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Construction of a Bacteriophage-Resistant Derivative of *Lactococcus lactis* subsp. *lactis* 425A by Using the Conjugal Plasmid pNP40

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***Lactococcus lactis* subsp. *lactis* 425A is an atypical strain which excretes a high concentration of α-acetolactate when grown in milk. The conjugative lactococcal plasmid pNP40, which encodes phage and nisin resistance, was introduced to strain 425A by conjugation, using resistance to phage and nisin as a selection. No phage-nisin resistance mutants were encountered. Transconjugants display complete resistance at both 21 and 39°C to those phage previously identified as lytic for 425A. Transconjugants lose their resistance characteristics when spontaneously cured of pNP40. The commercially important property of 425A—production of high levels of α-acetolactic acid—is unaffected by the presence of pNP40.**

Lactococcus lactis subsp. *lactis* 425A (formerly *Streptococcus lactis* subsp. *diacetylactis*) is a Cit^+ strain used in the manufacture of cultured butter. The traditional process relies on the fermentation of cream with mesophilic lactic acid bacteria followed by churning to give ripened butter and sour buttermilk. Strain 425A is used in the alternative NIZO process (21), in which a mixed-strain starter, designated 4/25, is used to produce large amounts of α-acetolactate (ALA) during fermentation of skim milk. Upon aeration with concentrated lactic acid (produced by fermentation of whey by *Lactobacillus helveticus*), a high concentration of diacetyl is produced through chemical decarboxylation of ALA. The diacetyl is subsequently worked into sweet-butter granules to give the characteristic ripened-butter flavor. Strain 425A has been identified as the major ALA-producing strain in the mixed starter 4/25 (14). A major problem associated with this economically important strain has been its sensitivity to bacteriophage attack. Because of the central role of this atypical strain in the NIZO process, it is not possible to control phage levels by following a rotation strategy or to replace it with a phage-unrelated strain with similar metabolic end products. One alternative is to expose the strain to its homologous phage and isolate bacteriophage-insensitive mutants (BIMs). This approach has been used to isolate BIMs of 425A (13), but these isolates have rapidly succumbed to phage when employed in an industrial situation.

Another approach which has been used successfully in the cheese industry has been to introduce a phage resistance plasmid into the sensitive strain and render the strain phage insensitive. This strategy was first described by Klaenhammer and his coworkers, who used the conjugative phage resistance plasmid pTR2030 (9, 15). They introduced pTR2030 to industrial strains to construct phage-insensitive transconjugants (19). The use of a conjugative rather than a nonconjugative plasmid has obvious advantages in that such a plasmid can be introduced into a sensitive strain by nonrecombinant techniques and can thereby develop, by natural means, resistant transconjugants which are acceptable to the food industry. The technique relies on a suitable selection process to distinguish transconjugants from donors, recipients, and, particularly, phage-resistant mutants, which may arise at unacceptably high frequencies. Sanders et al. (19) utilized a probe specific for pTR2030 sequences to detect transconjugants after phage selection. A similar strategy was employed by Jarvis et al. (12) to detect the transfer of the phage resistance plasmid pAJ1106.

A number of phage resistance plasmids in lactococci have been described (reviewed by Klaenhammer in reference 15). McKay and Baldwin (16) have described a conjugative 60-kb plasmid, pNP40, in *L. lactis* subsp. *lactis* DRC3. This plasmid differs from pTR2030 in that the mechanisms of resistance have been shown to be genotypically unrelated (11) and also in that pNP40 encodes resistance to nisin (16). This trait may allow the use of nisin and phage resistance as selection markers. In this report, we describe the introduction of pNP40 to 425A from a lactose-negative derivative of DRC3. The selection process relied on the insensitivity of transconjugants to two different phage isolated against 425A, resistance to nisin, and the ability to metabolize lactose. Transconjugants were recovered at low frequencies and were shown to be completely resistant to phage previously isolated against 425A and a BIM of 425A. Phage-resistant, nisin-resistant mutants were not encountered. ALA production was unaffected by the presence of pNP40.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture conditions. The bacterial strains used in this study are listed in Table 1. All strains were propagated routinely at 30°C in M17 (20), with the addition of 0.5% glucose (GM17) where appropriate. Modified M17 for the ALA assay contained 3% β-glycerophosphate and 10 mM triammonium citrate as described by Jordan and Cogan (14). Activity and stability tests were conducted in 10% reconstituted skim milk (RSM) at 21°C. Tryptone (0.5%) was added in the case of the proteolytically deficient strain 425A and its derivatives. Purified nisin [30,000 U/mg; R. B. Chemicals (Ireland) Ltd., Dublin] was resuspended in 0.02 N HCl and filter sterilized. When included in solid media, 1% Tween 20 was added to aid dispersion. All phage were isolated from cultures used

