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Alterations in PBP 1A Essential for High-Level Penicillin Resistance in *Streptococcus pneumoniae*

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High-level penicillin resistance in pneumococci is due to alterations in penicillin-binding proteins (PBPs) 2X, 2B, and 1A. We have sequenced the penicillin-binding domain of PBP 1A from penicillin-resistant South African pneumococcal isolates and have identified amino acid substitutions which are common to all the resistant isolates analyzed. Site-directed mutagenesis was then used to determine whether particular amino acid substitutions at specific positions in PBP 1A mediate penicillin resistance. PCR was used to isolate PBP 2X, 2B, and 1A genes from clinical isolate 8303 (penicillin MIC, 4 μg/ml). These wild-type PBP genes were cloned into pGEM-3Zf and were used as the transforming DNA. Susceptible strain R6 (MIC, 0.015 μg/ml) was first transformed with PBP 2X and 2B DNA, resulting in PBP 2X/2B-R6 transformants for which MICs were 0.25 μg/ml. When further transformed with PBP 1A DNA, 2X/2B/1A-R6 transformants for which MICs were 1.5 μg/ml were obtained. Site-directed mutagenesis of the PBP 1A gene from isolate 8303 was then used to reverse particular amino acid substitutions, followed by transformation of PBP 2X/2B-R6 transformants with the mutagenized PBP 1A DNA. For PBP 2X/2B/1A-R6 transformants, the introduction of the reversal of Thr-371 by Ser or Ala in PBP 1A decreased the MIC from 1.5 to 0.5 μg/ml, whereas the reversal of four consecutive amino acid substitutions (Thr-574 by Asn, Ser-575 by Thr, Gln-576 by Gly, and Phe-577 by Tyr) decreased the MIC from 1.5 to 0.375 μg/ml. These data reveal that amino acid residue 371 and residues 574 to 577 of PBP 1A are important positions in PBP 1A with respect to the interaction with penicillin and the development of resistance.

Penicillin inhibits the growth of pneumococci by the inactivation of penicillin-binding proteins (PBPs). Pneumococcal resistance to penicillin is due to the production of altered PBPs which have a decreased affinity for the antibiotic (7, 8, 12, 24). Barcus and coworkers (2) revealed that high-level penicillin resistance can be established by alterations only in PBPs 2X, 2B, and 1A. They cloned PBP 2X, 2B, and 1A genes from four clinical isolates (penicillin MICs, 1.5 to 16 μg/ml) and found that these three genes could transform susceptible strains so that they had full levels of penicillin resistance, identical to the resistance of the donor strains. PBPs 2X and 2B are primary PBP targets for penicillin (6, 17, 21, 24). We have investigated the role of PBP 1A in penicillin resistance and confirm that selection of a low-affinity PBP 1A requires the presence of low-affinity PBPs 2X and 2B and that alteration of PBP 1A plays a vital role in full penicillin resistance development.

We have sequenced the penicillin-binding domain (PBD)-encoding region of PBP 1A from 18 South African clinical pneumococcal isolates for which penicillin MICs ranged from 0.015 to 8 μg/ml. These data were used to identify amino acid alterations which are common to all resistant isolates and which would appear to be essential to the development of resistance. At present, the amino acid alterations in PBP 1A that are responsible for decreased penicillin affinity in clinical isolates are not well defined. We have used site-directed mutagenesis (SDM) to study some amino acid positions in PBP 1A, and we report on their importance for the interaction of PBP 1A with penicillin and the development of resistance.

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MATERIALS AND METHODS

Bacterial strains. Clinical isolates of pneumococci were obtained from the South African Institute for Medical Research, a reference center for pneumococci in South Africa. Pneumococci were routinely cultured at 37°C in 5% CO_2 on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% lysed horse blood.

