A Starter Culture Rotation Strategy Incorporating Paired Restriction/Modification and Abortive Infection Bacteriophage Defenses in a Single *Lactococcus lactis* Strain†

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Three derivatives of *Lactococcus lactis* subsp. *lactis* NCK203, each with a different pair of restriction/modification (R/M) and abortive infection (Abi) phage defense systems, were constructed and then rotated in repeated cycles of a milk starter culture activity test (SAT). The rotation proceeded successfully through nine successive SATs in the presence of phage and whey containing phage from previous cycles. *Lactococcus* cultures were challenged with 2 small isometric-headed phages, ϕ 31 and ϕ l36, in one rotation series and with a composite of 10 industrial phages in another series. Two native lactococcal R⁺/M⁺ plasmids, pTRK68 and pTRK11, and one recombinant plasmid, pTRK308, harboring a third distinct R/M system were incorporated into three NCK203 derivatives constructed separately for the rotation. The R⁺/M⁺ NCK203 derivatives were transformed with high-copy-number plasmids encoding four Abi genes, *abiA*, *abiC*, *per31*, and *per50*. Various Abi and R/M combinations constructed in NCK203 were evaluated for their effects on cell growth, level of phage resistance, and retardation of phage development during repeated cycles of the SAT. The three NCK203 derivatives chosen for use in the SAT exhibited additive effects of the R/M and Abi phenotypes against sensitive phages. In such combinations, phage escaping restriction are prevented from completing their infective cycle by an abortive response that kills the host cell. The rotation series successfully controlled modified, recombinant, and mutant phages which were resistant to any one of the individual defense systems by presenting a different set of R/M and Abi defenses in the next test of the rotation.

Since the first recognition of bacteriophages in dairy plants in the 1930s, control of phages has remained a serious problem for cheesemakers (5). In recent years, the economic significance of phages has increased dramatically because the demand for cheese has resulted in more mechanization, larger plants, and shorter manufacturing schedules. Worldwide, a number of strategies rely upon particular choices of starter strains in order to control phage. Starter cultures used in cheesemaking are known as undefined cultures, defined cultures with rotation, and defined cultures without rotation (5). Traditional undefined cultures, still used for small-scale cheesemaking in Europe today, contain multiple strains which are allowed to coevolve with phage, leading to successful milk fermentations (26). These starters are not suitable for large-scale cheese operations, however, because they produce an inconsistent product. Rotations of defined cultures are commonly used but are limited by the small number of phage-unrelated strains available. In recent years multiple-strain starters which contain two to five carefully selected cultures used continuously without rotation have been favored (38). When levels of 10⁵ to 10⁶ PFU of phage per ml are detected in whey samples, the strain affected by the phage is removed and replaced with a new strain or a phage-resistant derivative. While this system is widely used and maintains uniform prod-

uct quality, the availability of suitable replacement strains can limit its long-term use.

There are many potential advantages in using a single-strain starter system (36). Highly specialized strains, developed for specific fermentative or organoleptic qualities, may be used continuously. With a single strain in use, the phage diversity in cheese plants is reduced (39), and only one indicator strain is needed to monitor phage populations. Sing and Klaenhammer (36) described a rotation system based on several phage-resistant derivatives of a single strain. They tested various rotations systems with the Heap-Lawrence starter culture activity test (SAT), which evaluates the fermentative activity of a starter culture in milk over time and temperature profiles that mimic those used in commercial cheesemaking (11). Rotations of three *Lactococcus lactis* NCK203 derivatives containing different plasmid-encoded phage resistance mechanisms in successive cycles of SATs suppressed 10⁶ PFU of phage per ml from a commercial phage composite. Two of the NCK203 derivatives contained different plasmid-encoded restriction/modification (R/M) systems. The success of this rotation system was based on the inclusion of pTR2030, which encodes R/M and *abiA*, an abortive infection gene which interferes with phage DNA replication (14). The two defense systems are complementary, with R/M as the first line of defense against incoming phage DNA. If the phage DNA escapes restriction, the Abi defense aborts the infection, resulting in cell death and the prevention of release of modified phage progeny (21). The presence of the *AbiA* phenotype was crucial to the success of the system. Consequently, the system failed when *AbiA*-resistant phages were present at levels of 10³ PFU/ml in the phage composites.

The availability of numerous phage resistance plasmids encoding distinct R/M and Abi systems has expanded our ability

