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Self-Transferable Plasmids Determining the Hemolysin and Bacteriocin of *Streptococcus faecalis* var. *zymogenes*

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Strains of *Streptococcus faecalis* var. *zymogenes*, designated JH1 and JH3, produced a hemolysin and a bacteriocin. Hemolytic activity was lost from a low percentage of cells grown in broth at either 37 or 45 C. All nonhemolytic (Hly^-) variants had lost bacteriocin activity (Bcn^-), and those from strain JH3 had also lost resistance to the bacteriocin (Bnr^-). The majority of Hly^-, Bcn^- variants from JH1 retained bacteriocin resistance (Bnr^+). Strains JH1 and JH3 contained a plasmid deoxyribonucleic acid species of molecular weight 38×10^6 (plasmids pJH2 and pJH3, respectively), and strain JH1 also contained a 50×10^6 molecular weight plasmid (pJH1) which has previously been shown to carry the genes determining resistance to the antibiotics kanamycin, neomycin, streptomycin, erythromycin, and tetracycline. Hly^-, Bcn^-, Bnr^- variants of strain JH3 had completely lost plasmid pJH3. Hly^-, Bcn^-, Bnr^- variants of strain JH1 had completely lost plasmid pJH2 and retained plasmid pJH1, but Hly^-, Bcn^-, Bnr^+ variants had retained both plasmids pJH2 and pJH1. The Hly^+, Bcn^+, Bnr^+ traits from both parental strains were transferable to nonhemolytic *S. faecalis* strains during mixed incubation in broth at 37 C, and hemolytic recipient strains were found to have received plasmid pJH2 from strain JH1 and pJH3 from JH3. We conclude that the Hly^+, Bcn^+, Bnr^+ traits are borne on plasmid pJH2 in strain JH1 and pJH3 in strain JH3 and that, in Hly^-, Bcn^-, Bnr^+ variants of strain JH1, plasmid pJH2 has suffered a mutation affecting hemolysin and bacteriocin expression. We infer that the plasmids transfer by conjugation. Beta-hemolytic activity is the only property distinguishing the *zymogenes* variety from *S. faecalis*. Since we have shown that this activity is plasmid borne in strains JH1 and JH3, we endorse the view that the varietal status of *zymogenes* should be dropped.

In a previous report (12) we described a strain of *Streptococcus faecalis* var. *zymogenes*, JH1, that contained two plasmid deoxyribonucleic acid (DNA) species, pJH1 and pJH2. Plasmid pJH1 was shown to carry multiple antibiotic resistance genes and to be transferable to other *S. faecalis* strains. During this work it was observed that many clones of a nonhemolytic *S. faecalis* recipient strain, JH2-2, became capable of producing hemolysin after mixed incubation with hemolytic strain JH1. In this investigation we show that the hemolysin trait is linked to plasmid pJH2 in strain JH1 and to plasmid pJH3 in another *S. faecalis* var. *zymogenes* strain, JH3. Both plasmids transfer to other *S. faecalis* strains at high frequency, and we suggest that the transfer is by a conjugal mechanism. We describe other properties of the two plasmids and draw analogies between these and the properties of plasmids of the *Enterobacteriaceae*.

The beta-hemolytic activity of strains JH1 and JH3 is associated with a bacteriocin activity. The properties of the bacteriocin are typical of those found in *S. faecalis* var. *zymogenes* strains and described by Brock et al. (4). Brock and Davie (3) and Granato and Jackson (9) have shown that the hemolysin and bacteriocin are two activities of the same complex substance. We show that the bacteriocin trait, and also bacteriocin resistance, are linked to plasmids pJH2 and pJH3.

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. Recently the transfer by conjugation of bacteriocin and beta-hemolysin traits from an *S. faecalis* strain, classified as the variety *liquefaciens*, has been described (18).

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Except where stated, each wild

TABLE 1. *Bacterial strains used*

Strain	Phenotype[a]
S. faecalis var. *zymogenes*, JH1	Wild strain
S. faecalis, JH2	Wild strain
S. faecalis var. *zymogenes*, JH3	Wild strain
S. faecalis, JH6	Wild strain
S. faecalis var. *liquefaciens*, 31	Wild strain[b]
Staphylococcus aureus, Oxford strain	Wild strain[c]
S. faecalis, JH1-2	Thy^-, Gen
JH1-22	Thy^-, Rif
JH2-2	Fus, Rif
JH2-3	Fus, Gen
JH2-7	Thy^-, Fus, Rif
JH3-2	Fus, Rif
JH3-5	Thy^-, Fus, Rif
JH3-6	Fus, Gen
JH5-1	Fus

[a] Abbreviations: Thy, thymine; Fus, fusidic acid; Gen, gentamicin; Rif, rifampin.
[b] Obtained from L. N. Zimmerman (14).
[c] Strain NCTC 6571.

strain was isolated from a separate patient in Hammersmith Hospital. Strains JH1, JH2, JH3, and JH5 and the isolation of strain derivatives have been described (12). None of the *S. faecalis* strains fermented arabinose, in contrast to a previous statement made in error (12). The properties of strain JH6 will be described in a forthcoming paper.

Media, and the isolation and characterization of plasmid DNA by cesium chloride-ethidium bromide density gradient centrifugation and neutral sucrose gradient analysis. Media and plasmid DNA isolation and characterization were as previously described (12).

Detection of protease activity. The plate method described by Deible (6) was used.

Loss of hemolysin production. An 18-h broth culture of the hemolysin-producing strain (grown at 37 C) was diluted to 10^8 cells/ml in fresh broth and incubated with aeration for up to 4 h at either 37 or 45 C. Suitable dilutions were then plated on blood agar and incubated overnight at 37 C. Hemolysin-negative variants were purified on blood agar before further characterization.

