Plasmid-Mediated Mechanisms of Resistance to Aminoglycoside-Aminocyclitol Antibiotics and to Chloramphenicol in Group D Streptococci

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Genes conferring resistance to aminoglycoside-aminocyclitol antibiotics in three group D streptococcal strains, *Streptococcus faecalis* JH1 and JH6 and *S. faecium* JH7, and to chloramphenicol in JH6 are carried by plasmids that can transfer to other *S. faecalis* cells. The aminoglycoside resistance is mediated by constitutively synthesized phosphotransferase enzymes that have substrate profiles very similar to those of aminoglycoside phosphotransferases found in gram-negative bacteria. Phosphorylation probably occurs at the aminoglycoside 3'-hydroxyl group. Plasmid-borne streptomycin resistance is due to production of the enzyme streptomycin adenyltransferase, which, as in staphylococci and in contrast to that detected in gram-negative bacteria, is less effective against spectinomycin as substrate. Resistance to chloramphenicol is by enzymatic acetylation. The chloramphenicol acetyltransferase is inducible and bears a close resemblance to the type D chloramphenicol acetyltransferase variant from staphylococci.

In recent years it has become clear that many of the genes conferring resistance to antibiotics in group D streptococci are plasmid borne (4, 5, 13). The plasmids can transfer between group D streptococcal strains; some plasmids are conjugative (13), and nonconjugative plasmids can be mobilized by conjugative plasmids (9).

In gram-negative bacteria and *Staphylococcus* the mechanism of plasmid-mediated resistance involves the production of enzymes that inactivate the antibiotic by either modification or destruction of the antibiotic structure (8). This is in contrast to chromosomally determined antibiotic resistance, which is more usually due either to prevention of entry of the antibiotic into the cell or to a specific alteration of the antibiotic target site (8). In this report it is shown that, like the plasmid-borne resistance in the gram-negative bacteria and staphylococci, plasmid-linked resistances to aminoglycoside-aminocyclitol antibiotics and chloramphenicol in group D streptococci are also mediated by enzymatic modification of the antibiotic. The properties of the *Streptococcus faecalis* enzymes are compared with the similar enzymes of staphylococci and the gram-negative bacteria.

METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. Each wild strain was isolated from a separate patient in Hammersmith Hospital and characterized by biochemical and serological tests (12, 13). Derivative strains were isolated by selection of spontaneous mutants (13).

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

MIC determinations. The method of Steers et al. (18) was used to determine antibiotic minimal inhibitory concentrations (MICs).

Radiolabeling, isolation, and characterization of plasmid DNA. Plasmid DNA was isolated by cesium chloride-ethidium bromide density gradient ultracentrifugation and characterized by neutral sucrose gradient analysis as described previously (12, 13).

Calculation of plasmid molecular weight. The empirical formulae of Barth and Grinter (1) were used to calculate plasmid molecular weights.

Transfer of antibiotic resistance traits. The technique described by Jacob and Hobbs (13) was used in transferring antibiotic resistance traits.

Enzyme assays. (i) **Assays of aminoglycoside phosphorylation and streptomycin adenylation.** The extracts for assay were prepared as follows: 100 ml of an exponential culture in broth was harvested and washed once in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.2)-1 mM ethylenediaminetetraacetic acid, then resuspended in 1 ml

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of 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.2)-10 mM $MgCl_2$ -25 mM NH_4Cl -0.6 mM β -mercaptoethanol. The cells were treated for 60 min at 37°C with 500 μ g of lysozyme (Sigma) per ml and then subjected to five 60-s bursts from a sonicator at 70W output. The resulting suspension was centrifuged at $105,000 \times g$ for 45 min, and the supernatant was removed and used in assays performed as described previously (11).

