JOURNAL OF BACTERIOLOGY, Aug. 1980, p. 541-551
0021-9193/80/08-0541/11$02.00/0

Vol. 143, No. 2

Plasmid-Mediated Resistance to Aminocyclitol Antibiotics in Group D Streptococci†

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Streptococcus faecalis BM4100 was resistant to high levels of gentamicin, kanamycin, and structurally related antibiotics. The genes conferring resistance to aminocyclitols in this strain were carried by a plasmid, pIP800, self-transferable to other *S. faecalis* strains. The aminocyclitol resistance was mediated by constitutively synthesized phosphotransferase and acetyltransferase activities. It was inferred that phosphorylation occurred at the 2″-hydroxyl group and that acetylation occurred at the 6′-hydroxyl group of the aminocyclitols. The enzyme activities were not separable by gel filtration or by isoelectric focusing. Their apparent molecular weight was 31,000, and their isoelectric point was 5.3. With respect to substrate profile, size, and charge, the transferases from strain BM4100 resembled closely those with identical site specificity described in staphylococci. These results suggest that plasmid gene transfer may occur between the two pathogenic bacterial genera.

The role of plasmid-mediated aminocyclitol-modifying enzymes in the resistance of gram-negative bacteria towards these antibiotics has been extensively investigated (13). Since the initial report on the existence of plasmids in streptococci (10), it has become increasingly obvious that many of the genes mediating resistance to antibiotics (R determinants) are located on plasmids in this genus. These antibiotic resistance plasmids (R plasmids) are capable of self-transfer by conjugation (21) or can be mobilized by conjugative plasmids (15).

Group D streptococci are naturally resistant to low levels of aminocyclitols (minimal inhibitory concentrations [MICs] less than or equal to 250 μg/ml) (34). Clinical isolates of group D streptococci resistant to high levels (MICs greater than 2 mg/ml) of streptomycin and kanamycin or both have been reported (30). These resistances are mediated by aminocyclitol-modifying enzymes, and the corresponding genes are carried by self-transferable plasmids (12, 23, 29).

In this study we describe a *Streptococcus faecalis* strain resistant to high levels (greater than 2 mg/ml) of gentamicin, kanamycin, and structurally related antibiotics. (A preliminary report of part of this work was presented at the 4th International Symposium on Antibiotic Resistance, 4-8 June 1979, Smolenice, Czechoslovakia.) The properties of the responsible plasmid-borne enzymes are compared with those of similar enzymes of staphylococci and gram-negative bacteria.

† This work is dedicated to the memory of Michel Piechaud.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and properties of the streptococcal strains are listed in Table 1. *S. faecalis* subsp. *zymogenes* strain BM4100 was isolated from a patient in a general hospital in Paris.

Media. Brain heart infusion broth and agar (Difco) were used, except where stated. All incubations were at 37°C.

Determination of MICs. The method of Steers et al. (31) was used to determine MICs of the antibiotics.

Transfer of antibiotic resistance traits. Transfer was either in liquid medium (21) or on cellophane membranes (5). In the latter case, 0.1 ml each of donor and recipient cultures, grown to approximately 10^9 colony-forming units per ml, was mixed on a cellophane membrane placed on top of solid medium. After 1 h of incubation, cells were suspended in broth, and dilutions were spread on selective media.

Curing of antibiotic resistance. Curing of antibiotic resistance traits has been described in detail elsewhere (P. Courvalin, M.D. thesis, Faculté Necker-Enfants Malades, Paris, 1974). Bacteria were plated on agar medium containing serial dilutions of rifampin and incubated overnight. Bacteria from plates containing subinhibitory antibiotic concentrations were suspended in broth, replated, and screened for antibiotic-susceptible segregants by replica plating.

Tests for bacteriocin production and immunity. Tests for bacteriocin production and immunity were performed as described before (3).

Inactivation of sisomicin. Inactivation of sisomicin in the culture medium was determined as follows. Cells were grown for about 20 generations in broth containing 1 mg of sisomicin per ml. The medium was cleared by centrifugation, and the biological activity of the antibiotic in the supernatant was determined using a bioassay with *Staphylococcus aureus* strain 209P as the indicator organism.

TABLE 1. *Properties of S. faecalis strains*

Strain	Phenotype[a]	Enzyme activities present[b]	Plasmid content	Origin or source
JH2-2	Rif Fa			Spontaneous mutant; A. Jacob (30)
BM4100	Gen Kan Cm	2″-APH 6′-AAC	pIP800	Wild strain; A. Buu Hoï-Dang Van
	Em^c Hly Bcn		pIP801 pIP802 pIP803	
	Tc			
BM4100-1	Em^c Hly Bcn		pIP801 pIP802 pIP803	Curing of BM4100
	Tc			
BM4100-2			pIP802 pIP803	Curing of BM4100
	Tc			
JH2-100	Gen Kan Cm Em^c Hly Bcn	2″-APH 6′-AAC	pIP800 pIP801 pIP802	Transfer from BM4100
	Rif Fa			
JH2-101	Em^c Hly Bcn		pIP801 pIP802	Transfer from BM4100
	Rif Fa			
JH2-102	Gen Kan Cm	2″-APH 6′-AAC	pIP800 pIP802	Transfer from BM4100
	Rif Fa			
JH2-103	Cm		pIP800-1	Transfer from BM4100
	Rif Fa			

[a] Cm, Resistance to chloramphenicol; Em^c, constitutive resistance to marolide, lincosamide, and streptogramin B-type antibiotics; Fa, resistance to fusidic acid; Gen, resistance to gentamicin; Hly Bcn, production of hemolysin and production of and immunity to bacteriocin; Rif, resistance to rifampin; Tc, resistance to tetracycline, minocycline, and chelocardin.

[b] 2″-APH, aminocyclitol phosphotransferase modifying the hydroxyl group in the 2″ position; 6′-AAC, aminocyclitol acetyltransferase modifying the amino group in the 6′ position.

