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Penicillin-Binding Protein 1 of *Staphylococcus aureus* Is Essential for Growth

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***pbpA*, a gene encoding penicillin-binding protein (PBP) 1 of *Staphylococcus aureus*, was cloned in an *Escherichia coli* MC1061 transformant which grew on a plate containing 512 μg of vancomycin per ml. This gene encodes a 744-amino-acid sequence which conserves three motifs of PBPs, SXXK, SXN, and KTG. The chromosomal copy of *pbpA* could be disrupted only when RN4220, a methicillin-sensitive *S. aureus* strain, had additional copies of *pbpA* in its episome. Furthermore, these episomal copies of *pbpA* could not be eliminated by an incompatible plasmid when the chromosomal copy of *pbpA* was disrupted beforehand. Based on these observations, we concluded that *pbpA* is essential for the growth of methicillin-sensitive *S. aureus*.**

Staphylococcus aureus, one of the most medically important pathogens, causes both hospital- and community-acquired infections worldwide. Its resistance to multiple drugs, especially to β-lactam antibiotics, is a major therapeutic problem. *S. aureus* penicillin-binding proteins (PBPs), which synthesize its cell wall, are the targets of β-lactam antibiotics. Methicillin-sensitive *S. aureus* (MSSA) has four PBPs, PBP1 to -4, and methicillin-resistant *S. aureus* (MRSA) has an additional PBP (4, 10, 41), PBP2′, which is recognized as a major cause of its high resistance to β-lactam antibiotics. The gene encoding PBP2′, *mecA*, and several other genes have been shown to contribute to the β-lactam resistance of *S. aureus* (6, 7, 20, 21, 26, 36). The *mecA* gene was cloned by selecting a tobramycin-resistant *Escherichia coli* clone having a genomic fragment of MRSA in which sequences encoding methicillin and tobramycin resistance were linked (27). A gene encoding PBP2 was cloned from a λgt11 library by using antiserum which was raised against excised PBP2 from a sodium dodecyl sulfate (SDS)-polyacrylamide gel (31). The gene encoding PBP4 was cloned by Tn*551* insertion mutagenesis in a penicillin-resistant PBP4 overproducer (21). Here we describe another method for cloning the gene encoding PBP1 of *S. aureus* and genetically show the essentiality of this gene for the growth of MSSA.

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are summarized in Table 1. RN4220 (24) and BB255 (6) were kindly provided by R. P. Novick and B. Berger-Bächi, respectively.

Transformation of *E. coli* and *S. aureus*. *E. coli* MC1061 (11) and *S. aureus* RN4220 were transformed by electroporation by using a Gene Pulser system (Bio-Rad, Richmond, Calif.). Methods for the preparation of MC1061 competent cells and conditions of the transformation were described previously (15). A method for the preparation of RN4220 competent cells is described below. One fresh colony was inoculated into 5 ml of filtrated brain heart infusion (BHI) broth (Difco, Detroit, Mich.) and incubated at 37°C with gentle shaking. The cells were harvested at mid-log phase, i.e., optical density at 550 nm of <0.5, and rinsed twice with a buffer containing 1.1 M sucrose and 2 mM $MgCl_2$ at 4°C. After being concentrated 50 times by the rinse steps, the cells were transformed with DNAs by the application of current from a 25-μF condenser, which was charged to 2.0 kV, to a 1-mm-wide Gene Pulser cuvette (Bio-Rad), which was connected to a 200-Ω resistance in parallel. The cells were immediately mixed with 1 ml of BHI broth containing 1.1 M sucrose and incubated at 37°C for 1 h for the expression of antibiotic resistance markers. Then they were incubated on BHI agar containing appropriate concentrations of antibiotics at 37°C overnight. The DNAs used for electroporation were purified by the CsCl ultracentrifuge.

Antibiotics. Vancomycin was purchased from Shionogi (Osaka, Japan) and Sigma (St. Louis, Mo.). Tetracycline, erythromycin, and kanamycin were purchased from Sigma and used at concentrations of 2, 1, and 10 μg/ml, respectively, unless otherwise stated.