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TABLE 1. Bacterial strains and phage

L. lactis strain and DPC no.[a]	Phenotype	Phage sensitivity	Remarks
425A	Lac$^+$ Nis^s ϕ^s	ϕT, ϕD1, ϕD2, ϕ2, ϕ3	Phage-sensitive parent (13)
BIM21	Lac$^+$ Nis^r ϕ^s	ϕ2, ϕ3	Phage-resistant mutant of 425A (12)
DRC3	Lac$^+$ Nis^r ϕ^r	ϕdrc3	pNP40-containing strain (15)
DPC2839	Lac$^-$ Nis^r ϕ^r	ϕdrc3	Lac$^-$ derivative of DRC3 (this study)
DPC2723	Lac$^+$ Nis^r ϕ^r	ϕ3	pNP40 transconjugant of 425A (this study)
DPC2724	Lac$^+$ Nis^s ϕ^s	ϕT, ϕD1, ϕD2, ϕ2, ϕ3	DPC2723 cured of pNP40 (this study)
MG1614	Lac$^-$ Nis^s ϕ^s	ϕC2, ϕ712	Plasmid-free recipient (6)
DPC2844	Lac$^-$ Nis^r ϕ^r	None	DPC2723 × MG1614 (this study)
DPC3260	Lac$^-$ Nis^r ϕ^r	None	DRC3 × MG1614 (this study)

[a] Culture collection number, Dairy Product Centre, Moorepark, Fermoy, Ireland.

commercially in Ireland. Phage were propagated in M17 by the method of Terzhagi and Sandine (20).

DNA isolation and manipulations. Lactococcal plasmid DNA was isolated by the method of Anderson and McKay (1). Phage DNA was visualized by the rapid phage identification method of Hill et al. (10). DNA was digested according to the instructions provided by the supplier of the restriction enzymes.

Conjugation. The conjugation protocol and selection parameters developed during the course of this study are presented in Results.

Modified starter activity test. Phage susceptibility was determined by propagating the test culture in the presence of lytic phage. A 1% (vol/vol) inoculum of culture was added to 10 ml of RSM in duplicate. To one tube was added 200 μl of a phage suspension (10^7/ml, final titer). The other tube served as a control. The pH was measured after incubation at 21°C for 18 h. A fresh inoculum was prepared from stock for the second cycle, and 100 μl of phage suspension and 100 μl of filter-sterilized whey from cycle 1 were added. This was continued for five cycles. At the conclusion of the activity test, 10 μl of whey from cycle 5 was spotted onto an M17 plate which had been overlaid with the test culture to detect the presence of low levels of phage, which may not have affected the pH. The modified activity test was done in duplicate, and the pH figure represents the average of both tests.

Detection of ALA. ALA assays were conducted on cells grown in modified M17 at 30°C and in RSM at 21°C as described by Jordan and Cogan (14). The acetoin concentration was determined before and after treatment with HCl. The difference was used to calculate the amount of ALA in the sample. Standard curves were determined with purified acetoin (R. B. Chemicals).

Plasmid stability test. The stability of pNP40 in 425A grown in 10% RSM at 21°C was determined as follows. RSM was inoculated with 425A(pNP40) and incubated at 21°C overnight. Individual random colonies from M17 were spotted onto a plate which had been seeded with high-titer stocks of ϕ2 and ϕD_1. The plates were incubated overnight at 21°C. The number of resistant isolates (cells giving rise to a colony) was determined.

