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Promoter and Transcription Analysis of Penicillin-Binding Protein Genes in *Streptococcus gordonii*[▽]

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An optimally cross-linked peptidoglycan requires both transglycosylation and transpeptidation, provided by class A and class B penicillin-binding proteins (PBPs). *Streptococcus gordonii* possesses three class A PBPs (PBPs 1A, 1B, and 2A) and two class B PBPs (PBPs 2B and 2X) that are important for penicillin resistance. High-level resistance (MIC, ≥2 μg/ml) requires mutations in class B PBPs. However, although unmutated, class A PBPs are critical to facilitate resistance development (M. Haenni and P. Moreillon, Antimicrob. Agents Chemother. 50:4053–4061, 2006). Thus, their overexpression might be important to sustain the drug. Here, we determined the promoter regions of the *S. gordonii* PBPs and compared them to those of other streptococci. The extended −10 box was highly conserved and complied with a σ^A-type promoter consensus sequence. In contrast, the −35 box was poorly conserved, leaving the possibility of differential PBP regulation. Gene expression in a penicillin-susceptible parent (MIC, 0.008 μg/ml) and a high-level-resistant mutant (MIC, 2 μg/ml) was monitored using luciferase fusions. In the absence of penicillin, all PBPs were constitutively expressed, but their expression was globally increased (1.5 to 2 times) in the resistant mutant. In the presence of penicillin, class A PBPs were specifically overexpressed both in the parent (PBP 2A) and in the resistant mutant (PBPs 1A and 2A). By increasing transglycosylation, class A PBPs could promote peptidoglycan stability when transpeptidase is inhibited by penicillin. Since penicillin-related induction of class A PBPs occurred in both susceptible and resistant cells, such a mutation-independent facilitating mechanism could be operative at each step of resistance development.

We have recently shown that *Streptococcus gordonii* carries five high-molecular-weight penicillin-binding proteins (PBPs), including three transglycosylase-transpeptidase class A enzymes (PBPs 1A, 1B, and 2A) and two transpeptidase class B enzymes (PBPs 2B and 2X) (10, 12). Inactivation of these genes showed that both PBP classes had physiological and/or morphological implications (12). PBP 2X was essential, as it is in *Streptococcus pneumoniae* and a few other bacteria (7, 17, 28). Inactivation of PBP 1A resulted in an altered cell shape and peptidoglycan structure, inactivation of PBP 2A in increased bacterial chaining, and inactivation of PBP 2B in abnormal septation and increased penicillin-induced lysis. Only the PBP 1B mutant did not show obvious phenotypic changes.

PBPs were also found to be critical for the development of penicillin resistance. Exposure of *S. gordonii* to penicillin in the laboratory resulted in the progression towards penicillin resistance following two main consecutive phases: first, a non-PBP-mutation phase during which the MIC of penicillin increased progressively by ca. 100 times (from 0.008 μg/ml to 0.5 to 1 μg/ml) and second, a PBP mutation phase during which the MIC further increased by another 4 to 8 times (from 0.5 to 1 μg/ml to 2 to 4 μg/ml) and mutations occurred in class B PBPs 2X and 2B (13), as also was observed in *S. pneumoniae* (11).

However, although class A PBPs were dispensable in the absence of penicillin and did not undergo mutations in penicillin-resistant mutants, their presence (particularly that of PBP 1A and 2A) greatly facilitated the initial steps of penicillin resistance development (13). Indeed, mutants inactivated in PBP 1A or PBP 2A could hardly increase their MICs and did not develop mutations in PBP 2X and PBP 2B, even after prolonged exposure to the drug. This suggests that class A PBPs could mutually compensate for their functions to sustain growth of the inactivated mutants in the absence of penicillin but not in the presence of even low concentrations of the drug. Hence, a decreased activity of class A PBPs might be responsible for the difficulty of PBP-inactivated mutants to initiate resistance, and thus the function of class A PBPs might be a limiting factor in the emergence of penicillin resistance.

Consequently, the bacterium might require increased expression of class A PBPs to develop penicillin resistance, at least in the initial steps of the process. Therefore, we determined the promoter sequences of the five PBP genes of *S. gordonii* and studied their expression in the absence or in the presence of increasing concentrations of penicillin. The results indicate that while all PBPs were constitutively expressed in the absence of the drug, class A PBP 2A was distinctively overexpressed when penicillin was added to the culture, both in the susceptible parent and in a high-level-penicillin-resistant mutant of *S. gordonii*, whereas PBP 1A expression increased only in the resistant isolate.

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MATERIALS AND METHODS

Microorganisms, growth conditions, and reagents. The bacterial strains used in this study are described in Table 1. Streptococci were grown at 37°C either in brain heart infusion (BHI) broth (Oxoid Ltd, Hampshire, England) without