Construction of a plasmid library. A total of 5 μg of genomic DNA of NCTC8325 (33) was partially digested by *Sau*3AI (New England Biolabs, Beverly, Mass.), and fragments ranging from 3 to 5 kb in length were enriched by agarose gel electrophoresis. One hundred nanograms of pAW119 was digested with *Bam*HI (New England Biolabs), treated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), and ligated with the genomic fragments of NCTC8325. These DNAs were used for the transformation of MC1061. At 37°C, the transformants were selected on LB agar (Lennox; Difco) containing 512 μg of vancomycin and 10 μg of tetracycline per ml. After 18 h, the colonies obtained were streaked on duplicate LB plates containing 10 μg of tetracycline per ml; one set of the plates was incubated at 37°C, and the other set was incubated at 42°C for 18 h. Clones which grew at 37°C but which could not grow or which grew very slowly at 42°C were analyzed for their inserts. An introduced plasmid of each clone was digested with *Hin*cII (New England Biolabs) or was doubly digested with *Eco*RI (New England Biolabs) and *Pst*I (New England Biolabs), and the clones were compared to each other by agarose gel electrophoresis. Five clones were selected (see below), and their PBP activities were studied.

PBP assay. Detailed methods for the PBP assay were described previously (41). In brief, PBPs were labeled with [benzyl-^{14}C]penicillin (Amersham, Buckinghamshire, England), separated by SDS–8% polyacrylamide gel electrophoresis, and detected by fluorography.

***pbpA* disruption and its analysis.** To obtain *pbpA* disruptant AW304, RN4220 was first transformed with pPBPA to obtain AW303 and then AW303 was transformed with 2 μg of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and relevant markers[a]	Source or reference
Strains		
E. coli MC1061		11
S. aureus		
NCTC8325	Type strain of *S. aureus*	33
BB255	NCTC8325, penicillinase plasmid cured	6
RN4220	NCTC8325, penicillinase plasmid cured, *hly* mutant, restriction minus	24
AW302	RN4220(pAW11)	This study
AW303	RN4220(pPBPA)	This study
AW304	AW303 *pbpA*::pΔPBPA	This study
AW305	AW302(pAW12)	This study
AW306	AW303(pAW12)	This study
AW307	AW304(pAW12)	This study
Plasmids		
pAW119	Cloning shuttle vector, pMB1 ori for replication in *E. coli*, pAMα1 ori for replication in gram-positive organisms, Tet^r	This study
pAW9	Suicide vector in *S. aureus*, p15A ori for replication in *E. coli*, Tet^r	This study
pAW10	Small (3.9-kb) shuttle vector, p15A ori for replication in *E. coli*, pAMα1 ori for replication in gram-positive organisms, Tet^r	This study
pAW11	Cloning shuttle vector, pMB1 ori for replication in *E. coli*, pAMα1 ori for replication in gram-positive organisms, Em^r	This study
pAW12	Cloning shuttle vector, the same origins as pAW11, Km^r	This study
pPBPA	pAW11 with a *Kpn*I-*Kpn*I 3.1-kb fragment containing orf2 and *pbpA*	This study
pΔPBPA	pAW9 with a *Hin*dIII-*Hin*dIII 936-bp fragment containing a middle part of *pbpA*	This study
pORF2	pAW9 with a *Sau*3AI-*Hin*dIII 993-bp fragment containing intact orf2	This study

[a] Tet^r, tetracycline resistant; Em^r, erythromycin resistant; Km^r, kanamycin resistant; ori, origin.

pΔPBPA and cultured with erythromycin and tetracycline. Plasmids of *S. aureus* clones were extracted with a Miniprep kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions except for adding lysostaphin (Sigma) to resuspension buffer to a final concentration of 100 μg/ml and for incubation at 37°C for 10 min at the resuspension step. For amplification of chromosomal fragments around *pbpA*, PCR was performed by using primers 5′-TACTGGACATTCTAATGGTC-3′ and 5′-TTCCGACTC TATCACTTGTC-3′, and by using TaKaRa LA Taq (Takara Shuzo Co., Kyoto, Japan). Thermal cycles of 96°C for 20 s, 58°C for 20 s, and 72°C for 10 min were repeated 20 times. For Southern hybridization analysis, the resulting amplified products were transferred to a Hybond-N+ membrane (Amersham) and probed with whole pAW9 DNAs, which were labeled with digoxigenin (DIG) by using DIG-High Prime (Boehringer Mannheim). CDP Star (Boehringer Mannheim) was used for chemiluminescence detection by anti-digoxigenin-AP Fab fragments (Boehringer, Mannheim).

