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Characterization of Two Tetracycline Resistance Determinants in *Streptococcus faecalis* JH1

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Streptococcus faecalis strain JH1 harbors two conjugative plasmids: pJH1, an R plasmid mediating resistance to kanamycin, streptomycin, gentamicin, erythromycin, and tetracycline, and pJH2, a hemolysin-bacteriocin plasmid. Studies of plasmid-cured derivatives of strain JH1 and of transconjugants obtained after mixed incubation of JH1 with the plasmid-free *S. faecalis* strain JH2-2 revealed the presence of two tetracycline resistance determinants in strain JH1. One determinant mediated constitutive resistance to 40 μg of tetracycline per ml and was associated with plasmid pJH1. The second determinant, either on the chromosome of strain JH1 or on an undetectable plasmid, was inducible by tetracycline and enabled the host strain, in the absence of pJH1, to grow in the presence of 80 μg of tetracycline per ml. One transconjugant, strain DL172, was resistant to 80 μg of tetracycline per ml, sensitive to kanamycin, streptomycin, and erythromycin, and hemolytic in the presence, but not in the absence, of tetracycline. A single plasmid, pDL172, from this strain consisted of plasmid pJH2 and a 17.8-kilobase segment of DNA homologous to total cell DNA from strain JH1 but did not contain plasmid pJH1. Whether the addition of heterologous DNA to plasmid pJH2 occurred by translocation of a 17.8-kilobase tetracycline resistance transposon or by classical recombination with pJH2 has not been determined.

Jacob and co-workers described the conjugal transfer of multiple antibiotic resistance, hemolysin production, and bacteriocin resistance, as well as production, from *Streptococcus faecalis* strain JH1 to *S. faecalis* strain JH2-2 (7, 8). Transconjugant clones were shown to harbor either plasmid pJH1 (multiple antibiotic resistance; see reference 8) or pJH2 (hemolysin-bacteriocin; see reference 7). These transconjugants were in turn able to transfer the plasmids and their associated traits to secondary recipients (7, 8). Because all of the resistance traits associated with strain JH1 were simultaneously transferred to strains receiving plasmid pJH1, it was clear that these properties were plasmid borne. However, the resistance of the original donor, strain JH1, to tetracycline was only slightly reduced when plasmid pJH1 was lost. It was suggested that strain JH1 might have a "natural" resistance to tetracycline (8).

We report here the existence and properties of two different tetracycline resistance determinants in *S. faecalis* strain JH1. One determinant was associated with plasmid pJH1, and the other appeared to be integrated into the chromosome of the host strain, although the presence of a third plasmid in strain JH1, refractile to isolation and carrying the second tetracycline resistance determinant, could not be ruled out. There was no detectable homology between the two determinants, and the regulation and levels of resistance mediated by each were different. One transconjugant of strain JH2-2, selected for resistance to tetracycline, contained a plasmid that was composed of plasmid pJH2 and a fragment of strain JH1 DNA containing the tetracycline resistance determinant. Unlike other transconjugants harboring plasmid pJH2, this isolate did not express hemolytic activity unless tetracycline was incorporated into the growth medium.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptococcus faecalis* subsp. *zymogenes* JH1 habors two transmissible plasmids, pJH1 and pJH2, and is sensitive to less than 5 μg of fusidic acid and rifampin per ml (7, 8). Plasmid pJH1 codes for resistance to kanamycin, streptomycin, neomycin, erythromycin, and tetracycline (8), whereas pJH2 mediates the production of a hemolysin (Hly^+) which causes beta-hemolysis of horse, but not sheep, erythrocytes and also has bacteriocin activity against several gram-positive organisms (7). Resistance to the bacteriocin is also mediated by pJH2 (7). *S. faecalis* JH2-2 is devoid of any detectable plasmids, is resistant to >25 μg of Fus and Rif per ml, and can

serve as a recipient for plasmids pJH1 and pJH2 from strain JH1 in conjugation experiments (8). All other bacterial strains used in this study are described below, and their relevant properties are listed in Table 2.

Culture conditions. Bacterial cultures were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) or in BHI supplemented with additional (10 mM) D-glucose (BHIG). Incubation was without aeration except in the conjugation experiments described below. Solid BHIG contained 1.5% agar (Difco). Blood agar consisted of 5% defibrinated horse blood or sheep blood in Columbia agar base medium (BBL Microbiology Systems, Cockeysville, Md.). All incubations were done at 37°C.