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TABLE 1. Strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristic(s)	Source or reference
<i>L. lactis</i> subsp. <i>lactis</i>		
NCK202	Lac ⁻ R ₁ ⁺ /M ₁ ⁺ <i>str-15</i> pTRK68; previously designated <i>L. lactis</i> subsp. <i>lactis</i> L2FA	19
NCK203	Lac ⁻ R ⁻ /M ⁻ <i>str-15</i> ; derivative of NCK202 lacking pTRK68; propagating host for phages ϕ 31 and ul36	14
NCK308	NCK203(pTRK308); R ₃ ⁺ /M ₃ ⁺ Em ^r	35
NCK346	Lac ⁺ R ₂ ⁺ /M ₂ ⁺ <i>str-15</i> ; NCK203 transconjugant containing pTRK11	36
NCK682	NCK203(pTRK375); Per31 ⁺ Cm ^r	This study
NCK683	NCK203(pTRK371); AbiC ⁺ Em ^r	This study
NCK687	NCK203(pTRK68, pTRK371); R ₁ ⁺ /M ₁ ⁺ AbiC ⁺ Em ^r	This study
NCK688	NCK203(pTRK11, pTRK18); R ₂ ⁺ /M ₂ ⁺ AbiA ⁺ Em ^r Cm ^r	This study
NCK689	NCK203(pTRK308, pTRK375); R ₃ ⁺ /M ₃ ⁺ Per31 ⁺ Em ^r Cm ^r	This study
<i>E. coli</i>		
DH5 α	Transformation host	GIBCO-BRL
MC1061	Transformation host	15
Plasmids		
pTRKH1	Shuttle cloning vector; Tc ^r Em ^r ; high copy number in <i>Lactococcus</i> spp.; 11 kb	31
pTRKH3	Shuttle cloning vector; Tc ^r Em ^r ; high copy number in <i>Lactococcus</i> spp.; 7.8 kb	31
pNZ18	Shuttle cloning vector; Km ^r Cm ^r ; high copy number in <i>Lactococcus</i> spp.; 5.7 kb	6
pTRK11	Lac ⁺ R ₂ ⁺ /M ₂ ⁺ Tra ⁺ ; $\approx$ 100 kb	35
pTRK18	pSA3 + 10.3-kb fragment of pTR2030; AbiA ⁺ (Hsp ⁺); medium copy number in <i>Lactococcus</i> spp.; Cm ^r Em ^r ; 20.5 kb	14
pTRK68	Resident plasmid of NCK202; R ₁ ⁺ /M ₁ ⁺	14
pTRK308	pSA34 + 20-kb fragment of pTRK30; R ₃ ⁺ /M ₃ ⁺ Em ^r ; 25 kb	35
pTRK319	pMG36e + 1.3-kb fragment of pTN20; AbiC ⁺ (Prf ⁺) Em ^r ; 5 kb	9
pTRK323	pTRKH1 + 1.6-kb fragment of phage ϕ 50; Per50 ⁺ ; high copy number in <i>Lactococcus</i> spp.; Em ^r ; 12.6 kb	29
pTRK361	pTRKH2 + 4.5-kb fragment of ϕ 31; <i>per31</i> ; high copy number in <i>Lactococcus</i> spp.; Em ^r ; 11.4 kb	29
pTRK363	pTRKH3 + 7.3-kb fragment of pTR2030; AbiA ⁺ (Hsp ⁺); high copy number in <i>Lactococcus</i> spp.; Em ^r ; 12.8 kb	7
pTRK371	pTRKH3 + 2.5-kb pTRK319 fragment; AbiC ⁺ (Prf ⁺), high copy number in <i>Lactococcus</i> spp.; Em ^r ; 10.3 kb	This study
pTRK375	pNZ18 + 4.5-kb fragment of ϕ 31, <i>per31</i> ; high copy number in <i>Lactococcus</i> spp.; Cm ^r Km ^r ; 10.2 kb	This study
Bacteriophages		
ϕ 31	Small isometric-headed; P335 species; 31.9 kb	1
ϕ 31.7	Per31-insensitive derivative of ϕ 31	28a
ul36	Small isometric-headed; P335 species; 28.8 kb	27
Composite of industrial phages	Combination of 10 individual lysates containing approximately 10 ⁷ PFU of each phage per ml	This study

to construct phage-resistant derivatives. Various combinations of defenses can now be designed for use in multicomponent defense systems (for reviews of phage resistance mechanisms, see references 12, 22, and 34). Our understanding of the effectiveness of defenses and combinations of defenses has also increased (20). Four Abi defense genes available in our laboratory are *abiA* (14, 23), *abiC* (9), *per31* (29), and *per50* (13). The mechanisms of action of *abiA* and *abiC* have not been elucidated, but in general *abiA* retards phage DNA replication, while *abiC* acts at a point in the phage infection cycle subsequent to DNA replication. Both *per31* and *per50* are cloned phage origins of replication. Each of these cloned phage origins is proposed to interfere with phage DNA replication by presenting false targets in *trans* for phage replication factors. The use of high-copy-number cloning vectors has shown that gene dosage relates directly to the level of resistance obtained for three different Abi genes (3, 7, 29).

The opportunity now exists to target different parts of the phage lytic system with different Abi defenses and to pair R/M defenses with more-effective, high-copy-number Abi systems. The intent of this study was to design and genetically construct improved defense systems and combinations and evaluate their effectiveness in a single-strain starter rotation system based upon *L. lactis* NCK203.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. *L. lactis* subsp. *lactis* strains were propagated at 30°C in M17 medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% glucose. Erythromycin and chloramphenicol were added as needed at 1.5 and 7.5 μ g/ml, respectively. When *L. lactis* NCK203 was propagated in milk, the 11% skim milk powder was fortified with 1% glucose, 0.25% Casamino Acids, and 0.25% yeast extract. NCK203 is a derivative of the industrial strain LMA12 which has been cured of a plasmid encoding lactose utilization and protease functions (14). *Escherichia coli* strains were propagated in LB medium (33) or brain heart infusion (Difco Laboratories) at 37°C with 200 μ g of erythromycin per ml or 10 μ g of chloramphenicol per ml as needed. Bacterial stock cultures were stored at -20°C in the appropriate medium with 10% (vol/vol) glycerol. Phage were propagated through *L. lactis* subsp. *lactis* NCK203, and their titers were determined by standard double-layer agar plate methods (37). The efficiency of plaquing (EOP) was obtained by dividing the phage titer on the test strain by the titer on the homologous, phage-sensitive host, NCK203. Marschall Products, Rhone-Poulenc, Madison, Wis., kindly provided 10 separate industrial high-titer single-phage lysates.

SAT. The Heap-Lawrence SAT (11) was conducted essentially as described by Sing and Klaenhammer (36). The test was carried out in tubes containing 10 ml of fortified milk (11% skim milk powder, 1% glucose, 0.25% Casamino Acids, 0.25% yeast extract). The fortified-milk tubes were steamed for 60 min, cooled in ice water, and kept at 4°C until used. Frozen bacterial stock cultures were first inoculated into fortified milk supplemented with antibiotics and then propagated at 30°C for 18 h prior to inoculation in the test. In the first SAT, tubes of fortified milk were inoculated with 200 μ l of the overnight bacterial culture and 200 μ l of a diluted phage preparation to yield about 10⁶ PFU/ml. The tubes were incubated in water baths at 30°C for 100 min, then at 40°C for 190 min, and finally

