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Drug Resistance Plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*

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Sixteen strains of *Lactobacillus reuteri* and 20 strains of *Lactobacillus acidophilus* were tested for resistance to 22 antibiotics by using commercially available sensitivity disks. Evidence suggesting linkage of these resistances to plasmids was obtained by "curing" experiments with acridine dyes and high growth temperatures. Examination of plasmid patterns by agarose gel electrophoresis provided further evidence of loss in plasmid DNA under curing conditions in some of the strains examined.

Soon after the discovery of sulfanilamide and antibiotics, the resistant strains were isolated (10, 17, 18). At that time, however, it was not realized that some resistances in microorganisms were caused by extrachromosomal elements. A breakthrough came with the discovery that antibiotic resistance is transmitted by mixed cultivation between drug-resistant and -sensitive strains independently of the donor strain chromosome.

Many studies of drug resistance and its extrachromosomal position have been carried out. Studies concerning *Enterobacteriaceae* and staphylococci were reviewed by Watanabe (27), Novick (20), Clowes (6), and Scioli et al. (24).

Very few reports exist on drug resistance in members of the *Lactobacillus* genus (21, 25) and its link with plasmids (10, 12). In a previous paper (26a), we have shown the presence of plasmids in strains of *Lactobacillus*, particularly in *L. acidophilus* strains and, for the first time, in *L. reuteri* strains, described by Kandler et al. (11).

To improve our knowledge of functional properties encoded by plasmids found in *L. acidophilus* and *L. reuteri*, we tested many strains belonging to these species for antibiotic resistance. Then we considered the effects of ethidium bromide, acriflavine, and high temperatures in eliminating these resistances. Finally, we chose the strains most sensitive to the "curing" agents and showed some differences in their plasmid patterns.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. Sixteen strains of *L. reuteri* and 20 strains of *L. acidophilus* were obtained from the culture collection maintained in our laboratory. These strains, isolated from pig feces (labeled S) and calf feces (labeled D and A), were previously identified phenotypically and by genetic tests (22, 23). All of the strains were grown in MRS broth (9) at 37°C in the presence of 20 mM D,L-threonine to improve lysis. As a plasmid weight marker we used *Escherichia coli* strain V517, received from the Plasmid Reference Center, (Stanford University, Stanford, Calif.) and grown to mid-logarithmic phase in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.). Eight plasmids are harbored in this strain; plasmid size, identity, and designation were described by Macrina et al. (13).

Antibiotic agents. Commercially available Sensi-Discs (BBL Microbiology Systems, Cockeysville, Md.) of 22 different antibiotics were used. The potency, in micrograms or units, is based on the Kirby-Bauer procedure (1).

Sensitivity test procedure. A 1-ml portion of actively growing bacterial culture was poured into a petri plate with 10 ml of MRS agar. The disks were placed on the solidified agar surface, and the plates were left overnight at 4°C and then anaerobically incubated at 37°C for 48 h. Resistance was defined according to the Kirby-Bauer method (1).

Curing methods. (i) Ethidium bromide. Elimination of antibiotic resistance by ethidium bromide (Sigma Chemicals, St. Louis, Mo.) was performed according to the method of Bouanchaud et al. (3), modified regarding doses as follows: 10, 20, and 40 μg per ml of broth were the subinhibitory doses we used for our strains.

(ii) Acriflavine. We used the curing agent acriflavine (Fluka AG, Buchs, Switzerland), as described by Watanabe and Fucasawa (28), Ishiwa and Iwata (10), and McKay et al. (15), at a concentration of 5 μg per ml of broth.

(iii) High temperature. We followed the curing method described by Collins and Harvey (7), May et al. (14), and Terawaki et al. (26). Bacterial cultures were grown overnight at temperatures of 46 to 49°C, which were subinhibitory for the different strains.

Plasmid analysis. For isolation of plasmid DNA from the strains of lactobacilli, the method of Currier and Nester (8), modified by Casse et al. (4), was used. The procedure used for the isolation of *E. coli* plasmid

TABLE 1. Antibiotic resistance patterns for *L. reuteri* strains[a]

