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Effect of Plasmid Incompatibility on DNA Transfer to *Streptococcus cremoris*

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Several *Streptococcus cremoris* strains were used in protoplast transformation and interspecific protoplast fusion experiments with *Streptococcus lactis* and *Bacillus subtilis*, with pGKV110, pGKV21, and ΔpAMβ1 as the marker plasmids. ΔpAMβ1 is a 15.9-kilobase nonconjugative, deletion derivative of pAMβ1, which is considerably larger than the pGKV plasmids (approximately 4.5 kilobases). In general, ΔpAMβ1 was transferred more efficiently than the pGKV plasmids. Using electroporation, we were able to demonstrate that failure of efficient transfer for the pGKV plasmids was, except for one case, caused by incompatibility of these plasmids with resident plasmids of the recipient strain.

Lactic acid streptococci, comprising strains of *Streptococcus lactis*, *Streptococcus lactis* subsp. *diacetylactis*, and *Streptococcus cremoris*, are important components of mesophilic starter cultures in the manufacture of cheese and other fermented dairy products. Several attempts are presently being made to improve existing dairy strains, both by conventional methods and by recombinant DNA technologies. In recombinant DNA technology, the success of creating improved strains critically depends on the availability of efficient plasmid-transfer systems. Several such systems have recently been described, such as transductional and conjugal transfer of plasmids in *S. lactis*, *S. lactis* subsp. *diacetylactis*, and *S. cremoris*, transformation of protoplasts by plasmid DNA, protoplast fusions between *S. lactis* strains, and interspecific protoplast fusion between *S. lactis* and *S. cremoris* (for a review, see reference 13).

A serious drawback of polyethylene glycol-induced protoplast transformation is its limited applicability. Although this system has been used successfully in *S. lactis* (12, 23) and *S. lactis* subsp. *diacetylactis* (9), transformation of various strains of *S. cremoris* was only achieved in one case (22). However, it has recently been reported that plasmid DNA can be introduced into *S. lactis* by electroporation (10).

In this report, we describe DNA transfer to several *S. cremoris* strains, using protoplast transformation and interspecific protoplast fusions involving *S. lactis* and *Bacillus subtilis*. We also describe the development of an electroporation procedure for *S. cremoris*, the main component of mesophilic starters, based on that described for *Streptococcus thermophilus* by Somkuti and Steinberg (*in* O. M. Neijssel, R. R. van der Meer, and K. C. A. M. Luyben, ed., *Proceedings of the 4th European Congress on Biotechnology*, vol. 1, mop-160, p. 412, 1987). With this system, we were able to transform a number of *S. cremoris* strains. It appeared that the frequency of transformation by electroporation was strongly dependent on plasmid incompatibility.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study are described in Table 1.

TY broth (21) was used for culturing *B. subtilis*. For plating, TY broth was solidified with 1.5% agar. *S. lactis* and *S. cremoris* were cultured and plated on glucose-M17 (GM17) (25) (Difco Laboratories, East Molesey, England) broth and agar. *S. cremoris* protoplasts were regenerated on GM17-sucrose (GSM17) plates (12) or streptococcal regeneration (SR) plates (16) containing 10 g of tryptone, 5 g of yeast extract, 200 g of sucrose, 10 g of glucose, 25 g of gelatin, 15 g of agar, 2.5 mM $MgCl_2$, and 2.5 mM $CaCl_2$ per liter (pH 6.8).

Chloramphenicol was added at a final concentration of 5 μg/ml for *B. subtilis*, *S. cremoris*, and *S. lactis*. Erythromycin was used at a final concentration of 5 μg/ml for *B. subtilis*, *S. lactis*, and *S. cremoris*. Rifampin and streptomycin were used at final concentrations of 50 and 500 μg/ml, respectively, for *S. cremoris*.

Isolation of plasmid DNA. Plasmid DNA was isolated from *S. cremoris* by the method of Ish-Horowicz and Burke (11) with some modifications (26). The cells were lysed at 0°C in TES buffer (50 mM Tris hydrochloride, 5 mM EDTA, 50 mM NaCl, pH 8.0) containing 20% sucrose, 5 mg of lysozyme, and 100 μg of mutanolysin per ml, followed by 15 min at 37°C.

Restriction enzyme reactions and gel electrophoresis. Restriction enzymes were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany) and used as recommended by the supplier. Digested DNA was analyzed in 0.8% horizontal agarose (Bio-Rad Laboratories, Richmond, Calif.) gels in TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA, 0.5 μg of ethidium bromide per ml, pH 8.0).

Transformation of *S. cremoris*. *S. cremoris* protoplasts were prepared by the method of Okamoto et al. (16) with some modifications (27). *S. cremoris* protoplasts were transformed as described for *S. lactis* by Kondo and McKay (12), except that protoplasts and DNA were incubated in 22.5% polyethylene glycol for 20 min at room temperature in the presence of liposomes consisting of cardiolipin and phosphatidylcholine at a molar ratio of 1 to 6, respectively. The end concentration of liposomes in the transformation mixture was 50 μg of lipids per ml. After transformation, the protoplasts were plated on SR plates containing the selective antibiotics.

