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Phenotypic Consequences of Altering the Copy Number of *abiA*, a Gene Responsible for Aborting Bacteriophage Infections in *Lactococcus lactis*†

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The *abiA* gene (formerly *hsp*) encodes an abortive phage infection mechanism which inhibits phage DNA replication. To analyze the effects of varying the *abiA* gene dosage on bacteriophage resistance in *Lactococcus lactis*, various genetic constructions were made. An IS*946*-based integration vector, pTRK75, was used to integrate a single copy of *abiA* into the chromosomes of two lactococcal strains, MG1363 and NCK203. In both strains, a single copy of *abiA* did not confer any significant phage resistance on the host except for one of the MG1363 integrants, NCK625, which exhibited a slightly higher level of resistance to phages sk1 and p2. Hybridization of the total cellular RNA from NCK625 to an *abiA*-specific probe indicated that the integration took place downstream of a promoter causing stronger expression of *abiA* in this integrant. Three *abiA*-containing plasmids of various copy numbers were introduced into both strains, and the recombinants were evaluated for resistance to phages c2, p2, sk1, and φ31. Plasmid pTRK18 has a copy number of approximately six (*cn* = 6) and caused a decreased plaque size for all phages evaluated. Integration of pTRK75 into a native plasmid of NCK203 generated pTRK362 (*cn* = 13), which caused a reduced efficiency of plaquing (EOP = 10^{-2}) and reduced plaque size. A high-copy-number *abiA* plasmid (pTRK363), based on the pAMβ1 origin of replication, was also constructed (*cn* = 100). Plasmid pTRK363 caused a significant reduction in EOP (10^{-4} to 10^{-8}) and plaque size for all phages tested, although in some cases, this plasmid caused the evolution of AbiA-resistant phage derivatives. Altering the gene dosage or expression level of *abiA* significantly affects the phage resistance levels.

The lactococci are important industrial microorganisms that are used widely in dairy starter cultures. Many industrially important characteristics of these organisms, such as phage resistance, proteinase production, and carbohydrate utilization, are encoded by genes carried on plasmids. Integration of these plasmid-borne genes into the lactococcal genome may stabilize these traits. To date, several genes have been integrated into the lactococcal genome by using a naturally integrating plasmid (9), by transduction (23), or by using integration vectors based on homologous recombination (4, 22).

Two studies demonstrate the integration of determinants encoding lactose metabolism. These genes were integrated by transduction in one case (23), and by using the naturally integrating plasmid pSK11L in the other case (9). No quantitative data comparing any differences in lactose fermentation between the determinants in single copy versus those on a multicopy plasmid were reported in either case. Genes encoding proteolytic enzymes have also been integrated into the chromosome by using an integration vector based on homologous recombination (22) or by transduction (23). Proteolytic activity was shown to be gene dosage dependent, but a single copy of the gene was sufficient for growth in milk (23) or whey (22). After amplification of the integrated plasmid, which allowed comparison of proteolysis at various copy numbers, Leenhouts et al. (22) found that proteolysis increases with increasing copy number of the genes. A bacteriophage resistance gene, *abi* from pCI829, which is identical to *abiA*, has also been integrated into the lactococcal genome by using an integration vector based on homologous recombination between Tn*919* sequences on the chromosome and on the vector (4). After amplification of the integrated plasmid, these authors observed a slight increase in phage resistance compared with that of the single-copy integrant.

Although integration of genes will help stabilize them in a population, in some cases, the low copy number may not be sufficient to impart the desired characteristics on the cell. In the present study, we examined the effect of increasing gene copy number on the phage resistance phenotype conferred by *abiA*. The *abiA* gene, formerly called *hsp* (20), encodes an abortive phage resistance mechanism. The *abiA* gene was originally identified (16) and sequenced (13) from the phage resistance plasmid pTR2030, which encodes at least one other phage resistance mechanism, the restriction and modification system *LlaI* (15). The *abiA*-encoded mechanism is effective against a variety of phages and acts in the early stages of phage infection by inhibiting phage DNA replication (12). In this study, we examined the effect that the copy number of *abiA* has on phage resistance in *Lactococcus lactis* subsp. *lactis* MG1363 and NCK203.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. *L. lactis* cultures were propagated at 30°C in M17 broth (33) supplemented with 0.5% (wt/vol) glucose (GM17). Erythromycin (1.5 μg/ml) was added to the broth

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TABLE 1. Bacteria, phages, and plasmids