Bacterial strain	β-Lactams				Aminoglycosides				Tetracyclines			Macrolides				Phenicols (chloramphenicol)	Rifamycines (rifamicyn)	Miscellaneous		Peptides		
	Penicillin	Ampicillin	Cloxacillin	Cephaloridine	Streptomycin	Neomycin	Kanamycin	Gentamicin	Tetracycline	Terramicin	Aureomycin	Erythromycin	Oleandomycin	Spiramycin	Staphylomicin			Novobiocin	Vancomycin	Polymixin B	Colistin	Bacitracin
DSM 20016[b]			*		*	*	*	*	*	*			*						*	*	*	
D27			*		*	*	*	*	*	*	*	*	*	*					*	*	*	*
D109			*		*	*	*	*	*	*	*	*	*	*		*			*	*	*	*
D110			*		*	*	*	*	*	*	*	*	*						*	*	*	
D111			*		*	*	*	*	*	*	*	*	*	*					*	*	*	*
D287			*		*	*	*	*	*	*	*		*						*	*	*	*
2S			*		*	*	*	*	*	*	*	*	*	*					*	*	*	*
3S			*		*	*	*	*	*	*	*	*	*	*					*	*	*	
29S			*		*	*	*	*	*	*	*	*	*	*					*	*	*	*
32S			*		*	*	*	*	*	*	*	*	*	*			*		*	*	*	
33S			*		*	*	*	*	*	*	*						*		*	*	*	
40S			*		*	*	*	*	*	*	*	*	*	*					*	*	*	*
47S			*		*	*	*	*	*	*	*	*	*	*			*		*	*	*	*
55S			*		*	*	*	*	*	*	*	*	*	*		*			*	*	*	
250S			*		*	*	*	*	*	*	*	*	*	*					*	*	*	*
319S			*		*	*	*	*	*	*	*	*	*	*		*			*	*	*	*

[a] Antibiotic resistance is denoted by *; unless so indicated, strains were sensitive.
[b] DSM, Deutsche Sammlung für Mikroorganismen.

DNA was according to the method of Clewell and Helinski (5) as described also by Macrina et al. (13). The examination of plasmid DNA was performed by slab gel vertical electrophoresis, using 0.8% agarose (Bio-Rad Laboratories, Richmond, Calif.) in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA) adjusted to pH 7.8 with acetic acid. To each sample (40 μl) was added 5 μl of a dye solution consisting of 25% sucrose, 5 mM sodium acetate, 0.05% bromophenol blue, and 0.1% sodium dodecyl sulfate (2). Electrophoresis was carried out at 40 mA and 60 V for 5 h. The developed gels were stained in Tris-acetate buffer (pH 7.8) containing ethidium bromide (1 μg/ml) for 0.5 h, washed, and photographed under UV illumination.

Molecular weights (MW) were determined according to the method of Meyers et al. (16). Covalently closed circular DNA, obtained by lysis of our strains, was converted to the open circular form by thermal hydrolysis for 2 h at 75°C (10) to compare the electrophoretic migration of the DNA samples before and after hydrolysis and to determine whether a given band was covalently closed, circular or open circular DNA. With this method, however, we eliminated large plasmids which remain at the bottom of the well and do not enter the gel. Migration distances of the DNA were measured directly from photographs of the gel with dividers and an engineer's ruler. A standard curve for size estimation was generated by using *E. coli* V517 as the reference strain (13).

RESULTS AND DISCUSSION

Interpretation of antibiotic tests. The results of our antibiotic resistance studies are summarized in Table 1 (*L. reuteri*) and Table 2 (*L. acidophilus*). The antibiotic resistances ranged from resistance to 6 to 19 drugs. All strains examined were resistant to the antibiotics of the aminoglycoside group. All, except one strain of *L. acidophilus*, were sensitive to penicillin. Most of the strains tested were sensitive to chloramphenicol. All *L. reuteri* strains were resistant to vancomycin and cloxacillin, whereas many of the *L. acidophilus* strains were sensitive to these antibiotics. Except for DSM 20016, all *L. reuteri* strains were resistant to the three tetracycline antibiotics we used. *L. reuteri* strains exhibited a widely distributed resistance to antibiotics of the peptide group. The resistance pattern of *L. reuteri* strains was rather homogeneous compared with *L. acidophilus* strains.

Loss of antibiotic resistance. In a second stage we considered the antibiotic resistance of our strains after applying the three curing methods. Five strains (two *L. reuteri* and three *L. acidophilus*) did not grow even at the minimum concentration of ethidium bromide used. On the contrary, the concentration of acriflavine did not have any inhibitory effect. As regards curing by temperature, our data reflect the maximum level of temperature the strains could tolerate.

Our results did not allow us to choose one curing method as the most effective, and we think the choice must be made specifically for each individual strain. The effects of curing in most of our strains, with the elimination of several antibiotic resistances, were of interest in confirming the presence of drug resistance plasmids in *L. acidophilus* and *L. reuteri*.

