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Antiseptic and Antibiotic Resistance Plasmid in *Staphylococcus aureus* That Possesses Ability To Confer Chlorhexidine and Acrinol Resistance

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Plasmid pSAJ1 from a methicillin- and gentamicin-resistant strain of *Staphylococcus aureus* had a molecular size of 50 kilobases and conferred resistance not only to kanamycin, gentamicin, tobramycin, amikacin, benzalkonium chloride, acriflavin, and ethidium bromide but also to chlorhexidine. In addition, the cloned antiseptic resistance gene(s) manifested acrinol resistance in *Escherichia coli*.

With the clinical use of methicillin and related antibiotics, methicillin-resistant *Staphylococcus aureus* (MRSA) has appeared increasingly and has become a significant factor in nosocomial infections (2, 4, 11). It has also been reported that MRSA strains isolated in Australia and the United Kingdom manifest increased resistance to several antiseptics compared with methicillin-susceptible strains of *S. aureus* (1). Moreover, self-transmissible plasmids that confer resistance to both antibiotics (e.g., gentamicin) and antiseptics, including quaternary ammonium compounds (e.g., benzalkonium chloride), have been identified in MRSA strains (7, 8, 13). We isolated an antiseptic and antibiotic resistance plasmid from an MRSA strain which was isolated in Tokyo, Japan, and demonstrated plasmid-mediated resistance to chlorhexidine and acrinol.

Bacterial strains and chemicals. MRSA strain O3 (MIC of methicillin, ≥800 μg/ml) was isolated from a patient with chronic respiratory failure in Juntendo Hospital in 1986. *S. aureus* RN2677 (resistant to rifampin [100 μg/ml] and novobiocin [6.25 μg/ml]) and *S. aureus* 209P-Sm (resistant to streptomycin [500 μg/ml]) were used as plasmid-free strains. *Escherichia coli* HB101 was used as a host for recombinant plasmids. A minicell-producing *E. coli* strain, χ2207, was that previously used (14). Benzalkonium chloride, chlorhexidine gluconate (Hibitane), acriflavin, and acrinol were commercially available materials. The antibiotics used were kindly provided by their manufacturers. The ^{14}C-labeled amino acids mixture (15 amino acids) was purchased from Commissariat à L'Energie Atomique, Saclay, France.

Filter mating. Bacterial mating on membrane filters was done as previously described (10). After incubation for 18 h at 37°C, transconjugants were selected for both donor resistance marker (10 μg of gentamicin per ml) and recipient resistance marker (5 μg of novobiocin per ml for strain RN2677 and 50 μg of streptomycin per ml for strain 209P-Sm).

Susceptibility testing. Susceptibility testing of bacterial strains was done by using the twofold plate dilution method on Muller-Hinton agar (unsupplemented with salt; Becton Dickinson and Co., Madison, Wis.) by the standard procedure (5). One loopful (5 μl) of 10^6-CFU/ml suspensions of the test bacteria was streaked on the surface of the agar plates, and incubation was for 20 h at 37°C.

Plasmid DNA and DNA manipulation. Plasmid DNA in *E. coli* was isolated as previously described (15). When plasmid DNA was isolated from *S. aureus*, lysostaphin was used instead of lysozyme and the lysis of the cells was done at 37°C, as previously described (10). Buoyant density of plasmid DNA was determined by centrifugation of purified plasmid DNA in neutral CsCl gradients, as previously described (15). Cloning of *S. aureus* plasmid DNA fragments into pBR322 (specifying for resistance to ampicillin and tetracycline) or pACYC177 (specifying for resistance to ampicillin and kanamycin) was done as previously described (14).

Analysis of minicell products. Minicells from cultures of *E. coli* χ2207 cells with and without plasmids were isolated and lysed essentially by the method of Roozen et al. (9), as previously described (14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by using the Laemmli gel system (6); a 15% separation gel (pH 8.8) was used. Molecular weight standards were those from Boehringer GmbH, Mannheim, Federal Republic of Germany, and Sigma Chemical Co., St. Louis, Mo.

