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Conjugal Transfer of Plasmid-Borne Multiple Antibiotic Resistance in *Streptococcus faecalis* var. *zymogenes*

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A strain of *Streptococcus faecalis* var. *zymogenes*, designated JH1, had high-level resistance to the antibiotics streptomycin, kanamycin, neomycin, erythromycin, and tetracycline. These resistances were lost en bloc from approximately 0.1% of cells grown in nutrient broth at 45 C. The frequency of resistance loss was not increased by growth in the presence of the "curing" agents acriflavine or acridine orange, but after prolonged storage in nutrient agar 17% of cells became antibiotic sensitive. Covalently closed circular deoxyribonucleic acid (DNA) molecules were isolated from the parental strain and from antibiotic-sensitive segregants by using cesium chloride-ethidium bromide gradients. DNA molecular species were identified by using neutral sucrose gradients. Strain JH1 contained two covalently closed circular DNA species of molecular weights 50×10^6 and 38×10^6. An antibiotic-sensitive segregant, strain JH1-9, had lost the larger molecular species. A second sensitive segregant, strain JH1-5, had also lost the larger molecular species but a new molecular species of approximate molecular weight 6×10^6 was present. The antibiotic resistances that were curable from the parental strain were transferred to antibiotic-sensitive strains of *S. faecalis* and to strain JH1-9, during mixed incubation in nutrient broth at 37 C. Data to be described are interpreted to suggest that the transfer is by a conjugal mechanism. Analysis of the plasmid species in recipient clones showed that all had received the plasmid of molecular weight 50×10^6. Strain JH1-5 was not a good recipient. Analysis of one successful recipient clone of JH1-5 revealed that it had gained the 50×10^6 molecular weight plasmid but lost the 6×10^6 molecular weight species. These data are interpreted to mean that the multiple antibiotic resistance is borne by a transferable plasmid of 50×10^6 molecular weight, and that in clone JH1-5 this plasmid suffered a large deletion leaving only a 6×10^6 remnant which was incompatible with the complete replicon.

Our interest in the possibility that antibiotic resistance in *Streptococcus faecalis* was plasmid borne was stimulated by a personal communication (to Naomi Datta) from D. H. Smith of the Children's Hospital Medical Center, Boston, describing a strain of *S. faecalis* in which chloramphenicol resistance and resistance to certain heavy metal ions were unstable properties. Interest was enhanced by the report that since 1949 group D streptococci have become increasingly resistant to antibiotics, both in the level of resistance to particular drugs and in the multiplicity of this resistance (32). This increase has paralleled that observed in the *Enterobacteriaceae* and *Staphylococcus*, in which it is known that resistance to many antibiotics is plasmid borne.

The genetic properties and molecular structure of the plasmids of the *Enterobacteriaceae* and *Staphylococcus* have been extensively studied (7, 26). It is known that these plasmids exist as autonomously replicating deoxyribonucleic acid (DNA) molecules, physically separate from the host chromosome and that the plasmid DNA can be isolated in a covalently closed circular (CCC) form (15). Plasmid replication and segregation is precisely controlled so that each daughter cell contains at least one plasmid copy. Occasionally these mechanisms fail, resulting in the absence of a plasmid molecule from a daughter cell and therefore loss of the plasmid genetic traits. This spontaneous plasmid loss may be increased by growth under certain conditions and by growth in the presence of "curing" agents. Many plasmids are capable of self transfer by conjugation, and some may be transferred by bacteriophage vectors. In this investigation we have used these properties as criteria for determining whether the multiple antibiotic resistance of a strain of

S. faecalis var. *zymogenes*, isolated from a patient in Hammersmith Hospital, was plasmid-borne. Two plasmid DNA species have been isolated and characterized from this strain. Loss of multiple antibiotic resistance from the strain was observed after growth under curing conditions and the loss has been correlated with the absence of the larger plasmid DNA species. We report that the plasmid-borne resistances were transferable to several strains of *S. faecalis* and we describe experiments designed to discover the transfer mechanism. We show that resistance transfer was correlated with transfer of the larger plasmid. A preliminary report of this work was presented at a Society for General Microbiology Ordinary Meeting, 13-14 September 1973, at the University of Kent, England. The genetic traits borne by the smaller plasmid present in the *S. faecalis* var. *zymogenes* strain will be described in a forthcoming paper.

Conjugal transfer of either chloramphenicol resistance (isolated by selection on chloramphenicol containing agar) or bacteriophage resistance from a strain of *S. faecalis* var. *liquefaciens* has been reported previously (27). Recently, plasmids bearing a single antibiotic resistance have been isolated from an *S. faecalis* strain, but it is not known whether these plasmids are transferable (8).

MATERIALS AND METHODS

Media. Strains were grown in Oxoid nutrient broth, number 2, except where stated. This medium was supplemented with 2 μg of thymine per ml for the growth of thymine-requiring mutants. For curing experiments, Hirota's acridine orange (AO) broth was used (18). For isotope labeling of strains, Kornberg's modified M9 medium (23) was used, supplemented with 0.5% casein hydrolysate (Oxoid), 0.3% yeast extract (Difco), 20 μg of L-tryptophan per ml, and 250 μg of deoxyadenosine per ml. Deoxyadenosine was not included for the radiolabeling of thymine-requiring mutants.

Nutrient agar was prepared by the addition of Oxoid number 3 agar, at 1% concentration, to nutrient broth number 2. Blood agar was nutrient agar plus 4% horse blood. DST agar was Oxoid Diagnostic Sensitivity Test agar (code number CM 261). Lysed blood plates were DST agar supplemented with 4% saponin-lysed horse blood. The MacConkey agar used was Oxoid brand (code number CM7b). All incubations were at 37 C except where stated.

Bacterial strains. Lancefield group D streptococci isolated from clinical specimens were used exclusively in this study. Their primary identification on MacConkey agar was verified by Fuller's serological test (13). Species were classified by biochemical tests (29). The "zymogenes" and "liquefaciens" varieties of *S. faecalis* were distinguished by (i) hemolysis of blood agar and (ii) gelatin liquefaction. Sensitivity to antibiotics was determined by inoculating 50 to 100 colony-forming units onto nutrient agar plates containing doubling concentrations of an antibiotic, starting from either 1 μg/ml or 5 μg/ml, with a Steers' multiple inoculator apparatus (30). The MIC (minimum inhibitory concentration) was recorded as the lowest concentration of antibiotic preventing colony formation after 18 h of incubation. Some properties of the *S. faecalis* strains used in this study are listed in Table 1. Stock cultures were stored at 4 C and maintained by monthly subculture on blood agar plates. Stocks were preserved by storage in nutrient agar stabs at room temperature.

Isolation of mutants. Thymine-requiring mutants were isolated by a modification of Andrew's method (1), which takes advantage of the frequent association of trimethoprim resistance with thymine requirement. An 18-h broth culture was diluted 1:100 in broth supplemented with 10 μg of trimethoprim per ml. After 7 h of incubation with aeration the culture was diluted into broth containing thymine at 50 μg/ml and trimethoprim at 10 μg/ml, incubated 18 h, then plated on nutrient agar containing thymine (2 μg/ml) and trimethoprim (10 μg/ml). After 18 h of incubation two distinct sizes of colonies were observed of which the larger were found to require thymine.

Antibiotic-resistant mutants. Fusidic acid-resistant mutants were selected on nutrient agar containing 25 μg of fusidic acid per ml. Mutant colonies arose at a frequency of 4×10^{-9} per viable cell; those retained for the study had an MIC of 256 μg/ml (16-fold above the parental strain). Mutants resistant to rifampin and gentamicin were isolated after *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (NTG) mutagenesis. Cells in exponential phase of growth were resuspended in 0.5 volume of 0.1 M-trisodium citrate buffer, pH 5.6, containing 500 μg of NTG per ml. Cells were incubated for 30 min, harvested by centrifugation, washed once in broth, and resuspended in 1 volume of broth. After a further 2 h of incubation, the culture was plated on nutrient agar containing either gentamicin (50 μg/ml) or rifampin (500 μg/ml). Mutants having an MIC to rifampin of 1,280 μg/ml (16-fold above the parental strain) and to gentamicin of 80 μg/ml (eightfold above the parental strain) were used. A list of the derivative strains is presented in Table 2.

