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Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations

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J.L. RUIZ-BARBA, J.C. PIARD AND R. JIMENEZ-DIAZ. 1991. Plasmid profiles of 35 *Lactobacillus plantarum* strains isolated from different green olive fermentors were obtained. A large number of plasmids in the CCC form (from 5 to 16) were present in all the tested strains as confirmed by a second dimension electrophoresis of DNA. These plasmids, all of which remain cryptic, ranged from 2·0 to 68 kb in size. Novobiocin, sodium dodecyl sulphate and ethidium bromide were used as plasmid-curing agents but only novobiocin induced loss of extrachromosomal DNA at a high frequency in these strains.

INTRODUCTION

The genus *Lactobacillus* comprises an important group of lactic acid bacteria of much scientific and practical interest. They are primarily known for their ability to ferment a wide variety of food products as well as feed for animals.

The presence of plasmids within the species of *Lactobacillus* has been reported since they were first discovered in this genus (Chassy *et al.* 1976). Phenotypes such as bacteriocin production and immunity (Muriana & Klaenhammer 1987; Schillinger & Lücke 1989), lactose metabolism (Chassy *et al.* 1978) and drug resistance (Ishiwa & Iwata 1980; Vescovo *et al.* 1982; Morelli *et al.* 1983) have been linked to extrachromosomal DNA in several species of lactobacilli but most of the plasmids are, however, still cryptic.

Lactobacillus plantarum is one of the most important lactic acid bacteria in the production of fermented meats, grass and vegetables, including green olives. While the existence of plasmids in *Lact. plantarum* strains isolated from meat, cheese and silage has been demonstrated (Nes 1984; West & Warner 1985; Hill & Hill 1986; Mayo *et al.* 1989), no information is available about *Lact. plantarum* strains isolated from fermented green olives. As we are interested in this species in order to optimize its use as an inoculant in the lactic acid fermentation of olives, a genetic study of wild type strains used in this fermentation will be useful for this purpose. Therefore, the aim of this work was to demonstrate the presence of plasmid DNA in indigenous *Lact. plantarum* strains isolated from green olive fermentations and to study the stability of these plasmids in the presence of commonly used curing agents.

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MATERIALS AND METHODS

Strains and culture medium

Except for *Lact. plantarum* ATCC 8014 obtained from the Colección Española de Cultivos Tipo, the 35 *Lact. plantarum* strains used in the present research belong to our stock collection. They were isolated in MRS (De Man *et al.* 1960) medium originally from different Spanish-style green olive fermentations in three consecutive years and in four different locations. All of them were grown in MRS solid or liquid medium at 30°C as static cultures.

Plasmid extraction and analysis

For all *Lact. plantarum* strains the protocol of Anderson & McKay (1983) for isolating large plasmid DNA was followed. As a reference, *Streptococcus faecalis* JH2-2 containing the 30 kb plasmid pIP501 (Evans & Macrina 1983), *Streptococcus faecalis* BM4100WT containing 70·1, 53, 49 and 2 kb plasmids (Courvalin *et al.* 1980) and *Escherichia coli* V517 containing 56·4, 7·5, 5·8, 5·3, 4, 3·1, 2·8 and 2·2 kb plasmids (Macrina *et al.* 1978) were used. For *Strep. faecalis* strains, plasmids were extracted as described above for lactobacilli and the method of Grinsted & Bennett (1988) was followed for plasmid isolation from *E. coli*.

Plasmid profiles were obtained by electrophoresis in a submerged horizontal gel apparatus on 0·7% agarose gels in Tris-borate (Meyers *et al.* 1976) at 20 V for 1 h and then at 100 V for 3 h. Gels were stained for 30 min with 0·5 μg/ml

of ethidium bromide, destained for 5 min with 0·001 mol/l $MgSO_4$ and then visualized under u.v. light (302 nm) on a transilluminator. Photographs were taken with a Tri-X film 400 ASA (Kodak, Rochester, NY, USA) using both red (A002) and orange (A003) filters (Cokin, France).

Sizes of lactobacilli plasmids were determined according to the method of Meyers *et al.* (1976).

To distinguish CCC from OC and lineal forms of the plasmids, electrophoresis in a second dimension was used (Hintermann *et al.* 1981).

Plasmid curing

An 18 h culture in MRS broth of each of the *Lact. plantarum* strains used in this study was employed as an inoculum for fresh MRS broth (10 ml) containing ethidium bromide (EtBr), sodium dodecyl sulphate (SDS) or novobiocin. Concentrations ranging from 1·0 to 10 μg/ml (EtBr), 0·125 to 8 μg/ml (novobiocin) and 0·1 to 1·0 mg/ml (SDS) were used.