Strain, phage, or plasmid	Relevant characteristics[a]	Source or reference
Bacteria		
L. lactis subsp. *lactis*		
MG1363	Phage-sensitive host	10
NCK624 (integrant 1)	MG1363 (pTRK75 integrated into chromosome)	This study
NCK625 (integrant 5)	MG1363 (pTRK75 integrated into chromosome)	This study
NCK211	MG1363(pTRK18)	16
NCK626	MG1363(pTRK362)	This study
NCK627	MG1363(pTRK363)	This study
NCK203	Phage-sensitive host	15
NCK628	NCK203(pTRK75 integrated into chromosome)	This study
NCK279	NCK203(pTRK18)	This study
NCK629	NCK203(pTRK362)	This study
NCK630	NCK203(pTRK363)	This study
E. coli		
DH5α	Transformation host	GIBCO-BRL
SURE	Transformation host	Stratagene
Bacteriophages		
sk1	Small isometric phage for MG1363 ($AbiA^s$), species 936	26
p2	Small isometric phage for MG1363 ($AbiA^s$), species 936	11
c2	Prolate phage for MG1363 ($AbiA^s$), species c2	31
φ31	Small isometric phage for NCK203 ($AbiA^s$), species P335	1, 19
φ31.3	$AbiA^r$ $Per31^r$ derivative of φ31 resulting from φ31 infection of *L. lactis* NCK630	This study
Plasmids		
pSA3	*E. coli-Lactococcus* shuttle vector	7
pTRK18	$AbiA^+$ Em^r Cm^r	16
pTRK28	pSA3 with an IS*946* insertion, Em^r Cm^r Tc^r	28, 29
pTRK73	Ap^r pBluescript with the 456-bp *Xba*I internal *abiA* fragment	14
pTRK74	Em^r Cm^r Tc^r IS*946* *ori*$^-$	This study
pTRK75	Em^r Cm^r Tc^r IS*946* *ori*$^-$ *AbiA*$^+$	This study
pTRK362	Em^r Cm^r $AbiA^+$; cointegrate between pTRK75 and 7.5-kb NCK203 native plasmid; 20.3 kb	This study
pTRK363	Em^r $AbiA^+$; high-copy AbiA plasmid	This study
pTRK364	Cm^r IS*946* $AbiA^+$; contains 0.9 kb of MG1363 chromosomal DNA upstream from the pTRK75 insert in NCK625	This study
pTRK365	Reconstruction of pTRK75 containing *abiA* from NCK625	This study
pTRKH3	Em^r Tc^r; high-copy cloning vector	25

[a] Ap, ampicillin resistance; Em, erythromycin resistance; Cm, chloramphenicol resistance; Tc, tetracycline resistance; *ori*, presence or absence of a gram-positive origin of replication; $AbiA^{s/r}$, sensitivity/resistance to AbiA; $Per31^r$, resistance to Per31.

when appropriate. Bacteriophages were propagated and titrated by the method of Terzaghi and Sandine (33). *Escherichia coli* strains used for plasmid isolation and cloning were propagated at 37°C in LB medium (30). Plasmids were maintained in *E. coli* by addition of chloramphenicol (25 μg/ml) to the growth medium.

Transformation. *E. coli* cells were transformed by electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described by Dower et al. (8). *L. lactis* subsp. *lactis* MG1363 and NCK203 were grown with 4 and 1% glycine, respectively, before washing and electroporated by the protocol of Holo and Nes (17).

DNA isolation and cloning. Rapid and large-scale plasmid isolations from *E. coli* were performed by alkaline lysis (30). DNA was purified from agarose gels with the Prep-A-Gene DNA purification matrix kit (Bio-Rad Laboratories). Plasmid DNA was extracted from *L. lactis* strains by the method of Anderson and McKay (2). Total DNA was isolated from a 10-ml culture of *L. lactis* as described by Hill et al. (14). Phage DNA was extracted by the method of Raya et al. (27).

General procedures for DNA manipulations were performed as described before (30). Restriction endonucleases, T4 DNA ligase, and their corresponding buffers were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as specified by the manufacturer.

DNA-DNA hybridization. DNA from agarose gels was transferred to MSI Magnagraph nylon membranes (Micron Separations, Inc., Westboro, Mass.) as described by Southern (32). [^{32}P]dCTP-labeled probes were prepared by using a Multiprime DNA labeling kit (Amersham, Arlington Heights, Ill.). Radioactive hybridizations were performed with an Omni-blot apparatus (American Bionetics, Inc., Emeryville, Calif.). Densitometry of autoradiographs for assessment of plasmid copy number was performed with the IS-1000 Digital Imaging System (Innotech Scientific Corp., San Leandro, Calif.) as well as the LKB Ultrascan XL laser densitometer and the LKB Gel Scan program.

A nonradioactive DNA labeling (digoxigenin-dUTP label) and detection kit (Genius Kit; Boehringer Mannheim Biochemicals) was also used for probe preparation and hybridization. High-stringency conditions were used for all hybridizations.