Assay for aminocyclitol-modifying enzymes. To assay for aminocyclitol-modifying enzymes, the streptococcal extracts were prepared (12) and the enzymes were assayed (19) as described.

Preparation of plasmid DNA. Cells from 100 ml of an exponential broth culture were harvested and suspended in 10 ml of SET buffer (0.15 M NaCl, 0.1 M EDTA, 0.05 M Tris-hydrochloride; pH 8.0) containing lysozyme (1 mg/ml) and RNase A (500 μg/ml). This suspension was divided into 1-ml aliquots and incubated at 37°C for 1 h. The resulting protoplasts were lysed with 0.1% *N*-lauroylsarcosine (15 min at 37°C). The DNA was sheared, by passing the lysates five times through a 2-ml syringe, and pooled; the solution was adjusted to 32 g with SET buffer and mixed with 30 g of cesium chloride. Ethidium bromide was added to a final concentration of 150 μg/ml. The solution was centrifuged at 18°C for 14 h at 50,000 rpm in a Sorvall TV-850 rotor. Fractions containing plasmid DNA were pooled and banded a second time at 18°C for 6 h at 65,000 rpm in a Sorvall TV-865 B rotor. Fractions containing DNA were pooled, and ethidium bromide was removed by five consecutive extractions with isopropanol. The DNA was dialyzed three times against 1 liter of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5) and stored at 4°C.

Bacteriophage λ *c*I857 DNA was prepared as described (7).

Agarose gel electrophoresis of restriction endonuclease digests. Plasmid DNA was digested with restriction endonuclease *Eco*RI (9). Bacteriophage λ DNA was used as reference and cleaved by *Eco*RI and *Hin*dIII or by *Sma*I (32). The resulting DNA fragments were separated by electrophoresis in slab gels containing either a linear gradient (2.4 to 6%) of acrylamide (1) or 0.6, 0.8, or 1% agarose. Electrophoresis and staining were as described (28).

Gel filtration. A crude (100,000 × *g* centrifugal supernatant fluid; S100) enzyme preparation was applied to a column of Sephadex G100 superfine resin, equilibrated with buffer (50 mM Tris, 20 mM $MgCl_2$, 1 M NH_4Cl, 12 mM β-mercaptoethanol, adjusted to

pH 7.5 with acetic acid).

Isoelectric focusing in polyacrylamide gel. Isoelectric focusing was of S100 preparations in 5% polyacrylamide gels containing 2% ampholines. The gels were sliced, and each slice was cut in two. The determination in each slice was either of acetylation and pH or of acetylation and phosphorylation. The enzyme activities were determined (19) after incubation for 3 h at 4°C in buffer (20 mM Tris, 20 mM $MgCl_2$, 300 mM NH_4Cl, 12 mM β-mercaptoethanol, adjusted to pH 7.5 with acetic acid).

Enzymes. Restriction endonucleases *Eco*RI and *Sma*I were purified (32). Restriction endonuclease *Hin*dIII was kindly provided by J. Gardner and R. Jorgensen. Lysozyme and RNase A were from Sigma Chemical Co., St. Louis, Mo.

Chemicals. [1-^{14}C]acetylcoenzyme A, [γ-^{32}P]ATP (triethylammonium salt), and [*U*-^{14}C]ATP (ammonium salt) were obtained from the Radiochemical Centre, Amersham, England. The antibiotics were provided by the following laboratories: gentamicins (Gen) C1a, C1, C2, A, B, and Gen complex (26.3% C1a, 40.8% C1, 32.9% C2), sisomicin (Sis), 2″-deoxysisomycin (2″-DSis), and netilmicin (Net) from Schering; kanamycins (Kan) A, B, and C and amikacin (Ami) from Bristol; neamine (NeoA), neomycin B (NeoB), and spectinomycin (Spc) from Upjohn; paromomycin (Par) and butirosin (But) from Parke, Davis; tobramycin (Tob) and apramycin (Apr) from Lilly; lividomycin (Liv) A from Kowa; ribostamycin (Rib) from Meiji; streptomycin (Str) and tetracycline (Tc) from Pfizer; chloramphenicol (Cm) from Roussel; minocycline (Mn) from Lederle; erythromycin (Em) and chelocardin from Abbott; rifampin (Rif) from Lepetit; and fusidic acid (Fa) from Leo.

RESULTS

Plasmid-mediated characters expressed by *S. faecalis* BM4100 and their stability. *S. faecalis* BM4100 codes for resistance to gentamicin, kanamycin, and chloramphenicol; for resistance to macrolide, lincosamide, and streptogramin B-type antibiotics (Em^c); for tetracycline, minocycline, and chelocardin resistance; for hemolysin production (Hly); and for bacteriocin production and immunity (Bcn) (Table 1). In curing experiments some of these characters were lost en bloc, and two classes of variants were found: one had lost gentamicin, kanamycin, and chloramphenicol resistance (approximately 0.08% of colonies tested), and the other had also lost the Em^c, Hly, and Bcn traits (approximately 0.01%). One clone of each class was studied further (strains BM4100-1 and BM4100-2). No clone susceptible to tetracycline was obtained. The MICs of antibiotics for the parental strain and the two derivatives are shown in Table 2. Neither strain BM4100-1 nor BM4100-2 reverted to antibiotic resistance. The rate of reversion was less than 3.4×10^{-11} to kanamycin (2 mg/ml), gentamicin (2 mg/ml), and chloramphenicol (32 μg/ml) resistance for both strains and to erythromycin (1 mg/ml) resistance for strain BM4100-2.

