Promoter Identification and Transcription Analysis of Penicillin-Binding Protein Genes in *Streptococcus pneumoniae* R6

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Penicillin-binding proteins (PBPs) are membrane-associated enzymes, which are involved in the last two steps of peptidoglycan biosynthesis, and some of them are key players in cell division. Furthermore, they are targets of β-lactams, the most widely used antibiotics. Nevertheless, very little is known about the expression and regulation of PBP genes. Using transcriptional mapping, we now determined the promoter regions of PBP genes from the laboratory strain *Streptococcus pneumoniae* R6 and examined the expression profile of these six promoters. The extended −10 region is highly conserved and complies with a σ^A-type promoter consensus sequence. In contrast, the −35 region is poorly conserved, indicating the possibility for differential PBP regulation. All PBP promoters were constitutively expressed and highly active during the exponential and early stationary growth phase. However, the individual expression of PBP promoters varied approximately fourfold, with *pbp1a* being the highest and *pbp3* the lowest. Furthermore, the deletion of one nucleotide in the spacer region of the PBP3 promoter reduced *pbp3* expression ∼10-fold. The addition of cefotaxime above the minimal inhibitory concentration (MIC) did not affect PBP expression in the penicillin-sensitive R6 strain. No evidence for regulation of *S. pneumoniae* PBP genes was obtained.

Introduction

PENICILLIN-BINDING PROTEINS (PBPs) are modular membrane-bound enzymes catalyzing the final steps of bacterial cell wall synthesis. They are the targets of β-lactam antibiotics and play important roles in the division process. *Streptococcus pneumoniae* contains six PBPs, which are classified with respect to their molecular weight, domain structure, and enzymatic activities into three classes (for reviews, see Goffin and Ghuysen[1]; Sauvage *et al.*[2]; Zapun *et al.*[3]). All PBPs contain a DD-peptidase domain. *S. pneumoniae* PBP1a, PBP1b, and PBP2a are class A high-molecular weight (HMW) PBPs. They are bifunctional enzymes, since they polymerize the glycan chains by their N-terminal glycosyltransferase domain and crosslink the peptides by their DD-transpeptidase domain. The members of class B HMW PBPs (PBP2x and PBP2b) are mono-functional DD-transpeptidases and contain an N-terminal domain of unknown function. In addition, PBP2x contains a C-terminal domain consisting of two PASTA (PBP- and serine/threonine kinase-associated) domains.[4–6] The topol-ogy of all HMW PBPs consists of a cytoplasmic tail and a transmembrane anchor followed by two or three surface-exposed domains.[7] Finally, PBP3 (DacA) is a class C, low-molecular weight (LMW) PBP with DD-carboxypeptidase activity, hydrolyzing the C-terminal D-alanine moiety from the pentapeptides in the peptidoglycan (PG) chain.[8–10]

The genes encoding class A HMW PBPs can be deleted individually, demonstrating that none of them is essential for growth under laboratory conditions.[11,12] It is possible to isolate double mutants *pbp1b pbp2a* and *pbp1a pbp1b*, but *pbp1a pbp2a* double mutants are not viable.[12,11] Both class B PBPs, PBP2x and PBP2b, are essential in *S. pneumoniae*.[13–15]

S. pneumoniae grows and divides by alternating cycles of peripheral and septal PG synthesis (reviewed in Massidda *et al.*[16]). The genes *pbp2x* and *pbp2b* are located in the *dcw* (division and cell wall) cluster,[16,17] already indicating a role in the division process. The coordinated function of each PBP during the *S. pneumoniae* cell cycle remains largely un-known.[18] All HMW PBPs localize at mid cell,[19] the central growth zone, where new cell wall material is incorporated. Recently, the essential roles of PBP2x and PBP2b in cell

division were confirmed.[14–16,20,21] Depletion of *pbp2b* results in the formation of rounded and chained cells, indicating that PBP2b is essential for peripheral PG synthesis.[14,21] The depletion of *pbp2x* results in lemon-shaped or elongated cells often with pointed ends, showing that PBP2x is responsible for septal PG synthesis.[14,15,21] In contrast, LMW PBP3 does not localize at the division sites like the HMW PBPs, but is distributed over the entire cell surface[22,23] and at mid cell in some dividing cells.[23] Cells lacking PBP3 show heterogeneity in cell size and shape and display defects in septum placement[23–25] indicating an important role of PBP3 during cellular growth in general.

Beta-lactam antibiotics mimic the terminal D-Ala-D-Ala moiety of the PG pentapeptide stem and, therefore, are recognized as PBP suicide substrates.[26] PBPs are inhibited by β-lactams by forming a covalent PBP-β-lactam complex through the active site serine, which is enzymatically inactive. For most beta-lactams, this complex is very stable corresponding up to several generation times; kinetic parameters describing PBP-beta-lactam interactions can be found in Zapun *et al.*[3] The consequence is inhibition of bacterial growth and cell lysis. Mutations in PBPs of β-lactam-resistant strains reduce the protein affinity to the antibiotic, while leaving the enzymatic function apparently unaffected. Such mutated enzymes do not interact with β-lactams over a wide antibiotic concentration range and, therefore, PG synthesis and cell growth can continue. Mutations in *S. pneumoniae* PBP2x and PBP2b result in low-level resistance and additional alterations in PBP1a are required for high resistance levels. Occasionally, alterations in PBP2a, 1b, and 3 have been described in high-level-resistant strains (for review, see Hakenbeck *et al.*[7]). The interaction between PBP and β-lactam antibiotic is specific for each protein and for each antibiotic (for review, see Sauvage and Terrak[27]).[28] PBP2b does not interact with cefotaxime or other β-lactams with similar side chains.[29] In contrast to penicillins, which induce rapid lysis in *S. pneumoniae,* cells stop growing after cefotaxime treatment without significant lysis for several hours and are killed at a much lower rate. That is because cefotaxime induces a tolerant response.[29] PBP2b mutations can be selected with penicillin,[30] whereas cefotaxime selects for PBP2x mutations.[31–33]

