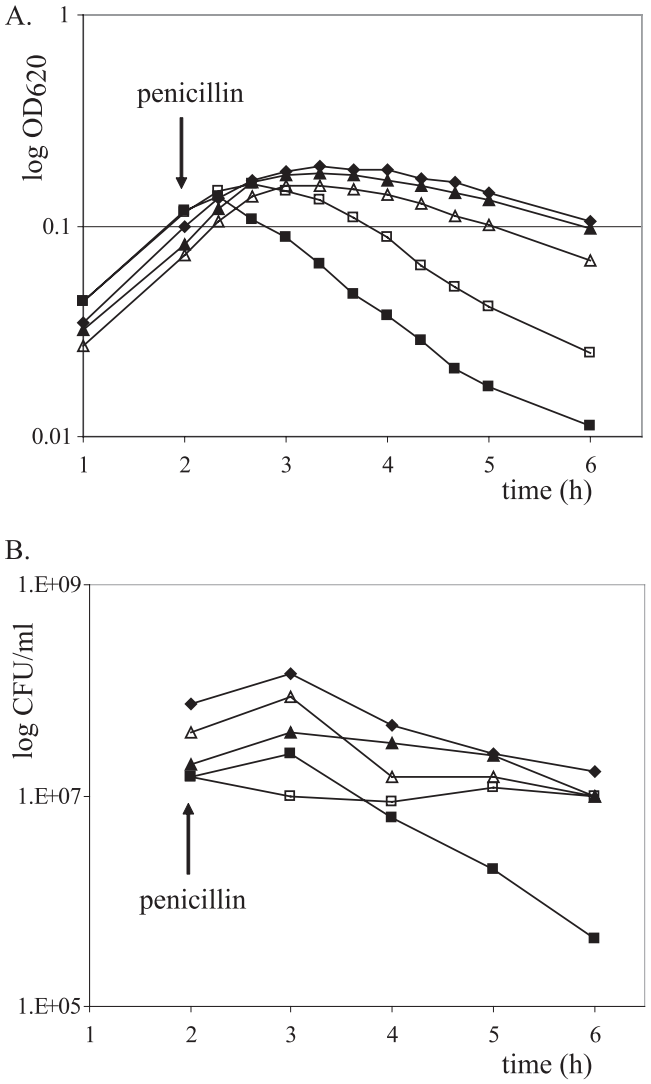

FIG. 5. Penicillin-induced early lysis (A) and time kill curves (B). The *S. gordonii* wild type (◆) and the deletion mutant PBP 1A (Δ), 1B (▲), 2A (□), or 2B (■) was treated with penicillin G (50-fold the MIC) at an OD_{620} of 0.15 (arrow). The data points represent the means for ≥3 independent experiments, with an interexperimental variation of ≤10%.

of OD) and killing but not to lysis induced by detergent or occurring spontaneously in the stationary phase of growth. Hyperosmotic conditions also restored its growth rate, most likely due to a protective effect of osmolarity on this lysis-prone mutant. This differential comportment suggests that penicillin-induced loss of OD was more likely related to penicillin-induced blockage of wall assembly, resulting in cell leakage and empty shells, than to overexpression of intrinsic autolysins. Nevertheless, exploring this issue using wall solubilization approaches (e.g., zymograms) failed, and this second possibility cannot be formally excluded.

A general feature of the present and previous observations is that bifunctional class A PBPs were more dispensable than monofunctional class B enzymes. This is somewhat counterintuitive since class A PBPs ensure transglycosylase, which is indispensable for glycan chain elongation. The difference between the class A and class B PBP essentialities is likely to rely on both gene redundancy and the functional localization of the enzymes during bacterial growth. First, there were three class A PBPs (PBPs 1A, 1B, and 2A), which could be deleted individually or in tandem, but no triple inactivated mutants were obtained. This suggests that the presence of at least one of these enzymes was necessary, presumably to ensure a minimal amount of transglycosylation. Second, an elegant series of experiments performed with *S. pneumoniae* indicated that class A PBPs had a certain plasticity in their physical location and could modify their migration depending on their presence in a parent or PBP-deleted genetic background (30). Such plasticity is an additional argument for the possibility of functional complementation between these enzymes. In contrast, the transpeptidase class B PBPs were precisely located at either the septum (PBP 2X) or the equatorial rings (PBP 2B). This implies less flexibility between these two enzymes, which might not replace each other in deleted mutants. This unique role also explains why they are the first PBPs to be mutated in penicillin-resistant mutants (20, 21).

In conclusion, this work and previous reports show that even if the majority of the PBPs are not individually essential, they each play a precise role inside the cell. The combination of those different roles leads to the formation of a normal and optimal peptidoglycan. The absence of one or two PBPs will generate a rearrangement of the remaining proteins in order to preserve the viability of the bacteria. However, even if the mutant survives, it suffers different anomalies, depending on the missing PBP, due to the fact that these enzymes are not completely redundant. Thus, the function of PBPs as transglycosylases and/or transpeptidases, their precise spatial localization, and their role in fine cell shape regulation were important enough to maintain the presence of each of them throughout evolution.

