Bacteriophage Resistance in *Lactococcus lactis* Engineered by Replacement of a Gene for a Bacteriophage Receptor

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ABSTRACT

The objective of this study was to construct a food-grade, phage-resistant strain of *Lactococcus lactis* by replacing a specific chromosomal gene with an allele that had been mutated in vitro. *Lactococcus lactis* contains a chromosomal gene (*pip*) that is required for infection by bacteriophages of the c2 species. A nonsense mutation in *pip* was constructed in vitro. The wild-type *pip* on the chromosome of strain LM2301 was exchanged for the mutated *pip*. The exchange left no antibiotic resistance genes or nonlactococcal DNA in the engineered strain (JK101). JK101 was resistant to the same phages as a strain that contains a spontaneous mutation in *pip*. JK101 grew as well as the *pip*$^+$ isogenic strain did in minimal or rich media.
(**Key words**: phage resistance, bacteriophage, *pip*, gene replacement)

Abbreviation key: **M17G** = M17 growth medium plus glucose, **M17G ery** = M17G plus erythromycin, **PCR** = polymerase chain reaction.

INTRODUCTION

Lactococcus lactis is an important industrial bacterium that is used to ferment milk to make Cheddar and other types of cheeses. Bacteriophage that contaminate the milk and industrial equipment pose a constant threat of failed fermentation and loss of milk. To avoid the negative economic impact of phage infections, US and New Zealand cheese factories routinely use a strategy of defined strain rotation (11, 14, 17, 18, 19, 31). This strategy uses a mixture of strains of *L. lactis*, each unrelated by phage host range, in the starter culture. The whey is monitored for plaque formation on each of the strains in the starter mixture, and any strain that forms plaques is replaced with either a phage-resistant derivative or an unrelated strain. Although this strategy and others have reduced the incidence of phage infections in the cheese factories, the problem still causes significant economic loss and has created a higher demand for phage-resistant strains.

Naturally occurring mechanisms of phage-resistance in *L. lactis* include restriction and modification systems (3, 5, 9, 25), abortive infection (10, 15, 16, 21), blockage of phage adsorption (20, 26, 27, 28), and another mechanism that apparently blocks phage penetration (6). In addition, mutations in the host DNA can prevent phage infection by altering the cell surface carbohydrates that act as phage receptors (8, 32, 34) and the plasma membrane components that are required for phage infection (33).

Our laboratory has cloned and sequenced a phage infection protein gene (*pip*) from *L. lactis* that is required for infection by many phages of the c2 phage species (7, 12). Southern hybridization analysis shows that all strains of *L. lactis* that have been examined, including subspecies *lactis* and *cremoris* and biovar *diacetylactis*, have a gene that is homologous to *pip* (1). Spontaneous phage-resistant mutants of *L. lactis* ssp. *lactis* strains C2 and ML3 were transformed with the cloned wild-type *pip* and were rendered phage-sensitive (1). These results together suggest a general role for *pip* in phage infections of *L. lactis*.

Although the cellular function of the *pip* gene product is unknown, an analysis of the deduced amino acid sequence predicts a membrane protein (7). During the early stages of phage infection, phage adsorbs to the Pip protein, causing the ejection of the phage genome from the phage head and (presumably) translocation of the phage DNA through the host membrane (22). Spontaneous mutations in *pip* render the host phage-resistant but otherwise the

Received October 7, 1996.
Accepted February 18, 1997.
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same as the phage-sensitive parental strain (7). Passage of a coculture of wild-type and *pip*⁻ strains in the presence of a composite of 10 phages fails to select for a mutant phage that might overcome the phage-resistant phenotype of the *pip*⁻ strain (1).

It would be advantageous to be able to move a mutant copy of *pip* into different strains of *L. lactis*. Not only would this move enable a strain to be engineered with a phage-resistance mechanism that is well characterized, but the requirement for *pip* in infections with other phages could be tested.