Isolation and fingerprinting of PBP genes. PBP genes were amplified from the chromosomal DNAs by PCR and were fingerprinted by methods that have been described previously (21). For PBP 2B and PBP 2X PCR, primers Pn2B up and Pn2B down were used as described by Dowson and coworkers (4), and primers Pn2X up and Pn2X down were used as described by Munoz and coworkers (15). For PBP 1A PCR, the following primers were used: Pn1A up (CGGCATTCG ATTTGATTCGCTTCT; positions 786 to 809 on the published PBP 1A gene sequence [14]) and Pn1A down (GTCGTACTATTATTTGTGCTTGGAGTG GTT; positions 2994 to 3023).

DNA sequencing. Double-stranded DNA (PCR products and plasmid DNA) were sequenced by the dideoxynucleotide method of DNA sequencing of Sanger et al. (19) with incorporation of the Sequenase enzyme (United States Biochemicals, Cleveland, Ohio). The protocol for sequencing was performed as described by the manufacturer of the Sequenase kit. The nucleotide sequences of both strands of the PBP genes were determined by sequencing with a series of oligonucleotides that primed at intervals of ±240 nucleotides along each strand. When the PCR products were sequenced, a minimum of two independent PCR products were sequenced in order to eliminate any errors introduced by PCR. The approximate frequency of errors by PCR with *Taq* DNA polymerase was <0.06%.

PBP 1A gene mutagenesis. All SDM experiments were performed with the PBP 1A gene isolated from isolate 8303 for which the penicillin MIC was 4 μg/ml. The megaprimer method of PCR-based SDM described by Smith and Klugman (20) was used to create mutants of this "resistance" gene. This PCR incorporates three primers and two amplification steps. The first PCR incorporates an internal mutagenic primer and a reverse primer. In a second PCR, the product of the first reaction is used as a megaprimer together with a forward primer annealing upstream of the mutagenic site, resulting in the amplification of the final mutagenic product. Pn1A up and Pn1A down were the forward and reverse primers, respectively. Independent PCRs were performed by incorporating mutagenic primer 1-GACTGGGGTTCTACTATGAAACCAA (positions 2044 to 2068) or mutagenic primer 2-AACCACATCAAGACCTCTCAATTTG

TAGCTCCAGAT (positions 2653 to 2688) (underscores indicate the positions at which mutagenesis occurred). Fifty-microliter PCR mixtures were set up. The first PCR contained 5 ng of PBP 1A DNA, 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1 μM mutagenic primer, 1 μM reverse primer, 200 μM (each) deoxynucleoside triphosphates (Boehringer GmbH, Mannheim, Germany), and 2 U of *Pwo* DNA polymerase (Boehringer). The PCR was performed in a Perkin-Elmer DNA thermal cycler 480 (Perkin-Elmer Corporation, Norwalk, Conn.) with 25 cycles of denaturation at 93°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min. The PCR product (megaprimer) was purified from agarose with Geneclean (Bio 101, Inc., La Jolla, Calif.) and was resuspended in 10 mM Tris-1 mM EDTA (pH 7.5; TE buffer). The second PCR contained 5 ng of PBP 1A DNA, 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 μM (each) deoxynucleoside triphosphates, 2 μg of megaprimer DNA, and 2 U of *Pwo* DNA polymerase, with five thermal cycles of 93°C for 1 min and 72°C for 3 min. While at 72°C, 1 μM forward primer was added and was gently mixed into the reaction mixture, and thermal cycling (25 times) was continued at 93°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The mutagenic PCR product was purified from agarose with Geneclean (Bio 101).

Cloning of PBP genes. PBP 2B, 2X, and 1A genes were cloned into the *Sma*I site of pGEM-3Zf(+) (Promega Corp., Madison, Wis.) by standard techniques. Recombinant plasmid DNA was extracted from transformed *Escherichia coli* JM109 by the alkaline lysis method (18).