Conjugation experiments. Donor (JH1) and recipient (JH2-2) strains were prepared for conjugation by transferring 0.1 ml of an exponentially growing culture to 10 ml of BHIG in a 125-ml-capacity screw-capped bottle and incubating it for 18 h with shaking (125 rpm; Aquatherm; New Brunswick Scientific Co., New Brunswick, N.J.). For broth matings, 0.1 ml of 18-h donor culture was mixed with 1 ml of 18-h recipient culture in a total volume of 10 ml of BHIG and incubated for 3 h with gentle shaking (100 rpm) in a 125-ml-capacity bottle. Filter matings were initiated by adding 0.2 ml each of the 18-h donor and recipient cultures to 1.6 ml of BHIG, mixing, and collecting the combined cultures on 0.45-μm filters (HAP; Millipore Corp., Bedford, Mass.). Filters were transferred, right-side up, to sheep blood agar and incubated for 3 h. Controls for both conjugation systems consisted of donor or recipient cells alone treated in the same manner as the mixed cultures. Conjugation frequencies and determinations of donor and recipient colony-forming units (CFU) at the end of the mating period were obtained by spreading dilutions on the appropriate selective media. For dilutions of the filter mating system, cells were first removed from the filters by blending in 2 ml of BHI in a Vortex mixer.

Donor cells were counterselected on solid medium containing fusidic acid and rifampin, each at 25 μg/ml. Transfer of hemolytic activity was determined on fusidic acid-rifampin horse blood agar. Medium for selecting tetracycline-resistant transconjugants, either BHIG or horse blood agar, contained 25 μg of tetracycline per ml in addition to fusidic acid and rifampin. All selection plates were scored after 24 and 48 h of incubation. Medium for detecting transconjugants resistant to other antibiotics contained 25 μg of Em, 1 mg of Sm, or 2 mg of Km per ml.

Plasmid isolation and purification. The acquisition of plasmid DNA after conjugation or the loss of plasmid DNA by spontaneous curing was confirmed by a rapid screening procedure for the detection of plasmids in streptococci (13).

Large quantities of purified plasmid DNA for restriction endonuclease digestions and hybridization experiments were obtained from 800- or 1600-ml cultures as follows. Cultures were grown to the midexponential phase in BHIG containing 10 mM L-threonine (1) and 0.05 μCi of [*methyl*-^{14}C]thymidine per ml (50 mCi/mmol; New England Nuclear Corp., Boston, Mass.). Cells were harvested by centrifugation and washed with an equal volume of 0.01 M sodium phosphate buffer (pH 7.0). After suspension in 50 mM Tris–10 mM EDTA (pH 8.2), at one-half the original culture volume, cells were prepared for lysis by incubation for 15 min in lysozyme (Sigma Chemical Co., St. Louis, Mo.) at 2 mg/ml. Lysozyme was removed by centrifugation, and the cells were resuspended in 50 mM Tris–10 mM EDTA at one-eighth the original culture volume. Lysis was accomplished in 4% sodium dodecyl sulfate at 55°C for 5 min. The lysate was enriched for plasmid DNA by shearing and NaCl-saturated phenol extraction according to Currier and Nester (4). Covalently closed circular plasmid DNA was separated from nicked plasmid and residual chromosomal DNA by dye buoyant density gradient centrifugation, as previously described (11). The isolated plasmid band, after extraction of ethidium bromide and removal of cesium chloride by dialysis (11), was further purified by a second banding in a dye buoyant density gradient, and dye and cesium chloride were again removed. This DNA was precipitated by the addition of 0.05 volume of 3 M ammonium acetate and 2 volumes of 95% ethanol. Precipitated DNA was stored at −20°C until needed. Plasmid yields by this procedure were between 30 and 40 μg from 800 ml of culture.

Purification of total cellular DNA. For the preparation of total cell DNA, 20-ml cultures were incubated overnight in the same medium as for the plasmid purification. Cells were harvested, washed, lysed, and the DNA sheared in the same manner as for the plasmid purification. DNA was extracted from sheared lysates with an equal volume of Tris-hydrochloride-saturated phenol (pH 8.0), the interface and phenol phase were washed with one-half volume of 50 mM Tris-10 mM EDTA, and the pooled aqueous phases were further extracted with an equal volume of chloroform-isoamyl alcohol (24:1). After the DNA was precipitated as described above, it was resuspended in 10 mM Tris-hydrochloride–10 mM EDTA–50 mM NaCl (pH 8.0). To 9 ml of DNA in this buffer, 10.61 g of cesium chloride was added and centrifuged to equilibrium in a 50 Ti rotor (Beckman Instruments Inc., Fullerton, Calif.) at 35,000 rpm and 20°C for 64 h. The gradient was fractionated, and aliquots were counted as previously described (11). Fractions containing the DNA were pooled and dialyzed against the buffer. Proteinase K (Sigma) was added to a final concentration of 0.5 mg/ml. After incubation for 90 min at 37°C, the DNA was extracted with Tris-hydrochloride-saturated phenol and chloroform-isoamyl alcohol and precipitated with ethanol as described above. Precipitated DNA was stored at −20°C until needed.

Restriction endonuclease digestion and agarose gel electrophoresis. *Eco*RI endonuclease (Bethesda Research Laboratories, Rockville, Md.) digestions were carried out on purified plasmid DNA or total cell DNA, according to the instructions of the supplier, at a ratio of 20 U of enzyme to 1 μg of DNA. All reactions were incubated at 37°C for 60 min and were terminated by the addition of stop mix (Bethesda Research Laboratories).

