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Cloning and characterization of the determinant for abortive infection of bacteriophage from lactococcal plasmid pCI829

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The genetic determinant for abortive infection of bacteriophage (Abi) from the lactococcal plasmid pCI829 was cloned on a 6·2 kb *StuI* fragment in *Escherichia coli* using the shuttle vector pSA3. In *Lactococcus lactis* subsp. *lactis* MG1363Sm the resulting recombinant plasmid pCI816 conferred complete insensitivity to the small isometric-headed phage 712 and a reduced plaque size in the case of the prolate-headed phage c2. The determinant was further localized by subcloning and nuclease Bal31 deletion analysis; approximately 2·0 kb of DNA was essential for the expression of the Abi$^+$ phenotype. Nucleotide sequence analysis of this region revealed a putative open reading frame of 1887 base pairs preceded by a putative promotor sequence and ribosome-binding site which exhibited similarity to consensus *E. coli* and *Bacillus subtilis* transcription/translation signals. Hybridization experiments indicated that this region was not homologous to the *abi* determinant from the phenotypically similar lactococcal plasmid pCI750.

Introduction

Production of many cultured dairy products relies on the efficient formation of lactic acid by lactic acid bacteria, including species of the genus *Lactococcus*. Bacteriophages are a significant cause of inhibition of starter cultures and as a result, phages and phage insensitivity have become a major focus of research interest. Current studies on aspects of phage insensitivity in lactococci have resulted in the identification of several plasmids which inhibit the proliferation of phage. These are discussed in some detail in recent reviews by Klaenhammer (1987, 1989), Daly & Fitzgerald (1987) and Sanders (1988). Subsequent reports on plasmid-mediated phage insensitivity include those by Josephen & Vogensen (1989), Murphy *et al.* (1988), Dunny *et al.* (1988), Jarvis (1988), Jarvis *et al.* (1989) and Steele *et al.* (1989). The insensitivity mechanisms that have been observed include adsorption inhibition, restriction and modification (R/M) and abortive infection (Abi). Recently, considerable effort has been focussed on the localization of the genetic determinants for phage insensitivity. These efforts have been successful particularly in the case of the plasmid pTR2030, where R/M and abortive infection (designated Hsp in this case) determinants were cloned on a 13·8 kb DNA fragment and further localized using Tn*5* mutagenesis and deletion analysis (Hill *et al.*, 1989*a*, *b*). Additional reports on the cloning of phage insensitivity determinants include those concerning pCI750 (Steele *et al.*, 1989), pIL105 (Simon & Chopin, 1988), and pKR223 (McKay *et al.*, 1989). In this laboratory a 27 MDa conjugative plasmid designated pCI829 which encodes Abi has been described (Coffey *et al.*, 1989). pCI829 was originally transferred by conjugation from *Lactococcus lactis* subsp. *lactis* UC811 to the plasmid-free strain *L. lactis* subsp. *lactis* MG1363Sm, where it conferred total insensitivity to small isometric-headed phages and a greatly reduced burst size in the case of prolate-headed phages. Plasmid pCI829 was found to be capable of stably co-existing with a derivative of pCI750 which encodes a similiar phage insensitivity mechanism (Baumgartner *et al.*, 1986). A strain containing both plasmids showed significantly greater insensitivity to phages than did one containing either plasmid on its own (Coffey *et al.*, 1989).

This report describes the cloning, subcloning, nuclease Bal31 treatment and nucleotide sequence analysis of the pCI829-associated genetic determinant for Abi.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M63080.

Table 1. *Bacterial strains and bacteriophages*

Strain	Sensitivity to phage 712*	Plasmid content	Origin/derivation
L. lactis subsp. *lactis*			
MG1363Sm	S†	None	Sm^r plasmid-free derivative of *L. lactis* subsp. *lactis* 712 (Gasson, 1983)
AC002	I	pCI829	Lac^- derivative of *L. lactis* subsp. *lactis* AC001 (Coffey *et al.*, 1989)
AC816	I	pCI816	Transformants of *L. lactis* subsp. *lactis* MG1363Sm (this study)
AC817	S	pCI817	
AC815	S	pCI815	
AC813	S	pCI813	
AC814	S	pCI814	
AC812	S	pCI812	
AC8147	I	pCI8147	
AC8144	I	pCI8144	
MM1	I	pMM1	Steele *et al.* (1989)
AC719	I	pB719	Deletion derivatives of pCI8147 (this study)
AC718	I	pB718	
AC721	S	pB721	
AC408	I	pB408	Deletion derivatives of pCI8144 (this study)
AC426	I	pB426	
AC433	S	pB433	
E. coli			
DB11(pSA3)	–	pSA3	Dao & Ferretti (1985)
HB101	–	None	Boyer & Roulland-Dussoix (1969)
TG1	–	pUC18/19 clones	Courtesy of W. deVos, NIZO, Ede, The Netherlands
AC810	–	pCI810	Deletion derivative of pCI816 (this study)
AC8147	–	pCI8147	Subclones for Bal31 analysis (this study)
AC8144	–	pCI8144	

* S, sensitive; I, insensitive.
† MG1363Sm also sensitive to phages c2 and ml3.