(ii) **Assays of chloramphenicol acetylation.** The techniques employed for the detection of [^{14}C]-chloramphenicol acetylation by bacterial cell suspensions have been described previously (16). Chloramphenicol acetyltransferase activity in cell-free extracts was determined by the spectrophotometric method of Shaw and Brodsky (17). The induction of chloramphenicol acetyltransferase by the parent antibiotic and by gratuitous inducers has been described (21). In the present experiments, *S. faecalis* strains were grown for 18 h in brain heart infusion broth at 37°C without shaking. The overnight cultures were then diluted with 4 volumes of fresh medium, after which they were

allowed to grow for an additional 4 h under the same conditions, before addition of inducers at the concentrations specified. Cells were collected by centrifugation after 2 h of further incubation and were suspended for lysis by sonic disruption as previously described (16). The immunological techniques for the characterization of chloramphenicol acetyltransferase employed both agar diffusion tests with and neutralization by antisera prepared against chloramphenicol acetyltransferase from *Staphylococcus aureus* or *Escherichia coli* (17, 21). Controls run in each instance with pre-immunization sera failed to yield either precipitation lines or inactivation of enzyme. The techniques used for polyacrylamide gel electrophoresis and for the localization of enzyme activity on cylindrical gels have been reported previously (17).

Antibiotics. The antibiotics were provided by the following laboratories: neomycin B and spectinomycin, Upjohn; kanamycins A, B, and C and amikacin, Bristol; paromomycin, butirosin, and chloramphenicol, Parke-Davis; lividomycin A, Kowa; ribostamycin, Meiji; tobramycin, Lilly; streptomycin, Pfizer; erythromycin, Abbott; fusidic acid, Leo; tetracycline, Lederle; rifampin, Ciba-Geigy.

TABLE 1. *Bacterial strains used*

Strain	Relevant phenotype ^a	Reference
<i>S. faecalis</i>		
JH1	Wild strain, Kan, Str, Em, Tc	13
JH2	Wild strain	12
JH2-7	JH2 derivative, Thy ⁻ , Fus, Rif	13
JH6	Wild strain, Kan, Str, Cm, Tc, Em	
<i>S. faecium</i>		
JH7	Wild strain, Kan, Str, Em	

^a Abbreviations: Kan, kanamycin; Str, streptomycin; Em, erythromycin; Tc, tetracycline; Fus, fusidic acid; Rif, Rifampin; Thy, thymine.

RESULTS

Location of the genes determining antibiotic resistance in *S. faecalis* JH1 and JH6 and *S. faecium* JH7. The genes determining resistance to kanamycin (Kan), streptomycin (Str), erythromycin (Em), and tetracycline (Tc) in strain JH1 have previously been shown to be carried by a conjugative plasmid, pJH1 (13). The MICs of these antibiotics for inhibition of strain JH1 and of a recipient strain, JH2-7, that has received plasmid pJH1 (strain JH2-15) are shown in Table 2.

The genes determining resistance to chlor-

TABLE 2. *MIC of antibiotics for inhibition of group D streptococci*

Strain	MIC of antibiotics (μ g/ml) ^a							
	Resistances determined by wild strains					Introduced chromosomal resistances ^b		
	Kan	Str	Cm	Tc	Em	Rif	Fus	Str
JH1	32,768	8,192	4	64	8,192	1	<4	
JH2	256	256	4	<0.25	0.25	4	<4	
JH2-7	128	256	4	<0.5	0.25	2,048	256	
JH2-15	>65,536	16,384	4	8	8,192	2,048	256	
JH6	65,536	>65,536	64	128	4,096	4	<4	
JH7	32,768	4,096	2	<0.5	8,192	<0.125	<4	
JH6-1	512	512	2	32	0.5	4	<4	
JH7-2	128	64	2	<0.5	1	0.5	<4	
JH2-12	>65,536	65,536	64	8	4,096	1,024	512	
JH2-14	>65,536	>65,536	4	0.5	8,192	2,048	256	
JH2-24	128	128	2	0.5	0.125	1,024	1,024	
JH2-22	256	512	NT	NT	0.5	NT	NT	
JH2-18	256		4	<0.25	0.125	2,048	<4	8,192

^a Abbreviations are as listed in Table 1, footnote a. NT, Not tested.

