Abortive Phage Resistance Mechanism AbiZ Speeds the Lysis Clock To Cause Premature Lysis of Phage-Infected Lactococcus lactis

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Received 23 June 2006/Accepted 19 September 2006

The conjugative plasmid pTR2030 has been used extensively to confer phage resistance in commercial Lactococcus starter cultures. The plasmid harbors a 16-kb region, flanked by insertion sequence (IS) elements, that encodes the restriction/modification system LlaI and carries an abortive infection gene, abiA. The AbiA system inhibits both prolate and small isometric phages by interfering with the early stages of phage DNA replication. However, abiA alone does not account for the full abortive activity reported for pTR2030. In this study, a 7.5-kb region positioned within the IS elements and downstream of abiA was sequenced to reveal seven additional open reading frames (ORFs). A single ORF, designated abiZ, was found to be responsible for a significant reduction in plaque size and an efficiency of plaquing (EOP) of 10^{-6}, without affecting phage adsorption. AbiZ causes phage φ31-infected Lactococcus lactis NCK203 to lyse 15 min earlier, reducing the burst size of φ31 100-fold. Thirteen of 14 phages of the P335 group were sensitive to AbiZ, through reduction in either plaque size, EOP, or both. The predicted AbiZ protein contains two predicted transmembrane helices but shows no significant DNA homologies. When the phage φ31 lysis and holin genes were cloned into the nisin-inducible shuttle vector pMSP3545, nisin induction of holin and lysis caused partial lysis of NCK203. In the presence of AbiZ, lysis occurred 30 min earlier. In holin-induced cells, membrane permeability as measured using propidium iodide was greater in the presence of AbiZ. These results suggest that AbiZ may interact cooperatively with holin to cause premature lysis.

The susceptibility of starter cultures to bacteriophage infection remains a problem in the cheese industry, especially with increasing reliance on defined starters and the high turnover in factories (8). Analysis of phage resistance mechanisms in starters has led to the identification of four categories of natural bacteriophage resistance in lactococci: (i) interference with phage adsorption, (ii) interference with phage DNA injection, (iii) DNA restriction/modification (R/M), and (iv) abortive infection. Numerous plasmids encoding one or more of these systems have been isolated from commercial starter strains (2, 7, 8). Abortive infection defenses (Abi) cause a disruption of phage development postinfection, resulting in a decrease in the number of infective particles released and death of the infected cells (7, 8). Nonsterile milk fermentations provide an evolutionary proving ground, in which lactic acid bacteria are exposed to constant challenge from bacteriophages. Phage defense mechanisms are often encoded on plasmids, many of which are conjugative, facilitating their transfer within starter culture populations.

There are three established groups of phages infecting L. lactis: groups c2 and 936 are composed of lytic phages which are highly homologous within the groups, while the P335 group contains both lytic and lysogenic members and is characterized by mosaic genomes showing evidence of many genetic exchanges (7, 8). The 20 Abi systems identified thus far target one, two, or all three phage groups (7, 8). Defense mechanisms include interference with phage DNA replication (AbiA, AbiF, AbiK, AbiP, AbiR, and AbiT), capsid production (AbiC), or packaging (AbiE, AbiF, and AbiQ). AbiB is an RNase, AbiD1 interferes with a phage RuvC-like endonuclease, and AbiU delays phage transcription. Recently, a new three-gene system linked with R/M activity that requires an associated methylase to protect the host from AbiR action has been described (55).