Hemolysin-negative variant mutation type. The hemolysin-negative variant mutation type was tested by the method of Granato and Jackson (9), except that horse blood was used in place of sheep blood.

Tests for bacteriocin activity and resistance, and chloroform and heat sensitivity of bacteriocin. These tests were performed as described by Brock et al. (4).

Inactivation of bacteriocin activity by trypsin. The method of Tomura et al. (18) was used. Inactivation of hemolysin activity was tested by using blood agar as the overlay.

Transfer of hemolysin activity. Strains were grown overnight in broth, supplemented with thymine where appropriate, standing at 37 C. After dilution to 10^8 cells/ml with fresh broth, the donor and recipient cultures were mixed in the ratio 1:10 and incubated at 37 C with gentle shaking. After an appropriate time, dilutions of the mixture were plated on nutrient agar containing an antibiotic to select for the donor and on blood agar containing an antibiotic to select for the recipient and to indicate recipient clones producing hemolysin.

Anaerobic fermentation of glycerol. Anaerobic fermentation of glycerol was examined using the method of Deible et al. (8), with the exception that anaerobic fermentation was achieved in an atmosphere of 10% CO_2 and H_2.

Reagents. Enzymes and analytical reagent grade chemicals were purchased from standard commercial sources, and radioisotopes were from the Radiochemical Centre, Amersham. Ethidium bromide was a gift from Boots Pure Drug Co. Ltd. and Sarkosyl NL97 was a gift from Geigy Chemicals. Rifampin (Rimactane) was a gift from Ciba Laboratories.

RESULTS

Some properties associated with the zymogenes variety of S. faecalis. Sherman (16) showed that beta-hemolytic activity was the major distinctive property of the *zymogenes* variety. Fermentation of sucrose and anaerobic fermentation of glycerol were almost always associated with the variety, but only occasionally with *S. faecalis*, whereas protein hydrolysis was occasionally associated with *zymogenes* but never with *S. faecalis*. The results of testing these properties in two *S. faecalis* var. *zymogenes* strains (JH1 and JH3) and one *S. faecalis* strain (JH2) are shown in Table 2. All three strains ferment sucrose and glycerol, but only the *zymogenes* strains have beta-hemolytic activity and hydrolyze gelatin.

Characterization of a bacteriocin activity. Brock et al. (4) and, more recently, Tomura et al. (18) have surveyed the bacteriocins of group D streptococci and showed that all strains of *S. faecalis* var. *zymogenes* produced a similar bacteriocin. The bacteriocin, designated type 1 by Brock et al. (type II by Tomura et al.), had the unique property, among group D streptococcal bacteriocins, of being active against a wide variety of gram-positive organisms, including all the enterococci except other *zymogenes* strains. The bacteriocin activity was inacti-

TABLE 2. *Properties associated with the zymogenes variety of S. faecalis*

Strain	Variety	Beta hemolysis[a]	Gelatin hydrolysis	Anaerobic glycerol fermentation	Sucrose fermentation
JH2		−	−	+	+
JH1	*zymogenes*	+	+	+	+
JH3	*zymogenes*	+	+	+	+

[a] Symbols: +, positive reaction; −, negative reaction.

vated by chloroform vapors and by heating at 80 C for 20 min, but was resistant to 10 min of heating at 80 C and to protease activity.

We have shown that the *zymogenes* strains JH1 and JH3 each produce a bacteriocin which has the properties listed in Table 3. Although bacteriocin activity was apparently sensitive to 10 min of heating at 80 C, the hemolytic activity was resistant to this treatment. In addition to being resistant to trypsin activity, the bacteriocin was resistant to the protease produced by strains JH1, JH3, and *S. liquefaciens* strain 31. We conclude that the bacteriocin activity of strains JH1 and JH3 is typical of that described by Brock et al. (4) and Tomura et al. (18) for strains of *S. faecalis* var. *zymogenes*.

Genetic stability of traits associated with zymogenes strains. We tested the stability of the hemolysin trait of derivatives of the two *zymogenes* strains by screening for the appearance of hemolysin-negative (Hly^-) clones on horse blood agar after growth in broth at either 37 or 45 C for 4 h (Table 4). The hemolysin trait is clearly an unstable property of these strains. We tested the Hly^- clones obtained in the above experiments, and others obtained from experiments in which growth in nutrient broth was for a shorter time before screening, for bacteriocin (Bcn) activity and bacteriocin sensitivity. 42/42 Hly^- clones of strain JH1-2, obtained after 37 C incubation, had lost bacteriocin activity toward strain JH2 and *Staphylococcus aureus*. A similar result was found for 21 Hly^- JH1-2 and 12 Hly^- JH3-2 clones obtained after 45 C incubation. All of the Hly^-, Bcn^- clones of JH3-2 had become sensitive to the bacteriocin of the parental strain and to that of strain JH1-2. In contrast, all of the JH1-2 Hly^-, Bcn^- clones obtained after 37 C incubation retained their bacteriocin resistance (Bnr^+) and only five of 21 Hly^-, Bcn^- clones of this strain isolated after 45 C incubation had become bacteriocin sensitive (Bnr^-). Several clones which were chosen for further study are listed in Table 5. None of the Hly^- variants had lost the capacity to ferment sucrose or glycerol (anaerobically) or to hydrolyze gelatin.

We examined three Hly^- variant clones, JH1-14, JH1-15, and JH3-3, for spontaneous reversion to hemolysin activity; no reversion was observed (frequency $<5 \times 10^{-8}$/cell for each strain).