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TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Relevant genotype	Construction	Source or reference
Plasmids			
pGEM-T-easy	Cloning vector, Amp^r		Promega
pJDC9	Integrative vector for *S. gordonii*, Em^r		6
pGEM-T-easy-*luc*	Expresses luciferase, Amp^r	Insertion of the *luc* gene in the poly(T) site	This work
pJDC9_1Aluc	*'pbp1A-luc*	Cloning of a *pbp1A* 878-nt fragment fused to the *luc* gene into pJDC9	This work
pJDC9_1Bluc	*'pbp1B-luc*	Cloning of a *pbp1B* 498-nt fragment fused to the *luc* gene into pJDC9	This work
pJDC9_2Aluc	*'pbp2A-luc*	Cloning of a *pbp2A* 528-nt fragment fused to the *luc* gene into pJDC9	This work
pJDC9_2Bluc	*'pbp2B-luc*	Cloning of a *pbp2B* 456-nt fragment fused to the *luc* gene into pJDC9	This work
pJDC9_2Xluc	*'pbp2X-luc*	Cloning of a *pbp2x* 516-nt fragment fused to the *luc* gene into pJDC9	This work
Ppbp2B_1	*Ppbp2B_1-luc*	Cloning of 579 nt (located 28 nt upstream of the *pbp2B* start codon) fused to the *luc* gene into pJDC9	This work
Ppbp2B_2	*Ppbp2B_2-luc*	Cloning of 597 nt (located 10 nt upstream of *pbp2B* start codon) fused to the *luc* gene into pJDC9	This work
Ppbp2B_3	*Ppbp2B_3-luc*	Cloning of 634 nt (located 37 nt downstream of *pbp2B* start codon) fused to the *luc* gene into pJDC9	This work
S. gordonii strains			
Challis (parent)	Wild type		24
SG103	Challis *arc*::*luc-erm*	Parent Ω pJDC9_arcluc, Em^r	3
SG_1Aluc	*luc* fused to *pbp1A*	Parent Ω pJDC9_1Aluc, Em^r	This work
SG_1Bluc	*luc* fused to *pbp1B*	Parent Ω pJDC9_1Bluc, Em^r	This work
SG_2Aluc	*luc* fused to *pbp2A*	Parent Ω pJDC9_2Aluc, Em^r	This work
SG_2Bluc	*luc* fused to *pbp2B*	Parent Ω pJDC9_2Bluc, Em^r	This work
SG_2Xluc	*luc* fused to *pbp2X*	Parent Ω pJDC9_2Xluc, Em^r	This work
SG_Ppbp2B_1	*luc* fused 3 nt upstream the putative −10 region of *Ppbp2B*	Parent Ω pJDC9_2Bluc, Em^r	This work
SG_Ppbp2B_2	*luc* fused 9 nt downstream the putative −10 region of *Ppbp2B*	Parent Ω pJDC9_2Bluc, Em^r	This work
SG_Ppbp2B_3	*luc* fused 46 nt downstream the putative −10 region of *Ppbp2B*	Parent Ω pJDC9_2Bluc Em^r	This work
PR1_2evolved	Penicillin resistant (MIC, 2 μg/ml)		13
SGPR_1Aluc	*luc* fused to *pbp1A*	PR1_2evolved Ω pJDC9_1Aluc, Em^r	This work
SGPR_1Bluc	*luc* fused to *pbp1B*	PR1_2evolved Ω pJDC9_1Bluc, Em^r	This work
SGPR_2Aluc	*luc* fused to *pbp2A*	PR1_2evolved Ω pJDC9_2Aluc, Em^r	This work
SGPR_2Bluc	*luc* fused to *pbp2B*	PR1_2evolved Ω pJDC9_2Bluc, Em^r	This work
SGPR_2Xluc	*luc* fused to *pbp2X*	PR1_2evolved Ω pJDC9_2Xluc, Em^r	This work

aeration or on Columbia agar (Oxoid) supplemented with 3% blood. *Escherichia coli* strain DH5α was used as host for plasmids pGEM-T-easy (Promega) and pJDC9 and their derivatives (Table 1). *E. coli* was grown at 37°C either in Luria-Bertani (LB) medium (Difco) with aeration or on LB agar plates. Growth of the cultures was followed by optical density at 620 nm (OD_{620}) using a spectrophotometer (Novaspec II; Amersham Biosciences, Piscataway, NJ). Whenever appropriate, erythromycin was added to the media at final concentrations of 5 μg/ml for *S. gordonii* and 500 μg/ml for *E. coli*. Bacterial stocks were stored at −80°C in broth supplemented with 10% (vol/vol) glycerol. For the experiments on gene regulation, various concentrations of penicillin G were added to cultures at an OD_{620} of 0.12.

DNA techniques. Molecular techniques were performed using standard methods (26) 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 2. Genetic transformation of *S. gordonii* was performed as previously described with about 1 μg of linear recombinant DNA (24).

Construction of the *pbp-luc* transcriptional fusions. A promoterless firefly luciferase gene carrying its own ribosome-binding site (RBS) (18) was amplified with the primers luc_BamHI_5′ and luc_PstI_3′ (Table 2) and cloned into the 3′-T site of the pGEM-T-easy vector. The resulting recombinant vector (pGEM-T-easy-*luc*) was used as a basis for the construction of the transcriptional fusions (Table 1), as exemplified here for the PBP 1A gene. The 3′ end of *pbp1A* was amplified by PCR from chromosomal DNA with the primers pbp1A_fusion_5′ and pbp1A_fusion_3′ (Table 2). The PCR product was digested with SalI and BamHI and ligated to the same sites in pGEM-T-easy-*luc*. The 2.1-kb *pbp1A-luc* segment of this construct was amplified by PCR with oligonucleotides pbp1A_KpnI_5′ and luc_PstI_3′, digested with KpnI/PstI, and subcloned into the corresponding sites of the suicide vector pJDC9 (6). The resulting plasmid, pJDC9_1Aluc, was transformed into wild-type *S. gordonii*, and the transformants were selected for erythromycin resistance. Correct plasmid integration into *pbp1A* of erythromycin-resistant transformants designated SG_1Aluc was assessed by PCR with one primer on the luciferase 3′ end (luc_PstI_3′) and one primer situated outside the construct on *pbp1A* (pbp1A_control).

Determination of light emission. Light emission was measured by slight modifications of a published method (15, 18). Tubes containing fresh prewarmed BHI broth were inoculated with 1/100 (vol/vol) of an overnight culture and growth was followed as described previously. At several times during logarithmic growth or after antibiotic addition, 100-μl samples of the cultures were removed and added to 2-ml Eppendorf tubes containing 250 μl of sodium citrate (pH 5.5). Immediately before light measurement, 50 μl of 1 mM beetle D-luciferin (Promega

