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Isolation, cloning and characterisation of the *abiI* gene from *Lactococcus lactis* subsp. *lactis* M138 encoding abortive phage infection

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Abstract

Plasmid pND852 (56 kb) encodes nisin resistance and was isolated from *Lactococcus lactis* ssp *lactis* (*L. lactis*) M138 by conjugation to *L. lactis* LM0230. It conferred strong resistance to the isometric-headed phage ϕ 712 and partial resistance to the prolate-headed phage ϕ c2. A 2.6 kb *HpaII* fragment encoding phage resistance was cloned into the streptococcal/*Bacillus* hybrid vector pGB301 to generate pND817. The mechanism of phage resistance encoded by pND817 involved abortive infection and this was illustrated by a reduction in burst size from 166 to 6 at 30°C and from 160 to 90 at 37°C. Partial resistance was therefore retained at 37°C. DNA sequencing revealed that the abortive infection was encoded by a single open reading frame (ORF), designated *abiI*, encoding a 332 amino acid protein. Neither *abiI* nor the predicted product showed significant homology to any existing sequence in the GenBank database. Frame shift mutation at the unique *EcoRI* site within the ORF resulted in loss of the *Abi*⁺ phenotype, confirming that the ORF is responsible for the encoded phage resistance.

Keywords

Lactococcus lactis; Plasmid pND852; *abiI* gene; Phage resistance; Abortive infection

1. Introduction

Lactic acid bacteria are used extensively in the dairy food industry for the production of dairy products including cheeses and yoghurts. Lactic acid bacteria can, however, succumb to phage infection during the production of fermented products thus compromising the effectiveness of the fermentation process. Improved resistance to phage is an ongoing challenge and is an extensively researched area in the lactococcal dairy starter industry (Klaenhammer, 1991). Several independent phage resistance mechanisms have been identified in lactic acid bacteria: adsorption inhibition, prevention of phage injection, restriction and modification and abortive infection (Klaenhammer and Fitzgerald, 1994). Abortive infection inhibits phage proliferation and is characterised by the death of the infected cell prior to the release of viable phage progeny. This mechanism is considered the most powerful mechanism of phage resistance identified thus far in lactococci (Klaenhammer and Fitzgerald, 1994). A number of lactococcal genes encoding abortive

phage infection have been cloned and sequenced and these include *abiA* (Hill et al., 1990; Coffey et al., 1991), *abiB* (Cluzel et al., 1991), *abiC* (Durmaz et al., 1992), *abiD* (McLandsborough et al., 1995), *abiD1* (Anba et al., 1995), *abiE* and *abiF* (Garvey et al., 1995), *abiG* (O'Connor et al., 1996) and *abiH* (Prevots et al., 1996). In this paper we report a new plasmid encoded abortive infection gene *abiI*

2. Materials and methods

2.1. Bacterial strains, plasmids, phages and media

The strain and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani medium (LB) (Sambrook et al., 1989) or in M63 minimal medium (Sambrook et al., 1989) supplemented with glucose (0.2%, wt./vol.). *Lactococcus lactis* strains were grown at 30°C in M17 (Terzaghi and Sandine, 1975) supplemented with 0.5% (wt./vol.) glucose (M17G). Lactose utilisation, proteinase activity and nisin resistance were determined by growth on solid media for 48 h. When appropriate, antibiotics were added as follows: for *E. coli*, 50 µg ampicillin ml⁻¹; for *L. lactis*, 5 µg erythromycin ml⁻¹, 5 µg chloramphenicol ml⁻¹, 300 IU nisin ml⁻¹, 50 µg fusidic acid ml⁻¹ and 500 µg streptomycin ml⁻¹.

2.2. Conjugation and transformation

Conjugation was carried out by filter-matings (Lai et al., 1977). *L. lactis* was transformed by electroporation as described by Powell et al. (1988). For *E. coli*, the CaCl₂ transformation method of Dagert and Ehrlich (1979) was used but without the extended pre-incubation in CaCl₂.