Protoplast fusion. *B. subtilis* protoplasts were prepared as described by Chang and Cohen (2). The protoplasts were concentrated 100-fold to approximately 10^{10} protoplasts per

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TABLE 1. Bacterial strains and plasmids

Strain designation	Chromosomal marker(s)[a]	Plasmid	Plasmid marker(s)[a]	Source or reference
Bacillus subtilis				
168	*trpC2*	None		1
168	*trpC2*	ΔpAMβ1	Em^r	26
Cu403		None		19
Cu403		pGKV21	Em^r Cm^r	This work
PSL1		pGKV110	Em^r	27
PSL1		pGKV21	Em^r Cm^r	27
Streptococcus lactis				
IL1403		None		4
IL1403		ΔpAMβ1	Em^r	26
IL1403		pGKV21	Em^r Cm^r	This work
MG3020		pAMβ1	Em^r	M. J. Gasson
Streptococcus cremoris				
F16	Rif^r	Cryptic plasmid complement		16
F16	Rif^r	Cryptic plasmid complement plus ΔpAMβ1	Em^r	This work
F16	Rif^r	Cryptic plasmid complement plus pGKV21	Em^r Cm^r	This work
F16	Rif^r	Cryptic plasmid complement plus pGKV110	Em^r	This work
H61	Rif^r	Cryptic plasmid complement		17
H61	Rif^r	Cryptic plasmid complement plus ΔpAMβ1	Em^r	This work
H61	Rif^r	Cryptic plasmid complement plus pGKV110	Em^r	This work
Wg2L	Rif^r	Cryptic plasmid complement		18
Wg2L	Rif^r	Cryptic plasmid complement plus ΔpAMβ1	Em^r	This work
Wg2L	Rif^r	Cryptic plasmid complement plus pGKV110	Em^r	This work
Wg2L-1	Rif^r	Cryptic plasmid complement, minus pWV01		This work
Wg2L-1	Rif^r	Cryptic plasmid complement, minus pWV01, plus pGKV110	Em^r	This work
Wg2L-1,2	Rif^r	Cryptic plasmid complement, minus pWV01 and pWV02		This work
Wg2L-1,2	Rif^r	Cryptic plasmid complement, minus pWV01 and pWV02, plus pGKV110	Em^r	This work
NZ1245	Str^r	None		NIZO[b]
NZ1245	Str^r	pAM1	Em^r	This work
NZ1250	Rif^r Str^r	None		NIZO
NZ1250	Rif^r Str^r	pGKV110	Em^r	This work
4847		None		6
4847		pGKV110	Em^r	This work
E8L		Cryptic plasmid complement		28
E8L		Cryptic plasmid complement plus pGKV110	Em^r	This work
HpL		Cryptic plasmid complement		8
HpL		Cryptic plasmid complement plus pGKV110	Em^r	This work
Undefined lactic *Streptococcus* sp.				
NZ1240		Cryptic plasmid complement		NIZO
NZ1240		Cryptic plasmid complement plus pAMβ1	Em^r	This work
NZ1240		Cryptic plasmid complement plus ΔpAMβ1	Em^r	This work
NZ1240		Cryptic plasmid complement plus pGKV110	Em^r	This work
NZ1240	Rif^r	Cryptic plasmid complement		NIZO
NZ1240	Rif^r	Cryptic plasmid complement plus pGKV21	Em^r Cm^r	This work
NZ1240	Rif^r	Cryptic plasmid compolement plus ΔpAMβ1	Em^r	This work

[a] Em^r, Erythromycin resistant; Cm^r, chloramphenicol resistant; Rif^r, rifampin resistant; Str^r, streptomycin resistant.
[b] Netherlands Institute for Dairy Research.

ml in S_{25}MM (25% sucrose, 0.02 M maleic acid, 0.02 M $MgCl_2$). For fusions between *S. cremoris* and *S. lactis*, 250 μl of protoplast suspension of donor and recipient were mixed and incubated in 37.5% polyethylene glycol plus 10 μg of DNase per ml for 20 min at room temperature. After dilution with 5 ml of S_{25}MM, the protoplasts were harvested by centrifugation and suspended in 1 ml of GSM17 broth. After expression was allowed for 2 h at 30°C, the protoplasts were plated on SR plates containing selective antibiotics.

For fusions between *S. cremoris* and *B. subtilis*, the same procedure was used, except that 420 μl of *S. cremoris* protoplast suspension was mixed with 80 μl of *B. subtilis* protoplast suspension.

Electroporation of *S. cremoris*. The Bio-Rad Gene Pulser transfection apparatus was used for electroporation. Overnight cultures of *S. cremoris* were diluted 100-fold in 20 ml of GM17 medium supplemented with 40 mM DL-threonine. Cells were grown to an optical density at 660 nm of 0.2, harvested, and washed in 5 ml of electroporation buffer containing 5 mM potassium phosphate (pH 7.4), 0.3 M sucrose, and 1 mM $MgCl_2$. The cells were resuspended in 1 ml of electroporation buffer and held on ice for 15 min.

Subsequently, 0.8 ml of cell suspension and 5 μg of plasmid DNA were mixed and electroporation was carried out at 5,000 V/cm. The capacitance (*C*) used was 25 μF.