Isolation of plasmid-specific DNA from strains JH1-2 and JH3-2 and some hemolysin-negative variants. In a previous report (12), we showed that strain JH1-2 contained covalently closed circular (CCC) DNA molecules characteristic of plasmids. This DNA was separated from chromosomal DNA by dye-buoyant density centrifugation. We have now found that strain JH3-2 also contains CCC DNA (data not shown), with the yield, expressed as percent of chromosomal DNA yield, varying between 1.98 to 2.63% in six experiments.

We examined several hemolysin-negative variants to establish whether plasmid-specific DNA had been lost from these strains. For this analysis we mixed cultures of each variant, labeled with [^{3}H]thymine, with the appropriate ^{14}C-labeled parental strain before lysis and dye-buoyant density centrifugation. This ena-

TABLE 3. *Characteristics of bacteriocin*

Strain	Treatment with[a]				Activity against[a]			
	$CHCl_3$	Trypsin	80 C, 10 min[b]	80 C, 20 min	*Staphylococcus aureus*	*S. faecalis*	*S. liquefaciens*	*S. zymogenes*
JH1	S	R	S	S	+	+	+	−
JH3	S	R	S	S	+	+	+	−

[a] Symbols: S, sensitive; R, resistant; +, active; −, inactive.
[b] Hemolytic activity was resistant to this treatment.

TABLE 4. *Effect of incubation temperature on stability of hemolysin trait*

Strain	Incubation at 37 C			Incubation at 45 C		
	No. screened	No. of Hly^- clones	% Hly^-	No. screened	No. of Hly^- clones	% Hly^-
JH1-2	4,754	38	0.80	2,003	1	0.05
JH3-2	3,827	0		2,137	10	0.47

TABLE 5. *Variant clones chosen for further study*

Strain	Phenotype	Origin
JH1-2 variants		
JH1-14	Hly⁻, Bcn⁻, Bnr⁺	After 37 C incubation
JH1-15	Hly⁻, Bcn⁻, Bnr⁺	After 37 C incubation
JH1-16	Hly⁻, Bcn⁻, Bnr⁺	After 37 C incubation
JH1-17	Hly⁻, Bcn⁻, Bnr⁺	After 37 C incubation
JH1-18	Hly⁻, Bcn⁻, Bnr⁻	After 45 C incubation
JH1-19	Hly⁻, Bcn⁻, Bnr⁻	After 45 C incubation
JH1-20	Hly⁻, Bcn⁻, Bnr⁺	After 45 C incubation
JH1-21	Hly⁻, Bcn⁻, Bnr⁺	After 45 C incubation
JH3-2 variants		
JH3-3	Hly⁻, Bcn⁻, Bnr⁻	After 45 C incubation
JH3-4	Hly⁻, Bcn⁻, Bnr⁻	After 45 C incubation

bled us to make an accurate comparison of yields of CCC DNA from the Hly⁻ variants 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. Neither of two Hly⁻ variants of JH3-2 (strains JH3-3 and JH3-4; Table 5) contained CCC DNA, although CCC DNA was successfully isolated from the parental JH3-2 strain mixed with each variant.

We analyzed eight Hly⁻ variants of JH1-2, with or without the Bnr trait (strains JH1-14 to -21; Table 5). The Bnr⁺ strains JH1-14, -16, -17, -20, and -21 showed little difference between their yield of CCC DNA and that of the parental strain, implying that there was no plasmid loss. The Bnr⁻ strains JH1-18 and -19 and Bnr⁺ strain JH1-15 yielded 42 to 52% of parental CCC DNA, implying that one of the two plasmids of JH1-2 was lost.

Identification and molecular weight determination of CCC DNA species. We have previously described the analysis of CCC DNA isolated from strain JH1-2 by sedimentation through 5 to 20% neutral sucrose gradients (12). We identified which DNA bands were CCC DNA and which were open circular (OC; formed by spontaneous breakdown of CCC DNA) by analyzing the characteristic changes in sedimentation rate of these DNA tertiary forms after treatment with a low concentration of deoxyribonuclease I, which converts CCC DNA to the OC form (12, 19). Two plasmid DNA species (pJH1 and pJH2) were characterized. Their molecular weights were 50×10^6 and 38×10^6, respectively, and plasmid pJH1 was shown to carry determinants for resistance to kanamycin, neomycin, streptomycin, erythromycin, and tetracycline. We have now similarly analyzed the CCC DNA isolated from strain JH3-2 from all the Hly⁻ variants described above and listed in Table 5.

Figure 1A shows the result of sedimenting ^{3}H-labeled CCC DNA from strain JH3-2, together with ^{14}C-labeled JH1-2 CCC DNA to act as a sedimentation reference, for 105 min. Sedimentation was from right to left. Plasmid pJH1 CCC DNA peaked at approximately fraction 9 and that of pJH2 at fraction 14. The broad band peaking at fraction 25 represents the unresolved OC forms of the two plasmids. A ^{3}H-labeled DNA band cosedimented with pJH2 CCC DNA and a second DNA band peaked at fraction 26. The relationship between these bands was discovered by incubating the mixture with a low concentration of deoxyribonuclease I. Figure 1B shows the result of sedimenting this treated DNA for 105 min. The DNA