Restriction fragments were separated on vertical 0.8% agarose slab gels in Tris-acetate buffer (12) at 60 mA and 80 V for 4 to 5 h. *Eco*RI, *Hin*dIII, and *Kpn*I digests of phage λ DNA were included in some gels as molecular weight markers. Gels were stained overnight in 2 liters of water containing 0.5 μg of ethidium bromide per ml. Bands were viewed with a long-wavelength lamp (Ultraviolet Products, San Gabriel,

Calif.) and photographed with a Polaroid MP-4 camera with type 57 film. Total plasmid DNA isolated by the rapid screening procedure was separated into bands in 0.7% agarose gels under the same conditions as described above.

Appropriate restriction endonuclease fragments were extracted from agarose gels by the method of Vogelstein and Gillespie (16).

DNA blotting and hybridization. Fragments from *Eco*RI digests of plasmid DNA or total cell DNA were transferred from agarose gels to sheets of nitrocellulose paper by the method of Southern (15). The DNA in the gels was denatured by soaking in 0.5 M NaOH–1.5 M NaCl for 2 h and then neutralized for 2 h in 0.5 M Tris-hydrochloride–3.0 M NaCl (pH 7.0). The DNA was transferred to nitrocellulose paper in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight. Blots were air dried for 1 h and then baked at 80°C for 1 to 2 h. Before hybridization, the blots were soaked in 3× SSC containing 0.08% bovine serum albumin, 0.08% polyvinylpyrrolidone, 0.08% Ficoll 400, and 10 μg of whole yeast RNA per ml (preincubation buffer) at 67°C for 4 h. Whole plasmid DNA or *Eco*RI fragments were labeled in vitro by nick translation (14) with a translation kit (Bethesda Research Laboratories) and ^{32}P-labeled dATP and dGTP (Amersham Corp., Arlington Heights, Ill.). Heat-denatured (100°C for 5 min) ^{32}P-labeled probe DNA (approximately 10^7 cpm) was hybridized with nitrocellulose blots in 30 ml of preincubation buffer containing unlabeled, sheared (300 to 500 base pairs) salmon sperm DNA (10 μg/ml) for 18 h at 67°C in heat-sealed plastic bags (Scotch Kapak; 3M Co. St. Paul, Minn.). The nitrocellulose sheet was then washed three times in 100 ml of 0.1× SSC–0.1% sodium dodecyl sulfate at 52°C for 30 min and three times in 0.1× SSC under the same conditions. After drying for 2 h at 80°C, the hybridized blot was placed against Kodak XR-2 film to produce an autoradiogram.

Induction of tetracycline resistance. The regulation (inducible or constitutive) of tetracycline resistance was determined as follows. A tetracycline-resistant (Tc^r) strain, growing exponentially in BHIG, was divided into three subcultures, A, B, and C. Tetracycline was added to B at a final concentration of 25 or 50 μg/ml (challenge culture) and to C at 1 μg/ml (induction culture), and A received no antibiotic (unchallenged control). A and B were incubated, and their growth (optical density at 660 nm) was monitored at 10-min intervals for 90 min to determine uninduced (time 0 [T_0]) growth rates in the presence and absence of tetracycline challenge. Subculture C was incubated for 4 h and diluted periodically with BHIG containing 1 μg of tetracycline per ml to maintain exponential growth. This culture was then divided into subcultures D and E, which were treated in the same manner as subcultures A and B, respectively, to establish induced (4 h [T_4]) growth rates in the presence and absence of tetracycline challenge. The difference between the ratio of growth rate (doubling of optical density at 660 nm per h) of challenged culture to growth rate of unchallenged culture, at T_0 and T_4, was taken as a measure of induction of tetracycline resistance. Because T_0 challenged cultures were unaffected by tetracycline during the first 20 min of incubation and because the growth rates of challenged, inducible strains at T_0 increased after 60 min of incubation, all doubling times were calculated from growth curves between 20 and 60 min of incubation.

Determination of antibiotic resistance levels and hemolysin production. The levels of resistance of the *S. faecalis* strains to kanamycin, streptomycin, erythromycin, and tetracycline were determined as follows. Plates were poured with BHIG agar containing increasing concentrations of kanamycin or streptomycin at 250, 500, 1,000, 2,000, 5,000, 10,000, and 20,000 μg/ml and of erythromycin at 1, 2, 4, 8, 10, 25, 50, 100, 500, 1,000, and 2,000 μg/ml and doubling concentrations of tetracycline from 1 to 320 μg/ml. Cell suspensions (10 μl) containing approximately 100 CFUs from an exponential-phase BHIG culture were spotted on each of the above plates. To maintain identical conditions, we spotted all strains being compared on the same plate at each concentration of antibiotic. All plates were incubated at 37°C, and the highest concentration of antibiotic permitting the appearance of at least one colony in 18 to 36 h was taken as the level of resistance for each strain.