^b Strains with these resistances are derivatives of strain JH2, into which the resistances were introduced by the sequential selection of a spontaneous mutant.

amphenicol (Cm), Kan, Str, Em, and Tc in strain JH6 and to Kan, Str, and Em in strain JH7 are also plasmid borne. These resistances are spontaneously lost en bloc from cells of each strain after growth in broth; approximately 0.75% (6/789) and approximately 0.25% (2/832) of JH6 and JH7 clones, respectively, had lost the antibiotic resistances. The MICs of antibiotics against the parental strains, JH6 and JH7, and one antibiotic-susceptible variant of each (strains JH6-1 and JH7-2, respectively) are shown in Table 2. Neither strain JH6-1 nor JH7-2 had a high rate of reversion to antibiotic resistance: reversion was $<9.6 \times 10^{-11}$ per bacterium to Kan, 2 mg/ml, for both strains; 9.1×10^{-10} per bacterium to Str, 2 mg/ml, for strain JH6-1; and $<9.1 \times 10^{-11}$ and 1.1×10^{-7} per bacterium to Em, 1 mg/ml, for strains JH6-1 and JH7-2, respectively. Clones that reverted to an antibiotic resistance regained neither the level of resistance of the parental strain nor resistance to other antibiotics.

The genes conferring resistance to Kan, Str, Cm, Em, and Tc in strain JH6 and to Kan, Str, and Em in strain JH7 are transferable en bloc from each parental strain to a derivative of the antibiotic-sensitive strain JH2, strain JH2-7, during mixed incubation in broth. Selection for transfer of any one of the above resistances resulted in the cotransfer of all resistances (except that Tc resistance of strain JH6 was occasionally not cotransferred). The MICs of the antibiotics against strains JH2 and JH2-7 and recipients that had received all the resistances from JH6 (strain JH2-12) or from JH7 (strain JH2-14) are shown in Table 2.

Strains JH2 and JH2-7 are plasmid-free (12, 13). By using cesium chloride-ethidium bromide ultracentrifugation, both strains JH2-12 and JH2-14 were shown to contain covalently closed circular DNA (data not shown). Neutral sucrose gradient analysis of the covalently closed circular DNAs revealed the presence of a single plasmid species in strain JH2-14 and two in strain JH2-12. These analyses, in which differentially radiolabeled plasmid pJH2 DNA (12) was used as the internal molecular-weight standard, are shown in Fig. 1. Figure 1A shows the covalently closed circular DNA tertiary forms of the ^3H -labeled plasmid from strain JH2-14 (designated pJH4) and of ^{14}C -labeled pJH2 with peaks at fractions 17 and 10, respectively, and the open circular forms of these plasmids, formed by spontaneous single-strand scission, at fractions 27 and 23, respectively. Figure 1B illustrates the ^3H -labeled covalently closed circular DNA forms of the larger and smaller plasmid species of strain JH2-12 at fractions 17 and 24, respec-

tively, and that of ^{14}C -labeled pJH2 at fraction 24. The corresponding open circular forms have peaks at fractions 29, 32, and 32, respectively. By using the molecular-weight value of 42×10^6 for plasmid pJH2 (revised value from that given in reference 12), the molecular weight of plasmid pJH4 was calculated to be 26×10^6 , that of the larger strain JH2-12 plasmid to be 76×10^6 (designated pJH5), and that of the smaller strain JH2-12 plasmid to be 41×10^6 (pJH6).

The transferable antibiotic resistances were also lost spontaneously en bloc from strains JH2-12 and JH2-14. No plasmid DNA could be found in either of two antibiotic-susceptible variants of strain JH2-14 (strains JH2-22 and JH2-23), and pJH5 of strain JH2-12 had been lost from the antibiotic-susceptible variant strain JH2-24. The antibiotics MICs for strains JH2-22 and JH2-24 are shown in Table 2.