Antibiotic resistance curing procedures. Growth in broth at different temperatures was as follows: an 18-h broth culture was diluted 1:10 in broth and incubated with aeration for 6 h at either 37 or 45 C before plating. Curing by chemical agents was as follows: cultures grown to late exponential phase in Hirota's AO broth were diluted to 10^4 cells/ml in AO broth containing a range of doubling concentrations, from 1.25 μg/ml to 40 μg/ml, of either acriflavine or acridine orange. After 18 h of incubation, cultures which contained a concentration of agent just subinhibitory to cell growth were plated. Control cultures, grown in AO broth with no chemical agent added, were also plated. Curing by ultraviolet (UV) irradiation was as follows: cells from an 18-h broth culture were harvested by centrifugation, resuspended in 0.9% sodium chloride at 2×10^7 cells/ml, and irradiated with a Phillips

TABLE 1. *Strains of S. faecalis*

Strain no.[a]	Characteristics[b]			Variety	MIC of antibiotics (μg/ml)[d]							
	β hemolysis	Bacteriocin production[c]	Gelatin liquefaction		Km	Sm	Nm	Tc	Em	Nal	Pmn	Sul[e]
JH1	+	+	+	Zymogenes	40,000	2,560	>40,000	320	5,000	5,000	320	20,000
JH2	−	−	−		20	40	40	0.625	0.125	5,000	320	20,000
JH3	+	+	+	Zymogenes	40	80	40	0.625	1	5,000	1280	20,000
JH4	+	+	−	Zymogenes	40	40	40	0.625	0.5	5,000	640	>20,000
JH5	−	−	−		10	40	40	0.625	1	2,560	320	20,000

[a] Each strain was isolated from a separate patient in Hammersmith Hospital.
[b] All strains ferment arabinose and mannitol and tolerate 0.04% Tellurite.
[c] Bacteriocin is active against other group D streptococci and *S. aureus*.
[d] Abbreviations: Km, kanamycin; Sm, streptomycin; Nm, neomycin; Tc, tetracycline; Em, erythromycin; Nal, nalidixic acid; Pmn, polymixin; Sul, sulphadimidine.
[e] MIC to sulphadimidine was determined in DST agar.

TABLE 2. *Derivatives of S. faecalis strains*

Strain no.	Parental strain	Phenotype[a]
JH1-2	JH1	Thy⁻, Gen
JH2-2	JH2	Fus, Rif
JH2-3	JH2	Fus, Gen
JH3-2	JH3	Fus, Rif
JH4-2	JH4	Fus, Rif
JH5-1	JH5	Fus

[a] Phenotypes introduced by mutagenesis and by selection of spontaneous mutants. Abbreviations: Thy, thymine; Gen, gentamicin; Fus, fusidic acid; Rif, rifampin.

TUV 15-W lamp to 1% survival (100 s at 50 cm). Treated cultures were screened for antibiotic-sensitive segregants by replica plating and, after purification, the antibiotic MICs for these segregants were determined.

Labeling, lysis, and cesium chloride-ethidium bromide centrifugation. Cells were grown for 17 h in supplemented M9 medium, with aeration. They were then diluted to approximately 5×10^7 cells/ml in fresh supplemented M9, containing either 0.06 ml of [*methyl*-^{3}H]thymidine (1 mCi/ml, 22 Ci/mmol) or 0.2 ml of [2-^{14}C]thymidine (50 μCi/ml, 62 mCi/mmol) for 8 ml of medium. For labeling of thymine-requiring mutants the medium was not supplemented with deoxyadenosine and either 0.06 ml of [*methyl*-^{3}H]thymidine plus 0.08 ml of thymine (250 μg of stock solution per ml) or [2-^{14}C]thymine (0.124 Ci/ml, 62 mCi/mmol) was added. The cultures were grown to 10^9 cells/ml, with aeration.

Lysis and dye-buoyant density centrifugation of the cultures were effected by the methods described by Bazaral and Helinski (5), modified for use with streptococci: 8 ml of radiolabeled culture was washed twice in TES buffer (0.05 M NaCl + 0.05 M tris(hydroxymethyl)aminomethane [Tris] + 0.005 M Na_2 ethylenediaminetetraacetic acid [EDTA], pH 8.0) at 4 C. When mixed lysates of two differentially labeled cultures were to be prepared, 4 ml of each culture were mixed together after the first wash in TES buffer. The cell pellets were resuspended in 0.25 ml of TES buffer containing 100 mg of sucrose per ml and then 0.25 ml of TES buffer was added containing 2 mg of lysozyme per ml, 1 mg of ribonuclease per ml, and 100 mg of sucrose per ml. The suspension was incubated for 30 min at 37 C, and cooled for 5 min in ice. The cells were lysed by the addition of 0.25 ml of a Sarkosyl solution (2.4% in water) and gently mixed by drawing the solution through a 1-ml pipette. After the addition of 0.5 ml of TES buffer at room temperature, the lysate was slowly drawn in and out of a 1-ml pipette 20 times to shear the DNA.

The sheared lysate (1.15 ml) was added to a mixture of cesium chloride (4.609 g) dissolved in 3 ml of TES buffer and 1 ml of ethidium bromide solution (4.8 mg/ml in TES buffer). The samples were then centrifuged for 60 h at 37,000 rpm (100,000 × *g*) at 20 C in an MSE 10 place fixed-angle titanium rotor. Tubes were punctured and 0.1-ml fractions were collected in test tubes with an apparatus designed by Tan (31). A 10-μliter sample of each fraction was pipetted onto a glass fiber disk (Whatman, GF/C), dried, washed, and counted for radioactivity. Significant fractions were pooled and ethidium bromide was removed by two extractions with an equal volume of 2-propanol (9). After dialysis against four changes of TES buffer over 24 h, the DNA samples were stored frozen at −70 C.

Cesium chloride density centrifugation. An 8-ml culture was labeled with [*methyl*-^{3}H]thymidine and lysed, as described above. DNA was extracted from the lysate by using the phenol method of Hickson, Roth, and Helinski (17), and was dialyzed against TES buffer. The purified DNA solution (0.17 ml) was then mixed with a solution of cesium chloride (6.233 g) in TES buffer (4.7 ml), centrifuged to equilibrium, and fractions were collected and counted for radioactivity as described above.

Preparation of ^{14}C-labeled λ bacteriophage

DNA. ^{14}C-labeled λ bacteriophage was isolated by using the method of thymine-less induction of *Escherichia coli* K-12, W3110 Thy$^-$ (λ) lysogen as described by Korn and Weissbach (22), from 100 ml of culture grown in broth containing 10 μM [2-^{14}C]thymine (20 mM; 124 μCi/ml). Phage was purified by low- and high-speed centrifugation followed by CsCl buoyant density centrifugation (21), and λ DNA was isolated by using the phenol method of Young and Sinsheimer (35). λ DNA was dialyzed against two changes of TES buffer (21) over 24 h and was stored frozen at −20 C.

Linear monomers of λ DNA were prepared immediately before use by heat treatment of the DNA at 75 C for 10 min followed by rapid cooling in ice (16).

DNase I treatment of DNA. A volume of deoxyribonuclease (DNase) I solution (5×10^{-4} μg/ml in 25 mM $MgSO_4$) was added to an equal volume of DNA solution, and incubated at 25 C. At an appropriate time, deoxyribonuclease activity was stopped by the addition of 1 volume of 60 mM Na_2 EDTA (pH 8.0), and the sample was immediately loaded onto a sucrose gradient. As a control, the DNA solution was treated in a similar manner with 25 mM $MgSO_4$ replacing the DNase solution.

Sucrose gradient velocity centrifugation and determination of plasmid molecular weight. Linear 5 to 20% neutral sucrose gradients were prepared according to the method of Baxter-Gabbard (3), modified by P. T. Barth and N. J. Grinter (to be published) as follows: cellulose nitrate centrifuge tubes (Beckman, number 305050) containing 4.6 ml of sucrose solution (12.5% in TES buffer) were frozen and stored at −70 C. To prepare a gradient, a tube was thawed in iced water for 1 h and then allowed to stand for 1 h at room temperature. After this time a sucrose gradient had been formed which was linear in increasing sucrose concentration, from the meniscus to approximately 0.5 ml (five fractions) from the tube bottom, where the gradient became slightly steeper.