This paper reports the exchange of the wild-type, chromosomal *pip* for a mutated *pip* in *L. lactis* subsp. *lactis* LM2301. The *pip*-exchanged strain is viable and resistant to phage.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Media

L. lactis subsp. *lactis* LM2301, a plasmid-free derivative of strain C2 (35), and its phage c2-resistant derivative, BG101 (7), were grown and maintained on M17 medium (30) that was supplemented with 0.5% glucose (**M17G**) at 30°C, except as indicated. pGhost6 [(2); obtained from Appligene Co., Pleasanton, CA] and pKG1 were maintained in lactococcal strains on M17G with 5 μg/ml of erythromycin (**M17G ery**). *Escherichia coli* DH5αmcr was used for all cloning; the *E. coli* was grown at 37°C in LB medium (24) with 20 μg/ml of chloramphenicol or 100 μg/ml of ampicillin for maintenance of pRB04 or pUC19, respectively. DH5αmcr(pKG1) was grown on brain-heart infusion (Difco Laboratories, Detroit, MI) with 250 μg/ml of erythromycin and 100 μg/ml of ampicillin.

Lactococcal bacteriophage stocks were prepared from single plaques and assayed as described (32). Phages were stored in M17G medium containing 20% glycerol at –70°C.

Growth rates were determined from triplicate cultures incubated at 30°C in an orbital shaker at 300 rpm. Optical densities were measured at 600 nm every 30 min for 5 h in a Pharmacia Novaspec II spectrophotometer (Pharmacia, Piscataway, NJ). Growth in minimal medium was evaluated by culturing in BL medium (13). Mean doubling times and standard errors (n = 3) are expressed.

Site-Directed Mutagenesis

All recombinant DNA procedures were performed according to methods described by Sambrook et al. (24), except as noted. Site-directed mutagenesis of *pip* was done with polymerase chain reaction (**PCR**) and uracil DNA glycosylase as described (23) using pRB04 as template for both reactions. The PCR was conducted with an air thermocycler (Idaho Technology 1605 (Idaho Falls, ID); according to the instructions of the manufacturer). *Taq* polymerase was purchased from Promega Corp. (Madison, WI). Oligonucleotide primers were synthesized at the Center for Gene Research and Biotechnology (Oregon State University, Corvallis). One reaction amplified the 5′ end of *pip* beginning 246 bp upstream of the first nucleotide of *pip* through nucleotide 1604. This reaction was programmed with a nonmutagenic primer ATAT*TCTAGA*TTTCCCTCTTTTTTAT-CAAATAAA, which included an *Xba*I restriction sequence (italicized) plus four nucleotides at the 5′ end to improve restriction efficiency. The mutagenic primer in the first reaction was AUAUUGUUAAU-AAA*TTA*TTTATTTAAACTATTTATTTGTT, which included base substitutions that created an in-frame nonsense codon (underlined). The second reaction amplified the 3′ end of *pip* beginning with nucleotide 1593 and ending 180 nucleotides downstream from *pip*. This reaction was programmed with a nonmutagenic primer ATAT*TCTAGA*TAATGGCGTTCATTT-TATCATAATCT, which included an *Xba*I restriction sequence (italicized). The mutagenic primer in the second reaction was UAUUAACAAUAU*GGTAC-C*ACAAAGAGAAGCAATT, including base substitutions that created a *Kpn*I recognition sequence (italicized). The PCR products were isolated from agarose gels, digested with *Xba*I, mixed, and ligated to pUC19 that had been linearized by *Xba*I and treated with alkaline phosphatase. The ligation mixture was treated with uracil DNA glycosidase (37°C for 30 min), heated to 65°C for 20 min, slowly cooled to 20°C, and transformed into DH5αmcr. Transformants were screened by plasmid isolation and restriction analysis with *Xba*I.

The *Xba*I fragment, containing the *pip* allele with the nonsense mutation and *Kpn*I restriction site, was cloned into the *Xba*I site of pGhost6 and designated pKG1. The identity of the mutated *pip* allele in pKG1 was confirmed by DNA sequencing at the Center for Gene Research and Biotechnology.