Plasmid transfer and plasmid-mediated resistance. MRSA strain O3 was mated with *S. aureus* RN2677 on membrane filters. Gentamicin-resistant transconjugants were obtained at a transfer frequency (number of selected transconjugants per donor) of 6.9×10^{-7}. Those transconjugants (designated as RN2677-G3) manifested resistance to kanamycin, gentamicin, tobramycin, amikacin, ethidium bromide, benzalkonium chloride, and acriflavin (Table 1), as had been reported with *S. aureus* transconjugants which possessed a self-transmissible plasmid (pSK1) originating in an Australian MRSA strain (7, 8, 12). However, RN2677-G3 manifested resistance to chlorhexidine gluconate as well (Table 1). This phenotype was confirmed by using transconjugants which were obtained by mating between RN2677-G3 and 209P-Sm (Table 1); the transfer frequency was 1.1×10^{-5}. Upon the conjugal transfer, transconjugants lacking the ability to manifest resistance to chlorhexidine gluconate, benzalkonium chloride, acriflavin, and ethidium bromide (and a related compound, propidium iodide) were spontaneously isolated at a frequency of 0.026. Plasmid (pSAJ1) DNA, isolated from transconjugants RN2677-G3 and 209P-Sm (pSAJ1), had a molecular size of ca. 50 kilobases (kb) and a buoyant density of 1.6955 g/cm^3, corresponding to a guanine-plus-cytosine content of 36.2 mol%.

Gene cloning and expression. The *Hin*dIII-digested fragments of pSAJ1 DNA were inserted into the *Hin*dIII site of

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TABLE 1. Resistance levels of MRSA strain O3 and transconjugants obtained by filter mating[a]

Antimicrobial agent	MIC (μg/ml) for:				
	O3	RN2677-G3[b]	RN2677	209P-Sm(pSAJ1)[c]	209P-Sm
Chlorhexidine gluconate	6.25	**3.13**	1.56	**3.13**	1.56
Benzalkonium chloride	12.5	**6.25**	3.13	**6.25**	3.13
Acriflavin	800	**800**	6.25	**400**	12.5
Acrinol	800	400	400	400	400
Ethidium bromide	200	**200**	3.13	**200**	3.13
Kanamycin	1,600	**800**	1.56	**800**	0.78
Gentamicin	400	**100**	0.2	**50**	0.1
Tobramycin	800	**25**	0.2	**12.5**	0.05
Amikacin	50	**12.5**	0.78	**6.25**	0.39

[a] Resistance manifested by transconjugants is in boldface.
[b] Transconjugant obtained by filter mating between strains O3 (donor) and RN2677 (recipient), with selection for gentamicin and novobiocin resistance. It carried the plasmid pSAJ1 originating in strain O3.
[c] Transconjugant obtained by filter mating between strains RN2677-G3 (donor) and 209P-Sm (recipient), with selection for gentamicin and streptomycin resistance.

pBR322, and ampicillin- and acriflavin-resistant transformants of *E. coli* HB101 were obtained. Such clones had a recombinant plasmid (designated as pBRJ1) consisting of a 5.9-kb *Hin*dIII fragment of pSAJ1 and a 4.4-kb pBR322 *Hin*dIII fragment (Fig. 1). Ampicillin- and kanamycin-resistant transformants were also obtained. They had a recombinant plasmid (designated as pBRJ2) consisting of a 2.5-kb *Hin*dIII fragment of pSAJ1 and a 4.4-kb pBR322 *Hin*dIII fragment. As shown in Table 2 (experiment 1), pBRJ1