TABLE 2. Primers used for PCR amplification

Purpose and primer name	Sequence[a]	
	Forward (5′)	Backward (3′)
Luciferase fusion and control		
luc_BamHI_5′	CGCGGATCCTCCGGATCCTCGAGGAGG	
luc_PstI_3′		CCCTGCAGTTACAATTTGGACTTTCCG
pbp1A_fusion	ACGCGTCGACTGACATCCGGAACAGGTACA	GCGGATCCTAAACCTTAACGCTGGCCGTTATTAGTC
pbp1B_fusion	ACGCGTCGACTGGATAGGACACGACGACAA	CGGGATCCTTTTAATTTGTCTGACTTTGACTAGAA
pbp2A_fusion	ACGCGTCGACTCAACTGCGAAAAAGATGACC	CGGGATCCAACTTACCAACCAAACCAGCTC
pbp2B_fusion	ACGCGTCGACGCAGCTACAGTCGCGAATAA	CGGGATCCTCCTTTCTAATTCATAGGGTGTAGTTG
pbp2X_fusion	ACGCGTCGACGTGACAGTGAAGCAGCCTGA	CGGGATCCTGCATCTTACTCTCCTAGTGTTATTG
pbp1A_KpnI_5′	CGGGTACCTGACATCCGGAACAGGTACA	
pbp1B_KpnI_5′	GGGGTACCTGGATAGGACACGACGACAA	
pbp2A_KpnI_5′	GGGGTACCTCAACTGCGAAAAAGATGACC	
pbp2B_KpnI_5′	GGGGTACCGCAGCTACAGTCGCGAATAA	
pbp2X_KpnI_5′	GGGGTACCGTGACAGTGAAGCAGCCTGA	
pbp1A_control_5′	CTGACGCTCAAAAGCAACTG	
pbp1B_control_5′	GCTGAGGATGCCATGTATCA	
pbp2A_control_5′	ATCAGGCCAGTATGCAGGTT	
pbp2B_control_5′	ACCCGCAAACTGGAGCTATT	
pbp2X_control_5′	AACTCAGCGCCCAAGTTTTA	
Determination of the *pbp2B* promoter		
Ppbp2B_5′	ACGCGTCGACACTTTCAACGTTTGGCTCGT	
Ppbp2B_1		GCGGATCCACAAAAAGATAAAAAATAATCTGGGAA AGAAGCCCAG
Ppbp2B_2		GCGGATCCTTTCTTCTATTCTACCACAAAAAGAT
Ppbp2B_3		GCGGATCCTTCTTTCTTAGGCATAATTTCTCTCA
Ppbp2B_KpnI_5′	ACGGTACCACTTTCAACGTTTGGCTCGT	
5′-RACE amplification		
pbp1A_race_1		GAATAGAGTCCACTCCACGGTGATT
pbp1B_race_1		GCTAAGGAATCGATGATTTCCGTTG
pbp2A_race_1		GGCATCCTCTACTCCCCAGACACCA
pbp2B_race_1		CCGTACGAATATTTCCCGTCTCGAA
pbp2X_race_1		TCAGATTTTTCCCCACTTGTTTTCG
pbp1A_race_2		AGAAAGAGTGAGATAAGGCCGCTTGCA
pbp1B_race_2		CGCAGAGCTGTCCACAGTAACCCTTAG
pbp2A_race_2		GAAGGAGCGGTCTTCAGTTGCAATAAC
pbp2B_race_2		CAGACAGTCGGTTTCCATCTGAGTCAA
pbp2X_race_2		AACCACGTTCTCGCTCATCTAATTGGG
pbp1A_race_3		TGGATTTCCCACCCTTATCA
pbp1B_race_3		CCCAGAAACAAAAGGACGAA
pbp2A_race_3		CGTTGACATTGGTGGTCTTG
pbp2B_race_3		AATCAGCAATCTGGCGTTTT
pbp2X_race_3		CAATAGCTGTCGCATCCTGA

[a] Engineered restriction sites are underlined. BamHI, GGATCC; KpnI, GGTACC; PstI, CTGCAG; SalI, GTCGAC.

Corporation, Madison, WI) diluted in GB buffer (25 mM glycylglycine, 15 mM $MgSO_4$) was added to the mixture. Luminescence was measured on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) for a period of 10 s with a delay of 2 s at 22°C. The specific bioluminescence was calculated by normalizing the relative light units to the OD_{620} of the culture, and the results are presented as means ± standard deviations for at least three samples.

RNA extraction and mapping of the transcriptional start sites. Total RNA was extracted by using the RNeasy Protect Bacteria Mini Prep kit (QIAGEN). Briefly, 5 ml of RNA Protect Bacteria reagent (QIAGEN) was added to 2.5-ml aliquots of *S. gordonii* cultures at an OD_{600} of 0.5 and vortexed for 5 s. After 5 min of incubation at room temperature, the suspension was centrifuged at 3,200 × *g* for 10 min. The pellet was immediately resuspended in 100 μl of Tris-EDTA buffer containing 15 mg of lysozyme per ml and incubated at room temperature for 15 min with tube inversion every 2 min. The manufacturer's protocol was followed from this point.

The 5′ end of each PBP transcript was mapped with the BD Smart rapid amplification of cDNA ends (RACE) kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The method is exemplified here for *pbp1A*. The gene-specific first-strand cDNA, synthesized from 1.2 μg total RNA with *pbp*-specific 5′-CDS primers pbp1A_race_1 (Table 2), was tailed with the BD SMART II A oligonucleotide. The products were then amplified with the nested gene-specific primer pbp1A_race_2 and the Universal Primer A Mix in a PCR involving 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 3 min. The PCR product was purified with the GFX Gel Band purification kit (Amersham Biosciences) and sequenced using primer pbp1A_race_3 (Synergene Biotech GmbH, Schlieren, Switzerland).

RESULTS

Determination of PBP genes promoters by 5′-RACE amplification. First, the 5′ ends of the PBP transcripts, corresponding to the transcription initiation site, were determined using the 5′-RACE PCR system. Second, promoter-like regions were localized by analyzing their upstream regions in silico in comparison to the *S. gordonii* sequence (www.tigr.org). For *pbp1A*, *pbp1B*, *pbp2A*, and *pbp2X*, a band corresponding to a specific

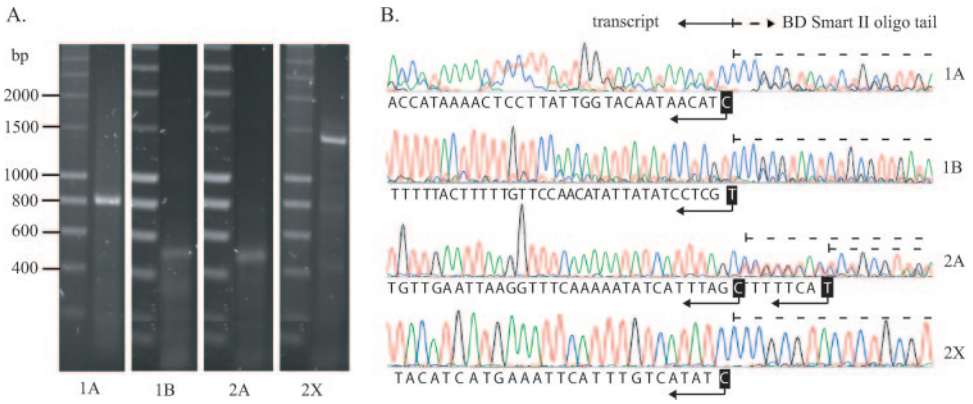

FIG. 1. Determination of the transcriptional start sites of *pbp1A*, *pbp1B*, *pbp2A*, and *pbp2X* by 5′-RACE PCR. (A) Agarose gel electrophoresis of PCR-amplified cDNA tailed with BD SMART II oligonucleotide. DNA was stained with SYBR Safe and visualized under UV light. (B) A chromatogram from the sequencing of the 5′-RACE PCR products. The BD SMART II oligonucleotide tails (dashed arrow) and the nucleotides complementary to the transcript beginnings (solid arrow) are shown.