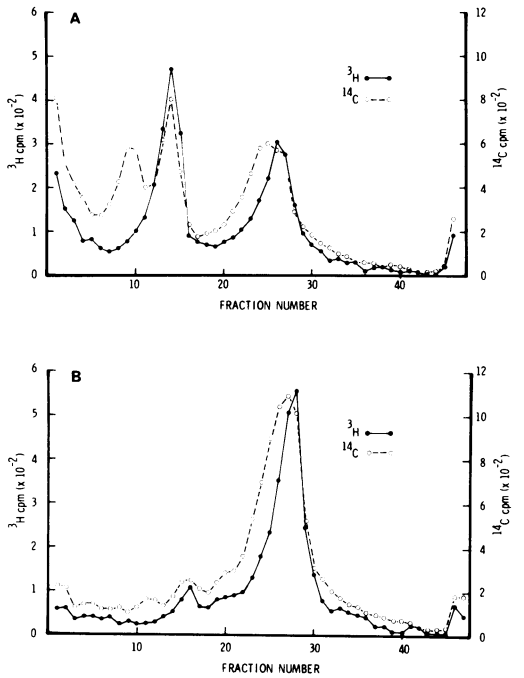

FIG. 1. *Neutral sucrose gradient analysis of plasmid DNA from strain JH3-2. Plasmid DNA was isolated from a mixed lysate of ^{3}H-labeled JH3-2 and ^{14}C-labeled JH1-2 by CsCl-EB density gradient centrifugation. After removal of ethidium bromide and dialysis, a sample was layered onto a 5 to 20% sucrose gradient and centrifuged at 100,000 × g at 20 C for 105 min. Fractions (0.1 ml) were collected directly onto glass-fiber disks through a hole pierced in the tube bottom and were assayed for ^{3}H and ^{14}C radioactivity after drying and washing. (A) Untreated sample; (B) sample treated with 1 volume of deoxyribonuclease I solution (5×10^{-4} μg/ml in 25 mM $MgSO_4$) for 7 min at 25 C, followed by 1 volume of 60 mM Na_2 ethylenediaminetetraacetic acid to stop enzyme activity.*

band which peaked at fraction 14 (Fig. 1A) now sediments with the DNA band peaking at fraction 27. This defines the fast sedimenting DNA band as being CCC DNA and the slow sedimenting band as being its OC form. Therefore, a single plasmid, designated pJH3, is present in strain JH3-2 which, since it cosediments with pJH2, has a molecular weight of approximately 38×10^6. The ^3H- and ^{14}C-labeled DNA in fractions 1 to 5, seen in Fig. 1A and in other gradients, possibly resulted from concentration of the leading edges of the fast sedimenting bands at the tube bottom. It did not represent another plasmid DNA species, since this DNA neither formed a discrete band nor sedimented to the gradient bottom when sedimented for only 85 min (data not shown), and no OC form of a plasmid of molecular weight greater than 50×10^6 was observed in Fig. 1B.

Using the molecular weight value of 38×10^6 for pJH3, the yields of CCC DNA determined from the dye-buoyant density gradients and the estimate of 1.47×10^9 for the molecular weight of the *S. faecalis* chromosome (1), we calculated that approximately 0.76 to 1.02 copies of plasmid pJH3 per chromosome had been isolated in the CCC DNA form.

The two Hly$^-$ variants JH3-3 and JH3-4 had lost the CCC DNA, and therefore loss of Hly$^+$, Bcn$^+$, Bnr$^+$ traits from strain JH3-2 correlates with loss of plasmid pJH3.

The two Hly$^-$, Bcn$^-$, Bnr$^-$ variant strains of JH1-2 examined (JH1-18 and JH1-19) had lost plasmid pJH2. Figure 2A shows the result of sedimenting CCC DNA isolated from the mixed lysate of ^3H-labeled JH1-19 and ^{14}C-labeled JH1-2 for 105 min. We interpret this result as indicating that the Hly$^+$, Bcn$^+$, Bnr$^+$ traits are carried by plasmid pJH2. This plasmid has also been lost from the Hly$^-$, Bcn$^-$, Bnr$^+$ strain JH1-15, and no small-molecular-weight remnant of it could be detected (Fig. 2B). In contrast, the other five Hly$^-$, Bcn$^-$, Bnr$^+$ strains (Table 5) had retained plasmid pJH2, with no measurable change in its molecular weight (data not shown). The possible significance of these data will be discussed later.

Transfer of hemolytic and bacteriocin activity and bacteriocin resistance from strains JH1-2 and JH3-2 to S. faecalis, strain JH2-2. We have shown that the Hly$^+$, Bcn$^+$, Bnr$^+$ traits of strains JH1-2 and JH3-2 are transferable to strain JH2-2 during mixed incubation in nutrient broth and have found that the traits are also transferable from this recipient to a second one (strain JH2-3). Thus, the transfer mechanism is not dependent upon the original host strain.

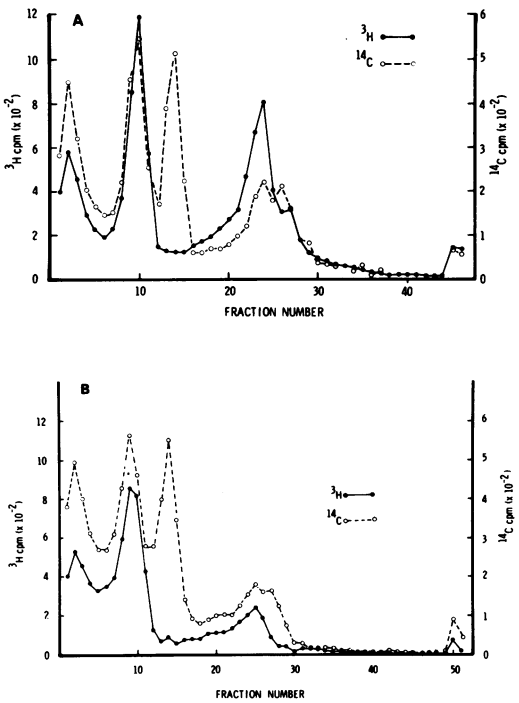

FIG. 2. *Neutral sucrose gradient analysis of plasmid DNA from hemolysin-negative variants of strain JH1-2. Plasmid DNA was isolated from a mixed lysate of the ^3H-labeled Hly$^-$ variant and ^{14}C-labeled JH1-2 and sedimented through a 5 to 20% sucrose gradient as described in the legend to Fig. 1. (A) ^3H-labeled JH1-19 plus ^{14}C-labeled JH1-2; (B) ^3H-labeled JH1-15 plus ^{14}C-labeled JH1-2.*