RNA isolation and hybridization. RNA was isolated from lactococcal cells as described by van der Vossen et al. (34) with the following modifications. Cells were harvested at an optical density at 600 nm (OD_{600}) of 0.6 and resuspended in 25%

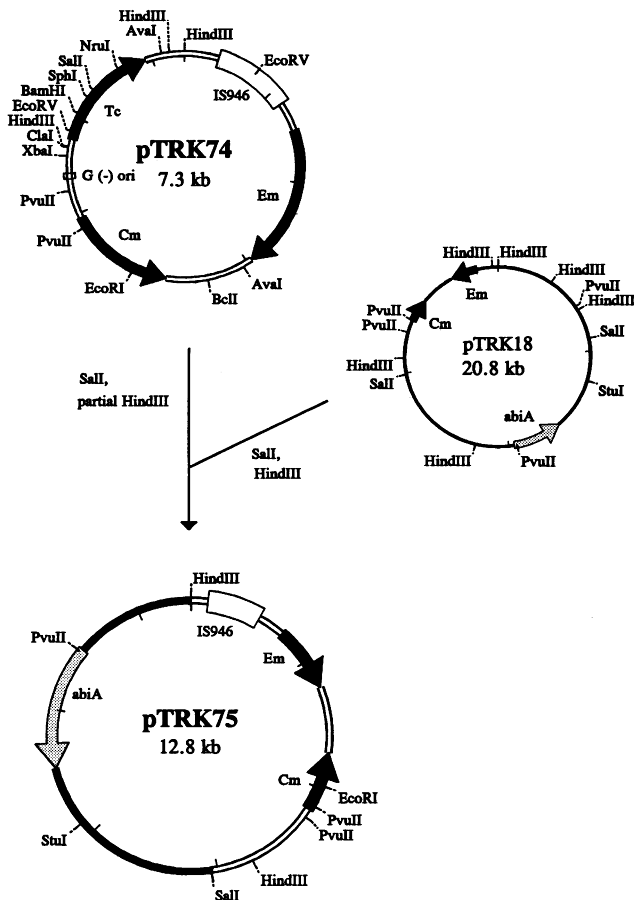

FIG. 1. Construction of pTRK75. Cm, chloramphenicol resistance; Em, erythromycin resistance; Tc, tetracycline resistance; G (−) *ori*, gram-negative origin of replication. Plasmids are not drawn to scale.

sucrose containing lysozyme (10 mg/ml). After lysis and phenol extraction as described before (34), RNA was precipitated with an equal volume of 6 M LiCl. The pellet was washed with 70% ethanol, dried under vacuum, and resuspended in water. The preparation was digested with RNase-free DNase I (Boehringer Mannheim Biochemicals) for 1 h at 37°C. The RNA concentration was determined spectrophotometrically (30). RNA samples were denatured and applied to a nylon membrane with a slot blot apparatus (Bio-Dot-SF; Bio-Rad) as described by Sambrook et al. (30). The RNA was UV cross-linked to the membrane and then hybridized to a ^{32}P-labeled *Xba*I internal *abiA* fragment (14). Densitometry of autoradiographs was performed with the IS-1000 Digital Imaging System (Innotech Scientific Corp.).

RESULTS

Construction of pTRK75, an integration vector containing *abiA*. Strains with a single copy of *abiA* per chromosome were constructed as a reference for plasmid copy number determinations as well as to evaluate the phage resistance conferred by this gene in single copy. In order to obtain these strains, an integration vector was constructed to allow insertion of *abiA* into the lactococcal chromosome.

The cloning of genes into an integration vector was facilitated by creating pTRK74 (Fig. 1). This vector was constructed by partial *Hin*dIII digestion of pTRK28 (pSA3 containing an IS*946* insert) (28, 29). The structure of pTRK74 was confirmed by restriction enzyme analysis. The partial digestion left all of the markers intact for cloning and selection while removing the gram-positive origin of replication. Therefore, maintenance of plasmid sequences and expression of plasmid genes in *Lactococcus* strains depend on random integration of the plasmid into the host genome via transposition of IS*946* (29).

Plasmid pTRK75, an integration vector containing the abortive phage resistance gene *abiA*, was constructed as follows (Fig. 1). The 6.6-kb *Sal*I-*Hin*dIII fragment from pTRK18 (16), which contains *abiA*, was ligated with the 6.2-kb fragment resulting from a *Sal*I-partial *Hin*dIII digestion of pTRK74. The ligation mixture was electroporated into *E. coli* DH5α, and one Tc^s clone which contained a plasmid of the expected size (12.8 kb) was recovered and designated pTRK75. Hybridization with the *Xba*I internal *abiA* fragment from pTRK73 (14) confirmed the presence of *abiA* in this plasmid. Restriction enzyme analysis confirmed the pTRK75 structure and indicated which *Hin*dIII site had been cut in the partial digestion. The restriction map of pTRK75 is shown in Fig. 1.

