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Batch cultures of recombinant *Lactococcus lactis* subsp. *lactis* in a stirred fermentor

I. Effect of plasmid content on bacterial growth and on genetic stability in pure cultures

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Summary. The effect of plasmid introduction into *Lactococcus lactis* subsp. *lactis* IL2661 on the growth of this strain and on plasmid stability was studied in pure batch cultures. The plasmids used (coding for erythromycin or chloramphenicol resistance) were the following: pIL205 (42 kb), pIL252 (4.6 kb, 6-9 copies), pIL253 (4.8 kb, 45-85 copies) and pE194 (inserted in the chromosome). Growth and acidification of *L. lactis* subsp. *lactis* IL2661 were similar to those of the derived recombinant lactococci. The maximal population at the end of the fermentation (9 h) was about $1.1 \pm 0.3 \times 10^{10}$ cfu/ml, and maximal growth rate $0.92 \pm 0.07\ h^{-1}$. Growth yield and lactic acid concentrations were $3.9 \pm 0.8 \times 10^{11}$ cfu/g lactose consumed and 25.6 ± 2.3 g/l, respectively. Different levels of plasmid stability were detected. Plasmid pE194, and plasmids pIL252 and pIL253 in the absence of pIL205, were stable after 10 h of culture. A slight loss (1-2%) of pIL205 was observed in all strains. In the presence of pIL205, plasmids pIL252 and pIL253 were maintained in only 56-95% of the cells. This result suggested an incompatibility between pIL205 and pIL252 or pIL253.

Introduction

Lactic acid bacteria constitute a very important group of microorganisms in the dairy industry. Their utilization, however, is never free of problems (acidification faults, bacteriophage attacks). As a result of progress in molecular biology and particularly genetic engineering techniques, it is now possible to construct new strains (Kondo and McKay 1985; Sandine 1987) in order to improve their technological characters. The problem of bacteriophage attack on lactic acid bacteria can be resolved by the use of strains improved in terms of their phage resistance (De Vos 1989; Sandine 1989). Cloning the genes coding for proteolytic enzymes (Kok and Venema 1988; Hayes et al. 1990) and peptidases (Nardi et al. 1991) would contribute to the improvement of proteolysis and growth of lactic acid bacteria in milk.

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The use of recombinant lactic acid bacteria on an industrial scale, however, requires a detailed investigation in order to study:

1. A possible change in the characteristics of these bacteria due to the introduction of exogenous genetic elements.
2. The instability of these elements.

The presence of certain plasmids has a deleterious effect on growth of *Escherichia coli* strains, especially highly amplified plasmids (Uhlin et al. 1979; Padan et al. 1983; Betenbaugh et al. 1989). The replication of high-copy-number plasmids represents an additional "metabolic burden" on the normal chromosome-directed metabolism of the bacterial cell. This can affect growth characteristic parameters of the recombinant strains.

Plasmid stability is often defined as the ability of recombinant cells to maintain their plasmid during growth (Imanaka and Aiba 1981). There are two major types of plasmid instability. The first, segregational instability, refers to the loss of the complete plasmid due to defective partitioning during cell division. The second, structural instability, refers to the change in plasmid structure due to deletion, insertion or rearrangement of DNA, which can result in the loss of plasmid-encoded function. In comparison with results reported for *Bacillus subtilis* (Harington et al. 1988), *E. coli* (Marin-Iniesta 1988; Tolentino and San 1988) and *Saccharomyces cerevisiae* (Caunt et al. 1989), relatively little work has been done on plasmid stability in lactic acid bacteria.

The purpose of this study was to quantify the influence of plasmid content on *Lactococcus lactis* growth and acidification and on plasmid stability. In order to maintain as much similarity as possible in all genetic and metabolic factors, the same host strain was used. The plasmids introduced into this strain differed in their size or their copy number; one of them was inserted in the chromosome. The experiments were performed under conditions of starter production, i.e. during batch cultures with controlled pH.

Materials and methods

Bacterial strains and plasmids. Table 1 lists *L. lactis* subsp. *lactis* IL2661 and the derived recombinant strains. All derived from the strain *L. lactis* subsp. *lactis* IL1403 (Chopin et al. 1984) and were protease-negative (prt$^-$). They were obtained from the collection of the Microbial Genetics Laboratory (Jouy-en-Josas, France). *L. lactis* subsp. *lactis* IL1776 (pIL205) (lactose-negative), obtained from the same collection, was used as reference strain in agarose gel electrophoresis.