The transferable antibiotic resistances could also transfer from the primary recipient strains JH2-12 and JH2-14 to other group D streptococcal strains after mixed incubation in broth.

We conclude that the genes conferring resistance to the antibiotics Kan, Str, Cm, Em, and Tc in strain JH6 are linked to the transferable plasmid pJH5 and that the genes conferring resistance to Kan, Str, and Em in strain JH7 are borne by the transferable plasmid pJH4.

Isolation of a streptomycin-resistant variant of *S. faecalis* JH2. A streptomycin-resistant mutant of strain JH2 was selected by growth of an overnight culture in broth containing 1 mg of streptomycin per ml. Since strain JH2 is plasmid free (12), the gene(s) conferring resistance to streptomycin is presumed to be located on the chromosome. The MICs of antibiotics against this strain, designated strain JH2-18 (which also has the additional phenotypes of resistance to rifampin and growth requirement for thymine), are shown in Table 2.

Mechanisms of plasmid-mediated resistance to aminoglycoside-aminocyclitol antibiotics. Strain JH2-7 and its derivatives that carry plasmids pJH1 (strain JH2-15), pJH4 (strain JH2-14), and pJH5 (strain JH2-12) were examined. The latter three strains were found to contain both aminoglycoside phosphotransferase and streptomycin adenylyltransferase activities. The aminoglycoside-aminocyclitol substrate profiles of enzymes extracted from these strains are shown in Fig. 2.

The fact that both butirosin and lividomycin A were phosphorylated indicates that the enzymes are type III aminoglycoside phosphotransferases, although the possibility that two enzymes (aminoglycoside phosphotransferase types I and II) are present (20) cannot be elimi-

FIG. 1. Neutral sucrose gradient analysis of plasmids isolated from *S. faecalis* JH2-12 and JH2-14. ^3H -labeled plasmid DNA was mixed with ^{14}C -labeled plasmid pJH2 DNA, layered onto a 4.6-ml 5 to 20% sucrose gradient, and centrifuged at 33,000 rpm and 20°C in an MSE 65 swinging bucket rotor (6 by 5.5 ml). Fractions (0.1 ml) were collected onto glass-fiber disks and, after being dried, washed, and redried, were assayed for radioactivity. Sedimentation was from right to left. (A) ^3H -labeled plasmid DNA from strain JH2-14 plus ^{14}C -labeled pJH2 DNA, sedimentation for 110 min. (B) ^3H -labeled plasmid DNA from strain JH2-12 plus ^{14}C -labeled pJH2 DNA, sedimentation for 70 min. $\bullet$, ^3H ; $\circ$, ^{14}C .

FIG. 2. Substrate profiles of enzymes extracted from *S. faecalis* strains JH2-12, JH2-14, and JH2-15. Phosphorylation (plain area) or adenylation (hatched area) are expressed relative to neomycin B or streptomycin as 100%, respectively. The abbreviations are as in Table 3, footnote a.

nated. Since kanamycin A is modified but tobramycin is not, it is inferred that the 3'-hydroxyl group is the site of phosphorylation.

In addition, crude extracts of these strains were found to contain a streptomycin-adenylylating enzyme that was much less effective against spectinomycin. Phosphorylation of streptomycin was not detected.

In all strains studied, the phosphorylating and adenylylating enzymes were expressed constitutively, since activity was detected in cells grown in the presence or absence of antibiotic.

The MICs of a variety of aminoglycoside-aminocyclitol antibiotics for inhibition of strain

JH2-7 and its three plasmid-carrying derivatives are shown in Table 3. These are consistent with the substrate ranges of the modifying enzymes.

Strain JH2-18, which has a chromosomally determined streptomycin resistance, showed no detectable streptomycin adenylyltransferase or phosphotransferase enzyme activities.