Allele Replacement

Lactococcus lactis LM2301 was transformed with pKG1 as described (4) except that 40 m*M* DL-threonine and 0.5% glucose were included in the growth medium until harvest. Gene replacement of chromosomal *pip* by the mutated *pip* on the pGhost6 plasmid was initially done as described (2) except that the integrants were selected by long-term culture (at least 26 generations) at 40°C. The identity of the

integrants as LM2301 was verified by measuring their sensitivities to phage c2 as described (7) except that the growth temperature was 40°C. A single colony integrant was randomly chosen and grown for 13 generations at 30°C without erythromycin to enrich for cells that excised the pKG1 from the chromosome. One hundred single colonies were scored for sensitivity to erythromycin and resistance to phages c2 and sk1 by streaking colonies to test plates as described (7). A second method was used to confirm the sensitivity of the isolates to phage sk1 and resistance to phage c2. Early exponential phase cultures (OD_{600} = 0.1) of each isolate were infected with phages c2 and sk1 separately. Optical density readings were made hourly for 5 h. Isolates were scored as being resistant to phage if, 5 h postinfection, the $OD_{600} \geq 1.1$. Isolates were scored as being sensitive to phage if, after 5 h postinfection, the $OD_{600} \leq 0.1$.

A second procedure for gene replacement was designed to minimize the exposure of cells to high temperatures. An overnight culture of *L. lactis* LM2301(pKG1) in M17G plus erythromycin (**M17G ery**) was diluted 1:100 (vol/vol) and was incubated for 2 hr in M17G ery at 30°C. The culture was then shifted to 37°C, incubated for 2 h, again diluted 1:100 in M17G ery, incubated 4 h, again diluted 1:100 (vol/vol) in M17G ery, and incubated overnight. The overnight culture was diluted 1:100 (vol/vol) in M17G and incubated at 30°C until the stationary phase was reached (about 12 h). This last step was repeated three times, and the final culture was diluted and plated on M17G. One hundred colonies were tested for erythromycin resistance and phage sensitivity as previously described (7).

Analytical PCR

Reactions were programmed with 1 μM of each primer and 5 mg/ml of template DNA. Chromosomal DNA was prepared as described previously (7) except that 0.1 mg/ml RNase A was added for 1 h at 37°C prior to the addition of ammonium acetate.

To verify the replacement of the wild-type *pip* with the mutated *pip*, a PCR product was generated from the following primers that are homologous to internal regions of *pip*: CGGCTTGCAAATCATCAATCAAGTC-CAAAA and GCCCGATTTGATTTCAGGCCAGT-CATTTT. The predicted length of the PCR product is 1006 nucleotides, beginning with nucleotide 927 through nucleotide 1932 of *pip*. The product is predicted to span the mutated region of *pip*, including the *Kpn*I site. The PCR product was purified (QIAquick® PCR purification kit from Qiagen, Inc., Chatsworth, CA) and sequenced as just described.

The primers described in the preceding paragraph were also paired with the following primers, which are homologous to regions that normally flank *pip* in the chromosome, but were not cloned in pKG1: primers TGCAAGTAATGGTGAAGATTTAACAAAT-GAA and CAGTTAAATCTCCCATTCTTTTCATTG-TTTTA were used in separate PCR reactions to produce products of predicted sizes 2227 and 1991 when paired with the primers described in the preceding paragraph.

The PCR analysis of the ampicillin resistance gene (*bla*) and flanking sequence on pGhost6 was done with primers GGTGGCGAAACCCGACAGGAC-TATAA and GGTCGCCGCATACACTATTCTCAG-AAT. The predicted length of the product is 1297 nucleotides, beginning with nucleotide 2592 through nucleotide 3888 (numbering according to Genbank accession VB0001 for sequence of pBR322).

Analysis of the erythromycin resistance gene (*ermC*) was done with primers GAAAAGAGATAAGAATTGTTCAAAGCTAATATT and GTATTTTTGTAATCAGCACAGTTCATTATCAA. The predicted length of the product is 814 nucleotides, beginning with nucleotide 2031 through nucleotide 2844 (numbering according to Genbank accession VB0131 for sequence of pE194).