Lactococcus lactis ME2 is a prototype phage-resistant strain which has been used successfully in the cheese industry (31). ME2 contains at least three plasmids which encode distinct phage defenses (2, 31). Plasmid pME0030 interferes with phage adsorption. Two self-transmissible plasmids, pTR2030 and pTN20, each encode at least one R/M system and one Abi system. Plasmid pTR2030 has previously been partially subcloned and sequenced to reveal an R/M system, LlaI, and the abortive gene abiA (originally designated hisp) (26, 39). However, testing of AbiA and R/M subclones of pTR2030 suggested that a third mechanism could be encoded on this plasmid (27, 32, 39, 42). Additional subcloning and sequencing of pTR2030 revealed a novel abortive resistance mechanism downstream of abiA. This study describes the genetic and phenotypic characterization of this new Abi mechanism on pTR2030.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Table 1 lists the strains, plasmids, and bacteriophages used in this work. L. lactis strains were grown at 30°C in M17 medium (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose (M17G) and erythromycin (Em) (1.5 μg/ml) or chloramphenicol (Cm) (5 μg/ml) as needed. Escherichia coli was grown in Luria-Bertani medium (43) or brain heart infusion medium (Difco Laboratories) supplemented with Em (50 μg/ml) or Cm (15 μg/ml) as needed. Phages were propagated on L. lactis, and titers were determined by standard methods (50). Efficiencies of plaquing (EOPs) were calculated by dividing the number of PFU per ml for each phage plated on a phage-resistant strain by the number of PFU per ml on the sensitive
indicator strain. Plaque diameters were measured with calipers and expressed as the average size of 10 individual plaques. Individual plaques were propagated by picking into 3.5 to 5 ml of M17G containing 10 mM CaCl2 and an inoculum of 35 to 50 /H9262l from an overnight culture of the sensitive L. lactis host. The tubes were incubated at 30°C until lysis occurred and then were centrifuged to pellet debris. Phage lysates were then filtered through a 0.45- /H9262m-pore-size syringe filter (Nalgene Co., Rochester, NY). Cell survival following phage infection (at a multiplicity of infection [MOI] of 2) and phage adsorption determinations (MOI of 10 /H110024) were conducted as described previously (4, 44, 47). Lysis curves were conducted by growing 9-ml cultures of lactococcal cells to an optical density at 600 nm (OD 600) of 0.4 to 0.5 and adding phage lysate at an MOI of 1350 /H113505 and CaCl2 to a concentration of 10 mM. Cells and phage were incubated at 30°C, and OD600 was determined at 15-min intervals. Center-of-infection assays and single-step growth curves were conducted as described previously (47).

### DNA isolation and manipulations

E. coli plasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN Inc., Valencia, CA) or the Perfectprep plasmid minikit (Eppendorf, Westbury, NY). L. lactis plasmid DNA was isolated using a modified alkaline lysis procedure (38) or using the QiAprep spin mini-prep kit as described previously (16).

PCR products were generated by using Taq DNA polymerase, the Expand high-fidelity PCR system, or the Expand Long Template PCR system obtained from Roche Applied Science (Indianapolis, IN), as directed by the manufacturer. PCR primers were designed using Primer Designer software (Scientific and Educational Software, Durham, NC) and obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Reactions were performed using a Perkin-Elmer model 2400 GeneAmp PCR system (Perkin-Elmer, Foster City, CA), with an initial denaturation step at 94°C for 5 min and then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 to 8 min. A final extension step for 8 to 15 min was at 68°C. PCR products were purified by using the QiAquick PCR purification kit (QIAGEN).

Restriction endonuclease digestions were performed as described by Sam-brook et al. (43). For gene cloning, DNA fragments were isolated from agarose gel slices with the Zymoclean Gel DNA recovery kit (Zymo Research, Orange, CA). Ligations were carried out using the Fast-Link DNA ligation and screening protocol provided by the manufacturer.

### TABLE 1. Strains, plasmids, and bacteriophages used in this work

<table>
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<th>Bacterial strain, plasmid, or phage</th>
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* Ap', ampicillin resistance; Cm', chloramphenicol resistance; Em', erythromycin resistance; Kn', kanamycin resistance.
Kit (Epicenter Technologies, Madison, WI). Electroporation of *E. coli* and *L. lactis* cells was performed as described previously (14). In some cases competent *E. coli* cells were prepared using the Zymo Research Z-Competent *E. coli* transformation buffer set according to the manufacturer's directions. Cloning ligation mixes were initially transformed into *E. coli*. Plasmid DNA was extracted from isolates and examined by sequencing or by restriction enzyme digestion to confirm the correct size of insert DNA and then was transformed into lacticoccal strains for plating and phenotypic evaluation.

**Construction of subclones.** Initial subclones of the pTR2030 region downstream of *abiA* (Fig. 1) were constructed in pTRKH2 with PCR-amplified inserts. Genomic DNA from NCK391 was used as the template, with the right primer GATCCGATCCAGGCGGACCAATTTTATTTT (subclone A), GATCCGATCCAGGCGGACCAATTTTATTTT (subclone B), GATCCGATCCAGGCGGACCAATTTTATTTT (subclone C), and GATCCGATCCAGGCGGACCAATTTTATTTT (subclone D). PCR products were digested with BamHI/PstI and inserted into similarly digested pTRKH2. Subclones E and F were constructed from subclone D through partial digestion and religation using restriction enzymes EcoRV and BglII, respectively. The second set of subclones shown in Fig. 2 was constructed similarly digested pTRKH2. Plasmid DNA was extracted and digested with BamHI/XbaI and ligated into similarly digested pMSP3545.