Integration of pTRK75 into the genomes of *L. lactis* subsp. *lactis* MG1363 and NCK203. pTRK75 was electroporated into *L. lactis* MG1363 by the protocol of Holo and Nes (17). Integrants were selected on GM17 agar containing erythromycin at 1.5 μg/ml. On average, 10 integrants were recovered per μg of DNA. Eighteen integrants, designated MG1363 integrants 1 to 18, were chosen for further analysis. To confirm single-copy integration of pTRK75, total genomic DNA was isolated from these integrants and digested with *Eco*RI, which cuts pTRK75 once. Hybridization of these digests to a digoxigenin-labeled pTRK75-specific probe (plasmid pSA3) showed two junction fragments in each lane that varied in size among the integrants (Fig. 2). This indicates that a single copy of pTRK75 is present in different locations in each of the 18 integrants. Total DNA from these integrants was also digested with *Pvu*II and *Stu*I and hybridized to a digoxigenin-labeled *Xba*I internal *abiA* fragment. The results demonstrated that all integrants contained the *abiA* gene (data not shown).

Plasmid pTRK75 was also introduced into *L. lactis* NCK203, which contains three native plasmids. After repeated attempts to electroporate this strain with pTRK75, only two erythromycin-resistant colonies were recovered. The reason for the low recovery of integrants with this plasmid in this strain is unknown, since other IS*946*-based vectors insert at higher frequencies (29). Hybridization of *Eco*RI-digested total DNA from these two isolates to digoxigenin-labeled pSA3 also gave two junction fragments, indicating a single-copy insertion (data not shown).

Examination of the plasmid profiles of the integrants showed that in one of the isolates, NCK629, the insertion had occurred in the 7.5-kb resident plasmid (Fig. 3). The resulting cointegrate plasmid, designated pTRK362 (20.3 kb), hybridizes to pSA3 and *abiA* (data not shown). In the other isolate, NCK628, the pTRK75 insert was in the chromosome.

Isolation of pTRK362 and introduction into *L. lactis* MG1363. Isolation of pTRK362 in *E. coli* was simplified by the presence of a gram-negative origin of replication and chloramphenicol resistance marker in the pTRK75 portion of the plasmid. A plasmid preparation from NCK203 (pTRK362) was electroporated into *E. coli* DH5α, and chloramphenicol-resistant colonies were selected. Transformants contained a plasmid of the expected size which hybridized to pSA3 and *abiA* (data not shown). Plasmid pTRK362 was then isolated from *E. coli* and electroporated into *L. lactis* MG1363. In this background, it replicated extrachromosomally because of the *ori*

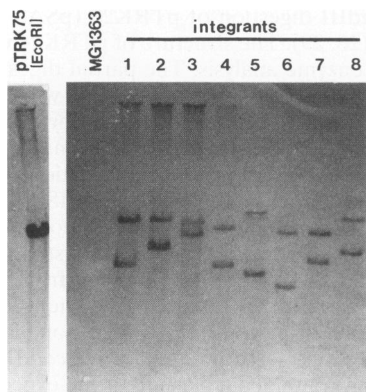

FIG. 2. Junction fragments from 8 of the 18 MG1363 pTRK75 integrants. Chromosomal DNA from the integrants was digested with *Eco*RI, which cleaves pTRK75 once. A digoxigenin-labeled pSA3 probe (specific for pTRK75) identified two junction fragments in different positions for each integrant, indicating random single-copy integration into the chromosome.

contributed by the 7.5-kb plasmid from NCK203 and conferred erythromycin resistance linked to the pTRK75 portion.

Construction of pTRK363. Plasmid pTRK363 was constructed by cloning *abiA* into pTRKH3. Plasmid pTRKH3 is high copy in lactococci and medium copy in *E. coli* (25). The *Eco*RI-*Sal*I fragment containing *abiA* from pTRK18 (Fig. 1) (16) was cloned into pTRKH3 cut with the same enzymes. Since the gram-negative *ori* was removed from pTRKH3 with this strategy, the ligation mixture was electroporated directly into *L. lactis* MG1363 and NCK203, and Em^r colonies were selected. Plasmid isolations and restriction analysis confirmed that the expected clone was present in both strains.

Copy number analysis. The copy numbers of three plasmids, pTRK18, pTRK362, and pTRK363, in MG1363 were determined. pTRK18 was described previously (16) and consists of a fragment from pTR2030 cloned into the shuttle vector pSA3. This plasmid has a deletion which inactivated the restriction and modification activities on pTR2030, leaving AbiA as the only active phage resistance mechanism on pTRK18. The copy numbers of the three plasmids in MG1363 were determined by isolating total DNA from MG1363(pTRK18), MG1363 (pTRK362), MG1363(pTRK363), and two pTRK75 chromosomal integrants, NCK624 (integrant 1) and NCK625 (integrant 5), as single-copy references. These DNAs were cut with *Pvu*II and *Stu*I, which yields the same 2.9-kb *abiA*-containing fragment in all strains. The digests were electrophoresed through an 0.8% agarose gel. The amount of DNA was adjusted so that each lane contained approximately the same amount of chromosomal DNA, as judged visually and confirmed by densitometry of portions of the gel containing only chromosomal fragments (Fig. 4A). Dilutions of each sample (1:10 and 1:100) were also electrophoresed (not shown) to aid in quantification of the higher-copy-number plasmids.