The DNA sample, usually between 0.03 and 0.2 ml in volume, was layered onto a gradient and overlaid with liquid paraffin. When required as a sedimentation reference, 0.03 ml of linear monomers of ^{14}C-labeled λ bacteriophage DNA was added to the plasmid DNA sample immediately before layering onto the gradient. Centrifugation was performed in an MSE swinging-bucket rotor (3 by 5 ml), using an MSE 65 preparative ultracentrifuge, at 35,000 rpm (100,000 × *g*), 20 C. Fractions of 0.1 ml were collected directly on glass fiber disks, dried, washed, and counted as above.

Sedimentation coefficients of plasmid DNA were determined by cosedimentation with λ DNA (34.4*S*; ref. 12), and application of the formula $S_1/S_2 = D_1/D_2$ (6), where S_1, S_2 are the sedimentation coefficients, and D_1, D_2 are the distances of the peaks from the meniscus, of the plasmid and λ DNA, respectively. The sedimentation coefficient of plasmid linear forms (S_3) was approximated using the relationships between this and the sedimentation coefficient of the sedimentation coefficient of the CCC and open circular (OC) forms determined by Freifelder (12) and Hudson, Clayton and Vinograd (19), and presented in graphical form by Clowes (7, p. 371). The approximate molecular weight of the plasmid linear form was then calculated, using the formula $S_2/S_3 = (M_2/M_3)^{0.38}$ (12) where M_2 is the molecular weight of λ DNA, which is 30.8×10^6 (11), and M_3 the molecular weight of the plasmid linear form. Generally, a more accurate molecular weight was then calculated, using the approximate molecular weight value to determine an accurate conversion factor for the calculation of the plasmid linear DNA sedimentation coefficient from that of the CCC and OC forms (7, p 371).

Radioisotope counting. Disks containing samples were washed with cold 5% trichloroacetic acid followed by acetone. They were then dried and counted in 5 ml of scintillation medium (7 g of butyl-PBD/liter of toluene) in glass scintillation vials. Counting was performed in an Intertechnique ABAC SL40 liquid scintillation spectrometer by using the manufacturer's preset discriminator levels for single and dual isotope counting.

Reagents. Analytical reagent grade chemicals were purchased from BDH Chemicals Ltd. Enzymes were purchased from Sigma Chemical Co., London, and radioisotopes were from the Radiochemical Centre, Amersham. Ethidium bromide was a gift from Boots Pure Drug Co. Ltd. and Sarkosyl NL 97 was a gift from Geigy Chemicals. Rifampin (Rimactane) was a gift from Ciba Laboratories. All other chemicals and antibiotics were purchased from standard commercial sources.

RESULTS

Antibiotic resistances of the group D streptococcal strains. The spectrum of antibiotic resistance of 100 group D streptococci, each isolated from a separate patient in Hammersmith Hospital, was evaluated with sensitivity disks. We used Oxoid urine multodisks (code number 30-44K) or Oxoid multodisks made to our specification (code number 3089E) on lysed blood plates. All the strains were resistant to polymixin (200 μg disk), nalidixic acid (30 μg), and sulphafurazole (500 μg). These resistances are typical of group D streptococci (14). Many were resistant to at least one of the following disks: kanamycin (30 μg), neomycin (25 μg), streptomycin (25 μg), and tetracycline (50 μg). Few were resistant to erythromycin (10 μg) or chloramphenicol (30 μg) and none was resistant to ampicillin (10 μg). Ten strains were sensitive to all of the antibiotics listed except polymixin, sulphafurazole, and nalidixic acid. An *S. faecalis* var. *zymogenes* strain, designated JH1, which was resistant to kanamycin (Km), neomycin (Nm), streptomycin (Sm), erythromycin (Em), and tetracycline (Tc), and four *S. faecalis* strains relatively sensitive to these antibiotics were chosen for further study. The MICs of the antibiotics for these strains are shown in Table 1.

Genetic stability of antibiotic resistance traits in S. faecalis var. zymogenes, strain

JH1. We tested the stability of the antibiotic resistance traits in strain JH1 by screening for the appearance of antibiotic-sensitive segregant clones by using the replica plating technique. We used culture conditions, and growth in the presence of chemical agents, that are known to cure some plasmid-borne resistances from strains of the *Enterobacteriaceae* and *Staphylococcus* (26). The results from several experiments are summarized in Table 3. After growth at 37 C in broth no loss of multiple resistances was detected. Increasing the incubation temperature to 45 C resulted in the simultaneous loss of resistance to Km, Nm, Sm, Em, and Tc from several clones. Similar antibiotic-sensitive clones were isolated after growth in Hirota's AO broth (18) either in the presence of 1.25 μg of acriflavine per ml or of 5 μg of acridine orange per ml. However, more sensitive segregants were isolated from the control, grown in Hirota's AO broth, than from the culture grown in AO broth with acridine orange. UV irradiation, at a dose which gave 99% killing, resulted in two clones (strains JH1-10 and JH1-11) which had lost partial resistance to some of the antibiotics. After storage of strain JH1 in a nutrient agar stab for 12 months at room temperature approximately 17% (10/60) of the clones had lost resistance to Km, Nm, Sm, Em, and Tc. We conclude from these data that the antibiotic resistance traits are unstable properties in strain JH1 and that the various curing procedures, with the exception of prolonged storage, did not significantly increase the frequency of antibiotic resistance loss.

TABLE 3. *Effect of curing agents on the stability of antibiotic resistance of S. faecalis var. zymogenes, strain JH1.*

Treatment	No. colonies screened	No. antibiotic-sensitive colonies found[a]	Antibiotic-sensitive colonies (%)
Incubation in broth:			
At 37 C	2,724	0	
At 45 C	2,077	3	0.14
Incubation with chemical curing agents:			
Acriflavine (1.25 μg/ml)	1,343	1	0.07
Control[b]	1,286	0	
Acridine orange (5 μg/ml)	2,251	1	0.04
Control[b]	2,484	5	0.20
UV irradiation:			
Irradiation to 1% survival	1,262	2[c]	0.16
No irradiation	1,012	0	

[a] All colonies had lost resistance to Km, Sm, Nm, Tc, and Em, except for those marked with footnote c.

[b] Control cultures were treated in the same way as the test cultures, but without the curing agent.

[c] Both colonies had lost partial resistant to some antibiotics, as explained in the text.

The MICs of antibiotics for several sensitive segregants chosen for further study, together with the parental strain, JH1, and some derivatives of the segregants, are listed in Table 4. The table illustrates the loss of resistance to Km, Nm, Sm, and Em, a slight loss of resistance to Tc, and retention of resistance to polymixin, sulphadimidine, and nalidixic acid. All sensitive segregants, except strains JH1-10 and JH1-11, had MICs within one doubling concentration or dilution of the values shown.

One sensitive segregant, strain JH1-3, was examined for spontaneous reversion to high-level resistance. No reversion to Km, Nm and Sm (2,000 μg/ml) or to Em (10 μg/ml) resistance was observed (reversion $< 1.6 \times 10^{10}$ for each antibiotic in nutrient agar).

Isolation of plasmid-specific DNA from strain JH1 and some antibiotic-sensitive segregants. We have analyzed strain JH1-2 (a derivative of strain JH1, Table 2) and three antibiotic-sensitive segregant derivatives, JH1-5, JH1-6, and JH1-9 (Table 4), for the presence of CCC DNA molecules, by using a dye-buoyant density centrifugation method (5). We found that all four strains contained a CCC DNA component that banded separately from chromosomal DNA. Illustrated in Fig. 1 is the banding profile of the DNA from strain JH1-2. Two bands were seen: a small, denser, CCC DNA band and a large chromosomal DNA band. The ratio of counts in the CCC DNA band to the chromosomal DNA band counts was 5.5%.