After the electric pulse, the cells were held on ice for 15 min. Then the cells were diluted 10-fold in GSM17 medium and, after 2 h of expression at 30°C, plated on GSM17 plates containing selective antibiotics. Colonies of transformed cells became visible after 48 h of incubation at 30°C.

Blot hybridization. After electrophoresis, DNA fragments were transferred to nitrocellulose filters from agarose gels by the protocol of Southern (24) as modified by Chomczynski and Qasba (3). Nick-translated ^{32}P-labeled (20) DNA was denatured for 7 min at 100°C. The filters were placed in a solution of 1× Denhardt solution (7), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 1% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA. The hybridization and washing steps were done at 64°C by standard procedures, followed by autoradiography on X-ray films (14).

Conjugation. Solid-surface matings between *S. cremoris* strains were performed by modifications of the method of McKay et al. (15) as described before (26).

RESULTS

Protoplast formation, regeneration, and transformation. The repeated failure to obtain transformed *S. cremoris* cells by protoplast transformation prompted us to investigate whether poor regeneration of the protoplasts on GSM17 medium might underlie this failure. Protoplasts of several *S. cremoris* strains (listed in Table 2) were prepared by treatment of the cells with lysozyme. After protoplast formation, the cells were suitably diluted in GSM17 medium and plated on GSM17 or SR medium. Regenerants were obtained after an incubation period of 2 to 5 days at 30°C.

The regeneration of seven different *S. cremoris* strains on GSM17 medium was generally poor, except for *S. cremoris* 4847 and F16, and all strains regenerated more efficiently on SR medium than on GSM17 medium (Table 2).

We next investigated the transformability of a number of *S. cremoris* strains which were regenerated on the two types of regeneration media. Protoplasts of *S. cremoris* F16, *S. cremoris* H61, *Streptococcus* strain NZ1240, and *S. cremoris* Wg2L were transformed with ΔpAMβ1, a 15.9-kilobase deletion derivative of pAMβ1, and pGKV110. Whereas only a few transformants were obtained for *S. cremoris* F16 and *Streptococcus* strain NZ1240 when the protoplasts were regenerated on GSM17 medium, ΔpAMβ1 produced transformants of all *S. cremoris* strains tested when regenerated on SR medium, although with varying efficiencies (Table 3).

TABLE 2. Regeneration frequencies of various *S. cremoris* strains on different media[a]

Strain	Regeneration frequency (%) on:		
	GSM17 (A)	SR (B)	B/A
S. cremoris H61	1.2×10^{-4}	2.9	24,000
Streptococcus strain NZ1240	0.14	6.8	49.4
S. cremoris NZ1250	$<10^{-9}$	2.6×10^{-4}	>3,200
S. cremoris Wg2L	2.2×10^{-2}	0.3	12.7
S. cremoris 4847	0.7	2.8	3.9
S. cremoris F16	0.9	11.0	12.5
S. cremoris E8L	1.6×10^{-2}	0.2	10

[a] Regeneration frequencies were determined as the ratio of number of colonies growing on regeneration plates and number of viable cells before protoplasting.

TABLE 3. Transformation of various *S. cremoris* strains on different regeneration media

Strain	Transformation efficiency[a] with plasmid:				
	pGKV110		ΔpAMβ1		pAMβ1 (SR)
	GSM17	SR	GSM17	SR	
S. cremoris F16	300	3,900	10	140	ND[b]
S. cremoris H61	<1	15	<1	10	ND
Streptococcus strain NZ1240	<1	<1	18	1,000	2
S. cremoris Wg2L	<1	<1	<1	2	ND

[a] The transformation efficiencies are expressed as the number of transformants per microgram of DNA. <1, No transformants found.
[b] ND, Not determined.

Because *Streptococcus* strain NZ1240 showed the highest transformation efficiency with ΔpAMβ1, we also tested this strain for transformation with pAMβ1 to examine the effect of plasmid size on transformation. Transformation was dramatically affected by the size of the plasmid (Table 3).

To ascertain the presence of pAMβ1 and ΔpAMβ1 in the transformed cells and exclude the possibility that erythromycin-resistant (Em^r) colonies had been produced through spontaneous mutation, which might be possible, especially in the case of the weakly transformable *S. cremoris* H61 and Wg2L, we analyzed the Em^r cells obtained with pAMβ1 and ΔpAMβ1 by blot hybridization. This showed that all *S. cremoris* strains tested carried ΔpAMβ1 or, in the case of *Streptococcus* strain NZ1240, pAMβ1 (data not shown). The presence of intact pAMβ1 in *Streptococcus* strain NZ1240 was confirmed by conjugation experiments. With *Streptococcus* strain NZ1240 (pAMβ1) as the donor and *S. cremoris* NZ1245 as the recipient, pAMβ1 was transferred with a frequency of 7.0×10^{-3} transconjugants per recipient. This value was comparable to that obtained when *S. lactis* MG3020 (pAMβ1) was used as the donor (3.4×10^{-3} transconjugants per recipient).