Beta-hemolysis on horse blood agar was variable unless assayed in the same manner each time. An overnight culture (1 ml) in BHI was transferred to 9 ml of BHIG in a 15-ml-capacity screw-capped tube. Incubation was continued until the culture reached an optical density at 660 nm of 0.5 (1×10^8 to 2×10^8 CFU/ml). A 0.1-ml portion of a 10^{-5} dilution of the culture in BHI was spread on a horse blood agar plate, with or without 25 μg of tetracycline or erythromycin per ml or 1 mg of kanamycin or streptomycin per ml, incubated for 18 to 24 h, and scored for zones of total clearing around colonies.

RESULTS

Conjugation experiments. We attempted to separate the two plasmids, pJH1 and pJH2, harbored by *S. faecalis* strain JH1 by transfer into a plasmid-free strain, JH2-2, during mixed incubation in broth culture. Transconjugants that received pJH2 could be scored as hemolytic colonies on horse blood agar containing fusidic acid and rifampin as counterselective markers. The results suggested that the Hly^+ phenotype was readily transmissible to strain JH2-2 at a frequency of 5.2×10^{-3} per donor CFU (Table 1). Several colonies were picked from the selection plates and tested for the expression of phenotypic traits associated with pJH1 (resistance to kanamycin, streptomycin, erythromycin, and tetracycline) and pJH2 (Hly^+) and for plasmid content. All of these Hly^+ isolates were sensitive to the four antibiotics and contained a single 35-megadalton plasmid that migrated at the same rate as pJH2 from strain JH1 in agarose gels.

Transconjugants that received only pJH1 would be expected to produce nonhemolytic colonies on horse blood agar containing fusidic acid, rifampin, and tetracycline. However, in medium containing tetracycline, only Hly^+ colonies were detected, at a frequency of 6.9×10^{-5}.

TABLE 1. Conjugation experiments between *S. faecalis* strains JH1 and JH2-2

Conjugation Conditions	Antibiotic(s) in selective medium[a]	Hly phenotype observed	Frequency of conjugation	Properties of transconjugants[b]	Plasmids present (megadaltons)
Broth culture	Fusidic acid and rifampin	Hly^+	5.2×10^{-3}	Hly^+	35
Broth culture	Tetracycline, fusidic acid, and rifampin	Hly^-	$<1.5 \times 10^{-8}$		
Broth culture	Tetracycline, fusidic acid, and rifampin	Hly^+	6.9×10^{-5}	Hly^+, multiply resistant Hly^+, multiply resistant Hly^+, Tc^r	35 and 50 ~85 ~45
Membrane filters	Fusidic acid and rifampin	Hly^+	2.4×10^{-3}	Hly^+	35
Membrane filters	Tetracycline, fusidic acid, and rifampin	Hly^-	2.0×10^{-6}	Hly^-, multiply resistant	50
Membrane filters	Tetracycline, fusidic acid, and rifampin	Hly^+	1.9×10^{-6}	Hly^+, multiply resistant Hly^+, multiply resistant	35 and 50 ~85

[a] Antibiotics were added to horse blood agar at concentrations described in the text.
[b] Multiply resistant, resistant to kanamycin, streptomycin, erythromycin, and tetracycline.

This result suggested that simultaneous transfer of the hemolysin plasmid was required to detect transfer of the resistance plasmid. Of 96 Hly^+, Tc^r colonies examined, 95 were also resistant to kanamycin, streptomycin, and erythromycin. Twenty-four of these multiply resistant, Hly^+ isolates were screened for plasmid content. Twenty-two isolates contained two plasmids each with sizes corresponding to pJH1 and pJH2, and two isolates each had a single plasmid of approximately 85 megadaltons. The one Hly^+, Tc^r isolate that remained sensitive to kanamycin, streptomycin, and erythromycin (strain DL172) contained a single plasmid that was intermediate in size between pJH1 and pJH2.

We suspected that since the production of and resistance to a bacteriocin to which strain JH2-2 is sensitive (7) is mediated by pJH2, such production by strain JH1 during incubation in broth culture may have resulted in the killing of virtually all pJH1-containing transconjugants of JH2-2 which had not also received pJH2 or a recombinant molecule composed of pJH1 plus pJH2. This interpretation was supported by the observation that the viable population of JH2-2 in the mixed culture had decreased by nearly two orders of magnitude from the beginning of incubation (1.4×10^8 CFU) to the end (1.7×10^6 CFU). Because of previous success in the transfer of other plasmids by mixed incubation on membrane filters (10, 12) and because of the assumption that any bacteriocin produced would be diluted by diffusion into the supporting agar base, we repeated the above conjugation experiment with filters in place of broth culture. The frequencies of pJH2 transfer were similar in the two different systems (Table 1). With membrane filters, however, more than half of the multiply resistant isolates obtained on tetracycline-containing selective medium were nonhemolytic and harbored a single plasmid of the same size as pJH1. In this system, the population of JH2-2 actually increased from the beginning of mixed incubation to the end, from 1.0×10^8 CFU to 3.0×10^8 CFU.