For analysis of the antibiotic-sensitive segregants we mixed cultures of a ^{3}H- (or ^{14}C-) labeled sensitive segregant and ^{14}C- (or ^{3}H-) labeled JH1-2 before lysis. The use of these lysates in the dye-buoyant density centrifugation enabled us to make an accurate comparison of yields of CCC DNA from the sensitive segregants and parental strain, since we assumed that loss of CCC DNA during the isolation procedure would be the same for both strains in the mixture. The ratio of counts in the CCC band of the antibiotic-sensitive segregant strain JH1-9 to its chromosomal band counts was 3.0%, whilst that of strain JH1-2 was 6.6%. This indicated that approximately 55% of CCC DNA

TABLE 4. *Some antibiotic-sensitive segregants and their derivatives*

Strain no.	Source	MIC to antibiotics (μg/ml)									
		Km	Sm	Nm	Tc	Em	Nal	Pmn	Sul[b]	Fus	Rif
JH1	Parental strain	40,000	2,560	>40,000	320	5,000	2,560	320	20,000	4	1.25
JH1-3	Incubation at 45 C ...	40	80	20	160	1	2,560	320	20,000	4	1.25
JH1-6	Incubation in AO	40	80	20	160	0.5	2,560	320	20,000	4	1.25
JH1-7	Incubation in AO broth with acridine orange	40	80	40	160	0.5	2,560	320	20,000	4	1.25
JH1-10	UV irradiation	640	160	320	320	4	2,560	320	20,000	4	1.25
JH1-11	UV irradiation	1,280	2,560	10,000	160	640	2,560	320	20,000	4	1.25
Derivatives[a]											
JH1-5		40	80	20	160	0.5	2,560	320	20,000	256	1280
JH1-9		40	80	40	160	1	2,560	320	10,000	256	1280

[a] Resistance to fusidic acid and rifampin introduced by mutagenesis and by selection of spontaneous mutants.

[b] MIC to sulphadimidine determined in DST agar.

present in strain JH1-2 was lost from strain JH1-9. A similar result was found for the other sensitive segregants examined.

We centrifuged phenol-purified DNA from strain JH1 to equilibrium in a cesium chloride density gradient (data not shown). Only one DNA band was detected, indicating that the guanine plus cytosine contents of the plasmid-specific CCC DNA and chromosomal DNA were similar.

Identification and molecular weight determination of CCC DNA species using neutral sucrose gradients. We analyzed CCC DNA isolated from strain JH1-2 and the three antibiotic-sensitive segregants by sedimentation through 5 to 20% neutral sucrose gradients. Figure 2A shows the result of sedimenting CCC DNA from JH1-2 for 105 min. Two fast-sedimenting bands, with peaks at fractions 9 and 14, were seen as well as poorly resolved, slower sedimenting bands peaking at fractions 25 and 27. The DNA in fractions 1 to 5, seen in this and other gradients, possibly resulted from concentration of the leading edges of the two fast-sedimenting bands at the tube bottom. It did not represent a third fast-sedimenting band since such a band was not observed after 85 min of sedimentation (data not shown). To discover the relationship between the fast- and slow-sedimenting bands we incubated the CCC DNA with a low concentration of DNase I, an endonuclease that causes single-strand breaks in DNA and which therefore converts CCC DNA to the OC form (4, 33). The effect of this treatment on sedimentation of the DNA is illustrated in Fig. 2B and C. A progressive decrease in size of the two fast-sedimenting bands and a corresponding increase in size of the slow-sedimenting bands was observed, with increasing time of incubation with DNase. This behavior defines the two fast-sedimenting bands as being CCC DNA species and the slow bands as being their OC forms. Linear DNA forms, which would sediment slower than the OC forms (33), were not generated by these DNase treatments. Resolution of the two OC forms was achieved by sedimentation for a longer time, as shown in Fig. 3. Using ^{14}C-labeled λ DNA as a sedimentation reference, we calculated the $S_{20,w}$ of the larger plasmid (pJH1) OC form to be 47.6*S* and that of the smaller plasmid (pJH2) to be 42.8*S*. From a similar experiment (data not shown) the $S_{20,w}$ value of the CCC DNA form of plasmids pJH1 and pJH2 was estimated to be 74.6 and 65.7*S*, respectively. Using the formulas described in Materials and Methods, the molecular weight of the plasmids was calculated to be approximately 50×10^6 for pJH1 and 38×10^6 for pJH2.

For analysis of the antibiotic-sensitive segregants we used CCC DNA isolated from the mixed lysates of the sensitive segregant and resistant parental strain. Figure 4 illustrates the sedimentation of CCC DNA from antibiotic segregant strain JH1-9 and from the parental derivative strain JH1-2. It shows the resolution of plasmids pJH1 and pJH2 CCC DNA from strain JH1-2, peaking at fractions 10 and 14, respectively, and the loss of plasmid pJH1 CCC DNA from strain JH1-9. Resolution of the OC forms of JH1-2 plasmids pJH1 and pJH2 and the loss of plasmid pJH1 OC form from JH1-9 is shown in Fig. 5.

Plasmid pJH1 was also lost from the two

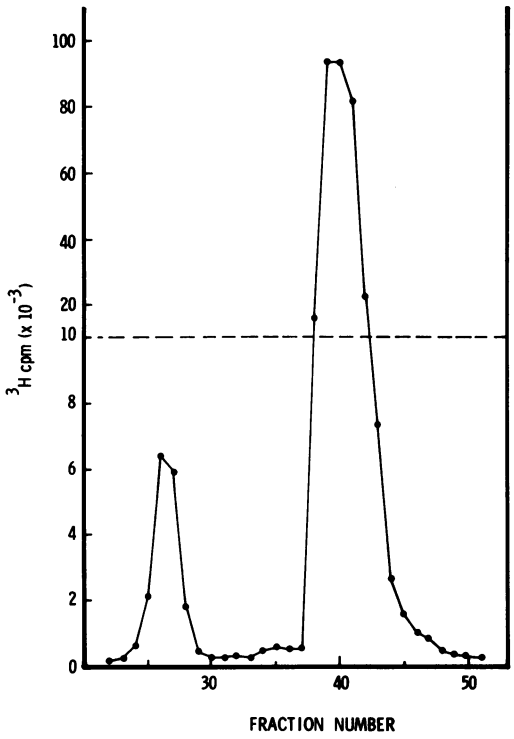

FIG. 1. *Fractionation of a CsCl-EB equilibrium density gradient containing ^{3}H-labeled DNA from S. faecalis var. zymogenes, strain JH1-2. Cells from an 8-ml culture, grown in medium containing ^{3}H-thymidine, were harvested, lysed with lysozyme-EDTA and Sarkosyl, and centrifuged to equilibrium in a CsCl-EB density gradient. Fractions (0.1 ml) were collected through a hole punctured in the tube bottom and 10 μliters of each was assayed for ^{3}H radioactivity. Fractions containing significant radioactivity were plotted as shown. Note the change in scale at 10^4 counts/min.*

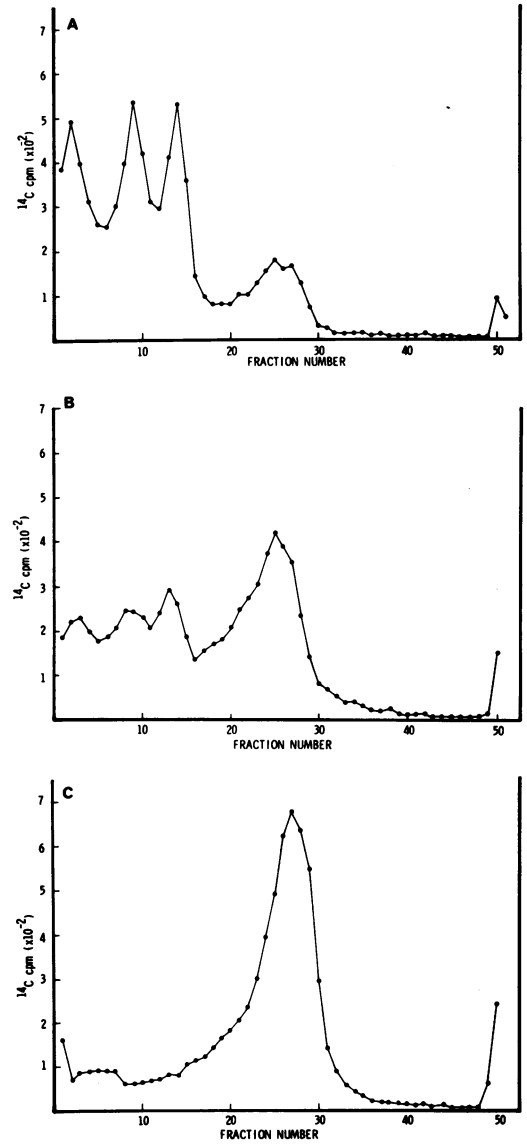

FIG. 2. *Neutral sucrose gradient analysis of DNase I degradation of plasmid DNA from strain JH1-2. Samples (0.03 ml) of ^{14}C-labeled JH1-2 plasmid DNA were treated with an equal volume of 5×10^{-10} g of DNase I per ml in 25 mM $MgSO_4$ at 25 C for the times specified below. To stop the DNase I activity 60 mM Na_2EDTA was then added. Samples were layered onto 5 to 20% sucrose gradients and centrifuged at 100,000 × g, 20 C, for 105 min. Fractions (0.1 ml) were collected directly onto glass fiber disks through a hole punctured in the tube bottom and were assayed for ^{14}C radioactivity after drying and washing. A, Control; plasmid DNA treated with 25 mM $MgSO_4$ for 7 min at 25 C followed by Na_2EDTA addition. B, DNA treated with DNase for 2 min. C, DNA treated with DNase for 7 min.*

