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The Plasmid Complement of *Lactococcus lactis* UC509.9 Encodes Multiple Bacteriophage Resistance Systems

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***Lactococcus lactis* subsp. *cremoris* strains are used globally for the production of fermented dairy products, particularly hard cheeses. Believed to be of plant origin, *L. lactis* strains that are used as starter cultures have undergone extensive adaptation to the dairy environment, partially through the acquisition of extrachromosomal DNA in the form of plasmids that specify technologically important phenotypic traits. Here, we present a detailed analysis of the eight plasmids of *L. lactis* UC509.9, an Irish dairy starter strain. Key industrial phenotypes were mapped, and genes that are typically associated with lactococcal plasmids were identified. Four distinct, plasmid-borne bacteriophage resistance systems were identified, including two abortive infection systems, AbiB and AbiD1, thereby supporting the observed phage resistance of *L. lactis* UC509.9. AbiB escape mutants were generated for phage sk1, which were found to carry mutations in *orf6*, which encodes the major capsid protein of this phage.**

Lactococcus lactis strains have been exploited for millennia for the fermentation of dairy products. In modern fermentations, the species is used mostly in defined starter mixes to produce fermented dairy products with consistent organoleptic properties (1).

It is widely believed that the original niche of *L. lactis* strains is plant based (2). Adaptation to the nutrient-rich dairy environment is reflected in the genomes of industrially exploited strains. Reduced genome sizes, in addition to a higher number of pseudogenes and transposase-encoding genes, highlight the extent of genome decay among industrial lactococcal genomes compared to their plant-associated brethren (2–4). The process of adaptation to the dairy environment via genome decay was recently experimentally demonstrated by monitoring sequence changes in the chromosome of a lactococcal plant isolate used in a dairy fermentation (2). Reductive evolution appears to be especially pronounced for *L. lactis* subsp. *cremoris* strains, which tend to have smaller genomes than *L. lactis* subsp. *lactis* strains (5) and which are almost exclusively found in dairy fermentation environments, with rare reports of their isolation from plant material (5–7).

Another feature of industrial lactococcal isolates is their extensive plasmid complement (8). Important dairy-associated phenotypes, such as lactose utilization and casein hydrolysis, have long been known to be carried on plasmids (9). A study of 150 *L. lactis* dairy strains from New Zealand showed that they all possessed a substantial number of plasmids, typically between 6 and 14 (5). The recent availability of the entire extrachromosomal sequence data for several strains has revealed multiple large plasmids (up to 80 kb) that carry genes for a diverse range of functions, including plasmid conjugation and mobilization, exopolysaccharide (EPS) production, bacteriophage resistance, heavy metal resistance, and citrate utilization (10–13).

Bacteriophages that infect *L. lactis* have been extensively studied due to their associated negative impact on dairy fermentations (14). Owing to their application potential, lactococcal phage resistance systems are among the most intensely characterized antiphage systems (15). Restriction/modification (R/M) and abortive infection (Abi) systems appear to be common bacteriophage resistance mechanisms within *L. lactis* and are frequently carried on plasmids (16). In contrast, CRISPR-cas systems appear to be very rare in *L. lactis*, with the only known representative being carried on a plasmid (17).

While individual modes of action remain unknown for many Abi systems, their defining feature is preventing phage proliferation by interfering with some critical aspect of the phage lytic cycle (15, 16). Their mode of action not only impacts phage proliferation, but also leads to the death of the host. This limits the number of progeny produced, thereby protecting the wider bacterial population. There are currently over 20 described lactococcal Abi systems, which affect different stages of phage multiplication, such as major capsid protein production (in the case of AbiC) (18), DNA replication (for AbiA) (19), and transcription (in the case of AbiG) (20). Since these Abi proteins display low (if any at all) levels of similarity to other proteins, their mode of action is usually difficult to predict. Genome sequencing of bacteriophage Abi escape mutants has led to the identification of the molecular triggers, modes of action, and possible interaction sites for several Abi proteins, such as AbiD1 (21), AbiQ (22), AbiT (23), and AbiV (24).

L. lactis subsp. *cremoris* UC509 is an Irish industrial starter strain isolated in the 1980s from a mixed starter culture (25). It is the lysogenic host to the well-studied lactococcal P335 group phage Tuc2009 (26–28). Recently, the genome and entire plasmid complement of its Tuc2009-cured derivative, *L. lactis* UC509.9, were sequenced (4). Here, we provide a detailed analysis of the eight plasmids of *L. lactis* UC509.9 and its plasmid-carried bacteriophage resistance systems.