Despite the important roles of PBPs in bacterial cell wall synthesis, cell division processes and development of β-lactam resistance, very little is known about the expression and regulation of PBP genes. To get insights into the expression and potential regulatory mechanisms, we identified the promoters of PBP genes and studied their expression during growth in a penicillin-sensitive strain in the absence and presence of cefotaxime below, at, and above the minimal inhibitory concentration (MIC) values. We show that all PBP promoters are constitutively expressed and the presence of cefotaxime does not influence their transcriptions. In addition, we show that a deletion in the spacer region between the −35 and −10 elements in the PBP3 promoter has a very strong effect on the promoter activity.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

All *S. pneumoniae* strains are derivatives of *S. pneumoniae* R6 strain.[34] The strains and plasmids used in this study are

TABLE 1. BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

	Relevant genotype or description[a]	Source or reference
Strains		
R6	Unencapsulated, nonvirulent descendent of D39	34
RP200	R6, *bgaA*::*tetM-lacZ,* Tet[R]	42
RKL44	R6, *bgaA*::*tetM*-P$_{vegW}$-*lacZ,* Tet[R]	42
801	Derivative of R6, containing *hexB* mutation	43
KP01	R6, *bgaA*::*tetM*-P$_{pbp1a}$-*lacZ,* Tet[R]	This study
KP02	R6, *bgaA*::*tetM*-P$_{pbp1b}$-*lacZ,* Tet[R]	This study
KP03	R6, *bgaA*::*tetM*-P$_{pbp2a}$-*lacZ,* Tet[R]	This study
KP04	R6, *bgaA*::*tetM*-P$_{pbp2b}$-*lacZ,* Tet[R]	This study
KP05	R6, *bgaA*::*tetM*-P$_{pbp2x}$-*lacZ,* Tet[R]	This study
KP06	R6, *bgaA*::*tetM*-P$_{pbp3}$-*lacZ,* Tet[R]	This study
KP09	R6, *bgaA*::*tetM*-P$_{pbp3-R801}$-*lacZ,* Tet[R]	This study
Plasmids		
pPP2	Integrative promoter probe plasmid (*bgaA*::*tetM-lacZ*), Amp[R], Tet[R]	42
pPP2$_{vegW}$	pPP2 derivative, carries P$_{vegW}$-*lacZ* fusion	42
pPP2$_{1a}$	pPP2 derivative, carries P$_{pbp1a}$-*lacZ* fusion	This study
pPP2$_{1b}$	pPP2 derivative, carries P$_{pbp1b}$-*lacZ* fusion	This study
pPP2$_{2a}$	pPP2 derivative, carries P$_{pbp2a}$-*lacZ* fusion	This study
pPP2$_{2b}$	pPP2 derivative, carries P$_{pbp2b}$-*lacZ* fusion	This study
pPP2$_{2x}$	pPP2 derivative, carries P$_{pbp2x}$-*lacZ* fusion	This study
pPP2$_3$	pPP2 derivative, carries P$_{pbp3}$-*lacZ* fusion	This study
pPP2$_{3M}$	pPP2 derivative, carries P$_{pbp3-R801}$-*lacZ* fusion	This study

[a]Antibiotic resistance marker.
Amp, ampicillin; Tet, tetracycline.

described in Table 1. *S. pneumoniae* strains were grown at 37°C without aeration in C medium[35] supplemented with 0.1% yeast extract (C + Y medium), in brain heart infusion broth (BHI, Broth), or on D-agar[36] plates containing 3% defibrinated sheep blood. Growth in liquid culture was monitored by nephelometry and is given in nephelo units [N].

Escherichia coli DH5α was used as a host for cloning and propagation of plasmids. *E. coli* was grown at 37°C either in LB medium with aeration or on LB agar plates.[37] Growth of *E. coli* was followed by measuring the optical density (OD) at 600 nm.

Transformation and DNA manipulations

Transformation of *S. pneumoniae* was performed according to a published procedure.[38] Transformants were selected on D-agar supplemented with 3% defibrinated sheep blood and 3 μg/ml tetracycline. *E. coli* DH5α was transformed according to Hanahan[39] and transformants were selected in the presence of 100 μg/ml ampicillin.

All DNA techniques were performed using standard methods.[37] Plasmid DNA was purified using the Nucleo-Bond AX-100 (MACHEREY-NAGEL) Kit. PCR reactions were performed using GoldStar Taq DNA polymerase (Eurogentec) or high-fidelity iProof DNA polymerase (Bio-Rad Laboratories) according to the manufacturer's instructions. PCR products and DNA recovered after restriction with endonuclease were purified using a JetQuick DNA Purification Kit (Genomed). DNA-modifying enzymes were purchased from New England BioLabs or Invitrogen and used as described by the manufacturer. DNA oligonucleotides used in this study are listed in Table 2 and were obtained from Eurofins, MWG or Operon. RNA oligonucleotides were obtained from Biomers GmbH.

RNA isolation

To isolate total RNA from *S. pneumoniae*, the cells were grown in C + Y medium to a density of N = 70, pelleted by 8.000 rpm at 4°C, and frozen in liquid nitrogen. For all subsequent solutions, diethylpyrocarbonate-treated water (DEPC; Ambion) was used. RNA was extracted as described previously.[40] Residual DNA was digested by the addition of 5 units (U) DNase (New England BioLabs) and incubation for 15 min at 37°C. The RNA was purified further using the RNeasy Midi Kit (QIAGEN) according to the manufacturer's instructions.