The plasmids of the strains used in this study are listed in Table 2. Plasmid pIL9 enables lactose to be utilized. Plasmid pIL205 (27.3 kb; chloramphenicol-resistant, Chl^r) was derived from plasmid pIP501 (Horodniceanu et al. 1976). Plasmids pIL252 (4.6 kb, 6–9 copies) and pIL253 (4.8 kb, 45–85 copies), derived from plasmid pAMβ1 (Clewell et al. 1974), confer erythromycin resistance (Ery^r). Plasmid pE194 (conferring Ery^r) was inserted in the chromosome by a Campbell-like recombination (strain IL3223) or by double crossing-over with deletion (strain IL3224) (Galleron, personal communication). Insertions were like those described for *L. lactis* subsp. *lactis* IL1403 (Chopin et al. 1989).

Fermentation conditions. Strains were stored at $-20°$C in M17 broth (Biokar, Beauvais, France) supplemented with the appropriate antibiotic. Erythromycin, chloramphenicol and streptomycin (Sigma, St. Louis, Mo., USA) were added at concentrations of 5, 10 and 2000 μg/ml, respectively.

After thawing, the strains were grown for 8 h at 30° C, after which they were transferred to an antibiotic-free medium sterilized at 110° C for 10 min and containing 100 g/l of skim milk (Elle-et-Vire, Union Laitière Normande, Condé-sur-Vire, France), 5 g/l of yeast extract (Difco, Detroit, Mich., USA), 5 g/l of bacto-peptone (Difco) and 1 ml/l of anti-foam silicone (Prolabo, Paris, France). Yeast extract and peptone were added in order to allow a good growth of the prt$^-$ strains. This medium was also used as culture medium in the fermentor. After overnight incubation at 30° C, the resulting culture was used to inoculate the fermentor at 5%.

Pure cultures of *L. lactis* subsp. *lactis* IL2661 and of the recombinant strains were carried out in duplicate. A 15-l LSL-Biolafitte fermentor (useful volume 10 l) was used for all fermentations, with control of temperature (30° C), agitation speed (200 rpm) and pH (6.5) by the addition of 10 M sodium hydroxide. Fermentations lasted 10 h.

Table 1. *Lactococcus lactis* subsp. *lactis* IL2661 and derived recombinant strains

Strain	Plasmid content
IL2661	pIL9
IL2673	pIL9, pIL252
IL2668	pIL9, pIL253
IL2674	pIL9, pIL205
IL2682	pIL9, pIL252, pIL205
IL2683	pIL9, pIL253, pIL205
IL3223	pIL9, pE194[a]
IL3224	pIL9, pE194[b]

[a] Plasmid pE194 was inserted in the chromosome by Campbell-like recombination
[b] Plasmid pE194 was inserted in the chromosome by double crossing-over with deletion

Monitoring fermentations. Samples were taken hourly. The bacterial population in the fermentor was determined by plating on M17 agar (Biokar) with or without antibiotics. Lactose and lactic acid concentrations were determined by HPLC (Waters Associates) on an Aminex HPX-87H column (Bio-Rad, Richmond, Calif., USA) with 0.1 M sulphuric acid as mobile phase at a flowrate of 0.6 ml/min.

Data processing. The bacterial enumeration data were mathematically smoothed using the Weibull function (Lebreton and Millier 1982) modified as follows. This is a non-linear regression applied to the increase in the natural logarithm of the bacterial population (X) as a function of culture time (T):

$$\mathrm{Ln}\,X = a-(a-b)\exp(-\exp(-c)\exp(d\,\mathrm{Ln}(T)))$$

where a, b, c and d are constants.

The growth rate (μ) was determined by calculating the first derivative of this equation. Its maximum value (μ_{max}) was reached when the derivative was cancelled with reference to time. Changes in the lactose and lactic acid concentrations with time were also smoothed with the Weibull function.

