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NOTES

Plasmid-Related Transmissibility and Multiple Drug Resistance in *Streptococcus faecalis* subsp. *zymogenes* Strain DS16

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Streptococcus faecalis subsp. *zymogenes* strain DS16 was found to harbor two plasmids, designated pAD1 (35 megadaltons) and pAD2 (15 megadaltons). pAD1 is transmissible and determines a hemolysin-bacteriocin, whereas pAD2 is nonconjugative and determines resistance to erythromycin, streptomycin, and kanamycin. pAD2 could be mobilized by pAD1, but usually involved formation of a pAD1-pAD2 cointegrate.

The recent use of *Streptococcus faecalis* subsp. *zymogenes* strain DS16 in relation to several ongoing plasmid studies in our laboratory (4, 11; P. K. Tomich, F. Y. An, and D. B. Clewell, Cold Spring Harbor Symp. Quant. Biol., in press) has warranted a general description of the plasmid-related properties of this strain. Strain DS16 is a multiple-drug-resistant clinical isolate obtained from St. Joseph's Mercy Hospital, Ann Arbor, Mich. The specific resistances and the corresponding minimum inhibitory concentrations in Penassay broth (Difco Laboratories) were as follows: tetracycline (Tc) resistance, 30 μg/ml; erythromycin (Em), streptomycin (Sm), and kanamycin (Km) resistances, all greater than 2 mg/ml. Em resistance was found to be inducible by using a previously described procedure (18). It also represented the MLS phenotype; that is, a single determinant confers resistance to macrolides, lincosamides, and streptogramin B-type antibiotics (17). The strain was clearly hemolytic (on horse blood agar [10]), as well as bacteriocinogenic, using the sensitive strain JH2-2 (13) as an indicator (10). As shown by others (2, 12), the hemolysin and bacteriocin of *S. faecalis* subsp. *zymogenes* are probably the same protein.

TABLE 1. *Properties of plasmids originating in strain DS16*

Plasmid	Mol wt (× 10^6)			Copy no.[d]	Genetic markers
	Sccc[a]	EM[b]	AGE[c]		
pAD1	35	34.8 ± 0.7	35	1-2	hem-bac, conjugative factors
pAD2	15	15.5 ± 0.3	15	1-2	resistance to Em, Sm, Km
Cointegrate	50	51.4 ± 0.8	ND[e]	0.05-0.10	hem-bac, conjugative factors, resistance to Em, Sm, Km
pAD1::Tn*917*	38	ND	ND		hem-bac, conjugative factors, resistance to Em

[a] Molecular weights were determined from the sedimentation rates of covalently closed circular (Sccc) molecules in a neutral sucrose density gradient, using an appropriate marker (1).

[b] Contour length measurements from electron micrographs (EM) (7) were used to calculate molecular weights (14), except for pAD1::Tn*917*.

[c] Agarose gel electrophoresis (AGE) (15) with the plasmid markers pAMγ1 (35 Mdal), pAMβ1 (18 Mdal), and pAMα1 (6 Mdal) (5) were used to determine molecular weights.

[d] Plasmid copy number per chromosome gene equivalent was calculated as described previously (5). pAD1::Tn*917* cannot be resolved in strain DS16.

[e] ND, Not determined.

Table 1 shows that strain DS16 harbors two plasmids. These were designated pAD1 and

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pAD2 and were found to have molecular masses of approximately 35 and 15 megadaltons (Mdal), respectively. Each was present to the extent of one to two copies per chromosomal genome equivalent.

On the basis of the following observations, we were able to show that pAD1 determines hemolysin-bacteriocin (hem-bac) production and that pAD2 determines resistance to Em, Sm, and Km. Storage of cells for several months on a slant gave rise to hem-bac-negative variants devoid of pAD1 but still containing pAD2. These occurred at a frequency of about 0.1%. Overnight cultures grown in broth containing acridine orange (30 μg/ml) yielded variants (at a frequency of 0.5%) simultaneously susceptible to Em, Sm, and Km (but not to Tc) which were shown to be devoid of pAD2 but not of pAD1. In addition, mating experiments using the plasmid-free strain JH2-2 as a recipient gave rise to the types of transconjugants shown in Table 2. hem-bac$^+$ (drug-susceptible) transconjugants were found to harbor pAD1 but not pAD2. Transconjugants selected for Em resistance, but which were hem-bac negative, were simultaneously resistant to Sm and Km (but not to Tc) and harbored only pAD2. Recipients that had become hem-bac$^+$ and resistant to Em, Sm, and Km harbored a single plasmid with a size equivalent to the sum of the molecular weights of pAD1 and pAD2, apparently representing a cointegrate. The latter was stable for a number of serial transfers to new recipients. pAD1 was found to be transmissible, as it could be transferred in secondary matings; however, cells harboring only pAD2 could not transfer antibiotic resistance. Thus, pAD2 is nonconjugative but may be mobilized by pAD1. In the majority of cases, mobilization involves a cointegrate, but this may not be strictly required since transconjugants harboring only pAD2 appear at a low frequency. (Generally, more than 90% [20 separate isolates] of drug-resistant transconjugants are hem-bac$^+$, representing cointegrates.) It is noteworthy that in strain DS16 a 50-Mdal plasmid has frequently been observed in small amounts (up to 5%, based on sucrose density gradient analyses) in plasmid preparations from dye-CsCl buoyant density gradients.