MATERIALS AND METHODS

Strains used in this study and growth conditions. Bacterial strains used in this study are listed in Table 1. *L. lactis* strains were grown in M17 broth or agar (Oxoid, United Kingdom), supplemented with 5 g/liter glucose

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TABLE 1 Strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or phage	Relevant feature(s)	Reference(s) or source
Lactococcus lactis subsp. *cremoris* strains		
UC509.9	Harbors plasmids pCIS1 to -8, host to bacteriophages Tuc2009 and c2	4, 25
UC509.9S1	*L. lactis* UC509.9 derivative cured of pCIS3 and pCIS4	This work
UC509.9S2	*L. lactis* UC509.9 derivative cured of pCIS1, pCIS3, and pCIS4	This work
NZ9000	*L. lactis* MG1363 derivative containing *nisRK*; host to sk1, jj50, 712, c2	29
158	Alternative host to bacteriophage Tuc2009	64
NZ7000	Nisin-producing *L. lactis* strain	29
Escherichia coli One Shot TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ* ΔM15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pPTPL	*E. coli-L. lactis* promoter-probe vector, Tet^r	34, 35
pPTPL*abiD1*$_{UC509.9}$	pPTPL containing *abiD* from pCIS8	This work
pPTPi	*E. coli-L. lactis* shuttle vector, P*nisA*, Tet^r	35
pPTPi*abiB*$_{UC509.9}$	pPTPi containing *abiB* from pCIS8	This work
Bacteriophages		
Tuc2009	P335 species, propagated on 158	26
c2	c2 species, propagated on NZ9000	65
sk1	936 species, propagated on NZ9000	61
jj50	936 species, propagated on NZ9000	66
712	936 species, propagated on NZ9000	66
SK1833-3	sk1 derivative, AbiB escape mutant	This work
SK1833-4	sk1 derivative, AbiB escape mutant	This work
SK1833-5	sk1 derivative, AbiB escape mutant	This work
SK1833-6	sk1 derivative, AbiB escape mutant	This work
SK1833-7	sk1 derivative, AbiB escape mutant	This work
SK1833-8	sk1 derivative, AbiB escape mutant	This work

and incubated overnight at 30°C. Where necessary, tetracycline (Sigma, United Kingdom) was added to growth media at a concentration of 5 μg/ml. For induction of genes that were placed under the transcriptional control of a nisin-inducible promoter (see below), growth medium was supplemented with a 1:2,000 dilution of the cell-free supernatant of the nisin-producing strain *L. lactis* NZ9700 (29).

Bacteriophage assays. Bacteriophages used in this study are listed in Table 1. Bacteriophages were propagated on their respective host strains as previously described, and lysates were maintained at 4°C (30). Spot assays and plaque assays were performed using the overlay method (31). Center-of-infection assays and one-step growth curves were performed in triplicate as previously described (22).

Cloning. Construction of all plasmids was performed in *Escherichia coli* One Shot TOP 10 (Invitrogen). All primers were ordered from Eurofins MWG (Ebersberg, Germany). The predicted *abi* genes, *abiB*$_{UC509.9}$ (*uc509_p8051*) and *abiD1*$_{UC509.9}$ (*uc509_p80062*), identified from the *L. lactis* UC509.9 plasmid complement, were amplified using KOD DNA polymerase (Invitrogen). Primers for amplification of *abiB*$_{UC509.9}$ and *abiD1*$_{UC509.9}$ (Table 1) contained BamHI (forward primer) and SphI (reverse primer) restriction sites to allow insertion into the low-copy-number, nisin-inducible vector pPTPi (to generate plasmid pPTPi*abiB*$_{UC509.9}$) or plasmid pPTPL (to generate plasmid pPTPL*abiD*$_{UC509.9}$), respectively (Table 1) (32, 33). The generated recombinant plasmids were then introduced into *L. lactis* NZ9000 by electrotransformation.

Plasmid DNA isolation. Plasmid DNA was isolated using the GeneJET plasmid prep kit (Thermo Scientific, Ireland) with the following modifications for isolation from *L. lactis*. Cells from a 10-ml overnight culture were harvested (10 min, 3,600 relative centrifugal force [RCF]), resuspended in 250 μl of protoplast buffer (20 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.75 M sucrose, 10 mg/ml lysozyme [Sigma, United Kingdom], 50 U/ml mutanolysin [Sigma, United Kingdom]), and incubated for 30 min at 37°C. Treated cells were harvested at 3,600 RCF for 10 min, the supernatant was discarded, and the procedure was continued as recommended by the GeneJET kit manufacturer. DNA was visualized via UV transillumination of 0.6% agarose gels stained with ethidium bromide.

Plasmid curing. Protoplast-induced plasmid curing (34) was carried out as follows: 20-ml cell cultures (optical density at 600 nm, ~0.35) were harvested by centrifugation (3,600 RCF, 10 min) and washed once in sterile, deionized water. The pellet was resuspended in 5 ml of SGM17 (GM17 plus 0.3 M sucrose), lysozyme was added to a final concentration of 10 mg/ml, and the mixture was incubated for 30 min at 37°C. The cells were harvested by centrifugation at 3,600 RCF for 10 min and washed twice in SGM17. The final pellet was resuspended in 1 ml SGM17, and serial dilutions were plated on SGM17 agar plates. Plates were incubated for 48 h at 30°C. Individual colonies were then picked and screened for plasmid loss by plasmid DNA isolation and subsequent agarose gel electrophoresis as described above. Confirmation of plasmid curing was performed by PCR analysis, using plasmid-specific primers (primer combinations pcis1F [GATATTCCATTTATTCGTTCTG] and pcis1R [AATTTCCTTGTCCACCTTG] to detect plasmid pCIS1; pcis3F [CAAGCCCTAGACCAATTCAG] and pcis3R [GACTCCCAGGTTGTCCA] to detect plasmid pCIS3; pcis4F [CAGAAACTTGGCTTGGATAG] and pcis4R [ATGGCCCGTACTGGATCG] to detect pCIS4. Plasmid-curing PCR confirmation conditions were 95°C for 10 min followed by 30 cycles of 95°C for 15 s, 51°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 7 min.