Plasmid incompatibility. AW305, AW306, and AW307 were cultured at 37°C in 5 ml of LB broth (Lennox; Difco) containing kanamycin for AW305 and AW306 and containing kanamycin and tetracycline for AW307. Every 12 h, each clone was diluted 1,000-fold with the same medium. At times zero and at 4, 8, 12, 24, and 46 h after the first inoculation, AW305 and AW306 were harvested and plated on LB agar (Lennox; Difco) with or without erythromycin. AW307 was harvested at the same times and was plated on LB agar (Lennox; Difco) containing tetracycline with or without erythromycin. After the incubation of each clone at 37°C for 18 h, the number of colonies on each plate was counted, and ratios of plating efficiencies with erythromycin to those without erythromycin were calculated.

Nucleotide sequencing. DNA sequences were determined by automatic DNA sequencers ABI PRISM 373A and 310 (Perkin-Elmer Applied Biosystems) with a DyePrimer and/or a DyeTerminator cycle sequencing kit (Perkin-Elmer Applied Biosystems).

Cloning of *pbpA*. Previously, we found that the plating efficiency of an *E. coli* MC1061 strain having *mecA* in its episome was about 1,000 times higher than that of a strain having a control plasmid when they were cultured with 512 μg of vancomycin per ml at 37°C. At 42°C, the strain having *mecA* grows very slowly, while the growth rate of the control strain at 42°C did not remarkably differ from that at 37°C (unpublished data). Based on these findings, we speculated that the PBP gene(s) of *S. aureus* might be cloned by positive selection of *E. coli* MC1061 transformants by using vancomycin at 37°C and by subsequent negative selection at 42°C. As the first screening step, a NCTC8325 genomic library containing about 20,000 transformants of MC1061 was plated on LB agar containing 512 μg of vancomycin and 10 μg of tetracycline per ml. After incubation at 37°C for 18 h, 71 transformants were obtained. Thirty-two transformants could not grow or grow very slowly at 42°C. In 12 of these 32 transformants, five common restriction patterns were observed in inserts of their plasmids (data not shown). To check the penicillin-binding activities of these transformants, we performed a PBP assay for five representative transformants, each of which showed one of the five common restriction patterns of the inserts. As shown in Fig. 1, one transformant, which had a 4.9-kb genomic fragment of NCTC8325, had extra PBP activities (lane 2). These activities were not observed in a control *E. coli* clone having pAW119 (lane 1). The mobility of the strongest band of this clone was the same as that of PBP1 of *S. aureus* BB255 (lanes 2 and 3). We determined the nucleotide sequence of the 4.9-kb fragment and found a total of five open reading frames (ORFs); three ORFs are located inside of the fragment and the other two, lacking N-terminal or C-terminal amino acid sequences, are located at each side of the fragment (Fig. 2). A part of the

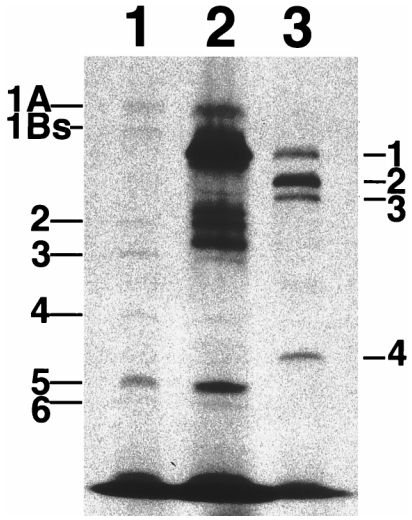

FIG. 1. Fluorography of PBPs labeled with [benzyl-^{14}C]penicillin after separation by SDS–8% polyacrylamide gel electrophoresis. Lane 1, an *E. coli* MC1061 transformant having pAW119; lane 2, an *E. coli* MC1061 transformant having a 4.9-kb genomic fragment of *S. aureus* NCTC8325; lane 3, *S. aureus* BB255. Designations of PBPs of *E. coli* and *S. aureus* are indicated at the left and right sides of the fluorography, respectively. On each lane, 100 μg of proteins of the membrane fraction was applied.