Transformation. Nonencapsulated, penicillin-susceptible pneumococcal strain R6 (a laboratory strain derived from Rockefeller University strain R36A [1]) was used as the recipient in transformation studies. Cloned PBP genes were used as transforming DNA. Pneumococcal strain R6 was made competent as follows. Bacteria were cultured in C medium (22) until the late exponential phase (optical density at 620 nm, 0.25) and, after the addition of glycerol to 10%, were frozen at −70°C in 1-ml aliquots. For transformation, 4 μg of recombinant plasmid DNA was added to 1 ml of competent cells, which was then incubated at 30°C for 1 h and at 37°C for 2 h. Eighty-microliter amounts were then plated onto Mueller-Hinton–blood agar containing increasing concentrations of penicillin (0.03 to 4 μg/ml), and the plates were incubated at 37°C for 48 h. Transformants were picked from the plates containing the highest penicillin concentration possible. Transformation frequencies were calculated as the number of resistant transformants per milliliter of transformation mixture divided by the total number of cells per milliliter of transformation mixture.

MIC determination. The penicillin MICs for pneumococci were determined by the agar dilution method specified by the National Committee for Clinical Laboratory Standards (16). Benzylpenicillin (Sigma Chemical Co., St. Louis, Mo.) was incorporated into Mueller-Hinton agar supplemented with 5% horse blood, with plates containing antibiotic at concentrations of 0.03, 0.06, 0.125, 0.25, 0.375, 0.5, 1, 1.5, 2, 3, and 4 μg/ml. The bacteria were cultured at 37°C in serum broth (South African Institute for Medical Research, Johannesburg, South Africa) until a turbidity equivalent to that of a McFarland 0.5 standard was obtained, and then 1- to 2-μl aliquots of this inoculum were applied to an agar surface with an inoculum-plating device. The agar plates containing the different antibiotic concentrations were inoculated, starting with the agar containing the lowest antibiotic concentration. Once the inoculum had been absorbed into the agar, the plates were inverted and were incubated at 35°C for 16 h. The MIC was recorded as the lowest concentration of antibiotic that completely inhibited bacterial growth on the agar.

Nucleotide sequence accession numbers. The PBP 1A sequence data for strain R6 appears in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under the accession number M90527, while data for the following resistant isolates, which are also listed in Table 1, appear under the indicated accession numbers: Isolate 63509, AF046238; isolate 8303, AF046230; isolate M11, AF046238; isolate 35193, AF046230; isolate 56739, AF046234; isolate 65654, AF046231; isolate N94, AF046236; isolate 43, AF046232; isolate 64429, AF046233; isolate 7851, AF046235; and isolate 17619, AF046237.

RESULTS AND DISCUSSION

Analysis of PBP 1A sequence. The PBD of high-molecular-weight PBPs, such as PBP 1A, is believed to start ±60 amino acid residues before the active-site serine residue that is acylated by penicillin and ends ±60 residues after the conserved Lys-Thr-Gly motif (10). We have sequenced the PBD-encoding region of PBP 1A from 18 South African clinical pneumococcal isolates for which penicillin MICs ranged from 0.015 to 8 μg/ml. The nucleotide sequence of the PBP 1A gene from penicillin-susceptible reference strain R6, which was determined and which was found to agree with the published sequence (14), was used as the basis for comparison with clinical isolates.

The PBD-encoding region of PBP 1A from isolates for which MICs were <0.25 μg/ml revealed up to seven nucleotide substitutions and a single amino acid substitution differing from that of strain R6. Because this amino acid substitution (Glu-388 by Asp) also occurs in susceptible strains, it probably does not confer penicillin resistance, although it also occurs in proteins from all the resistant isolates analyzed. Widespread alterations in the PBD-encoding region of PBP 1A were seen only in isolates for which MICs were ≥0.25 μg/ml (Table 1). Our sequence analysis therefore suggests that an MIC of 0.25 to 0.5 μg/ml represents a breakpoint in resistance. At this breakpoint PBP 1A starts participating in the development of resistance as a result of significant alterations in its PBD. These data can be compared to those from previous phenotypic studies on PBPs. The disappearance of PBP 1A from PBP profiles (fluorography) of transformants as they reach resistance levels of 0.4 μg of penicillin per ml has suggested that an altered PBP 1A with a decreased affinity for penicillin occurs only in isolates for which MICs are approximately 0.4 μg/ml and higher (24). Studies with clinical isolates of pneumococci have revealed that PBP 1A is absent from the fluorograms for isolates for which penicillin MICs are ≥0.25 μg/ml (13). Furthermore, Kell and coworkers (11) transformed a penicillin- and cefotaxime-resistant strain (MICs, 4 and 2 μg/ml, respectively) with inactivated PBP 1A DNA and successfully obtained growth of the transformant, revealing the tolerance of the pneumococcus to the loss of PBP 1A. The penicillin and cefotaxime MICs for the transformant were reduced to 0.5 μg/ml. This resultant decrease in the MIC accompanying the inactivation of PBP 1A supports the idea that PBP 1A plays a role in the development of penicillin and cefotaxime resistance when MICs are >0.5 μg/ml.