We measured the kinetics of acquisition of hemolysin activity by JH2-2 from strain JH1-2. Cultures (18 h) 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 1 donor to 10 recipients, and incubated with gentle shaking at 37 C. At intervals during a 4-h incubation, dilutions were plated on horse blood agar, supplemented with thymine where appropriate and containing an antibiotic to select for the donor or recipient strains. Plates were incubated for 24 h before colonies were counted and the number of hemolytic recipients scored. To prove that transfer occurred, we purified some hemolytic recipient colonies and tested them on antibiotic ditch plates for the unselected chromosomal antibiotic resistances. All the clones tested were sensitive to gentamicin (20 μg/ml) and resistant to rifampin (640 μg/ml) and fusidic acid (128 μg/ml). The transfer kinetics are shown in Fig. 3. The number of

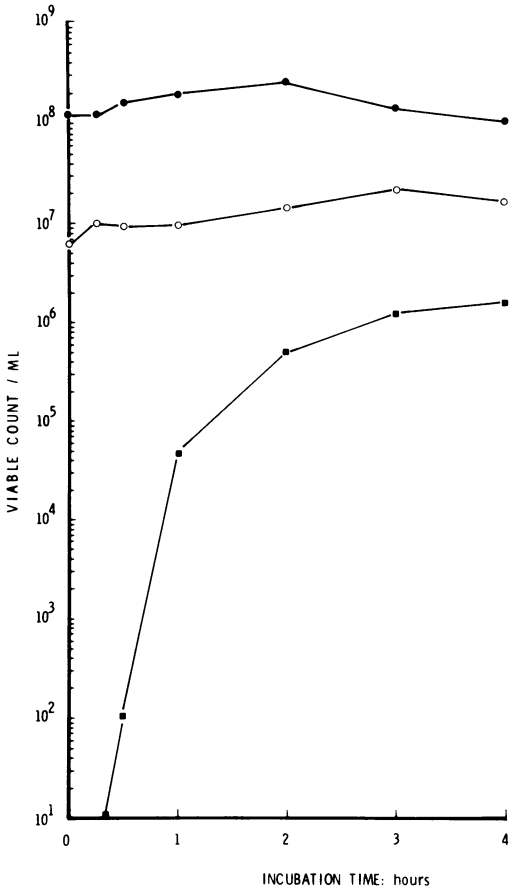

FIG. 3. *Kinetics of acquisition of the transferred hemolysin trait by strain JH2-2 from JH1-2. Overnight broth cultures of donor strain JH1-2 and recipient strain JH2-2 were diluted to 10^8 cells/ml and mixed in the ratio of 1 donor to 10 recipients. The mixed culture was incubated with aeration at 37 C, and at the times indicated samples were withdrawn and suitable dilutions were spread onto agar plates to select for donors* (○) *or recipients* (●) *and to indicate those recipients having acquired hemolytic activity* (■).

hemolytic recipients increased very markedly during the first 120 min, but in the next 120 min the increase was only twofold. After 4 h of mixed incubation the frequency of hemolytic activity transfer was 9.3×10^{-2}/donor. The transfer frequency of hemolytic activity from strain JH3-2 to JH2-2 after 4 h was 3.1×10^{-3}/donor, during which time the recipient viable count decreased fivefold, presumably owing to killing by the donor bacteriocin.

Ten out of ten hemolytic recipient clones derived from each donor were found to produce the type 1 bacteriocin and had become bacteriocin resistant. None had received the ability to hydrolyze gelatin. We could not test for transfer of the sucrose and anaerobic glycerol fermentation characteristics, since the recipient strain was positive for these traits (Table 2).

Isolation and identification of plasmid DNA species from JH2-2 recipient clones after receipt of hemolysin and bacteriocin activities and bacteriocin resistance. We have isolated CCC DNA species from clones of strain JH2-2 that had received the Hly^+, Bcn^+, Bnr^+ traits from strain JH1-2 (strains JH2-6 and JH2-8) and from JH3-2 (strains JH2-10 and JH2-11), by using dye-buoyant density centrifugation. Since strain JH2-2 itself does not contain CCC DNA (12), we concluded that plasmid DNA had been transferred to these recipient clones. The species of transferred plasmid(s) were identified by sedimentation through neutral sucrose gradients. Figure 4A illustrates the sedimentation of ^{3}H-labeled JH2-8 CCC DNA,