Mapping of transcriptional start sites

The transcriptional start point of each PBP transcript was determined by rapid amplification of cDNA ends (5′-RACE) as described previously[41,42] using the same RNA adapter and gene-specific oligonucleotides (Table 2). Briefly, 15 µg of total cellular RNA was incubated with and without 25 U Tobacco Acid Pyrophosphatase (TAP; Epicentre Biotechnologies) in the buffer supplied by the manufacturer at 37°C for 60 min in the presence of 20 U SUPERaseIN RNase inhibitor (Ambion). Subsequently, the reaction mixture was extracted with phenol:chloroform (1:1) and precipitated with ethanol. RNA pellets were resuspended in 55 µl DEPC-treated water (Ambion), mixed with 500 pmol RNA adapter (5′ RACE-Adapter; Table 2) (Biomers GmbH) and heat-denatured at 95°C for 5 min. Ligation of the adapter was carried out at 17°C overnight with 100 U T4 RNA ligase (New England BioLabs) in the supplied buffer and in the presence of 80 U SUPER-aseIN (Ambion) in a total reaction volume of 100 µl. The reaction mixture was extracted with phenol:chloroform (1:1), ethanol precipitated, and the pellet was resuspended in 25 µl DEPC-treated water. Subsequently, 5 µl of ligated RNA were reverse transcribed with a gene-specific oligonucleotide (Table 2) using the First-Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche) according to the instructions of the manufacturer in a volume of 20 µl. Then, 2 µl of cDNA were amplified by PCR using the nested gene-specific oligonucleotides (Table 2) and the RACE-PCR_5′ oligonucleotide,

TABLE 2. OLIGONUCLEOTIDES USED IN THIS STUDY

Oligonucleotides	Sequence (5′-3′)
For 5′-RACE amplification	
pbp1a_RACE_1	AAGCTAATGCTCAGATACTTGATTAGG
pbp1a_RACE_2	ATTCCTTGTTGGGCAAGGAC
pbp1b_RACE_1	CACGAATCACCGCCTTGGGTACTACAC
pbp1b_RACE_2	AAGTGCGCAACAAATCACTC
pbp2a_RACE_1	GGTAATGGTAGAGCCACCACCTGAACG
pbp2a_RACE_2	CGGCCATAGTTAATCCCGTC
pbp2x_RACE_1	TTCAGCCGGCGATTTCCGATTTTTGG
pbp2x_RACE_2	CTCACGCTGGTCCAATTGAG
pbp2b_RACE_1	CTGATGCTCACATAAGTCAGTAACTTT
pbp2b_RACE_2	ACGCGTAAAGGAAACAACCTGCTTTAAC
pbp3_RACE_1	ATTGACAACAGTGGCATCCTGAATTCC
pbp3_RACE_2	TCTCAGCTAGGGCAATAGCG
RACE-PCR_5′	GATATGCGCGAATTCCTG
5′ RACE-adapter[a]	GAUAUGCGCGAAUUCCUGUAGAACGAACACUAGAAGAAA
For promoter probe cloning[b]	
pbp1a_ppf	AGATGCATGCGATCCTTATCACAAAATAAACCC
pbp1a_ppr	GCGG<u>GGATCC</u>ACATCACTTTATTATACCATAATTTG
pbp1b_ppf	ACA<u>TGCATG</u>CCAAAAATGTGCATGTGTTTTTTCTCC
pbp1b_ppr	GCGG<u>GGATCC</u>ACTTTATCTATTATACCACAAAAGGG
pbp2a_ppf	ACA<u>TGCATG</u>CTGAATTGCATACAGATATTGTAAC
pbp2a_ppr	GCGG<u>GGATCC</u>TTCATGCGTTTATTTTATCATCTTC
pbp2x_ppf	ACA<u>TGCATG</u>CGTCAAGTATTTTTGACATATTTTTTG
pbp2x_ppr	GCGG<u>GGATCC</u>ATATCTTATCTATTTTACCACAAATC
pbp3_ppf	ACA<u>TGCATG</u>CTGGTCCAAAAAAATAATTTATTTCTAC
pbp3_ppr	GCGG<u>GGATCC</u>CATAGTAGTCTTATTCTATCATAAAG
pbp2b_ppf	ACA<u>TGCATG</u>CGGGAATAGTCACACTTCATTATAAC
pbp2b_ppr	GCGG<u>GGATCC</u>ATCTTACTCTTAATTGTACCACAATTTG

[a]RNA oligonucleotide obtained from Biomers GmbH.
[b]*Sph*I and *Bam*HI restriction sites are underlined.
RACE, rapid amplification of cDNA ends.

which is complementary to the RNA adapter sequence. The PCR was performed using the GoldStar Taq DNA polymerase (Eurogentec) for 40 cycles at an annealing temperature of 50°C. Subsequently, PCR fragments were analyzed on a 2% agarose gel and sequenced.

The sequenced PCR fragments of *pbp1b*, *pbp2a*, and *pbp2b* showed in electropherograms a weak additional sequence in the region of the RNA adapter sequence, which overlaps with high-quality sequence and suggests the presence of the multiple sequences. To exclude the possibility that two transcription starts are present, the PCR fragments were ligated into the pGEM-T-Easy (Promega) vector and 15 colonies were analyzed by PCR followed sequencing of the respective PCR products (see text for details).

Construction of plasmids and strains

Transcription was monitored using the promoter probe plasmid pPP2.[42] Promoter regions were amplified by PCR from *S. pneumoniae* R6 genomic DNA using oligonucleotide pairs, which were named according to the PBP genes as listed in Table 2 (for example, pbp1a_ppf and pbp1a_ppr for the *pbp1a* promoter). The PCR products were cleaved with *Sph*I and *Bam*HI and inserted into the same sites in pPP2, generating the plasmids pPP2$_{1a}$, etc. Cloning was performed using the *E. coli* DH5α strain. After sequencing the insertions, plasmids were transformed into *S. pneumoniae* R6. The promoter probe plasmid cannot replicate in *S. pneumoniae* and promoter fusions were integrated into the endogenous β-galactosidase gene *bgaA* leading to its inactivation.[42] Correct integration occurred by double crossover and was confirmed by PCR and DNA sequencing. The resulting *S. pneumoniae* strains, harboring different promoter-*lacZ* fusions, were designated KP01–KP06 (Table 1).

The reporter plasmid pPP2$_{3M}$ was constructed following mainly the same procedure, except that the promoter fragment of *pbp3* was amplified by PCR using genomic *S. pneumoniae* 801 DNA.[43] The PCR fragment was cleaved with *Sph*I and *Bam*HI and ligated into the same sites into the pPP2 vector, generating the plasmid pPP2$_{3M}$. The resulting *S. pneumoniae* strain, harboring P$_{pbp3\text{-}R801}$-*lacZ* fusion, was designated KP09.