TABLE 1. Properties of South African isolates of pneumococci[a]

Isolate	Source	Serogroup	Penicillin MIC (μg/ml)	Origin and date of isolation (mo/yr)
63509	N/S	19	8	SH, 8/1987
8303	SPT	23	4	BH, 10/1995
M11	N/S	6	4	HH, 9/1989
35193	B/C	6	2	BH, 1/1991
56739	SPT	19	1	RXH, 3/1990
65654	CSF	6	0.25	RXH, 11/1988
N94	SPT	6	0.5	RXH, 11/1989
43	SPT	6	0.25	BH, 8/1988
64429	B/C	6	0.25	BH, 6/1989
7851	B/C	6	0.5	CH, 10/1988
17619	B/C	19	0.25	CWH, 7/1989

[a] Abbreviations: B/C, blood culture; SPT, sputum; CSF, cerebrospinal fluid; N/S, nasal swab; BH, Baragwanath Hospital; RXH, Red Cross Hospital; CH, Coronation Hospital; CWH, Clairwood Hospital; HH, Hillbrow Hospital; SH, Somerset Hospital.

Figure 1 exhibits the prominent amino acid substitutions identified in the PBD of PBP 1A from resistant isolates. These substitutions were found to be common to all isolates and may therefore be essential to the development of resistance. For isolates for which MICs were from 0.25 to 1 μg/ml, nucleotide and amino acid alterations were essentially confined to an area surrounding the Lys-557–Thr–Gly motif (from amino acid residue 533 to the end of the PBD). This included six amino acid substitutions which were common among all resistant isolates: Thr-574 by Asn, Ser-575 by Thr, Gln-576 by Gly, Phe-577 by Tyr (four consecutive residues), Leu-583 by Met or Thr, and Ala-585 by Val (Fig. 1, line B). As the level of penicillin resistance among isolates increased above MICs of 1 μg/ml, the number of nucleotide and amino acid alterations also increased, such that the entire PBD was included. The PBD-encoding region of PBP 1A from high-level-resistant isolates