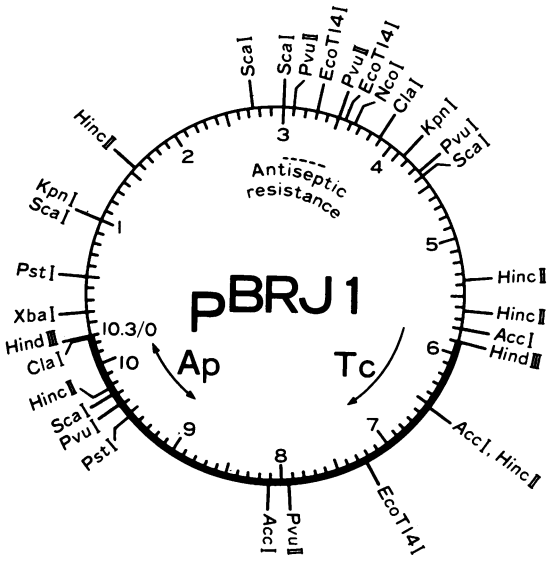

FIG. 1. Restriction endonuclease cleavage map of antiseptic resistance-specifying recombinant plasmid pBRJ1. The thin line represents the 5.9-kb *Hin*dIII fragment originating in *S. aureus* plasmid pSAJ1, and the thick line represents the pBR322 *Hin*dIII fragment. Numbers are DNA sizes in kilobases. Ap, Ampicillin resistance; Tc, Tetracycline resistance (in pBRJ1, the pBR322 promoter for the Tc gene is located opposite to the Tc structural gene drawn in this figure). The antiseptic resistance (ASR) region indicates the location of a gene(s) for resistance for chlorhexidine gluconate, benzalkonium chloride, acrinol, acriflavin, and ethidium bromide (and propidium iodide). The 5.9-kb *Hin*dIII fragment had no cleavage sites for *Bam*HI, *Xma*I, *Xma*III, *Bal*I, *Xho*I, *Sal*I, *Eco*RV, *Ava*I, *Sph*I, *Nde*I, *Sac*I, *Bgl*II, *Stu*I, *Mlu*I, *Hpa*I, *Bss*HII, *Sac*II, or *Nru*I.

conferred upon its *E. coli* host resistance not only to chlorhexidine gluconate, benzalkonium chloride, acriflavin, and ethidium bromide (and propidium iodide) but also to acrinol, to which *S. aureus* carrying pSAJ1 manifested no detectable resistance (Table 1); this (antiseptic) resistance phenotype mediated by pBRJ1 was referred to as ASR. Kanamycin or gentamicin resistance levels conferred by pBRJ2 were considerably low (Table 2, experiment 1) compared with those of *S. aureus* carrying pSAJ1 (Table 1).

Attempts were made to delete the 0.38-kb *Pvu*II-*Pvu*II region on the 5.9-kb *Hin*dIII fragment of pBRJ1 (Fig. 1). For this, the 5.9-kb *Hin*dIII fragment was purified and then inserted into the *Hin*dIII site of pACYC177, which had no *Pvu*II sites. DNA of the resultant ampicillin and acriflavin resistance recombinant plasmid (designated as pACJ1) was subjected to complete *Pvu*II digestion, ligation, and transformation of *E. coli* HB101. The ampicillin resistance plasmid thus obtained (designated as pACJ1d) exactly lacked the 0.38-kb *Pvu*II-*Pvu*II region (possessing only one *Pvu*II site), and it lost the ability to manifest the ASR phenotype; the original pACJ1 plasmid manifested the ASR phenotype to the same or an even greater extent than pBRJ1, as shown in Table 2 (experiment 2). These results strongly indicated that the ASR phenotype is coded for by a single gene(s) located on the *Pvu*II site(s) (Fig. 1).

Products from the recombinant plasmids were analyzed in *E. coli* minicells (Fig. 2). Only a protein with a molecular weight of 38,000 (38K) correlated well with the ASR phenotype in such a way that it was prominent in ASR^+ cells (lanes 3, 5, and 7) but not in ASR^- cells (lanes 1, 2, 4, 6, and 8). The 38K protein was a major product in the case of pBRJ1-carrying minicells (lane 3). The conclusion that the 38K protein is a product from the ASR region needs further experiments. Very faint protein bands running around 47,000- to 50,000-molecular-weight positions were found only in ASR^+ samples (lanes 3, 5, and 7). A protein with 41,000 molecular weight was found to be synthesized from the 5.9-kb *Hin*dIII fragment irrespective of the ASR phenotype (lanes 3 and 5 to 8); the gene for the 41K protein must be located outside the 0.38-kb *Pvu*II-*Pvu*II region on the 5.9-kb *Hin*dIII fragment. In the cases of the recombinant plasmid-carrying minicell samples, some of β-lactamase-related polypeptides (a precursor or a degraded polypeptide of β-lactamase; 3) disappeared (lanes 3 and 5 to 8); the reason for this is not known.