Characters transferred by BM4100. The genes conferring resistance to gentamicin, kanamycin, and chloramphenicol and the traits Em^c and Hly-Bcn were transferred from the parental strain to strain JH2-2 during mixed incubation. Transfer of tetracycline resistance was never observed. Selection for transfer of gentamicin, kanamycin, or chloramphenicol resistance revealed cotransfer of all three (in 61 clones studied), except that chloramphenicol resistance

TABLE 2. *MICs of various antibiotics against S. faecalis strains*

Antibiotic	MIC[a] (μg/ml) against strain:							
	JH2-2	BM4100	BM4100-1	BM4100-2	JH2-100	JH2-101	JH2-102	JH2-103
Gentamicin	16	>4,096	8	16	>4,096	32	>4,096	32
Sisomicin	8	>4,096	4	8	>4,096	16	>4,096	32
Netilmicin	8	1,024	4	8	512	8	2,048	8
Kanamycin A	64	>4,096	16	64	>4,096	64	>4,096	256
Kanamycin B	32	>4,096	16	32	>4,096	64	>4,096	64
Amikacin	128	512	32	128	1,024	256	1,024	256
Tobramycin	32	>4,096	8	16	2,048	32	2,048	32
Neamine	128	>4,096	128	256	4,096	256	4,096	256
Neomycin B	128	128	32	64	512	512	512	512
Paromomycin	256	256	128	256	512	512	512	512
Lividomycin A	512	512	256	512	2,048	2,048	2,048	4,096
Butirosin	128	256	128	256	256	256	256	256
Ribostamycin	128	>4,096	64	128	4,096	256	4,096	256
Apramycin	256	256	64	128	512	512	512	512
Streptomycin	128	256	256	256	64	128	256	256
Spectinomycin	8	32	32	16	32	32	16	8
Chloramphenicol	8	64	8	8	64	16	64	64
Tetracycline	1	64	64	64	2	2	2	2
Minocycline	0.25	32	32	32	0.25	0.25	0.25	0.25
Erythromycin	0.125	4,096	4,096	0.125	4,096	8,192	0.125	0.125

[a] MICs were determined in Mueller-Hinton agar medium.

TABLE 3. *Frequency of transfer between S. faecalis strains*[a]

Selected resistance from donor	Unselected characters from donor	Frequency[b]
Gen	Kan Cm	4.8×10^{-7}
Gen	Kan Cm Em[c] Hly Bcn	3.2×10^{-7}
Em	Hly Bcn	2.3×10^{-3}
Em	Hly Bcn Gen Kan Cm	2.2×10^{-3}
Tc		$<6.8 \times 10^{-8}$

[a] Donor strain, BM4100; recipient, JH2-2. Counterselection was with 20 μg of rifampin and 10 μg of fusidic acid per ml.

[b] Frequencies are expressed relative to 6.8×10^{8} bacterial donors after mating on cellophane membrane (5) for 1 h.

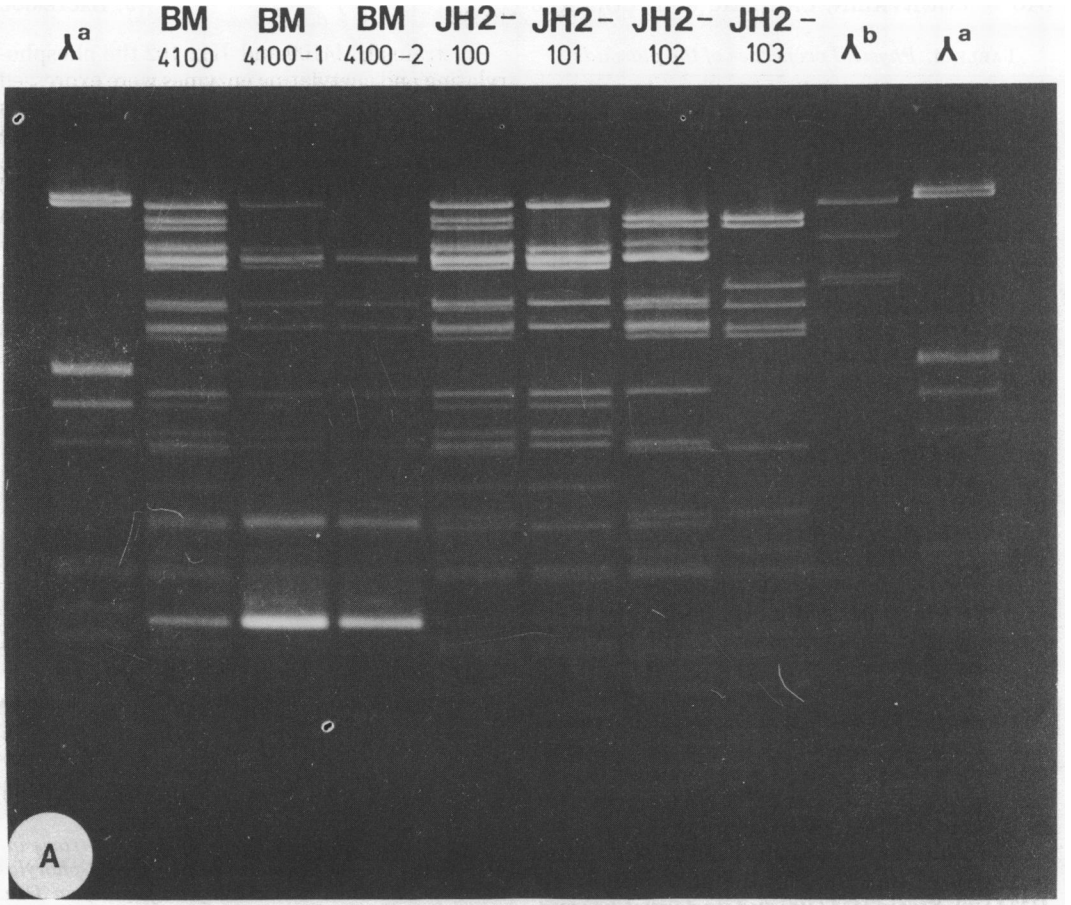
alone was transferred once and selection for transfer of erythromycin resistance revealed cotransfer of the Hly and Bcn traits (in 11 clones studied). Hemolysin and bacteriocin are possibly identical proteins (2). Cotransfer of all resistance characters, with the exception of tetracycline resistance as mentioned above, was observed most frequently (1,049 clones studied). The frequencies of transfer are shown in Table 3. Ten clones of each class of transcipients were tested for the synthesis of aminocyclitol-modifying activities (19). One transcipient of each class was studied further. The MICs of the antibiotics for strains JH2-2 and the transcipients that had received (i) all the transferable characters from BM4100 (strain JH2-100), (ii) Em[c] and Hly-Bcn traits (strain JH2-101), (iii) gentamicin, kanamycin, and chloramphenicol resistances (strain JH2-102), and (iv) chloramphenicol resistance alone (strain JH2-103) are shown in Table 2. The MICs of aminocyclitols and of chloramphenicol and erythromycin for the wild-type strain and its derivatives and for JH2-2 and the transcipients were all high when the respective R determinants were present; they were all low when the R determinants were absent. The transferable characters were transferred from the primary recipient strain to other group D streptococcal strains after mixed incubation.