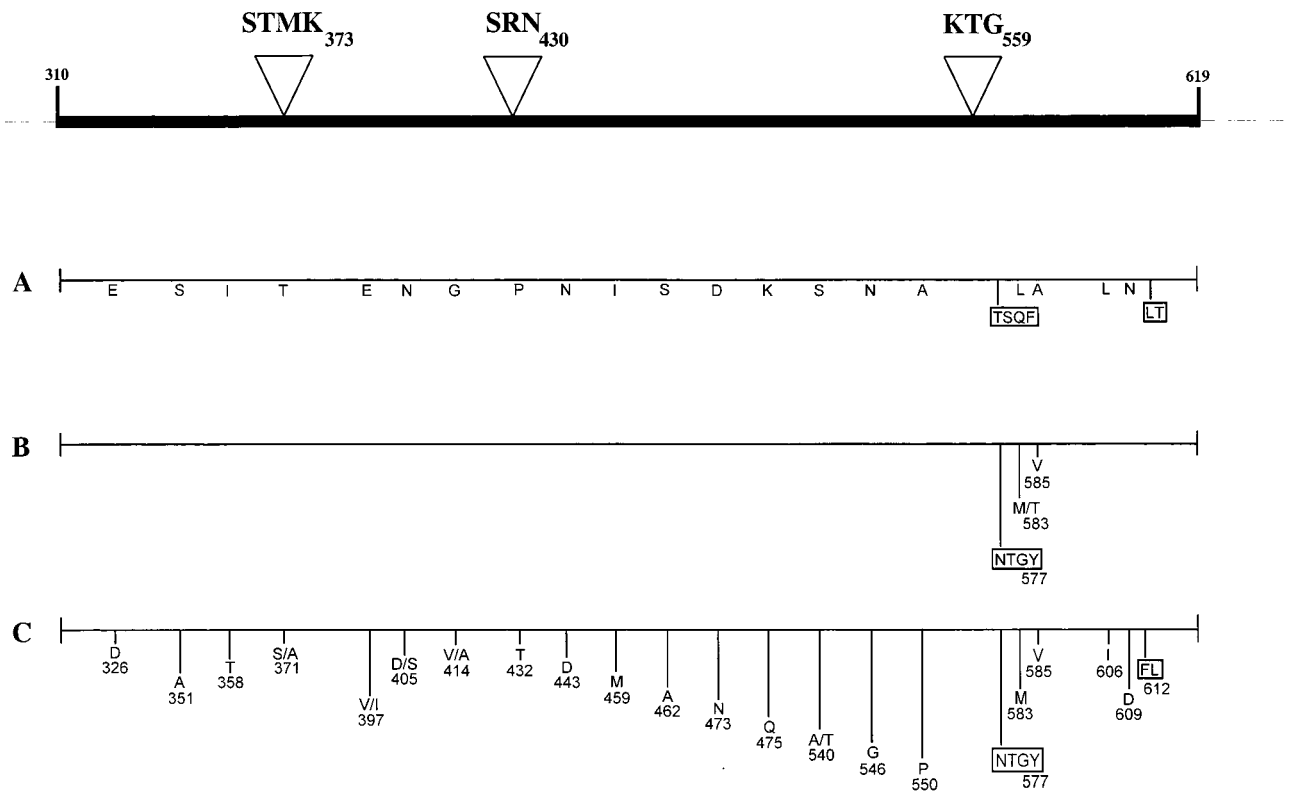

FIG. 1. Prominent amino acid substitutions in the PBD of PBP 1A from penicillin-resistant pneumococcal isolates compared to the sequence of susceptible strain R6 (two or more consecutive substitutions are boxed). The positions of the three conserved amino acid sequence motifs (STMK, SRN, and KTG) are indicated. (A) Amino acid sequence data for strain R6. (B) Amino acid substitutions for isolates for which MICs were from 0.25 to 1 μg/ml. (C) Amino acid substitutions for isolates for which MICs were ≥2 μg/ml. The data are numbered according to the published sequence of Martin and coworkers (14).

(MICs, ≥2 μg/ml) revealed the most extensive nucleotide sequence divergence (up to 21%) from strain R6, resulting in up to 43 amino acid alterations which spanned the entire PBD.

Only high-level-resistant isolates (MICs, ≥2 μg/ml) had amino acid alterations within the locality of the Ser-370–Thr–Met–Lys and Ser-428–Arg–Asn motifs of PBP 1A (Fig. 1, line C). An interesting substitution was that of Thr-371 by Ser or Ala which occurred within the Ser-370–Thr–Met–Lys motif adjacent to the active-site serine residue. This substitution was seen only in PBPs 1A from isolates for which MICs were ≥2 μg/ml. It would therefore appear that this substitution may be of particular importance in mediating the higher levels of resistance. The importance of this amino acid position is supported by previous studies. Hedge and Spratt (9) isolated a number of cephalexin-resistant laboratory mutants of *E. coli* in which resistance was shown to be due to altered PBP 3, the major killing target for β-lactams in *E. coli*. One class of mutants showing the highest levels of resistance (eightfold increase compared to that for the parental strain) revealed a single amino acid substitution of Thr-308 by Pro, which occurred within the second position of the active-site Ser-307–X–X–Lys motif (9). By SDM, a threonine-to-serine mutation at the second position of the Ser-X-X-Lys motif of a TEM β-lactamase was shown to significantly decrease the catalytic activity of the enzyme (3).