The DNA was transferred to a nylon membrane and hybridized to the ^{32}P-labeled *Xba*I internal *abiA* fragment, resulting in one 2.9-kb hybridizing band in each lane (Fig. 4B). The density of each band was determined by scanning the autoradiogram with a densitometer. The relative copy numbers of each plasmid were estimated by comparing the density of the single-copy *abiA* bands from NCK624 and NCK625 with the densities of the bands from the three plasmids (Table 2). Copy

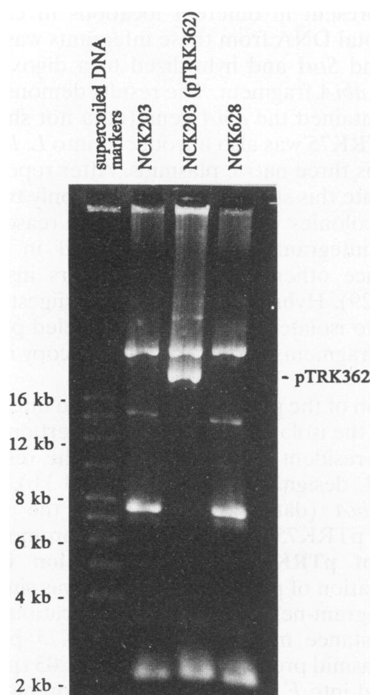

FIG. 3. Plasmid preparations from *L. lactis* NCK203, NCK203 (pTRK362), and NCK628. Plasmid pTRK75 (12.8 kb) integrated into the 7.5-kb native plasmid of NCK203 to create pTRK362 (20.3 kb). NCK628 has pTRK75 integrated into its chromosome and therefore has no alteration in plasmid profile.

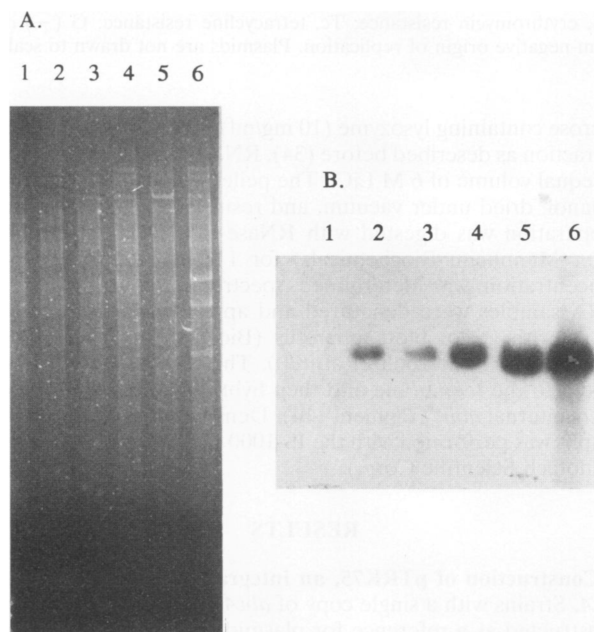

FIG. 4. Copy number determination for pTRK18, pTRK362, and pTRK363 in *L. lactis* MG1363. Total DNA was digested with *Pvu*II and *Stu*I (A) and then probed with the ^{32}P-labeled *Xba*I internal *abiA* fragment (B). Lane 1, MG1363; lane 2, NCK624; lane 3, NCK625; lane 4, MG1363(pTRK18); lane 5, MG1363(pTRK362); lane 6, MG1363 (pTRK363).

TABLE 2. Estimated copy numbers of three plasmids bearing *abiA* in *L. lactis* MG1363

Plasmid	Copy no.[a]	Original source of gram-positive origin of replication (reference)	Mode of replication (reference)
pTRK18	6	pIP501 (18) (*Streptococcus agalactiae*)	Theta, unidirectional (21)
pTRK362	13	7.5-kb native plasmid from *L. lactis* NCK203	Unknown
pTRK363	100	pAMβ1 (5) (*Enterococcus faecalis*)	Theta, unidirectional (3)

[a] Copy numbers were estimated from the results of at least two independent experiments.

number was not determined in the NCK203 background, although phenotypic data suggest that the relative copy numbers are comparable.