**Cloning activity.** The subclone pTRKH686abiZ was constructed with a PCR product amplified from NCK391 genomic DNA with the primers GATCTCTAGACGGGCGGAGTTGATTACCATGTG and GATCTCTAGATTGTATGATTATGAATATATTATATTT. The PCR product was digested with XbaI and ligated into similarly digested pMSP3545. A series of left primers: GATCTCTAGACGGGCGGAGTTGATTACCATGTG and GATCTCTAGATTGTATGATTATGAATATATTATATTT. The PCR product was digested with XbaI and ligated into similarly digested pTRKH686 plasmid DNA. To combine pTRKH686abiZ with the nisin-inducible holin and lysin plasmids, competent NCK203(pTRK686abiZ) cells were prepared and electroporated with the pMSP3545 plasmids. AbiZ activity was confirmed in CmR and EmR transformants by using plaque assays.

**Sequencing.** Partial sequencing of pTR2030 was carried out the North Carolina State University Genome Research Laboratory as part of a larger project. The region of pTR2030 downstream of *abiA* was PCR amplified using primers homologous to the C-terminal end of *abiA* (CGTAAGTCACTCTAGTGCTG TCTTACTGCA) and to IS946 (AGTAACCAACAGCGACAATCACAT TCTTACTGTG). The 7.5-kb product was separately digested with restriction enzyme ApoI, RsaI, or Sau3A1 and shotgun cloned into EcoRI-, EcoRV-, or BamHI-digested pZErO-2 (Invitrogen, Carlsbad, CA), respectively. The ABI Prism TOP10 transformation transformation isolates were initially picked into 384-well plates by a Genetix Qbot robot (Boston, MA). Plasmid DNA was prepared in a 96-well plate format using a QIAGEN BioBot 9600 liquid-handling robot (Valencia, CA), followed by cycle sequencing on a Perkin-Elmer 9700 thermocycler (Applied Biosystems, Foster City, CA). The ABI Prism Ready Reaction sequencing kit was used according to the manufacturer's instructions with the T7 promoter primer (20-mer; Promega, Madison, WI). Sequencing reaction products were purified with QIAGEN DyeEX Dye-Terminator removal system and read on the Perkin-Elmer Prism 3700 sequencer. After sequence assembly using Phred and Consed software (17, 18, 21), gaps between the initial contigs were closed using PCR products produced from the ends of the contigs. All identified ambiguities or single-stranded regions were also resolved to obtain the complete double-stranded sequence of the 7.5-kb region. The sequence was analyzed using Clone Manager 6.0 and GAGAAAACACCCGCGGAAGC. The PCR products were digested with NcoI and XbaI and ligated into similarly digested pMSP3545.

**Subclone EOP Plaque Size**

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<th>Plaque Size</th>
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<tr>
<td>B</td>
<td>9 x 10⁻⁴</td>
<td>variable</td>
</tr>
<tr>
<td>C</td>
<td>1 x 10⁻⁴</td>
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<tr>
<td>D</td>
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</tr>
<tr>
<td>E</td>
<td>4 x 10⁻⁶</td>
<td>pinpoint</td>
</tr>
<tr>
<td>F</td>
<td>1.0</td>
<td>normal</td>
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</tbody>
</table>