With pGKV110, transformants were only obtained in *S. cremoris* F16 and H61 (Table 3). Apparently, transformation is strongly dependent on the type of plasmid DNA which is used as a donor molecule. Intriguingly, the efficiencies of transformation of *S. cremoris* H61 with pGKV110 and ΔpAMβ1 were similar, despite the fact that ΔpAMβ1 is approximately 3.5 times larger than pGKV110.

Interspecific protoplast fusions. It has been reported that in some cases (5) in which protoplast transformation was not productive, protoplast fusion could be used as an alternative for plasmid transfer. To examine whether plasmids were transferable to *S. cremoris* in this way, protoplasts of rifampin-resistant (Rif^r) mutants of *S. cremoris* F16, *S. cremoris* H61, *Streptococcus* strain NZ1240, and *S. cremoris* Wg2L were fused to protoplasts of *B. subtilis* Cu403(pGKV21), *B. subtilis* 168(ΔpAMβ1), *S. lactis* IL1403(pGKV21), and *S. lactis*(ΔpAMβ1). The results are presented in Table 4 and show that compared with the other strains, *S. cremoris* F16 acquired the plasmids most efficiently in the fusion experiments, probably because of its superior regeneration capacities. Similarly, the failure to obtain fusants of *S. cremoris* Wg2L is probably due to its poor capacity to regenerate to complete cells.

In *Streptococcus* strain NZ1240, interspecific protoplast fusion involving pGKV21 was more successful than protoplast transformation with pGKV110 (compare Tables 3 and

TABLE 4. Plasmid transfer by interspecific protoplast fusion of various strains of *S. cremoris* with *B. subtilis* and *S. lactis*[a]

Recipient	Frequency of plasmid transfer[b] with the following donor:			
	B. subtilis Cu403(pGKV21)	*S. subtilis* 168(ΔpAMβ1)	*S. lactis* IL1403	
			pgKV21	ΔpAMβ1
S. cremoris F16	0.5×10^{-5}	1.3×10^{-5}	3.0×10^{-5}	3.6×10^{-4}
S. cremoris H61	$<2.5 \times 10^{-7}$	$<2.5 \times 10^{-7}$	$<2.5 \times 10^{-7}$	5.8×10^{-4}
Streptococcus strain NZ1240	2.4×10^{-7}	3.1×10^{-6}	9.4×10^{-6}	3.3×10^{-5}
S. cremoris Wg2L	$<2.9 \times 10^{-7}$	$<2.9 \times 10^{-7}$	$<2.9 \times 10^{-7}$	$<2.9 \times 10^{-7}$

[a] Fusions were performed according to the precedures described in Materials and Methods.
[b] The frequency of plasmid DNA transfer is expressed as the number of fusants per recipient.

4). Although *Streptococcus* strain NZ1240 could not be transformed with pGKV110, fusants containing pGKV21 could be obtained with low frequency, indicating that protoplast fusion can be used as a successful alternative to protoplast transformation.

Analysis of the plasmid content of a number of independently obtained fusants showed that all had lost several plasmids of the *Streptococcus* strain NZ1240 plasmid complement (Fig. 1) when the fusions were done with donors carrying pGKV21. However, when ΔpAMβ1 was introduced, no loss of components of the endogenous plasmid population was observed (data not shown). Figure 1 also shows that in the fusants which had acquired pGKV21, an increased amount of a resident plasmid was present. However, the observation that the introduction of pGKV21 was accompanied by the loss of plasmids suggests that plasmid incompatibility is involved in the relatively poor acquisition of pGKV plasmids by *S. cremoris* strains. Strong support in favor of this supposition is provided below.

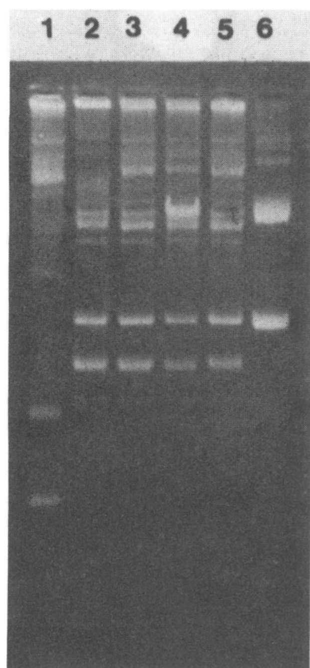

FIG. 1. Plasmid content of *Streptococcus* strain NZ1240 (pGKV21) obtained after fusions with *S. lactis*(pGKV21) and *B. subtilis*(pGKV21). Lane 1, Plasmid content of *Streptococcus* NZ1240 (before fusion). Lanes 2 to 5, Plasmid content of independently obtained *Streptococcus* strain NZ1240(pGKV21) after fusions with *S. lactis*(pGKV21) (lanes 2 and 3) and *B. subtilis*(pGKV21) (lanes 4 and 5). Lane 6, pGKV21.

Electroporation of *S. cremoris* Wg2L. The data above show that plasmids are transformable to *S. cremoris* by protoplast transformation and protoplast fusion. However, in general, the frequencies of transfer were rather low. To investigate whether alternative methods would produce higher frequencies of transfer, we examined the possibility of electroporation of *S. cremoris* Wg2L. This strain was chosen because it was refractory to protoplast transformation and did not yield fusants.