Plasmid content of BM4100 and its derivatives and transcipient strains. Strains BM4100, BM4100-1, BM4100-2, JH2-100, JH2-101, JH2-102, and JH2-103 were shown by ultracentrifugation in cesium chloride-ethidium bromide to contain covalently closed circular DNA. The plasmid DNA from each strain was purified, digested with *Eco*RI endonuclease, and analyzed by acrylamide gel (data not shown) or agarose gel electrophoresis (Fig. 1 and Tables 1 and 4).

Comparative analysis of the phenotypes (Table 1) and the *Eco*RI-generated fragment patterns of plasmid DNA (Fig. 1) in the individual strains led us to conclude that the wild-type strain BM4100 harbors four plasmids (Table 4). Plasmid pIP800 encoded gentamicin, kanamycin, and chloramphenicol resistance; it had a molecular size of 70.1 kilobases (kb) and 14 *Eco*RI-generated DNA fragments, which were numbered in order of decreasing size. pIP801 encoded the Em[c] and Hly-Bcn traits; it had a molecular size of 53 kb and nine fragments, designated with letters A to I. pIP802 was cryptic, with a molecular size of 49 kb and 11 fragments (α to λ). pIP803 had a molecular size of 2 kb, and it had no *Eco*RI site. The possibility that bands α to λ originated from two (or more) cryptic plasmids was ruled out by electron microscopic analysis (11) of BM4100-2 plasmid DNA (results not shown). The molecular size of pIP802 by electron microscopy was 50.5 kb, and that of pIP803 was 2.5 kb.

Strains BM4100-1 and BM4100-2 were obtained after curing of BM4100 with rifampin. BM4100-1 was susceptible to gentamicin, kanamycin, and chloramphenicol and had lost pIP800. BM4100-2 was further susceptible to erythromycin, was Hly-Bcn negative, and lacked plasmids pIP800 and pIP801. Matings between BM4100 and the plasmid-free strain JH2-2 yielded transcipients JH2-100 through JH2-103. JH2-100 was resistant to gentamicin, kanamycin, chloramphenicol, and erythromycin and was Hly-Bcn positive after acquisition of plasmids pIP800, pIP801, and pIP802. JH2-101 was resistant to erythromycin, was Hly-Bcn positive, and had acquired pIP801 and pIP802. JH2-102 was resistant to gentamicin, kanamycin, and chloramphenicol and had plasmids pIP800 and pIP802. JH2-103 was resistant to chloramphenicol only and contained plasmid pIP800-1. Plasmid pIP800-1 had a molecular size of 67.3 kb (fragments numbered 1 to 14; Fig. 1). Its *Eco*RI-generated pattern was indistinguishable from that of pIP800 except that fragment 3 (10.6 kb) was replaced by fragment 3′ (7.8 kb), presumably arising from a 2.8-kb deletion in pIP800 fragment

FIG. 1. *Analysis of plasmid DNA content of S. faecalis BM4100 and its derivatives and transcipients by agarose gel electrophoresis. (A) Plasmid DNA (2 to 3 μg) was digested with EcoRI. Fragments obtained by digestion of λ DNA, 48 kb (16), with restriction endonucleases EcoRI + HindIII (λ[a]; references 1, 26, 33) and SmaI (λ[b]; reference 27) were used as molecular size standards. Electrophoresis was carried out in a 0.8% agarose horizontal slab gel (18 by 13 by 0.4 cm) for 12 h at 3 V/cm. (B) Schematic representation of (A) fragments from plasmids pIP800, pIP800-1 (——), pIP801 (– – –), pIP802 (· · · ·), and pIP803 (–· –·). Numbers and letter designations are as explained in the text. SC, Supercoiled; L, linear; OC, open circles. The molecular size of the reference λ DNA fragments is expressed in kilobases. Plasmids pIP800 and pIP800-1 EcoRI fragment 14 were detected on acrylamide gels only and are arbitrarily positioned in the graph.*