other sensitive segregants examined. In one of these, strain JH1-5, a new CCC DNA band, pJH1-1, was observed (Fig. 6), which peaked at fraction 35 and had an $S_{20,w}$ of 29.9*S*. Its molecular weight was calculated to be approximately 6.1×10^6.

Number of plasmid copies per chromosome. We used the above data to estimate the number of copies of plasmids pJH1 and pJH2 per chromosome. From the results obtained from the dye-buoyant density centrifugation of the JH1-2 plus JH1-9 mixed lysate we determined that the yields of CCC DNA from each strain, expressed as the percentage of chromosomal DNA yield, was 6.6 and 3.0%, respectively. Neutral sucrose gradient analysis of this DNA showed that plasmid pJH1 was lost from JH1-9. Therefore, the yield of plasmid pJH2

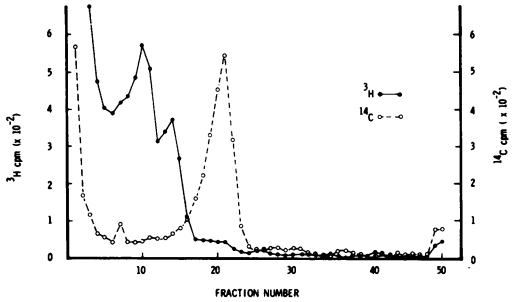

FIG. 3. *Neutral sucrose gradient analysis of OC DNA forms of strain JH1-2 plasmids. A 0.06-ml sample of ^{3}H-labeled JH1-2 plasmid DNA was treated with DNase I for 7 min and sedimented through a 5 to 20% sucrose gradient, as described in the legend to Fig. 2. Sedimentation was for 180 min. Linear monomers of ^{14}C-labeled λ DNA was added as a sedimentation reference (34.4S).*

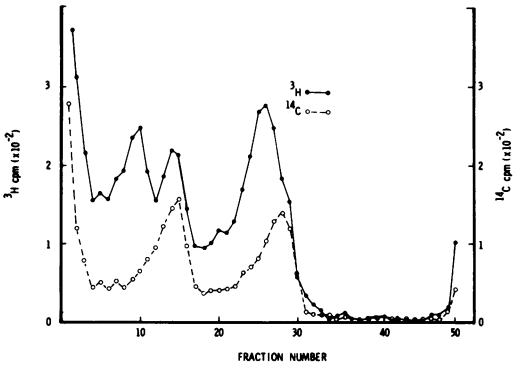

FIG. 4. *Neutral sucrose gradient analysis of plasmid DNA from strain JH1-9. Plasmid DNA was isolated from a mixed lysate preparation containing ^{14}C-labeled JH1-9 and ^{3}H-labeled JH1-2, by using a CsCl-EB density gradient. After removal of EB and dialysis, 0.07 ml was layered onto a 5 to 20% sucrose gradient and centrifuged at 100,000 × g at 20 C, for 110 min. Fractions (0.1 ml) were collected, the ^{3}H and ^{14}C radioactivity was assayed, and the results were plotted as shown.*

DNA from JH1-2 was approximately 3.0% and plasmid pJH1 DNA was approximately 3.6%. The molecular weight of the *S. faecalis* chromosome was estimated to be 1,470 × 10^6 (2). Using the values of 50 × 10^6 and 38 × 10^6 for the molecular weights of plasmids pJH1 and pJH2, and assuming that the specific radioactivity of CCC DNA and chromosomal DNA was the same, we calculated that approximately 1.1 copies per chromosome of each plasmid was isolated in the CCC DNA form from strain JH1-2.

Transfer of multiple antibiotic resistance to other S. faecalis strains. We tested the ability of derivatives of four antibiotic-sensitive strains of *S. faecalis*, JH2-2, JH3-2, JH4-2, and JH5-1 (Table 2), to act as recipients for the transfer of antibiotic resistance from strain JH1-2. We found that resistance to Km, Nm, Sm, Em, and Tc was transferred to each recipient during mixed incubation in nutrient broth at 37 C and have shown that the multiple resistances were also transferable from the primary recipient to a second one (strain JH2-3), indicating that the transfer mechanism was not dependent upon the original host strain.

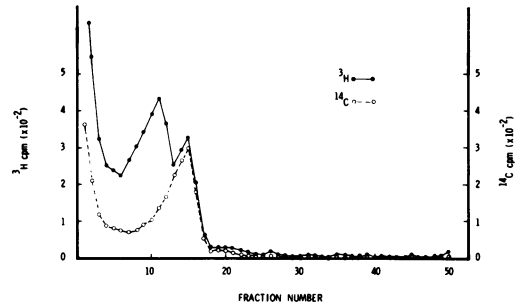

FIG. 5. *Neutral sucrose gradient analysis of the OC form of plasmid DNA from JH1-9. Mixed plasmid DNA from ^{14}C-labeled JH1-9 and ^{3}H-labeled JH1-2 was isolated as described in the legend to Fig. 4. A 0.07-ml sample was treated with DNase I for 7 min and then sedimented through a 5 to 20% sucrose gradient, as described in the legend to Fig. 2. Sedimentation was for 180 min. Fractions (0.1 ml) were collected, the ^{3}H and ^{14}C radioactivity was assayed, and the results were plotted as shown.*

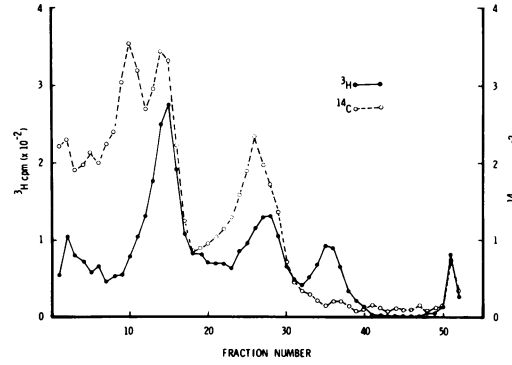

FIG. 6. *Neutral sucrose gradient analysis of plasmid DNA from strain JH1-5. Plasmid DNA was isolated from a mixed lysate preparation containing ^{3}H-labeled JH1-5 and ^{14}C-labeled JH1-2, by using a CsCl-EB density gradient. After removal of EB and dialysis, 0.07 ml was layered onto a 5 to 20% sucrose gradient and centrifuged at 100,000 × g at 20 C, for 105 min. Fractions (0.1 ml) were collected, the ^{3}H and ^{14}C radioactivity was assayed, and the results were plotted as shown.*

We have determined the kinetics of acquisition of resistance by one recipient strain, JH2-2. Derivative strains were used to allow unambiguous strain selection and recognition. Eighteen-hour cultures of the donor and recipient strains, both grown in broth supplemented with 2 μg of thymine per ml, were diluted in fresh supplemented broth to 10^8 cells/ml, mixed in the ratio of 1 donor to 10 recipients, and incubated with gentle shaking at 37 C. At intervals during a 4-h incubation period dilutions were plated on DST agar plates supplemented with thymine where appropriate, containing antibiotics to estimate the donor and recipient viable counts and the number of recipients having received an antibiotic resistance. Plates were incubated for 24 h before colonies were counted. To prove that transfer occurred we purified colonies that grew on the transfer-selection plates and tested them on antibiotic ditch plates for the presence of unselected resistances that may have been transferred, and for unselected chromosomal resistances to determine strain type. For complete characterization, we estimated the MICs of the antibiotics for some of these colonies. The results are presented in Fig. 7 and Table 5. Figure 7 shows that a 1,000-fold increase in the number of resistant recipients occurred during the first 30 min, but during the next 210 min the increase was less than twofold. After 4 h of mixed incubation the frequency of resistance transfer was approximately 10^{-5} per recipient (10^{-4} per donor). The MICs of antibiotics for the donor strain JH1-2, the recipient strain JH2-2 and strain JH2-4, a typical recipient clone that had received multiple antibiotic resistance, are listed in Table 5. The MICs of the antibiotics for which there are transferable resistances are shown, together with those of the antibiotics to which chromosomal resistance had been introduced by mutagenesis.