Mutagenesis and transformation studies. Penicillin-susceptible pneumococcal strain R6 was used as the recipient in transformation studies. Cloned PBP 2X, 2B, and 1A genes from penicillin-resistant isolate 8303 were used as the transforming DNA. Strain R6 was transformed with individual PBP genes as well as different combinations of PBP 2X, 2B, and 1A genes. DNA fingerprinting and DNA sequencing were used to confirm the introduction of these "resistance" genes into strain R6. The results of these transformation experiments are summarized in Table 2. When strain R6 was transformed with PBP 2B DNA or PBP 1A DNA, or a combination of both, mutants were selected on plates containing 0.03 μg of penicillin per ml, with subsequent MICs calculated to be 0.03 μg/ml. Analysis of the PBP genes of these R6 mutants revealed unaltered PBP 2B and 1A genes. Identical mutants were selected in control (vector-only) transformations. This finding of low-level penicillin resistance associated with unaltered PBPs supports and extends previous studies which have described non-PBP-related low-level cefotaxime and piperacillin resistance in laboratory mutants obtained after several selection steps on increasing concentrations of antibiotic (5, 23). Transformation of strain R6 with PBP 2X DNA or PBP 2X plus PBP 1A DNA resulted in transformants (transformation frequency, 4×10^{-5}) selected on plates containing 0.03 μg of penicillin per ml, with subsequent MICs calculated to be 0.06 μg/ml. These transformants revealed integrated PBP 2X "resistance" genes only. This altered PBP 2X together with non-PBP-related mechanisms would account for this low-level resistance. Transformants for which MICs were greater than 0.06 μg/ml were obtained only when strain R6 was transformed with a combination of PBP 2X and PBP 2B DNA. These PBP 2X/2B-R6 transformants (frequency, 9×10^{-7}) were selected on plates containing 0.125 μg of penicillin per ml (MICs for the transformants, 0.25 μg/ml) and showed integrated PBP 2X and 2B "resistance" genes. Transformation with a combination of PBP

TABLE 2. Pneumococcal transformation studies

Recipient	Transforming DNA[a]	Penicillin MIC for transformants (μg/ml)	Integration of altered PBPs in transformants
Strain R6	PBP 2B	0.03	No
Strain R6	PBP 1A	0.03	No
Strain R6	PBPs 2B and 1A	0.03	No
Strain R6	PBP 2X	0.06	Yes; PBP 2X
Strain R6	PBPs 2X and 1A	0.06	Yes; PBP 2X
Strain R6	PBPs 2X and 2B	0.25	Yes; PBPs 2X and 2B
Strain R6	PBPs 2X, 2B, and 1A	0.25	Yes; PBPs 2X and 2B
2X/2B-R6 mutant	PBP 1A	1.5	Yes; PBPs 2X, 2B, and 1A
2X/2B-R6 mutant	Mutagenized PBP $1A^{(371)}$	0.5	Yes; PBPs 2X, 2B, and $1A^{(371)}$
2X/2B-R6 mutant	Mutagenized PBP $1A^{(574–577)}$	0.375	Yes; PBPs 2X, 2B, and $1A^{(574–577)}$

[a] Cloned PBP 2X, 2B, and 1A "resistance" genes from penicillin-resistant isolate 8303 were used as transforming DNA. The superscript numbers indicate the amino acid positions at which mutagenesis occurred.

2X, 2B and 1A DNA produced the same results as transformation with PBP 2X and 2B DNA. Further steps of transformation and selection were required for the introduction of an altered PBP 1A. Therefore, an altered PBP 2X appears to be essential to allow the selection of transformants with altered PBPs 2X and 2B. Only within this genetic background of altered PBPs 2X and 2B will the selection of transformants with an altered PBP 1A be possible. These results therefore confirm the ordered multistep process of penicillin resistance development first suggested by Zighelboim and Tomasz (24), which starts with an alteration of PBP 2X, followed by alteration of PBP 2B and then PBP 1A.