TABLE 2. EOPs for phage resistance plasmids in NCK203

Plasmid(s) in NCK203	Phenotype ^a	EOP (mean $\pm$ SD)	
		ϕ 31	ul36
pTRK68	R ₁ ⁺ /M ₁ ⁺	(1 $\pm$ 0.8) $\times$ 10 ⁻⁴	(1 $\pm$ 0.6) $\times$ 10 ⁻⁴
pTRK371	AbiC ⁺	1 $\pm$ 0.08	(3 $\pm$ 2) $\times$ 10 ⁻²
pTRK68 + pTRK371	R ₁ ⁺ /M ₁ ⁺ + AbiC ⁺	(2 $\pm$ 0.9) $\times$ 10 ⁻³	(7 $\pm$ 7) $\times$ 10 ⁻⁶
pTRK11	R ₂ ⁺ /M ₂ ⁺	(1 $\pm$ 0.3) $\times$ 10 ⁻²	(2 $\pm$ 0.7) $\times$ 10 ⁻³
pTRK18	AbiA ⁺	0.5 $\pm$ 0.15	(6 $\pm$ 0.6) $\times$ 10 ⁻²
pTRK11 + pTRK18	R ₂ ⁺ /M ₂ ⁺ + AbiA ⁺	(5 $\pm$ 0.8) $\times$ 10 ⁻³	(2 $\pm$ 1) $\times$ 10 ⁻⁵
pTRK308	R ₃ ⁺ /M ₃ ⁺	(2 $\pm$ 1) $\times$ 10 ⁻⁴	(1 $\pm$ 0.3) $\times$ 10 ⁻⁴
pTRK375	Per31 ⁺	(7 $\pm$ 2) $\times$ 10 ⁻⁷	0.8 $\pm$ 0.2
pTRK308 + pTRK375	R ₃ ⁺ /M ₃ ⁺ + Per31 ⁺	<2 $\times$ 10 ⁻⁸	(1 $\pm$ 0.7) $\times$ 10 ⁻⁴
pTRK323	Per50 ⁺	0.9 $\pm$ 0.2	(8 $\pm$ 3) $\times$ 10 ⁻⁵
pTRK361	Per31 ⁺	(8 $\pm$ 4) $\times$ 10 ⁻⁷	0.8 $\pm$ 0.1
pTRK363	AbiA ⁺	(4 $\pm$ 1) $\times$ 10 ⁻⁴	(4 $\pm$ 3) $\times$ 10 ⁻⁶

^a The R/M systems encoded by pTRK68, pTRK11, and pTRK308 are distinct in their specificities of restriction and modification activities.

at 30°C for 100 min. Upon completion of the third incubation period, the pH of each sample was determined. To determine the titer of the phage produced, whey samples were prepared by adding 0.5 ml of 10% lactic acid to each tube, vortexing, and then centrifuging 1.5 ml in a microcentrifuge to pellet the milk curd. Whey samples were then stored at -20°C. For the actual phage assays, the whey samples were thawed and diluted, and titers were determined by spotting 10- μ l aliquots onto a lawn of NCK203 cells. The lowest level of detection was 10² PFU/ml. In subsequent tests of the rotation, each tube was inoculated with 200 μ l of overnight culture as in the first test, but 100 μ l of whey from the previous test was added to each tube in place of diluted phage stock. The SAT rotation was continued for nine tests with transfer of whey from the previous test, except that the rotation series was discontinued when cultures failed to lower the pH of the milk below 6.0 and allowed phage propagation of 10⁸ to 10⁹ PFU/ml. In some nine-test rotations, 10⁵ PFU of phage per ml was added in tests 2 to 9 in addition to whey.

Phage growth curves in milk. Phage growth on NCK203 and related strains was characterized in fortified milk. Overnight milk cultures were inoculated (200 μ l) into 10 ml of fortified milk and incubated for 2 or 3 h at 30°C until they reached a pH of approximately 6.0 (yielding about 10⁸ CFU/ml). To 900 μ l of culture, 100 μ l of diluted phage was added to yield 10⁶ PFU/ml at a multiplicity of infection of 0.01. The tubes were held at room temperature for 10 min to allow phage adsorption and then centrifuged for 3 min in a microcentrifuge. All supernatant was pipetted off, and the cell pellet was washed twice with fortified milk. The pellet was finally resuspended in 1 ml of fortified milk, and 100 μ l was transferred to 10 ml of fortified milk. This point was taken as time zero. The tubes were incubated at 30°C, and samples were taken periodically for phage titration on NCK203.

Gene cloning. Isolation of plasmids from *L. lactis* cells was as described by O'Sullivan and Klaenhammer (30), except that ethidium bromide was not used. *E. coli* plasmid DNA was isolated by standard alkaline lysis procedures or with the Qiagen Plasmid Kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions. Endonuclease restrictions and ligations were performed as described by Sambrook et al. (33). Vector fragments were dephosphorylated with shrimp alkaline phosphatase (U.S. Biochemical Corp., Cleveland, Ohio) prior to electrophoresis. DNA fragments were isolated from electrophoresis gel slices by using the GeneClean II kit (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions. When two fragments were isolated for ligations, separate gel slices containing the vector and insert fragments were purified with GeneClean together in one tube, according to the manufacturer's suggestions, and then ligated.

Electroporations of both *L. lactis* and *E. coli* cells were carried out as described by Dower et al. (8) with the Bio-Rad Gene Pulser (Bio-Rad, Richmond, Calif.) set at 25 μ F, 2.0 kV, and 200 Ω and with 0.2-cm cuvettes. The electroporation protocol described by O'Sullivan et al. (29) was employed to transform some R⁺/M⁺ hosts. Plasmid clones in *E. coli* were evaluated for the acquisition of insert DNA by restriction enzyme digestion and then electroporated into lactococcal hosts for evaluation of phage resistance phenotypes. DNA from lactococcal transformants was isolated and digested with restriction enzymes to confirm the presence and identity of transformed plasmids. Copy numbers of recombinant plasmids in NCK203 were estimated as high, medium, or low on the basis of previous data (7, 29) and direct comparisons of band brightnesses with those of NCK203 resident plasmids in ethidium bromide-stained agarose gels of plasmid DNA preparations.

RESULTS

Cloning of Abi genes to high-copy-number shuttle vectors. Three high-copy-number Abi plasmids, each with a different

abi gene cloned into one of the pTRKH family of vectors (31), were already available: pTRK323 (*per50*), pTRK361 (*per31*) (29), and pTRK363 (*abiA*) (7). Phage resistance phenotypes exhibited by these high copy-number plasmids are shown in Table 2. *abiC* was cloned into pTRKH3 by the following strategy. *abiC* along with the P32 promoter of pMG36e (40) was cloned from pTRK319 (9). Plasmid pTRK319 was digested with *Hind*III, and the two approximately equal-size fragments were shotgun cloned into *Nru*I-digested pTRKH3 (Fig. 1A). *E. coli* transformants were selected on erythromycin (200 μ g/ml)-brain heart infusion agar plates, and their DNA was evaluated for inserts containing *abiC* by digestion with *Eco*RI. The resulting clone, pTRK371, was transformed into NCK203. Phage ul36 plaqued at an EOP of 3 $\times$ 10⁻² on NCK203(pTRK371), confirming the presence of the functioning *abiC* gene (Table 2). Phage ϕ 31 is not affected by *abiC*.