An overnight culture of *S. cremoris* Wg2L was diluted 100-fold in GM17 medium supplemented with 40 mM DL-threonine to weaken the cell wall by incorporation of the D-isomere. When DL-threonine was omitted, no transformants could be obtained. After the culture grew to an optical density of 0.2 at 660 nm, the cells were washed and concentrated 20-fold in electroporation buffer. After the suspension was chilled on ice for 15 min, electroporation was carried out.

The effect of the field strength on the frequency of transformation is presented in Fig. 2 and shows that in the trajectory from 3,000 to 3,750 V/cm, the number of CFU increases. Microscopic analysis of the culture showed that this increase is caused by breaking up of the streptococcal chains. The decrease of CFU observed in the trajectory from 2,750 to 5,000 V/cm is probably caused by lethal damage inflicted on the cells during the electroporation procedure. Figure 2 also shows that the frequency of transformation increased proportional to the field strength and that the highest number of transformants, 1,100 transformants per μg of ΔpAMβ1, was obtained at 5,000 V/cm.

We also examined the effect of multiple pulses on the frequency of transformation and viability. Just one pulse at 5,000 V/cm gave the best results (data not shown).

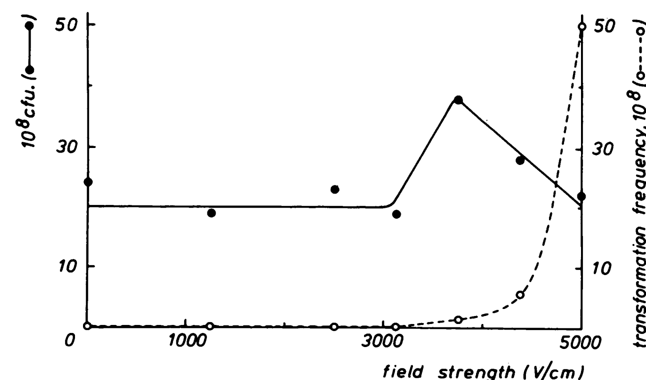

FIG. 2. Transformation by electroporation of *S. cremoris* Wg2L. Effect of field strength on viability and transformation efficiency. Electroporation was done at a capacitance of 25 μF.

TABLE 5. Transformation by electroporation of various *S. cremoris* strains[a]

Strain	No. of transformants per μg of DNA
S. cremoris F16	4,000
S. cremoris H61	50
Streptococcus strain NZ1240	<1
S. cremoris Wg2L	20
S. cremoris E8L	300
S. cremoris HpL	60
S. cremoris NZ1250	760
S. cremoris 4847	1,200

[a] Electroporation was done at 5,000 V/cm and C = 25 μF, with pGKV110 as the marker plasmid.

Effect of plasmid incompatibility on transformation. To examine whether the electroporation procedure developed for *S. cremoris* Wg2L was generally applicable, we electroporated various other strains of *S. cremoris* in the presence of pGKV110. The results (Table 5) show that some strains, such as *S. cremoris* H61, *Streptococcus* strain NZ1240, *S. cremoris* Wg2L, and *S. cremoris* HpL, transformed relatively poorly with pGKV110, whereas other strains, such as *S. cremoris* F16 and 4847, were efficiently transformed. With the exception of *Streptococcus* strain NZ1240, electroporation was superior to protoplast transformation and protoplast fusion as a means to transfer plasmid DNA to *S. cremoris*.

Analysis of the plasmid content of eight pGKV110-transformed *S. cremoris* Wg2L colonies showed that, except for one case, all transformants had lost the endogenous plasmid pWV01 (data not shown). Apparently, the introduction of pGKV110, which is derived from pWV01, one of the cryptic plasmids of *S. cremoris* Wg2L (27), leads to the loss of pWV01. Interestingly, when pGKV21, also derived from pWV01 (27), was introduced into *Streptococcus* strain NZ1240 by protoplast fusion, this also led to the loss of several plasmids (Fig. 1). This observation, together with the fact that those strains which lose components of the plasmid complement upon introduction of pGKV vectors are poorly transformable, suggested that the transformability of *S. cremoris* is intimately related to plasmid incompatibility. On the basis of this hypothesis, we expected that in other strains, also difficult to transform with pGKV110, plasmids showing homology with pGKV110 should be present. This was analyzed by blot hybridization.

The results shown in Fig. 3 indicate that in *S. cremoris* Wg2L-1(pGKV110) (lane 3), pGKV110 was present, whereas pWV01 was absent. As expected, in *S. cremoris* Wg2L (lane 4), pWV01 showed strong homology with pGKV110. However, pWV02 also gave a hybridization signal, indicating that homology existed between pWV02 and pGKV110. In *S. cremoris* HpL (lane 7), also poorly transformable with pGKV110, a large plasmid was present which also showed homology with pGKV110. This plasmid also disappeared after the introduction of pGKV110 [*S. cremoris* HpL(pGKV110) (lane 8)]. In *Streptococcus* strain NZ1240 (lane 11), several plasmids were present showing strong homology with pGKV110. Inspection of Fig. 1 (lanes 2 to 5) indicates that these plasmids had also disappeared in *Streptococcus* strain NZ1240 as a consequence of the introduction of pGKV21. Further support for our hypothesis that