Lactobacillus plantarum strains were inoculated (about 10^2–10^4 cfu/ml) and incubated at 30°C for at least 72 h. After incubation, the culture containing the highest concentration of curing agent which still allowed visible growth was taken, treated cells were diluted in saline and appropriate dilutions (0·1 ml) were plated on MRS agar. After incubation at 30°C, isolated colonies were randomly selected for further plasmid analysis.

As a control, MRS broths without any curing agent were inoculated with each of the *Lact. plantarum* strains studied, incubated at 30°C for the same time as for the curing treatments and then plated on MRS agar. Single colonies were randomly selected for plasmid identification as described above.

RESULTS

The presence of extrachromosomal DNA in 35 different strains of *Lact. plantarum* isolated from Spanish-style green olive fermentations was investigated. Figure 1 shows the plasmid profiles of some of these wild type strains. As summarized in Table 1, all of these lactobacilli appeared to contain several plasmid bands. The number of plasmids present in a single strain varied from 5 to 16 ranging between 68 and 2 kb in size but in general low molecular weight plasmids predominated over the high molecular weight ones.

Overestimation of the number of plasmids harboured by

Table 1 Plasmid profiles of *Lactobacillus plantarum* wild type strains

Strains	No. of plasmids	Size (kb)
LPC1, LPC7	8	60; 45; 24; 15; 10·3; 8·4; 3·4; 2·5
LPC4, LPC5, LPC10, LPC13, LPC14, LPC17	6	50; 13; 12; 10; 3·4; 2·5
LPC16	5	50; 13; 12; 10; 3·4
LPC19	7	49; 30·5; 24·5; 15·5; 9; 25; 2
LPC22	8	45; 25; 20; 14; 10·5; 8·4; 3·4; 2·1
LPC25	16	50; 40; 30; 24; 21; 18·5; 12; 10·5; 10; 9; 8·6; 8; 4; 3·4; 2·5; 2
FB/2	6	31; 24; 15·5; 11·5; 8·8; 2
6N/1	8	40; 25; 10; 8·2; 5·6; 3·3; 2·5; 2
BOM/1	10	50; 34; 25; 12·5; 11·5; 10·8; 8·1; 3·4; 3·3; 2
144/1	9	50; 30·5; 15; 13; 10; 8·2; 5·6; 5·4; 3·5
15N/1, 15N/2	7	49; 37; 25; 10·5; 8·6; 7·6; 3·8
128/1	11	68; 50; 37; 15·5; 12·5; 11·5; 10·5; 5; 3·5; 3·3; 2·1
128/2	7	45; 25; 10·5; 8·4; 5·6; 5·4; 3·5
LPS1, 1/112/1	5	68; 12; 11·5; 8·4; 2·4
LPS5	6	68; 25; 12; 10·5; 8·4; 2·4
LPS10, 1/43/1	7	52; 30; 15; 12·5; 9; 8·4; 3·2
LPS15, LPS20, 1/91/1	8	68; 45; 35; 23; 16; 13; 12; 2·4
LPCO1, LPCO6	9	52; 44; 30; 19; 16·5; 12; 8·4; 4·8; 2·4
LPCO10	9	49; 35; 27; 18; 16·5; 12; 8·4; 4·8; 2·4
2/43/2	6	68; 45; 23; 22; 16; 2·4
2/112/1, 2/91/1, 2/43/1	7	68; 49; 25; 12; 10·5; 8·4; 2·4