PCR product was revealed by agarose gel electrophoresis (Fig. 1A) and sequenced. For *pbp2B*, no specific band was observed, possibly due either to secondary structures hindering the progression of the reverse transcriptase or to RNA-RNA interactions. An alternative technique was used to determine the promoter, as described below.

The transcriptional starts of *pbp1A*, *pbp1B*, and *pbp2X*, shown in Fig. 1B, were located 621 nucleotides (nt), 13 nt, and 1,285 nt upstream, respectively, of the putative ATG start codon. For *pbp2A*, two transcriptional starts were identified. One of them corresponded to the putative translational start codon, as predicted by comparison with the *S. pneumoniae* PBP 2A sequence (accession number NP_359415.1), and the second was located 7 nt downstream. This overlap between the transcriptional and translational starts implies that the mRNA does not display any RBS upstream from the initiation codon. Although rare, this phenomenon has already been described for the *S. pneumoniae polA* gene (19), as well as for mycobacteria and streptomycetes (8, 29). Similarly, the spacer between the transcriptional and putative translational initiation sites of *pbp2X* is too short to accommodate an RBS (see Fig. 3), and *pbp2B* is not preceded by an obvious RBS (Fig. 2). It is therefore possible that the translation initiation of *pbp2A*, *pbp2B*, and *pbp2X* involves a noncanonical mechanism.

The transcriptional start of the *pbp2B* gene could not be determined by 5′-RACE. The localization of its promoter was thus deduced using transcriptional fusions. Plasmid pJDC9 containing the promoterless *luc* gene was integrated into the *S.*

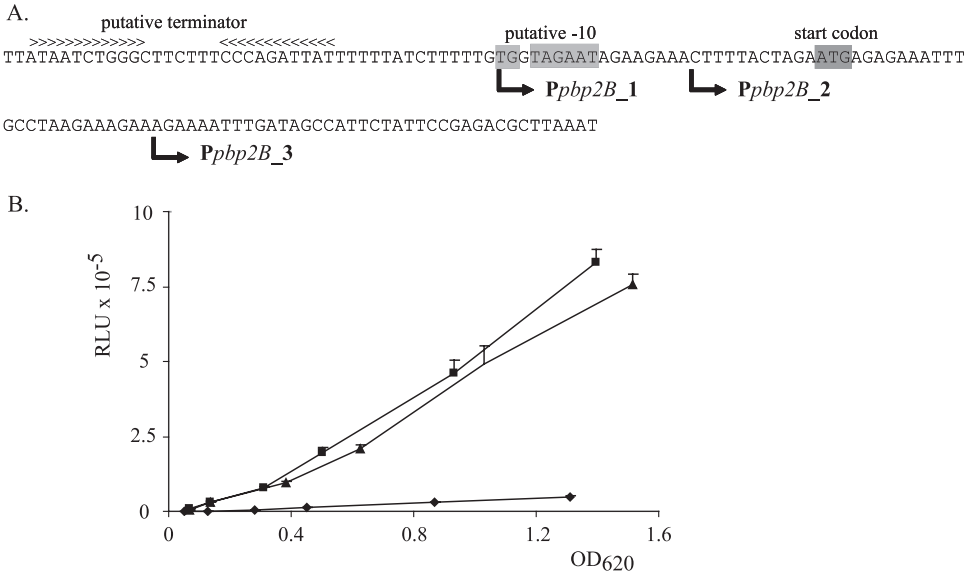

FIG. 2. Determination of the transcriptional start of *pbp2B* by using specific fusions with the luciferase reporter gene. (A) Specific localization of the fusions. (B) Expression profiles of Ppbp2B_1 (◆), Ppbp2B_2 (▲), and Ppbp2B_3 (■) transcriptional fusions. Bacteria were grown in BHI medium, and at different time points, samples were withdrawn for the determination of the OD_{620} and bioluminescence. Relative luciferase units (RLU) are plotted against the OD_{620}. Data from a representative experiment are shown as the means of triplicate values, with error bars indicating standard deviations.

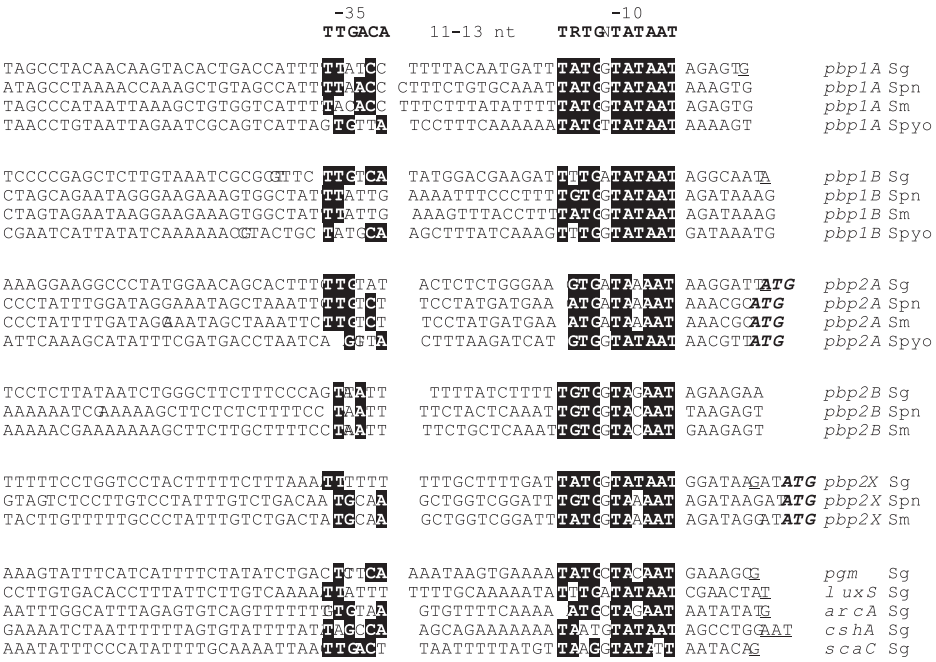

FIG. 3. Alignment of putative promoter sequences of *S. gordonii* and other streptococci. *S. gordonii* promoter sequences were deduced from 5′-RACE (*pbp1A*, *pbp1B*, *pbp2A*, *pbp2X*, and *pgm*), primer extension (*luxS*, *arcA*, *cshA*, and *scaC*), and transcriptional *luc* fusion (*pbp2B*) analyses (1, 2, 9, 16, 21). Residues identical to the consensus of the −35 and the extended −10 regions, indicated above the alignment, are highlighted with black boxes. Experimentally determined transcriptional start points are underlined. Sg, *S. gordonii*; Spn, *S. pneumoniae*; Sm, *S. mitis*; Spyo; *S. pyogenes*.