In experiments with the four Abi genes cloned on high-copy-number vectors, it was found that NCK203(pTRK361) and NCK203(pTRK363) grew and produced acid very slowly in fortified milk, while growth in M17-0.5% glucose was not affected (data not shown). Therefore, although both plasmids expressed high levels of phage resistance, they could not be included in combinations intended for the SAT. Only two Abi clones in the pTRKH family of vectors, pTRK323 and pTRK371, were suitable for inclusion in the SAT rotation.

Plasmid pNZ18 (6) was chosen as an alternate shuttle vector for cloning of the Abi genes because of its high copy number in lactococci. pNZ18 (5.7 kb) contains a multiple cloning site, replicates by the rolling circle mechanism, and encodes chloramphenicol resistance. The 4.5-kb *Sal*I-*Sma*I fragment of pTRK361 encoding *per31* was cloned into the large *Sal*I-*Nru*I fragment of pNZ18 (Fig. 1B). This plasmid, designated pTRK375, limited the plaquing efficiency of phage ϕ 31 to 7 $\times$ 10⁻⁷ when transformed into NCK203 (Table 2). *abiC* was also introduced into pNZ18, but the resulting plasmid was structurally unstable when transformed into NCK203. *per50* was cloned into pNZ18 as well, but resistance against phage ul36 was marginal (EOP $\approx$ 1) in this construction. Of the three Abi genes cloned into pNZ18, only pTRK375 containing *per31* was suitable for inclusion in the SAT rotation.

Combining Abi-encoding and R/M-encoding plasmids in NCK203. R/M and Abi plasmids were combined in NCK203 by electroporating the Abi-encoding plasmids carrying antibiotic resistance markers into cells containing the R/M-encoding plasmids. The following NCK203 derivatives were used as transformation hosts: NCK202, the parent strain of NCK203, containing R₁⁺/M₁⁺ (pTRK68) (14, 19); NCK346, containing

FIG. 1. Cloning strategies for pTRK371 (A) and pTRK375 (B). Plasmid maps are not drawn to scale. Abbreviations: *Em^r*, erythromycin resistance; *Tc^r*, tetracycline resistance; *Km^r*, kanamycin resistance; *Cm^r*, chloramphenicol resistance; *ori* (G+), *ori* (G-), and *ori*, replication origins that function in gram-positive bacteria, gram-negative bacteria, or both, respectively. P32-ATG indicates the P32 promoter.

TABLE 3. Effect of addition of phages ϕ 31 and ul36 on *L. lactis* NCK203 activity in fortified milk

SAT ^a	Plasmids present	Relevant functions ^b	pH	PFU/ml ^c
1	— ^d		6.5	7×10^6
	none	R^-/M^-	6.4	6×10^9
	pTRK68, pTRK371	R_1^+/M_1^+ , AbiC ⁺	5.2	5×10^5
2	pTRK11, pTRK18	R_2^+/M_2^+ , AbiA ⁺	5.4	$<10^2$
3	pTRK308, pTRK375	R_3^+/M_3^+ , Per31 ⁺	5.3	$<10^2$
4	pTRK68, pTRK371	R_1^+/M_1^+ , AbiC ⁺	5.1	4×10^2
5	pTRK11, pTRK18	R_2^+/M_2^+ , AbiA ⁺	5.2	$<10^2$
6	pTRK308, pTRK375	R_3^+/M_3^+ , Per31 ⁺	5.3	$<10^2$
7	pTRK68, pTRK371	R_1^+/M_1^+ , AbiC ⁺	5.3	$<10^2$
8	pTRK11, pTRK18	R_2^+/M_2^+ , AbiA ⁺	5.1	$<10^2$
9	pTRK308, pTRK375	R_3^+/M_3^+ , Per31 ⁺	5.1	$<10^2$

^a Numbers indicate the number of sequential subcultures of phage in whey. For SAT 1, 10^6 PFU/ml was added. For SATs 2 to 9, 10^5 PFU each of phages ϕ 31 and ul36 per ml and 100 μ l of whey from the previous SAT were added to each culture at the beginning of the SAT.

^b R^+/M^+ indicates a functional R/M system for DNA. Subscripts 1, 2, and 3 indicate different R/M systems. AbiA and AbiC are abortive infection defense systems. Per31 is an abortive-type system based on a cloned phage origin of replication present in *trans* during the phage infection.

^c Determined at the conclusion of the SAT by spotting aliquots of diluted whey on NCK203 as an indicator.

^d Milk-only control.

R_2^+/M_2^+ (pTRK11) (36); and NCK308, containing R_3^+/M_3^+ (pTRK308) (35) (Table 1). Both pTRK11 (R_2^+/M_2^+) and pTRK68 (R_1^+/M_1^+) are native lactococcal plasmids which do not encode antibiotic resistance markers, whereas pTRK308 is a recombinant R^+/M^+ plasmid encoding erythromycin resistance. pTRK308 (R_3^+/M_3^+) was combined with pTRK375, encoding *per31* and chloramphenicol resistance. The additive effect of R/M and Abi defenses of this NCK203 derivative lowered the EOP of phage ϕ 31 to undetectable levels and that of ul36 to 10^{-4} (Table 2). Separate NCK202 transformants with functional Abi genes were obtained with plasmids pTRK371, pTRK375, and pTRK361. The pTRK68-pTRK371 combination (R_1^+/M_1^+ , AbiC⁺) was chosen for the SAT rotation. The EOP for ϕ 31 was 2×10^{-3} with the combination of

pTRK68 and pTRK371, compared with 1×10^{-4} for pTRK68 alone, but the effect against ul36 was additive, lowering the EOP to 7×10^{-6} . Last, NCK346, containing pTRK11 (R_2^+/M_2^+), was electroporated with pTRK18 (14), a medium-copy-number *abiA*-encoding plasmid. NCK203(pTRK363) bearing high-copy-number *abiA* on pTRKH1 was retarded in its capability for growth in fortified milk and thus was not used in the SAT rotations. The combination of pTRK11 and pTRK18 (R_2^+/M_2^+ , AbiA⁺) decreased the EOP of ϕ 31 to 5×10^{-3} and that of ul36 to 2×10^{-5} (Table 2).