TABLE 2. Antibiotic resistance patterns for *L. acidophilus* strains[a]

Bacterial strain	β-Lactams				Aminoglycosides				Tetracyclines			Macrolides				Phenicols (chloramphenicol)	Rifamycines (rifamicyn)	Miscellaneous		Peptides		
	Penicillin	Ampicillin	Cloxacillin	Cephaloridine	Streptomycin	Neomycin	Kanamycin	Gentamicin	Tetracycline	Terramicin	Aureomycin	Erythromycin	Oleandomycin	Spiramycin	Staphylomicin			Novobiocin	Vancomycin	Polymixin B	Colistin	Bacitracin
A229					*	*	*	*												*	*	
A284					*	*	*	*	*	*										*	*	
7S					*	*	*	*	*	*	*	*		*	*					*	*	*
10S			*		*	*	*	*	*	*	*	*	*	*						*	*	
26S					*	*	*	*	*	*	*									*	*	
147S					*	*	*	*	*	*	*	*	*	*						*	*	
168S	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*		*	*	*	*	
188S			*		*	*	*	*	*	*	*									*	*	*
196S			*		*	*	*	*	*	*	*	*			*					*	*	
247S					*	*	*	*	*	*	*	*	*	*						*	*	*
265S					*	*	*	*	*	*	*									*	*	*
301S					*	*	*	*	*	*	*	*	*	*						*	*	
D137			*		*	*	*	*	*	*	*								*	*	*	
D160			*		*	*	*	*	*	*	*	*	*	*					*	*	*	
D328					*	*	*	*	*	*	*	*	*	*					*	*	*	*
D179			*		*	*	*	*	*	*	*	*	*	*						*	*	*
A274					*	*	*	*												*	*	
A275					*	*	*	*												*	*	
ATCC 4356					*	*	*	*												*	*	
LA3					*	*	*	*												*	*	

[a] Antibiotic resistance is denoted by *; unless so indicated, strains were sensitive.

To obtain further evidence of the link between plasmids and antibiotic resistance, we chose seven strains (four *L. reuteri* and three *L. acidophilus*) as representative. Plasmid profiles of these strains are shown in Fig. 1, and their antibiotic patterns and curing sensitivity are summarized in Tables 3 and 4. Plasmid profiles before and after curing are given in Figs. 2, 3, and 4.

***L. reuteri* strains.** The four *L. reuteri* strains tested (D109, 47S, D111, and D287) showed different sensitivities to the curing agent. Strain D109, after ethidium bromide treatment, lost resistance to bacitracin, acriflavine treatment had no curing effect, and, after growth at 49°C, D109 lost resistance to gentamicin, aureomycin, chloramphenicol, and bacitracin. Strain 47S had the same sensitivity to ethidium bromide and growth at 48°C, losing bacitracin and novobiocin resistances, but with acriflavine there was no curing effect. Plasmid profiles of these two strains are shown in Fig. 2, in which we can see the loss of all of the plasmids harbored by strain D109 (MW: 6.2×10^6, 3.8×10^6, 3.00×10^6, 2.75×10^6, 2.3×10^6, and 1.9×10^6) and two (MW: 4.10×10^6 and 2.55×10^6) of the eight plasmids harbored by strains 47S. Strain D111 had the same sensitivity to ethidium bromide and acriflavine curing, as it lost, after both of these treatments, resistance to erythromycin, oleandomycin, and spiramycin. After growth at 49°C, this strain lost the same antibiotic resistances plus bacitracin resistance, and it lost four plasmids (MW: 44.11×10^6, 40.80×10^6, 3.00×10^6, and 2.00×10^6) harboring only the 7.58-megadalton plasmid (Fig. 3).

The last *L. reuteri* strain we studied, D287, lost only resistance to oleandomycin when treated with ethidium bromide and acriflavine, whereas after growth at 49°C, resistances missing were those to oleandomycin and bacitracin. After curing in this strain, three plasmids were lost (MW: 8.31×10^6, 2.80×10^6, and 2.08×10^6).