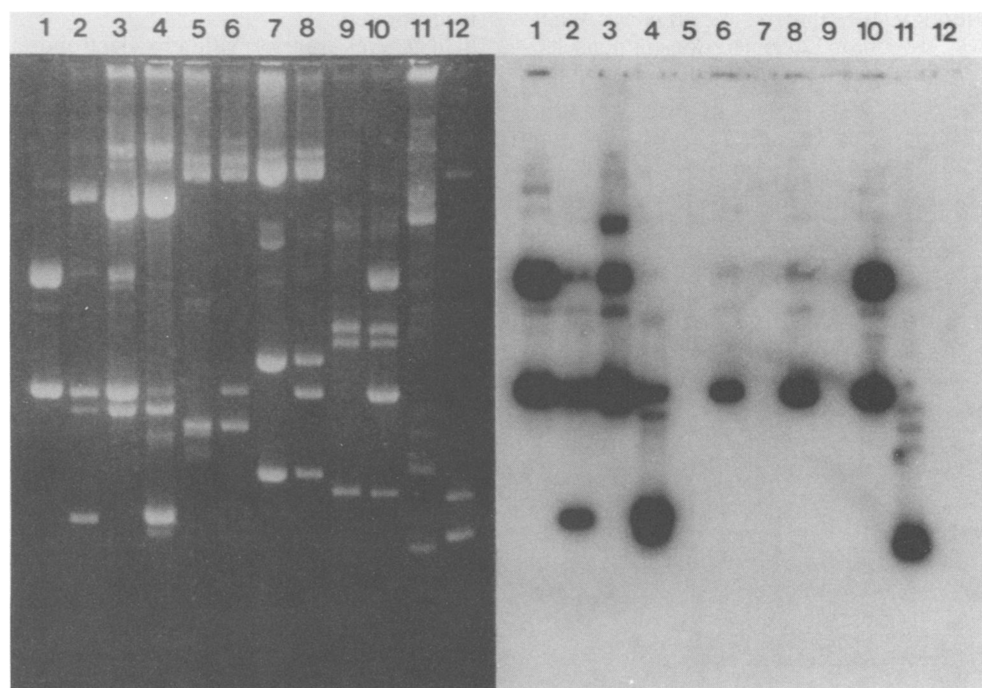

FIG. 3. Hybridization analysis of several *S. cremoris* strains for homology with pGKV110. ^{32}P-labeled pGKV110 was hybridized to pGKV110 (lanes 1) and to the plasmid complement of *S. cremoris* Wg2L(pGKV110) (lanes 2), *S. cremoris* Wg2L-1(pGKV110) (lanes 3), *S. cremoris* Wg2L (lanes 4), *S. cremoris* E8L (lanes 5), *S. cremoris* E8L(pGKV110) (lanes 6), *S. cremoris* HpL (lanes 7), *S. cremoris* HpL(pGKV110) (lanes 8), *S. cremoris* F16 (lanes 9), *S. cremoris* F16(pGKV110) (lanes 10), *Streptococcus* strain NZ1240 (lanes 11), and *S. cremoris* H61 (lanes 12). Left panel, Plasmid profiles; right panel, Southern blot of left panel.

plasmid transformation is dependent on plasmid incompatibility was derived from the observation that *S. cremoris* E8L and F16, lacking plasmids which hybridized to pGKV110 (Fig. 3, lanes 5, 6, 9, and 10), were highly transformable with this plasmid. The only exception was *S. cremoris* H61, which was poorly transformable despite the absence of homology between pGKV110 and any of the plasmids of the plasmid complement of this strain. Apparently, factors other than plasmid incompatibility determine the poor transformability of this strain.

The incompatibility between pWV01 and pGKV110 was studied in more detail. *S. cremoris* Wg2L(pGKV110) still containing pWV01 (Fig. 3 lane 2) was grown on GM17 medium without antibiotics. After 20 and 120 generations, the cells were plated on GM17 plates without antibiotics and replica plated on GM17 plates containing erythromycin. After both 20 and 120 generations, the ratios of Em^r and Em^s colonies were the same: 25% were Em^s and 75% were Em^r. Apparently, 20 generations of growth in the nonselective medium sufficed to eliminate pGKV110 from 25% of the cells. Plasmid analysis confirmed that pGKV110 was lacking from the Em^s colonies and also showed that pWV01 was still present. Plasmid analysis of the Em^r colonies showed that pWV01 had been lost (data not shown). These results indicate that the competing activity of pGKV110 is superior to that of pWV01.

Plasmid analysis of Em^r colonies obtained after 120 generations of growth in the nonselective medium showed that, in addition to loss of pWV01, in 60% of the cases investigated, pWV02 was also absent [*S. cremoris* Wg2L-1,2(pGKV110)] (Fig. 4). This shows that pWV02 is also incompatible with pGKV110, which is in accordance with the observation that this plasmid showed homology to pGKV110 (Fig. 3, lane 4). To investigate whether this incompatibility might also result in the loss of pGKV110, *S. cremoris* Wg2L-1(pGKV110), that is, *S. cremoris* Wg2L lacking pWV01, was grown for 120 generations in nonselective medium. No Em^s colonies were detected, indicating that the incompatibility between pGKV110 and pWV02 was unidirectional, in the sense that pGKV110 was retained, but pWV02 was lost.