Isolation and genome sequencing strategy for AbiB escape mutants. To isolate AbiB$_{UC509.9}$ escape mutants of phage sk1, a 1×10^8 PFU per ml sk1 lysate (propagated on *L. lactis* NZ9000) was used to challenge nisin-induced *L. lactis* NZ9000 harboring pPTPi*abiB*$_{UC509.9}$ by plaque assay. Plaques with a normal appearance (i.e., 2 mm in size and clear, as opposed to pin-point and hazy) were isolated from the resulting overnight lawns,

and phages from these plaques (representing presumed sk1-derived Abi-$B_{UC509.9}$ escape mutants) were propagated on cultures of nisin-induced *L. lactis* NZ9000 pPTPi*abiB*$_{UC509.9}$ until complete lysis had occurred. This procedure was repeated three times for each of the six independently isolated mutants (Table 1).

Genome sequencing of one of the Abi$B_{UC509.9}$ escape mutants of sk1, named SK1833, was performed by using a Sanger sequencing approach (performed by Eurofins MWG, Ebersberg, Germany), employing the bacteriophage sk1 genome sequence (accession number NC_001835.1) for primer design and overlapping, PCR-generated sections of the SK1833 genome as templates. All PCR products were generated using high-fidelity KOD DNA polymerase (Invitrogen), and each amplified region was sequenced at least twice from two independent PCRs. Reads were assembled and mutations identified by using the SeqMan package (version 5.0; DNAStar Lasergene). Mutations were confirmed by sequencing the same region of the predecessor sk1 phage.

Bioinformatic analysis. The nucleotide sequences of the plasmids of *L. lactis* UC509.9 were accessed through GenBank (accession numbers CP003165 [pCIS1], CP003164 [pCIS2], CP003163 [pCIS3], CP003162 [pCIS4], CP003161 [pCIS5], CP003160 [pCIS6], CP003159 [pCIS7], and CP003158 [pCIS8]) (4). Putative open reading frames (ORFs) were identified by using Prodigal version 2.0 ORF prediction (35) and BLASTP (36), and the resulting identified ORFs were inspected by using Artemis (37) with manual checking and editing. Domain searches and functional searches of each ORF were performed using the Pfam (38), KEGG (39), and COG databases (40).

Nucleotide sequence accession number. The genome sequence of SK1833 was submitted to GenBank and assigned accession number KF676640.

RESULTS

General plasmid features. *L. lactis* UC509.9 was previously shown to contain eight plasmids, named pCIS1 to pCIS8, ranging in size from 4.3 kb to over 80.5 kb and representing the largest lactococcal plasmid complement sequenced to date, with pCIS8 representing the largest lactococcal plasmid sequenced to date (4). The 6.1-kb plasmid of *L. lactis* UC509.9, named pCIS3, had previously been sequenced and characterized, revealing that it carries a magnesium transporter and a type I restriction-modification HsdS specificity subunit (41). Total G+C content ranged between 30.1% and 38.5%, which is similar to the GC content of the *L. lactis* UC509.9 genome (35.8%) and other lactococcal plasmids (8). The total plasmid content represents 202,302 bp of extrachromosomal DNA harboring 193 ORFs, 127 of which can be assigned a putative function, while 25 appear to harbor mutations that render such ORFs pseudogenes. In terms of the total genome (chromosome and plasmids), the plasmid complement represents 9% of total genomic DNA (and 8.5% of the total number of protein-encoding genes).

The majority of these ORFs display high homology with genes located on other lactococcal plasmids or chromosomes. The identified ORFs on plasmids pCIS2 and pCIS4 encode, aside from replication functions (see below), hypothetical proteins with no assignable phenotypic traits. Functions frequently identified on other lactococcal plasmids, such as those encoding EPS production, cold shock proteins, and heavy metal transport (8), are absent from the *L. lactis* UC509.9 plasmid complement. No single plasmid appears to specify a complete set of proteins required for conjugation or mobilization, suggesting that none of the *L. lactis* UC509.9 plasmids is (self-)transmissible.

All *L. lactis* UC509.9 plasmids are predicted to replicate via a theta-type replication mechanism, based on sequence similarity with known theta-type replicons (10, 42). Ten highly homologous theta-type replication protein-encoding genes with associated origins of replication were detected on the eight plasmids, with three such replication functions carried by the biggest plasmid, pCIS8, suggesting that pCIS8 is the result of cointegration of three smaller plasmids. Eight of the replicons belong to the Rep_3 superfamily (PF01051) and RepB_C (PF06430) pfam families and possess characteristic 2.5 22-bp sequence repeats located upstream of the corresponding start codon of the replication protein-encoding gene, whereas the remaining two replicons (represented on pCIS4 and pCIS7) belong exclusively to the Rep_3 superfamily, where the replication protein-encoding gene is preceded by 5 22-bp sequence repeats.