**FIG. 1.** Partial map of pTR2030 showing the region between the two copies of IS946. The previously sequenced region including the R/M operon and *abiA* (ORFs are shown with dark gray arrows) (GenBank accession no. U17233) is included, along with the 7.5-kb downstream region in pTR2030 sequenced for this study (ORFs are shown with light gray arrows). Putative gene functions and the 310-base-pair region sharing homology with regions near other *abi* genes are indicated. The lengths and widths of the black bars shown above the sequence indicate the lengths and relative strengths of RNA transcripts across the region as determined by Northern blotting and slot blotting. Subcloned regions are indicated with lines below the map. EOPs and plaque morphologies for the subclones are shown.
Sequence walking on the phage φ31 genome was accomplished using phage DNA isolated as described previously (14) and digested with restriction enzyme EcoRV or HindIII. The digested DNA was heated for 15 min at 75°C to inactivate the enzymes and then separately ligated with pBluescript KS(+) plus mid digested with the same enzymes. The ligation reaction products were used as templates for PCRs with a primer homologous to the end of the known φ31 sequence (ATTGCTTAGGAAGATGCCGGTGTG) and the M13 forward or reverse primer homologous to pBluescript. PCR products were purified from gel bands using the Zymoclean Gel DNA recovery kit (Zymo Research, Orange, CA) according to the manufacturer's instructions and were sequenced by Davis Sequencing (Davis, CA). Additional primers were ordered as needed for continued walking using the ligation mixes as templates. The additional sequence has been used to update GenBank record AF022773.

**RNA isolation and hybridization.** Control L. lactis NCK203(pTRK2H2) cells and NCK203(pTRK2H2 subclone D) cells were cultured to an OD600 of 0.6 to 0.7 in 15 ml of M17G broth. A 6-ml aliquot was removed from each culture, pelleted, and quick frozen for RNA extraction. Total cellular RNA was isolated using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) as described previously (10). PCR products for making probes were amplified using the following primer pairs: abeA, GATCCTCTAGATTTTGAGCTAATGTTA and ACCCAT CTATTAACCTTTCTCTTC; integrase, GATCGGATGCAAATTTTCTTGTTCT; ORF I, GGGGCTGAGGCTATCTCTCCT; ORF X, GATCGGATCCAATATCC; ORF Y, GATCGGATCTTTTGGTAGCTAAAATGGTA and ACCCAT CTATTAACCTTTCTCTTC. PCR products were amplified using the following primer pairs: abeA, GATCCTCTAGATTTTGAGCTAATGTTA and ACCCAT CTATTAACCTTTCTCTTC; integrase, GATCGGATGCAAATTTTCTTGTTCT; ORF I, GGGGCTGAGGCTATCTCTCCT; ORF X, GATCGGATCCAATATCC; ORF Y, GATCGGATCTTTTGGTAGCTAAAATGGTA and ACCCAT CTATTAACCTTTCTCTTC. PCR products were amplified using the following primer pairs: abeA, GATCCTCTAGATTTTGAGCTAATGTTA and ACCCAT CTATTAACCTTTCTCTTC; integrase, GATCGGATGCAAATTTTCTTGTTCT; ORF I, GGGGCTGAGGCTATCTCTCCT; ORF X, GATCGGATCCAATATCC; ORF Y, GATCGGATCTTTTGGTAGCTAAAATGGTA and ACCCAT CTATTAACCTTTCTCTTC. Restriction enzyme digestion of the PCR products was used to confirm PCR product identities. PCR products were purified from gel bands using the Zymoclean Gel DNA recovery kit (Zymo Research, Orange, CA) according to the manufacturer's instructions and were sequenced by Davis Sequencing (Davis, CA). Additional primers were ordered as needed for continued walking using the ligation mixes as templates. The additional sequence has been used to update GenBank record AF022773.

Sequencing of pTR2030 in the region between abiA and IS946. In a previous study, a 13.6-kb BglII fragment of pTR2030 was cloned into shuttle vector pSA3 to produce plasmid pTK6 (30). This fragment encodes the phage resistance determinants for the LlaI restriction/modification operon and also carries the abortive gene abiA (26, 39). However, phage resistance from pTK6 (EOP of 10−8) with phage φ31 is expressed at a lower level than that from pTR2030 (EOP of <10−10 with phage φ31) (32). The R/M operon and abiA gene encoded on pTK6 have been sequenced (Fig. 1) (25, 39). As a starting point for identification of another phage resistance element, we sought to determine the remaining sequence of the region which is shown in Fig. 1, between the two copies of IS946 in pTR2030.