***L. acidophilus* strains.** Of the three *L. acidophilus* strains studied (D137, 168S, and A274) strain D137 showed the same sensitivity to the three curing methods, which caused the loss of cloxacillin and aureomycin resistances. Strain 168S exhibited the greatest number of antibiotic resistances of the strains tested. In fact, it was resistant to 19 of the 22 antibiotics used and behaved differently towards the three curing methods (Table 4). The difference concerned either the number or the kind of antibiotic resistances lost. Plasmid patterns of D137 and 168S are shown in Fig. 4. Whereas all four plasmids (MW: 17.3×10^6, 6.6×10^6, 3.31×10^6, $2.50 \times$

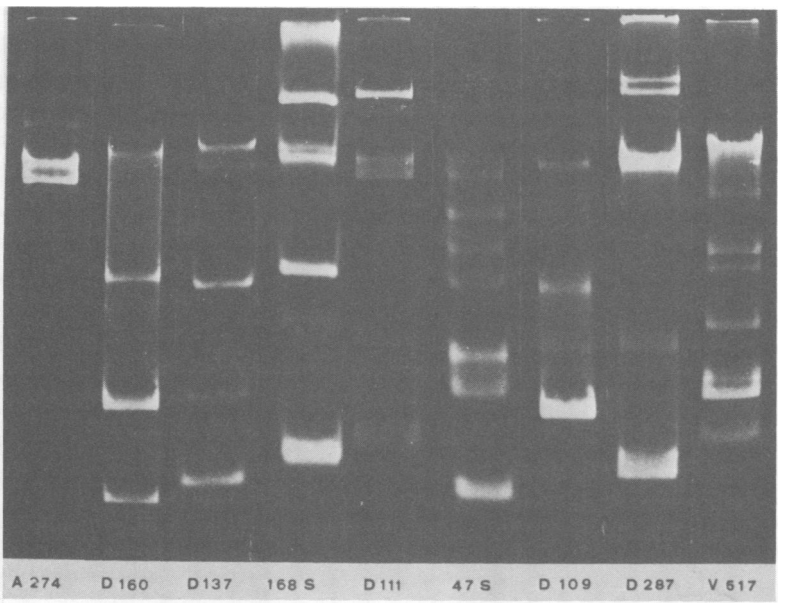

FIG. 1. Plasmid profiles of *L. acidophilus* and *L. reuteri* strains (from left): *L. acidophilus* A274, D160, D137, and 168S; *L. reuteri* D111, 47S, D109, and D287. In the last well are *E. coli* V517 reference mobility plasmids (35.84, 4.82, 3.67, 3.39, 2.63, 2.03, and 1.79 megadaltons). The last band is the open circular form of the 1.36-megadalton plasmid.

TABLE 3. Antibiotic resistance patterns for cured strains of *L. reuteri*[a]

Bacterial strain	β-Lactams				Aminoglycosides				Tetracyclines			Macrolides				Phenicols (chloramphenicol)	Rifamycines (rifamicyn)	Miscellaneous		Peptides		
	Penicillin	Ampicillin	Cloxacillin	Cephaloridine	Streptomycin	Neomycin	Kanamycin	Gentamicin	Tetracycline	Terramicin	Aureomycin	Erythromycin	Oleandomycin	Spiramycin	Staphylomycin			Novobiocin	Vancomycin	Polymixin B	Colistin	Bacitracin
D109	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+
D109 + EB[b]	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	−
D109 + acriflavine	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+
D109 at 49°C	−	−	+	−	+	+	+	−	+	+	−	+	+	+	−	−	−	−	+	+	+	+
47S	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	+	+	+
47S + EB	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−
47S + acriflavine	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+
47S at 48°C	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	−
D111	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+
D111 + EB	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+
D111 + acriflavine	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+
D111 at 49°C	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−
D287	−	−	+	−	+	+	+	+	+	+	+	−	+	−	−	−	−	−	+	+	+	+
D287 + EB	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+
D287 + acriflavine	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+
D287 at 49°C	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−

[a] Antibiotic resistance is denoted by +; loss of antibiotic resistance is denoted by −.
[b] EB, Ethidium bromide.

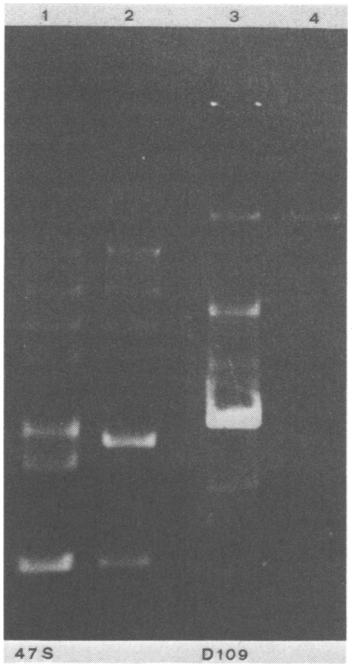

FIG. 2. Plasmid profiles of *L. reuteri* strains 47S and D109 before and after curing. (Well 1) 47S grown at 37°C; (well 2) 47S grown at 48°C; (well 3) D109 grown at 37°C; (well 4) D109 grown at 49°C.