Important technological phenotypes. Many traits are desirable for *L. lactis* starter strains to proliferate in the dairy environment, several of which have been described previously (8). Table 2 describes identified *L. lactis* UC509.9 plasmid-carried properties with industrial significance.

Two essential phenotypes are required for rapid growth in milk by *L. lactis* strains: lactose metabolism and casein metabolism (43). The full set of genes that are predicted to specify the lactose-specific phosphoenolpyruvate-phosphotransferase system (PEP-PTS), i.e., *lacABCDFEGX*, and the putative divergently transcribed repressor-carrying gene, *lacR*, are located on the largest plasmid, pCIS8. The ability to degrade and metabolize casein is dependent on a cell wall-associated protease and an oligopeptide transport system (44). Similar to other lactococcal plasmid complements (10), this ability is carried across two plasmids, pCIS6 and pCIS8, which harbor genes for the oligopeptide permease (Opp) (45) and the proteinase/maturase system (PrtP/M) (46), respectively.

Flavor development and cheese ripening are dependent on various intracellular enzymes, such as peptidases and amino acid decarboxylases, acting upon casein degradation products once they have been transported into the cell by the oligopeptide system (47). Multiple putative peptidases are carried on the *L. lactis* UC509.9 plasmid complement. In addition to the chromosomally located *pepF*, a second copy of this gene, which encodes oligoendopeptidase F, is located on pCIS8 (*uc509_p8028*). Genes for proteins with peptidase family S51 and dipeptidase E domains are located on pCIS8 (*uc509_p8069*) and pCIS6 (*uc509_p6010*), respectively. Also carried on pCIS6 is a predicted pyroglutamyl-peptidase (*uc509_p6011*) that is responsible for the removal of pyroglutamate from the N termini of peptides.

Bacteriocins are small, ribosomally synthesized antimicrobial peptides produced by lactic acid bacteria (48). Interestingly, the sequence of pCIS7 reveals the presence of two clusters that specify two putative bacteriocins and their associated export and immunity functions; however, only one cluster (*uc509_p7029* to *uc509_p7033*) appears to be intact, since the second cluster (*uc509_p7038* to *uc509_p7042*) contains a bacteriocin-processing protein gene, *lcnC* (*uc509_p7042*), in which an internal stop codon is present, thus representing a pseudogene. The intact cluster contains ORFs with homology to the lactococcin A (LcnA)-specifying gene cluster present in *L. lactis* WM4 (49). This is comprised of four genes, which govern bacteriocin synthesis (*lcnA*), processing and secretion (*lcnCD*), and immunity (*lciA*). Consistent with the above findings, *L. lactis* UC509.9 was shown to produce at least one protease-sensitive antibacterial activity, which was effective against the bacteriocin-sensitive indicator strain, *L.*

TABLE 2 Important dairy phenotypes encoded by the plasmid complement of *L. lactis* UC509.9[a]

Plasmid	Gene(s)	Product(s)	Function
pCIS1	*hsdS*	Type I restriction enzyme specificity subunit	Bacteriophage resistance
pCIS3	*hsdS*	Type I restriction enzyme specificity subunit	Bacteriophage resistance
	corA	CorA family magnesium and cobalt transporter	Increased Mg^{2+} uptake
pCIS5	*uc509_p5006*	2-Dehydropantoate 2-reductase	
pCIS6	*uc509_p6010*	Peptidase E	Degradation of casein products
	uc509_p6011	Pyrrolidone-carboxylate peptidase	Degradation of casein products
	corA	CorA family magnesium and cobalt transporter	Increased Mg^{2+} uptake
	dld	D-Lactate dehydrogenase	ATP conversion
	uc509_p6021	Pyridine nucleotide-disulfide oxidoreductase	
	prtP/M	Cell envelope proteinase and maturase	Degradation of casein
	uc509_p6021[b]	Glycosylhydrolase	Oligosaccharide degradation
	uc509_p6030	Major facilitator family transporter	ATP generation
pCIS7	*umuC(1)*	Low-fidelity polymerase	Stress response
	uc509_p7009/10	*para*-Aminobenzoate synthase component I/II	Folate precursor biosynthesis
	umuC(2)	Low-fidelity polymerase	Stress response
	uc509_p7029-33	Lactococcin A biosynthesis and transport	Bacteriocin production
pCIS8	*mntH*	Mn^{2+}/Fe^{2+} transporter	Increased Mn^{2+} uptake
	uspA	Universal stress protein	Stress response
	lacABCDFEGX	Lactose PEP-PTS utilization operon	Lactose fermentation
	pepF	Oligopeptidase F	Degradation of casein products
	oppOACBFD	Oligopeptide uptake	Degradation of casein products
	aldC	α-Acetolactate decarboxylase	α-Acetolactate degradation
	abiB	Abortive infection protein B	Bacteriophage resistance
	uc509_p8059[a]	CHW repeat/cell adhesion domain peptidase	Degradation of casein products
	abiD1	Abortive infection protein D1	Bacteriophage resistance
	uc509_p8069	Peptidase E	Degradation of casein products
	uc509_p8086	BRCT domain protein	Stress response

[a] Plasmids pCIS2 and pCIS4 carry genes mostly harboring hypothetical proteins or pseudogenes and therefore were not included in the table.
[b] Not previously associated with *L. lactis* plasmids.

lactis HP (data not shown). In addition, pCIS8 also harbors a gene for a putative EntA_Immun (PF08951) family protein that is thought to confer broad range class II bacteriocin immunity (50).