Southern Hybridization Analysis

Southern hybridization analysis (29) was done as described (24). Genomic DNA was prepared as described (7) and restricted with *Xba*I; 1 μg was applied to each lane of the gels. Three micrograms of the 1006-bp PCR product from *pip* that was described in the preceding section was labeled with digoxigenin with the Genius Kit according to the instructions of the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN). The probe was specific for *pip* and did not contain sequences that flank *pip* or vector sequences.

RESULTS

Construction of a Mutated *pip* Allele

A Tn*5* insertional mutation in *pip* had been isolated previously (7) and was located by sequencing to nucleotide 1311 of *pip*. A mutated *pip* allele was created by site-directed mutagenesis to create an in-frame, nonsense codon (TAA) beginning at nucleotide 1198. The location for the nonsense codon was selected because the location is close to the site at which the Tn*5* insertion caused inactivation of *pip* (7). In addition, a unique *Kpn*I restriction site was introduced at nucleotide 1215 to provide a means to

detect the mutated allele. The mutated *pip* was cloned into pGhost6 (pKG1).

Exchange of the Wild-Type for the Mutated *pip*

pKG1 was transformed into *L. lactis* LM2301. Integration of pKG1 into the chromosome was selected by growth in the presence of erythromycin at a temperature (40°C) that was not permissive for pKG1 replication. Subsequent resolution of the integrated pKG1 occurred during culture at 30°C in the absence of erythromycin selection. Forty-four of 100 colonies tested were sensitive to erythromycin, indicating the loss of the integrated pKG1. Twenty-eight of the 44 colonies were resistant to phage c2.

To ascertain that the 28 phage c2-resistant isolates were *L. lactis* and not contaminants, each isolate was tested by two different methods for sensitivity to phage sk1. Phage sk1 does not require *pip* for infection (1, 33). Twenty-seven of the 28 phage c2-resistant isolates were sensitive to phage sk1.

Because the method of integration resulted in an unexpected selection that was unrelated to *pip* (see next section), a second experiment to exchange *pip* was conducted under conditions that minimized exposure to the elevated temperature. Of the 100 colonies screened in the second exchange, 18 were sensitive to erythromycin. Eight of the 18 were resistant to phage c2. All 18 were sensitive to phage sk1.

PCR Analysis of the Exchanged *pip*

Four of the isolates that were resistant to phage c2 and sensitive to phage sk1 during the first exchange were picked at random for further analysis. Three isolates that were resistant to phage c2 and 3 that were sensitive to phage c2 from the second exchange were also analyzed further. Because all phage c2-resistant isolates gave identical results, only the results from 1 isolate from the first exchange (KG101) and 1 isolate from the second exchange (JK101) are shown. All isolates that were sensitive to phage c2 gave the same results as LM2301 (data not shown).

The PCR were programmed with chromosomal DNA from LM2301, KG101, JK101, or pKG1. Primers were designed to make a 1006-bp product that included the unique *Kpn*I site engineered into the mutated *pip*. The *Kpn*I site would be 292 bp from one end of the predicted product from the reaction programmed with DNA from either KG101 or JK101. The results show that each reaction produced a single product that was the same size (Figure 1). The products from reactions that were programmed with DNA from LM2301 (lane 2), KG101 (lane 4), and pKG1 (lane 6) were about 1000 bp. The product from the reaction that was programmed with LM2301 DNA was not cleaved (lane 3), but the products from the reactions programmed with KG101 DNA or pKG1 were cleaved by *Kpn*I into fragments of about 300 and 700 bp (lanes 5 and 7). All of the product from KG101 appears to have been cleaved with *Kpn*I, suggesting that only the mutant allele is present in KG101. The results with JK101 were identical to those shown for KG101 (data not shown).