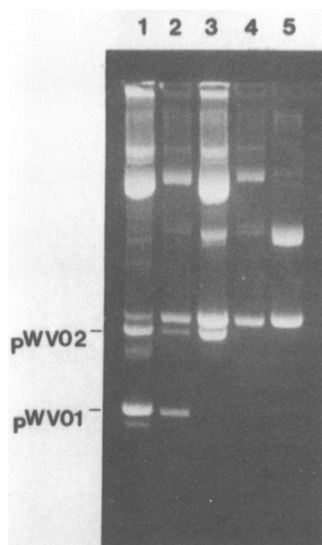

FIG. 4. Plasmid content of Em^r colonies from *S. cremoris* Wg2L(pGKV110) after growth on GM17 medium without selective pressure. Lanes: 1, plasmid content of *S. cremoris* Wg2L; 2, plasmid content of *S. cremoris* Wg2L(pGKV110); 3, plasmid content of *S. cremoris* Wg2L-1(pGKV110); 4, plasmid content of *S. cremoris* Wg2L-1,2(pGKV110); 5, pGKV110.

DISCUSSION

To improve *S. cremoris*, the major component of mesophilic starter cultures, by recombinant DNA technology, efficient systems for the transfer of recombinant DNAs are required. However, the various strains of *S. cremoris* are poorly, if at all, transformable by naked DNA. The present study shows that the regeneration efficiency is an important factor in the efficiency of protoplast transformation. All strains of *S. cremoris* used in this study gave increased frequencies of transformation when the cells were regenerated on SR medium, concomitant with increased regeneration capacities.

As an alternative to transferring plasmid DNA to *S. cremoris*, protoplast fusions with *S. lactis* and *B. subtilis* were used. Whereas in all fusions tested, except for *S. cremoris* Wg2L, ΔpAMβ1 was readily transferable, although with varying efficiencies, the transfer of pGKV21 was successful only with *S. cremoris* F16 and *Streptococcus* strain NZ1240. It is not immediately clear why the considerably larger ΔpAMβ1 should be transferred more easily than pGKV21. Rather, the contrary would be expected. However, the more efficient transfer of ΔpAMβ1 may be related to its higher copy number in *B. subtilis* and *S. lactis*. ΔpAMβ1 has a copy number of about 80 in both *B. subtilis* and *S. lactis*, whereas pGKV21 had a copy number of only 4 in both species. The less efficient transfer of plasmids involving fusions of *S. cremoris* with *B. subtilis* as compared with fusions with *S. lactis* may be caused by the decreased efficiency of regenerating protoplasts having a cytoplasmic membrane in which pieces of unrelated membrane are incorporated.

In general, transformation by electroporation proved to be superior over protoplast transformation and the transfer of plasmids by protoplast fusion, but again the transfer of ΔpAMβ1, at least in *S. cremoris* Wg2L, was much more efficient than that of the pGKV vectors.

Analysis of the plasmid content of *S. cremoris* Wg2L after introduction of pGKV110 by electroporation showed that most transformants had been cured of pWV01. Loss of plasmids was also observed in *Streptococcus* strain NZ1240 after the introduction of pGKV21. We conjectured that plasmid incompatibility might be a decisive factor with respect to transformation efficiency. If this hypothesis is correct, one would expect that other poorly transformable strains like *S. cremoris* HpL and H61 would harbor plasmids showing incompatibility, and thus homology, with pGKV vectors. Blot hybridization proved that, indeed, the plasmid complement of *S. cremoris* Wg2L, *Streptococcus* strain NZ1240, and *S. cremoris* HpL contained components which were homologous to pGKV110. Moreover, after introduction of pGKV vectors, these plasmids had disappeared (Fig. 3), indicating plasmid incompatibility.

The copy number of the resident incompatible plasmid also appears to affect the efficiency of DNA transformation. In *S. cremoris* HpL in which the incompatible plasmid was present in low copy number, the effect of plasmid incompatibility seemed to be less dramatic than in *S. cremoris* Wg2L and *Streptococcus* strain NZ1240, in which the endogenous incompatible plasmids were present in high copy number.

For *S. cremoris* H61, no homology was observed of components of the plasmid complement with pGKV vectors.

In accord with this was the observation that after introduction of pGKV110 in *S. cremoris* H61, no loss of plasmids was observed (data not shown). Nevertheless, the strain continued to be poorly transformable, pointing at the existence of other factors, preventing the stable introduction of pGKV110. These factors also prevented the transfer of ΔpAMβ1 from *B. subtilis* by heterospecific protoplast fusion.

To follow the fate of pGKV110 introduced by electroporation in *S. cremoris* Wg2L, we grew the transformed recipient for 20 and 120 generations in the absence of selective antibiotics. After both 20 and 120 generations, pGKV110 was lost with a frequency of 25% from the population. However, the colonies which were still resistant to erythromycin after 120 generations not only had lost pWV01, but in 6 of 10 cases had also lost pWV02, indicating that this plasmid is also incompatible with pGKV110. This is in accordance with the observation that this plasmid gave a hybridization signal with pGKV110 (Fig. 3, lane 4).