Characterization of bacteriophage resistance in *L. lactis* UC509.9. *L. lactis* UC509.9 is a highly bacteriophage-resistant strain that is sensitive to infection by bacteriophages Tuc2009 and c2 while being insensitive to all tested 936 group phages currently in our collection (51). The observed bacteriophage resistance may be partly due to plasmid-harbored bacteriophage restriction systems. Based on similarity searches, we identified four bacteriophage resistance systems on three *L. lactis* UC509.9 plasmids (see below). These resistance systems are present in addition to two predicted chromosomally encoded phage resistance systems, representing a predicted, yet undefined type I R/M system and the type II ScrFI (100% amino acid identity [52]) R/M system. Several experiments were performed in order to define the contribution of the plasmid-carried systems to the overall phage resistance of *L. lactis* UC509.9.

Stacking of type I R/M systems. Two genes encoding predicted HsdS specificity subunits of type I restriction-modification systems are located on pCIS1 and pCIS3, the latter of which has previously been studied in detail (41). Type I restriction systems consist of a HsdR restriction subunit, HsdM methylation subunit and a HsdS specificity subunit, all three of which are chromosomally-encoded in *L. lactis* UC509.9 as three adjacent genes (*uc509_0476* to *uc509_0478*). The holoenzyme of such a Type I R/M system consists of a heterooligomer of HsdM and HsdR subunits, and may utilize different HsdS subunits to broaden its specificity and phage-resistance efficiency (41, 53, 54).

To determine the effect of (loss of) multiple type I specificity subunits on bacteriophage efficiency of plaquing (EOP), plasmid curing by protoplast formation was undertaken to generate strains lacking one or both plasmid-carrried *hsdS* genes (Fig. 1). Confirmation of plasmid loss was performed by plasmid-specific PCR (data not shown). These derivatives were then challenged with bacteriophage Tuc2009 or c2, which had been propagated on *L. lactis* 158 and *L. lactis* NZ9000, respectively, to remove DNA methylation, which would render the bacteriophages insensitive to *L. lactis* UC509.9 R/M systems. The obtained results (Table 3) showed that the loss of pCIS3 (*L. lactis* UC509.9S1) leads to a 1,000-fold increase in the EOP for Tuc2009 and a modest 10-fold increase in the EOP for c2, compared to the EOP on *L. lactis* UC509.9 harboring the complete plasmid complement. The combined effect of the loss of both plasmids/*hsdS* genes (*L. lactis* UC509.9S2) further increased the c2 E.O.P. by 100-fold (i.e., the EOP was 1,000-fold higher than that obtained for *L. lactis* UC509.9), whereas the combined effect on Tuc2009 was minimal (Table 3). Additionally, pCIS4 was lost from both *L. lactis* UC509.9S1 and UC509.9S2 during plasmid curing (Fig. 1). The loss of pCIS4 does not appear to have any effect on Tuc2009 or C2

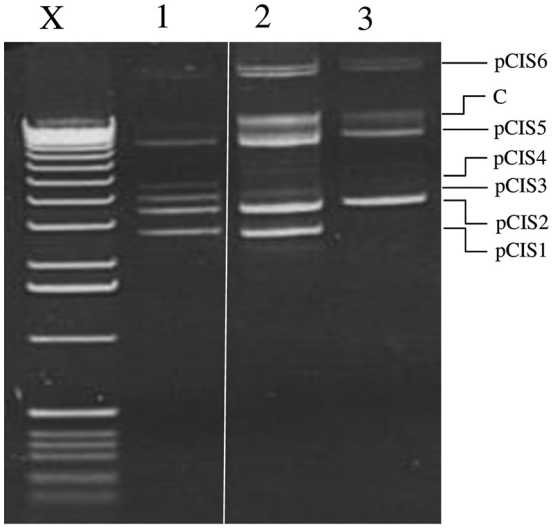

FIG 1 Plasmid profiles of *L. lactis* UC509.9 and its plasmid-cured derivatives. X, molecular weight marker X (Roche, Germany); lane 1, UC509.9; lane 2, UC509.9S1 (cured of pCIS3 and pCIS4); lane 3, UC509.9S2 (cured of pCIS1, pCIS3, and pCIS4). C, chromosomal DNA.

infection (data not shown), which is consistent with the observation that pCIS4 does not carry any obvious bacteriophage resistance mechanisms. Confirmation of failure to cure the larger plasmids, pCIS7 and pCIS8, which are not visible by means of standard agarose gel electrophoresis (Fig. 1), was determined with pCIS7- and pCIS8-specific PCR primers (data not shown).