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REFERENCES

1. **Amsterdam, D.** 1996. Susceptibility testing of antimicrobials in liquid medium, p. 52–111. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams and Wilkins, Baltimore, Md.
2. **Bischoff, M., M. Roos, J. Putnik, A. Wada, P. Glanzmann, P. Giachino, P. Vaudaux, and B. Berger-Bachi.** 2001. Involvement of multiple genetic loci in *Staphylococcus aureus* teicoplanin resistance. FEMS Microbiol. Lett. **194:**77–82.
3. **Bizzini, A., P. A. Majcherczyk, S. Beggah-Möller, B. Soldo, J. Entenza, M. Gaillard, P. Moreillon, and V. Lazarevic.** Submitted for publication.
4. **Cabeen, M. T., and C. Jacobs-Wagner.** 2005. Bacterial cell shape. Nat. Rev. Microbiol. **3:**601–610.
5. **Chen, J.-D., and D. A. Morrison.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. Gene **64:**155–164.
6. **Cooper, J. D., G. Ogden, J. McIntosh, and D. C. Turnell.** 1984. The stability of the o-phthalaldehyde/2-mercaptoethanol derivatives of amino acids: an investigation using high-pressure liquid chromatography with a precolumn derivatization technique. Anal. Biochem. **142:**98–102.
7. **Costa, M., I. Pecci, B. Pensa, M. Fontana, and D. Cavallini.** 1989. High-performance liquid chromatography of cystathionine, lanthionine and aminoethylcysteine using o-phthaldialdehyde precolumn derivatization. J. Chromatogr. **490:**404–410.
8. **Daniel, R., A. Williams, and J. Errington.** 1996. A complex four-gene operon containing essential cell division gene *pbpB* in *Bacillus subtilis*. J. Bacteriol. **178:**2343–2350.