gordonii chromosome at three different sites in the vicinity of the putative promoter (Fig. 2): first, after the putative terminator of the preceding gene (strain SG_Ppbp2B_1 in Table 1); second, 8 nt downstream of the putative −10 region (strain SG_Ppbp2B_2); and third, 23 nt after the putative start codon of the gene (strain SG_Ppbp2B_3). The expression of luciferase (Fig. 2B) was very low in the strain bearing the *luc* gene upstream of the putative promoter (strain SG_Ppbp2B_1). In contrast, when the *luc* gene was inserted immediately after the putative transcriptional start (SG_Ppbp2B_2), luciferase activity was highly induced and comparable to what was measured in the strain bearing the *luc* fusion after the putative start codon. Together with in silico analysis, this demonstrates that the transcriptional start was most likely located 12 nt upstream of the putative start codon (Fig. 2B) and was preceded by an extended −10 region, as described below.

Consensus sequence of the *S. gordonii* promoters and in silico comparison with other species. At a distance of 5 to 7 nt, the transcriptional starts of the *pbp* genes are preceded by a sequence exhibiting a strong similarity (at least 8/10 nt) to the so-called extended −10 consensus sequence (TRTGNTAT AAT) of *Bacillus subtilis* and *S. pneumoniae* σ^A-type promoters (Fig. 3) (14, 25). In contrast, the putative −35 consensus region of the *pbp* genes displayed a poor match (2/6 to 3/6 nt), both among themselves and with other genes described for *S. gordonii* (with the exception of *pbp1B*) (1, 2, 9, 16, 21). The same observation was made when the putative PBP promoters of *S. pneumoniae* R6, *Streptococcus mitis* NCTC 12261, and *Streptococcus pyogenes* M1 GAS were included in the alignment (Fig. 3). It is noteworthy that we did not find any homologue of PBP 2B in *S. pyogenes* and also did not find any obvious −10 and −35 consensus region for PBP 2X.

Operon organization of the *pbp* genes. The operon organization was inferred by combining (i) promoter localization identified either by RACE or with luciferase transcriptional fusions and (ii) sequence analyses, including the presence of stem-loop structures that may form transcriptional terminators as well as gene orientation. *pbp1A* apparently forms a two-gene operon with the upstream gene *recU*. *pbp2B* probably also forms an operon with the downstream *recR* gene. *pbp2X* forms an operon with two upstream genes (homologues of *mraW* and *ftsL*) and most likely with the downstream *mraY* gene, as in the *S. pneumoniae* cell wall gene cluster (20). Finally, the transcripts of *pbp1B* and *pbp2A* appear to be monocistronic.

The overall gene organization appears to be relatively well conserved between *S. gordonii*, *S. pneumoniae*, *S. mitis*, and *S. pyogenes*, as revealed by in silico comparisons (data not shown). In all four organisms, *pbp1A*, *pbp1B*, and *pbp2X* share the same genetic environment. The organization is also highly similar for *pbp2B*, except for *S. pyogenes*, in which no homologue of this gene was found. Finally, in *S. pneumoniae*, *S. mitis*, and *S. pyogenes*, *pbp2A* is part of a putative operon (with *rpmG*, *secE*, and *nusG*), whereas in *S. gordonii* it seems to be monocistronic and separated from *rpmG-secE-nusG* by a divergently transcribed gene. The presence of a terminator between the three genes and *pbp2A* was thus sought in *S. pneumoniae*, *S. mitis*, and *S. pyogenes*, but only a weak candidate was found.

Expression of PBPs in wild-type *S. gordonii*. To monitor the expression of the control *arc* and the *pbp* genes, *luc* transcrip-