RESULTS

Selection parameters to detect conjugal transfer of pNP40. Both DRC3 and 425A are phenotypically similar in that both are Lac$^+$ Cit$^+$ mesophilic lactococci. To preserve the food-grade status of these strains, it was important not to introduce unacceptable genetic markers such as drug resistance to distinguish between donor and recipient. A number of selection criteria were examined in an attempt to detect plasmid transfer, including phage resistance, nisin resistance, and the ability to metabolize lactose.

A number of phage against 425A have been isolated during industrial use. Three phage, ϕT, ϕD_1, and ϕD_2, against 425A were isolated in two different factories at different times. Initially, 425A was exposed to ϕT, and a BIM, designated BIM21, was isolated (13). BIM21 is also resistant to ϕD_1 and ϕD_2. When this isolate was introduced to industrial use, it rapidly succumbed to phage. This phage, designated ϕ2, is lytic for both BIM21 and 425A. The restriction patterns of all four phage were examined by using the rapid-identification protocol described by Hill et al. (10). The results obtained with *Eco*RI are shown in Fig. 1. Phage ϕ2 is clearly different from the other three phage, which are obviously highly related if not identical. Similar results were obtained with other restriction enzymes. Given these data and the different host ranges, it was concluded that ϕ2 and the group ϕT, ϕD_1, and ϕD_2 represented two phage types capable of attacking strain 425A. Assuming that pNP40 confers resistance to both phage types, the use of both phage types for selection would not be expected to decrease the viability of pNP40 transconjugants. However, it could be expected that both phage types would significantly affect the incidence of phage-resistant mutants.

Plasmid pNP40 has been shown to encode resistance to nisin (3, 4, 16). This characteristic provided an additional selection parameter for detecting transconjugants. The resistance levels to nisin exhibited by DRC3 and 425A were

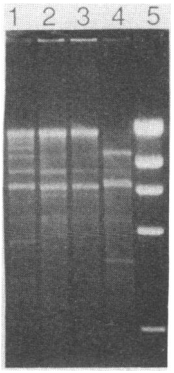

FIG. 1. Agarose gel of *Eco*RI-digested DNA extracted from *L. lactis* subsp. *lactis* 425A 45 min after infection with ϕD_1 (lane 1), ϕD_2 (lane 2), ϕT (lane 3), or ϕ2 (lane 4). Lane 5 contains λ-*Hin*dIII size standards.

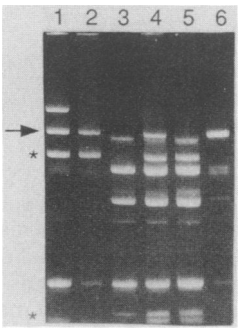

FIG. 2. (A) Agarose gel electrophoresis of plasmid DNA from DRC3 (lane 1), DPC2839 (DRC3 Lac$^-$) (lane 2), 425A (lane 3), DPC2723 [425A(pNP40)] (lane 4), and DPC2724 (lane 5). Lane 6 contains pNP40 DNA (intense band). The position of pNP40 is indicated by an arrow. The two cryptic plasmids referred to in the text are also indicated (*).

indistinguishable on M17 (lactose 0.5%) plates supplemented with Tween 20; both strains were reduced to <1% of the initial CFU on plates containing 15 U of nisin per ml. Direct selection for the transfer of pNP40 was not possible on solid medium because we were unable to predict whether the presence of pNP40 in 425A would lead to elevated nisin resistance. When 425A was grown in broth, nisin at a level of 10 U/ml completely inhibited the culture. This concentration did not affect DRC3, which was undisturbed by nisin concentrations of ≤30 U/ml. Therefore, rather than include nisin in the selection media, a preenrichment step of growth in broth containing 15 U of nisin per ml was included to allow the inhibition of 425A prior to plating of the strain on solid media.