We have also tested the ability of the two antibiotic-sensitive segregants JH1-5 and JH1-9 to act as recipients. After 4 h of mixed incubation, the frequency of transfer of multiple resistance to strain JH1-9 was 2.9×10^{-6} per recipient (1.7×10^{-5} per donor) whilst that to strain JH1-5 was $<6 \times 10^{-10}$ per recipient ($<4 \times 10^{-9}$ per donor).

Isolation and identification of plasmid DNA species from recipient strains after receipt of multiple antibiotic resistances. We have isolated CCC DNA species from the antibiotic-resistant recipient clone JH2-4 (Table 5) and two other clones of recipient JH2-2 that had received multiple antibiotic resistance, by using dye-buoyant density centrifugation. Strain JH2-2 itself did not contain a CCC DNA band, as shown in Fig. 8A. In the same figure the profile of DNA from strain JH1-2, differentially labeled and mixed with strain JH2-2 before lysis and centrifugation, is shown and indicates that CCC DNA was successfully isolated from this strain. Figure 8B illustrates the dye-buoyant density centrifugation of strain JH2-4 and shows a CCC DNA band now present in the recipient, of similar (but not identical) buoyant density to that of the differentially labeled JH1-2 CCC DNA band. The two other antibiotic-resistant recipients tested also had CCC DNA present.

The species of plasmid(s) transferred to the antibiotic-resistant recipients were identified using neutral sucrose gradients. Figure 9 shows resolution of the CCC DNA forms of the plasmids isolated from strain JH2-4 and the differentially labeled strain JH1-2. All three resistant recipients had similar sedimentation profiles, which showed that each had received plasmid pJH1 DNA. These data are interpreted to confirm that the resistances to Km, Nm, Sm, Em, and Tc are borne by this plasmid. Although it is clear that Tc resistance is plasmid-borne, resistance of the original host strain, JH1, to Tc is only slightly reduced upon loss of the plasmid (Table 4). This may suggest that strain JH1 has a natural Tc resistance.

We have also analyzed one clone each of the antibiotic-sensitive segregant strains JH1-5 and JH1-9 that had received multiple antibiotic resistance after transfer (strains JH1-12 and JH1-13 respectively). Both had received plasmid pJH1 and retained plasmid pJH2. However, strain JH1-12 had lost the small plasmid, pJH1-1, which is present in strain JH1-5.

Mechanism of transfer. We have attempted to discover the mode of transfer by using tests designed to distinguish between the known transfer mechanisms: conjugation, transformation, and transduction.

(i) Sensitivity of transfer to bacterial filtration. We prepared cell-free filtrates of donor strain JH1-2 by filtering an overnight broth culture either through a membrane filter (type HA, 0.45 μm pore size; Millipore Corp.) or through a Seitz filter (Carlson-Ford, grade HP/EKS) at 37 C. One volume of filtrate was added to nine volumes of an overnight broth culture of recipient strain JH2-2 diluted to 10^8 cells/ml with fresh broth, and incubated with gentle shaking for 4 h before plating to select for donor, recipient, and recipients having acquired antibiotic resistance. The recipient strain did not receive any transferable resistances from either of the filtrates (transfer frequency $< 3 \times 10^{-9}$ per recipient for each filtrate).

(ii) Sensitivity of transfer to chloroform. We treated an overnight broth culture of strain JH1-2 with chloroform by adding 0.2 ml to 10 ml of culture and shaking for 15 min at 37 C. Remaining chloroform was removed by a further incubation for 30 min with occasional vigorous aeration by blowing into the culture via a pipette, and aspirating off the chloroform vapor. The chloroform-treated donor culture was then mixed and incubated with recipient JH2-2 as described above. No transfer of resistances to the recipient was detected (transfer frequency $< 3 \times 10^{-9}$ per recipient). As a control to show whether any residual chloroform would affect transfer, nutrient broth was similarly chloroform treated and added to donor and recipient cultures before they were mixed. The transfer frequency after 4 h was 3.5×10^{-5} per donor, indicating that residual chloroform did not affect transfer.

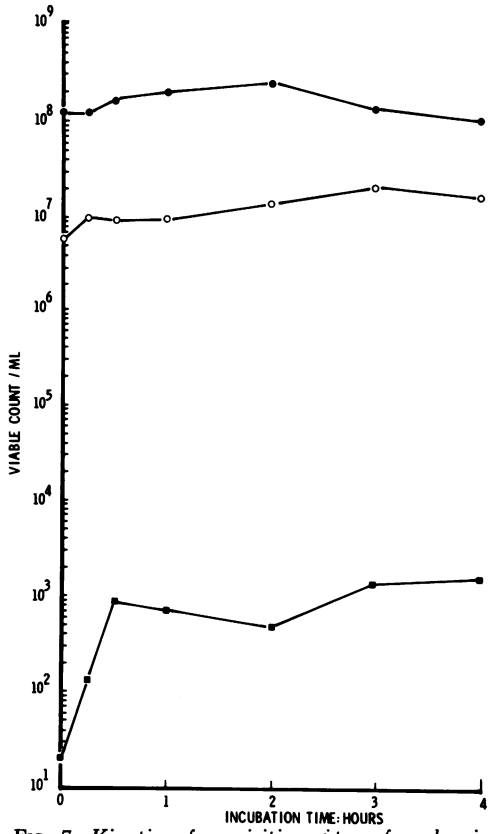

FIG. 7. *Kinetics of acquisition of transferred resistance by strain JH2-2. Eighteen-hour broth cultures of donor strain JH1-2 and recipient strain JH2-2 were diluted to 10^8 cells/ml and mixed in the ratio of 1 donor cell to 10 recipients. The mixed culture was incubated with aeration at 37 C and at the times indicated samples were withdrawn and suitable dilutions were spread onto agar plates to select for donors* (○), *recipients* (●), *and recipients having acquired antibiotic resistance* (■).

(iii) Sensitivity of transfer to deoxyribonuclease I. We incubated an overnight broth culture of strain JH1-2 with 10 μg of DNase I per ml and 5 mM $MgSO_4$ at 37 C for 5 min, before mixing it with a JH2-2 recipient culture similarly treated with DNase I and $MgSO_4$. After 4 h of mixed incubation the transfer frequency in the DNase-treated mixture was 8.6×10^{-5} resistant recipients per donor (1.3×10^{-5} per recipient), whereas that for a control mixed incubation was 7.6×10^{-5} resistant recipients per donor (1.0×10^{-5} per recipient).

In some experiments we included DNase I and $MgSO_4$ in the saline used for cell dilution and in the selective agar plates, to avoid the possibility of transformation occurring on the plates. No difference in the transfer frequency between the test and control mixed cultures was observed.

(iv) Production of bacteriophage by strain JH1-2. We inoculated drops of the cell-free filtrates, the chloroform-treated donor culture, a supernatant of JH1-2 obtained after centrifugation at 20,000 × *g* for 20 min, and a JH1-2 culture grown for 18 h, onto plates overlaid with agar containing a presumptive indicator strain, and incubated overnight at 37 C. The four strains which acted as recipients in resistance transfer (Table 2) were tested as indicators, but no plaques were observed on any.