λa
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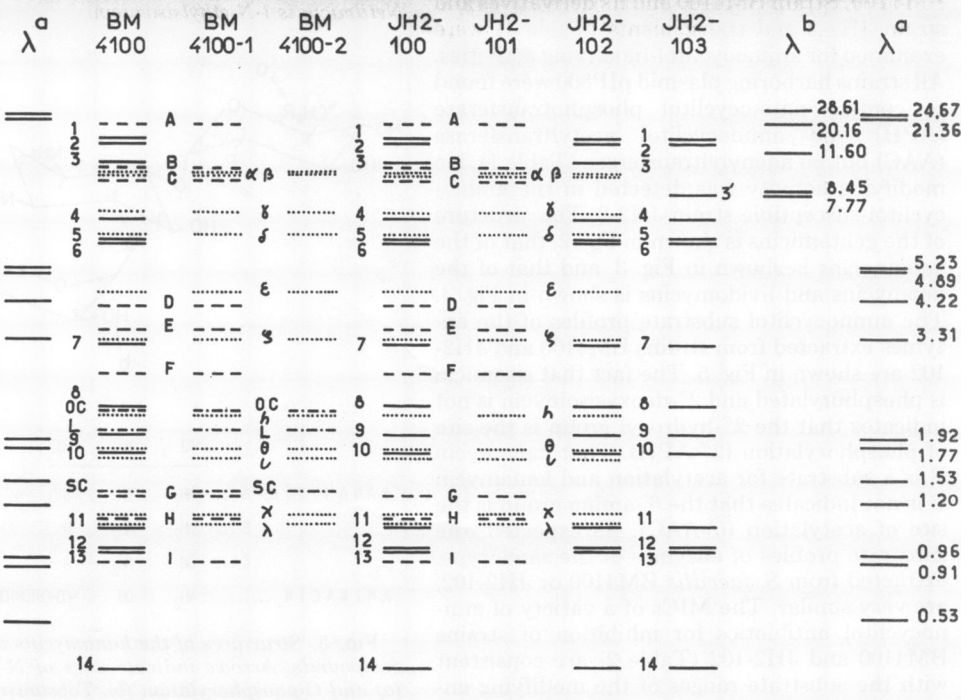
BM 4100-1
BM 4100-2
JH2-100
JH2-101
JH2-102
JH2-103
λb
λa
A


λa
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BM 4100-1
BM 4100-2
JH2-100
JH2-101
JH2-102
JH2-103
λb
λa
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20.16
11.60
8.45
7.77
24.67
21.36
5.23
4.69
4.22
3.31
1.92
1.77
1.53
1.20
0.96
0.91
0.53
B

TABLE 4. *Physical properties of the plasmids*

Plasmid	Phenotypic characters	Molecular size (kb)[a]
pIP800	Gen Kan Cm	70.1
pIP801	Em[c] Hly Bcn	53
pIP802		49
pIP803	(Tc?)[b]	2
pIP800-1	Cm	67.3

[a] Molecular size was estimated after agarose gel electrophoresis; the values are means from two determinations.

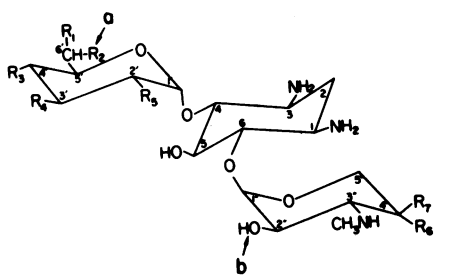
[b] The presence of the Tc R determinant on pIP803 is questionable (see the text).

3. This enabled us to assign at least part of the gene(s) determining gentamicin and kanamycin resistances to fragment 3. The low rate ($<6.5 \times 10^{-11}$) of reversion of strain JH2-103 to kanamycin or gentamicin high-level resistance is consistent with the existence of a deletion. The deleted sequence would possess sufficient coding capacity for the enzymes responsible for these resistances (cf. properties of the aminocyclitol-modifying enzymes).

Since plasmid pIP803 was present in the derivatives of BM4100 that are tetracycline resistant, but absent from all transcipients, which are tetracycline susceptible, it is not known whether the tetracycline resistance is encoded by this plasmid or by the BM4100 chromosome.

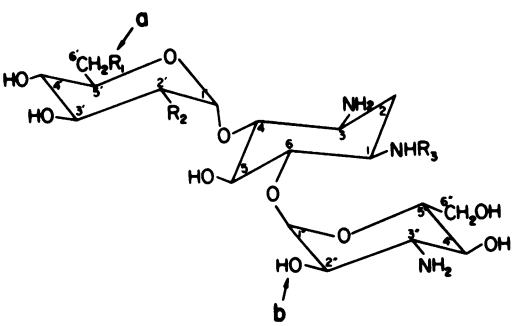
Mechanisms of plasmid-mediated resistance to aminocyclitol antibiotics in BM4100. Strain BM4100 and its derivatives and strain JH2-2 and transcipients (Table 1) were examined for aminocyclitol-modifying activities. All strains harboring plasmid pIP800 were found to contain aminocyclitol phosphotransferase (APH) and aminocyclitol acetyltransferase (AAC) but no adenylyltransferase (Table 1). No modifying activity was detected in the aminocyclitol-susceptible strain JH2-2. The structure of the gentamicins is shown in Fig. 2, that of the kanamycins is shown in Fig. 3, and that of the neomycins and lividomycins is shown in Fig. 4. The aminocyclitol substrate profiles of the enzymes extracted from strains BM4100 and JH2-102 are shown in Fig. 5. The fact that sisomicin is phosphorylated and 2″-deoxysisomycin is not indicates that the 2″-hydroxyl group is the site of phosphorylation (2″-APH). That kanamycin B is a substrate for acetylation and kanamycin C is not indicates that the 6′-amino group is the site of acetylation (6′-AAC). As expected, the substrate profiles of enzymes of the same type, extracted from *S. faecalis* BM4100 or JH2-102, are very similar. The MICs of a variety of aminocyclitol antibiotics for inhibition of strains BM4100 and JH2-102 (Table 2) are consistent with the substrate ranges of the modifying enzymes (Fig. 5).

In strains BM4100 and JH2-102 the phosphorylating and acetylating enzymes were expressed constitutively, as they were detected in cells grown in the absence of antibiotic. When these strains were grown in the presence of sisomicin, which is a substrate for 2″-APH and 6′-AAC (Fig. 5), no gross inactivation of the antibiotic in the medium was found by a microbiological assay.