Resistance to $\phi 2$, ϕD_1, and nisin are characteristics expected to be associated with the desired transconjugants. However, strain DRC3 exhibits the same phenotypes. In order to readily distinguish between donor and transconjugant, a Lac$^-$ derivative of DRC3 was isolated by growth at 39°C. Twelve of 12 Lac$^-$ isolates were shown by plasmid analysis to have lost a single plasmid of approximately 90 kb. All 12 retained pNP40. One such derivative, DPC2839 (Fig. 2), was used as a donor in subsequent experiments. When plated on lactose indicator agar (17), the donor (small white colonies) was easily separated from 425A (large yellow colonies).

Conjugation results. The successful mating protocol utilized all the parameters discussed in the previous section. Strains 425A (recipient) and DPC2839 (donor) were grown to mid-log phase, mixed at a ratio of 2:1, plated on milk agar, and incubated overnight at 30°C. Cells were suspended in 1 ml of Ringer's solution, and 100 μl of the mating mix was inoculated into 5 ml of M17 containing 15 U of nisin per ml, 10^7 ϕD_1 per ml, 10^7 $\phi 2$ per ml, and 20 mM $CaCl_2$ and incubated at 30°C for 2 h. The cells were plated on lactose indicator agar. After 48 h, donor counts (small white colonies) were estimated and presumptive transconjugants (large yellow colonies) were picked, purified, and challenged with nisin, ϕD_1, and $\phi 2$ to confirm the phage-nisin resistance phenotype. Putative pNP40 transconjugants were detected at a frequency of 3×10^{-8} per donor (Table 2). In control experiments with the recipient alone, no yellow colonies were detected. The use of ϕD_1, $\phi 2$, or nisin singly was not completely selective, and resistant mutants arose at various frequencies (Table 2). Plasmid profiles of 16 of the Lac$^+$ Nis^r ϕ^r strains confirmed that they were 425A derivatives. In a number of instances, the transfer of pNP40 was accompanied by the transfer of other cryptic plasmids from DRC3. One such transconjugant, DPC2723, contained two cryptic plasmids of DRC3 origin in addition to pNP40 (Fig. 2). All 16 strains also exhibited complete resistance to $\phi 2$ and ϕT and grew normally in broth containing 15 U of nisin per ml. After two overnight transfers at 30°C in GM17, a number of spontaneously phage-sensitive derivatives of DPC2723 were detected (two of five isolates examined). The reversion to phage sensitivity was accompanied by the loss of nisin resistance and the loss of pNP40 (one example, DPC2724, is shown in Fig. 2). The additional cryptic plasmids originating from DRC3 were present in DPC2724, confirming that the nisin and phage resistance phenotypes were both associated with pNP40. Confirmation of the integrity of pNP40 in DPC2723 was obtained after second-round matings to a plasmid-free recipient, *L. lactis* subsp. *lactis* MG1614. Transfer was detected by selecting for resistance to two phages ($\phi C2$ and $\phi 712$, at a final titer each of 10^7/ml) and to nisin (15 U/ml). Again, transconjugants containing additional cryptic plasmids from both donors and a number of transconjugants possessing only pNP40 were frequently observed (data not shown). By means of a similar strategy, pNP40 was conjugally transferred from DRC3 to MG1614. Representative transconjugants from both matings were chosen, and the plasmid DNAs were isolated and compared by restriction analysis. The restriction patterns were identical for a number of restriction enzymes, and no detectable rearrangements were observed (data not shown).

TABLE 2. Effect of selection parameters on incidence of resistant variants

Strain	Selection	No. of Lac$^+$ colonies[a]
425A	None	5.8×10^{10}
	Nisin	1.0×10^5
	$\phi 2$	2
	ϕD_1	3
	Nisin, $\phi 2$, ϕD_1	0
425A + DPC2839	Nisin, $\phi 2$, ϕD_1	202[b]

[a] Large yellow colonies on lactose indicator agar, counted after 48 h.
[b] pNP40 transconjugants.