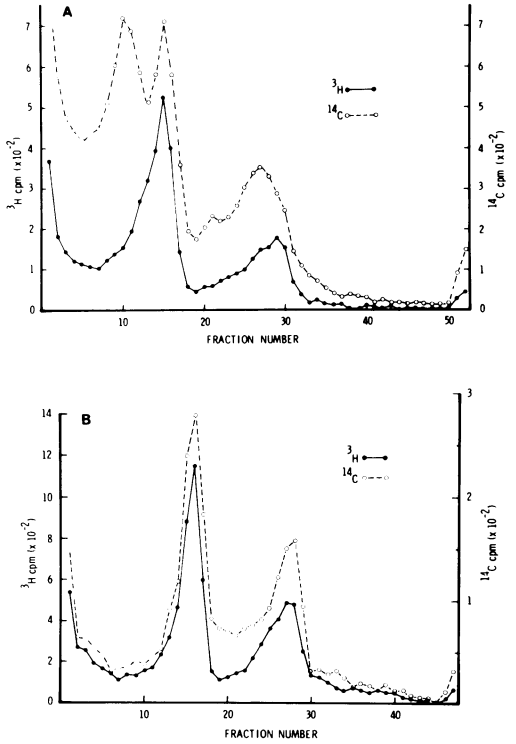

FIG. 4. *Neutral sucrose gradient analysis of plasmid DNA from recipient clones that received the hemolysin trait. Plasmid DNA was isolated from a mixed lysate of the ^{3}H-labeled recipient and ^{14}C-labeled donor strains and sedimented through a 5 to 20% sucrose gradient as described in the legend to Fig. 1. (A) ^{3}H-labeled JH2-8 plus ^{14}C-labeled JH1-2; (B) ^{3}H-labeled JH2-10 plus ^{14}C-labeled JH3-2.*

together with ^{14}C-labeled JH1-2 CCC DNA, for 105 min and shows that strain JH2-8 had received plasmid pJH2. Figure 4B shows the sedimentation of ^{3}H-labeled JH2-10 CCC DNA and ^{14}C-labeled JH3-2 CCC DNA. The differentially labeled DNA bands cosedimented, indicating that plasmid pJH3 had been transferred to strain JH2-2.

We also found that the transfer of the Hly$^+$, Bcn$^+$, Bnr$^+$ traits from a primary recipient (JH2-8) to a secondary one (strain JH2-3) was correlated, in one clone examined, with the transfer of plasmid pJH2. These data confirm that the Hly$^+$, Bcn$^+$, Bnr$^+$ traits are borne by plasmid pJH2 in strain JH1 and plasmid pJH3 in strain JH3.

Further examination of those Hly$^-$, Bcn$^-$, Bnr$^+$ variants of strain JH1-2 that retained plasmid pJH2. Although we have shown that the Hly$^+$, Bcn$^+$, Bnr$^+$ traits of strain JH1 are borne by plasmid pJH2, we have isolated five Hly$^-$, Bcn$^-$, Bnr$^+$ clones which retained the plasmid. We have attempted to show whether loss of the hemolysin and bacteriocin activities from these clones was due to a mutation in the host strain which prevented their expression. If this were so, and assuming that the plasmid transfer mechanism itself was not affected, then hemolysin activity would be detected upon transfer of plasmid pJH2 from the clones to a new host. We attempted to transfer hemolysin activity from each of the clones to recipient JH2-2, but without success (transfer frequencies per donor $<3.6 \times 10^{-8}$).

Granato and Jackson (9–11) have shown that the hemolytic activity of *S. faecalis* var. *zymogenes*, strain X-14, is composed of two macromolecular components, an activator substance, A, and a catalytic molecule, L. Two classes of Hly$^-$ mutants were identified; one class had lost the capacity to produce A substance and the other, L substance. Cross-streaking of one mutant class with the other on blood agar plates resulted in formation of spur-shaped zones of hemolytic activity at the junction of the two streaks. We examined the possibility that loss of hemolytic activity from the five clones was due to loss of either A or L substance. We cross-streaked each of the clones against the others, but no zone of hemolysis was observed. We conclude that these clones had either lost both components or were all members of the same mutant class.

Efficiency of Hly$^-$ variants as recipients of the plasmid-borne hemolysin trait. We have tested five Bnr$^-$, Hly$^-$, Bcn$^-$ variants and 17 Bnr$^+$, Hly$^-$, Bcn$^-$ variants of strain JH1-2 and two Bnr$^-$, Hly$^-$, Bcn$^-$ variants of strain JH3-2 to determine whether they would act as recipients for the transfer of the Hly$^+$, Bcn$^+$, Bnr$^+$ plasmids pJH2 and pJH3 from derivative strains of JH1 and JH3. The frequency of Hly$^+$ transfer after 4 h of mixed incubation was determined, and the results for those variants listed in Table 5 are recorded in Table 6.

All the Bnr$^-$ variants from strains JH1-2 and JH3-2 received plasmid pJH2 or pJH3 at a frequency similar to that of the efficient recipient strain JH2-2. None of the Bnr$^+$ variants of strain JH1-2 showed recipient ability for plasmid pJH2; no Hly$^+$ recipient clones were recognized. In contrast, four out of four of these Bnr$^+$ variants received plasmid pJH3 with normal frequency from strain JH3-2. The Bnr$^+$ strains therefore exerted superinfection inhibition (Sin) against plasmid pJH2 but not pJH3.

All of the Bnr$^-$ variants examined for plasmid DNA content had lost the plasmid carrying the Hly$^+$, Bcn$^+$, Bnr$^+$ properties. Five out of six Hly$^-$, Bcn$^-$, Bnr$^+$ variants of JH1-2 retained plasmid DNA with the molecular weight of plasmid pJH2 (Bnr$^+$ strain JH1-15 had lost this DNA). We conclude that the Sin trait is carried by plasmid pJH2 and that in the Bnr$^+$ strains plasmid pJH2 was probably in a mutant form that no longer determined the Hly$^+$, Bcn$^+$ characters but only the Bnr$^+$ and Sin traits. The

TABLE 6. *Transfer frequency of hemolysin trait to Hly$^-$ variants*

Donor strain	Recipient strain	Frequency of hemolytic recipients per donor
JH1-22	JH1-18	1.5×10^{-2}
	JH1-19	1.5×10^{-1}
	JH1-14	$<5.3 \times 10^{-8}$
	JH1-15	$<2.1 \times 10^{-7}$
	JH1-16	$<5.7 \times 10^{-8}$
	JH1-17	$<5.6 \times 10^{-8}$
	JH1-20	$<4.5 \times 10^{-7}$
	JH1-21	$<5.1 \times 10^{-7}$
JH3-2	JH1-18	2.5×10^{-3}
	JH1-14	3.9×10^{-2}
	JH1-15	3.7×10^{-2}
	JH1-16	3.9×10^{-2}
	JH1-20	4.0×10^{-2}
JH1-2	JH3-3	5.0×10^{-2}
	JH3-4	4.8×10^{-1}
JH3-6	JH3-3	2.9×10^{-2}
	JH3-4	1.1×10^{-2}
JH1-2	JH2-2	9.3×10^{-2}
JH3-2	JH2-2	3.1×10^{-3}

superinfection inhibition was specific to pJH2 itself and was not effective against pJH3.