Six parameters characteristic of growth and acidification were determined: the initial bacterial population, X_0 (cfu/ml), the maximum bacterial population, X_{max} (cfu/ml), the time, T_{max} (h), at which X_{max} was reached, the maximum growth rate, μ_{max} (h^{-1}), the yield of biomass, Y_{max} (cfu/g), and the final lactic acid concentration, LA_{max} (g/l). The last two parameters were determined at time T_{max}, based on smoothed values. The biomass yield was the ratio between the maximum population and lactose consumed.

Determination of plasmid stability. The stability of plasmids pIL205, pIL252, pIL253 and pE194 was studied in cultures of the recombinant lactococci derived from *L. lactis* subsp. *lactis* IL2661. The percentage of clones retaining their plasmid was determined in each culture sample by replica-plating 100 colonies from M17 agar to M17 agar supplemented with the antibiotic corresponding to the plasmid being tested. Plasmid content of the strains was verified by agarose gel electrophoresis.

Extraction of plasmid DNA and agarose gel electrophoresis. Plasmid DNA was extracted as described by Anderson and McKay (1983). Electrophoresis was carried out on a horizontal slab of 0.5% agarose gel (Seakem, FMC Bioproducts, Rockland, Maine, USA) containing 0.04 μg/ml of ethidium bromide. Electrophoresis was at constant voltage (60 V) for 15 h in TBE buffer (89 mM TRIS-borate, 2 mM EDTA).

Table 2. Plasmids of the strains used in this study

Plasmid	Plasmid marker[a]	Copy number	Size (kb)	Reference
pIL9	Lac$^+$	1–2	42.0	Chopin et al. (1984)
pIL252	Ery^r	6–9	4.6	Simon and Chopin (1988)
pIL253	Ery^r	45–85	4.8	Simon and Chopin (1988)
pIL205	Chl^r	1–2	27.3	Langella (1988)
pE194	Ery^r	1	3.7	Horinouchi and Weisblum (1982)

[a] Lac$^+$, lactose utilization; Ery^r, erythromycin resistance; Chl^r, chloramphenicol resistance

Results and discussion

Growth and acidification characteristics

The growth and acidification characteristics of the recombinant strains of *L. lactis* subsp. *lactis* were determined in order to study their behaviour in the stirred fermentor. These characteristics were compared to those of *L. lactis* subsp. *lactis* IL2661 (host strain). The example of a pure culture of *L. lactis* subsp. *lactis* IL2668 was chosen to illustrate the progression of a typical fermentation. Changes in the bacterial population (Fig. 1A) and in lactose consumption and lactic acid production (Fig. 1B) show that the experimental values are very close to the value smoothed by the Weibull function. Lactic acid production started soon after the onset of bacterial growth and continued after the cessation of cell growth, during the stationary phase. The growth rate versus fermentation time (the derivative of the Weibull function) is also shown in Fig. 1A. It increased very rapidly at the beginning of the culture, reaching μ_{max} and then progressively decreasing until reaching a null value in the stationary phase.

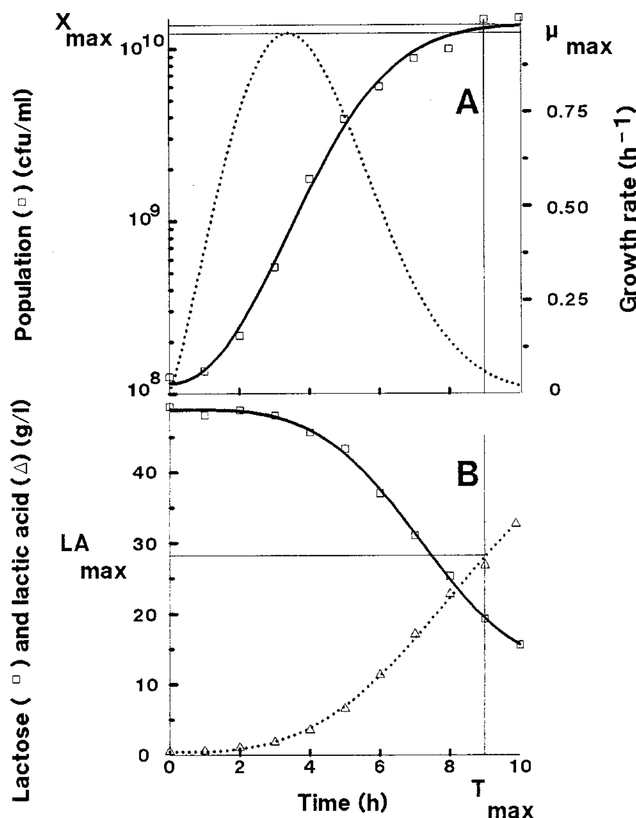

Fig. 1A, B. Fermentation kinetics of *Lactococcus lactis* subsp. *lactis* IL2668. **A** Bacterial population (□) and growth rate (···). **B** Lactose (□) and lactic acid concentration (△): —, ··· are smoothed curves. (T_{max}, X_{max}, μ_{max}, LA_{max}: for explanation, see Materials and methods)