Susceptibility of pNP40 transconjugants to phage. The ability of the transconjugant DPC2723 to withstand a modified Heap-Lawrence phage challenge (8) was also examined. Since the NIZO production method involves growth only at 21°C, all growth was in RSM at this temperature. Initially, 425A and DPC2723 were challenged with a high titer (10^7/ml) of those phages (ϕD_1 and $\phi 2$) isolated against 425A and BIM21 (Table 3). Strain 425A lost activity on the first cycle, whereas DPC2723 was able to grow normally for five cycles. Whey from the fifth cycle was spotted on a plate overlaid with DPC2723 to allow detection of any phage present at low levels. No such low-level phage were detected (Table 3). At the end of each cycle involving DPC2723, spot tests on the parental 425A were also negative (data not shown). Therefore, growth of the transconjugant in a system heavily contaminated with phage active against 425A removes those phage from the environment, presumably by allowing adsorption and possibly injection of phage DNA, but does not generate viable progeny. A more rigorous examination of DPC2723 was performed by replacing the defined mix of phage with a phage cocktail containing an unknown number

TABLE 3. Resistance of 425A and DPC2723 to phage challenge in RSM at 21°C

Strain	Phage[a]	Final pH[b] after cycle:					Spot test
		1	2	3	4	5	
425A	None	4.76					
	$\phi 2$ and ϕD_1	6.62					+
	Phage cocktail	6.58					+
DPC2723	None	4.80	4.78	4.79	4.79	4.90	−
	$\phi 2$ and ϕD_1	4.80	4.79	4.82	4.78	4.91	−
	Phage cocktail	4.79	4.80	4.80	6.06	6.51	+[c]

[a] Phages $\phi 2$ and ϕD_1 were added at a final concentration of 10^7/ml.
[b] Initial pH of milk was 6.65.
[c] Phage were detected at 21°C but not at 30°C.

of phages isolated over a number of years in the cheese industry. Again, 425A lost activity during the first cycle, confirming that the phage cocktail contained a high number of phages active against this background. However, DPC2723 was unaffected until cycle 4 (Table 3). The phage against DPC2723 detected at this point, designated $\phi 3$, was unable to infect either parent or transconjugant at 30°C but gave rise to small hazy plaques at 21°C. The transconjugant was unaffected by $\phi 3$ when cycle 4 or cycle 5 was carried out at 30°C. After a single cycle at 30°C, spot tests at 21°C showed no phage. This phenomenon is not plasmid linked, since the same results were obtained with 425A. The restriction profile of $\phi 3$ DNA was unlike those of ϕD_1 and $\phi 2$ (data not shown). In milk activity tests, $\phi 3$ was able to disrupt growth only at 25, 21, and 18°C. No disruption was observed at 30°C or above.

Effect of pNP40 on production of ALA. If DPC2723 is to be used as an alternative to 425A in the production of cultured butter, it is critical to demonstrate that the presence of pNP40 does not affect growth in milk or affect production of high levels of ALA. As is the case for the proteolytically deficient 425A, DPC2723 was unable to clot 10% RSM unless supplemented with tryptone. This indicates that neither pNP40 nor the two DRC3 cryptic plasmids can complement the Prt^- phenotype. The pH of milk supplemented with tryptone was reduced to identical levels by both 425A and DPC2723 in the absence of phage (Table 3). Similarly, the ability to reduce the pH of modified M17 was identical in parent and transconjugant (data not shown). Levels of ALA produced in broth and milk were determined. Strains 425A and DPC2723 produced 4.33 and 2.9 mM ALA in milk and broth, respectively. These values are in good agreement with those previously determined for 425A (14). It can be concluded that pNP40 does not interfere with the production of this important metabolite.

Stability of pNP40 in transconjugants. The transconjugants were grown under conditions which would mimic commercial conditions, i.e., growth in RSM at 21°C. Cells were grown for 25 generations and plated at 21°C on M17. Individual colonies were assessed for resistance to both ϕD_1 and $\phi 2$. In all, 100 colonies were spotted on plates seeded with high titers (10^8/ml) of $\phi 2$ and ϕD_1. All 100 grew normally to form colonies, indicating the presence of pNP40. Control 425A was unable to form a colony on phage plates. The effect of high temperatures on the phage resistance phenotype was also examined, since it has been reported that the pNP40 mechanism is heat sensitive (16). Strain DPC2723 was resistant to phage $\phi 2$ and ϕD_1 challenge at 39°C in M17 and RSM, whereas under the same conditions, 425A was rapidly lysed.