2.3. Phage techniques

Cross-streaking was employed for the initial screening of phage-resistant and phage-sensitive isolates. Phage preparations were titrated by a standard plaque assay (Adams, 1959). Efficiency of plating (EOP) was calculated by dividing the phage titre obtained, on the host being tested, by the phage titre obtained on the sensitive or control host. Phage adsorption was investigated using the method of Gautier and Chopin (1987). Cell survival was determined as described by Behnke and Malke (1978). The percent cell death was calculated as $100 \times [(CFU \text{ per millilitre of cultures without phage}) - (CFU \text{ per millilitre of cultures with phage})] / CFU \text{ per millilitre of cultures without phage}$. One step growth curves and burst size determinations were carried out according to the procedure described by Klaenhammer and Sanozky (1985). Burst sizes were calculated as PFU ml⁻¹ after release of progeny phage divided by PFU ml⁻¹ present during the latent period.

2.4. Plasmid DNA techniques

Lactococcal plasmid DNA was isolated by the method of Anderson and McKay (1983). Plasmids from *E. coli* were isolated as described by Birnboim and Doly (1979). Plasmid DNA was purified by caesium chloride-ethidium bromide density gradient centrifugation (Sambrook et al., 1989) and desalted by dialysis in 1x TE buffer (10 mM Tris and 1 mM EDTA). Restriction digestion and molecular cloning were as described in Sambrook et al. (1989). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim (Biochemicals, IN) and used as recommended by the manufacturer. Recovery of DNA fragments from agarose gels was performed using a Qiaex gel extraction kit (Qiagen, CA).

2.5. Nucleotide sequencing and analysis

Both DNA strands were sequenced using an Applied Biosystems (CA) 377 DNA sequencer, following the manufacturer's protocol. Sequencing of the phage resistance determinant was initiated using the T7 and SP6 primers of pGEM-7Zf(+) (Promega Corporation, Madison, WI). Based on the sequences obtained, 20-mer oligonucleotide primers were then synthesised and used to 'walk' along the DNA template. Recording and analysis of the nucleotide sequence were carried out using the AutoAssembler™ DNA sequence assembly software (Applied Biosystems) and the Angis Software System operated by the Australian Genomic Information Centre, University of Sydney.

2.6. Nucleotide sequence accession number

The GenBank accession number for the DNA sequence of the *abiI* gene encoding abortive phage infection from *L. lactis* M138 is U38973.

3. Results

3.1. Characterisation of the wild type strain

L. lactis subsp. *lactis* M138 was selected to screen for plasmid-encoded phage resistance since this strain had shown good phage resistance during commercial use. M138 was found to be capable of utilising lactose and the milk protein casein as carbon and nitrogen sources, respectively, and to be resistant to nisin. Plasmid profile of M138 showed six plasmids of the following approximate sizes: 72, 56, 9.4, 8.3, 3.8 and 3.4 kb.

3.2. Identification of a plasmid that encodes phage resistance

M138 was used as a conjugation donor to transfer Nis^r to *L. lactis* LM0230Sm^r. Sm^r Nis^r transconjugants were obtained at a frequency of 10³ ml⁻¹ of mating mixture. They were screened for resistance to ϕ 712 and ϕ c2 by the cross-streaking method. Strong resistance to ϕ 712 and partial resistance to ϕ c2 was observed. Plasmid analysis of five Nis^r and phage resistant (ϕ^r) transconjugants showed that all of them harboured a plasmid of approximately 56 kb. Additional low molecular weight plasmids were also observed in some of these transconjugants. Subsequent conjugal transfer of the 56 kb plasmid verified that it encoded Nis^r and ϕ^r . This plasmid was designated pND852.

3.3. Subcloning of DNA fragments encoding abortive infection from pND852

pND852 contains nine *HpaII* sites. Using a shot gun cloning technique, attempts were made to clone the fragments into the streptococcal vector, pGB301, containing a unique *HpaII* site. pND852 was partially digested with *HpaII* and pGB301 was digested to completion with the same enzyme. The digests were precipitated and ligated by T4 DNA ligase. The mixture was then electroporated into LM0230. Transformants were selected on M17G containing sucrose and erythromycin and tested for phage resistance by cross-streaking against ϕ 712 and ϕ c2. A variety of plasmids of different sizes were isolated from the phage-resistant transformants and analysed by restriction endonuclease digestion. The smallest plasmid which encoded phage resistance contained a 2.6 kb *HpaII* fragment and was named pND817. The 2.6 kb *HpaII* fragment was subcloned into the unique *ClaI* site in pGEM-7Zf(+) and the ligation mixture transformed into competent *E. coli* NM522 cells. The resulting plasmid was designated pND821 and used for DNA sequencing.