The region between abiA and the downstream copy of IS946 was PCR amplified, sequenced, and analyzed. Seven predicted open reading frames were identified in the region between
abiA and IS946 (Fig. 1). In BLAST searches, the 5′ end of the DNA sequence was identical to sequence from the 60-kb conjugative, bacteriocin-producing plasmid pMRC01 from L. lactis DPC3147, with homology to hypothetical ORFs 24 and 25 of that sequence (12). The ORF designated ORF 25 in pMRC01 encodes a potential shikimate kinase (clusters of orthologous groups of proteins, AroK) with an N-terminal double-glycine peptidase domain indicative of ABC-type exporters. In pTR2030, following the region of pMRC01 homology, are two hypothetical ORFs, ORFs X and Y. The predicted 222-amino-acid ORF X protein (pI 4.85) had homology to PcrL, a hypothetical protein of *Enterococcus faecalis* V583 (1e−34). The predicted ORF Y protein (432 amino acids; pI, 5.2) had homology to a hypothetical protein of *Hahella chejuensis* KCTC2396 (6e−11). There are numerous inverted repeats located in the region between these two ORFs. A very large 46-bp inverted repeat follows ORF Y, which encodes two putative helix-turn-helix motifs (Center for Biological Sequence Analysis, Technical University of Denmark; http://www.cbs.dtu.dk/services/TMHMM-2.0/). The transmembrane helices were positioned at residues 297 to 319 and 334 to 356 within the ORF Y predicted protein. While the GC content of the entire 16.4-kb pTR2030 region was 32.9%, ORF X had a GC content of 35.8%, whereas ORF Y had 29.6% GC. The next two ORFs comprises genes for a putative two-component transposase, followed by a putative integrase gene and *abiA*.

**Subcloning of *abiA* and downstream ORFs.** The region including *abiA* and downstream genes was subcloned into the lactococcal shuttle vector pTRKH2, and the resulting plasmid (subclone D in Fig. 1) was transformed into NCK203. When challenged with phage ϕ31, pinpoint plaques were observed but were uncountable, allowing only an estimate of an EOP of <3 × 10^{−8} (Fig. 1). In contrast, phage ϕ31 plaqued on pTRKH2-abiA at an EOP of 8 × 10^{−4}. Subclones A, B, C, E, and F were constructed in order to localize the source of the additional phage resistance. Subclone E had an EOP of 4 × 10^{−6}, whereas subclone F had an EOP of 1. Thus, an additional phage resistance determinant was positioned in the region of ORF Y. Northern blotting and RNA slot blotting were performed to determine the lengths and relative intensities of transcripts from the region (Fig. 1). All ORFs except that for the putative integrase were expressed on five separate transcripts, with the highest level of expression from the ORF 24-ORF 25 and ORF Y transcripts.

A second set of subclones of the ORF X-ORF Y region was constructed in pTRKH2 to determine the smallest fragment encoding the phage resistance activity located around ORF Y (Fig. 2). The 1.8-kb noncoding region between the two genes contains a 310-base-pair region with homology to several lactococcal sequences, including 309-bp direct repeats flanking *abiGi* and *abiGii* (35). The same region of homology is found in close proximity to cadC genes of pAH82 (36) and two *Streptococcus thermophilus* cadC coding regions (a genomic island of strain CNRZ368 [40] and *S. thermophilus* strain 4134 [46]). The region is also found upstream of lactococcal phage resistance genes *abiTi* (5) and *abiLi* (9) and in plasmid pNZ4000 (51, 52). Upstream of the 310-bp region in pTR2030 there are three stem-loops with energies of −25.1, −19.2, and −12.3 kcal, and a stem-loop with an energy of −23.0 kcal lies just downstream of ORF Y (Fig. 2). Subclones of the region were designed to determine whether these various features had an effect on phage resistance. Subclones A1, A2, and B2, which excluded ORF Y, had no phage resistance activity (EOP = 1). Conversely, subclones B3, D3, D4, E3, and E4 containing ORF Y reduced the EOP of phage ϕ31 to 10^{−5} to 10^{−6}. The presence of the 310-bp region upstream of the stem-loop downstream of ORF Y did not significantly enhance activity. The smallest subclone with activity was E4, which carried ORF Y and had an EOP of 5 × 10^{−6} for ϕ31. This subclone was selected for use in further analysis of the phage resistance phenotype encoded in ORF Y. ORF Y is expressed constitutively from its native promoter.