The PCR product of the reaction with DNA from KG101 was sequenced. The results showed that the sequence of *pip* had been changed from the wild type to the mutated sequence as predicted, including the in-frame nonsense codon and the *Kpn*I restriction site.

Southern Hybridization Analysis

The *Xba*I-digested chromosomal DNA from LM2301 and KG101 was analyzed by Southern hybridization using a probe that was homologous to nucleotides 927 through 1932 of *pip*. The results

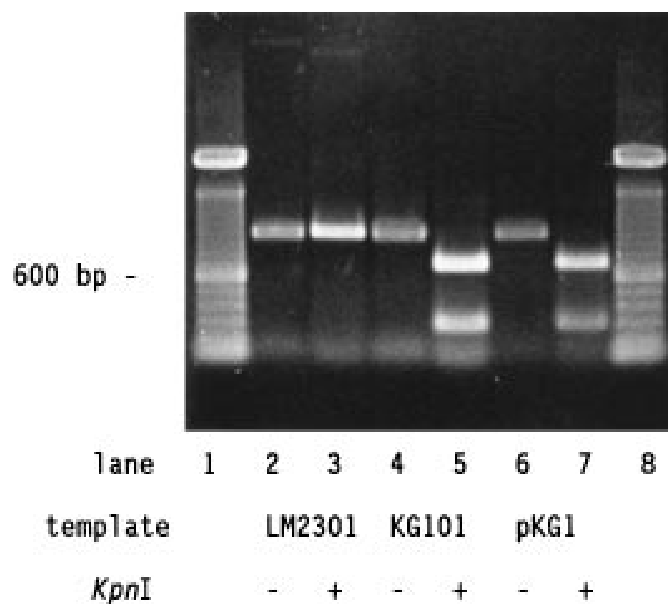

Figure 1. The electrophoretic gel of polymerase chain reaction (PCR) products stained with ethidium bromide. The PCR reactions were each programmed with one of the indicated template DNA and primers homologous to *pip*. Following the PCR reaction, half of each reaction was restricted with *Kpn*I (lanes 3, 5, and 7), and the other half was incubated without *Kpn*I (lanes 2, 4, and 6). The products were electrophoresed on a 0.8% agarose gel. Lanes 1 and 8 are 100-bp ladder size standards.

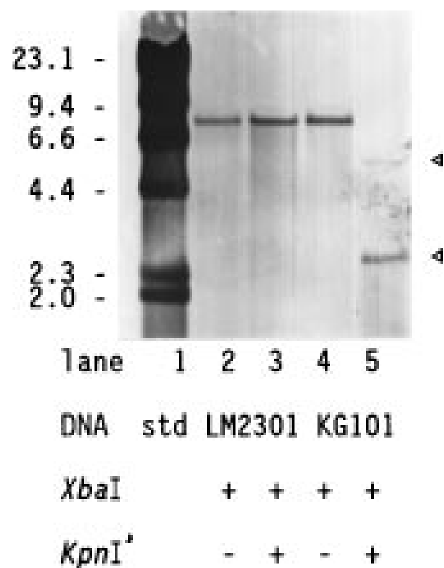

Figure 2. Southern hybridization analysis with a labeled probe for *pip*. Chromosomal DNA from LM2301 (lanes 2 and 3) and KG101 (lanes 4 and 5) was restricted with *Xba*I (lanes 2 and 4) or *Xba*I and *Kpn*I (lanes 3 and 5) and then analyzed by the method of Southern (29). A probe that was specific for *pip* was used for detection. λ Phage DNA restricted with *Hin*dIII (lane 1) was used as a size standard (std), and the fragment lengths are indicated in kilobase.

(Figure 2) show that *Xba*I-digested DNA of LM2301 (lane 2) and KG101 (lane 4) each contain one 8.6-kb fragment. When chromosomal DNA was digested with both *Xba*I and *Kpn*I, *Kpn*I had no effect on the *Xba*I fragment from LM2301 DNA (lane 3), but cleaved the *Xba*I fragment of KG101 DNA (lane 5, arrowheads) into 5.7- and 2.9-kb fragments. The actual number of base pairs between the *Kpn*I site and 1 of the *Xba*I sites is known from sequencing to be 2658 bp (7). These results confirm that KG101 contains only the mutated *pip* and suggest that no vector sequences remain in the engineered strain.