Interestingly, a small percentage (2 to 8%) of hem-bac$^+$, Em-resistant transconjugants were susceptible to all of the other drugs. Examination of a number of such strains showed in each case the presence of a single plasmid with a molecular weight of 38×10^6, or 3 Mdal larger than pAD1. Subsequent matings demonstrated that Em resistance now transfers at relatively high frequency (10^{-3} to 10^{-2} per donor) and is

TABLE 3. *Inactivation of Sm and Km by sterile DS16 filtrate*[a]

Antibiotic tested	Additions	Inactivation at antibiotic concn (mg/ml) of:	
		2	0
Sm	Filtrate	+	+
	Heated filtrate	−	+
	Control	−	+
Km	Filtrate	+	+
	Heated filtrate	−	+
	Control	−	+
Em	Filtrate	−	+
	Heated filtrate	−	+
	Control	−	+

[a] DS16, grown to 80 Klett units (using a no. 54 filter), was removed from culture fluid by centrifugation, and the supernatant was passed through a sterile membrane filter (Millipore Corp., type GS, 0.22 μm) and is called "filtrate." Heated filtrate represents sterile culture fluid heated at 90°C for 10 min. The filtrate and heated filtrate were added to individual culture tubes containing the three individual antibiotics such that the final concentration was 0 or 2 mg/ml. The tubes were then seeded with a loopful of JH2-2 (susceptible to the drugs) and incubated for 12 h at 37°C. Controls were similarly prepared tubes, but with sterile medium in place of the filtrate added to medium (in table), and filtrate added to medium without JH2-2 to test for the sterility of the filtrate (no growth). Symbols: +, growth of JH2-2; −, no growth.

TABLE 2. *Various transconjugants obtained from a mating of DS16 and JH2-2*[a]

Phenotype of transconjugant	Frequency-donor	Mol wt ($\times 10^6$) of plasmid present	Plasmid
Hemolytic (Em Sm Km)^s	10^{-3}–10^{-2}	35	pAD1
(Em Sm Km)^r	10^{-9}–10^{-8}	15	pAD2
Hemolytic, Em^r (Sm Km)^s	10^{-8}–10^{-7}	38	pAD1::Tn*917*
Hemolytic, (Em Sm Km)^r	10^{-7}–10^{-6}	50	Cointegrate

[a] JH2-2, a plasmid-free strain resistant to rifampin and fusidic acid, was used as a recipient in 4-h Penassay broth matings at 37°C with DS16 (9). Transconjugants containing only pAD1 were selected on plates containing horse blood, rifampin, and fusidic acid each at 25 μg/ml. Transconjugants containing pAD2 or the cointegrate were selected on the same type of plates, except Em at 50 μg/ml was also added. The superscripts r and s represent, respectively, resistance and susceptibility to the specific antibiotic. Several transconjugants of each class were purified and characterized. The frequencies include the range of numerous mating experiments. The plasmid pAD1::Tn917 and the cointegrate are described in the text.

linked with the hem-bac marker. Further experiments have confirmed that Em resistance is located on a transposon which we have designated Tn*917* (Tomich et al., in press).

pAD2 differs from another *S. faecalis* nonconjugative R-plasmid that we have studied, pAMα1 (10), in that its mobilization by a resident conjugative plasmid occurs only at a very low frequency (10^{-7} to 10^{-6}). pAMα1 can be mobilized by pAMγ1 or pPD1 (two independent 35-Mdal hemolysin plasmids) at frequencies of up to 10^{-2} per donor and higher; in this case, cointegrates do not appear to be involved in transfer (9). The reasons for these differences are not obvious. Conceivably, pAD2, in contrast to pAMα1, could lack certain determinants normally required for efficient mobilization (cf. the case for ColE1 in *Escherichia coli* [16]).

Whereas the mechanism of Em resistance is generally associated with an N^6-dimethylation of adenine in 23S ribosomal ribonucleic acid (17), resistance to Sm and Km frequently involves enzymatic inactivation of the drugs (6, 8). Table 3 shows that culture filtrates of strain DS16 are capable of inactivating high concentrations (2 mg/ml) of both Sm and Km, indicating that drug-modifying enzymes are excreted. (The inactivation could be prevented by heating the filtrate to 90°C for 10 min.) As expected, filtrates had no effect on Em.

The Tc resistance determinant of strain DS16 is located on the chromosome. When the cells are grown in the presence of subinhibitory concentrations of Tc, pAD2 has repeatedly been observed to integrate into the chromosome (4). The reason for this is not clear; however, we suspect that it is related to a Tc resistance amplification phenomenon (3). The Tc determinant has also been observed to transpose onto pAD1, after which it will transfer to recipient strains at a relatively high frequency (11). The transposition of the Tc resistance determinant (tentatively designated Tn*916*) and the Em resistance determinant (designated Tn*917* [Tomich et al., in press]) and the Tc-related amplification phenomenon are currently under investigation in our laboratory.

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