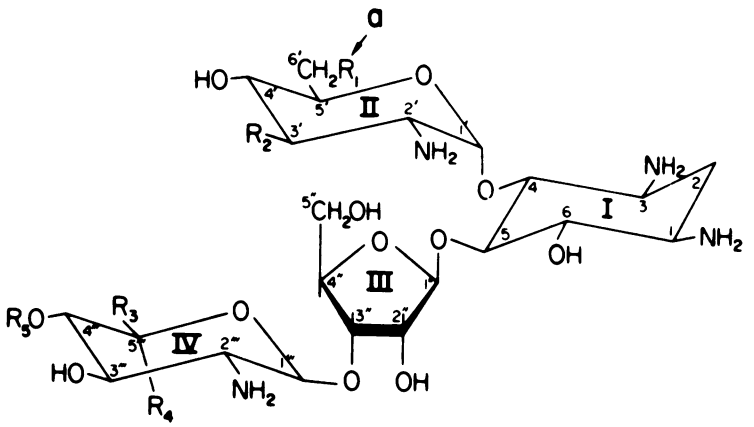
	R1	R2	R3	R4	R5	R6	R7
GENTAMICIN A	H	OH	OH	OH	NH_2	H	OH
B	H	NH_2	OH	OH	OH	OH	CH_3
C_{1A} ..	H	NH_2	H	H	NH_2	OH	CH_3
C_2 ..	CH_3	NH_2	H	H	NH_2	OH	CH_3
C_1 ..	CH_3	$NHCH_3$	H	H	NH_2	OH	CH_3

FIG. 2. *Structures of the gentamicins. Arrows indicate sites of N-acetylation (a) and O-phosphorylation (b). Sisomicin is 4′,5′-dehydrogentamicin C1a. Netilmicin is 1-N-ethylsisomicin.*



	R1	R2	R3
KANAMYCIN A ...	NH_2	OH	H
B ...	NH_2	NH_2	H
C ...	OH	NH_2	H
AMIKACIN	NH_2	OH	-CO-CH(OH)CH_2-CH_2NH_2

FIG. 3. *Structures of the kanamycins and related compounds. Arrows indicate sites of N-acetylation (a) and O-phosphorylation (b). Tobramycin is 3′-deoxykanamycin B.*



	R1	R2	R3	R4	R5
NEOMYCIN B ...	NH_2	OH	H	CH_2NH_2	H
C ...	NH_2	OH	CH_2NH_2	H	H
PAROMOMYCIN ..	OH	OH	H	CH_2NH_2	H
			CH_2NH_2	H	
LIVIDOMYCIN A	OH	H	H	CH_2NH_2	MANNOSE
B	OH	H	H	CH_2NH_2	H

FIG. 4. *Structures of the neomycins and lividomycins. Arrow indicates site of N-acetylation (a). Neomycin A lacks rings III and IV, ribostamycin lacks ring IV of neomycin B, and butirosin is 1-N hydroxyaminobutyryl-ribostamycin.*

Properties of the aminocyclitol-modifying enzymes. The pH optimum (Fig. 6) for the phosphorylation of gentamicin C1a was between 7.5 and 8, depending upon the buffer used for the enzyme assay, and it was 5.5 for the acetylation of the same compound.

The two enzyme activities, 2″-APH and 6′-AAC, could not be separated. They were eluted in one peak after gel filtration, and their apparent molecular weight was 31,000. In several instances, but not consistently, a small amount of 2″-APH activity was found to precede the peak of 2″-APH and 6′-AAC activities (Fig. 7). After isoelectric focusing in polyacrylamide gels the same isoelectric point, 5.3, was estimated for both enzyme activities (Fig. 8).

DISCUSSION

S. faecalis subsp. *zymogenes* strain BM4100 was isolated from a clinical specimen. This strain is resistant to gentamicin, kanamycin, chloramphenicol, erythromycin, and tetracycline and codes for hemolysin production and for bacteriocin production and immunity. These characters, with the possible exception of tetracycline resistance, are carried by transferable plasmids, as is evident from their instability and the correlation of their loss or acquisition with the absence or presence of plasmid DNA.

Plasmid-mediated high-level resistance to aminocyclitols in group D streptococci has been described (12, 21, 23, 29). The resistance to streptomycin was mediated by an adenylyltransferase, and resistance to kanamycin was mediated by a 3′,5″-phosphotransferase (3′-APH, type III) (12, 23, 29). High-level resistance to gentamicin is novel in streptococci, and so is the occurrence of 2″-APH and of 6′-AAC in this genus.

Enzymes with identical site specificity have been described, 2″-APHs and 6′-AACs in staphylococci (7, 14, 20, 24) and 6′-AACs in gram-negative bacteria (13). The substrate profiles of the respective enzymes, 2″-APH and 6′-AAC, from streptococci (Fig. 5) and staphylococci (7, 20, 24) are very similar. However, the substrate profiles of the 6′-AAC from these gram-positive

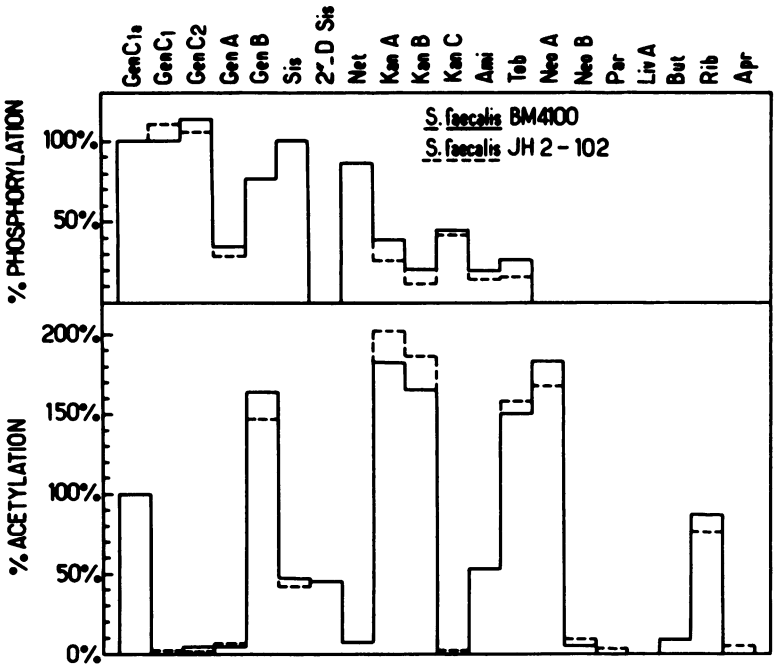


FIG. 5. *Substrate profiles of enzymes extracted from S. faecalis strains BM4100 (solid lines) and JH2-102 (dashed lines). Phosphorylation and acetylation are expressed relative to gentamicin C1a as 100%.*