***L. lactis* UC509.9 abortive infection systems.** The largest *L. lactis* UC509.9 plasmid, pCIS8, harbors clear homologs of *abiD1* (21) and *abiB* (55) (whose protein products exhibit 58 and 94% amino acid identity, respectively); for this reason, they are designated $abiD1_{UC509.9}$ and $abiB_{UC509.9}$, respectively. As *L. lactis* UC509.9 is not susceptible to 936 group bacteriophages of our collection, which is possibly (and partially) due to the presence of these Abi systems, we attempted to cure pCIS8 from *L. lactis* UC509.9. However, this could not be achieved. Therefore, we employed an alternative strategy whereby the individual *abi* genes were cloned and expressed in the 936 group-sensitive host *L. lactis* NZ9000 to determine their effectiveness.

The $abiD1_{UC509.9}$ gene and its associated upstream region, previously shown to be important for the AbiD abortive infection phenotype (56), were cloned into the low-copy-number vector pPTPL to generate the plasmid pPTPL$abiD1_{uc509.9}$. *L. lactis* NZ9000 harboring pPTPL$abiD1_{uc509.9}$ was challenged with 936 group bacteriophages and was found to provide very moderate, but noticeable and reproducible, resistance against sk1 and 712 (Table 4).

TABLE 3 EOP values for *L. lactis* 158-propagated Tuc2009 and *L. lactis* NZ9000-propagated c2 on *L. lactis* UC509.9 (two plasmid-carried copies of *hsdS*) and plasmid-cured derivatives UC509.9S1 (one plasmid-carried copy of *hsdS*) and UC509.9S2 (no plasmid-carried *hsdS*)[a]

Strain	EOP	
	Tuc2009	c2
NZ9000	NA	1 ± 0.2
158	1 ± 0.1	NA
UC509.9	$(4.1 \pm 0.1) \times 10^{-4}$	$(1.7 \pm 0.6) \times 10^{-5}$
UC509.9S1	0.1 ± 0.6	$(1.3 \pm 0.6) \times 10^{-4}$
UC509.9S2	0.9 ± 0.2	$(6.8 \pm 0.2) \times 10^{-3}$

[a] Results are means ± standard errors and are representative of three independent repeat experiments. NA, not applicable.

TABLE 4 EOP values for various 936 bacteriophages on *L. lactis* NZ9000 harboring pPTPL or pPTPL$abiD1_{UC509.9}$[a]

Phage	pPTPL		pPTPL$abiD1_{UC509.9}$	
	EOP on NZ9000	Plaque size (mm)	EOP on NZ9000	Plaque size (mm)
sk1	1 ± 0.1	1.5 ± 0.6	0.65 ± 0.3	≤0.2
712	1 ± 0.1	1.5 ± 0.5	0.74 ± 0.1	≤0.2
c2	1 ± 0.1	3 ± 0.6	0.65 ± 0.1	2 ± 0.5

[a] Results are means ± standard errors and are representative of three independent repeat experiments.

Transcription of *abiB* is dependent on a constitutive promoter (57), while overexpression of AbiB from a high-copy-number plasmid has previously been described as toxic for *L. lactis* (16). To avoid similar problems with uncontrolled expression of $AbiB_{UC509.9}$, the corresponding gene was cloned into the low-copy-number, nisin-inducible vector pPTPi. Nisin-induced transcription of $abiB_{UC509.9}$ from pPTPi did not appear to cause any bacteriostatic effect (data not shown). *L. lactis* NZ9000 expressing $AbiB_{UC509.9}$ was challenged with 936 group phages (Table 1). The $AbiB_{UC509.9}$ system was shown to be highly effective at interfering with sk1 and jj50 proliferation (Table 5), reducing the EOP by approximately 10^5-fold. Curiously, 936 group bacteriophage 712 appears unaffected by $AbiB_{UC509.9}$.

Characterization of an $AbiB_{UC509.9}$ escape mutant. Six sk1 escape mutants resistant to $AbiB_{UC509.9}$ were randomly isolated and propagated in a host that expresses $AbiB_{UC509.9}$. All six mutants exhibited near-complete insensitivity to $AbiB_{UC509.9}$ (Table 5).

One escape mutant, SK1833, was further examined to assess its ability to circumvent the action of $AbiB_{UC509.9}$. One-step growth curves were performed, and the number of effective centers of infection of SK1833 was calculated on *L. lactis* NZ9000 cells with and without the nisin-induced expression of $AbiB_{UC509.9}$. The burst size of sk1 on *L. lactis* NZ9000 harboring pPTPi$AbiB_{UC509}$ without induction and thus not expressing $AbiB_{UC509.9}$ was 153 ± 16 virions (mean ± standard error), with an associated latent period of 40 ± 1 min. Similar results were obtained for escape mutant SK1833, with a burst size of 192 ± 17 and a latent period of 41 ± 3 min. Due to aborted infection, the burst size and latent period of sk1 on *L. lactis* NZ9000 expressing $AbiB_{UC509.9}$ could not be measured. SK1833, on the other hand, was capable of replication on *L. lactis* NZ9000 expressing $AbiB_{UC509.9}$, though with a somewhat reduced burst size of 95 ± 18 and a latent period of 42 ± 1 min. The number of effective centers of infection was also determined for SK1833: in the presence of $AbiB_{UC509.9}$, the majority of infected cells released virions (80% ± 28%), whereas this number was determined to be 100% (± 10%) in the absence of $AbiB_{UC509.9}$. These results suggest that mutations obtained by SK1833 as a result of a selective pressure to bypass the antiphage activities of $AbiB_{UC509.9}$-expressing cells have a relatively small impact on bacteriophage fitness.

