Synergistic Effects of *abiE* or *abiF* from pNP40 When Cloned in Combination with *abiD* from pBF61[1]

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ABSTRACT

Two fragments conferring partial phage resistance were located on plasmid pNP40 from *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* DRC3 and cloned. A 2.3-kb *Pst*I fragment from pNP40 containing *abiF* conferred partial phage resistance to prolate-headed phage c2, and a 4.8-kb *Pst*I fragment of pNP40 containing *abiE* conferred partial phage resistance to small isometric-headed phage sk1. When each of the two fragments was cloned individually into a plasmid containing the abortive phage infection gene *abiD* from *L. lactis* ssp. *lactis* KR5, phage resistance was enhanced. When cloned with *abiD*, the 2.3-kb *Pst*I fragment enhanced the resistance against prolate-headed phages, as was indicated by a 400-fold decrease in the efficiency of plating compared with that of *abiD* alone. When the 4.8-kb *Pst*I fragment of pNP40 was cloned with *abiD*, resistance to small isometric-headed phages was enhanced, as was indicated by a greater than 50-fold decrease in efficiency of plating compared with that of *abiD* alone. The 4.8-kb *Pst*I fragment of pNP40 cloned with *abiD* showed a large decrease (500- to 1000-fold) in efficiency of plating against prolate-headed phages, even though the 4.8-kb *Pst*I fragment of pNP40 by itself conferred no resistance to the prolate-headed phages. (**Key words**: bacteriophage, phage resistance, abortive infection, *Lactococcus lactis*)

Abbreviation key: **EOP** = efficiency of plating, **Em** = erythromycin (**r** = resistant), **M17G** = M17 broth containing 0.5% glucose.

INTRODUCTION

Lactococci are the principal acid-producing bacteria used in cheese manufacture. The final quality of cheese is dependent on the ability of the starter culture to grow and produce lactic acid during manufacture. Failure of the starter culture to produce the required lactic acid content during manufacture has been attributed to various factors (15), but the major contributor is bacteriophage infection. It has long been known that naturally occurring phage-resistant lactococcal strains exist, and the resistance of these strains can be linked to the presence of plasmid DNA. The mechanisms of phage resistance identified in lactococci include inhibition of phage adsorption, inhibition of phage DNA penetration, restriction-modification systems, and abortive infections (7, 15). Many strains that are not sensitive to phage naturally contain two to three different bacteriophage resistance mechanisms either on the same plasmid or on separate plasmids (2, 3, 5, 6, 9, 10, 11, 14, 15, 21), which may have developed to aid survival in an environment with a diverse phage population.

The phage resistance plasmid pBF61 was originally isolated from a commercial strain designated *Lactococcus lactis* ssp. *lactis* KR5, and an abortive-type phage resistance *abiD* was cloned and sequenced from this strain (18). The *abiD* conferred a greater degree of resistance to small isometric-headed phages than to prolate-headed phages. Plasmid pNP40, originally isolated from *L. lactis* ssp. *lactis* biovar *diacetylactis* DRC3, was shown to confer complete resistance to prolate-headed phages (17) and has been shown to encode two abortive-type resistance mechanisms (6). The two abortive-type phage resistances from pNP40 include AbiE, which is encoded by two overlapping open reading frames, and AbiF, which is a single protein that has 26% identity with AbiD (6). This paper describes the cloning of each abortive-type phage resistance from pNP40 in conjunction with the *abiD* gene and the enhanced phage resistance phenotype conferred by the recombinant plasmids. This report is the first of enhanced

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resistance after abortive phage resistance from different strains of lactococci are combined on a single plasmid.

MATERIALS AND METHODS

Cultures, Plasmid DNA Extractions, and Electroporation

The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were stored at –20°C in 5.5% (wt/vol) reconstituted nonfat milk. Lactococcal cultures were grown in M17 broth (pH 7.0) containing 0.5% glucose (**M17G**; Difco Laboratories, Detroit, MI) (22) at 30°C. Lactococcal strains containing pIL253-derived plasmids were propagated in M17G containing 10 μg of erythromycin (**Em**)/ml.

Lactococcal plasmids were isolated using the method of Anderson and McKay (1). Restriction endonucleases, dephosphorylations (bacterial alkaline phosphatase), and subsequent ligations (T4 DNA ligase) were performed according to the instructions of the manufacturer (Gibco-BRL Life Technologies, Gaithersburg, MD). Electroporation of plasmid DNA into lactococci was performed as described by McIntyre and Harlander (16).