SAT rotations. The following rotation strategy was implemented in each round of SATs: in SATs 1, 4, and 7, NCK203 (R_1^+/M_1^+ , AbiC⁺) was used; in SATs 2, 5, and 8, NCK203 (R_2^+/M_2^+ , AbiA⁺) was used; and in SATs 3, 6, and 9, NCK203 (R_3^+/M_3^+ , Per31⁺) was used. In the initial rotation experiment, lysates were added to NCK203(R_1^+/M_1^+ , AbiC⁺) to obtain 10^6 PFU/ml each for phages ϕ 31 and ul36. In the eight subsequent SATs, the three strains were rotated as described above with 100 μ l of whey from the previous SAT carried over to the next SAT. The control, NCK203, failed the first SAT, but the rotation of the three NCK203 derivatives continued successfully for nine successive SATs (Table 3). Following SAT 1, proliferation of the two phages was at or below 10^2 PFU/ml, the minimum level of detection for phage in the whey plaquing assay.

The second experiment was similar to the first except that 10^5 PFU each of ϕ 31 and ul36 per ml was added to SATs 2 to 9 in addition to 100 μ l of whey. In three repetitions of this experiment, the nine-test rotation was completed successfully (Table 4). Additional controls included any one strain used continuously in repeated SATs. These controls were treated the same as the rotation strains, with whey carried over from each test to the next. Three of the four controls failed in the first 2 days: NCK203, NCK203(R_1^+/M_1^+ , AbiC⁺), and NCK203(R_2^+/M_2^+ , AbiA⁺). Surprisingly, NCK203(R_3^+/M_3^+ , Per31⁺) used continuously did not fail for the nine successive SATs.

In order to further investigate the effectiveness of NCK203(R_3^+/M_3^+ , Per31⁺), the effect of *per31* alone was evaluated in a second experiment in which NCK203(pTRK375, Per31⁺) was challenged with ϕ 31. In SAT 1, 10^6 PFU of ϕ 31 per ml was added to NCK203(pTRK375). In subsequent SATs,

TABLE 4. pH of fortified milk cultured with *L. lactis* NCK203 and a rotation series of NCK203 containing different R/M and abortive infection (Abi⁺, Per31⁺) plasmids when challenged with phages ϕ 31 and ul36

Control or rotation series	pH ^a in SAT no. ^b :								
	1	2	3	4	5	6	7	8	9
Controls ^c									
— ^d	6.5 (2×10^7)	6.5 (1×10^7)	6.5 (3×10^7)	6.5 (2×10^6)	6.5 (1×10^5)	6.5 (6×10^5)	6.5 (2×10^5)	6.5 (2×10^5)	6.5 (2×10^5)
No plasmid	6.3 (1×10^{10})								
R_1^+/M_1^+ , AbiC ⁺	5.2 (4×10^7)	6.4 (2×10^9)							
R_2^+/M_2^+ , AbiA ⁺	5.1 (1×10^7)	6.4 (2×10^9)							
R_3^+/M_3^+ , Per31 ⁺	5.1 (2×10^5)	5.3 (9×10^4)	5.3 (2×10^4)	5.4 (8×10^3)	5.2 (6×10^3)	5.2 (6×10^2)	5.1 (8×10^3)	5.5 (1×10^3)	5.2 (1×10^4)
Rotation series ^e									
1	5.2 (4×10^7)	5.2 (2×10^5)	5.2 (6×10^2)	5.1 (1×10^3)	5.2 (1×10^3)	5.2 ($<10^2$)	5.0 (8×10^2)	5.3 (3×10^2)	5.2 (3×10^2)
2	5.3 (4×10^3)	5.3 (1×10^3)	5.4 (1×10^3)	5.2 (1×10^6)	5.4 (2×10^5)	5.4 (2×10^2)	5.3 (2×10^6)	5.3 (2×10^5)	5.3 (3×10^3)
3	5.5 (5×10^7)	5.5 (6×10^6)	5.4 (1×10^3)	5.5 (5×10^5)	5.4 (6×10^5)	5.3 (2×10^3)	5.2 (2×10^7)	5.4 (3×10^5)	5.3 (1×10^4)

^a The numbers in parentheses denote the titer of phage (PFU per milliliter on NCK203) in the whey from each sample.

^b SAT numbers indicate the number of sequential tests. For SAT 1, 10^6 PFU each of phages ϕ 31 and ul36 per ml was added to the cultures initially. For SATs 2 to 9, 10^5 PFU each of phages ϕ 31 and ul36 per ml and 100 μ l of whey from the previous SAT was added to each culture.

^c The plasmids present are indicated.

^d —, the same phage mix as was added to the rotation strain was added to the control tube with milk only.

^e The results of three separate repetitions of the rotations are given. The following plasmids were used: SATs 1, 4, and 7, R_1^+/M_1^+ , AbiC⁺; SATs 2, 5, and 8, R_2^+/M_2^+ , AbiA⁺; SATs 3, 6, and 9, R_3^+/M_3^+ , Per31⁺ (see Results).

100 μ l of whey from the previous SAT and 10^5 PFU/ml of a ϕ 31 lysate were added at the beginning of each test. The experiment was continued successfully for 16 tests and finally failed in test 17.

A whey sample from SAT 9 of this test was examined to determine the types of phage present. The phage content of the whey was assayed on NCK203(pTRK375), and 10 single-plaque isolates were purified. DNA from the 10 isolates was digested with *Hind*III, and the fragmentation pattern was compared with those of ϕ 31 and several ϕ 31 recombinant variants isolated recently in our laboratory (28b, 29). All 10 phage isolates revealed a pattern distinct from that of ϕ 31 but identical to that of ϕ 31.7, a recombinant phage isolated following challenge of NCK203(pTRK361) with ϕ 31 (data not shown). Phage ϕ 31.7 is partially resistant to *per*31 [EOP of 0.6 on NCK203(pTRK375) with a reduced plaque size].

The proliferations of phages ϕ 31 and ϕ 31.7 were compared on NCK203, NCK203(pNZ18), and NCK203(pTRK375) during growth in milk (Fig. 2). The burst size of ϕ 31.7 (60 on NCK203 [standard deviation, 40]) is significantly smaller (1% level) than that of ϕ 31 (300 on NCK203 [standard deviation, 100]). The propagation of ϕ 31 is greatly suppressed by the presence of pTRK375 but not by that of pNZ18, the vector alone (Fig. 2A). The development of ϕ 31.7 appears to be slightly retarded by pTRK375 after the first burst, and a slight effect of pNZ18 (significant at 100 min) is negligible overall (Fig. 2B). The ability of NCK203(pTRK375) to suppress this mutant phage is most likely due to the initial low frequency of appearance of the phage (29) plus the relatively smaller burst size of ϕ 31.7 compared with that of ϕ 31.