Biochemical basis of chloramphenicol resistance in *S. faecalis* JH6. Preliminary experiments revealed that the growth of *S. faecalis* JH6 in the presence of chloramphenicol resulted in the inactivation of the antibiotic as measured by the absence of growth-inhibiting activity from culture filtrates (data not shown). Since

the chloramphenicol inactivation phenotype determined by plasmid-linked genes is often associated with the presence of the enzyme chloramphenicol acetyltransferase, an experiment was performed to determine the fate of chloramphenicol when added to cultures of *S. faecalis* JH6 and the susceptible derivative strain JH6-1. Figure 3 depicts the conversion of [14 C]chloramphenicol to its corresponding 1-acetoxy and 3-acetoxy esters by a culture of strain JH6, but not a JH6-1 culture, following a 20-min incubation of exponentially growing cultures of broth strains with a subinhibitory concentration (2.5 μ g/ml) of the antibiotic. The radioactive products were shown to be chromatographically identical with the mono-acetates produced by R factor-bearing strains of chloramphenicol-resistant *E. coli* known to contain the acetylating enzyme.

Chloramphenicol acetyltransferase activity was assayed directly in cell-free extracts prepared from strains JH6 and JH6-1, with the results shown in Table 4. The extracts prepared from the susceptible strain (JH6-1) had no detectable chloramphenicol-acetylating activity, whereas the extracts from the resistant parent contained amounts of enzyme that were a function of the growth conditions employed. Low levels of chloramphenicol acetyltransferase were noted when strain JH6 was inoculated in the absence of antibiotic, whereas substantial levels were noted after exposure of strain JH6 to chloramphenicol or to an analog (3-deoxychloramphenicol) that is an effective gratuitous inducer of chloramphenicol acetyltransferase synthesis in staphylococci (21). The response of *S. faecalis* JH6 is similar to that observed with chloramphenicol-resistant staphylococci in that enzyme synthesis is dependent on the concentration of chloramphenicol or its 3-deoxy analog (which is neither a substrate for the enzyme nor an inhibitor of protein synthesis).

To gain some insight into the evolutionary relationship of the plasmid-linked chloramphenicol acetyltransferase of *S. faecalis* strain JH6 to

analogous systems it was necessary to use the techniques of polyacrylamide gel electrophoresis and enzyme neutralization by specific antibody. Since four electrophoretically distinct types of chloramphenicol acetyltransferase have been described in staphylococci, it was of interest to compare the strain JH6 enzyme with a representative of each of the known *S. aureus* variants (15). Figure 4 shows the results of such a comparison. Since the type D staphylococcal enzyme and that from strain JH6 had not been purified, the detection method in each instance was the specific histochemical stain for the presence of chloramphenicol acetylation (15, 17). The presence of enzyme in each band so located was confirmed by slicing unstained gels run in parallel and measuring the enzyme directly following elution (after freezing and thawing) from each 2-mm segment. Both types of experiment revealed that the electrophoretic mobility of the enzyme from *S. faecalis* JH6 is identical with that of the type D staphylococcal variant. Analogous experiments were performed (data not shown) comparing the *S. faecalis* enzyme with the three known variants of chloramphenicol acetyltransferase mediated by R factors in gram-negative bacteria (10) and with similar enzymes from seven other sources (Y. Zaidenzaig and W. V. Shaw, Eur. J. Biochem., in press). The electrophoretic mobility of the *S. faecalis* JH6 enzyme was distinct from all known variants save for the staphylococcal D type.