Determination of β-galactosidase activity

Determination of β-galactosidase activity in cell extracts of *S. pneumoniae* strains carrying *E. coli* lacZ gene fusions was performed as described previously.[44,45] The strains were grown in C + Y or BHI media and the β-galactosidase activity was measured at various time points during the exponential growth up to the beginning of the stationary phase. The sample size was adjusted to contain the equivalent of 2 ml cells at N = 90. Specific β-galactosidase activities are expressed in nmoles of nitrophenol released per min per mg of protein. Protein concentrations were determined using the standard method of Bradford.[46] Experiments were carried out in triplicates. Student's *t*-test was applied to check the significance of the results.

Prediction of promoters and terminators

The promoters were predicted by two consensus hexamers: −10 (5′-TATAAT-3′) and −35 (5′-TTGACA-3′) located upstream of the transcriptional start sites. Rho-independent transcription terminators were identified using the Mfold program,[47] which can be accessed through the The mfold Web Server. The consensus sequence covering the promoter region was designed using the web-based application WebLogo.[48,49]

Results

Genetic organization of the PBP genes in S. pneumoniae R6 genome

The genes encoding PBPs in *S. pneumoniae* are localized at six distinct loci (Fig. 1) scattered throughout the genome.
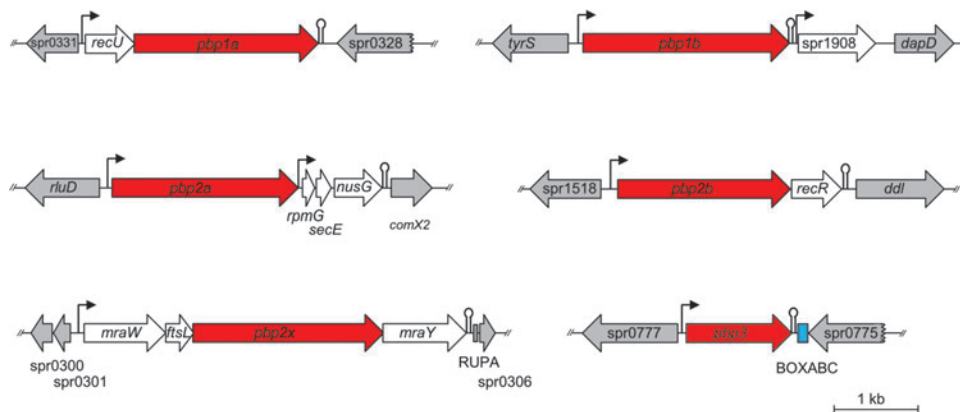
FIG. 1. Organization of genomic regions containing *Streptococcus pneumoniae* R6 PBP genes. The coding regions and their directions of transcription are indicated by *arrows* and drawn in scale as indicated by the bar representing the size of 1 kb. PBP genes are shown in *red*, genes organized in operons with PBP genes are *white,* and flanking genes are imaged in *gray*. BOX and RUP elements are depicted in *blue*. Putative promoters are indicated by small *black arrows* and putative transcriptional terminators are shown as stem-loop structures. Spr numbers represent *S. pneumoniae* R6 genes. GI number of the *S. pneumoniae* R6 chromosome sequence in the NCBI Nucleotide database is GI:15902044; *pbp1a* (*pbpA*) identified in the NCBI Gene database (GeneID:934791), *pbp1b* (GeneID:934893), *pbp2a* (GeneID:933569), *pbp2b* (GeneID:933948), *pbp2x* (*pbpX*, GeneID:934744), and *pbp3* (*dacA*, GeneID:934315). GI, GenInfo identifier; PBP, penicillin-binding protein.

We searched for putative promoter regions and putative transcriptional terminators to define the genetic organization of *S. pneumoniae* R6 genomic regions containing the PBP genes.

The PBP1a gene is apparently cotranscribed together with the upstream RecU gene. The transcription starts at a promoter upstream of *recU* and ends at a strong transcriptional terminator behind *pbp1a*. The two genes overlap by four nucleotides (nt). This transcriptional unit was also identified by genomic tiling arrays.[50] The RecU gene essential in *S. pneumoniae*[51] encodes the Holliday junction resolvase RecU, which in *Staphylococcus aureus* is required for chromosome segregation and DNA damage repair.[52]

The transcript of *pbp1b* is monocistronic. Although the region between *pbp1b* and the downstream-located gene spr1908 encoding a hypothetical protein is only 64 nt long, it contains a putative terminator followed by the spr1908 promoter.

The genetic organization of the *pbp2a* locus strongly suggests that *pbp2a* is part of a putative operon, including *rpmG*, *secE*, and *nusG* encoding a putative competence-specific global transcription modulator, the SecE subunit of the preprotein translocase, and a transcription termination/antitermination factor. A promoter has been identified upstream of *pbp2a* gene and only a very weak terminator was found downstream of *pbp2a*. The presence of an additional promoter downstream of *pbp2a* gene indicates that *rpmG-secE-nusG* can be independently transcribed. Coexpression of *rpmG-secE* was identified in the *S. pneumoniae* TIGR4 strain by genomic tiling arrays.[50]

Furthermore, the PBP2b gene forms an operon with *recR* located downstream. A promoter was predicted in front of *pbp2b* and a putative transcriptional terminator downstream of the RecR gene encodes a protein, which may play a role in DNA repair. No information is available regarding the essentiality of RecR in *S. pneumoniae*.