Table 3 lists the growth and acidification characteristics of *L. lactis* subsp. *lactis* IL2661 and of the recombinant strains. From duplicated cultures, average and standard deviation were determined for every parameter (except T_{max}). Variation coefficients were then calculated and were less than 62% for X_0 and X_{max}, 16% for μ_{max}, 47% for Y_{max} and 23% for LA_{max}. Those high values could be related to inaccuracy of bacterial enumeration (at least 30%) and of determination of the lactose and lactic acid concentrations (at least 8%).

Taking into account errors due to the determination of growth and acidification characteristics, the results show that the growth of *L. lactis* subsp. *lactis* IL2661 was similar to that of the recombinant strains derived from it. For similar inoculation levels (0.9 and 1.2 (±0.3) 10^8 cfu/ml, respectively), the X_{max} were nearly the same (1.1 (±0.1) and 1.1 (±0.3) 10^{10} cfu/ml). The same was true for μ_{max} (1.03 (±0.01) and 0.91 (±0.07) h^{-1}) and Y_{max} (3.4 (±0.3) and 3.9 (±0.9) 10^{11} cfu/g). Similar LA_{max} were produced at T_{max} (28 (±6.4) and 25.2 (±2.2) g/l).

Plasmids pIL205, pIL252 and pIL253 differ by their size (27.3 kb [pIL205] and 4.6–4.8 kb [pIL252–pIL253]) and their copy number (6–9 [pIL252] and 45–85 [pIL253] copies). The introduction of these plasmids (alone or in pairs) into *L. lactis* subsp. *lactis* IL2661 had apparently no significant effect on the growth and acid-

Table 3. Growth and acidification characteristics[a] of *L. lactis* subsp. *lactis* strains

Strain	T_{max} (h)	X_0 ($\times 10^8$) (cfu/ml)	X_{max} ($\times 10^{10}$) (cfu/ml)	μ_{max} (h^{-1})	Y_{max} ($\times 10^{11}$) (cfu/g)	LA_{max} (g/l)
IL2661	8	0.9±0	1.1±0.1[b]	1.03±0.01	3.4±0.3	28.0±6.4
IL2673	9	0.9±0.4	0.6±0.2	0.89±0.11	2.3±0.7	21.4±2
IL2668	9	1.1±0.1	1.1±0.1	0.89±0.11	4.2±1.1	26.5±6.2
IL2674	9	1.1±0.6	0.8±0.4	0.91±0.15	3.2±1.5	23.3±2.6
IL2682[c]	9	1.3±0.2	1.2±0	0.85±0.06	4.3±0	26.6±0.8
IL2683[c]	8	1.3±0.8	1.3±0.2	1.05±0.1	4.5±0.1	27.1±4.4
IL3223	9	1.1±0.2	1.2±0.1	0.87±0.04	4.7±0.2	24.4±1.5
IL3224	9	1.7±0.1	1.3±0.2	0.89±0.02	4.3±0.8	27.1±0.7

[a] T_{max}, final time of the fermentation; X_0, initial population; X_{max}, maximal population; μ_{max}, maximum growth rate; Y_{max}, maximum growth yield on lactose; LA_{max}, final concentration of lactic acid. For culture conditions, see Materials and methods
[b] Average and standard deviation for two experiments
[c] After 10 h of culture, 56–95% of the cells contained plasmid pIL252 (strain IL2682) or pIL253 (strain IL2683). The μ_{max} of derived strains IL2682 and IL2683, having lost pIL252 or pIL253 respectively, were 0.85 and 1.09 h^{-1}

ifying activity of this strain. The same was true for the insertion of plasmid pE194 in the chromosome.