All four known types of chloramphenicol acetyltransferase from staphylococci cross-react immunologically when tested by the precipitin reaction or by neutralization (15). There is, however, no cross-reaction of the *E. coli* R factor-mediated variants of chloramphenicol acetyltransferase with those from staphylococci. Crude extracts of fully induced *S. faecalis* JH6 were analyzed with both a goat antiserum prepared against the type I (*E. coli*) R factor variant (10) and rabbit antibody raised against purified staphylococcal enzyme (15). The streptococcal enzyme gave a reaction of identity (no spur

TABLE 3. MIC of aminoglycoside-aminocyclitol antibiotics for inhibition of *S. faecalis* JH2-7 and plasmid-carrying derivatives

Strain	MIC of antibiotics (μ g/ml) ^a :											
	Neo B	Par	Liv A	But	Rib	Kan A	Kan B	Kan C	Ami	Tob	Str	Spc
JH2-7	256	512	1,024	256	256	128	128	256	512	32	128	64
JH2-12	<4,096	<8,192	<8,192	4,096	<4,096	<4,096	<4,096	<1,024	1,024	32	8,192	64
JH2-14	<4,096	<8,192	<8,192	4,096	<4,096	<4,096	<4,096	<1,024	512	32	8,192	64
JH2-15	<4,096	<8,192	<8,192	4,096	<4,096	<4,096	<4,096	<1,024	1,024	32	8,192	64

^a Abbreviations: Neo B, neomycin B; Par, paromomycin; Liv A, lividomycin A; But, butirosin; Rib, ribostamycin; Kan A, kanamycin A; Kan B, kanamycin B; Kan C, kanamycin C; Ami, amikacin; Tob, tobramycin; Str, streptomycin; Spc, spectinomycin. MICs were determined using Mueller-Hinton agar medium.

FIG. 3. Autoradiographic demonstration of [^{14}C]-chloramphenicol acetylation by *S. faecalis* JH6. The results on the right (II) were obtained with the chloramphenicol-susceptible derivative JH6-1. Thin-layer chromatography was performed on silica-gel using chloroform-methanol (97.5:2.5 by vol.) as described elsewhere (16). Radioactive chloramphenicol was the *N*-dichloroacetamido 1- ^{14}C -labeled product of the Radiochemical Centre, Amersham, England, and was used at a final specific activity of 1 mCi/mmol. The origin (Ori) of application of the ethylacetate extracts of the culture supernatant is indicated, as are the location of chloramphenicol (CM) and the 1-acetyl (1-Ac) and 3-acetyl (3-Ac) derivatives.

formation) with all *S. aureus* variants when tested by double diffusion in agar against the homologous antiserum. No precipitin lines were observed when the antiserum specific for the R factor (*E. coli*) variant of chloramphenicol acetyltransferase was tested against the enzyme from strain JH6 or from staphylococci (data not shown). The immunological cross-reactivity of the *S. faecalis* enzyme with the type D variant from *S. aureus* is shown in Fig. 5. The neutralization curves are similar but not identical, whereas the R factor variant (type I) of chloramphenicol acetyltransferase is unaffected by the antiserum. The reciprocal experiment with antibody against the latter shows inactivation of the homologous enzyme but not neutralization of the strain JH6 or type D (*S. aureus*) enzyme.

DISCUSSION

S. faecalis JH1 and JH6 and *S. faecium* JH7 were isolated from separate clinical specimens. Genes determining resistance to aminoglycoside-aminocyclitol antibiotics in these strains and to chloramphenicol in strain JH6 are borne by transferable plasmids. Strain JH1 has previously been shown to contain a conjugative plasmid, pJH1, that carries the genes for aminoglycoside-aminocyclitol, Tc, and Em resistances (13). The evidence that in strains JH6 and JH7 the antibiotic resistance genes are plasmid linked is the spontaneous instability of these traits and the correlation between their loss and acquisition and the absence and presence of plasmid DNA. Plasmid pJH4, from *S. faecium* JH7, has a molecular weight of 26×10^6 and codes for erythromycin and aminoglycoside-aminocyclitol antibiotic resistances. Plasmid pJH5 determines resistance to Cm, aminoglycoside-aminocyclitol antibiotics, Em, and Tc and has a molecular weight of 76×10^6 . Like plasmid pJH1, plasmids pJH4 and pJH5 probably transfer by a conjugal mechanism (A. E. Jacob, unpublished data).