The genetic organization of the *pbp2x* locus indicates that the four genes constitute an operon, which was described as cell wall gene cluster by Massidda *et al.*[17] A promoter has been identified in front of the MraW gene (encoding a *S*-adenosyl-methyltransferase) and a terminator downstream of *mraY* (encoding a phospho-*N*-acetylmuramoyl-pentapeptide-transferase) followed by a RUPA repeat element.[53] PBP2x is the third gene in this operon downstream of *ftsL* (encoding a cell division protein of unknown function), in which all genes are essential and their products are involved in PG biosynthesis.[16,51,54]

Finally, the transcript of *pbp3* appears to be monocistronic ending at a transcriptional terminator just before a BOXABC element.[55] A promoter structure with an extended −10 region was found upstream of the PBP3 gene.

Determination of the transcription initiation site

To determine the transcriptional start sites of the six *pbp* promoters, 5′-RACEs were performed using oligonucleotides located in the downstream genes of the predicted promoters (Fig. 1 and Table 2). RNA of the R6 strain was treated with and without the TAP enzyme, which converts the 5′-triphosphate of RNA to a monophosphate and thereby enables ligation of the RNA to an adapter oligonucleotide. The adaptor allows specific amplification of the 5′-end sequence subsequent to reverse transcription. Finally, the PCR fragments were sequenced and the start points were determined for the six transcripts that include the PBP genes (Fig. 2). The DNA sequence electropherograms of six PCR products showed high-quality sequences and thereby clearly identified the transcription start sites (Fig. 2). The transcriptional start of *pbp1a* is located 620 nt upstream from the *pbp1a* start codon and 27 nt upstream of the *recU* start codon, which is the first gene in operon (Fig. 1). For *pbp1b*, the transcription start was mapped to the adenine residue
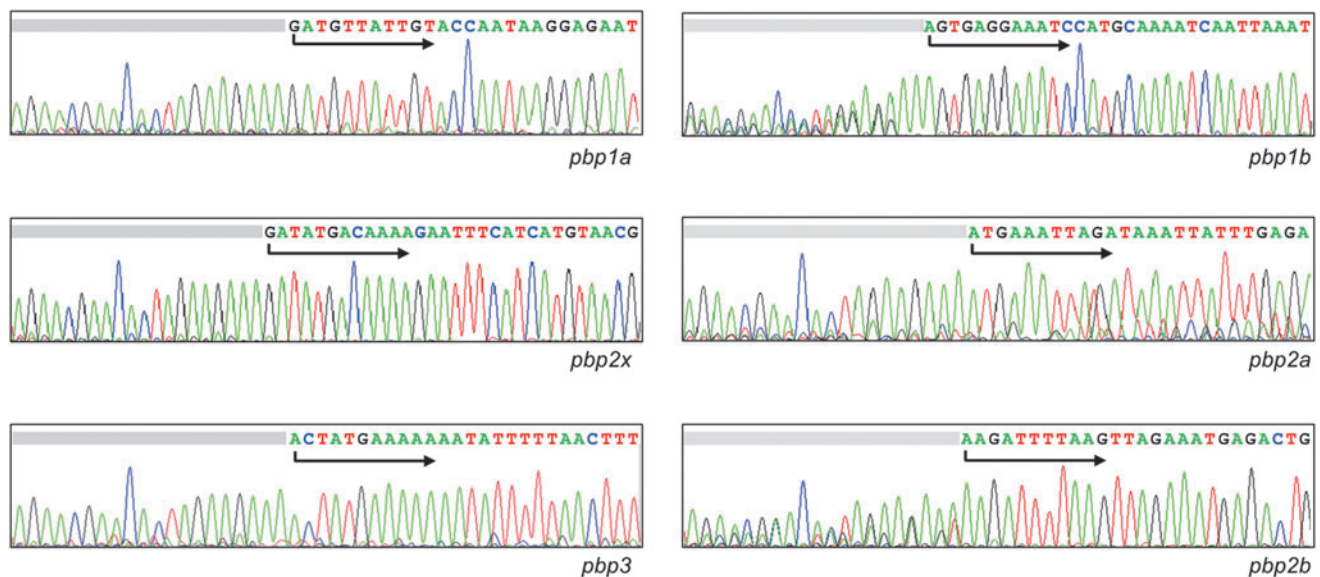
FIG. 2. Determination of transcriptional start sites of PBP genes using 5′-RACE. Electropherograms from the DNA sequencing reactions of the 5′-RACE PCR products are shown. The sequences of the RNA adaptors are shown as *gray bars*. The nucleotides complementary to the 5′ ends of mRNA are shown and the directions of the transcription are indicated by *arrows*. The first nucleotide represents the transcription initiation site. RACE, rapid amplification of cDNA ends.

located 13 nt upstream of the ATG start codon. The *pbp2a* transcriptional start overlaps with the putative translational start codon, which implies that the mRNA is leaderless; no ribosomal binding site (RBS) was detected upstream of the start codon. The transcriptional start of *pbp2x* was mapped 1,286 nt upstream from the *pbp2x* start codon and is located only 3 nt upstream of the *mraW* start codon. Similarly, the transcriptional start of *pbp3* was mapped as the adenine residue located 3 nt upstream of the *pbp3* start codon. The spacer between the transcriptional and putative translational initiation sites for both, *pbp2x* (*mraW*) and *pbp3*, is also too short to accommodate an RBS. Finally, for *pbp2b*, the transcripts initiate at an adenine residue located 17 nt from the first putative start codon of *pbp2b*.

In addition to high-quality sequence profiles, the electropherograms of three PCR products *pbp1b*, *pbp2a*, and *pbp2b* (Fig. 2, right) show weak sequences, in particular, noticeable in the area of the sequence complementary to the RNA adaptor. An explanation for these results could be that mRNA molecules are degraded by endonuclease. To exclude the possibility that two transcription starts are present, the PCR fragments were cloned into the pGEM-T-Easy vector and 15 colonies were analyzed by PCR and sequencing. For *pbp1b*, one second transcription start, and for *pbp2a*, two additional transcription starts were identified. In all cases, transcription starts were located within the coding region with no obvious −10 region and −35 consensus regions close by, therefore, they were not considered in subsequent experiments. In case of *pbp2b*, no additional transcriptional starts were found.