All of the multiply resistant, Hly^+ transconjugants obtained by either conjugation system were beta-hemolytic on horse blood agar in the presence or absence of antibiotic. Strain DL172, the single Hly^+, Tc^r isolate that was obtained from the broth culture and harbored a plasmid intermediate in size between pJH1 and pJH2, was hemolytic only if tetracycline was added to horse blood agar (Fig. 1).

The transconjugants obtained from these experiments were used to provide evidence for the existence of two different tetracycline resistance determinants in strain JH1 and to study their regulation.

Evidence for two tetracycline resistance determinants in strain JH1. *S. faecalis* strains are naturally resistant to relatively high levels of aminoglycoside antibiotics such as kanamycin and streptomycin, but are highly sensitive to macrolides, such as erythromycin, and to tetracyclines. The natural response, in vitro, of *S. faecalis* isolates devoid of R plasmids is typified by strain JH2-2 (Table 2). Many strains of *S. faecalis* that have been recently isolated from clinical sources have demonstrated high levels of resistance to tetracycline and erythromycin

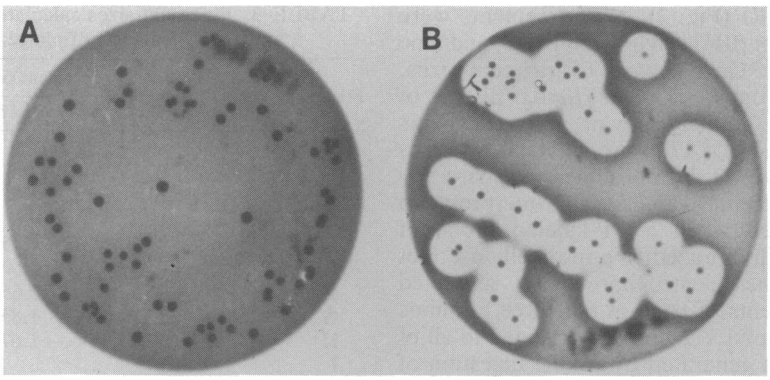

FIG. 1. Hemolytic activity of *S. faecalis* strain DL172 in the presence and absence of tetracycline. Colonies were obtained on Columbia agar base medium containing 5% horse blood without (A) and with (B) tetracycline (25 μg/ml). Incubation conditions were as described in the text.

and resistance to the aminoglycosides considerably higher than normal (2, 3, 9). This has clearly resulted from the presence of R plasmids in these isolates and is exemplified by strain JH1. The increased resistance of strain JH1 to kanamycin, streptomycin, and erythromycin was shown by Jacob and Hobbs to be due entirely to plasmid pJH1, but the resistance of this strain to tetracycline was only partially explained by the presence of this plasmid (8). We have confirmed the results of these authors, relative to tetracycline resistance, and expanded them as illustrated in Table 2 and by subsequent experiments described below. As seen in Table 2, a transconjugant of JH2-2 which received pJH1, strain DL77, was able to grow in the presence of the same levels of kanamycin, streptomycin, and erythromycin as the donor strain, JH1, but was considerably less resistant to tetracycline. Plasmid pJH2 could not account for the additional level of tetracycline resistance, because transconjugant strain DL76 was equally sensitive to this antibiotic as strain JH2-2. In addition, the different levels of resistance to tetracycline could not be explained on the basis of strain differences for expression of pJH1-determined resistance, because an isolate of JH1 cured of plasmid pJH1, strain DL75, was still resistant to 80 μg of tetracycline per ml. Furthermore, the level of tetracycline resistance of strain DL172 was the same as that expressed by strain DL75. This result suggested that the tetracycline resistance determinant in strain DL172 was not acquired from pJH1.

Origin of the tetracycline resistance determinant in strain DL172. Plasmids pJH1, pJH2, and pDL172 were isolated from strains DL77, DL76, and DL172, respectively. After purification by two bandings in dye buoyant density gradients, each plasmid was digested with restriction endo-

TABLE 2. Properties of *S. faecalis* strains used to provide evidence for the existence of two tetracycline resistance determinants in strain JH1

Strain	Level of resistance (μg/ml) to:				Hemolysis		Plasmids present	Reference or derivation
	Kanamycin	Streptomycin	Erythromycin	Tetracycline	Without tetracycline	With tetracycline		
JH2-2	1,000	500	1	<1	−	NG[a]	None	8
JH1	10,000	10,000	1,000	160	+	+	pJH1, pJH2	7 and 8
DL77	10,000	10,000	1,000	40	−	−	pJH1	JH1 × JH2-2 transconjugant (this paper)
DL76	1,000	500	1	<1	+	NG[a]	pJH2	JH1 × JH2-2 transconjugant (this paper)
DL75	250	250	1	80	+	+	pJH2	JH1 derivative missing pJH1 (this paper)
DL172	1,000	500	<1	80	−	+	pDL172	JH1 × JH2-2 transconjugant (this paper)