A final SAT rotation (Table 5) was conducted with a composite of industrial phages provided by Marshall Products. NCK203 was sensitive to 3 of the 10 phages. Two of the three phages which plaqued on NCK203 were resistant to R_1/M_1 encoded by pTRK68 because they were likely isolated on the industrial starter which bears pTRK68 (data not shown). New lysates of these two phages prepared on NCK203 were sensitive to NCK203(pTRK68). The lysates for these two phages, with DNA modification removed, were used in the SAT rotation. A combined lysate was made by mixing 0.5 ml of a 10^8 -PFU/ml titer of each of the 10 phages to produce a 5.0-ml composite lysate, except that one phage, supplied at a lower titer by Marshall Products, was present at only 10^7 PFU/ml. The rotation was conducted as in previous tests, with a total of 10^6 PFU of the composite lysate per ml present in SAT 1 and 100 μ l of whey and 10^5 PFU of the composite lysate per ml present in SATs 2 to 9. Nine tests of the rotation were successfully completed (Table 5). The continuous controls lasted 1 day (NCK203), 2 days [NCK203(R_1^+/M_1^+ , AbiC $^+$) and NCK203(R_3^+/M_3^+ , Per31 $^+$)], or 4 days [NCK203(R_2^+/M_2^+ , AbiA $^+$)].

DISCUSSION

In recent years the growing number of phage resistance plasmids isolated from naturally phage-resistant strains of lactococci has provided a diverse genetic pool (reviewed by Klaenhammer and Fitzgerald [22]). Recent efforts have exploited high-copy-number vectors to increase the expression of phage resistance genes in lactococci and integrate them in the chromosome (4, 7, 29). The intent of this study was to create new combinations of phage resistance genes, some of which are strongly expressed from high-copy-number vectors. Various combinations of these genes were used to create unique derivatives of a single strain, and these were evaluated in a starter

FIG. 2. Growth curves for phages ϕ 31 (A) and ϕ 31.7 (B) on NCK203 and derivatives growing in fortified milk. $\bullet$, NCK203; $\blacktriangledown$, NCK203(pNZ18); $\blacksquare$, NCK203(pTRK375). The points represent three independent determinations. Bars represent standard deviations.

rotation system based on the phage defense rotation strategy developed previously in our laboratory (36).

In order to take full advantage of the benefits of R/M and Abi combinations, each of the derivatives generated for this study was designed to contain both types of phage resistance. In the majority of cases, combinations of R/M and Abi systems resulted in additive gains in the level and/or spectrum of phage resistance. However, some combinations of Abi systems or of Abi and R/M systems were not additive. While in most cases it was possible to construct the genetic combinations, alterations in one or both phenotypes were periodically observed. These alterations may arise from the type of vector used, the specific defense combinations employed, or the effects of the recombinant plasmids on the bacterial host. While none of these were investigated to clarify specific causes and effects, the results show that the selection and combination of phage defenses must be empirically addressed until more understanding

TABLE 5. pH of fortified milk cultured with *L. lactis* NCK203 and a rotation series of NCK203 containing different R/M and abortive infection (Abi⁺, Per31⁺) plasmids when challenged with a composite of industrial phages

Control or rotation series	pH ^a in SAT no. ^b :								
	1	2	3	4	5	6	7	8	9
Controls ^c									
— ^d	6.5 (2 × 10 ⁵)	6.5 (1 × 10 ⁶)	6.5 (1 × 10 ⁵)	6.5 (9 × 10 ⁴)	6.5 (2 × 10 ⁵)	6.5 (7 × 10 ⁴)	6.5 (2 × 10 ⁵)	6.5 (2 × 10 ⁵)	6.5 (1 × 10 ⁵)
No plasmid	6.3 (2 × 10 ⁹)								
R ₁ ⁺ /M ₁ ⁺ , AbiC ⁺	5.5 (3 × 10 ⁷)	6.1 (6 × 10 ⁸)							
R ₂ ⁺ /M ₂ ⁺ , AbiA ⁺	5.4 (4 × 10 ⁵)	5.7 (2 × 10 ⁷)	5.6 (9 × 10 ⁷)	6.0 (1 × 10 ⁸)	6.1 (2 × 10 ⁸)				
R ₃ ⁺ /M ₃ ⁺ , Per31 ⁺	5.4 (4 × 10 ⁷)	6.3 (2 × 10 ⁸)							
Rotation series ^c	5.4 (3 × 10 ⁷)	5.5 (4 × 10 ⁶)	5.4 (5 × 10 ⁵)	5.6 (1 × 10 ⁷)	5.5 (3 × 10 ⁵)	5.3 (3 × 10 ⁵)	5.3 (2 × 10 ⁷)	5.3 (3 × 10 ⁴)	5.3 (2 × 10 ⁶)

^a The numbers in parentheses denote the titer of phage (PFU per milliliter on NCK203) in the whey from each sample.

^b SAT numbers indicate the number of sequential tests. For SAT 1, 10⁵ PFU each of 10 phages per ml was added to the cultures initially. For SATs 2 to 9, 10⁴ PFU each of 10 phages per ml and 100 µl of whey from the previous day's test was added to each strain.

^c The plasmids present are indicated.

^d —, the same phage mix as was added to the rotation strain was added to the control tube with milk only.

^e The following plasmids were used: SATs 1, 4, and 7, R₁⁺/M₁⁺, AbiC⁺; SATs 2, 5, and 8, R₂⁺/M₂⁺, AbiA⁺; SATs 3, 6, and 9, R₃⁺/M₃⁺, Per31⁺ (see Results).

of defense mechanisms, points of action, and genetic controls is developed.

In the prototype phage-resistant strain *L. lactis* subsp. *lactis* ME2, both *abiA* and *abiC* are present and are located on separate plasmids. Initially, we attempted to combine two distinct *abi* genes on a single plasmid for potential use in the starter rotation experiments. Two combinations were constructed on the basis of the high-copy-number vectors pTRKH3 (*abiA-abiC* combination) and pTRKH1 (*per31-per50* combination). In these combinations, AbiA and Per31 resistance remained at high levels, whereas the AbiC and Per50 resistance phenotypes were significantly lower than expected at a high copy number. Although many naturally phage-resistant strains contain plasmids encoding more than one type of phage resistance (12, 22), the only native plasmid characterized to date that encodes more than one Abi gene is pNP40 (10, 12).