Consensus sequence of S. pneumoniae promoters

Based on the mapping results described above, we aligned the promoter regions of PBPs loci and analyzed the sequences (Fig. 3A). At a distance 6–8 nt upstream of the

transcriptional start site, almost all sequences exhibit an extended −10 promoter element (5′-TRTGNTATAAT-3′), which was originally identified in *Bacillus subtilis*,[56] and which is common in *S. pneumoniae*.[57] The extended −10 element 5′-TRTG-3′ is located 1 nt upstream of the −10 hexamer, which consists of the 6 nt TATAAT and is absolutely essential for transcription. The 5′-TG-3′ motif of the extended −10 promoters is positioned at −15/−14 with respect to the transcriptional start site. The extended −10 element is highly conserved in all promoters of the PBP loci (Fig. 3B). In contrast, the −35 element is not well conserved and is difficult to define. Only the two promoters upstream of *pbp1a* and *pbp1b* contain, respectively, four and five matches to consensus −35 hexamer. However, in both cases, the length of the spacer between the promoter −10 and −35 elements is not optimal and contains 19 or 15 nt, respectively. For *E. coli*, it has been shown that the most frequent spacer region length is 17 nt.[58] The promoter of *pbp2a* contains four matches to the consensus −35 hexamer sequence and has a 17 nt long spacer. The other three promoters displayed a poor match to the −35 hexamer with two or three matches only. Figure 3B depicts a DNA sequence logo generated from the alignment of *pbp* promoters from Fig. 3A. The best conserved promoter element is clearly the −10 sequence.

No other regulatory motifs common to some or all promoter regions of the PBP loci were found, and thus it is not likely that one regulatory mechanism directs the expression of all PBP genes.

PBP promoter activity in the sensitive R6 strain

To evaluate *pbp* promoter activity, the integrative promoter probe system pPP2 was used.[42] PBP promoter fragments were cloned upstream of the promoterless *E. coli* β-galactosidase LacZ gene. In the next step, the reporter

FIG. 3. Alignment of putative *S. pneumoniae* R6 PBP promoters. **(A)** The alignment shows the conserved sequences located upstream of the six PBP loci. The −35 regions marked in *orange*; the extended −10 region is shown in *green* within the *gray* area. Experimentally determined transcriptional start points are shown in *blue*. The first translation start codons (ATG) are shown in *red*. **(B)** The WebLogo[48,49] representation of the position weight matrices derived from the sequences depicted in **(A)** is shown.

FIG. 4. Activity of the PBP promoters in *S. pneumoniae* R6 strain during growth in C + Y medium. Growth curves and β-galactosidase activities of one representative experiment are shown. Growth was monitored by nephelometry (N, nephelo units). β-galactosidase activities were determined in 30 min intervals and are given in nmol nitrophenol produced per min and mg of protein.

constructs were stably integrated into the *S. pneumoniae* R6 genome by homologous recombination at the *bgaA* locus encoding the endogenous β-galactosidase. Upon integration by double crossover, most of the BgaA gene was deleted and the background β-galactosidase level was eliminated. As controls, the RP200 and RKL44 strains were used, which carry a promoterless-*lacZ* and a P$_{vegW}$-*lacZ* fusion, respectively. The *vegW* promoter served as a nonregulated control for regulation studies in *S. pneumoniae* R6 derivatives. P$_{vegW}$ is a derivative of P$_{vegII}$ and was constructed by reducing the spacing between the −10 and the −35 region from 18 to 17 nt, thereby deleting a G.[42] Subsequently, β-galactosidase activities were determined throughout the growth of the strains in C + Y medium. As shown in Fig. 4, all PBPs promoters are constitutively expressed and highly active during the exponential growth and early stationary growth phase as well, similar to the constitutive promoter P$_{vegW}$, which showed a β-galactosidase activity of 200 U throughout; the promoterless control construct gave values below 1 U (data not shown). All data were validated by the Student's *t*-test implying no growth phase-specific regulation of PBP expression in the sensitive R6 strain. Interestingly, the individual expression activities of the PBP promoters varied. Lowest promoter activities were observed for PBP3 and PBP1b and the strongest promoter was PBP1a, leading to the following hierarchy of the PBP promoter activities PBP3 < PBP1b < PBP2x = PBP2a < PBP2b < PBP1a (Fig. 4). Promoter activities were also determined in cells grown in BHI medium since this medium is easy to prepare and the measurements of β-galactosidase activities provide

stable results. Consistently, the same relative expression patterns were observed, but the levels of expression were ranging between 2.1- and 2.8-fold higher than in C + Y medium (data not shown). The expression of P$_{vegW}$-*lacZ* also increased to the same degree (400 U). A higher expression of a variety of promoters in cells grown in BHI medium has been reported[45]; the reason for this phenomenon is unclear.

Response of promoter activities to cefotaxime

Beta-lactams interact with the DD-peptidase domain of PBPs resulting in growth inhibition of *S. pneumoniae*.[2,7,28] Cefotaxime was chosen since this compound induces a tolerant response and the cells do not lyse for many hours.[29] In contrast, most other beta-lactams induce a rapid lytic response in this organism and consequently a different experimental setup would be required to prevent cellular lysis during the sampling period. However, even during nonlytic conditions beta-lactam-treated cells shed membrane vesicles and precursors of cell wall polymers into the medium,[29,59] and the interpretation of results under such artefact prone conditions causes severe problems.