**Characterization of the new phage resistance phenotype.** Several experiments were conducted to determine the type of phage resistance encoded by ORF Y. The survival of NCK203 (pTRKH2) and NCK203(pTRKH2:E4) was determined to be 3.4% and 11%, respectively, after infection with phage ϕ31, indicating that most infected cells die even though the cells with AbiZ restrict plaquing to ϕ31. Adsorption of ϕ31 was not affected by ORF Y, as phage adsorption to both strains was 98%. Phage ϕ31 propagated in broth on NCK203(pTRKH2: E4) was not resistant in subsequent plaquing on the same strain, indicating that an R/M system was not involved. Comparable amounts of replicating phage ϕ31 DNA were visible on agarose gels of digested DNA isolated at timed intervals from ϕ31-infected NCK203 cells with and without pTRKH2:E4 (data not shown). We conclude that the new phage resistance encoded in ORF Y is not due to failure of adsorption or injection of ϕ31 DNA or to a restriction/modification system. The new phage resistance gene, designated *abiZ*, encodes an abortive infection resistance which results in cell death after infection and appears to limit the production of progeny phage.

**Range of AbiZ activity.** EOPs and plaque sizes of several P335 group phages were determined on NCK203(pTRKH2: E4). The results are summarized in Fig. 3. In general, the P335 group phages exhibited a range of sensitivities for AbiZ, as observed by reductions in plaque size and/or EOP. Interestingly, phages ϕ31.1 and ϕ31.2, which are identical to phage ϕ31 except in the origin region (14), showed sensitivities different from that of ϕ31. This information indicates that sensitivity to AbiZ is at least partially determined by early or middle phage genes. The closely related phages ul36 (EOP = 6 ×
and ul37 (EOP = 0.95) also showed marked differences. To determine the effect of AbiZ on other groups of lactococcal phages, *L. lactis* MG1363 was transformed with pTRKH2:E4 and tested with the 936 group phages sk1 and p2 and the c2 group phage c2. Both of the 936 group phages had EOPs of 0.8 and slightly reduced plaque sizes. Phage c2 plated at an EOP of 1 with normal plaque sizes. In contrast, the two 936 group phages and phage c2 are sensitive to AbiA (Fig. 3). Therefore, while AbiZ is effective primarily against P335 type phages rather than the 936 or c2 types, AbiA is effective against phages of all three types.

**Mechanism of AbiZ.** To investigate phage propagation with and without AbiZ, single-step growth curves were conducted. The average burst size of φ31 on NCK203(pTRKH2) of 316 ± 68 was reduced by two logs to 3 ± 1.4 for NCK203(pTRKH2:abiZ) (Fig. 4, left). The efficiency of center-of-infection assays was reduced to 0.75. Both the single-step growth curves and lysis curves (Fig. 4, right) indicated that φ31-infected NCK203(pTRKH2:E4) lysed earlier than NCK203(pTRKH2). Therefore, AbiZ appeared to be different from all Abi systems identified previously, as none of the abortive genes so far have been reported to cause early lysis.

When phage φ31 is plaqued on NCK203(pTRKH2:E4), small plaques dominate the cell lawn. Two large and two small plaques picked from lawns of NCK203(pTRKH2:E4) were propagated on NCK203 and used to challenge NCK203(pTRKH2:abiZ). Phage isolated from the two small plaques remained sensitive to AbiZ. However, phage isolated from the two large plaques were resistant, at EOPs of 1 (isolate φ31-M2) and 0.02 (isolate φ31-M1). Lysis curves were produced by following the OD₆₀₀ and EOP₉₀₀ after infection of NCK203(pTRKH2) and NCK203(pTRKH2:E4) with φ31 and the two phage mutants. In the presence of AbiZ, lysis curves for φ31-M1 and φ31 were almost identical. However, φ31-M2 lysis was more accelerated (Fig. 5, left). In the presence of AbiZ (Fig. 5, right), the results were much more variable. Notably, both φ31 and φ31-M1 started to lyse the cells earlier, between 15 and 30 min after infection, whereas phage φ31-M2 showed a lysis curve similar to that of the AbiZ control. These data suggested that in the presence of AbiZ, phages φ31 and φ31-M1, but not φ31-M2, lysed prematurely.

As a result of the differences in lysis onset between the three phages in the presence of AbiZ, sequence walking was employed to determine the complete sequence of the lysin and holin genes. Phage φ31 and the mutants, φ31-M1 and φ31-M2 had identical sequences across the holin and lysin genes (data not shown).