nucleotide sequence of this 4.9-kb fragment and its deduced amino acid sequences are shown in Fig. 3a. The third ORF encodes a 744-amino-acid sequence, which shows a high degree of homology to those of PBP2B of *Bacillus subtilis* (45) (50.6% similarity and 40.6% identity), SpoVD of *B. subtilis* (12) (40.8% similarity and 31.5% identity), PBP2X of *Streptococcus pneumoniae* (25) (37.9% similarity and 32.4% identity), and PBP3 of *E. coli* (32) (33.2% similarity and 26.3% identity). The amino acid sequence of the product of the third ORF of *S. aureus* and those of all these PBPs, including that of spore-making protein SpoVD, share the conserved motifs of PBPs, SXXK, SXN, and KT(S)G (Fig. 3b). In addition, the calculated molecular size of the deduced amino acid sequence of the product of the third ORF was 82.7 kDa, which corresponded to the molecular size of PBP1 of *S. aureus* as estimated by its mobility in a gel used for the PBP assay (41). Based on these observations, we concluded that the gene encoding PBP1 of *S. aureus* NCTC8325 has been cloned in the 4.9-kb fragment. We propose the name *pbpA* because its product has the highest molecular weight of any of the PBPs of *S. aureus*. A pulsed-field gel electrophoresis analysis revealed that this gene is located on the *Sma*I-B fragment of the NCTC8325 chromosome (35, 42) (data not shown).

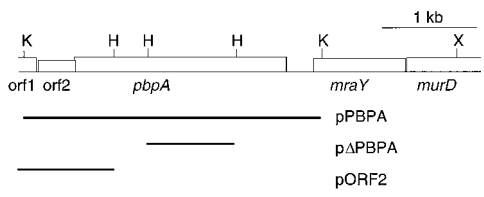

FIG. 2. A restriction map of a 4.9-kb genomic fragment of *S. aureus* NCTC8325 containing five ORFs, which are shown by boxes. The name of each ORF is indicated under the map. Each thick line under the map corresponds to an insert of each plasmid, whose name is indicated at the right side of each line. K, *Kpn*I; H, *Hin*dIII; X, *Xba*I.

Essentiality of *pbpA*. To test the essentiality of *pbpA* for growth of *S. aureus* genetically, we tried to disrupt this gene by homologous recombination. For its high level of competency, we adopted RN4220, a restriction-minus derivative of NCTC8325, and optimized conditions for transforming this strain by electroporation. With the conditions described in Materials and Methods, 5×10^5 transformants per μg of pAW10 (3.9 kb in length) can usually be obtained. In addition, we constructed pΔPBPA, which cannot replicate in *S. aureus* and which has a 936-bp *Hin*dIII-*Hin*dIII fragment of a middle portion of *pbpA* as an insert (Fig. 2, 3a, and 4a). Because this fragment lacks codons for both N-terminal and C-terminal sequences of PBP1, it will disrupt *pbpA* by a single homologous recombination (Fig. 4a). As a control, we constructed pORF2, which is another suicide plasmid having a 993-bp fragment containing complete orf2 (Fig. 2 and 3a) located upstream of *pbpA* and which will not disrupt orf2 on the chromosome. In the first experiment, we tried to transform RN4220 with 5 μg of pΔPBPA or pORF2. Table 2 shows the results of three independent experiments. A total of 147 transformants were obtained from pORF2, while no transformant could be obtained from pΔPBPA. A Southern hybridization analysis of randomly selected transformants of pORF2 showed that pORF2 was integrated into the chromosomal copy of orf2 by homologous, not by illegitimate, recombination (data not shown). In the next experiment, we tried to transform AW302 and AW303 with pΔPBPA. AW302 had control plasmid pAW11 and had only one copy of *pbpA* on its chromosome, while AW303 had extra copies of *pbpA* in its episome (Table 1). In this experiment, transformants were easily obtained from AW303, while no transformant could be obtained from AW302. One of 11 analyzed transformants of AW303, AW304, had a plasmid showing the same electrophoretic mobility as that of pPBPA (Fig. 4b). However, the length of the PCR-amplified fragment from the chromosome around *pbpA* of AW304 increased from 4.1 to 7.6 kb; the difference corresponds to the length of pΔPBPA (Fig. 4a and c). A whole fragment of pAW9, an original suicide plasmid used to make pΔPBPA (Table 1), hybridized to this 7.6-kb fragment of AW304 (Fig. 4c). These results showed that the chromosomal copy of *pbpA* of AW304 was disrupted by homologous recombination with pΔPBPA, while the episomal copies of *pbpA* on pPBPA were unaffected. In the other 10 transformants of AW303, the episomal copies of *pbpA* on pPBPA were disrupted and the chromosomal copy of *pbpA* was intact (data not shown). We think that this observed frequency was reasonable, because the copy number of pPBPA, estimated by Southern hybridization analysis, was about 10 (data not shown). In the third experiment, we tried to eliminate pPBPA from AW304 by using incompatible plasmid pAW12, which has the same replication origin as pPBPA but which has a different selection marker (Km^r; Table 1). At first, we transformed AW302, AW303, and AW304 with pAW12 and selected them with both erythromycin and kanamycin to obtain AW305, AW306, and AW307, respectively. In the presence of erythromycin and kanamycin, AW305, AW306, and AW307 can tentatively retain pAW11 and pAW12, pPBPA and pAW12, and pPBPA and pAW12, respectively (34). Then, these clones were cultured without erythromycin. When AW305 and AW306 were cultured with kanamycin alone, the numbers of erythromycin-resistant clones decreased logarithmically (Fig. 5). However, almost all populations of AW307 retained resistance to erythromycin even though they were cultured without erythromycin for 46 h (Fig. 5). This observation means that the extrachromosomal *pbpA* on pPBPA cannot be eliminated if the chromosomal copy of *pbpA* is disrupted beforehand. Based on the