We tested whether the presence of cefotaxime in BHI medium affects the expression of PBP genes. Bacterial growth was inhibited already at a concentration of 0.01 μg/ml (0.5 × MIC) cefotaxime and the cells stopped growing at a cell density of N = 30 (Fig. 5A). The expression of PBP genes was determined at 30, 60, and 150 min after the addition of cefotaxime at a concentration of 0.04 μg/ml (2 × MIC). No significant effect on the expression of PBP genes was
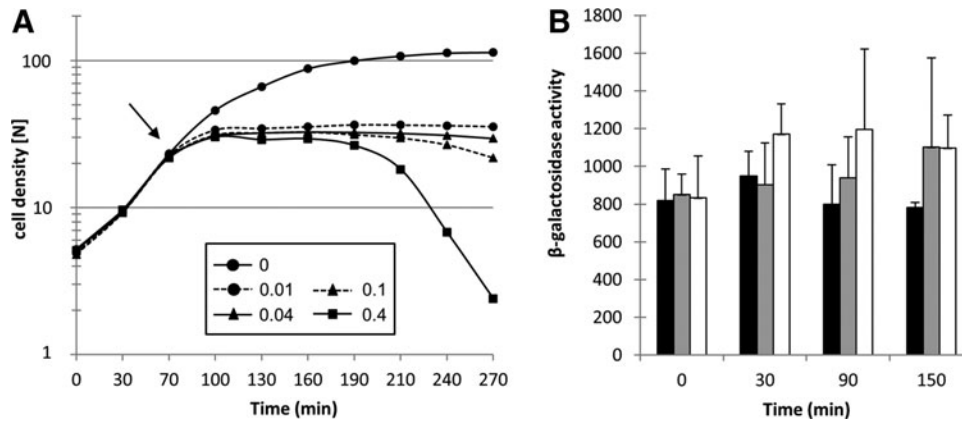
FIG. 5. Growth and *pbp2x* promoter activity in response to cefotaxime treatment. **(A)** Growth of *S. pneumoniae* R6 in BHI medium was followed by nephelometry (N). Cefotaxime was added to the exponentially growing cultures at the concentrations (μg/ml) at the time point as indicated by the *arrow*. **(B)** Activity of the *pbp2x* promoter at different time points after the addition of cefotaxime. 0 indicates the time immediately before the cefotaxime addition. β-galactosidase activities were measured after 30, 90, and 150 min and are given in nmol nitrophenol produced per min and mg of protein. *Black bars*: control, no cefotaxime; *gray bars*: 0.04 μg/ml cefotaxime; *white bars*: 0.4 μg/ml cefotaxime. The results are mean value ± SD of three independent experiments. SD, standard deviation.

observed according to Student's *t*-test. The expression of *pbp2x* is shown as an example in Fig. 5B, and expression of all PBP genes at 2×MIC cefotaxime are shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/mdr). The addition of cefotaxime at a concentration of 0.4 μg/ml (20×MIC) did not affect the expression level of *pbp2x* (Fig. 5B), but R6 cells start to lyse two hours later. After 30 min growth in the presence of 0.4 μg/ml, only PBP2b could be labeled with BOCILLIN™FL, documenting that all other PBPs were acylated (not shown).

A mutation in the promoter region affects the amount of PBP3

While investigating the physiological role of PBP3 and its effect on cefotaxime resistance, Selakovitch-Chenu *et al.*

used the laboratory strain 801, an R6 derivative, which produces a reduced amount of PBP3.[43] They described that this strain carries a deletion of 1 nt in the upstream sequence of the PBP3 gene.[60] We sequenced the promoter region of the *pbp3* gene from mutant 801 to identify the position of this mutation within the promoter region defined in the present study. The deletion of 1 nt is located in the space region between the −35 and the extended −10 elements (Fig. 6A). It is well established that the length of the spacer region in *E. coli* is critical.[61] Usually, the −35 sequence is located 17 nt upstream of the −10 element.[58] In case of the 801 strain, the length of the PBP3 promoter spacer region is 16 nt. Therefore, we decided to evaluate the promoter activity and cloned this promoter in the plasmid pPP2. The resulting plasmid pPP$_{3M}$ was transformed into the *S. pneumoniae* R6 and the reporter construct was integrated into the

FIG. 6. Effect of the mutation in the PBP3 promoter of *S. pneumoniae* 801 on PBP3 expression. **(A)** Comparison of the *pbp3* promoter region between *S. pneumoniae* R6 and the 801 mutant. The −35 (*orange*) and extended −10 promoter (*green* within the *gray* area) regions are boldfaced. The transcriptional start point is shown in *blue* and the translation start codon of *pbp3* is indicated in *red*. The deletion of one nucleotide in 801 is highlighted by the *red box*. **(B)** β-galactosidase activities expressed from the promoters *pbp3*$_{R6}$ (*gray*) and *pbp3*$_{R801}$ (*black*) in KP06 and KP09 strains. Strains were grown in C+Y medium. β-galactosidase activities were determined at four different time points and are given in nmol nitrophenol produced per min and mg of protein. The results are mean value ± SD of three independent experiments.
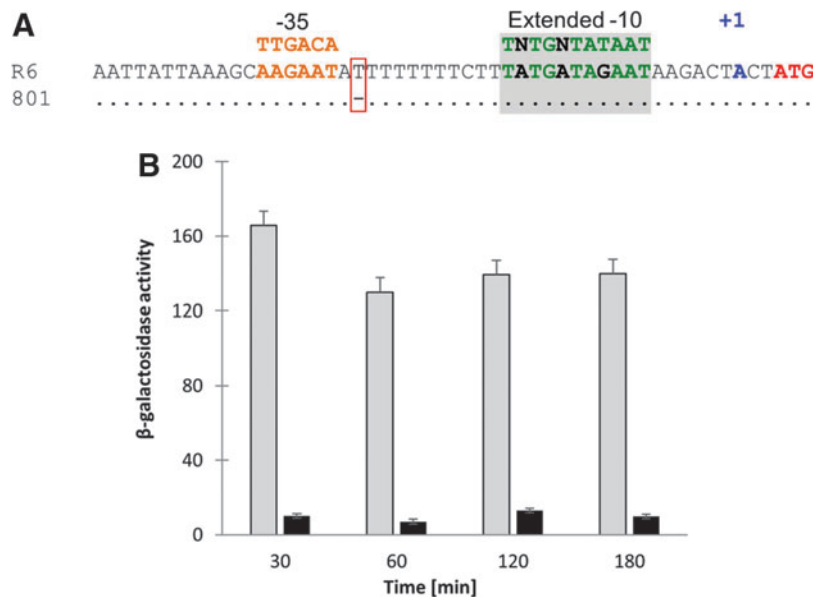
genome by homologous recombination. Subsequently, β-galactosidase activities were measured during growth at four time points (Fig. 6B). The promoter activity of the mutated PBP3 was reduced 10-fold in comparison to wild-type promoter indicating that the deletion of 1 nt in the space region greatly affects the promoter strength, in agreement with the lower PBP3 protein production in strain 801.