[a] NG, No growth.

nuclease *Eco*RI (Fig. 2). Nine fragments were produced from pJH1, eight from pJH2, and nine from pDL172. On the basis of fragment sizes, determined from *Eco*RI and *Hin*dIII digests of phage λ DNA (data not shown), seven fragments from pJH2 and pDL172 were identical, whereas pJH1 and pDL172 did not appear to share any common fragments (Table 3). This interpretation was confirmed when a ^{32}P-labeled probe of pDL172 was hybridized to a nitrocellulose blot containing the electrophoretically separated *Eco*RI fragments of each of the three plasmids (Fig. 3). The pDL172 probe hybridized to all of the fragments generated by *Eco*RI digestion of pJH2, but it shared absolutely no homology with pJH1. The results illustrated in Fig. 2 and 3 suggested that a segment of heterologous DNA containing a tetracycline resistance determinant had inserted into *Eco*RI fragment D of pJH2. This heterologous DNA, 17.8 kilobases (kb) in size and containing a single internal *Eco*RI site, was present in the *Eco*RI fragments A* and D of pDL172. It was also clear from these results that

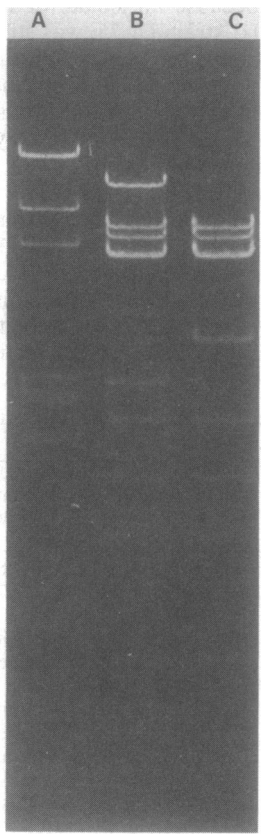

FIG. 2. *Eco*RI digests of plasmids pJH1 (A), pJH2 (C), and pDL172 (B). Fragment patterns were obtained by agarose gel electrophoresis.

TABLE 3. Fragment sizes calculated from *Eco*RI and *Hin*dIII digests of phage λ DNA

Fragment	Fragment size (kb)		
	pJH1	pDL172	pJH2
A*		19.0	
A	27.0	12.5	12.5
B	15.0	11.2	11.2
C	11.0	9.6	9.6
D	4.45	4.25	5.45
E	4.05	3.50	3.50
F	3.95	2.65	2.65
G	3.25	1.85	1.85
H	1.95	1.67	1.67
I	1.87		

the tetracycline resistance determinant was not derived from pJH1. For determining the origin of the inserted DNA, the *Eco*RI fragment A* of pDL172, which had to include most of the heterologous DNA, was extracted from an agarose gel (16) and labeled with ^{32}P (14) for use as a probe in hybridization reactions with blotted *Eco*RI digests of pJH1, pJH2, and pDL172 and of total cellular DNA from strains JH1, JH2-2, and DL75 (Fig. 4). As expected, the ^{32}P-labeled A* fragment hybridized to the A* fragment of pDL172 and to the D fragment of pJH2, but it did not have any detectable homology with pJH1. *Eco*RI digests of total cellular DNA from strains JH1 and DL75, a derivative of JH1 which had lost pJH1, both exhibited homology with fragment A* in an area of the blot equivalent to a fragment of 23 kb. There was no detectable homology between this pDL172 fragment and the *Eco*RI digest of DNA from strain JH2-2. These results suggested that the heterologous DNA in pDL172 originated from a unique site on the chromosome of strain JH1 or from an undetectable plasmid in this strain.

Regulation of Tc resistance in strains DL77 and DL172. Strain DL172 expressed hemolytic activity only in the presence of tetracycline. This requirement suggested that resistance to tetracycline must be an inducible property of this strain. We examined the regulation of tetracycline resistance in strain DL172, as well as in the pJH1-containing transconjugant, strain DL77 (Table 4). The growth rate of an uninduced DL172 culture challenged with 25 μg of Tc per ml was approximately one-third that of an unchallenged control culture. This was improved nearly 2.5 times after a 4-h induction period in the presence of a subinhibitory concentration (1 μg/ml) of tetracycline. The improvement in the growth rate after induction was even more dramatic, greater than seven-fold, when the challenge concentration of tetracycline was increased to 50 μg/ml. These results clearly