KG101 and JK101 Lack Plasmid Marker DNA

The absence of vector plasmid DNA in the engineered strains was ascertained as follows. Oligonucleotides that were homologous to DNA flanking the coding region of *pip* were used as PCR primers to show that there was no added DNA in the pip^{-} mutants KG101 and JK101. A primer upstream of *pip* was paired with a primer internal to *pip*, and the reaction produced a PCR product of the same size when programmed with DNA from LM2301, KG101, or JK101 (Figure 3A). When PCR reaction products were treated with *Kpn*I, the products from KG101 and JK101 were cleaved. Similarly, a primer downstream of *pip* was paired with a primer internal to *pip*, and the reaction produced a PCR product of the same size when programmed with DNA from either LM2301, KG101, or JK101 (Figure 3B). Because the two PCR products overlap, *pip* is represented completely by the two PCR products. These results suggest that, within the limits of detection of this kind of analysis, no additional DNA remains from the gene exchange.

Both KG101 and JK101 were analyzed further for the presence of pGhost6 vector DNA; a PCR was programmed with DNA from either KG101 or JK101 and oligonucleotide primers that are homologous to the erythromycin resistance gene (*ermC*) on pKG1. The predicted size of the product is 814 bp. As a positive control, DNA was prepared from the strain with pKG1 integrated in the chromosome. The results (Figure 4) show that no PCR product was generated with the KG101 DNA (lane 11), but a product of about 800 bp was produced from the strain with the integrated form of pKG1 (lane 9). Additional controls show that the same size (800 bp) product was generated in a reaction programmed with pKG1 (lane 7). No product was detected in reactions programmed with pUC19 (lane 8) or chromosomal DNA from LM2301 (lane 10). Results were similar when oligonucleotide primers were used that are homologous to the ampicillin resistance gene (Figure 4) except that the predicted size of the product was 1297 bp. Identical 1.3-kb products were generated in reactions that were programmed with pKG1 (lane 1), pUC19 (lane 2), and chromosomal DNA from the strain with integrated pKG1 (lane 3); no product was formed in reactions programmed with chromosomal DNA from either LM2301 (lane 4) or KG101 (lane 5). The results from reactions that were programmed with DNA from JK101 were identical to those of KG101 (data not shown). These results show that nonlactococcal sequences of the pGhost6 integration vector are absent from KG101 and JK101.

Phage Resistance of KG101, JK101, and a Spontaneous *pip* Mutant

The efficiencies of plating of phages c2, ml3, and sk1 on KG101 and JK101 were compared with efficiencies on LM2301 and BG101. Phages c2 and ml3 formed no plaques on KG101, JK101, or BG101, even when 10^7 to 10^9 pfu were applied per plate. This result shows that KG101 and JK101 are as resistant to the phages c2 and ml3 as the spontaneous *pip*