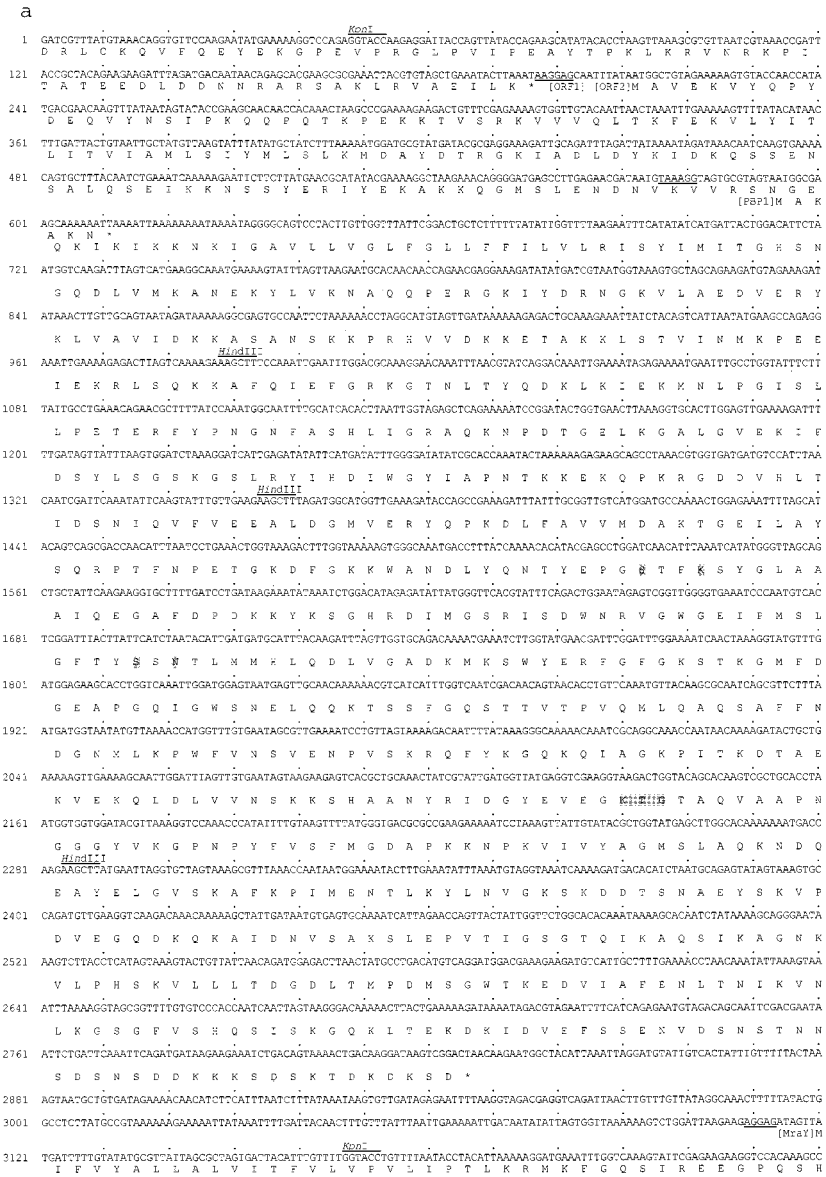

FIG. 3. (a) Nucleotide sequence of a part of the 4.9-kb fragment of NCTC8325 shown in Fig. 2, and its deduced amino acid sequence. The putative ribosome binding site of each ORF is underlined. The conserved motifs of PBPs are shown by shading in the amino acid sequence of PBP1. *Kpn*I and *Hin*dIII sites used for constructing the plasmids shown in Fig. 2 are indicated above the nucleotide sequence. (b) Amino acid sequence alignment of PBPs of various organisms including PBP1 of *S. aureus*. Common motifs of PBPs are shown by shadowing PBP1, PBP1 of *S. aureus*; PBP2B, PBP2B of *B. subtilis*; SPOVD, SpoVD of *B. subtilis*; PBP2X, PBP2X of *S. pneumoniae*; PBP3, PBP3 of *E. coli.*

results of these three experiments (the failure to obtain transformants from RN4220 with pΔPBPA, the success in obtaining a *pbpA* disruptant in the presence of pPBPA, and the observed retention of pPBPA in a chromosomal *pbpA* disruptant AW304), we concluded that *pbpA* of *S. aureus* (MSSA) is essential for its growth.

As described in this report, a total of four genes encoding PBP1, PBP2′ (27), PBP2 (31), and PBP4 (21) have been cloned and sequenced from *S. aureus*. As mentioned above, all genes other than that of PBP2 were cloned without purification of PBPs. To clone *pbpA*, encoding PBP1 of *S. aureus*, we used vancomycin for selecting *E. coli* MC1061 transformants. A population analysis of an MC1061 clone having pPBPA (Table 1, Fig. 2) showed vancomycin resistance similar to that conferred by *mecA* (data not shown). It remains unknown, however, whether *pbpA* or orf2 or both