Cloning Strategy

Plasmid pLS1 was constructed by cloning both *Pst*I phage resistance fragments from pNP40 into vector pIL253. The 4.8-kb fragment conferred partial resistance to small isometric-headed phage sk1, and the 2.3-kb fragment conferred partial resistance to prolate-headed phage c2. The cloning strategy that was used to generate plasmids pMCLS2, pMCLS3a, and pMCLS3b is shown in Figure 1. Plasmid pLS1 was restricted with *Pst*I to release the phage resistance fragments and then restricted with *Bam*HI to prevent pIL253 from religating. Plasmid pMC65 was restricted at a single *Pst*I site upstream from the open reading frame of *abiD* (18) and dephosphorylated with alkaline phosphatase to prevent self-ligation. The ligation mixture was transformed into LM0230 by electorporation, and transformants were selected by resistance (**r**) to Em (Em^r).

Duplicate Spot Test

Duplicate plates containing different phages were used to screen transformants for increased phage resistance. One milliliter of phage diluted in phage broth (17) (1×10^4 to 1×10^6 pfu/ml) was added to 50 μl of 1 *M* $CaCl_2$ in 2.5 ml of tempered (45°C), molten M17G soft agar (4.5 g of agar/L of M17G, pH 7.15). The mixture was poured onto M17G base plates (10 g of agar/L of M17G, pH 7.15). The plates were dried at 37°C for 5 to 8 h. Overnight growth of cultures was spotted (10 μl) onto the plates and incubated at 30°C for 16 to 18 h.

TABLE 1. Bacterial strains, plasmids, and bacteriophages.

Strain, phage, or plasmid	Relevant characteristic	Source or reference
Bacterial strain[1]		
LM0230	Plasmid-free derivative of strain C2	(19)
MC65	LM0230 containing pMC65	(19)
LS1	LM0230 containing pLS1	This study
LS2	LM0230 containing pLS2	This study
LS3	LM0230 containing pLS3	This study
MCLS2	LM0230 containing pMCLS2	This study
MCLS3a	LM0230 containing pMCLS3a	This study
MCLS3b	LM0230 containing pMCLS3b	This study
Plasmid		
pNP40	Self-transmissible phage resistance plasmid, 60 kb	(18)
pIL253	High copy number lactococcal vector; Em^r	(20)
pMC65	2.5-kb Abi^+ fragment of pBF61 cloned into pIL253	(19)
pLS1	pIL253 containing 2.3-kb and 4.8-kb *Pst*I fragments from pNP40	This study
pLS2	pIL253 containing a 2.3-kb *Pst*I fragment from pLS1	This study
pLS3	pIL253 containing a 4.8-kb *Pst*I fragment from pLS1	This study
pMCLS2	pMC65 with the same 2.3-kb fragment as pLS2	This study
pMCLS3a	pMC65 with the same 4.8-kb *Pst*I fragment as pLS3	This study
pMCLS3b	Same as pMCLS3a with the 4.8-kb fragment cloned in the opposite orientation	This study
Bacteriophage		
c2	Prolate-headed phage species c2	(19)
sk1	Small isometric-headed phage species 936	(19)

[1]All are *Lactococcus lactis* ssp. *lactis*.