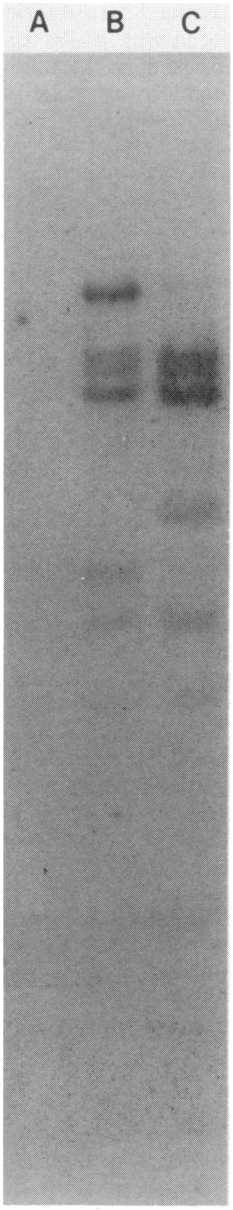

FIG. 3. Autoradiogram of *Eco*RI digests of plasmids pJH1, pJH2, and pDL172 hybridized to ^{32}P-labeled pDL172 DNA. Blots contained DNA from agarose gel electrophoresed with 0.5 μg of digested pJH1 (lane A), pDL172 (lane B), and pJH2 (lane C). Digestion, electrophoresis, blotting, ^{32}P-labeling, and hybridization conditions were as described in the text.

suggested that tetracycline resistance in strain DL172 was an inducible property. In contrast, the growth rate of strain DL77, which contained the tetracycline resistance determinant associated with pJH1, did not change significantly in the presence of 25 μg of tetracycline of per ml after induction. This strain did not grow at all in the presence of 50 μg of tetracycline per ml either before or after induction, a result consistent with the level of resistance determined on solid media. Plasmid pJH1 thus appears to mediate tetracycline resistance constitutively. The behavior of strain DL75, a derivative of JH1 which had lost pJH1 but retained the chromosomal tetracy-

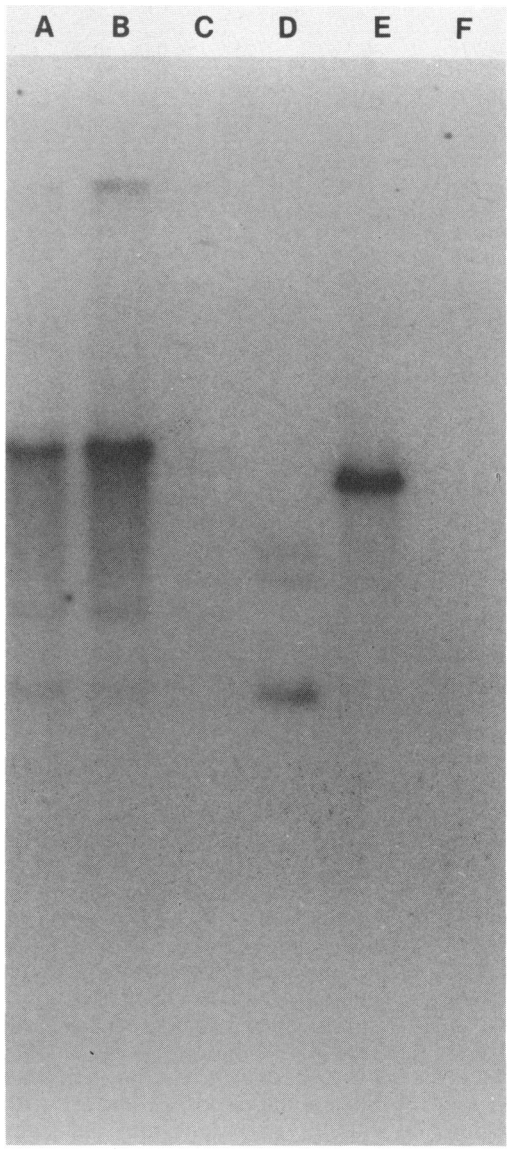

FIG. 4. Autoradiogram of *Eco*RI digests of plasmids and cellular DNA hybridized to ^{32}P-labeled fragment A* from pDL172. All experimental conditions were as described in the text. *Eco*RI-digested cellular DNA was obtained from strains JH1 (lane A), DL75 (lane B), and JH2-2 (lane C). Plasmid digests were pJH2 (lane D), pDL172 (lane E), and pJH1 (lane F).

TABLE 4. Regulation of tetracycline resistance in *S. faecalis* strains DL77(pJH1) and DL172(pDL172)[a]

Strain	Level of resistance to tetracycline (μg/ml)	Tetracycline challenge (μg/ml)	Ratio of growth rate of challenged culture to growth rate of unchallenged culture at[b]		T_4/T_0
			T_0	T_4	
DL77	40	25	0.08	0.09	1.06
		50	0	0	
DL172	80	25	0.31	0.73	2.35
		50	0.04	0.28	7.06

[a] Induction experiments were performed as described in the text.