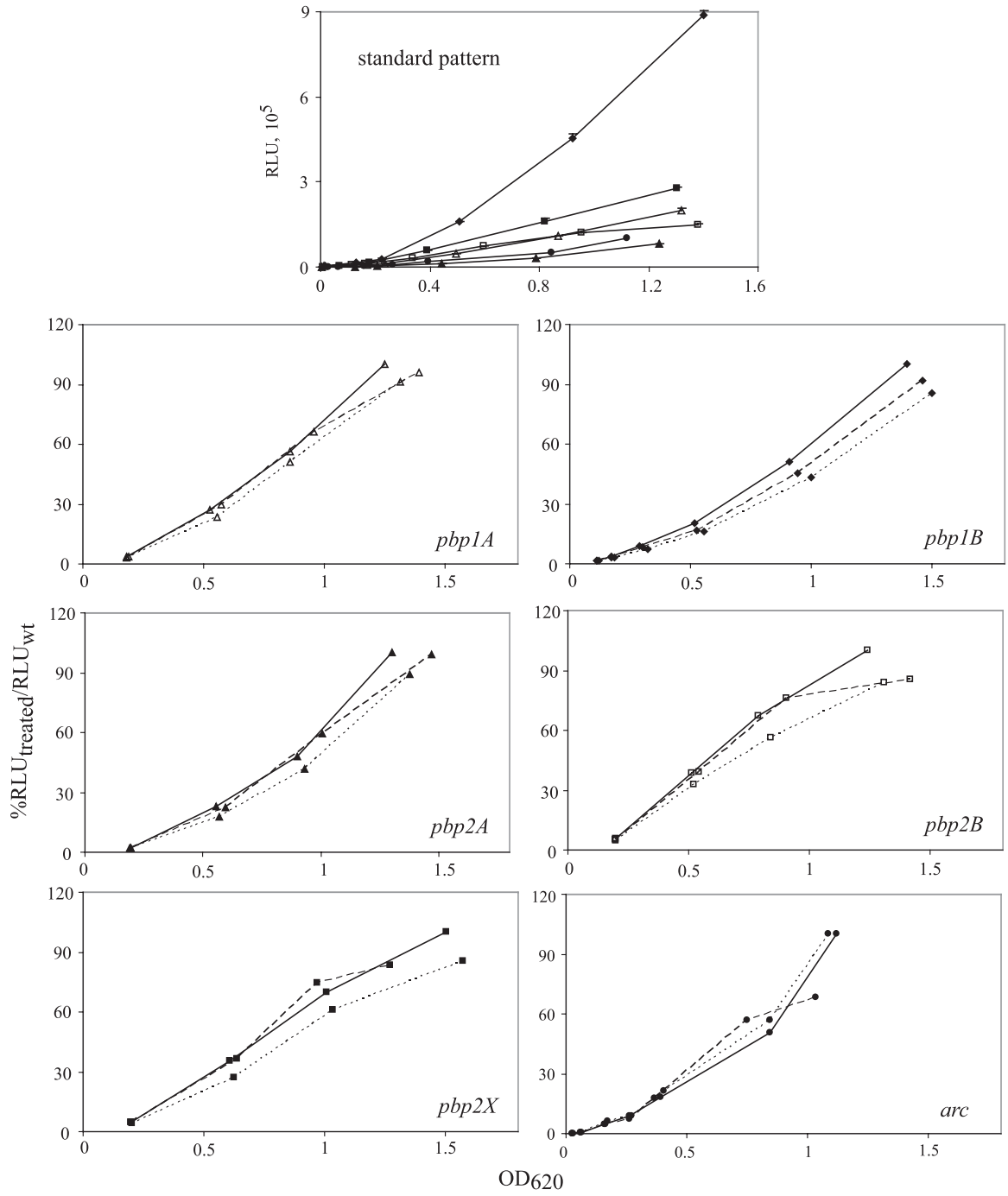

FIG. 4. Expression profiles of the *pbp* genes and of the control *arc* gene transcriptional fusions in the susceptible wild-type *S. gordonii*. Cultures were grown under standard conditions (—) or in the presence of subinhibitory concentrations of penicillin G. Penicillin was added at an OD_{620} of 0.12, and concentrations corresponded to 1/2× the MIC (– –) or 1/8× the MIC (- - -). The expression of *arc* (●), *pbp1A* (△), *pbp1B* (◆), *pbp2A* (▲), *pbp2b* (□), and *pbp2x* (■) was monitored as described in the legend to Fig. 2B. The expression of all genes is shown in the same graph and expressed in relative luciferase units (RLU) (general pattern) for the sake of global comparison. In all other graphs, data are expressed as a percentage of the value for the nontreated control. The maximal expression of each gene is considered 100%. Data are then expressed as the percentage of the expression of the corresponding gene under standard conditions.

tional fusions were used. In the wild-type background under standard conditions, the luciferase activity increased during the exponential phase (Fig. 4). Experiments were stopped at the end of the exponential phase, since luciferase measurements were shown to be nonreliable in stationary phase (18, 27). All *pbp* genes exhibited a similar pattern. The luciferase activity was highly reproducible over >5 separate experiments, and its levels systematically followed the PBP 1B > PBP 2X > PBP 1A = PBP 2B > PBP 2A hierarchy.

Cell wall-active antibiotics disturb the function of PBPs and

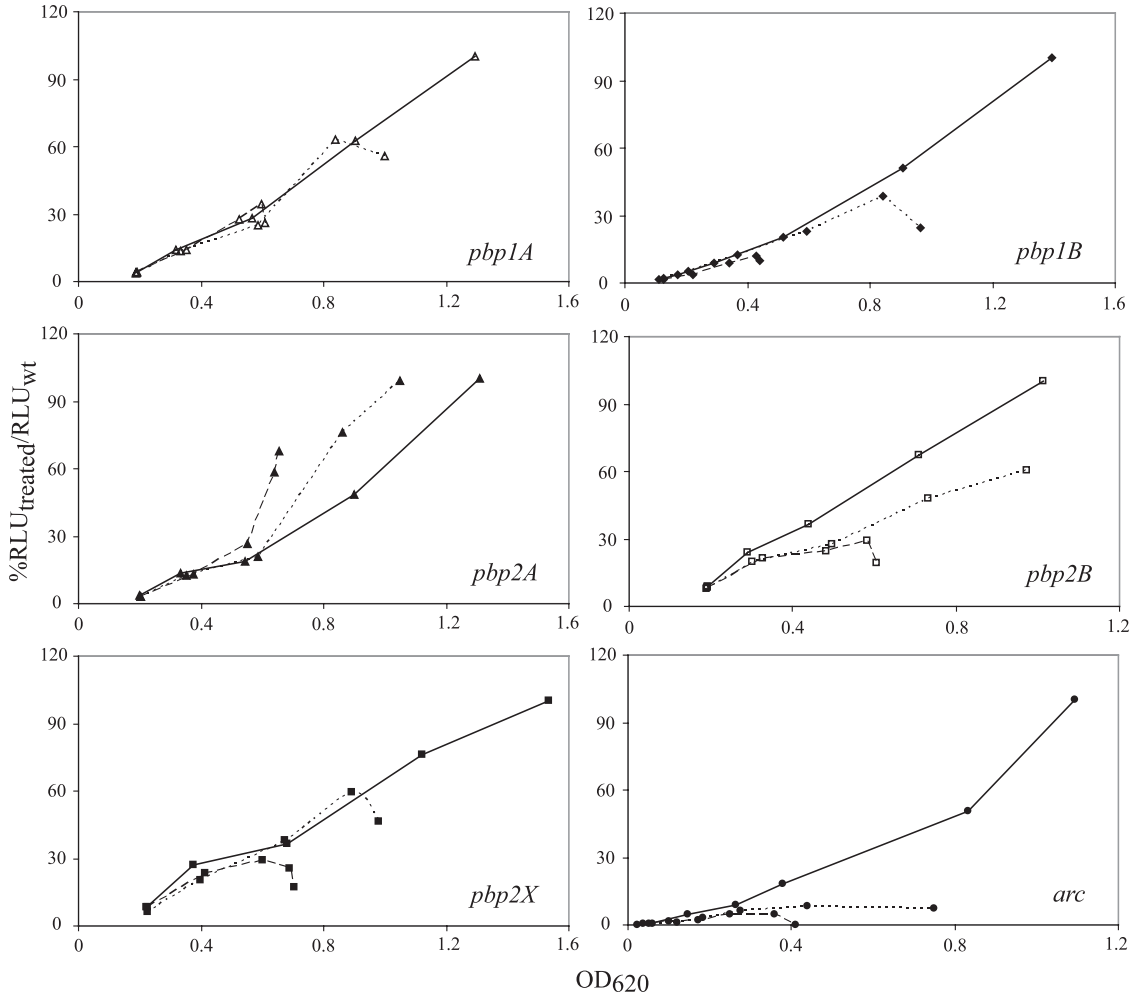

FIG. 5. Expression profiles of the wild-type *pbp* genes and of the control *arc* gene transcriptional fusions in presence of suprainhibitory concentrations of penicillin G. Cultures were grown under standard conditions (—) or in the presence of penicillin at 2× the MIC (- - -) or 8× the MIC (– –). The expression of *arc* (●), *pbp1A* (△), *pbp1A* (◆) *pbp2A* (▲), *pbp2b* (□), and *pbp2x* (■) was monitored as described in the legend to Fig. 2B. Data are expressed as a percentage of the nontreated control, as described in the legend to Fig. 4. RLU, relative luciferase units.