cocci differ from those of the corresponding enzymes in gram-negative bacteria (20, 24). The 6'-AACs from *S. faecalis* and *S. aureus* modify neamine much more efficiently than the structurally related compound neomycin B. They also acetylate gentamicin C1a more efficiently than sisomicin and much more so than netilmicin. By contrast, these three compounds are equally good substrates for the corresponding enzymes from *Escherichia coli* or *Pseudomonas aeruginosa* (13). Comparable differences between the 3'-APH enzymes of gram-positive (6, 23, 29; P. Courvalin et al., *in* Mitsuhashi et al., ed., *Medical and Biological Aspects of Resistant Strains,* in press) and gram-negative (13) organisms have been observed. The apparent confinement of 2"-APH to streptococci and staphylococci and the fact that the 6'-AAC enzymes exist in a remarkable variety in gram-negative bacteria (13), but not so in gram-positive cocci, may be another reflection of the similarities among the enzymes observed within the gram-positive cocci and the distinction from those of the gram-negative organisms.

The 2"-APH and 6'-AAC activities in *S. aureus* strains Palm and BM4600 could not be separated by using the same techniques as in *S. faecalis* BM4100 (Courvalin et al., in press). Furthermore, the apparent molecular weight (31,000) and isoelectric point (5.3) of the two enzymes in these strains were identical. Le Goffic et al. (24) had reported a molecular weight of 28,000 and an isoelectric point of 5.7 or 5.8 for the two enzymes in *S. aureus* Palm. Dowding (14), studying *S. aureus* C1, separated a 2"-APH from a 6'-AAC electrophoretically. It can therefore be assumed that at least one of the enzymes in strain C1 is different from those described in Fig. 7 and 8. We conclude that the 2"-APH and the 6'-AAC from *S. faecalis* BM4100 and from *S. aureus* strains Palm and BM4600 are very similar. It remains to be determined whether the model of one polyfunctional enzyme proposed for the two staphylococcal activities (25) holds true for their streptococcal equivalents.

The pH optima for the activity of 6'-AAC and 2"-APH from *S. faecalis* BM4100 were 5.5 and 8, respectively (Fig. 6). These differ from the optima obtained for the corresponding activities of *S. aureus*, which were approximately 7 for the 6'-AAC activity and not distinct for the 2"-APH activity (Courvalin et al., in press). It seems, then, that the 6'-AACs, as well as the 2"-APHs, from *S. faecalis* and *S. aureus* are not identical.

The striking similarity of the substrate profile, molecular weight, and isoelectric point of the streptococcal and staphylococcal 2"-APH and 6'-AAC activities could reflect a structural homology analogous to that between the *S. faecalis* and *S. aureus* 3'-APH enzymes. This ho-

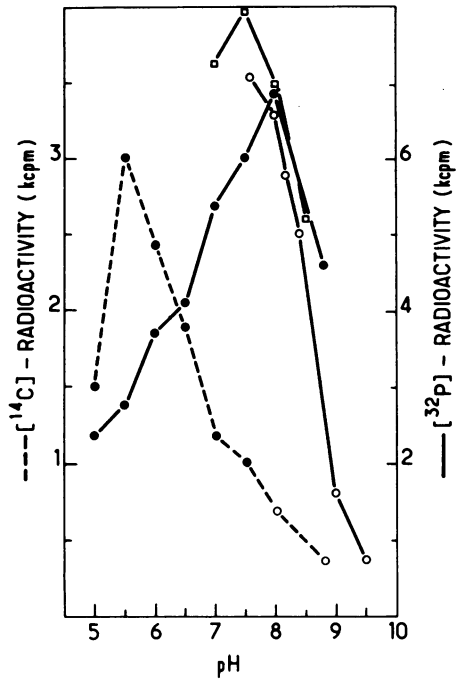


FIG. 6. *pH optimum for aminocylitol-phosphorylating and -acetylating activities of S. faecalis JH2-102. The reaction was carried out in the presence of gentamicin C1a and [γ-^{32}P]ATP or [^{14}C]acetylcoenzyme A. The buffers were: 60 mM Tris-60 mM maleic acid-45 mM $MgCl_2$-450 mM NH_4Cl-1.5 mM dithiothreitol, adjusted with NaOH (●); 150 mM glycine-45 mM $MgCl_2$-450 mM NH_4Cl-1.5 mM dithiothreitol, adjusted with NaOH (○); 50 mM Tris-20 mM $MgCl_2$-100 mM NH_4Cl-1.5 mM dithiothreitol, adjusted with acetic acid (□).*

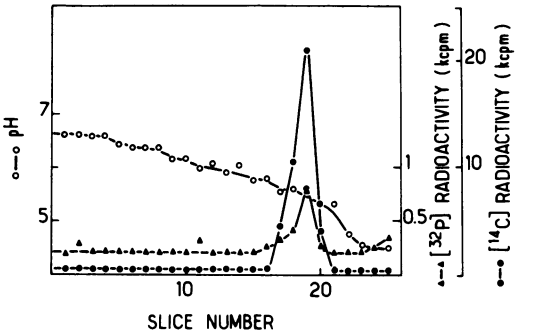


FIG. 8. *Isoelectric focusing of a crude enzyme preparation from strain JH2-102. Isoelectric focusing was of 30 μl of an S100 preparation in 5% polyacrylamide gels (cylinders, 0.3 by 10 cm) containing 2% ampholines (pH range, 3.5 to 8) for 16 h at 400 V and for 1 h at 800 V. The gels were sliced as indicated, and every slice was cut in two. The determination in every slice was either of acetylation and pH or of acetylation and phosphorylation. The enzyme activity was determined (19) using gentamicin C1a as substrate and [γ-^{32}P]ATP or [^{14}C]acetylcoenzyme A as cofactor after incubation (3 h at 4°C) of every half-slice in 40 μl of the following buffer: 20 mM Tris, 20 mM $MgCl_2$, 0.3 M NH_4Cl, 12 mM β-mercaptoethanol, adjusted to pH 7.5 with acetic acid.*

mology had been revealed by cross-immunoelectrophoresis (T. J. White, D. I. Smith, S. Rosenthal, and J. Davies, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no. 287, 1978) and by DNA-DNA hybridization experiments (Courvalin et al., in press) of the plasmid genes. No homology was found between either enzymes (White et al.,