Phages were titrated on LM0230Sm^r, LM0230Sm^r (pND852) and LM0230(pND817) to measure the expression of the cloned abortive infection system (Table 2). pND817 conferred a higher level of resistance to ϕ 712 and ϕ c2 than the parent plasmid. The presence of

pND817 in LM0230 essentially eliminated plaque formation by ϕ 712 and reduced the diameter of ϕ c2 plaques even further when compared LM0230Sm^r(pND852).

3.4. Mechanism of phage resistance encoded by pND817

Adsorption of ϕ 712 and ϕ c2 to LM0230 (control) was 99 and 98% respectively. The corresponding values for LM0230(pND817) were 97% and 99%. This indicated that phage resistance from pND817 was not due to prevention of phage adsorption. Phage picked from the hazy pinpoint plaques on LM0230(pND817) did not infect the same strain more efficiently, indicating that host-controlled modification was not the encoded mechanism. Further experiments were carried out to determine if abortive infection was the mechanism encoded by pND817. Cell survival assay indicated that 97% of LM0230(pND817) died after infection by ϕ 712 and 92% of LM0230(pND817) died after infection by ϕ c2 (Table 2). One-step growth curves and burst size measurements were carried out at 30°C using LM0230(pND817) (Fig. 1). For ϕ 712 in LM0230 the latent period was 25 min, and the average burst size was 166 phages per cell. In LM0230(pND817), the latent period was 30 min and phage release was slower. The burst size was substantially reduced to 5.6. Plaques produced by ϕ 712 on LM0230(pND817) were very hazy and not easily visible, presumably due to the reduced burst size. These characteristics of reduced burst size and hazy plaques are consistent with an abortive infection mechanism. Compared to 30°C no substantial difference in latent period and burst size was observed for the control strain at 37°C (Fig. 1). For LM0230(pND817) a similar latent period was observed at 37°C compared to 30°C. However, the burst size increased to 90 phages per cell and pin-point plaques, 0.5 mm in diameter, were observed. This result indicates that some resistance is retained at 37°C.

ϕ 712 was infected into LM0230 and LM0230(pND817), and phage DNA was extracted (Hill and Klaenhammer, 1991), digested by *Hind*III then checked on agarose gel at various time intervals to determine the stage the abortive mechanism may operate (Fig. 2). It was found that phage DNA was formed at approximately the same rate in both strains, however, it appeared that the pND817 encoded abortive system reduced the rate or prevented the phage DNA from being packaged. The phage DNA content dropped significantly in the control strain after 90 min incubation, but was still at maximum levels in the test strain after 90 min incubation.

3.5. Nucleotide sequencing

The nucleotide sequence of the 2.6 kb DNA insert in pND821 was determined (Fig. 3). Examination of the sequence indicated the presence of two large open reading frames (ORF) on opposite strands. The first ORF represents a truncated nisin resistance gene since nucleotides 1–814 share 98% homology with the nisin resistance gene encoded by pNP40 (Froseth and McKay, 1991) and the derived amino acid sequence shows 99% homology. The large intact ORF was composed of 996 nucleotides beginning with an ATG start codon and ending at a TAA stop codon. The ORF has the potential to encode a protein of 332 amino acids with a predicted molecular mass of 39 349 kDa. Neither the nucleotide sequence nor its predicted protein product showed significant homology to any other existing sequences in the GenBank (release 91) and EMBL databases (release 43). This search included lactococcal sequences as well as sequences from other organisms. To comply with the nomenclature suggested by Coffey et al. (1991) this ORF was designated *abil*. Examination of the DNA sequence for transcriptional and translational regulatory sequences revealed that 8 bp upstream from the ATG codon is a sequence resembling Shine-Dalgarno sequences that have been reported for *L. lactis* (Guchte et al., 1992). This putative ribosome binding site has a free energy of $-7.2 \text{ kcal mol}^{-1}$ with the 3' end of the 16s rRNA of *L. lactis*. At 23 bp upstream of the putative Shine-Dalgarno sequence are probable -10 and -35 sequences which exhibited similarity to consensus *E. coli* and *Bacillus* promoters,

the -10 sequence being identical to these while four of the six nucleotides of the -35 region are the same. In addition the -10 region is preceded by a TGy sequence frequently observed in lactococcal promoters (Guchte et al., 1992). Directly downstream of the terminator codon was a region of dyad symmetry that could form a stem/loop structure of up to 24 bp followed by five consecutive T residues. The free energy of the putative hairpin loop was calculated to be $-18.9 \text{ kcal mol}^{-1}$. This symmetrical region may represent a rho-independent terminator.