Interestingly, the combination of pTRK68 (R₁⁺/M₁⁺) with Abi defenses seemed to have a slight but statistically significant effect on R/M effectiveness. First, ϕ 31 is not affected by AbiC, but the presence of pTRK371 in combination with pTRK68 raised the EOP for ϕ 31 approximately 1 log unit above the EOP for pTRK68 alone. Second, there was a slight but reproducible difference between the EOPs for ϕ 36 challenging NCK203(pTRK68) or NCK203(pTRK68, pTRK361) and challenging NCK203(pTRK68, pTRK375), even though Per31 normally has no effect on ϕ 36 (data not shown). In this case the vector on which *per31* is cloned seems to affect EOPs for the R/M system. These effects seen with pTRK68 were the only exception to the trend of predictable additivity when two functioning R/M and Abi defenses were combined.

The most obvious difference between the pTRKH family of vectors and pNZ18 is the method of plasmid replication. The pAM β 1 gram-positive origin of the pTRKH vectors replicates by unidirectional theta replication (25), while pNZ18 replicates by the rolling circle mechanism (6). For high-copy-number plasmids, the method of replication may affect growth in milk as well as increase the energy requirements for plasmid DNA replication. Both *abiC* and *per50* seem to be affected by the type of high-copy-number vector in which they are cloned. An *abiC*/pNZ18 plasmid was constructed but was not stable in NCK203, while *abiC*/pTRKH3 (pTRK371) and *per31*/pNZ18 (pTRK375) plasmids were stable (data not shown). In addition, the phage specificity of *per50* varied for *per50*/pNZ18 and *per50*/pTRKH1 (pTRK323) clones (data not shown).

The optimized rotation used in the SATs consists of three NCK203 derivatives in which the effects of different R/M and

Abi defenses are additive. The rotation was successfully continued for 9 days under challenge from a high-titer phage, even when the phage was added to each SAT along with whey from the previous SAT. The SAT is an accurate predictor of the longevities of starter cultures in cheese plants. In New Zealand, strains to be used as starters must survive five cycles of the SAT (18). Survival through 6 to 10 cycles correlates well with the performance of the culture over extended periods in the industry (11, 24). Potential sources of phages in cheese plants are raw and pasteurized milk, starters, whey, finished products, factory air, whey aerosols, and factory equipment (26). DNA homology studies performed by Jarvis (16, 17) indicated that the release of temperate phages was unlikely to be the predominant source of lytic phages in cheese plants and that lytic phages originate from only a few distinct phage types. Casey et al. (4) found that DNAs from four phages active against four cultures isolated over a 4-year period in a cheese plant did not hybridize with chromosomal DNAs from the starter strains. In contrast, we have recently discovered that host-encoded sequences do contribute to the appearance of new recombinant phages. A series of new recombinant phages was isolated after NCK203 containing high-copy-number Abi-encoding plasmids was challenged with phage ϕ 31 or ϕ 36 (28, 29). These recombinant sequences are currently being characterized in an attempt to determine if they originate from defective phages, noninducible or undetected NCK203 prophages, or bacterial DNA.

In the present study, one recombinant phage, ϕ 31.7, a variant of ϕ 31 (28a), was found in a whey sample in the ninth consecutive SAT when NCK203(pTRK375) was challenged continuously with ϕ 31. The recombinant phage is *per31* resistant. Nevertheless, plasmid pTRK375 was able to suppress domination of the culture by phage ϕ 31.7 through 17 consecutive SATs. This effect was likely due to the low frequency of appearance of ϕ 31.7, its lower burst size compared with that of ϕ 31, and its inability to propagate sufficiently on NCK203 bearing pTRK375.

In addition to recombinant phages, resistant phages expected to carry minor mutations are commonly isolated against Abi defenses (2, 9, 28b, 32). Also, phages escaping R/M systems are modified and no longer subject to restriction. The starter rotation system proposed here is designed to overcome all types of phage DNA modifications and mutations by the rotation of three derivatives of a single host strain, each of which presents any modified or recombinant phages with a new combination of R/M and Abi defenses. Since these are intra-

cellular defense systems, new virulent phages readily adsorb to the cell surface and inject their DNA. Once the genome has entered the cell, action by either the restriction or abortive system destroys or traps the phage and thus eliminates its genetic potential to contribute to new virulent types. Therefore, any new phages appearing in whey from the previous SAT's fermentation which are sensitive to the new defense combinations should be removed from the system. For example, in a successful rotation scheme (Table 3), it was noted that phage numbers present initially dropped dramatically, and new virulent phage did not appear or dominate the fermentation. The designed rotation system completed 9 days of consecutive tests when challenged continuously with high titers of phage lysates, both the ϕ 31-ul36 combination and a composite of 10 industrial phage isolates, as well as whey from the previous day's test. These results indicate that similarly designed systems could be successfully used in the dairy industry.