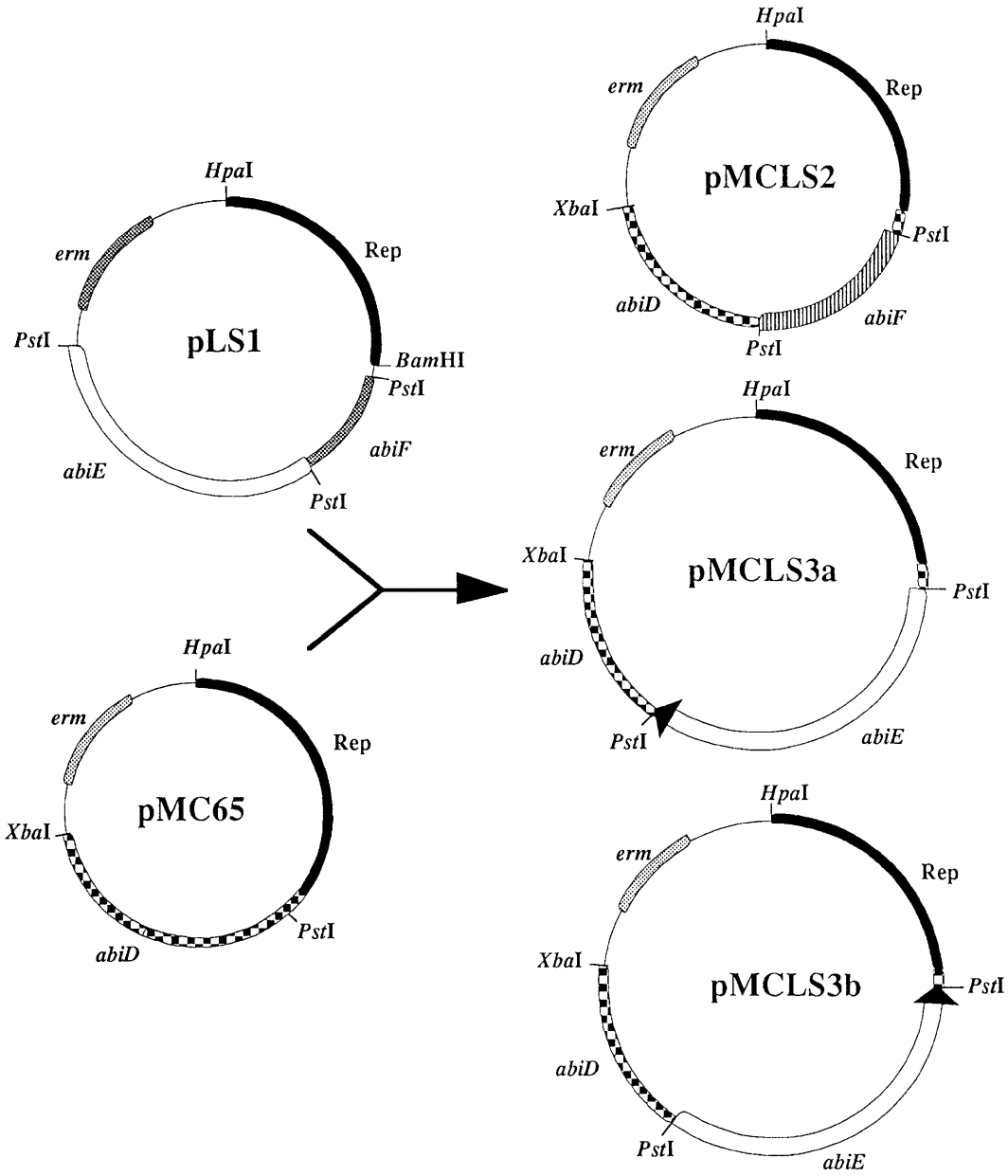

Figure 1. Cloning strategy used to insert the phage resistance fragments from pNP40 (cloned in pLS1) into the phage resistance plasmid pMC65. Plasmid pMC65 contained *abiD* from pBF61 and confers an abortive-type phage resistance. Plasmid pLS1 contained two *Pst*I fragments from pNP40; each conferred resistance to phages with different specificities. The 2.3-kb *Pst*I fragment, containing *abiF*, imparts a resistance to prolate-headed phages, and the 4.8-kb *Pst*I fragment containing *abiE* confers resistance to small isometric-headed phages. Plasmid pLS1 was restricted with *Pst*I to release the phage resistance fragments and then restricted with *Bam*HI to prevent the self-ligation of this plasmid. The mixture was added to pMC65 that had been restricted with *Pst*I and dephosphorylated with bacterial alkaline phosphatase to prevent self-ligation. The restricted DNAs were ligated and transformed into plasmid-free LM0230. Transformants were identified by their resistance to erythromycin. All transformants were tested for phage resistance using a spot test. Phage-resistant transformants were lysed, and the plasmid DNA was restricted to confirm fragment size. Three strains were isolated, each with a plasmid containing one of the *Pst*I fragments from pLS1. One strain had a plasmid with the prolate-headed phage resistance *abiF* gene fragment in pMC65 and was designated pMCLS2. Two strains had a plasmid with the small isometric-headed phage resistance *abiE* gene fragment within pMC65. This 4.8-kb fragment was in different orientations as confirmed by restriction enzyme analysis. These two plasmids were designated pMCLS3a and pMCLS3b. Rep = DNA fragment required for replication; *erm* = DNA fragment containing the erythromycin resistance gene. The plasmids were not drawn to scale.

TABLE 2. Efficiency of plating against phages c2 and sk1.