Concerning the effect of plasmid size on the growth of a recombinant strain, our results were similar to those of Copella and Dhurjati (1989). These authors have determined the effects of plasmid size (6.3 kb and 14.3 kb) on the growth of *S. cerevisiae* strains during batch culture without selective pressure. They showed that plasmid size had no effect on the μ_{max} and Y_{max} of the host strain. Moreover, our results showed that there was no effect of the plasmid copy number on the growth of a *L. lactis* subsp. *lactis* strain. This result was inconsistent with that from Seo and Bailey (1985), who studied the effect of introduction of closely related plasmids with a different copy number on the growth of *E. coli* HB101. The recombinant strains bearing plasmids at 12, 24, 60, 122 and 408 copies had respective decreases in their μ_{max} of 8%, 9%, 13%, 18% and 23%, compared to that of the plasmid-free strain. The difference between the two results could be due to the precision of the method used for the determination of growth parameters. Our study did not allow us to detect variations in μ_{max} lower than 16%. Therefore, the effect of plasmid copy number might not be statistically significant.

Plasmid stability

Plasmid stability of pIL205, pIL252, pIL253 and pE194 was investigated in pure cultures of recombinant *L. lactis* subsp. *lactis* with no selection pressure. Three levels of stability were detected:

1. Plasmids pIL252 (small copy number) and pIL253 (high copy number) were conserved in strains IL2673 and IL2668. The stability of these plasmids was 100% from the beginning to the end of the fermentation (10 h). Regardless of the mode of insertion in the chromosome (Table 1), plasmid pE194 was also conserved in strains IL3223 and IL3224. Our study on plasmid stability was carried out in batch cultures involving a small number of generations (maximum ten). Simon and Chopin (1988) have studied the stability of plasmids pIL252 and pIL253 in the plasmid-free strain *L. lactis* subsp. *lactis* IL1403. They observed, after 28 generations, that 99% of the cells carried plasmid pIL253. On the other hand, plasmid pIL252 was markedly unstable, the percentage of cells bearing this plasmid being 34% after nine generations. The presence of plasmid pIL9 could contribute to a better stabilization of plasmid pIL252.
2. Plasmid pIL205 in the presence of only plasmid pIL9 (strain IL2674) was stable in the first test and showed a very slight loss (1–2%) in the second. In the presence of pIL9 and plasmids pIL252 (strain IL2682) and pIL253 (strain IL2683), pIL205 was conserved in 98–99% of the cases (Fig. 2). The small percentage loss appeared only between hours 9 and 10 in strains IL2682 and IL2683. Clones of these strains having lost pIL205 conserved plasmids pIL252 and pIL253, respectively.
3. Plasmids pIL252 (strain IL2682) and pIL253 (strain IL2683) were less stable in the presence of pIL9 and pIL205. The percentage of cells bearing these plasmids decreased during fermentation, reaching 56–95% after 10 h of culture. Figure 2 shows such a loss of plasmid pIL252 in a culture of *L. lactis* subsp. *lactis* IL2682. Instability was already observed in the pre-culture. The maximum growth rates of derived strains IL2682 and IL2683, which had lost pIL252 or pIL253, were 0.85 and 1.09 h^{-1}, respectively.

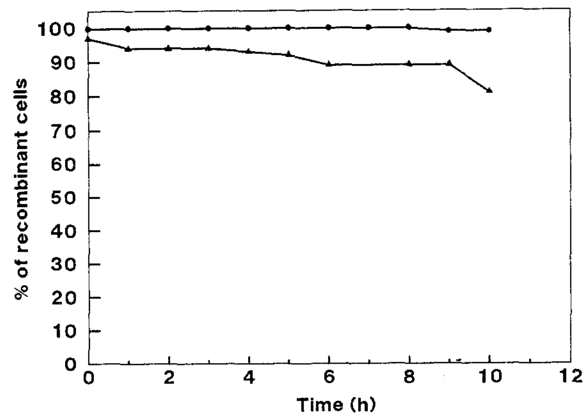

Fig. 2. Stability of plasmids pIL205 and pIL252 in *L. lactis* subsp. *lactis* IL2682 during a batch culture. Recombinant cells bearing pIL205 (●) or pIL252 (▲)

The instability of plasmids pIL252 and pIL253 in strains IL2682 and IL2683 may be explained either by a continuous loss of these plasmids during the growth of the strains or by the more rapid growth of the strains having lost pIL252 or pIL253. The first hypothesis is likely to be the better one, since derived strains IL2682 and IL2683 having lost plasmids pIL252 and pIL253 had μ_{max} values comparable to those of strains having conserved these plasmids (Table 3).