Aminoglycoside phosphotransferase and streptomycin adenyltransferase activities have

TABLE 4. Induction of chloramphenicol acetyltransferase synthesis in *S. faecalis* JH6 by chloramphenicol and 3-deoxychloramphenicol

Addition to medium ^a	Chloramphenicol acetyltransferase (U/mg of protein)	
	<i>S. faecalis</i> JH6	<i>S. faecalis</i> JH6-1
None	0.01	<0.005
Chloramphenicol (10 $\mu\text{g}/\text{ml}$)	0.20	<0.005
Chloramphenicol (25 $\mu\text{g}/\text{ml}$)	0.41	<0.005
3-Deoxychloramphenicol (15 $\mu\text{g}/\text{ml}$)	0.63	<0.005

^a Both compounds were of the D, threo configuration. Induction was performed for 2 h on exponentially growing cells as described in the text.

been detected in the three *S. faecalis* strains, JH2-15, JH2-12, and JH2-14, that have received plasmids from strains JH1, JH6, and JH7, respectively. Since the antibiotic-susceptible recipient strain *S. faecalis* JH2-7 showed neither

FIG. 4. Electrophoretic mobility of *S. faecalis* JH6 chloramphenicol acetyltransferase. Results are presented as a tracing of the actual gels stained either for protein by Coomassie blue (A through D) or for enzyme activity (D' and S) as described previously (16, 17). The faint and variable formazan deposit formed enzymatically is readily visible on the actual gels but is virtually impossible to detect in printed illustrations made from photographs. Tubes A through D contained, respectively, the purified variants of chloramphenicol acetyltransferase for *S. aureus*. Tubes D' and S contained, respectively, crude extracts of the type D enzyme for *S. aureus* and the *S. faecalis* JH6 extract. The protein marker (M) was human hemoglobin A. Migration was downward towards the anode at pH 9 as previously described (15, 17, 21). DF indicates the position of the dye front.

FIG. 5. Neutralization of chloramphenicol acetyltransferase activity by antibody. Equal volumes (100 µl) of crude cell-free extract (5 mg of protein per ml) of *S. faecalis* JH6, *S. aureus* (type D), or *E. coli* (type I) were mixed with an equal volume of tris(hydroxymethyl)aminomethane-hydrochloride (50 mM, pH 7.8) in 100 mM sodium chloride containing the quantities of antiserum indicated. Each sample was allowed to stand for 1 h at 4°C prior to assay for chloramphenicol acetyltransferase by the spectrophotometric method. (A) Crude extracts of the *S. faecalis* JH6 (○) and *S. aureus* type D (●) enzymes were reacted against rabbit antiserum raised against the staphylococcal enzyme. The antiserum failed to neutralize the type I chloramphenicol acetyltransferase specified by an *E. coli* R factor (■). In each case, 100% was equivalent to approximately 0.3 U of chloramphenicol acetyltransferase per ml. (B) Goat antiserum specific for type I R factor chloramphenicol acetyltransferase was used to titrate the homologous enzyme used as the antigen (●) or the *S. faecalis* JH6 (○) and *S. aureus* type D (■) extracts.

of these enzyme activities, the enzyme production is plasmid determined.

The aminoglycoside antibiotics are assumed to be modified at the 3'-hydroxyl group; none of the 3'-deoxy aminoglycosides (e.g., tobramycin, Fig. 2) is a substrate for phosphorylation by the *S. faecalis* extracts. The aminoglycoside substrate profiles of phosphotransferases extracted from the *S. faecalis* strains studied are very similar to those of plasmid-directed aminoglycoside phosphotransferases found in gram-negative bacteria such as *E. coli* (7). In both species, as opposed to the situation in *S. aureus* (6), amikacin (1-N-AHBA kanamycin A) is a poor substrate for the phosphorylating enzymes; the cells are susceptible to the drug.