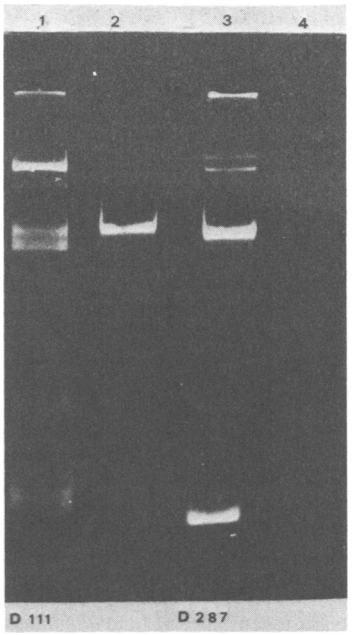

FIG. 3. Plasmid profiles of *L. reuteri* strains D111 and D287 before and after curing. (Well 1) D111 grown at 37°C; (well 2) D111 grown at 49°C; (well 3) D287 grown at 37°C; (well 4) D287 grown at 49°C.

TABLE 4. Antibiotic resistance pattern for cured strains of *L. acidophilus*[a]

Bacterial strain	β-Lactams				Aminoglycosides				Tetracyclines			Macrolides				Phenicols (chloramphenicol)	Rifamycines (rifamicyn)	Miscellaneous		Peptides		
	Penicillin	Ampicillin	Cloxacillin	Cephaloridine	Streptomycin	Neomycin	Kanamycin	Gentamicin	Tetracycline	Terramicin	Aureomycin	Erythromycin	Oleandomycin	Spiramycin	Staphylomycin			Novobiocin	Vancomycin	Polymixin B	Colistin	Bacitracin
168S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−
168S + EB[b]	−	−	+	−	−	+	−	−	+	+	+	+	+	+	−	−	−	−	−	+	+	−
168S + acriflavine	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	−
168S at 48°C	−	−	+	−	−	+	+	−	+	+	−	+	+	+	−	−	−	−	+	+	+	−
D137	−	−	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−
D137 + EB	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−
D137 + acriflavine	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−
D137 at 48°C	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−
A274	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−
A274 + acriflavine	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−
A274 at 46°C	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−

[a] Antibiotic resistance is denoted by +; loss of antibiotic resistance is denoted by −.
[b] EB, Ethidium bromide.

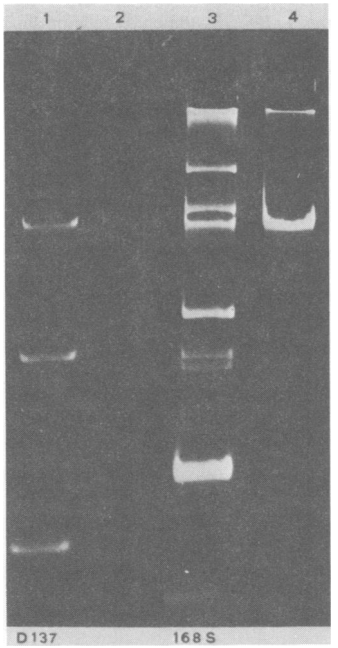

FIG. 4. Plasmid profile of *L. acidophilus* strains D137 and 168S before and after curing. (Well 1) D137 grown at 37°C; (well 2) D137 grown at 49°C; (well 3) 168S grown at 37°C; (well 4) 168S grown at 48°C.

10^6, and 1.65×10^6) of D137 were lost, strain 168S lost five of the six plasmids it harbors (MW: 15.6×10^6, 5.12×10^6, 2.9×10^6, 2.7×10^6, and 1.75×10^6), retaining only the 42-megadalton plasmid.

Strain A274 was chosen because, although it was not sensitive to the curing agents, electrophoretic migration showed a loss of two plasmids (MW: 14.12×10^6 and 7.58×10^6) which were not related to the antibiotic resistances of this strain. Probably these resistances have another genetic location.

Our results show that many of the *L. reuteri* and *L. acidophilus* strains examined have a wide range of antibiotic resistances. The linkage of antibiotic resistance to plasmid DNA was based on the curing results and on the electrophoretic patterns of the strains before and after curing.

We are presently conducting transformation experiments to link each single plasmid to a determined antibiotic resistance.

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