Methods

Bacterial strains, bacteriophages and media. The bacterial strains and bacteriophages used in this study are listed in Table 1. Strains of lactococci were routinely grown in M17 medium (Terzaghi & Sandine, 1975) at 30 °C with lactose replaced by glucose when necessary (GM17). Strains of *Escherichia coli* were grown in LB medium (Davis *et al.*, 1980) at 37 °C with shaking. Solid media contained 1·5% (w/v) agar (Oxoid no. 3). The antibiotics erythromycin (Em), chloramphenicol (Cm), tetracycline (Tc) and ampicillin (Ap) were added to selective media for strains of *E. coli* at levels of 250, 25, 12·5 and 50 μg ml^{-1}, respectively. For lactococci, Em was added at 2 μg ml^{-1}.

Isolation of plasmid DNA. Large-scale and rapid isolation of plasmid DNA from lactococci was achieved using the method described by Anderson & McKay (1983). For *E. coli*, the method of Birnboim & Doly (1979) was used. Purification of plasmid DNA by caesium chloride/ethidium bromide buoyant density gradients was performed as described by Maniatis *et al.* (1982).

Restriction endonuclease analysis, molecular cloning techniques and nucleotide sequence analysis. Restriction endonucleases were purchased from Boehringer. DNA digestion and general cloning procedures were as outlined by Maniatis *et al.* (1982). Restriction analysis was performed as described previously (Coveney *et al.*, 1987). For Bal31 deletion analysis the plasmids pCI8147 and pCI8144 were linearized at the *Bam*HI site and treated with various concentrations of the enzyme for 30 min. DNA fragments from recombinant plasmids containing the *abi* determinant were cloned in pUC18 or pUC19. The nucleotide sequence was determined by sequencing double-stranded plasmid DNA in two orientations by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the T7 DNA Sequencing Kit (Promega Corp. USA). Synthetic 17-mer DNA primers were prepared on a DNA synthesizer (Beckman model 200A).

Transformation procedures. Strains of *E. coli* were transformed using the method of Mandel & Higa (1970). Lactococci were transformed by electroporation using a Gene Pulser apparatus and a pulse controller unit (Bio-Rad). The culture was grown for 15 h at 21 °C in GM17 broth and then diluted (1 : 50) in fresh broth and grown at 30 °C for 2·5 h. Cells were harvested by centrifugation at 4 °C, washed twice in ice-cold 10% (w/v) sucrose (Sigma), resuspended in 0·05 culture volumes of ice-cold 10% sucrose and held on ice until required. Up to 0·1 vol. of plasmid DNA solution was thoroughly mixed with 200 μl of cell suspension in a chilled Gene Pulser cuvette (electrode separation of 2 mm). Cells were exposed to a single electric pulse (peak voltage, 2·5 kV; capacitance, 25 μF; resistance 200 Ω), which generated a peak field strength of 12·5 kV cm^{-1}. Immediately after delivery of the pulse the cells were added to 1 ml GM17 broth and incubated at 30 °C for 2 h before spread-plating on GM17-Em. Colonies were visible after 24 h.

Detection of clones exhibiting the Abi phenotype. Following ligation and transformation into *E. coli*, recombinant plasmids were purified and transformed into *L. lactis* subsp. *lactis* MG1363Sm by electroporation. Lactococcal electro-transformants were grown for 12 to 18 h at 21 °C in GM17-Em and challenged with MG1363Sm-specific phages 712 and c2 by the standard plaque assay technique. Plaque sizes were compared to those of the same phages on *L. lactis* subsp. *lactis* MG1363Sm containing vector alone and *L. lactis* subsp. *lactis* AC002. Plasmid DNA from clones exhibiting insensitivity to phage 712 and a reduction in plaque size with phage c2 as compared to MG1363Sm was isolated and analysed to verify the presence of pCI829-derived DNA.

Southern hybridization. DNA was restricted with the appropriate restriction enzyme, electrophoresed on 0·8% agarose gels, transferred to nitrocellulose filters by the method of Southern (1975) as modified by Wahl *et al.* (1979), and hybridized with plasmid DNA labelled with digoxigenin-dUTP. After hybridization to the target DNA using high-stringency procedures, the hybrids were detected by enzyme-linked immunoassay using an antibody conjugate and subsequent enzyme-catalysed colour reaction. All components and protocols were as suggested by the suppliers (Boehringer).