are known to exert a selective pressure on these enzymes. We thus tested whether the presence of penicillin in the medium would alter the expression of *pbp* genes. Subinhibitory concentrations of penicillin (1/8× or 1/2× the MIC) did not alter bacterial growth and had no significant effect on the expression of the five genes studied (Fig. 4). On the other hand, suprainhibitory concentrations of penicillin (2× and 4× the MIC) progressively inhibited bacterial growth, which came to a stop at OD_{620}s of 0.6 (4× the MIC) and 1 (2 × the MIC), respectively, and interfered with the luciferase assay (Fig. 5). It is unclear whether the rapid decrease in light emission was due to the blockage of gene expression or was a consequence of growth arrest and ATP depletion (18). Nevertheless, during growth (at an OD_{620} of 0.5), the *pbp2A* gene showed a specific and transient induction related to the presence of the antibiotic. Moreover, the *pbp2A* response slope steepened in parallel with increasing drug concentrations in the medium.

Expression of PBPs in a penicillin-resistant isolate. The expression of PBP genes was also measured in a laboratory-generated penicillin-resistant mutant (PR1_2evolved) (13) which had an MIC of 2 μg/ml, i.e., 250 times greater than that of the parent. PR1_2evolved contains several mutations, notably in PBP 2B and PBP 2X, that are accompanied by a decrease in the affinity of these proteins for the drug (data not shown). Under standard conditions, the luciferase activities of PBPs were globally enhanced compared to those in the susceptible parent (Fig. 4), except for *pbp1B*, which was slightly reduced (Fig. 6). Maximal factors of increase were 1.55 ± 0.09 for *pbp1A*, 0.70 ± 0.06 for *pbp1B*, 1.59 ± 0.14 for *pbp2A*, 1.39 ± 0.1 for *pbp2B*, and 2.1 ± 0.11 for *pbp2X*. Differences were relatively small but highly reproducible, as presented here as the means from six separate experiments.

When exposed to subinhibitory concentrations of penicillin (1/8× or 1/2× the MIC) the expression pattern of the penicillin-resistant PR1_2evolved clearly differed from that of the susceptible parent (Fig. 6). The mutant grew normally in spite of the drug, thus allowing a valid comparison of the results. The expression of the five PBP genes varied and presented dissimilarities between class A PBPs (PBPs 1A, 1B, and 2A) and class B PBPs (PBPs 2B and 2X). Penicillin at 1/2× the

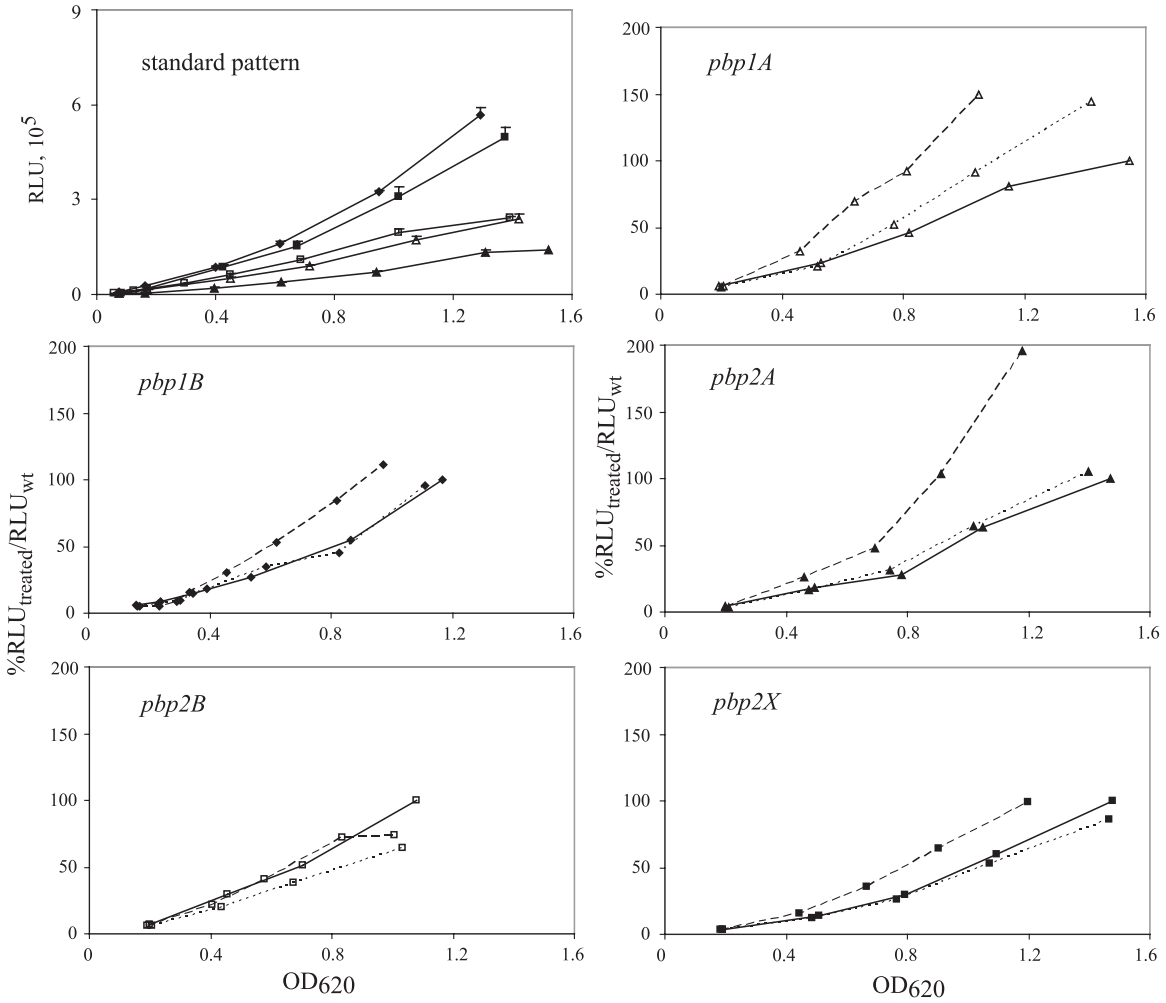

FIG. 6. Expression profiles of the penicillin-resistant PR1_2evolved *pbp* gene transcriptional fusions. Cultures were grown under standard conditions (—) or in the presence of subinhibitory concentrations of penicillin G. Penicillin was added at an OD of 0.12, and concentrations corresponded to 1/2× the MIC (– –) or 1/8× the MIC (- - -). The expression of *pbp1A* (△), *pbp1B* (◆), *pbp2A* (▲), *pbp2b* (□), and *pbp2x* (■) was monitored as described in the legend to Fig. 2B. Data are expressed as described in the legend to Fig. 4. RLU, relative luciferase units.