[b] Growth rates (as doubling times per hour) ranged from 1.08 to 1.29 for strain DL77 and from 0.92 to 1.18 for strain DL172.

cline resistance determinant, was similar to that of strain DL172 in that resistance was inducible (data not shown).

DISCUSSION

We conclude from the data presented in this communication that *S. faecalis* JH1 contains two different determinants coding for resistance to tetracycline. One of these mediates constitutive resistance to 40 μg of tetracycline per ml and is associated with the R plasmid, pJH1. The second determinant, on the chromosome of strain JH1 or on an undetectable plasmid, is inducible by tetracycline and permits a derivative of strain JH1 that is missing pJH1 to grow in the presence of 80 μg of the antibiotic per ml. This latter determinant can also become associated with plasmid pJH2, either by transposition or classical recombination and, in the example studied here, can alter the regulation of pJH2-mediated hemolytic activity.

Jacob and Hobbs were able to transfer plasmid pJH1, independent of plasmid pJH2, from strain JH1 to antibiotic-sensitive strains of *S. faecalis* and from primary recipients to a second recipient by mixed incubation in broth culture (8). When we incubated mixed cultures of strains JH1 and JH2-2 in broth, we did not obtain any pJH1-containing transconjugants that were not also hemolytic on horse blood agar. All of these Hly^+ multiply resistant transconjugants contained either both pJH1 and pJH2 or what appeared to be a larger plasmid species formed by cointegration of both plasmids. Although we cannot explain the discrepancy between our results and those of Jacob and Hobbs (8), we suspect that in our broth system, bacteriocin produced by strain JH1 killed any JH2-2 cells that had not obtained the bacteriocin resistance gene(s) associated with plasmid pJH2. This interpretation is based on the following observations. (i) The viable count of JH2-2 at the end of mixed incubation in broth was nearly two orders of magnitude less than at the start. (ii) In the filter mating system in which bacteriocin either may not be so readily produced or may be diluted by diffusion into the supporting agar base, the viable count of JH2-2 did not decrease. (iii) More than 50% of the multiply resistant transconjugants obtained in the latter system were nonhemolytic and contained only plasmid pJH1. Plasmid pJH2 was highly transmissible, independent of plasmid pJH1, in both mating systems.

One Hly^+, Tc^r transconjugant obtained by mixed incubation in broth culture was found to be sensitive to kanamycin, streptomycin, and erythromycin. This isolate, strain DL172, harbored a single plasmid, pDL172, intermediate in size between pJH1 and pJH2. Strain DL172 responded to tetracycline in the same manner as strain DL75, a derivative of JH1 which had lost plasmid pJH1, with regard to its level (80 μg/ml) and regulation (inducible) of resistance. Unlike the multiply resistant, Hly^+ transconjugants obtained by either conjugation system, strain DL172 was hemolytic only in the presence of tetracycline. Restriction endonuclease *Eco*RI digests and hybridization experiments revealed that pDL172 shared no detectable homology with pJH1 but contained all of pJH2 plus 17.8 kb of DNA shown to be homologous with a specific *Eco*RI fragment from the genome of strain JH1, as well as strain DL75. We interpret these results to mean that a fragment of JH1 DNA, originating from the chromosome or an undetected plasmid and containing the inducible tetracycline resistance determinant, had become integrated into plasmid pJH2, either before or during the conjugation process. The expression of pJH2-mediated hemolytic activity in the hybrid plasmid was now controlled by the regulatory gene(s) for expression of tetracycline resistance, probably due to the site or orientation of integration of the heterologous DNA or both.

Plasmid pDL172 may have been formed by a random recombinational event between the DNA of strain JH1 and pJH2 or as a result of transposition. Franke and Clewell (5, 6) have described a transmissible tetracycline resistance determinant integrated in the chromosome of *S. faecalis* strain DS16 and located on a 15-kb transposon (Tn916). This element can transfer, by conjugation, in the absence of plasmid DNA and can also insert into multiple sites of several different conjugative plasmids. Some insertions of Tn916 into pAD1, a hemolysin plasmid from strain DS16, have resulted in the inactivation of

hemolysin expression, and others have given rise to hyperexpression of hemolytic activity (5, 6). Preliminary results suggest that the 19-kb *Eco*RI fragment A* of plasmid pDL172 shares at least some homology with Tn916 (V. Burdett, personal communication).

We are currently attempting to determine if the 17.8 kb of heterologous DNA in pDL172 are transposable. In this regard, we have transferred plasmid pDL172, by conjugation, from strain DL172 to other *S. faecalis* strains. Derivatives of these transconjugants that have the following properties have been obtained: (i) expression of hemolytic activity in the absence of tetracycline; (ii) resistance to tetracycline; and (iii) the presence of a plasmid that migrates in agarose gels at the same rate as pJH2 rather than pDL172. We are attempting to determine if the tetracycline resistance determinant of pDL172 has integrated into the chromosomes of these transconjugant isolates. We are also trying to establish the degree of homology between this fragment and Tn916.

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