TABLE 5. *MIC of antibiotics to S. faecalis strains used in antibiotic-resistance transfer experiments*

Strain	MIC of antibiotics (μg/ml)							
	Transferable resistances					Chromosomal resistances[a]		
	Km	Sm	Nm	Tc	Em	Fus	Rif	Gen
JH1-2	40,000	10,000	>40,000	320	5,000	4	1.25	80
JH2-2	40	40	80	1.25	0.125	256	1,280	20
JH2-4	40,000	2,560	>40,000	40	2,500	256	1,280	20

[a] Resistances introduced by mutagenesis or by selection of spontaneous mutants in order to simplify strain selection and recognition.

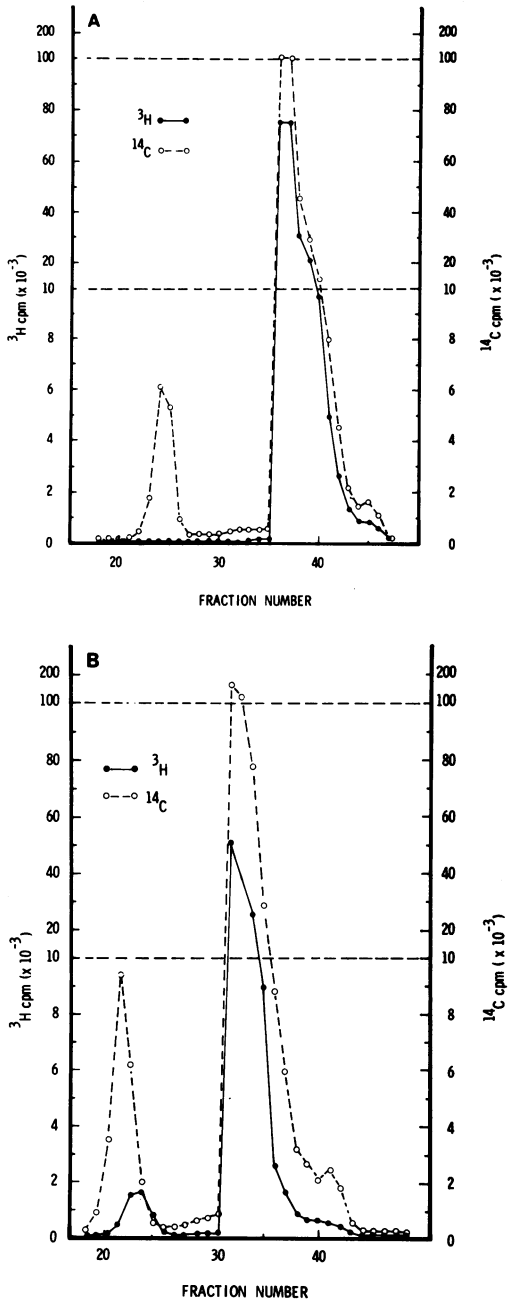

FIG. 8. *Equilibrium centrifugation in CsCl-EB density gradients of DNA from strain JH2-2 and antibiotic resistant strain JH2-4. ^{3}H-thymidine-labeled cells from 4-ml cultures of strains JH2-2 and JH2-4 were each mixed with a 4-ml culture of ^{14}C-thymine-labeled strain JH1-2. After harvesting and washing, the cells were lysed with lysozyme-EDTA and Sarkosyl and the lysates were centrifuged to equilibrium in CsCl-EB gradients. After fractionation and assay for ^{3}H and ^{14}C radioactivity, fractions containing significant radioactivity were plotted as shown. Note the changes in scale at 10^4 and 10^5 counts/min A, ^{3}H-JH2-2 DNA plus ^{14}C-JH1-2 DNA. B, ^{3}H-JH2-4 DNA plus ^{14}C-JH1-2 DNA.*

DISCUSSION

The major conclusion we draw from our data is that the genes determining resistance of *S. faecalis* var. *zymogenes*, strain JH1, to Km, Nm, Sm, Em, and Tc are borne by a transferable plasmid of approximately 50 × 10^6 molecular weight. The criteria we have used are those which have been accepted as proof that many antibiotic resistances of the *Enterobacteriaceae* and *Staphylococcus* are plasmid borne (26). The loss of resistances en bloc from strain JH1 at a high frequency during certain growth conditions (Table 3), and the very high loss during storage of the strain are indicative of plasmid-mediated resistance. The isolation and characterization of DNA species characteristic of plasmids from JH1 (Fig. 1 and 2) and the correlation between loss and attainment of resistance and the absence and presence of one of the plasmid species, pJH1 (Fig. 4 and 9) are all consistent with Novick's criteria and with our interpretation.

We have shown that multiple antibiotic resistance is transferred to a range of *S. faecalis* recipient strains and that the transfer mechanism is not uniquely dependent upon the original host strain, JH1. We interpret our data to infer that the mechanism of transfer is conjugation. By analogy with the conjugal transfer of R factors between the *Enterobacteriaceae*, it would be expected that cell-to-cell contact be-

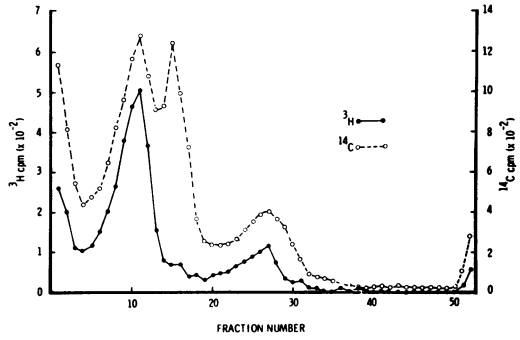

FIG. 9. *Neutral sucrose gradient analysis of plasmid DNA from strain JH2-4. A 0.08-ml amount of plasmid DNA, isolated from a mixed lysate of ^{3}H-labeled JH2-4 and ^{14}C-labeled JH1-2 by using a CsCl-EB density gradient, was layered onto a 5 to 20% sucrose gradient and centrifuged at 100,000 × g at 20 C, for 105 min. Fractions (0.1 ml) were collected, the ^{3}H and ^{14}C radioactivity was assayed, and the results were plotted as shown.*

tween a viable donor and a recipient cell would be essential for conjugation (34). Accordingly, our results showing that cell-free filtrates of a donor culture, or a suspension of nonviable (chloroform-treated) donor cells, do not transfer antibiotic resistance are consistent with it being a conjugal mechanism. The kinetics of acquisition of transferred resistance (Fig. 7) are difficult to interpret since we do not understand why the rate of transfer should markedly decrease after 30 min of mixed incubation. However, such an observation is not inconsistent with a conjugal transfer mechanism since a parallel observation has been noted for the conjugal transfer of several R factors in *E. coli* under similar experimental conditions in which cell division did not occur (10). The alternative interpretation, that the transfer vector is a bacteriophage, seems unlikely. In staphylococci, efficient transfer of antibiotic resistance plasmids in mixed culture has been shown to depend on carriage of a prophage and, in one instance, transducing phage particles are either very unstable or largely bound to the donor cells (24). We have found no evidence for the presence of a prophage in our donor streptococcus. If the donor is lysogenic, the phage produced fails to lyse the strains which act as transfer recipients. Transducing phage particles, if they exist, must be chloroform sensitive and adsorbed by Seitz and Millipore filters. Although we cannot completely rule out the possibility that such a bacteriophage is the streptococcal transfer vector, we believe transfer by conjugation to be the more likely mechanism. Transfer is not by DNA transformation since it is insensitive to DNase I activity, and DNA should not be affected by chloroform—indeed chloroform is often used to remove contaminating protein from DNA to be used as a transforming principle (25).