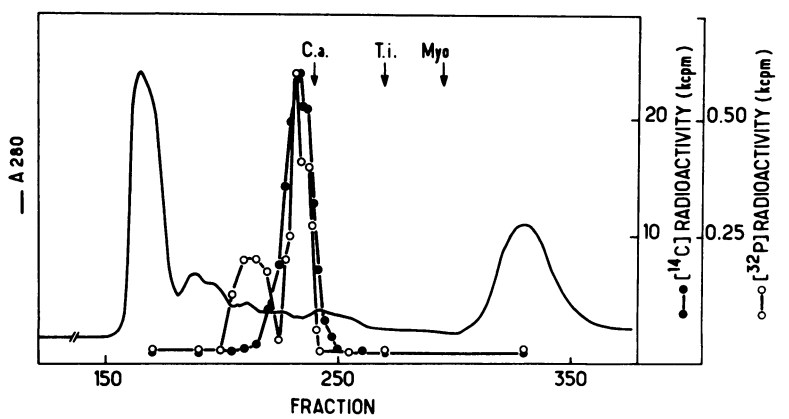


FIG. 7. *Gel filtration of a crude enzyme preparation from strain JH2-102. To 1 ml of an S100 preparation, 70 mg of solid NH_4Cl and 10 μl of β-mercaptoethanol were added. The mixture was filtered through a column (1.5 by 190 cm) of Sephadex G-100 superfine equilibrated with buffer as follows: 50 mM Tris, 20 mM $MgCl_2$, 1 M NH_4Cl, and 12 mM β-mercaptoethanol, adjusted to pH 7.5 with acetic acid. The flow rate was 3 to 4 ml/h, and 0.6-ml fractions were collected. The determination of the enzyme activity was as described (19) using gentamicin C1a as substrate and [γ-^{32}P]ATP or [^{14}C]acetylcoenzyme A as cofactor. The molecular weight markers used were: carbonic anhydrase (C.a.), 30,000; trypsin inhibitor (T.i.), 21,500; myoglobin (Myo), 17,200.*

18th ICAAC, abstr. no. 287) or genes (8; Courvalin et al., in press) from gram-positive and gram-negative bacteria. These findings are in favor of a common origin of R determinants in streptococci and staphylococci.

Despite the differences between the modifying enzymes from gram-positive and gram-negative organisms, there are features common to bacterial resistance towards aminocyclitols. The enzymes are synthesized constitutively, and there is no gross inactivation of the antibiotics in the culture medium (6, 12; Courvalin et al. in press; see Results).

S. faecalis BM4100 is resistant to macrolide, lincosamide, and streptogramin B-type antibiotics (Table 3). This resistance phenotype has been described in clinical isolates of *S. aureus* (4) and streptococci (10). N^6-Dimethylation of adenine in 23S rRNA has been identified as the chemical modification responsible for the resistance in these two bacterial genera (35). Nucleotide sequence homology between *S. aureus* and several streptococcal genes encoding macrolide, lincosamide, and streptogramin B-type resistance has been recently demonstrated (36). These observations were also interpreted in favor of a common origin of R determinants in streptococci and staphylococci (36).

S. faecalis BM4100 is also resistant to chloramphenicol (Table 3). Plasmid-linked high-level chloramphenicol resistance (MIC > 50 μg/ml) in streptococci (12), staphylococci (17), and gram-negative bacteria (18) is usually mediated by a chloramphenicol acetyltransferase. Again, in this resistance system, it was shown that the streptococcal enzymes resemble more closely the "family" of chloramphenicol acetyltransferase variants found in staphylococci rather than those in *E. coli* and other enteric bacteria (17).

Obviously, then, there exist common aspects within streptococcal and staphylococcal resistance towards aminocyclitols, chloramphenicol, and macrolide-lincosamide-streptogramin B-type antibiotics which have been implied, in one case (36), to support the notion of facile exchange of R determinants between these two bacterial genera. This is in fact a possibility, since there is recent material proof of direct plasmid transfer between streptococci and staphylococci (H. W. B. Engel et al., submitted for publication).

Serious group D streptococcal infections in humans (e.g., endocarditis) are treated with penicillin and aminocyclitol (22). Group D streptococci resistant to high levels of streptomycin or kanamycin or both are refractory to synergism of penicillin with these aminocyclitols (30). In addition to being resistant to kanamycin, strain BM4100 is highly resistant to gentamicin. This strain is also resistant to the combination of penicillin and gentamicin or related compounds (sisomicin, netilmicin; unpublished data). Considering the range of aminocyclitol-modifying enzymes detected now in streptococci and the existence of their genes on self-transferable plasmids, we anticipate the emergence of strains refractory to synergism of all possible combinations of β-lactam and aminocyclitol antibiotics.

ACKNOWLEDGMENTS

We thank E. Derlot for the determination of MICs, O. Rouelland for secretarial assistance, and Y. A. Chabbert for material support. The electron microscopy was carried out in the Laboratoire de Microscopie Electronique, Département de Virologie, and the photography was performed by P. Lemoine in the Service Photographique, Institut Pasteur.

This work was supported by a grant (C.R.L. 78.4.133.1) from the Institut National de la Santé et de la Recherche Médicale (INSERM); E.C. was Chargé de Recherche, INSERM.

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