A lower amount of PBP3 has been described also in another laboratory strain I41R.[62] This strain differs from R6 and its derivatives by expressing the *Dpn*II phenotype.[63] Sequencing of the *pbp3* promoter region showed that *S. pneumoniae* I41R carries the same mutation as strain 801 described above.

Discussion

The expression and potential regulatory mechanisms of PBPs are poorly understood in *S. pneumoniae*. We have now investigated the promoter sequences of the six PBP genes from *S. pneumoniae* strain R6 in detail and examined the PBP expression pattern under the particular conditions.

The DNA sequences 150 nt upstream and 100 nt downstream (data not shown) of the transcriptional start sites did not reveal any common regulatory motif. Therefore, it is unlikely that one common mechanism controls the expression of all PBP genes in *S. pneumoniae*. All PBP promoters contained a highly conserved extended −10 promoter element, but the −35 promoter element varied considerably. These results are similar to those obtained by Haenni *et al.*,[64] who investigated PBP promoters in *S. gordonii*. This organism also belongs to the Mitis group of viridans streptococci, but is only distantly related to *S. pneumoniae*.[65] Extended −10 promoter elements occur frequently in Gram-positive bacteria[66] and are also common in *S. pneumoniae*.[57] In Gram-negative bacteria, such as *E. coli*, the extended −10 element has a 5′-TG-3′ motif at promoter positions −14 and −15 corresponding to 1 nt upstream of the −10 hexamer.[67] In contrast, Gram-positive bacteria contain a highly conserved extended sequence with the consensus 5′-TRTG-3′, which is also known as the −16 region[68,69] (Fig. 3). The 5′-TRTG-3′ motif was found in five PBP promoters; only the *pbp2a* promoter contained a 5′-TG-3′ motif. The full −10 extension is at least four times more common in *S. pneumoniae* than in *E. coli*.[57] The impact of a full −10 extension compared with the 5′-TG-3′ motif is not well understood. For *E. coli*, it has been demonstrated that the 5′-TG-3′ motif further stabilizes the interactions between the RNA polymerase and DNA.[70]

Mitchell *et al.* showed that in *E. coli*, the extended −10 promoters tend to have longer spacer, have fewer matches to the consensus −35 hexamer, and contain short runs of T residues in the spacer region.[58] We note that three PBP promoters contain 2/6 or 3/6 matches to the consensus −35 hexamer (Fig. 3), whereas the *pbp2a* promoter shows the highest match (4 out of 6 nt). In all four promoters, the −35 sequence is located 17 nt upstream of the −10 element, which is the most frequent spacer distance in *E. coli*.[61] Two promoters, *pbp1a* and *pbp1b*, have four and five matches, respectively, to the consensus sequence, but in both cases the length of the spacer region is not optimal. Thus, in all PBP promoters the −35 element is not well conserved. In this context, it is important to note that this region plays an important role in the *pbp3* promoter activity, although it has only

2 nt matches to the consensus −35 hexamer. The deletion of only 1 nt in the spacer region of PBP3 promoter reduces *pbp3* expression ∼10-fold (Fig. 6); whether this is due to a shortened spacer between the −35 and −10 element, or whether the deletion of one thymidine is responsible for this effect remains to be clarified. In contrast, Sabelnikov *et al.* showed that the *S. pneumoniae dpnM* promoter,[57] which contains an extended −10 promoter element, can function without a −35 element. Taken together, the general importance of −35 elements in extended −10 promoters in *S. pneumoniae* bacteria remains unclear.

PBP expression analysis in *S. pneumoniae* R6 during different growth phases and in two different media did not reveal any specific PBP regulation, since all six *pbp* promoters were constitutively expressed in both media. However, the individual expression rate of the six PBP promoters differed by approximately a factor of four. It could be possible that different 5′ regions of the transcribed mRNA molecules influence the expression of the reporter LacZ. However, in the publication where the *lacZ*-fusion system was described, the authors also performed qPCR experiments and did not see any deviations when different promoter regions were tested in the *lacZ* assay.[44] Previously, it was demonstrated that in *E. coli*, the expression of the LMW PBP6a was 2- to 10-fold higher in stationary phase than in exponentially growing cells.[71] We did not see an alteration of the expression in early stationary growth phase. The expression pattern during late stationary growth was not followed. Nevertheless, no change in expression pattern was observed between exponential growth and early stationary phase, independent on the medium used in the experiments (C + Y vs. BHI medium) (see Fig. 4 for C + Y). This is in agreement with data obtained from microarray analysis assessing the responses of *S. pneumoniae* to penicillin.[72]

Haenni *et al.* showed that in the presence of low concentrations of penicillin, *pbp2a* of *S. gordonii* displayed increased expression, and in a penicillin-resistant mutant, penicillin treatment resulted in increased expression of *pbp1a* as well.[64] The authors suggested that an increased transglycosylase activity could stabilize a poorly crosslinked PG, and in doing so, promote bacterial survival at borderline penicillin concentrations. In contrast, the expression of all six *S. pneumoniae* PBPs was not affected upon addition of 0.04 μg/ml cefotaxime (2 × MIC) in BHI medium. This concentration already inhibits bacterial growth (Fig. 5), but does not affect the localization profiles of PBP2x tagged with GFP.[15]

Although we could not detect any significant changes in the expression pattern of the PBP promoters in this study, it cannot be ruled out that other regulatory mechanisms exist that control the PG biosynthesis machineries or components thereof during cellular growth directly or indirectly. Further analysis of PBP expression and biochemical data during treatment with different beta-lactam antibiotics and in resistant strains of *S. pneumoniae* and other, more closely related streptococci of the Mitis group, will help to shed more light on potential regulatory mechanisms of PBPs in these bacteria.

Disclosure Statement

No competing financial interests exist.

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