Phage[1]	Efficiency of plating							
	MC253	MC65 *abiD*	LS1 *abiE, abiF*	LS2 *abiF*	MCLS2 *abiD, abiF*	LS3 *abiE*	MCLS3a *abiD, abiE*	MCLS3b *abiD, abiE*
c2	1	2.9×10^{-1}	1.2×10^{-1}	3.0×10^{-1}	7.2×10^{-4}	1	2.4×10^{-4}	5.7×10^{-4}
sk1	1	1.9×10^{-5}	1.4×10^{-1}	8.5×10^{-1}	1.6×10^{-5}	5.9×10^{-1}	$<3.5 \times 10^{-7}$	$<3.5 \times 10^{-7}$

[1]Both phage types were propagated in strain MC253.

Preparation and Enumeration of Bacteriophages

Phage stocks were prepared on exponentially growing cultures of MCIL253 (LM0230 containing cloning vector pIL253) from single plaque isolates (17). Bacteriophages were enumerated as described by Terzaghi and Sandine (22). Assays were performed by combining 50 μl of 1 *M* $CaCl_2$, 0.25 ml of a log-phase culture, and 1 ml of bacteriophage diluted in phage broth. Phages were allowed to adsorb for 10 min, after which 2.5 ml of tempered, molten M17G soft agar overlay was added and then poured onto an M17G base plate. Plates were incubated at 30°C for 6 h. Efficiency of plating (**EOP**) was calculated by dividing the plaque-forming units per milliliter on the test strain by the plaque-forming units per milliliter on the strain on which the phage stock was propagated.

RESULTS

Plasmid pNP40 is a 60-kb, self-transmissible plasmid that codes for resistance to nisin and bacteriophage. Intact pNP40 confers complete phage resistance to prolate-headed phage c2 at 21 and 32°C (17). Enzyme *Pst*I restriction of pNP40 generated eight fragments that were ligated into vector pIL253 and transformed into LM0230. Fragments from pNP40 encoding partial phage resistance were identified by screening Em^r transformants by spot test. Previously, Garvey et al. (6) had cloned and sequenced these portions of pNP40. Our 2.3-kb *Pst*I fragment from pNP40 (strain LS2, Table 2) corresponded to *abiF*, and the 4.8-kb *Pst*I fragment (strain LS3, Table 2) corresponded to *abiE*. The 2.3-kb fragment conferred partial resistance to prolate-headed phage c2, as revealed by reduced plaque size and a small reduction in EOP (3.0×10^{-1}). The 4.8-kb *Pst*I fragment cloned from pNP40 (strain LS3) showed no reduction in EOP toward prolate-headed phage c2, but, against the small isometric-headed phage sk1, conferred a slight reduction in EOP (5.9×10^{-1}) and a reduction in plaque size. When cloned together (strain LS1), the EOP of each phage type was reduced slightly, and visible plaque size was also reduced (Tables 2 and 3).

In an attempt to increase the resistance of pMC65 (*abiD*), the pNP40 fragments from pLS1 were inserted into the *Pst*I site of pMC65 at 454 bp upstream from the *abiD* open reading frame (Figure 1). The ligation mixture was transformed into LM0230, and transformants were selected for Em^r. Broth growth was used for spot tests to identify transformants with increased phage resistance by spotting onto duplicate plates containing either bacteriophage c2 or sk1

TABLE 3. Observed plaque sizes for phages c2 and sk1 when plated upon recombinant strains.

Phage	Plaque morphology							
	MC253	MC65 *abiD*	LS1 *abiE, abiF*	LS2 *abiF*	MCLS2 *abiD, abiF*	LS3 *abiE*	MCLS3a *abiD, abiE*	MCLS3b *abiD, abiE*
c2	Large clear	Small fuzzy	Medium, irregular fuzzy center	Small plaques	Small on lawn of ppp[1]	Large clear	Small on lawn of ppp[1]	Small on lawn of ppp[1]
sk1	Medium clear	Small on lawn of ppp[1]	Small fuzzy	Medium clear	Small on lawn of ppp[1]	Small fuzzy	Uncountable ppp[2]	Uncountable ppp[2]

[1]At lower dilutions, small plaques were visible (counted and used to calculate EOP) on a lawn of uncountable pinpoint plaques (ppp); at high dilutions, no plaques were visible.

[2]Uncountable ppp were visible at low dilutions; at high dilutions, no plaques were visible.

(Figure 2). This spot test allowed for the differentiation of recombinant transformants that had better phage resistance than MC65 (spot 4f). Plasmid content was determined from transformants with a lack of visible plaques on the sk1 plates and a high reduction or elimination of plaques on the c2 plate (such as positions 1c, 5c, 6c, and 2e [Figure 2]). Transformants spotted at 6c and 2e were recombinants (later designated MCLS2 and MCLS3a, respectively). No transformants were isolated that contained both fragments from pLS1 in pMC65.