3.6. Confirmation that *abil* is responsible for the encoded phage resistance

The entire *abil* gene is flanked by *AluI* sites. The 1225 bp *AluI* fragment was cloned into the *SmaI* site of pMU1327, the ligation mixture transformed into LM0230 and Em^r transformants were screened for phage resistance by cross-streaking. Plasmid extraction showed that all phage resistant colonies harboured a plasmid of the expected size. This was designated pND835. pND835 was used to re-electroporate LM0230, and all the transformants exhibited the previously observed resistance to $\phi 712$ and $\phi 2$. Four base pairs, verified by DNA sequencing, were inserted into the unique *EcoRI* site in *abil* causing a frame shift. This was achieved by cutting pND817 with *EcoRI*, filling in the sticky ends with Klenow fragment followed by blunt end ligation. The resulting plasmid, designated pND839, when introduced into LM0230 did not impart phage resistance to $\phi 712$ or $\phi 2$. This indicated that this ORF is responsible for the encoded phage resistance.

4. Discussion

pND852 was identified as a 56 kb conjugative plasmid encoding both phage and nisin resistance. Two phage resistance plasmids, pNP40 and pTN1060, that also encode nisin resistance have been reported previously (McKay and Baldwin, 1984; Klaenhammer, 1989). pTN1060 was the result of cointegration between two plasmids (pTN20 and pTR1040) that were formed during conjugative mobilisation of pTR1040 by the conjugative plasmid pTN20 (Higgins et al., 1988). pNP40 was initially selected by lactose fermentation and also found to encode resistance to nisin and phages. Although pND852 and pNP40 share a very similar nisin resistance gene their phage resistance genes are different. Association of nisin resistance and phage resistance on a conjugative plasmid (pND852) can be exploited in construction of phage-resistant strains by simple conjugation.

When the 2.6 kb fragment from pND852 was cloned into pGB301 generating pND817, LM0230(pND817) showed greater phage resistance than LM0230 Sm^r (pND852). LM0230(pND817) was essentially resistant to $\phi 712$ as the hazy pinpoint plaques observed on LM0230 Sm^r (pND852) were usually not visible on LM0230(pND817). This increased phage resistance may be due to a higher copy number of the cloning vector pGB301 compared to pND852 or to enhanced expression of *abil* in pND817. Dinsmore and Klaenhammer (1994) reported that altering the gene dosage of *abiA* significantly affected phage resistance levels. Three *abiA*-containing plasmids, pTRK18, pTRK362 and pTRK363, of various copy numbers, were introduced into a phage-sensitive strain, and the recombinants evaluated for resistance to phages. The high copy number plasmid, pTRK363, caused a significant reduction in EOP (10^{-4} – 10^{-8}) and a reduction in plaque size compared to that of the other two lower copy number plasmids.

Frame-shift mutation in *abil* resulted in complete loss of phage resistance. Although it is possible that disruption of a regulatory gene could lead to a similar result, this can be ruled out since pND835 contains only one complete ORF yet still conferred phage resistance when introduced into LM0230, indicating that this ORF is responsible for the encoded phage resistance.

Although codon usage in *abiI* is typical for *L. lactis*, the GC content (29%) is lower than the average (37%) for lactococcal DNA, but comparable with the other *abi* genes (26–29%). Thus low GC content appears to be a feature of lactococcal *abi* genes although it is not clear whether this reflects their origin or function. Secondary structure of the deduced AbiI was analysed by the EMBL 'Predict Protein' program and no transmembrane domain was found, suggesting that it is a cytosolic protein. The amino acid sequence of AbiI was compared with other published Abi proteins by the Bestfit program. Identity was between 16 and 23% and similarity was between 41 and 50%. This is not as great as the 47% identity between AbiD1 and AbiF, but is close to 26% identity between AbiD and AbiF (Garvey et al., 1995).