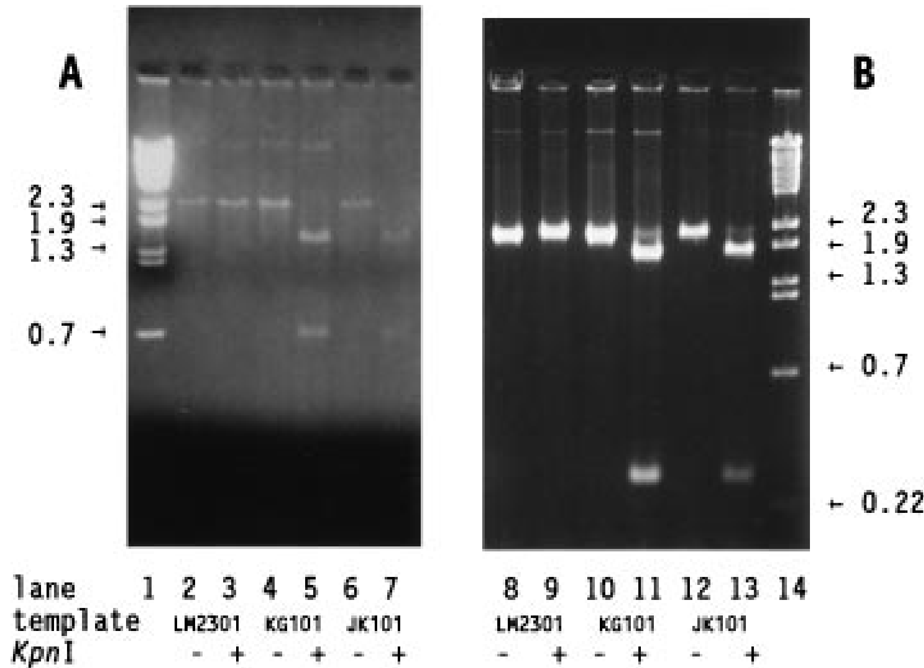

Figure 3. The electrophoretic gel of polymerase chain reaction (PCR) products stained with ethidium bromide. The PCR reactions were programmed with genomic DNA of LM2301 (lanes 2, 3, 8, and 9), KG101 (lanes 4, 5, 10, and 11), or JK101 (lanes 6, 7, 12, and 13). Primers for lanes 2 to 7 were derived from sequence upstream of that cloned in pKG1 and downstream of the location of the engineered *Kpn*I site. Primers for lanes 8 to 13 were derived from sequence downstream of that cloned in pKG1 and upstream of the location of the engineered *Kpn*I site. Following the PCR reaction, half of each reaction was treated with *Kpn*I (lanes 3, 5, 7, 9, 11, and 13). Samples were electrophoresed in 2% agarose gels. Lanes 1 and 14 contain DNA size standards. The sizes of some of the fragments are indicated in kilobase.

mutant BG101 that was described previously (7). The efficiency of plating of phage sk1 on KG101 and JK101 was 1.0, which was the same as that found for BG101.

Growth Characteristics

The doubling times of *pip*$^+$ and *pip*$^-$ isolates (including KG101) from the first gene exchange experiment were uniformly longer (about 10%) than that of LM2301 or BG101 (data not shown). In contrast, *pip*$^+$ and *pip*$^-$ isolates (including JK101) from the second gene exchange experiment did not have reduced growth rates relative to LM2301 (data not shown). The mean (±SEM) doubling times of LM2301, BG101, KG101, and JK101 in M17G were 42.9 ± 0.2, 43.2 ± 0.3, 49.8 ± 1.0, and 41.6 ± 0.4 min, respectively. LM2301, BG101 and JK101 grew in minimal medium, but KG101 did not.

DISCUSSION

The results show that wild-type *pip* in *L. lactis* was replaced with a mutant allele that was engineered in vitro. The growth characteristics of the mutant suggest that *pip* is not required for viability, although the truncated protein predicted from the sequence of the mutated *pip* may possibly be functional. However, we consider it unlikely that a protein lacking two-thirds of its sequence and its carboxy-terminal membrane anchoring region (7) would be functional.

A strain engineered with the mutated *pip* (JK101) is resistant to the same bacteriophages as the spontaneous phage-resistant *pip* mutant BG101. An important characteristic of JK101 and BG101 is that the level of phage resistance appears to be absolute, because no plaques have ever been detected (except for phage sk1) using these strains as indicators, even when as much as 10^9 pfu was applied per 100-mm assay plate. This result is consistent with previous data showing that bacteriophages were unable to overcome the resistance mechanism in a spontaneous *pip* mutant (RMC2/4), even after continuous challenge of a mixture of wild-type and *pip* strains for 100 generations with a composite of 10 phages (at 1×10^5 pfu/ml each) under simulated cheese-making conditions (1).