Analysis of plasmid DNA from an antibiotic-sensitive segregant strain, JH1-5, revealed that it contained a new plasmid species of approximate molecular weight 6.1×10^6, as well as retaining the plasmid of 38×10^6 molecular weight, pJH2 (Fig. 6). We also found that this strain was a very poor recipient for the transfer of plasmid-borne resistances from the parental strain, JH1-2; the transfer frequency per donor being $<0.02\%$ of that when using strain JH1-9 as a recipient. Plasmid analysis of one clone of JH1-5 that had been a successful recipient of antibiotic resistance showed it to have gained plasmid pJH1 but to have lost the 6.1×10^6 molecular weight plasmid. We infer from these data that plasmid pJH1 and the 6.1×10^6 molecular weight plasmid are incompatible. Incompatible plasmids are those which cannot stably coexist within the same host, and incompatibility is a property universally exhibited by plasmids isogenic for those functions, such as the maintenance genes, which are essential for autonomy (20, 26). We suggest that loss of the antibiotic resistances from strain JH1-5 was not the result of the complete loss of pJH1 DNA, but of an extensive DNA deletion from this plasmid. The remnant was the 6.1×10^6 molecular weight plasmid, which must have retained the pJH1 replication and maintenance functions since it was apparently a stable plasmid. The small plasmid was therefore isogenic with pJH1 for the autonomy functions and incompatible with it.

The amount of plasmid DNA isolated from strain JH1 as CCC DNA, expressed as a percentage of isolated chromosomal DNA, was found to range between 2.6 and 6.6% over 17 experiments. This corresponds to between 0.43 and 1.1 copies per chromosome each of plasmids pJH1 and pJH2. The highest value we obtained is still a minimum estimate of the number of plasmid copies per chromosome in vivo since it is most probable that some plasmid DNA exists in the OC form in vivo and undoubtedly we have lost some CCC DNA during isolation. Nevertheless, the near unitary relationship in copy number between the plasmids and chromosome suggests that replication of both plasmids is controlled so as to occur once per cell division cycle, and indicates that they are analogous to those R factors of the *Enterobacteriaceae* whose replication is under stringent control (7).

As separation of plasmid DNA from chromosomal DNA was not achieved by using a cesium chloride density gradient, the buoyant densities of these DNAs must be very similar. A value of 1.697 g/cm^3 has been published for the buoyant density of *S. faecalis* DNA, corresponding to a guanine plus cytosine content of 38% (28). An unexpected observation in many of our cesium chloride-ethidium bromide gradients was the slightly higher buoyant density found for CCC DNA labeled with a high specific radioactivity of [2-^{14}C]thymine relative to the same CCC DNA labeled with [*methyl*-^{3}H]thymidine, as illustrated in Fig. 8B. The density difference was also found with ^{14}C-labeled CCC DNA and ^{3}H-labeled CCC DNA isolated from isogenic hosts, and was apparently specific for the CCC DNA. We have no explanation for these observations.

The discovery of transferable plasmid-borne antibiotic resistance in *S. faecalis* may afford an explanation for the great increase in antibiotic resistance of group D streptococci (32). We shall

be testing this hypothesis by examining a range of group D streptococcal species from our collection for the presence of such plasmids, and we also anticipate examining the mechanisms of resistance.

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LITERATURE CITED

1. Andrew, M. H. E. 1973. Use of trimethoprim to obtain thymine-requiring mutants of *Streptococcus faecalis*. J. Gen. Microbiol. **74**:195-199.
2. Bak, A. L., C. Christiansen, and A. Stenderup. 1970. Bacterial genome sizes determined by DNA renaturation studies. J. Gen. Microbiol. **64**:377-380.
3. Baxter-Gabbard, K. L. 1972. A simple method for the large scale preparation of sucrose gradients. FEBS Lett. **20**:117-119.
4. Bazaral, M., and D. R. Helinski. 1968. Characterisation of multiple circular DNA forms of colicinogenic factor E1 from *Proteus mirabilis*. Biochemistry **7**:3513-3519.
5. Bazaral, M., and D. R. Helinski. 1968. Circular DNA forms of colicinogenic factors E1, E2, and E3 from *Escherichia coli*. J. Mol. Biol. **36**:185-194.
6. Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. Biophys. J. **3**:309-321.
7. Clowes, R. C. 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. **36**:361-405.
8. Courvalin, P. M., C. Carlier, and Y. A. Chabbert. 1972. Plasmid-linked tetracycline and erythromycin resistance in group D Streptococcus. Ann. Inst. Pasteur **123**:755-759.
9. Cuzin, F., M. Vogt, M. Dieckmann, and P. Berg. 1970. Induction of virus multiplication in 3T3 cells transformed by a thermosensitive mutant of polyoma virus. II. Formation of oligomeric polyoma DNA molecules. J. Mol. Biol. **47**:317-333.
10. Datta, N., A. M. Lawn, and E. Meynell. 1966. The relationship of F type piliation and F phage sensitivity to drug resistance transfer in R^+F^- *Escherichia coli* K12. J. Gen. Microbiol. **45**:365-376.
11. Davidson, N., and W. Szybalski. 1971. Physical and chemical characteristics of lambda DNA, p. 45-82. *In* A. D. Hershey (ed.), The bacteriophage lambda. Cold Spring Harbor Laboratories, New York.
12. Freifelder, D. 1970. Molecular weights of coliphages and coliphage DNA. IV. Molecular weights of DNA from bacteriophages T4, T5 and T7 and the general problem of determination of M. J. Mol. Biol. **54**:567-577.
13. Fuller, A. T. 1938. The formamide method for the extraction of polysaccharides from hemolytic streptococci. Brit. J. Exp. Pathol. **19**:130-139.
14. Garrod, L. P., and F. O'Grady. 1968. Antibiotic and chemotherapy. E. and S. Livingstone Ltd., Edinburgh.
15. Helinski, D. R., and D. B. Clewell. 1971. Circular DNA. Annu. Rev. Biochem. **40**:889-942.
16. Hershey, A. D., E. Burgi, and L. Ingraham. 1963. Cohesion of DNA molecules isolated from phage lambda. Proc. Nat. Acad. Sci. U.S.A. **49**:748-755.
17. Hickson, F. T., T. F. Roth, and D. R. Helinski. 1967. Circular DNA forms of a bacterial sex factor. Proc. Nat. Acad. Sci. U.S.A. **58**:1731-1738.
18. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **46**:57-64.
19. Hudson, B., D. A. Clayton, and J. Vinograd. 1968. Complex mitochondrial DNA. Cold Spring Harbor Symp. Quant. Biol. **33**:435-442.
20. Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**:329-348.
21. Kaiser, A. D. 1962. The production of phage chromosome fragments and their capacity for genetic transfer. J. Mol. Biol. **4**:275-287.
22. Korn, D., and A. Weissbach. 1962. Thymineless induction in *Escherichia coli* K12 (λ). Biochim. Biophys. Acta **61**:775-790.
23. Kornberg, A., S. B. Zimmerman, S. R. Kornberg, and J. Josse. 1959. Enzymatic synthesis of DNA. VI. Influence of bacteriophage T2 on the synthetic pathway of host cells. Proc. Nat. Acad. Sci. U.S.A. **45**:772-785.
24. Lacey, R. W. 1971. Transfer of tetracycline-resistance between strains of *Staphylococcus aureus* in mixed culture. J. Gen. Microbiol. **69**:229-237.
25. Marmur, J. 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. **3**:208-218.
26. Novick, R. P. 1969. Extrachromosomal inheritance in bacteria. Bacteriol. Rev. **33**:210-257.
27. Raycroft, R. E., and L. N. Zimmerman. 1964. A new mode of genetic transfer in *Streptococcus faecalis* var. *liquefaciens*. J. Bacteriol. **87**:799-801.
28. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of DNA from its buoyant density in CsCl. J. Mol. Biol. **4**:430-443.
29. Sharpe, M. E., T. F. Fryer, and D. G. Smith. 1966. Identification of the lactic acid bacteria, p. 65-79. *In* Gibbs, B. M., and F. A. Skinner (ed.), Identification methods for microbiologists, part A. Academic Press Inc., London.
30. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307-311.
31. Tan, K. B. 1972. A simple device for fractionating density gradients. Anal. Biochem. **45**:306-308.
32. Toala, P., A. McDonald, C. Wilcox, and M. Finland, 1969. Susceptibility of group D streptococcus (enterococcus) to 21 antibiotics *in vitro* with special reference to species differences. Amer. J. Med. Sci. **258**:416-430.
33. Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis. 1965. The twisted circular forms of polyoma viral DNA. Proc. Nat. Acad. Sci. U.S.A. **53**:1104-1111.
34. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. **27**:87-115.
35. Young, E. T., and R. L. Sinsheimer. 1967. Vegetative bacteriophage λ DNA I. Infectivity in a spheroplast assay. J. Mol. Biol. **30**:147-164.