on pPBPA are necessary for the *E. coli* clone to be resistant to vancomycin. The reason for the observed vancomycin resistance is difficult to explain. One hypothesis is that the PBPs of *S. aureus* may protect the peptidoglycan precursor from an attack by vancomycin at the periplasm of *E. coli*. Although PBP overproduction has been shown to be related to the glycopeptide resistance of *S. aureus* (30), it is not probable that the PBPs of *S. aureus* function in *E. coli*, and even the translocation of the PBPs of *S. aureus* from the cytoplasm to the periplasm of *E. coli* cannot be proven by the PBP assay used in this study (41). The second hypothesis is that the overproduced PBPs of *S. aureus* may alter the concentrations of various peptidoglycan precursors such as UDP-

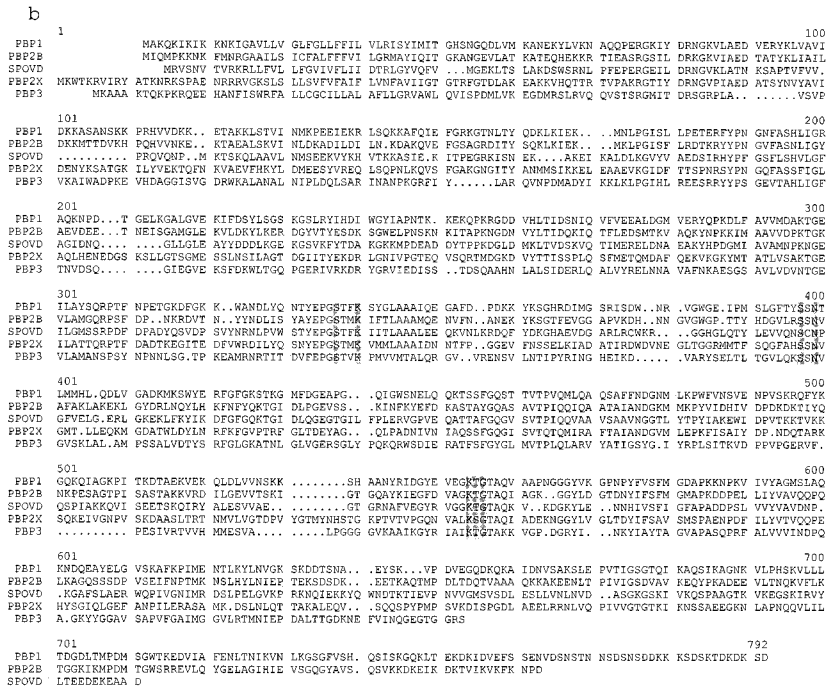

FIG. 3—*Continued.*

N-acetylmuramoyl-tri-, tetra-, and pentapeptides in the cytoplasm of *E. coli*. In enterococci, altered concentrations of these precursors were shown to be related to glycopeptide resistance (2). It is interesting to analyze the remaining four *E. coli* transformants, which grew with 512 μg of vancomycin per ml and which each had one of the common restriction patterns in the introduced plasmids but did not show detectable extra-PBP activity. They may contain a gene(s) involved in the synthesis or assembly of peptidoglycan precursors of *S. aureus*. On the other hand, we think that the negative selection at 42°C is less specific than vancomycin selection. This temperature may be toxic to an *E. coli* transformant in which PBP1 of *S. aureus* is overproduced (Fig. 2).