Phage resistance phenotypes conferred by *abiA* at various copy numbers. The 18 MG1363 chromosomal integrants and the NCK203 chromosomal integrant (NCK628) were challenged with appropriate phages to determine the phage resistance phenotype resulting from single-copy integration of *abiA* in the chromosomes of these strains. Both MG1363 and NCK203 containing the *abiA*-bearing plasmids pTRK18, pTRK362, and pTRK363 were also evaluated for phage resistance. The results of these analyses are summarized in Tables 3 and 4 and in the following paragraphs.

In the MG1363 background, most of the single-copy chromosomal pTRK75 integrants (integrants 1 to 4 and 6 to 18), when challenged with the small isometric-headed phage sk1, gave plaques only slightly smaller than those present on wild-type MG1363, but the halos surrounding the plaques on MG1363 were not present in these integrants (Table 3). One exception was observed: on NCK625 (integrants), sk1 gave plaques 50% smaller in diameter than those on MG1363. This integrant will be discussed further in the following section. The presence of pTRK18 caused a decrease in plaque size. Plasmid pTRK362 caused pinpoint plaques with a reduction in efficiency of plaquing (EOP) of phage sk1 to 6.0×10^{-2}. The highest level of phage resistance was observed with the high-copy-number plasmid pTRK363; no plaques were observed. Similar results were observed when these strains were challenged with phage p2 (Table 3).

The prolate-headed phage c2 was also affected by AbiA, but to a lesser degree (Table 3). Again, the highest level of resistance was observed with pTRK363. Small and large plaques appeared on MG1363(pTRK363) at a frequency of 1.0×10^{-5}. After propagation and further testing against MG1363(pTRK363), the phages recovered from large plaques had a reduced plaque size and an EOP of 0.5, while the phages recovered from small plaques had a reduced plaque size and an EOP of 2×10^{-2}. The restriction enzyme patterns of these partially AbiA-resistant c2 derivatives were identical to that of c2 (data not shown).

Similar results were observed in the NCK203 background (Table 4). When challenged with φ31, the plaques on chromosomal integrant NCK628 were only slightly smaller than those on wild-type NCK203. NCK203(pTRK18) showed a reduction in φ31 plaque size, and pTRK362 resulted in pinpoint plaques and a reduction in EOP to 6.6×10^{-2}. Plasmid pTRK363 caused a reduction in EOP to 3.0×10^{-4}, and large and small plaques were observed (Table 4). The phages recovered from both large and small plaques remained fully resistant to AbiA ($AbiA^r$) and retained their original plaque morphology after propagation. Restriction enzyme analysis of the genomes of the $AbiA^r$ phages showed that the majority of them had the same restriction enzyme pattern as φ31, indicating that a point mutation caused the resistance to AbiA. A phage was recovered at low frequency ($<10^{-2}$) from a small plaque which had an altered restriction enzyme pattern compared with that of φ31 (Fig. 5). This phage was designated φ31.3 and appears to be identical to φ31.1, which was isolated after φ31 infection of NCK203 containing the φ31 origin of replication cloned on a high-copy-number plasmid (Per31) (24). The φ31.3 genome is 1.5 to 2.0 kb smaller than the φ31 genome and contains several different restriction fragments. The origin of the "new" DNA and the event which contributed to its appearance are under investigation.

Analysis of NCK625 (MG1363 integrant 5). As mentioned previously, NCK625 (MG1363 integrant 5) exhibited higher phage resistance than the other 17 MG1363 chromosomal integrants even though it contained a single copy of the *abiA* gene. The following experiment was designed to clone the *abiA* gene from the NCK625 chromosome (designated *abiA**) and reintegrate it to determine whether the reduced plaque size resulted from a mutation in the *abiA* gene or its expression signals. The chromosomal DNA from NCK625 was digested with *Bcl*I, resulting in a fragment containing *abiA**, a chloramphenicol resistance gene, a gram-negative origin of replication, IS*946*, and 900 bp of the chromosomal sequence flanking the

TABLE 3. Phage resistance phenotypes resulting from *abiA* at several copy numbers in *L. lactis* MG1363 when challenged with phages sk1, p2, and c2[a]

Strain	*abiA* copy no.	sk1		p2		c2	
		Aps	EOP	Aps	EOP	Aps	EOP
MG1363	0	1.4 (2.2)	1.0	1.4 (2.5)	1.0	2.4 (4.0)	1.0
Integrants 1 to 4 and 6 to 18	1	1.3	1.0	1.3 (1.8)	0.7	2.4 (4.0)	1.0
Integrant 5 (NCK625)	1	0.8	0.8	1.0	0.7	2.1 (3.9)	0.9
MG1363(pTRK18)	6	0.8	0.8	1.0	0.9	1.8 (3.9)	0.8
MG1363(pTRK362)	13	<0.4	6.0×10^{-2}	<0.4	0.1	0.6	9.0×10^{-2}
MG1363(pTRK363)[b]	100	NA	$<1.0 \times 10^{-8}$	NA	$<1.0 \times 10^{-8}$	0.5,1.1[b]	1.0×10^{-5}

[a] All results are averages of at least four independent experiments. Aps, average plaque size (in millimeters). Numbers in parentheses indicate the diameter of the halo surrounding the plaque (in millimeters). NA, not applicable.
[b] Values denote two plaque sizes observed.