All SDM experiments were performed with the PBP 1A gene isolated from resistant isolate 8303. Strain R6 remained the recipient in transformation studies. For SDM of PBP 1A, we needed to obtain the correct genetic background with respect to PBPs 2X, 2B, and 1A. Therefore, strain R6 was first transformed with the PBP 2X and 2B "resistance" genes isolated from isolate 8303, resulting in transformants for which penicillin MICs were 0.25 μg/ml. When these PBP 2X/2B-R6 transformants were further transformed with the PBP 1A gene from isolate 8303, transformants (frequency, 4×10^{-6}) were selected on plates containing 1 μg of penicillin per ml, with MICs calculated to be 1.5 μg/ml. These results confirmed that an MIC of 0.25 to 0.5 μg/ml represents a breakpoint in resistance, a level at which PBP 1A starts to participate in penicillin resistance development. The MIC for the donor strain (4 μg/ml) was never reached in the crosses described above. This suggests that the mechanism of high-level penicillin resistance may still have new elements to be discovered, such as the possible participation of PBPs 1B and 2A.

The technique of SDM is used to introduce mutations into DNA and can therefore be used to investigate the importance of amino acid substitutions identified in altered PBPs. SDM of the altered PBP 1A gene from resistant strain 8303 was performed as follows. Particular mutations were inactivated (alterations were reversed back to the original sequence), the resistant strain was then transformed with the mutated gene, and then it was determined whether the reversal of the amino acid change resulted in transformants with decreased levels of resistance. This approach of SDM was chosen because multiple amino acid mutations have been identified in altered PBP 1A, and therefore, when a particular amino acid mutation is being analyzed, it is probably important that the remaining genetic background of the altered PBP be maintained. The substitution of Thr-371 by Ser or Ala, which was identified only in PBP 1A from high-level penicillin-resistant isolates, was the first substitution to be reversed in the PBP 1A gene from isolate 8303. The megaprimer method of PCR-based SDM, incorporating mutagenic primer 1, was used to create the mutagenized PBP 1A DNA. PBP 2X/2B-R6 transformants (MICs, 0.25 μg/ml) were transformed with this mutagenized PBP 1A DNA, resulting in PBP 2X/2B/1A-R6 transformants (frequency, 2×10^{-5}), and were selected on plates containing 0.25 μg of penicillin per ml; the MICs for these transformants were 0.5 μg/ml. For PBP 2X/2B/1A-R6 transformants having the original wild-type PBP 1A gene, MICs were 1.5 μg/ml; therefore, the reversal of Thr-371 by Ser or Ala in PBP 1A accounted for a decrease in the MIC from 1.5 to 0.5 μg/ml. Our second SDM experiment resulted in the reversal of four consecutive amino acid substitutions in PBP 1A (Thr-574 by Asn, Ser-575 by Thr, Gln-576 by Gly, and Phe-577 by Tyr) which were common among all resistant isolates for which MICs were ≥0.25 μg/ml. Mutagenic primer 2 was used to create the mutagenized PBP 1A DNA. When PBP 2X/2B-R6 transformants were transformed with the mutagenized PBP 1A DNA, the MICs for the resulting PBP 2X/2B/1A-R6 transformants (frequency, 3×10^{-6}), selected on plates containing 0.25 μg of penicillin per ml, were 0.375 μg/ml. The reversal of these four consecutive substitutions in PBP 1A therefore accounted for a decrease in the MIC from 1.5 to 0.375 μg/ml. For all PBP 2X/2B/1A-R6 transformants, DNA sequencing confirmed the introduction of mutagenized PBP 1A genes.

Concluding remarks. Our analysis has shown that amino acid residue 371 and residues 574 to 577 of PBP 1A are important with respect to the interaction with penicillin. Substitutions at residues 574 to 577 are common to all resistant isolates (MICs, ≥0.25 μg/ml) and have been shown to be critical to the development of penicillin resistance. In the presence of these four substitutions, an alteration at residue 371 allows the development of full resistance.

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