**Investigation of AbiZ-holin/lysin interactions.** In order to investigate the potential effect of AbiZ on the timing of lysis, the phage φ31 holin and lysin cassette was cloned into the
nisin-controlled expression vector pMSP3545, and abiZ was cloned for in trans expression into the Cm’ shuttle vector pTRK686 (49). Plasmid pMSP3545 contains a nisin-inducible promoter (PnisA) and genes encoding NisR and NisK, the two-component signaling mechanism for activating transcription in the presence of nisin (6). When induced with 25 ng/ml of purified nisin, NCK203(pMSP3545:HL) was subject to partial lysis in the absence of phage infection. The optical densities of induced cultures, with and without pTRK686:abiZ, were followed over time (Fig. 6, left). In addition, the cultures were plated to obtain numbers of viable cells (Fig. 6, right). No effects were observed when nisin was absent. However, nisin induction of holin and lysin resulted in detectable cell lysis. The measures of both optical density and viable cell count showed that holin- and lysin-induced lysis in the phage-free system occurred 30 min earlier when AbiZ was present. Therefore, the presence of AbiZ accelerates holin- and lysin-induced cell lysis.

In order to determine the effect of holin alone, the phage φ31 holin gene was cloned into pMSP3545 without the lysin gene. Cell membrane permeability was assayed by adding propidium iodide to cells induced, or not induced, with nisin for holin expression. Propidium iodide is a fluorescing nucleotide-binding probe, which enters only cells with damaged membranes (53). Nisin induction of holin resulted in a considerable increase in fluorescence over that of the controls. Moreover, AbiZ’ cells were more permeable than AbiZ cells after holin induction (Fig. 7). These results suggest that AbiZ may interact cooperatively with the phage φ31 holin to cause premature lysis.

**DISCUSSION**

To our knowledge, AbiZ is the first lactococcal phage resistance determinant found to induce premature lysis in phage-infected cells. Premature lysis causes the infected cells to burst and release the developing phage before many phage particles are completely assembled. Therefore, although the infected cells die, the number of infective particles released from a bursting cell is reduced by two logs. This is a significant reduction, which along with a reduction in numbers of infective centers of 0.75, resulted in an EOP of $10^{-6}$ for P335 group phage φ31. All but one of the other 13 P335 group phages tested were sensitive to AbiZ to some degree (Fig. 3). The two 936 group phages were largely resistant to AbiZ, as was c2 group phage c2. P335 group phages, which include both lytic and temperate members, are highly recombinogenic and have recently been isolated from cheese factories with increasing frequency (8).

Plasmid pTR2030 also encodes AbiA and the type IIA R/M system LlaI. The discovery of AbiZ positions three distinct defenses encoded within a region of 16 kb flanked by IS946.

The completed sequence between the IS elements also encodes a putative transposase and integrase. The presence of the putative mobility genes between two IS elements suggests that the entire region would likely function as a mobile phage resistance cassette, over and above the mobility conferred by the high-frequency conjugal plasmid pTR2030. However, no evidence exists for transfer of this phage resistance cassette per se, as closely homologous sequences of the entire region were not found in BLAST searches of DNA or protein databases. Interestingly, a 310-base-pair region adjacent to abiZ shares a high degree of homology with sequences located in close proximity to a number of other abortive resistance genes. However, the homologous region was not necessary for the function of AbiZ, nor did it enhance function. In addition, current analysis shows a significant alignment of abiA with the reverse transcriptase family of proteins pfam00078. The active domain identified in the search corresponds to the region previously iden-
tified as a leucine repeat motif (11). This region was essential for AbiA activity. Similar results have been obtained for AbiK, which has homology to AbiA (2e−05) (19).

The three phage defense systems of pTR2030 are expressed constitutively on separate transcripts. They target the complete range of phage development, from the hydrolysis of incoming infecting phage DNA (LiaI) to inhibition of phage DNA replication (AbiA) to accelerating the timing of lysis (AbiZ). AbiA and AbiZ are also complementary in that the sets of phages inhibited by each defense are separate but some phages overlap in their sensitivities to both Abis. Combinations of phage resistance defenses are commonly found in lacticoccal strains. L. lactis ME2, the original source of pTR2030, also contains pTN20, which encodes a R/M system and abortive defense (13), as well as pME0030, which encodes resistance to phage adsorption (31). The use of multiple barriers to phage infection and development within a starter culture strain or within a group of rotated cultures has long been used to control phages in cheese factories (8, 15, 33, 48).