TABLE 4. Phage resistance phenotypes resulting from *abiA* at several copy numbers in *L. lactis* NCK203 when challenged with phage φ31[a]

Strain	φ31	
	Aps[b]	EOP
NCK203	1.2	1.0
NCK203 (chromosomal integrant)	1.0	1.0
NCK203(pTRK18)	0.8	0.8
NCK203(pTRK362)	<0.4	6.6×10^{-2}
NCK203(pTRK363)	0.6,1.3[c]	3.0×10^{-4}

[a] All results are averages of at least four independent experiments.
[b] Aps, average plaque size (in millimeters).
[c] Values denote two plaque sizes observed.

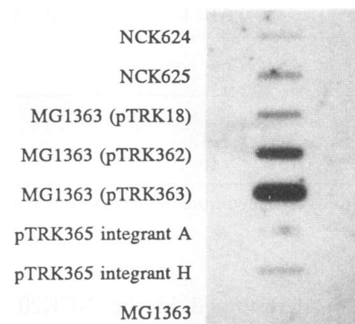

FIG. 6. Total RNA isolated from *abiA*-containing MG1363 derivatives and hybridized to the ^{32}P-labeled *Xba*I internal *abiA* fragment.

insert. The *Bcl*I digest was ligated in a large volume (100 μl) so as to encourage intramolecular ligation and then electroporated into *E. coli* SURE, in which only plasmids with the *abiA** fragment can replicate and confer chloramphenicol resistance. A plasmid was isolated from a Cm^r colony and digested with *Bcl*I to confirm that only one *Bcl*I fragment was included in the ligation. This plasmid, designated pTRK364, contains chromosomal sequences which might result in insertion by homologous recombination, and it does not contain the erythromycin resistance marker used for selection in lactococci. The *abiA** gene was therefore subcloned in the following manner to create another integration vector. Plasmid pTRK364 was digested with *Eco*RI and partially digested with *Hin*dIII, and the 8.6-kb *abiA*-NCK625 fragment was purified from an agarose gel. This fragment was ligated to the 4.2-kb *Eco*RI-*Hin*dIII fragment from pTRK145 (29), which contains IS*946* and an Em^r gene. The resulting integration vector, pTRK365, has the same restriction map and the same markers as pTRK75 except that the *abiA** gene is derived from NCK625.

Plasmid pTRK365 was electroporated into MG1363 two separate times, and a total of 12 Em^r integrants were obtained (six from each electroporation). Each contained a single pTRK365 insertion in different chromosomal locations, as confirmed by hybridization analysis (data not shown). When these integrants were challenged with the phage sk1, 7 of the

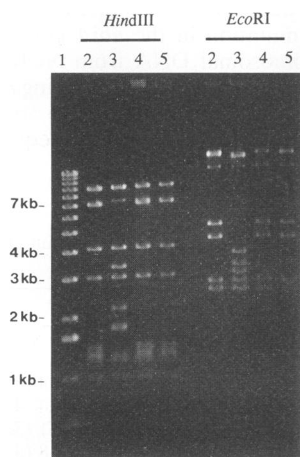

FIG. 5. Restriction enzyme patterns of φ31 and its AbiA^r derivatives. Lane 1, molecular size markers; lane 2, φ31 DNA; lane 3, φ31.3 DNA; lanes 4 and 5, AbiA^r φ31 point mutants derived from a small (lane 4) and a large (lane 5) plaque after phage φ31 infection of NCK360(pTRK363).

12 integrants (A to G) gave plaques similar in size to those of 17 of the original pTRK75 integrants (Table 3). The remaining five pTRK365 integrants (H to L) gave plaques that were reduced in size, characteristic of NCK625. If the *abiA* gene had been altered in NCK625, all of the pTRK365 integrants would exhibit the reduced plaque size seen in NCK625. Since this was not the case, these data eliminate the possibility that the increased phage resistance observed with NCK625 is due to a mutation in *abiA*.

Another possibility examined was that the pTRK75 insert in NCK625 occurred downstream of a strong promoter sequence in the MG1363 chromosome, causing increased transcription of the *abiA* gene. To study this, the level of transcription of *abiA* was examined by hybridizing the ^{32}P-labeled *Xba*I *abiA* internal fragment to total RNA from several *abiA*-containing MG1363 derivatives. Total RNA was isolated from strains MG1363, MG1363(pTRK18), MG1363(pTRK362), MG1363 (pTRK363), NCK624 integrant 1, NCK625 integrant 5, and pTRK365 integrants A and H. A standard amount of each total-RNA sample (20 μg) was applied to a nylon membrane with a slot blot apparatus and then hybridized to the *abiA*-specific probe.