There is one interesting property of the streptomycin adenylyltransferase of *S. faecalis*. As in *S. aureus* (6), and in contrast to the same type of activity detected in gram-negative bacteria (3), streptomycin is an excellent substrate for the enzyme but spectinomycin is not. Suzuki et al. (19) have described a streptomycin 6-adenylylating enzyme in *S. aureus* that does not modify spectinomycin. Such an enzyme may be present in the *S. faecalis* strains we have examined, since they are streptomycin resistant but spectinomycin susceptible.

In gram-positive bacteria such as *S. aureus*, plasmid-coded resistance to β -lactam antibiotics and chloramphenicol results from the inducible expression of modifying enzymes that detoxify all of the drug in the culture medium (2). By

contrast, in gram-negative bacteria (8) and in staphylococci (6), plasmid-mediated aminoglycoside-aminocyclitol-modifying enzymes do not express resistance by gross inactivation of the antibiotics; modification of a small amount of the drug apparently, by an unknown mechanism, prevents further uptake of the antibiotic (8). When *S. faecalis* JH2-7 harboring each of the three plasmids was grown in the presence of streptomycin or neomycin B, no detectable change in the antibiotic concentration was found by microbiological methods, and the antibiotic remained unchanged.

Measurements of aminoglycoside-aminocyclitol-adenylylating or aminoglycoside-phosphorylating enzyme activities in strains grown with or without antibiotic revealed the presence of substantial modifying activities in all cases. Thus, the aminoglycoside-aminocyclitol-modifying enzymes in *S. faecalis* (as well as in staphylococci [6]), like their counterparts in gram-negative bacteria, are synthesized constitutively; however, the possibility that superinduction of the constitutive level occurs in the presence of the drug cannot be eliminated.

The biochemical mechanism of chloramphenicol resistance in *S. faecalis* JH6 is clearly enzymatic acetylation. The streptococcal enzyme resembles more closely the family of chloramphenicol acetyltransferase variants found in staphylococci rather than those which are the product of R factor genes in *E. coli* and related enteric bacteria. The group of enzymes found in gram-positive bacteria are all inducible and immunologically related, whereas the presence of an R factor in *E. coli* promotes the constitutive synthesis of three known variants of chloramphenicol acetyltransferase, all of which are electrophoretically and immunologically distinct from the enzyme in *S. faecalis* JH6.

It might be expected on taxonomic grounds that the antibiotic-inactivating enzymes of streptococci would resemble the analogous enzymes in staphylococci more closely than those of the gram-negative bacteria. This is clearly so for chloramphenicol acetyltransferase, and also for streptomycin adenylyltransferase, but the aminoglycoside phosphotransferases of the streptococci have closer affinities with those of the gram-negative bacteria. The close resemblance of the streptococcal chloramphenicol acetyltransferase to a specific (type D) variant of the enzyme from staphylococci begs the question of their identity with one another and the extent of homology shared by the plasmid of *S. faecalis* JH6 and those plasmids of staphylococci that carry the type D enzyme determinant. More detailed studies to answer these questions are required; in particular, N-terminal sequence de-

terminations and peptide maps of enzymatic digests of the two proteins and measurements of DNA-DNA hybridization between the plasmid of *S. faecalis* JH6 and its presumptive counterpart in strains of staphylococci that synthesize the type D variant of chloramphenicol acetyltransferase. Such studies will have to be extended to the chloramphenicol resistance systems that have been described in other streptococci (14, 16).

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ADDENDUM IN PROOF

Antiserum prepared against the type III neomycin phosphotransferase (6) isolated from *S. aureus* RN450/pSH2 showed immunological cross-reactivity when tested with extracts of *S. faecalis* strains JH2-12 and JH2-14 by using the cross-immuno-electrophoresis technique (T. J. White and D. I. Smith, unpublished data).

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