The deduced amino acid sequence of *pbpA* of *S. aureus* shows high degrees of homology to various PBPs of other organisms (see above). At present, the functions of PBP3, encoded by *ftsI*, of *E. coli* have been analyzed more throughly than those of other PBPs. A temperature-sensitive mutant of *ftsI* is aseptate and filamentous at the restrictive temperature (40), and overproduction of PBP3 in the *ftsI* mutant results in the suppression of filamentation (16). A study using azthreonam, which inhibits the PBP3 of *E. coli* specifically, confirms the notion that PBP3 is a septum-making enzyme (17). A carboxy truncation mutant of PBP2B of *B. subtilis* is reported to be filamentous (45), and no disruptant of the associated gene can be obtained (14). The deletion of PBP2X of *S. pneumoniae* has been shown to be lethal too (23). It is most probable that PBP1 of *S. aureus* also contributes to the synthesis of the peptidoglycan septum. Further investigation by immuno- and/or fluorescence staining of PBP1 to show its localization in dividing cells of *S. aureus* will provide more information about the function(s) of PBP1 as such techniques did for PBP3 of *E. coli* (44).

Interestingly, four ORFs other than that of *pbpA* on the cloned 4.9-kb fragment (Fig. 2) also show homologies to the members of cell division gene cluster *mra* of *E. coli* (29), in which *ftsI* is located; deduced amino acid sequences corresponding to orf1, orf2, *mraY*, and *murD* of *S. aureus* (Fig. 2) have homologies to those corresponding to *yabC* (8, 9) (49.2% similarity to a partial sequence), *ftsL* (18) (31.8% similarity), *mraY* (22) (55.0% similarity), and *murD* (28) (37.3% similarity to a partial sequence) of *E. coli*, respectively. *B. subtilis* also has homologous genes in the neighborhood of *pbpB*, which encode PBP2B; deduced amino acid sequences corresponding to orf1, orf2, *mraY*, and *murD* of *S. aureus* (Fig. 2) have homologies to those corresponding to *yllC* (45) (73.8% similarity to a partial sequnece), *ftsL* (45) (39.3% similarity), *mraY* (13) (67.9% similarity), and *murD* (13) (55.0% similarity to a partial sequnece) of *B. subtilis*, respectively. All these genes of *E. coli* and *B. subtilis* are thought to be involved in the synthesis of peptidoglycan or its precursors (12–14, 16, 18, 22, 28, 29, 45). In addition, *E. coli* has other cell wall-synthesizing genes, *ftsZ* and *ddl*, within the *mra* region (8, 9). Our preliminary sequencing data spanning the 14 kb downstream of *pbpA* confirmed the presence of *ftsZ* of *S. aureus* (1) 5.4 kb from *pbpA*, but we could not find any ORFs homologous to the *ddl* or *van* genes of enterococci (3). This observation agrees with that in a recently published report (37), which described a nucleotide sequence spanning 12.1 kb around *pbpA*.

Several genetic methods have been established to show the essentiality of *E. coli* genes for its growth. These methods include obtaining a temperature-sensitive mutant (40), conditional expression with an *araC* promoter (18), and selection of a disruptant with *rpsL* (19) or *sacB* (38). A mutation in *rpsL* confers streptomycin resistance on *E. coli*, but this resistance is sensitive dominant (19). At first, we tried to apply this selection to *S. aureus* by using its own *rpsL* gene (43). This gene, however, does not work as well that of *E. coli*. So we tried to disrupt *pbpA* by high-efficiency transformation with a suicide plasmid and by a subsequent single homologous recombination. By