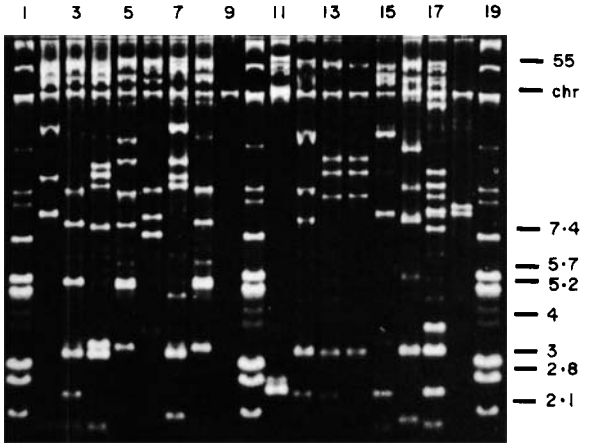

Fig. 1 Agarose gel electrophoresis of plasmid DNA from wild type *Lactobacillus plantarum* strains isolated from green olive fermentations: strain FB/2 (lane 2), strain 6N/1 (lane 3), strain BOM/1 (lane 4), strain 144/1 (lane 5), strain 15N/1 (lane 6), strain 128/1 (lane 7), strain 128/2 (lane 8), strain LPC1 (lane 12), strain LPC4 (lane 13), strain LPC16 (lane 14), strain LPC19 (lane 15), strain LPC22 (lane 16) and strain LPC25 (lane 17). *Escherichia coli* V517 (lanes 1, 10 and 19), *Streptococcus faecalis* JH2-2 (lane 9) and BM4100WT (lane 11) and *Lact. plantarum* ATCC 8014 (lane 18) were used as standards. The numbers on the right indicate the size of the standard plasmids from *E. coli* V517 (in kb)

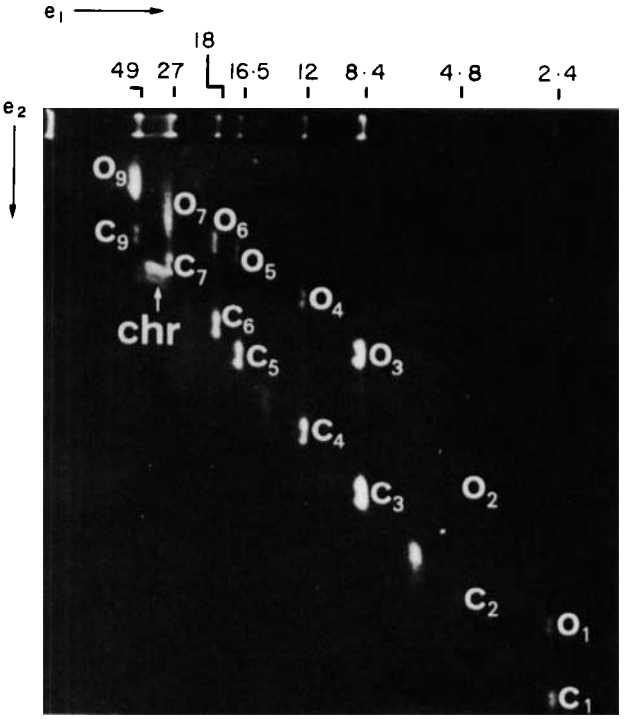

Fig. 2 Identification of *Lactobacillus plantarum* LPCO10 plasmids. Total DNA from this strain was separated on a 0·7% agarose gel (e_1) and then stained with 0·5 μg/ml EtBr. After 10 min of u.v. irradiation, the gel was subjected to a second electrophoresis (e_2), perpendicular to the first one (e_1). The numbers on the upper part of the photograph indicate the size of *Lact. plantarum* LPCO10 plasmids (in kb). The 35 kb plasmid was not present in this preparation. Symbols: O_1 to O_9, OC form of the corresponding plasmid 1 to 9; C_1 to C_9, CCC form of the corresponding plasmid 1 to 9; chr, chromosome

each strain was avoided by using the method of Hintermann *et al.* (1981). In Fig. 2, the second dimension electrophoresis of the *Lact. plantarum* strain LPCO10 plasmid DNA is illustrated.

The results obtained in curing experiments with SDS, novobiocin and EtBr in five selected strains of *Lact. plantarum* are summarized in Table 2. As is shown, novobiocin appeared to be the most effective of the three curing agents used, at concentrations ranging from 0·125 to 0·25 μg/ml in the culture medium. In the case of *Lact. plantarum* ATCC 8014, however, the plasmids were found to be resistant to the effect of SDS, EtBr and novobiocin and no cured derivatives were obtained.

The percentage of cured isolated colonies randomly tested from LPS5, 128/1, 2/112/1 and LPCO10 strains after treatment with novobiocin was almost 100% and also a high number of plasmids were eliminated in all cases. Nevertheless, certain plasmids were lost more frequently than others, as shown in Table 3. In general, whereas low molecular weight plasmids were eliminated in most cases, high molecular weight ones were lost at a lower frequency. However, there are some exceptions such as certain plasmids in *Lact. plantarum* LPCO10.