The results from three trials indicate that approximately 2.5 times more RNA is expressed from *abiA* in NCK625 and pTRK365 integrant H than from *abiA* in NCK624 and integrant A (Fig. 6). This suggests that a stronger promoter upstream of the *abiA* gene is directing increased expression of *abiA* in these strains, resulting in higher RNA levels and greater phage resistance. Figure 6 also shows the levels of RNA produced when *abiA* is present on multicopy plasmids.

DISCUSSION

The *abiA* gene was cloned from pTRK18 into the IS*946*-based integration vector pTRK74. The resulting plasmid, pTRK75, contains *abiA* and has the ability to integrate into the lactococcal genome. The effect of *abiA* on phage resistance when present at one copy per chromosome was determined by challenging chromosomal pTRK75 integrants of *L. lactis* MG1363 and NCK203 with phages. Three *abiA*-containing plasmids with various copy numbers were also introduced into both strains and evaluated for their effect on phage resistance. The level of phage resistance conferred by *abiA* to all phages tested is dependent on the number of copies of this gene present in the cell. As the *abiA* copy number increases, phage resistance increases.

The single-copy *abiA* phenotypes observed by Casey et al. (4) were similar to those in this study except for phage c2, for which we did not observe a reduction in plaque size. After

amplification of an *abiA*-containing plasmid (pCI194) which was integrated into the *L. lactis* chromosome, Casey et al. (4) observed little increase in resistance to phages 712 (EOP = 10^{-1}) and c2 (slightly reduced plaque size). The gene copy number was not determined after amplification of pCI194, but the data in the present study suggest that *abiA* was present in only a few copies.

In this study, we dramatically improved the effectiveness of AbiA by placing the *abiA* gene on a high-copy-number plasmid. Phages sk1 and p2 are completely inhibited by pTRK363, as they are by pTR2030, the lactococcal plasmid on which *abiA* was identified. The prolate-headed phage c2, whose EOP is reduced to only 0.3 by pTR2030, was significantly inhibited by pTRK363 (EOP = 1.0×10^{-5}). In the NCK203 background, pTRK363 inhibited ϕ31 to an EOP of 3.0×10^{-4}. Plasmid pTR2030 completely inhibits ϕ31, but this phage is also sensitive to the restriction and modification system present on this plasmid. Identical results have recently been obtained with a high-copy-number vector (same replicon as pTRK363) containing a 2.2-kb PCR product which consists only of the *abiA* structural gene and its expression signals. Therefore, sequences flanking *abiA* in pTRK363 are not contributing to the phenotypes observed.

In MG1363, some single-copy chromosomal integrants analyzed (NCK625 and pTRK365 integrants H to L) had a slightly higher level of phage resistance than others. Examination of the level of *abiA*-specific RNA produced in these strains indicates that the observed phenotype is due to increased expression of the gene, possibly due to a strong promoter located upstream of the insert. This indicates that future attempts to clone this and other phage resistance genes downstream from strong lactococcal promoters will increase the resistance level conferred.

The present study also demonstrates the utility of an IS*946*-based vector for marking and retrieving cryptic plasmids. A pTRK75 insertion into the native 7.5-kb plasmid of NCK203 allowed the isolation of the cointegrate plasmid (pTRK362) in *E. coli*. The IS*946*-based integration vectors constructed (pTRK75, pTRK74, and pTRK145 [29]) contain a gram-negative *ori* and a chloramphenicol resistance gene, which allow plasmids or cloned chromosomal sequences to be isolated in *E. coli* for further characterization. Also, integrating genes into native plasmids may provide a higher-copy alternative to chromosomal integration when attempting to stabilize genes within a cell.

The appearance of AbiA-resistant ϕ31 derivatives and partially AbiA-resistant c2 derivatives after infection of strains containing pTRK363 suggests that high-copy-number *abiA* plasmids, when used alone, may not increase the longevity of starter cultures. However, high-copy-number *abiA* in combination with other phage defense systems, which, ideally, act at different points of the lytic cycle, should contribute significantly to the effectiveness of defense systems engineered for dairy starter cultures.

As plasmids encoding phage resistance are discovered or constructed and subsequently used to create phage-insensitive strains of lactococci, it is important to consider the copy number of the plasmid. In this study, we have demonstrated that the copy number of the abortive phage resistance gene *abiA* can have a dramatic impact on the phage resistance phenotype observed. Increased expression of *abiA* also increases phage resistance. Future strategies for increasing phage resistance can incorporate both increased gene copy number and increased expression.

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