AbiI showed phage resistance towards both isometric phage 712 and prolate phage c2, and retained some function at 37°C. No reference has been made to temperature resistance in other published Abi systems, however, AbiA was reported sensitive to high temperature (Hill et al, 1990). The partial temperature-resistance phenotype of AbiI is commercially important because of the temperature profiles used in cheese manufacture. The AbiI system therefore provides a potentially useful phage resistance system for the construction of commercial lactococcal cultures with improved phage resistance.

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Fig. 1. Effect of pND817 on one step growth curves of $\phi 712$. LM0230 (○) and LM0230(pND817) (●) at 30°C. LM0230 (□) and LM0230(pND817) (■) at 37°C. Results are the mean of two experiments.

Fig. 2. Phage replication in LM0230 and LM0230(pND817). Lanes 1–5: phage DNA isolated from LM0230 after ϕ 712 infection of 0, 10, 20, 45 and 90 min. Lanes 6–10: phage DNA isolated from LM0230(pND817) after ϕ 712 infection of 0, 10, 20, 45 and 90 min.

Complete sequence of lactococcal *abiI* gene encoding abortive phage infection. The derived amino acid sequence is presented. Indicated are the putative Shine-Dalgarno sequence (double underline), -35 and -10 promoter sequences (underlined) and a possible transcriptional terminator (converging arrows).

Table 1

Bacterial strains, plasmids and phages

Strain, plasmid or phage	Relevant characteristics	Source or Reference
Bacteria		
<i>L. lactis</i> subsp. <i>lactis</i>		
M138	Industrial strain, Lac ⁺ Prt ⁺ Nis ^r	Mauri Laboratories, Moorebank, Australia
LM0230	Lac ⁻ Prt ⁻ , plasmid-free derivative of c2	Efstathiou and McKay, 1977
LM0230Sm ^r	Sm ^r derivative of LM0230	Duan et al., 1996
LM0230Fus ^r	Fus ^r derivative of LM0230	Duan et al., 1996
<i>E. coli</i>		
NM522	Transformation host	Gough and Murray, 1983
Plasmids		
pGB301	9.8 kb streptococcal vector, Em ^r , Cm ^r	Behnke and Gilmore, 1981
pGEM7Zf(+)	3 kb <i>E. coli</i> vector encoding Amp ^r and <i>lacZ</i> screening	Promega Corporation, Madison, WI
pMU1327	7.0 kb streptococcal vector, Em ^r	Achen et al., 1986
pND852	Native plasmid of M138, ϕ^r Nis ^r , 56 kb	This study
pND817	12.3 kb, 2.6 kb <i>HpaII</i> fragment from pND852 cloned into the <i>HpaII</i> site of pGB301	This study
pND821	5.6 kb, 2.6 kb <i>HpaII</i> fragment from pND817 cloned into the <i>ClaI</i> site of pGEM-7Zf(+)	This study
pND835	1225 bp <i>AclI</i> fragment encoding <i>abiI</i> cloned into pMU1327 at the <i>SmaI</i> site	This study
pND839	Frame-shift mutation at the unique <i>EcoRI</i> site of pND817	This study
Phages		
ϕ 712	Small isometric-headed phage propagated on LM0230	M.J. Gasson, AFRC Institute of Food Research, Norwich, UK
ϕ c2	Prolate-headed phage propagated on LM0230	L.L. McKay, University of Minnesota, Minn.

Amp, ampicillin; Cm, chloramphenicol; Em, erythromycin; Fus, fusidic acid; Nis, nisin; Sm, streptomycin; Lac, lactose utilisation; Prt, proteinase; ϕ^r , phage resistance.

Table 2

Expression of phage resistance from DNA encoding abortive infection from pND852

Host strain	Phage ϕ 712			Phage ϕ 2		
	EOP	D/M	Cell death (%)	EOP	D/M	Cell death (%)
LM0230Sm ^r	1.0	2 mm	100	1.0	2 mm	100
LM0230Sm ^r (pNDS52)	ND	HP	—	10 ⁻³	PP	—
LM0230(pND817) and LM0230Sm ^r (pND817)	<10 ⁻⁹	—	97	ND	HP	92

EOP, efficiency of plating; D/M, plaque diameter (mm) or plaque morphology; ND, not determined; HP, very faint hazy pin-point plaques; PP, clear pin-point plaques.