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REFERENCES

- Alatossava, T., and T. R. Klaenhammer. 1991. Molecular characterization of three small isometric-headed bacteriophages which vary in their sensitivity to the lactococcal phage resistance plasmid pTR2030. *Appl. Environ. Microbiol.* **57**:1346–1353.
- Bidnenko, E., C. Schouler, S. D. Ehrlich, and M.-C. Chopin. 1993. Identification of a phage DNA fragment conferring resistance to *abi-105* by complementation or recombination. *FEMS Microbiol. Rev.* **12**:p107.
- Casey, C. N., C. Daly, and G. F. Fitzgerald. 1992. Controlled integration into the *Lactococcus* chromosome of the pCI829-encoded abortive infection gene from *Lactococcus lactis* subsp. *lactis* UC811. *Appl. Environ. Microbiol.* **58**:3283–3291.
- Casey, C. N., E. Morgan, C. Daly, and G. F. Fitzgerald. 1993. Characterization and classification of virulent lactococcal bacteriophages isolated from a cheddar cheese plant. *J. Appl. Bacteriol.* **74**:268–275.
- Cogan, T. M., N. Peitersen, and R. L. Sellars. 1991. Starter systems, p. 16–23. In *Bulletin of the International Dairy Federation*, no. 263/1991. Practical phage control. International Dairy Federation, Brussels.
- De Vos, W. M. 1987. Gene cloning and expression in lactic streptococci. *FEMS Microbiol. Rev.* **46**:281–295.
- Dinsmore, P. K., and T. R. Klaenhammer. 1994. Phenotypic consequences of altering the copy number of *abiA*, a gene responsible for aborting bacteriophage infections in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **60**:1129–1136.
- Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *Escherichia coli* by high voltage electroporation. *Nucleic Acids Res.* **16**:6127–6145.
- Durmaz, E., D. L. Higgins, and T. R. Klaenhammer. 1992. Molecular characterization of a second abortive phage resistance gene present in *Lactococcus lactis* subsp. *lactis* ME2. *J. Bacteriol.* **174**:7463–7469.
- Garvey, P., G. F. Fitzgerald, and C. Hill. 1993. The conjugal plasmid pNP40 encodes multiple bacteriophage resistance mechanisms. *FEMS Microbiol. Rev.* **12**:p105.
- Heap, H. A., and R. C. Lawrence. 1976. The selection of starter strains for cheesemaking. *N. Z. J. Dairy Sci. Technol.* **11**:16–20.
- Hill, C. 1993. Bacteriophage and bacteriophage resistance in lactic acid bacteria. *FEMS Microbiol. Rev.* **12**:87–108.
- Hill, C., L. A. Miller, and T. R. Klaenhammer. 1990. Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis*. *J. Bacteriol.* **172**:6419–6426.
- Hill, C., K. Pierce, and T. R. Klaenhammer. 1989. The conjugative plasmid pTR2030 encodes two bacteriophage defense mechanisms in lactococci, restriction/modification (R/M) and abortive infection (Hsp). *Appl. Environ. Microbiol.* **55**:2416–2419.
- Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Construction and screening cDNA libraries in λ gt10 and λ gt11, p. 49–78. In D. M. Glover (ed.), *DNA cloning*, vol. I. IRL Press Ltd., Oxford.
- Jarvis, A. W. 1984. DNA-DNA homology between lactic streptococci and their temperate and lytic phages. *Appl. Environ. Microbiol.* **47**:1031–1038.
- Jarvis, A. W. 1987. Sources of lactic streptococcal phages in cheese plants. *N. Z. J. Dairy Sci. Technol.* **22**:93–103.
- Jarvis, A. W., H. A. Heap, and G. K. Y. Limsowtin. 1989. Resistance against industrial bacteriophages conferred on lactococci by plasmid pAJ1106 and related plasmids. *Appl. Environ. Microbiol.* **55**:1537–1543.
- Jarvis, A. W., and T. R. Klaenhammer. 1986. Bacteriophage resistance conferred on lactic streptococci by the conjugative plasmid pTR2030: effects on small isometric-, large isometric-, and prolate-headed phages. *Appl. Environ. Microbiol.* **51**:1272–1277.
- Klaenhammer, T. R. 1989. Genetic characterization of multiple mechanisms of phage defense from a prototype phage-insensitive strain, *Lactococcus lactis* ME2. *J. Dairy Sci.* **72**:3429–3443.
- Klaenhammer, T. R. 1991. Development of bacteriophage resistant strains of lactic acid bacteria. *Biochem. Soc. Trans.* **19**:675–681.
- Klaenhammer, T. R., and G. F. Fitzgerald. 1994. Bacteriophages and bacteriophage resistance, p. 106–168. In M. J. Gasson and W. de Vos (ed.), *Applied genetics of lactic acid bacteria*. Backie and Son, Ltd., Glasgow, United Kingdom.
- Klaenhammer, T. R., D. Romero, W. Sing, and C. Hill. 1991. Molecular analysis of pTR2030 gene systems that confer bacteriophage resistance to lactococci, p. 124–130. In G. M. Dunne, P. P. Cleary, and L. L. McKay (ed.), *Genetics and molecular biology of streptococci, lactococci, and enterococci*. American Society for Microbiology, Washington, D.C.
- Lawrence, R. C., H. A. Heap, and J. Gilles. 1984. A controlled approach to cheese technology. *J. Dairy Sci.* **67**:1632–1645.
- Le Chatelier, E., S. D. Ehrlich, and L. Janniere. 1993. Biochemical and genetic analysis of the unidirectional theta replication of the *S. agalactiae* plasmid pIP501. *Plasmid* **29**:50–56.
- Lodics, T. A., and L. R. Steenson. 1990. Characterization of bacteriophages and bacteria indigenous to a mixed-strain cheese starter. *J. Dairy Sci.* **73**:2685–2696.
- Moineau, S., J. Fortier, H. W. Ackermann, and S. Pandian. 1992. Characterization of lactococcal bacteriophages in Quebec cheese plants. *Can. J. Microbiol.* **38**:875–882.
- Moineau, S., S. Pandian, and T. R. Klaenhammer. 1994. Evolution of a lytic bacteriophage via DNA acquisition from the *Lactococcus lactis* chromosome. *Appl. Environ. Microbiol.* **60**:1832–1841.
- O'Sullivan, D. Personal communication.
- O'Sullivan, D., and P. K. Dinsmore. Personal communication.
- O'Sullivan, D. J., C. Hill, and T. R. Klaenhammer. 1993. Effect of increasing the copy number of bacteriophage origins of replication, in *trans*, on incoming-phage proliferation. *Appl. Environ. Microbiol.* **59**:2449–2456.
- O'Sullivan, D. J., and T. R. Klaenhammer. 1993. Rapid miniprep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. *Appl. Environ. Microbiol.* **59**:2730–2733.
- O'Sullivan, D. J., and T. R. Klaenhammer. 1993. High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* **137**:227–231.
- Parreira, R., R. Vallyasevi, S. D. Ehrlich, and M.-C. Chopin. 1993. Identification of a phage DNA fragment conferring resistance to *abi-416* by complementation. *FEMS Microbiol. Rev.* **12**:107.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sanders, M. E. 1988. Phage resistance in lactic acid bacteria. *Biochimie* **70**:411–421.
- Sing, W. D., and T. R. Klaenhammer. 1991. Characterization of restriction and modification plasmids from *Lactococcus lactis* subsp. *cremoris* and their effects when combined with pTR2030. *J. Dairy Sci.* **74**:1133–1144.
- Sing, W. D., and T. R. Klaenhammer. 1993. A strategy for rotation of different bacteriophage defenses in a lactococcal single-strain starter culture system. *Appl. Environ. Microbiol.* **59**:365–372.
- Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**:807–813.
- Thunell, R. K., and W. E. Sandine. 1985. Types of starter cultures, p. 127–144. In S. E. Gilland (ed.), *Bacterial starter cultures for foods*. CRC Press, Boca Raton, Fla.
- Thunell, R. K., W. E. Sandine, and F. W. Bodyfelt. 1981. Phage-insensitive, multiple-strain starter approach to cheddar cheese making. *J. Dairy Sci.* **64**:2270–2277.
- van de Guchte, M., J. M. B. M. van der Vossen, J. Kok, and G. Venema. 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* **55**:224–228.