TABLE 2. Resistance levels of *E. coli* HB101 carrying recombinant plasmids[a]

Antimicrobial agent	MIC (μg/ml) for:						
	Expt 1; vector, pBR322			Expt 2; vector, pACYC177			HB101
	HB101(pBRJ1)	HB101(pBRJ2)	HB101(pBR322)	HB101(pACJ1)	HB101(pACJ1d)	HB101(pACYC177)	
Chlorhexidine gluconate	**6.25**	1.56	1.56	**12.5**	1.56	1.56	1.56
Benzalkonium chloride	**400**	100	100	**400**	100	100	100
Acriflavin	**800**	100	100	**800**	100	100	100
Acrinol	**800**	100	50	**800**	100	100	100
Ethidium bromide	**800**	50	50	**800**	50	50	50
Kanamycin	1.56	**25**	1.56	ND[b]	ND	ND	3.13
Gentamicin	0.2	**1.56**	0.2	ND	ND	ND	0.39
Tobramycin	0.39	**1.56**	0.2	ND	ND	ND	0.39
Amikacin	0.78	0.78	0.78	ND	ND	ND	0.39

[a] Resistance conferred by recombinant plasmids is in boldface.
[b] ND, Not done.

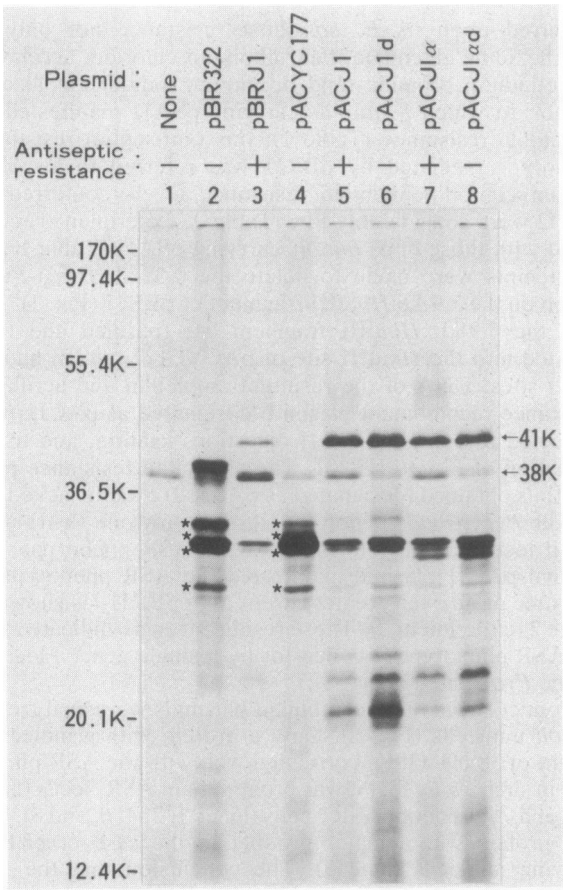

FIG. 2. Analysis of recombinant plasmid products in *E. coli* minicells. The recombinant plasmid pACJ1α is a pACYC177 derivative which was constructed by the insertion of the antiseptic resistance (ASR)-specifying 5.9-kb *Hin*dIII fragment into the *Hin*dIII site of pACYC177 in an opposite orientation from that in the case of pACJ1. pACJ1αd is a pACJ1α derivative which was constructed by the deletion of the 0.38-kb *Pvu*II-*Pvu*II region on the 5.9-kb *Hin*dIII fragment, just like pACJ1d (see the text). Products from the ampicillin resistance gene (β-lactamase and its related polypeptides; 3, 14) of pBR322 and pACYC177 are marked with asterisks (*).

The antiseptic and antibiotic resistance plasmid pSK1, with a molecular size of 28.4 kb, carries the gene (*qacA*) for resistance to benzalkonium chloride, acriflavin, ethidium bromide, propamidine isethionate, and diamidinodiphenylamine dihydrochloride, as well as the genes for gentamicin resistance and trimethoprim resistance (7, 8, 12). The gene for the ASR phenotype seems similar to the *qacA* gene (8) with respect to the location of the *Pvu*II site(s) and some patterns of the resistance phenotype, although the identity of the two genes is not known. pSAJ1, unlike pSK1, did not manifest trimethoprim resistance.

The present study demonstrated that the ASR phenotype (e.g., resistance to chlorhexidine gluconate and acrinol) was observed in *E. coli* more markedly than in *S. aureus* (Tables 1 and 2). This suggests that the gene for the ASR phenotype may have originated evolutionarily in bacterial species other than *S. aureus*.

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