In contrast to segregational instability of pGKV110 in the presence of pWV01, from which it was derived, the plasmid was completely stable in the absence of selective pressure after it had eliminated pWV01 from the recipient.

An interesting question concerns why pWV01, and ultimately pWV02, are lost more easily from *S. cremoris* Wg2L than pGKV110 in the absence of selective pressure (75% loss of pWV01 and 25% loss of pGKV110). With respect to the pWV01 moiety in pGKV110, a particular *Cla*I fragment is missing (27). One explanation for the superior competition of the vector is that this fragment contains a sequence on which a protein involved in the incompatibility phenomenon must act. A second point of interest concerns the observation that we have never seen loss of pWV01 or pWV02 in *S. cremoris* Wg2L, although pGKV110, and therefore pWV01, are weakly incompatible with pWV02. One possibility to explain this is that pWV01 and pWV02 weakly compete at the level of replication, but use different attachment sites during segregation over the daughter cells.

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LITERATURE CITED

1. **Anagnostopoulos, C., and J. Spizizen.** 1960. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
3. **Chomczynski, P., and P. K. Qasba.** 1984. Alkaline transfer of DNA to plastic membrane. Biochem. Biophys. Res. Commun. **122**:340–344.
4. **Chopin, A., M.-C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11**:260–263.
5. **Cocconcelli, P. S., L. Morelli, M. Vescovo, and V. Bottazzi.** 1986. Intergeneric protoplast fusion in lactic acid bacteria. FEMS Microbiol. Lett. **35**:211–214.
6. **Davey, G. P., R. D. Appleby, and L. E. Pearce.** 1986. Isolation of plasmid free *Streptococcus cremoris* strains by protoplast-induced curing. N.Z. J. Dairy Sci. Technol. **21**:73–75.
7. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
8. **Exterkate, F. A.** 1975. An introductory study of the proteolytic system of *Streptococcus cremoris* strain Hp. Neth. Milk Dairy J. **29**:303–318.
9. **Geis, A.** 1982. Transfection of protoplasts of *Streptococcus lactis* subsp. *diacetylactis*. FEMS Microbiol. Lett. **15**:119–122.
10. **Harlander, S. K.** 1987. Transformation of *Streptococcus lactis* by electroporation, p. 229–233. *In* J. J. Ferretti and R. Curtiss III (ed.), Streptococcal genetics. American Society for Microbiology, Washington, D.C.
11. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2999.
12. **Kondo, J. K., and L. L. McKay.** 1984. Plasmid transformation of *Streptococcus lactis* protoplasts: optimization and use in molecular cloning. Appl. Environ. Microbiol. **48**:252–259.
13. **Kondo, J. K., and L. L. McKay.** 1985. Gene transfer systems and molecular cloning in N streptococci: a review. J. Dairy Sci. **68**:2143–2159.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **McKay, L. L., K. A. Baldwin, and P. M. Walsh.** 1980. Conjugal transfer of genetic information of group N streptococci. Appl. Environ. Microbiol. **40**:84–91.
16. **Okamoto, T., Y. Fujita, and R. Irie.** 1983. Protoplast formation and regeneration of *Streptococcus lactis* cells. Agric. Biol. Chem. **47**:259–263.
17. **Okamoto, T., Y. Fujita, and R. Irie.** 1985. Interspecific protoplast fusion between *Streptococcus cremoris* and *Streptococcus lactis*. Agric. Biol. Chem. **49**:1371–1376.
18. **Otto, R., W. M. de Vos, and J. Gavrieli.** 1982. Plasmid DNA in *Streptococcus cremoris* Wg2: influence of pH on selection in chemostats of a variant lacking a protease plasmid. Appl. Environ. Microbiol. **43**:1272–1277.
19. **Reeve, J. N., N. H. Mendelson, S. I. Coyne, L. L. Hallock, and R. M. Cole.** 1973. Minicells of *Bacillus subtilis*. J. Bacteriol. **114**:860–873.
20. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**: 236–251.
21. **Rottlander, E., and T. A. Trautner.** 1970. Genetic and transfection studies with *Bacillus subtilis* phage SP50. J. Mol. Biol. **108**: 47–60.
22. **Simon, D., A. Rouault, and M.-C. Chopin.** 1985. Protoplast transformation of group N streptococci with cryptic plasmids. FEMS Microbiol. Lett. **26**:239–241.
23. **Simon, D., A. Rouault, and M.-C. Chopin.** 1986. High-efficiency transformation of *Streptococcus lactis* protoplasts by plasmid DNA. Appl. Environ. Microbiol. **52**:394–395.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
25. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
26. **van der Lelie, D., and G. Venema.** 1987. *Bacillus subtilis* generates a major specific deletion in pAMβ1. Appl. Environ. Microbiol. **53**:2458–2463.
27. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50**:540–542.
28. **Visser, S., F. A. Exterkate, C. J. Slangen, and G. J. C. M. de Veer.** 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{s1}-, β-, and κ-casein. Appl. Environ. Microbiol. **52**:1162–1166.