Because of the methods used to exchange *pip* alleles and the intended use in foods for human con-

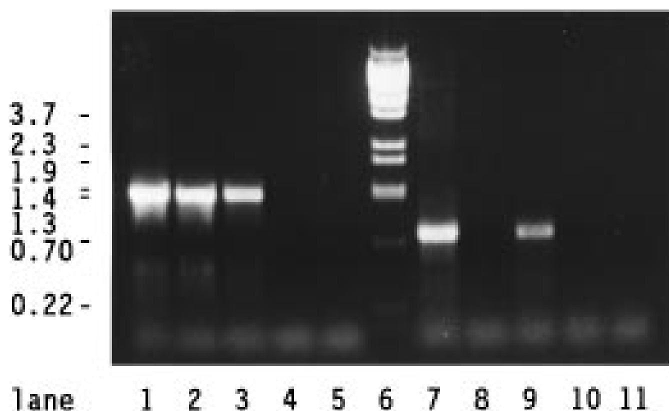

Figure 4. An electrophoretic gel of polymerase chain reaction (PCR) products stained with ethidium bromide. The PCR reactions were each programmed with pKG1 (lanes 1 and 7), pUC19 (lanes 2 and 8), or chromosomal DNA from a strain with integrated pKG1 (lanes 3 and 9), LM2301 (lanes 4 and 10), or KG101 (lanes 5 and 11). Primers homologous to *amp* (lanes 1 to 4) or *ermC* (lanes 7 to 11) were included. Reaction mixtures were electrophoresed on a 0.8% agarose gel, and stained with ethidium bromide. Lane 6 contains size standards. The sizes of some of the standards are indicated in kilobase.

sumption, it was important to verify that nonlactococcal sequences were removed from the engineered strains. To this end, KG101 and JK101 were sensitive to erythromycin, indicating a lack of vector sequences. This result was confirmed by analytical PCR (Figure 4), which showed that both KG101 and JK101 lacked the *ermC* and *bla* genes encoding antibiotic resistance of pGhost6. Analytical PCR also did not detect any additions or deletions in the sequences flanking the *pip* mutation or the region cloned in pKG1. Southern hybridization analysis showed that size of the *Xba*I-restricted chromosomal DNA of KG101 was identical to that from LM2301, indicating that no foreign sequence had been inserted in *pip*. Together, these results show that, within the limits of detection of the analyses, the vector sequences have been removed from JK101.

The reduced growth rate of KG101 and the inability of KG101 to grow on minimal medium were unrelated to the pip^- allele, as was shown by analyzing both pip^+ and pip^- isolates derived from the same integrant that was used to produce KG101. Because all isolates made during the isolation of KG101 showed the same growth defects, regardless of which *pip* allele they contained, the growth defects were unrelated to *pip*. The growth defect or defects were apparently the result of selection at the elevated temperatures used initially for integration of pKG1. An alternative procedure that minimized the exposure of the cells to elevated temperatures was used to produce JK101. JK101 and the spontaneous pip^- mutant BG101 have normal growth rates and grow in minimal medium. These results show that *pip* is not required for growth and viability of *L. lactis*. The function of the Pip protein in the cell remains an open question.

An understanding of the different mechanisms of phage resistance in *L. lactis* will enable the engineering of the next generation of phage-resistant strains. Most of the known genes from *L. lactis* that confer resistance to phage are not chromosomally encoded. Engineering phage resistance at the *pip* locus is advantageous because of its stable, chromosomal location, which obviates selective pressure required for many plasmid-borne systems. The replacement of *pip* in the plasmid-free derivative of strain C2 represents an important step in the genetic engineering of bacteriophage resistance in *L. lactis*. Additional research is needed, however, to apply this technology to commercially important strains.

ACKNOWLEDGMENTS

This work was supported by a grant from the Western Dairy Center.

We thank Richard Ivey for technical assistance; William Sandine, Todd Klaenhammer, Carl Batt, Jeff Broadbent, and Jim Steele for helpful discussions and advice; and Alan Bakalinsky for reviewing the manuscript.

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