The mode of action of AbiZ appears to be at least partially attributable to some sort of cooperative interaction with phage holin. Several lines of evidence are presented here to support this conclusion. First, AbiZ is responsible for premature lysis, seen in phage ϕ31 single step growth curves as well as lysis curves of ϕ31-infected NCK203. Premature lysis was also observed with a second P335 group phage, Q30 (data not shown). Second, the AbiZ-resistant ϕ31 mutant, ϕ31-M2, plaques normally and is not subject to premature lysis. Third, AbiZ enhances partial cell lysis caused by holin and lysis expressed in NCK203 in the absence of phage infection. Fourth, AbiZ enhances the permeability of cells in which holin alone was expressed.

The timing of phage lysis is controlled by the phage holin protein (54). Holins permeabilize the cell membrane, allowing the lysis protein access to the cell wall, followed immediately by cell wall destruction and cell lysis. Holin timing is critical for optimal phage development and is often regulated by protein inhibitors which may be encoded by separate genes or may be products of the holin gene itself, from a separate transcript initiated at a dual-start motif (54). The phage ϕ31 holin does not appear to carry a dual-start motif, so its regulatory gene, if present, is currently unknown. During the late stage of a phage infection, holin molecules are produced and accumulate in the cell membrane until a critical point is reached, at which the cell membrane potential suddenly collapses. The collapse triggers holin precipitation, allowing formation of an oligomeric complex of holin which disrupts the membrane, allowing the release of lysis (22). In coexpression studies, it has been shown that dissimilar holins, A S and T4 T, induce earlier lysis when they are produced together than when either is produced alone (41). Those authors speculate that the unrelated T and S holins physically interact at some point and act cooperatively to “kill without warning.” Sequencing of the holin genes from AbiZ-insensitive phages revealed no mutation, suggesting that there is no direct interaction between AbiZ and holin. Thus, the most likely explanation for AbiZ-induced premature lysis is that the membrane-bound protein AbiZ interacts cooperatively in some way with the phage holin or holin inhibitor to trigger holin precipitation prematurely. The varying sensitivities of the P335 group phages could be due in part to the degree to which AbiZ interacts with the different phage holins or inhibitors.

It is clear from the data presented here that some component of the early or middle genes of phage ϕ31 is also involved in AbiZ sensitivity. When the AbiZ sensitivities of ϕ31, ϕ31.1, and ϕ31.2 are compared, there are obvious differences (Fig. 3). Phages ϕ31.1 and ϕ31.2 are mutants of ϕ31, which arose through recombination with the NCK203 genome (14). The ϕ31 origin and early genes, a total of almost 9 kb of DNA, were replaced in the mutants, but the late genes of the three phages are the same. This information implies the involvement of an early or middle gene in AbiZ sensitivity. Likewise, the AbiZ-resistant ϕ31 mutants ϕ31-M1 and ϕ31-M2 were sequenced through the holin and lysin genes and found to be identical to phage ϕ31. The mutation(s) responsible for AbiZ resistance has not been located. Perhaps one of the as-yet-uncharacterized early or middle genes of phage ϕ31 encodes a holin regulatory protein. Holin regulators have been found to bind physically to holin proteins (23). A mutated or entirely different holin regulator could theoretically disrupt the cooperative action of AbiZ with the holin protein. Since mutants ϕ31-M1 and ϕ31-M2 have different levels of resistance to AbiZ, two different mutations are probably involved. The fact that ϕ31-M2 develops more quickly than M1 may help it evade the mechanism of AbiZ, resulting in almost complete loss of sensitivity.

Mutant phages resistant to a number of different abortive and R/M phage defense systems have been recovered (8). Thus, the need to characterize new defenses continues. Combinations of defenses through rotations and other strategies have worked well in recent years to minimize the problem of phage counterdefense (8). AbiZ is a highly effective and unique defense which complements AbiA and LiaI and may be useful in combination with other defense systems.

ACKNOWLEDGMENTS

This study was partially supported by Danisco, USA, Inc., and the North Carolina Dairy Foundation.

We thank the North Carolina State University Genome Research Laboratory for assistance with DNA sequencing. We thank Mick Callanan, Eric Altermann, Andrea Azcarate, and Rodolphe Barrangou for helpful discussions and Andrea Azcarate and Sarah O’Flaherty for reviewing the manuscript.

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