Location of bacteriocin resistance and Sin traits in clone JH1-15. Clone JH1-15 had lost plasmid pJH2 and the plasmid-borne hemolysin and bacteriocin activities, but had retained the bacteriocin resistance and Sin traits shown to be carried by plasmid pJH2. We have considered four possible explanations for these observations. (i) The Bnr$^+$,Sin traits were located on a small remnant of plasmid pJH2. We could not detect a small-molecular-weight plasmid species in this clone (Fig. 2B). (ii) The Bnr$^+$,Sin traits had been transposed onto the other plasmid present in the clone, plasmid pJH1, which carries determinants for antibiotic resistance. We have transferred plasmid pJH1 from strain JH1-15 to strain JH2-7, by selection for antibiotic resistance transfer, and tested four recipient clones for the co-transfer of the Bnr$^+$,Sin traits. None of these clones were resistant to the bacteriocin produced by strains JH1-2 and JH3-2, and all were efficient recipients of the Hly$^+$ trait from strain JH1-2. (iii) Strain JH1-15 had lost plasmid pJH2 and then acquired the bacteriocin resistance and Sin traits by spontaneous mutation(s) of the bacterial chromosome. (iv) Recombination occurred between the host chromosome and plasmid pJH2, which resulted in chromosomal integration of the Bnr$^+$,Sin traits and loss of autonomous pJH2 DNA. We have not distinguished between the last two possibilities.

Mechanism of transfer. We have shown that plasmid pJH1 probably transfers by a conjugal mechanism (12). We have used the same tests to determine the mechanism by which plasmids pJH2 and pJH3 transfer. (i) Cell-free filtrates of the donor strains JH1-2 and JH3-6, prepared 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), did not transfer hemolysin activity to recipient strain JH2-2 (transfer frequency $<4 \times 10^{-9}$/recipient for each filtrate after 4 h of incubation). (ii) Pretreatment of the donor cultures JH1-2 and JH3-6 with chloroform prevented transfer of plasmids pJH2 and pJH3. We treated overnight broth cultures of the two strains with chloroform by adding 0.1 ml to 5 ml of culture and shaking for 15 min at 37 C. Remaining chloroform vapor was removed by aspiration during a further 30 min of incubation. No transfer of hemolytic activity was detected after 4 h of mixed incubation of 1 volume of chloroform-treated donor culture with 9 volumes of recipient JH2-2 (transfer frequency $<6 \times 10^{-9}$/recipient for each donor). (iii) Pretreatment of overnight broth cultures of donor strains JH1-2 and JH3-6 and recipient strain JH2-2 with 10 μg of deoxyribonuclease I per ml and 5 mM $MgSO_4$ at 37 C for 5 min before mixed incubation had no significant effect on the frequency of transfer of hemolytic activity. The transfer frequencies for the deoxyribonuclease I-treated mixtures were 1.9×10^{-2}/JH1-2 donor in 4 h, and 3.9×10^{-2}/JH3-6 donor. Transfer frequencies in untreated mixtures were 5.8×10^{-3} and 4×10^{-2}, respectively. (iv) We have not been able to detect the production of bacteriophage by strains JH1-2 and JH3-6 using a spot lysis test. We inoculated drops of the cell-free filtrates, the chloroform-treated donor cultures, and supernatants of the two cultures, obtained after centrifugation at 20,000 $\times$ *g* for 20 min, onto plates overlaid with nutrient agar containing a presumptive indicator strain. Strains which acted as recipients of the Hly$^+$ plasmids (JH2-2, JH1-18, JH1-19, JH3-3, JH5-1, and JH6) were used as indicators, but no plaques were observed on any.

DISCUSSION

We conclude from our data that the genes determining the hemolysin and bacteriocin activities and bacteriocin resistance of strains JH1 and JH3 are borne by transmissible plasmids pJH2 and pJH3, respectively, both of which have a molecular weight of approximately 38×10^6. The instability of these traits and the correlation between their loss and acquisition and the absence and presence of plasmids pJH2 and pJH3 are the evidence for this interpretation.

Plasmids pJH2 and pJH3 transfer to other *S. faecalis* strains at a high frequency (generally greater than 10^{-2}/donor after 4 h of mixed incubation), and their transferability is not dependent upon the original host strains. The transfer mechanism of these plasmids has characteristics similar to those previously described for the transferable multiple antibiotic resistance plasmid pJH1, found in strain JH1 (12). This mechanism we have inferred to be conjugation. The lack of transfer from chloroform-treated donor cultures and from donor cell-free filtrates, the inability to detect bacteriophages capable of lysing those *S. faecalis* strains that act as efficient plasmid recipients, and the insensitivity of the transfer mechanism to the enzyme deoxyribonuclease I are properties in support of this conclusion. An alternative hypothesis is that the transfer was bacteriophage