To determine the underlying mutations incurred by the $AbiB_{UC509.9}$ escape mutant, the complete genome of SK1833 was

TABLE 5 EOP values and associated mutations of sk1 and sk1-derived $AbiB_{UC509.9}$ escape mutants on *L. lactis* NZ9000 cells with or without nisin-induced expression of $AbiB_{UC509.9}$

Phage[a]	EOP on NZ9000 pPTPi*abiB*$_{UC509.9}$		Mutation			
	Uninduced	Induced	Gene	Reference position[b]	Nucleotide change(s)	Substitution(s)
sk1	1 ± 0.2	(1.8 ± 0.2) × 10^{-5}	NA	NA	NA	NA
SK1833	1 ± 0.2	0.8 ± 0.2	*orf6*	4670	C → T	L110F
			orf6	4729	T → C	Synonymous
SK1834	1 ± 0.2	0.9 ± 0.1	*orf6*	4587	C → T	S82L
			orf6	4703	G → A	T121A
SK1835	1 ± 0.11	0.91 ± 0.1	*orf6*	4604	G → A	E88K
SK1836	1 ± 0.1	1 ± 0.2	*orf6*	4604	G → A	E88K
			orf6	4712	C → A	L124I
SK1837	1 ± 0.2	0.96 ± 0.1	*orf6*	4604	G → A	E88K
			orf6	4734	C → T	S131L
SK1838	1 ± 0.3	0.6 ± 0.3	*orf6*	4587	C → T	S82L
712	1 ± 0.1	1 ± 0.1	NA	NA	NA	NA
c2	1 ± 0.1	0.1 ± 0.2	NA	NA	NA	NA

[a] Bold type denotes a phage whose whole genome was determined. The EOP results are means ± standard errors and are representative of three independent repeat experiments. NA, not applicable.
[b] Nucleotide position, based on the sk1 reference genome (accession number NC_001835.1).

sequenced and compared to that of sk1, from which it was derived. Sequencing revealed two single-nucleotide changes in *orf6*, which encodes the major capsid protein (23) (Table 5). One of the mutations was synonymous, and therefore the amino acid sequence was unaffected. Sanger sequencing of *orf6* of sk1 confirmed that these mutations were not present in the ancestor phage. Sequencing of *orf6* from the five other $AbiB_{UC509.9}$ escape mutants all revealed either single or double mutations located at the 5′ end of *orf6* (Table 5).

DISCUSSION

L. lactis UC509.9, a prophage-cured derivative of an Irish dairy starter strain, possesses the smallest lactococcal chromosome currently known; however, the strain harbors an extensive plasmid complement, which includes pCIS8, the largest lactococcal plasmid sequenced to date. The number of plasmids is higher than the six suspected plasmids originally identified by plasmid profiling (41). This difference may be due to poor separation of similarly sized/large plasmids and/or to degradation of the large plasmids pCIS7 and pCIS8 during plasmid isolation. Prophage curing of UC509 was facilitated by mitomycin C exposure, which has long been known to induce plasmid loss (58). Upon generation of UC509.9, no phenotypic changes were observed between UC509 and UC509.9 (except for those related to the loss of Tuc2009), although this does not preclude the possibility that mutations among the resident plasmids may have occurred relative to UC509. Attempts to cure all plasmids from *L. lactis* UC509.9 by using protoplastation and other methods were unsuccessful, indicating that some of the UC509.9 plasmids carry genes for as-yet-uncharacterized stability functions.

L. lactis UC509.9 is a highly phage-resistant strain. To date, only two phages are known to successfully infect *L. lactis* UC509.9, the P335 group phage Tuc2009 and phage c2, the namesake of its group (59). No 936 group phage from our collection (51), which are ubiquitous in dairy manufacturing environments and cause the majority of dairy fermentation failures (14), have been found to infect *L. lactis* UC509.9. This latter phage insensitivity could be, in part, due to the multitude of phage resistance mechanisms both encoded by the *L. lactis* UC509.9 chromosome (see below) and carried on its plasmids. *L. lactis* UC509.9 is predicted to produce three type I HsdS restriction enzyme subunits, of which one is chromosomally encoded, while the remaining two are plasmid-borne (specified on plasmids pCIS1 and pCIS3). The HsdS subunit is known to determine the specificity of the type I restriction subunit HsdR, which together with the methylase (HsdM) subunit creates a holoenzyme restriction enzyme. The ability of lactococcal strains such as UC509.9 to "stack" *hsdS* subunits has been shown previously (41, 53, 54) and was corroborated here by plasmid curing, whereby multiple type I specificities increased the restriction capability, and thus phage resistance, of the strain (Table 3). The differences in sensitivity to the presence of HsdS subunits are likely due to a combination of factors. First, c2 and Tuc2009 were propagated on two different *L. lactis* strains which may have different indigenous R/M systems, and this may cause different protective methylation of these two phages. Second, there are likely differences in the frequencies of the unknown restriction sites of type I systems between the genomes of Tuc2009 and c2.