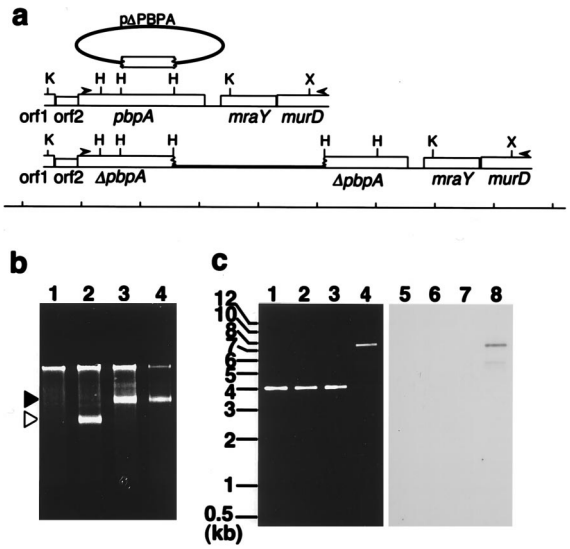

FIG. 4. (a) Restriction maps of the genomic fragment around *pbpA* before and after integration of suicide plasmid pΔPBPA. The name of each ORF is indicated under the maps, and the size marker is indicated at the bottom. Short vertical lines in the size marker are 1 kb apart. Arrowheads show the locations of the primers used for PCR shown in panel c. Abbreviations of the restriction enzymes are as defined in the legend for Fig. 2. (b) Plasmid profiles of *S. aureus* clones. The plasmid fraction of each clone was analyzed by agarose gel electrophoresis. Solid and open triangles indicate pPBPA and pAW11, respectively. Lane 1, RN4220; lane 2, AW302; lane 3, AW303; lane 4, AW304. (c) PCR-amplified fragments separated by agarose gel electrophoresis (lanes 1 to 4) and Southern hybridization analysis (lanes 5 to 8) by using pAW9 as a probe. Lanes 1 and 5, RN4220; lanes 2 and 6, AW302; lanes 3 and 7, AW303; lane 4 and 8, AW304. The sizes of size markers are indicated at the left side of the panel.

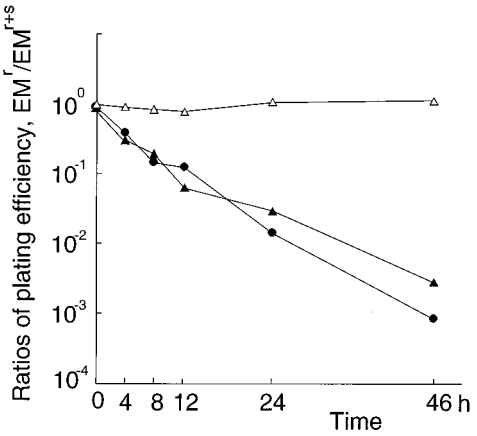

FIG. 5. Elimination of pAW11 and pPBPA by incompatible plasmid pAW12. After being cultured in LB broth with kanamycin at 37°C for the indicated numbers of hours, AW305 (●), AW306 (▲), and AW307 (△) were plated on LB agar with or without erythromycin. Calculated ratios of plating efficiency with versus without erythromycin were plotted at each harvesting time.

combining the data from the transformation and recombination experiments with the result of the plasmid incompatibility experiment, we could genetically show the essentiality of *pbpA* for the growth of MSSA. Our conclusion agrees with the previously postulated essentiality of PBP1 on the basis of PBP assays (5, 39).

Competitive PBP assays of MRSA have shown that β-lactam antibiotics much more easily bind to PBP1- to -4 than to PBP2′ (41), which leads to a well-accepted notion that PBP2′ can substitute for all other PBPs in the presence of β-lactam antibiotics. This notion, however, has not yet been proven genetically. A recent study by Pinho et al. shows that a Tn*551* insertion near the C terminus of PBP2 of a MRSA strain results in a reduction of its methicillin resistance (36). Our study shows that PBP1 disruption is lethal to MSSA, but its essentiality and even its contribution to the methicillin resistance of MRSA remain unknown. By using a phage 80α transduction system, whether PBP2′ can substitute for PBP1 or not is under investigation.

Nucleotide sequence accession numbers. The DNA sequence data described in this paper have been deposited in the GenBank/EMBL/DDBJ database with accession no. D28879 and AB007500.

TABLE 2. Result of transformation with suicide plasmids

Suicide plasmid	No. of transformants in expt:		
	1	2	3
pΔPBPA	0	0	0
pORF2	54	11	82

We thank R. N. Novick and B. Berger-Bächi for supplying *S. aureus* strain RN4220 and BB255, respectively.

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