The stability of plasmids pIL252 and pIL253 in the presence of plasmid pIL9 and the loss of stability of the same plasmids in the simultaneous presence of pIL9 and pIL205 raises the problem of incompatibility between plasmids. Incompatibility is the incapacity of two or several plasmids to coexist in a stable manner in a cell line (Novick and Hoppensteadt 1978). Plasmids pIP501 (parental plasmid of pIL205) and pAMβ1 (parental plasmid of pIL252 and pIL253) are homologous at the level of the replication gene (Evans and Macrina 1983; Leblanc and Lee 1984). This would explain an incompatibility between plasmids pIL205 and pIL252 or pIL253, the origin of which could be the replication or partition functions (Bergquist 1987).

The construction of plasmids pIL252 and pIL253 (respectively 4.6 and 4.8 kb), by deletions of plasmid pAMβ1 (26.5 kb) (Simon and Chopin 1988), could be responsible for the loss of some functions useful for plasmid stabilization, such as the resolvase gene (Swinfield et al. 1991). This gene maintains the plasmid population in the monomeric state, so lowering the probability of producing plasmid-free segregants. Since plasmid pIL205 (27.3 kb), obtained from plasmid pIP501 (30.2 kb), underwent only small deletions, it may have conserved some of these functions. This could explain the better stability of plasmid pIL205 compared to that of

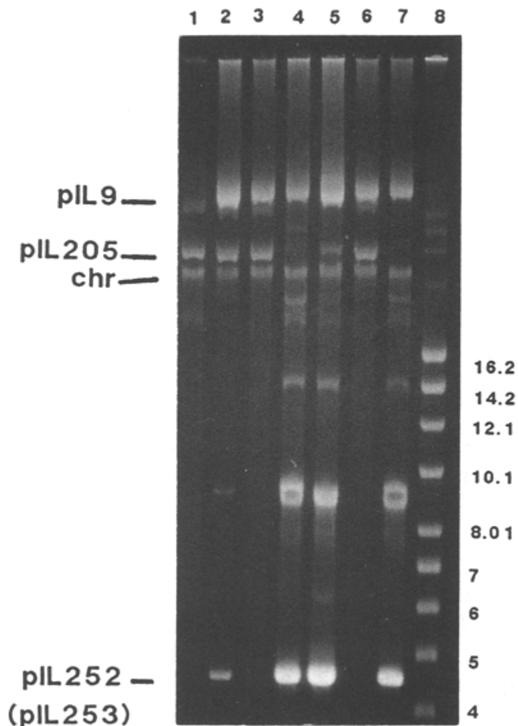

Fig. 3. Agarose gel electrophoresis of plasmid DNA isolated during batch cultures from *L. lactis* subsp. *lactis* IL1776 (*lane 1*); IL2682 (*lane 2*); derived strain IL2682 that had lost pIL252 (*lane 3*) or pIL205 (*lane 4*); IL2683 (*lane 5*); derived strain IL2683 that had lost pIL253 (*lane 6*) or pIL205 (*lane 7*). *Lane 8*: 1-kb supercoiled ladder (Bethesda Research Laboratories, Bethesda, Md., USA)

pIL252 (pIL253) in *L. lactis* subsp. *lactis* IL2682 (IL2683).

Clones were analysed by agarose gel electrophoresis (Fig. 3). The loss of plasmid pIL205 was thus observed in strains IL2682 (lane 4) and IL2683 (lane 7). The same was true for plasmids pIL252 (lane 3) and pIL253 (lane 6) of strains IL2682 and IL2683. These results showed segregation instability of plasmids pIL205, pIL252 and pIL253.

No structural instability was observed for plasmids pIL252 and pIL253. This is in total agreement with the high structural stability of plasmid pAMβ1 (parental plasmid of pIL252 and pIL253) (Jannière et al. 1990) due to its unidirectional theta replication (Bruand et al. 1991). It seems also that plasmid pIP501 replicates in the same way. This is characteristic of large plasmids of Gram-positive bacteria.

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