The phage resistance of each clone was evaluated by plaque morphology and EOP (Tables 2 and 3). Strains LS2 (pIL253 carrying the 2.3-kb *Pst*I fragment with *abiF*), LS3 (pIL253 carrying the 4.8-kb fragment with *abiE*), and LS1 (pIL253 carrying both *abiE* and *abiF*) were used as controls, along with MC253 (LM0230 with pIL253) and MC65. When the 2.3-kb *Pst*I prolate-headed phage resistance fragment (*abiF*) was combined with pMC65 (strain MCLS2), there was about a 400-fold decrease in EOP against phage c2 compared with the 2.3-kb fragment alone in strain LS2(pLS2) or to MC65(pMC65). In contrast to MC65, resistance of MCLS2 to small isometric-headed phages was not enhanced.

The 4.8-kb fragment (*abiE*) by itself in pIL253 (strain LS3) conferred a small reduction in EOP (5.9×10^{-1}) and reduced the plaque size of small isometric-headed phages. When the 4.8-kb fragment was cloned into pMC65 (pMCLS3a and pMCLS3b), it conferred a greater than 50-fold lower EOP against phage sk1 (EOP $<3.5 \times 10^{-7}$) than was observed with *abiD* (EOP 1.9×10^{-5}) and a greater than 10^6-fold reduction in EOP than did *abiE* alone (EOP 5.9×10^{-1}). In addition to the enhancement of resistance to small isometric-headed phages, when the 4.8-kb fragment was cloned in conjunction with pMC65 (strains MCLS3a and MCLS3b), the resistance to prolate-headed phage c2 was unexpectedly enhanced (EOP of 5.7×10^{-4} and 2.4×10^{-4} with MCLS3b and MCLS3a, respectively). These EOP are 500- to 1000-fold lower than those with *abiD* alone and were unexpected, because the 4.8-kb fragment alone (strain LS3) conferred no resistance to prolate-headed phages.

DISCUSSION

The cloning of two phage resistance fragments from pNP40, each in conjunction with *abiD* from pBF61, is described. We used the cloned fragments of pNP40 to study the influence of multiple abortive infection mechanisms upon the phage resistance encoded by *abiD*. The phenotypes we observed with the cloned abortive infection-encoding fragments from pNP40 differed in part from those reported by Garvey et al. (6). We observed the same phenotype for *abiE* (pLS3), which was a reduction in plaque size and EOP against small isometric-headed phage sk1 and