DISCUSSION

In this report, we describe the introduction of the phage resistance plasmid pNP40 into strain 425A. Plasmid pNP40 also encodes resistance to nisin, a substance generally regarded as safe which is permitted in the food chain. We show that the use of nisin and phage for selection is sufficient to distinguish transconjugants from phage-resistant mutants. In our hands, it was important to use nisin in broth and not to attempt to incorporate it into the solid selective medium. This was because of the similar levels of nisin resistance exhibited by 425A and DRC3 on solid media. We are unable to explain why this should be so, since nisin was suitable for selection in liquid medium. The period of preenrichment also enhances the selective effect of the added phage by allowing propagation to high numbers during outgrowth. The selective effect of phage used in this way is far greater than the usual procedure, in which phage are added to the mating mix only minutes prior to plating (data not shown). The use of a preenrichment step may positively influence the numbers of transconjugants detected. To attempt to minimize this problem, we have expressed the transconjugant frequency relative to the donor colony count, since this figure should also increase during preenrichment. The identification of two different phage types against 425A was also important. It has been our experience that BIMs of 425A isolated after challenge with ϕD_1 remain sensitive to $\phi 2$. However, pNP40 transconjugants are resistant to both phage types. Selection with two phages decreases the likelihood that phage-resistant mutants will arise at a frequency higher than the conjugation frequency. The use of a double selection, for phage and nisin, and outgrowth in broth removed the need to use hybridization techniques to isolate transconjugants, as was the case with both pTR2030 (18) and pAJ1106 (12). It was noteworthy that in almost all conjugative events involving pNP40, the transfer of other plasmids from the donor to the recipient was observed. The nature of the mobilization event is unclear, since there was no increase in size in the cotransferred plasmids, making insertion sequence involvement unlikely. We have utilized the ability of pNP40-mediated cotransfer to introduce both pNP40 and a nonconjugative restriction and modification plasmid into MG1614, rendering the strain phage resistant at both 30 and 37°C (7).

It has been reported that the pNP40 phage resistance mechanism is inoperative at a high temperature and also that the plasmid may be thermosensitive in its replication (16). In our hands, MG1614 bearing pNP40 showed no resistance to phage C2 at 37°C, confirming the results obtained by McKay and Baldwin (16) in the closely related strain *L. lactis* subsp. *lactis* LM2301. Strain DPC2723 readily lost pNP40 at 30°C, although both plasmid and phenotype were stable at temperatures of 39°C in the presence of a phage challenge. In contrast, 12 of 12 Lac^- derivatives of DRC3 isolated after growth at 39°C all retained pNP40 in the absence of selective pressure. This suggests a strain-dependent role in the effect of high temperatures on the maintainance of pNP40.

The usefulness of DPC2723 will not be established until it has undergone prolonged exposure in the industry. However, we are confident that it offers a significant advantage over 425A, since it possesses complete resistance to those phages most commonly encountered in the commercial environment. We will monitor for the rise of phages with the ability to overcome the pNP40-encoded resistance mechanism(s). That such phages exist is certain, since a phage (ϕdrc3) which can propagate on the original pNP40 host, DRC3, has been isolated (16), and a phage ($\phi 3$) which can

propagate on DPC2723 was detected in this study. Phage φ3 was incapable of disrupting cell growth at temperatures higher than 25°C and was removed from the medium by a single growth cycle at 30°C. In the event of other phage emerging in the industry, it may be possible to replace DPC2723 with an alternative transconjugant, e.g., a 425A derivative bearing pTR2030, since it has been shown that these two plasmids are unrelated (11). Alternatively, a number of plasmids with complementary mechanisms could be introduced to the same strain, as described by Coffey et al. (2) for pCI829 and pCI750. In order to understand the mechanism of action, we have cloned and expressed a pNP40 phage resistance determinant in lactococci (5). This work will allow a better appraisal of which of the other phage resistance plasmids described in the literature would provide the best alternative or addition to the pNP40-encoded system. Meanwhile, pNP40 shows considerable promise as a means of protecting strains of industrial significance, and we are continuing efforts to introduce this plasmid to other important strains.

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