9. **de Jonge, B., Y. Chang, D. Gage, and A. Tomasz.** 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. J. Biol. Chem. **267:**11248–11254.
10. **Denome, S. A., P. K. Elf, T. A. Henderson, D. E. Nelson, and K. D. Young.** 1999. *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. J. Bacteriol. **181:**3981–3993.
11. **Di Guilmi, A. M., A. Dessen, O. Dideberg, and T. Vernet.** 2003. Functional characterization of penicillin-binding protein 1b from *Streptococcus pneumoniae*. J. Bacteriol. **185:**1650–1658.
12. **Di Guilmi, A. M., N. Mouz, J.-P. Andrieu, J. Hoskins, S. R. Jaskunas, J. Gagnon, O. Dideberg, and T. Vernet.** 1998. Identification, purification, and characterization of transpeptidase and glycosyltransferase domains of *Streptococcus pneumoniae* penicillin-binding protein 1a. J. Bacteriol. **180:**5652–5659.
13. **Di Guilmi, A. M., N. Mouz, L. Martin, J. Hoskins, S. R. Jaskunas, O. Dideberg, and T. Vernet.** 1999. Glycosyltransferase domain of penicillin-binding protein 2a from *Streptococcus pneumoniae* is membrane associated. J. Bacteriol. **181:**2773–2781.
14. **Douglas, C. W., J. Heath, K. K. Hampton, and F. E. Preston.** 1993. Identity of viridans streptococci isolated from cases of infective endocarditis. J. Med. Microbiol. **39:**179–182.
15. **Frandsen, E. V., V. Pedrazzoli, and M. Kilian.** 1991. Ecology of viridans streptococci in the oral cavity and pharynx. Oral Microbiol. Immunol. **6:**129–133.
16. **Geourjon, C., and G. Deleage.** 1995. SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Comput. Appl. Biosci. **11:**681–684.
17. **Ghuysen, J. M.** 1991. Serine beta-lactamases and penicillin-binding proteins. Annu. Rev. Microbiol. **45:**37–67.
18. **Goffin, C., and J.-M. Ghuysen.** 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. **62:**1079–1093.
19. **Gordon, E., N. Mouz, E. Duee, and O. Dideberg.** 2000. The crystal structure of the penicillin-binding protein 2x from *Streptococcus pneumoniae* and its acyl-enzyme form: implication in drug resistance. J. Mol. Biol. **299:**477–485.
20. **Grebe, T., and R. Hakenbeck.** 1996. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob. Agents Chemother. **40:**829–834.
21. **Haenni, M., and P. Moreillon.** 2006. Mutations in penicillin-binding protein (PBP) genes and in non-PBP genes during selection of penicillin-resistant *Streptococcus gordonii*. Antimicrob. Agents Chemother. **50:**4053–4061.
22. **Hakenbeck, R., H. Ellerbrok, T. Briese, S. Handwerger, and A. Tomasz.** 1986. Penicillin-binding proteins of penicillin-susceptible and -resistant pneumococci: immunological relatedness of altered proteins and changes in peptides carrying the beta-lactam binding site. Antimicrob. Agents Chemother. **30:**553–558.
23. **Hardy, J. M., and G. H. Bowen.** 1974. The normal microbial flora of the mouth. Soc. Appl. Bacteriol. Symp. Ser. **3:**47–83.
24. **Hardy, J. M., and P. D. Marsh.** 1978. Streptococci and the human oral flora, p. 157–206. *In* F. A. Skinner and L. B. Quesnel (ed.), Streptococci. Academic Press, Inc., New York, N. Y.
25. **Higgins, M. L.** 1973. A fixation and imbedding procedure for thin-sectioning bacteria, p. 686–689. *In* A. I. Laskin and H. Lechevalier (ed.), CRC handbook of microbiology, vol. 1. CRC Press, Cleveland, Ohio.
26. **Hoskins, J., P. Matsushima, D. L. Mullen, J. Tang, G. Zhao, T. I. Meier, T. I. Nicas, and S. R. Jaskunas.** 1999. Gene disruption studies of penicillin-binding proteins 1a, 1b, and 2a in *Streptococcus pneumoniae*. J. Bacteriol. **181:**6552–6555.
27. **Kell, C. M., U. K. Sharma, C. G. Dowson, C. Town, T. S. Balganesh, and B. G. Spratt.** 1993. Deletion analysis of the essentiality of penicillin-binding proteins 1A, 2B and 2X of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **106:**171–175.
28. **Lau, P. C. Y., C. K. Sung, J. H. Lee, D. A. Morrison, and D. G. Cvitkovitch.** 2002. PCR ligation mutagenesis in transformable streptococci: application and efficiency. J. Microbiol. Methods **49:**193–205.
29. **Matsuhashi, M.** 1994. Utilization of lipid-linked precursors and the formation of peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation, p. 55–71. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier, Amsterdam, The Netherlands.
30. **Morlot, C., A. Zapun, O. Dideberg, and T. Vernet.** 2003. Growth and division of *Streptococcus pneumoniae*: localization of the high molecular weight penicillin-binding proteins during the cell cycle. Mol. Microbiol. **50:**845–855.
31. **Pagliero, E., L. Chesnel, J. Hopkins, J. Croize, O. Dideberg, T. Vernet, and A. M. Di Guilmi.** 2004. Biochemical characterization of *Streptococcus pneumoniae* penicillin-binding protein 2b and its implication in beta-lactam resistance. Antimicrob. Agents Chemother. **48:**1848–1855.
32. **Paik, J., I. Kern, R. Lurz, and R. Hakenbeck.** 1999. Mutational analysis of the *Streptococcus pneumoniae* bimodular class A penicillin-binding proteins. J. Bacteriol. **181:**3852–3856.
33. **Pares, S., N. Mouz, Y. Petillot, R. Hakenbeck, and O. Dideberg.** 1996. X-ray structure of *Streptococcus pneumoniae* PBP2x, a primary penicillin target enzyme. Nat. Struct. Biol. **3:**284–289.
34. **Podbielski, A., B. Spellerberg, M. Woischnik, B. Pohl, and R. Lutticken.** 1996. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). Gene **177:**137–147.
35. **Pozzi, G., R. A. Musmanno, P. M. Lievens, M. R. Oggioni, P. Plevani, and R. Manganelli.** 1990. Method and parameters for genetic transformation of *Streptococcus sanguis* Challis. Res. Microbiol. **141:**659–670.
36. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208–212.
37. **Ryter, A., E. Kellenberger, A. Birchandersen, and O. Maaloe.** 1958. Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. I. Les nucléoïdes des bactéries en croissance active. Z. Naturforsch B **13b:**597–605.
38. **Sambrook, J., E. J. Fritsch, and T. Maniatis.** 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Schlaeppi, J. M., O. Schaefer, and D. Karamata.** 1985. Cell wall and DNA cosegregation in *Bacillus subtilis* studied by electron microscope autoradiography. J. Bacteriol. **164:**130–135.
40. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA. **72:**2999–3003.
41. **Stingele, F., and B. Mollet.** 1996. Disruption of the gene encoding penicillin-binding protein 2b (*pbp2b*) causes altered cell morphology and cease in exopolysaccharide production in *Streptococcus thermophilus* Sfi6. Mol. Microbiol. **22:**357–366.
42. **Umagat, H., P. Kucera, and L. F. Wen.** 1982. Total amino acid analysis using pre-column fluorescence derivatization. J. Chromatogr. **239:**463–474.
43. **Williamson, R., R. Hakenback, and A. Tomasz.** 1980. *In vivo* interaction of beta-lactam antibiotics with the penicillin-binding-proteins of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **18:**629–637.