mediated. No transducing phages for the Hly$^+$, Bcn$^+$ traits of *S. faecalis* var. *zymogenes* have been found. Brock (2) isolated a bacteriophage from such a strain (which contained only one bacteriophage species) but this did not transduce the Hly$^+$, Bcn$^+$ traits, although it was capable of lysogenizing an *S. faecalis* strain. Transduction in our systems could only have been by chloroform-sensitive bacteriophages that were adsorbed by asbestos and cellulose ester filters and which failed to efficiently lyse presumptive indicator strains. In a recent, independent, study (18) beta-hemolysin and bacteriocin traits were shown to be transferable from a strain of *S. faecalis*, classified as the *liquefaciens* variety, to other *S. faecalis* strains. Neither a heat-killed culture nor a cell-free filtrate of this strain could act as a transfer donor. Transfer was unaffected by deoxyribonuclease treatment, but was completely inhibited by incubation with trypsin. Antiserum prepared against the donor bacterium severely inhibited transfer. The authors concluded that the traits were transferred by a conjugal mechanism.

Although plasmids pJH2 and pJH3 both carry Hly$^+$, Bcn$^+$, Bnr$^+$ traits and have similar molecular weights, we have found that they differ in several other properties. All Hly$^-$, Bcn$^-$ clones of strain JH3-2 had become bacteriocin sensitive, whereas the majority of Hly$^-$, Bcn$^-$ clones of JH1-2 retained bacteriocin resistance. All Hly$^-$, Bcn$^-$, Bnr$^-$ variants of both strains examined for plasmid DNA content had lost the plasmid bearing these traits. However, five out of six Hly$^-$, Bcn$^-$, Bnr$^+$ clones of strain JH1-2 had retained plasmid pJH2, with no detectable change in its molecular weight, and these strains did not revert to Hly$^+$, Bcn$^+$. Loss of the hemolysin and bacteriocin activities from these strains might have been due to a mutation in the host bacterium that prevented expression of Hly$^+$, Bcn$^+$ and also transfer activities. We think it more likely that Hly$^+$, Bcn$^+$ loss resulted from a point mutation or small deletion of the plasmid DNA. Strains possessing the mutated pJH2 plasmid did not act as recipients for plasmid pJH2 carrying the Hly$^+$, Bcn$^+$ traits, but would accept plasmid pJH3 at a transfer frequency normal for this plasmid (Table 6). This observation of transfer inhibition is analogous to the phenomenon of superinfection inhibition observed for many plasmids of the *Enterobacteriaceae* (13), in which the entry into or establishment of a transferable plasmid in a new host cell is prevented by a resident plasmid. The difference in behavior of plasmids pJH2 and pJH3 to superinfection inhibition may be explained either if the two plasmids can co-exist within a cell or if plasmid pJH3 displaces the resident plasmid pJH2 from the recipient.

Strain JH1-15 is an Hly$^-$, Bcn$^-$, Bnr$^+$ variant of JH1-2 and, like other Hly$^-$, Bcn$^-$, Bnr$^+$ clones, it was an efficient recipient of plasmid pJH3 but not pJH2. Unlike the other clones it had apparently lost all plasmid pJH2 extrachromosomal DNA (Fig. 2B). We have shown that these traits had not been transposed onto plasmid pJH1, but we have not distinguished between the possibilities that (i) strain JH1-15 had lost plasmid pJH2 and then acquired the Bnr$^+$,Sin traits by spontaneous mutation(s) of the bacterial chromosome or (ii) recombination occurred between the host chromosome and plasmid pJH2 that resulted in chromosomal integration of the Bnr$^+$,Sin traits and loss of autonomous pJH2 DNA. The latter seems the more likely, since the probability of three (presumably) independent mutation events occurring together must be very low. We hope to prove, by DNA-DNA hybridization studies, that plasmid DNA has integrated into the host chromosome.

We have previously shown (12) that the amount of plasmid pJH2, isolated as CCC DNA, corresponded to between 0.43 and 1.1 copies/bacterial chromosome. The CCC DNA of plasmid pJH3 corresponded to between 0.76 and 1.02 copies/chromosome. This suggests that replication of both plasmids is controlled so as to occur once per cell division cycle, and that they are analogous to those R plasmids of the *Enterobacteriaceae* whose replication is under stringent control (5). The buoyant density of plasmid pJH2 DNA is very similar to that of the host chromosomal DNA (12) and corresponds to a guanine plus cytosine content of 38% (15).

The beta-hemolytic activity of *S. faecalis* strains JH1 and JH3 characterizes them as being the *zymogenes* variety (16), and we have shown that the bacteriocin activity of these strains has the properties typical of the *zymogenes* variety (4). We have also shown that these properties are linked to a transferable plasmid and are lost spontaneously from a host cell at high frequency. Spontaneous loss of hemolytic activity is a property commonly observed for *zymogenes* strains (7, 17). Tomura et al. (18) have recently shown that another *S. faecalis* strain carried transferable hemolysin and bacteriocin traits. These data lead us to suggest that the beta-hemolysin trait of *S. faecalis* var. *zymogenes* strains will prove to be commonly, if not invariably, plasmid borne.

Since the hemolytic activity is the property that distinguishes the *zymogenes* variety from *S. faecalis* (16), we endorse Deible's view (7) that the varietal status of *zymogenes* should be dropped. All of the Hly$^-$, Bcn$^-$ variants we have examined had retained proteolytic activity, fermentation of sucrose, and anaerobic fermentation of glycerol, and we conclude that these traits are not plasmid linked in strains JH1 and JH3. If Sherman (16) is correct in his view that the protease and fermentation properties are very commonly associated with beta-hemolytic activity, it suggests that *S. faecalis* strains having the protease and fermentation traits are the preferred hosts of plasmids carrying the Hly$^+$, Bcn$^+$, Bnr$^+$ genes.

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