DISCUSSION

Our results corroborate previous reports (Nes 1984; West & Warner 1985; Hill & Hill 1986; Mayo *et al.* 1989; Klaenhammer 1984) in which the presence of a variety of plasmids was clearly demonstrated in strains of *Lact. plantarum* from different sources. In contrast with most of these reports, however, we detected plasmid bands in all of the studied strains and many of these plasmids were of considerably higher molecular weight than those observed previously. As the method of Anderson & McKay (1983) for the extraction of plasmid DNA was also followed by some of these authors (Hill & Hill 1986; Mayo *et al.* 1989), the differences observed are probably not due to the extraction procedure used but to the different source of the strains.

Although strains with the same plasmid profile are grouped in Table 1, it is not necessarily true that all those plasmids are identical. We do not know what these plasmids encode and they remain cryptic at present. However, we can state that some of the studied strains appear to be similar in both number of plasmids harboured and molecular weight of these plasmids. Moreover, those strains sharing a certain plasmid profile were isolated from different fermentors at the same location. The plasmid profiles

Table 2 Curing of plasmids from wild-type strains of *Lactobacillus plantarum* by using different curing agents

Curing agent	Concentration	Strain	% of cured isolated colonies	Total no. of cured plasmids	Size of lost plasmid (kb)
EtBr	8–10 μg/ml	LPS5	10 (1/10)*	1	25
		2/112/1	10 (1/10)	2	49; 12
		128/1	0 (0/10)	—	—
		LPCO10	ND	—	—
		ATCC 8014	0 (0/10)	—	—
Novobiocin	0·125–0·25 μg/ml	LPS5	100 (10/10)	4	68; 25; 10·5; 8·4
		2/112/1	100 (8/8)	1	8·4
		128/1	100 (10/10)	7	68; 12·5; 11·5; 10·5; 5; 3·5; 2·1
		LPCO10	94 (49/52)	7	49; 35; 27; 18; 16·5; 12; 8·4
		ATCC 8014	0 (0/10)	—	—
SDS	0·6–1 mg/ml	LPS5	10 (1/10)	1	68
		2/112/1	50 (5/10)	2	68; 49
		128/1	0 (0/10)	—	—
		LPCO10	ND	—	—
		ATCC 8014	0 (0/10)	—	—

* Colonies cured/colonies tested.
EtBr, Ethidium bromide; SDS, sodium dodecyl sulphate; ND, not determined.

appear to be conserved in these instances even when the strains were isolated in consecutive years. We also found many cases in which almost the complete profile was shared by different strains from the same location; from three to eight plasmid bands that were identical in electrophoretical motility, although at least one of the bands always differed. Taking into account the fact that the lactobacillus population in each fermentor used for the isolation of these strains was naturally occurring, it is possible that certain plasmids are widely distributed among the strains from a certain location and maintained for years in the same environment of olive processing due to, perhaps, some advantageous characteristics conferred by these plasmids or that the strains themselves share a common ancestry. We are at present carrying out restriction analysis and hybridization experiments with some of these plasmids in order to establish their level of similarity as well as to elucidate their possible role in the fermenting properties of *Lact. plantarum*.

Table 3 Curing efficiency of novobiocin on plasmids from *Lactobacillus plantarum*

Strain	Plasmid lost (kb)	Percentage of loss in isolated colonies
LPS5	68	10
	25	10
	10·5	30
	8·4	90
128/1	68	20
	12·5	30
	11·5	60
	10·5	70
	5	60
	3·5	50
	2·1	50
2/112/1	8·4	100
LPCO10	49	45·6
	35	2
	27	30·6
	18	73·5
	16·5	8·2
	12	10·2
	8·4	4·1

Novobiocin has not been extensively used as a plasmid curing agent in lactobacilli even though it has been successfully utilized to eliminate plasmids in other bacteria (McHugh & Swartz 1977; Taylor & Levine 1979). However, intercalating dyes such as acriflavine and EtBr, elevated growth temperature, SDS and other agents have been commonly applied for this purpose (Vescovo *et al.* 1982; Morelli *et al.* 1983; Vescovo *et al.* 1984). In our experience, neither EtBr nor SDS were as effective as novobiocin in curing plasmids from our strains, as shown in Table 2. On the other hand, our results are in agreement with those obtained by Danilevskaya & Gragerov (1980) and Cejka *et al.* (1982) in which they demonstrated a stronger effect of some novobiocin related compounds on smaller plasmids from *E. coli*. As our strains normally

harbour some small plasmids, this curing agent in combination with others will be useful to obtain plasmid-free *Lact. plantarum* variants. In the future we will use these variants in order to elucidate the functions encoded by the plasmids present in these *Lact. plantarum* strains.

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