MIC induced an early and increased expression of class A *pbp1A* and *pbp2A*, and the *pbp1A* expression was still increased at 1/8× the MIC. On the other hand, the global expression of the class B *pbp* genes did not increase, except for a slight augmentation of *pbp2X* in presence of the antibiotic at 1/2 × the MIC. The expression of *pbp2A* and *pbp2B* was also monitored using the quantitative reverse transcription-PCR technique and showed the same trend as in the luciferase experiments, although with a smaller amplitude (data not shown).

The question then arose as to whether the differential gene regulation in the resistant mutant was due to mutations in the promoter region. However, sequencing of these regions did not reveal any sequence variation.

DISCUSSION

This study determined the promoter regions of the five PBP genes of *S. gordonii* and assessed their expression in the absence or presence of penicillin. By sequence comparisons, *pbp* promoter regions in the related *S. pneumoniae*, *S. mitis*, and *S. pyogenes* were also identified. With a few exceptions, all these PBP promoters had a highly conserved −10 box which respected the TRTGNTATAAT consensus sequence of *B. subtilis* and *S. pneumoniae* σ^A-type promoters (14, 25). This is likely to exclude PBP regulation by specific stress-activated sigma factors such as the extracytoplasmic function sigma factor in *B. subtilis* (22) or σ^M in *S. aureus* (5). On the other hand, they all had quite variable −35 boxes, as shown for *S. pneumoniae* promoter regions (25), leaving the possibility of a differential regulation.

Importantly, the few exceptions included the PBP 2B gene, for which no homologue was found in *S. pyogenes*, and the PBP 2X gene, for which no promoter consensus sequence was found. These differences might be a hint as to the reason for the absence of penicillin-resistant *S. pyogenes* in spite of >50 years of drug exposure in the clinical environment (23). Indeed, PBPs 2X and 2B are the primary targets for penicillin resistance mutations in several streptococci (11, 13), and a difference in their structure or regulation might be a hindrance to resistance development. Molecular proof of this possibility will be important to assess.

In the absence of penicillin, all PBP genes were constitutively expressed in both the susceptible parent and the resistant mutant. Yet, there was a trend toward a greater global PBP expression in the resistant mutant, in which the luminescence was reproducibly 1.5 to 2 times higher than in the susceptible parent. Whether this global increase is a cause or a consequence of resistance still remains unknown. Yet, increased PBP production could allow out-competing of their blockage by penicillin. On the other hand, increased PBP concentrations might also be required to compensate for possible altered function of mutated enzymes.

In the presence of penicillin, the expression of class A PBP 2A was clearly increased in both the susceptible parent and the resistant mutant, and PBP 1A was increased in the resistant bacterium. In contrast, the expression of other PBPs was relatively unchanged. Selective induction of class A PBPs during penicillin exposure conformed to previous observations. First, PBP genes could be differentially regulated in response to specific environmental conditions, as hypothesized on the basis of their polymorphic promoter −35 box (see above). Second, microarray analyses assessing the responses of *S. aureus* and *B. subtilis* to cell wall inhibitors revealed drug-related induction of class A PBP genes, namely, *pbp2* in *S. aureus* and *ponA* in *B. subtilis* (4, 30). Third, an increased expression of class A PBPs in response to the drug is likely to facilitate the progression toward resistance. Indeed, if deletion of class A PBPs hindered resistance development, then their overexpression might facilitate it, as suggested by the present and previous reports (4, 13, 30).

A speculative model to explain this effect was proposed previously (13). An optimally interconnected peptidoglycan consists of a network of glycan chains cross-linked by peptide bridges. At high concentrations, penicillin blocks the whole transpeptidase apparatus and bacterial growth comes to a halt. At borderline penicillin concentrations, on the other hand, transpeptidase is only partially blocked and cross-linking continues at a reduced pace, allowing long glycan chains to undergo a minimal level of cross-linking, which might be critical for cell wall integrity. Since class A PBPs, but not class B PBPs, carry a transglycosylase domain in addition to the transpeptidase activity (10), they are logical candidates for such a compensatory effect. Recent gene deletion experiments support such a model. Indeed, deletion of class A PBP 1A shortened the length of the glycan chains by ca. 30% (12). Conversely, overexpression of such enzymes is expected to increase the relative length of these chains.

Eventually, penicillin-related overexpression of class A PBPs occurred within a narrow window of drug concentrations (from 1/8× the MIC for the susceptible parent to 8× the MIC for the resistant mutant). This overexpression might be considered not a resistance mechanism sensu stricto but rather a facilitator that improves bacterial survival and the chance of developing resistance mutations at borderline antibiotic concentrations. However, its impact should not be underestimated. The facilitating mechanism is likely to operate at each step of resistance selection, as penicillin-related induction of class A PBPs occurred in both susceptible and resistant cells.

In summary, examination of the promoters and gene regulation of the *S. gordonii* PBP genes highlighted the existence of differential regulatory pathways that may facilitate resistance development in the absence of mutations. The specific induction of class A PBPs by penicillin was consistent with previous observations suggesting that an increased transglycosylase activity could stabilize an otherwise poorly cross-linked peptidoglycan and promote bacterial survival at borderline penicillin concentrations. Genetic comparisons also provided a hint as to why *S. pyogenes* has not successfully developed penicillin resistance so far. Some observations remain unexplained, including the truncated architecture of the PBP 2A, PBP 2B, and PBP 2X gene regulatory regions. Such unusual structures were found in other microorganisms (8, 19, 29), but their consequence for protein expression is unclear. In addition to supporting the importance of drug-related gene regulation, as also shown by others (4, 30), this study shows the importance of establishing links between global gene regulation and its consequences at the functional and structural levels.

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