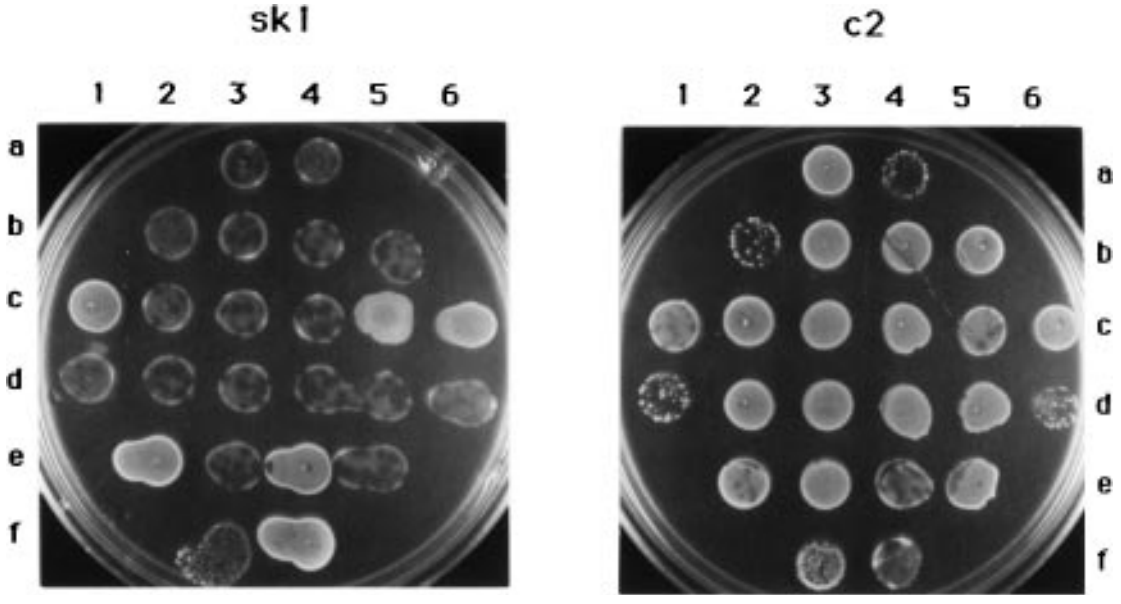

Figure 2. Spot test to screen transformants for decreased sensitivity to phages. Duplicate plates of soft agar overlay containing different phage species were spotted with 10 μl of each transformant. Phage resistance was evaluated by estimation of plaque size and was compared with positive control strain MC65 (spot 4f) and negative control LM0230 (spot 3f). Recombinant plasmids were isolated from transformants that appeared to exhibit better phage resistance than MC65.

no influence on prolate-headed phage c2. The difference occurred with the 2.3-kb *Pst*I fragment containing *abiF*. The EOP was slightly reduced (1.4×10^{-1}), plaque size of small isometric-headed phages was not reduced, and only a small effect upon EOP (1.2×10^{-1}) and reduction of plaque size toward prolate-headed phages was observed. Garvey et al. (6) found a significant reduction in EOP against both phage c2 (8.0×10^{-4}) and small isometric-headed phage 712 (3.0×10^{-6}). The differences may be due to different cloning vectors, plasmid copy number, host background, or length of cloned fragment.

Many investigators have observed that cloned fragments containing phage resistance mechanisms have a lower degree of resistance than does the native lactococcal plasmid (3, 12, 13). Although never studied specifically, this observed lower phage resistance after cloning has been attributed to differences in plasmid copy number, multiple phage resistance mechanisms present on the native plasmid, or both (3, 12, 13). In our study, we did not observe an enhancement in phage resistance when both phage resistance mechanisms from pNP40 were cloned into a single plasmid (pLS1). When *abiF* (2.3-kb *Pst*I fragment) was cloned in the same orientation as *abiD* (construct pMCLS2), no enhancement of resistance to small isometric-headed phage sk1 was observed, but resistance to prolate-headed phage c2 was enhanced significantly. If this increase was due to read-through of *abiD* from the *abiF* promoter, increased resistance against both phage species would be expected. Therefore, we interpret the increase in phage resistance only against phage c2 as an additive effect of two different abortive infection genes. The combination of *abiE* from pNP40 and *abiD* enhanced the resistance to both phage types. Although *abiE* cloned alone (pLS3) only exhibited a reduced EOP and plaque size against phage sk1, when cloned in conjunction with *abiD* (pMCLS3a and pMCLS3b), the resistances to both phage types were greatly enhanced. This enhanced resistance was observed, regardless of the orientation of the 4.8-kb fragment; therefore, it is unlikely that the enhanced resistance is due to transcriptional read-through from an upstream promoter. The sequence of *abiE* reveals a region encoding a RecA protein that is present on the cloned 4.8-kb fragment (8). The observed enhancement could be via interaction of the two Abi proteins, changes in *abiD* transcription by plasmid-encoded proteins such as RecA, enhancement of AbiD function at the protein level, another factor, or a combination of factors. The nature of the interaction between *abiD* and *abiE* is unknown and warrants future investigation.

Apparently, not all combinations of phage resistance mechanisms result in enhanced phage resistance. Durmaz and Klaenhammer (4) reported the combination of different Abi phenotypes and restriction-modification systems to create a rotation system for phage-resistant starter cultures. Those researchers reported that, when multiple Abi determinants (*abiA* and *abiC*) were cloned onto the same plasmid, the expression of one phenotype (AbiA) remained strong, but the phenotype of the second determinant (AbiC) was less than expected (4). This result was unexpected because both mechanisms function on different plasmids in the original parental strain (3, 4). In addition, in this study, the presence of *abiE* with *abiF* on the same plasmid did not appear to enhance greatly the resistance against either phage type. In contrast, *abiE* in conjunction with *abiD* enhanced resistance to both phage types tested, and *abiF* in conjunction with *abiD* enhanced resistance to prolate-headed phage. For this reason, it appears that not all combinations of phage resistance mechanisms produce a synergistic interaction against phage attack.

CONCLUSIONS

This paper reports the first combination of Abi-encoding fragments from different strains to exhibit enhancement of resistance when combined on the same plasmid. Identification of synergistic phage resistance is a first step toward the goal of constructing strains with multiple levels of phage resistance for use during commercial milk fermentations.

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