L. lactis UC509.9 harbors two plasmid-carried, functional Abi systems, AbiB and AbiD1. It has previously been reported that AbiD1 is effective against c2 and 936 group phages (21, 56), whereas AbiB has been reported to be effective against 936 group phages only (16). Our results demonstrated that $AbiB_{UC509.9}$ and$AbiD1_{UC509.9}$ are both functional and effective against 936 group phages. The EOPs of 936 group phages on $AbiB_{UC509.9}$ were severely reduced, whereas the decrease in the EOPs of 936 group phages on cells harboring $AbiD1_{UC509.9}$ was modest, with plaques appearing pin-point and hazy, suggesting that the burst size of the bacteriophage was affected, which is in agreement with previous results (56). The AbiD1 system is believed to be activated by the gene product of *orf1*, which is located in the middle region of 936 phage bIL66 (21). There are no homologs of bIL66 *orf1* in c2 or the P335 group phage Tuc2009, which may explain why these phages can still infect UC509.9, although it has been shown for AbiQ that unrelated gene products from different lactococcal phage groups

may play a recognition or activation role for a given Abi system (22).

Many Abi systems do not exhibit similarity to functionally characterized proteins (other than to other Abi systems) or domains, making postulations regarding their mode of action difficult (16). We generated and characterized bacteriophage escape mutants of sk1 that were insensitive to $AbiB_{UC509.9}$. Interestingly, previous attempts to obtain escape mutants for AbiB were not successful (16). Similar to the unrelated AbiT system, we found that $AbiB_{UC509.9}$ escape mutants harbor single- or double-nucleotide changes in *orf6*, the major capsid protein (23). Unlike AbiT-mediated escape mutants, where all identified mutations affected the C-terminal part of the translated product of the affected gene, $AbiB_{UC509.9}$-mediated escape mutants carry genetic alterations located within a 100-bp region of the 5′ portion of *orf6*.

It has previously been shown that infection of AbiB-expressing cells results in the degradation of all phage transcripts during expression of the structural region of 936 group phages, leading to the suggestion that AbiB either possesses a latent RNase activity or activates a cellular RNase (55). Additionally, blocking of phage protein synthesis during infection of cells harboring *abiB*, by using chloramphenicol, demonstrated that a phage protein is required during the early stages of infection to confer the AbiB phenotype (16, 60). Considering these previously reported findings, and the fact that multiple degradation products were detected by Parreira and colleagues (55), in combination with our results, which revealed single- or double-nucleotide mutations in *orf6*, clearly suggests that insensitivity to AbiB is not due to mutations in RNase restriction sites. Therefore, it is possible that synthesis and/or activation of $AbiB_{UC509.9}$ is facilitated by (a portion of) the major capsid protein. Previous analysis of phage transcripts during infection of AbiB cells detected extensive degradation of phage transcripts 15 min postinfection. *orf6* resides in the late sk1 transcript region, which is predicted to be expressed 15 min postinfection on the basis of Northern analysis, therefore correlating well with previous data (61).

Abi systems that require activation by either enhancement of translational stability or unmasking of a latent activity have previously been described. As mentioned above, expression of AbiD1 is positively regulated by the presence of the bIL66 *orf1* gene product, which stabilizes *abiD1*-encompassing mRNA, a phenomenon that does not occur in escape mutants of bIL66 (21). Activation of latent Abi mechanisms in the presence of a phage gene product has also been demonstrated in *E. coli*. Induction of latent anticodon RNase activity of the Abi tRNA ribotoxin PrrC is regulated by a small phage T4-carried gene product, termed Stp (62). PrrC-mediated escape mutants of phage T4 were shown to carry mutations in *stp*. Further experiments aimed at elucidating the $AbiB_{UC509.9}$ mechanism of activation and subsequent action are ongoing.

As mentioned above, the parent strain of *L. lactis* UC509.9, *L. lactis* UC509, harbors the prophage Tuc2009 (27). Tuc2009 carries genes for a well-characterized superinfection exclusion protein (Sie_{2009}), which confers resistance to certain phages belonging to the 936 group (63). In addition to these, the chromosomally encoded type II restriction enzyme ScrF1 (52) endows *L. lactis* UC509 with a sixth phage resistance mechanism. Based on the combined results of our EOP experiments (Tables 3, 4, and 5), we predict that these resistance mechanisms make the strain virtually impenetrable to 936 group bacteriophages, although additional, chromosomally or plasmid-carried phage resistance systems can of course not be ruled out.

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