Results

Localization of determinants encoding Abi on pCI829

To facilitate genetic analysis of the pCI829-associated phage insensitivity mechanism, attempts were made to localize the genetic determinants for Abi. *L. lactis* subsp. *lactis* AC002 was used as a source of pCI829. This plasmid (see the upper part of Fig. 3) was mapped with a number of restriction enzymes, some of which were subsequently used to clone fragments into various *E. coli–L. lactis* shuttle vectors. When pCI829 was digested with *Stu*I, fragments of approximately 37 and 6·2 kb were generated. These fragments were mixed with pSA3 which had been linearized with *Nru*I and the ligation mix transformed into *E. coli* HB101. Analysis of transformants did not yield recombinant plasmids containing the larger of the two fragments. However, a large number of transformants contained a plasmid of 16·4 kb which was subsequently identified as pSA3 containing a 6·2 kb insert originating from pCI829. Further restriction analysis showed that this insert was present in both orientations in various isolates of the 16·4 kb plasmid. Following electroporation into *L. lactis* subsp. *lactis* MG1363Sm, this plasmid was found to mediate the inhibition of replication of phages c2 and 712 irrespective of insert orientation; a representative plasmid was designated pCI816.

Characterization of Abi on pCI816

The pCI816-containing strain, designated *L. lactis* subsp. lactis AC816 was compared with MG1363Sm (containing pSA3) and AC002 (containing pCI829) with regard to sensitivity to phages c2 and 712. The presence of pSA3 alone in MG1363Sm did not affect phage replication. With phage 712, MG1363Sm exhibited plaques 0·5 mm in diameter. This phage failed to form plaques on either AC002 or AC816 although in the case of the latter strain there was some slight inhibition of growth on the 10^0 and 10^{-1} dilutions in the plaque assay when a phage stock of 10^8 to 10^9 p.f.u. ml^{-1} was used. Phage c2 produced plaques of 3·0 to 3·5 mm on MG1363Sm, 0·5 mm on AC002 and 1·0 to 1·5 mm on AC816 (Fig. 1). There was

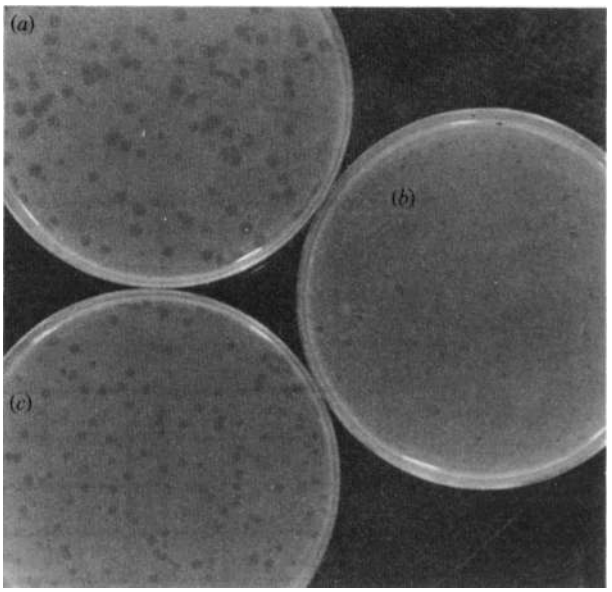

Fig. 1. Comparison of plaque morphology of phage c2 on *L. lactis* subsp. *lactis* MG1363Sm (*a*), *L. lactis* subsp. *lactis* AC002 containing pCI829 (*b*), and *L. lactis* subsp. *lactis* AC816 containing pCI816 (*c*).

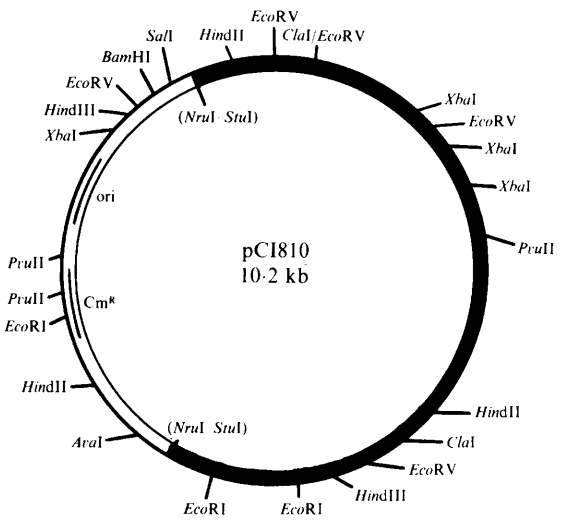

Fig. 2. Restriction map of pCI810, the deletion derivative of pCI816. DNA from pCI829 is shown in black. The remainder is pACYC184 DNA. The junction sites, *Nru*I and *Stu*I, are indicated.

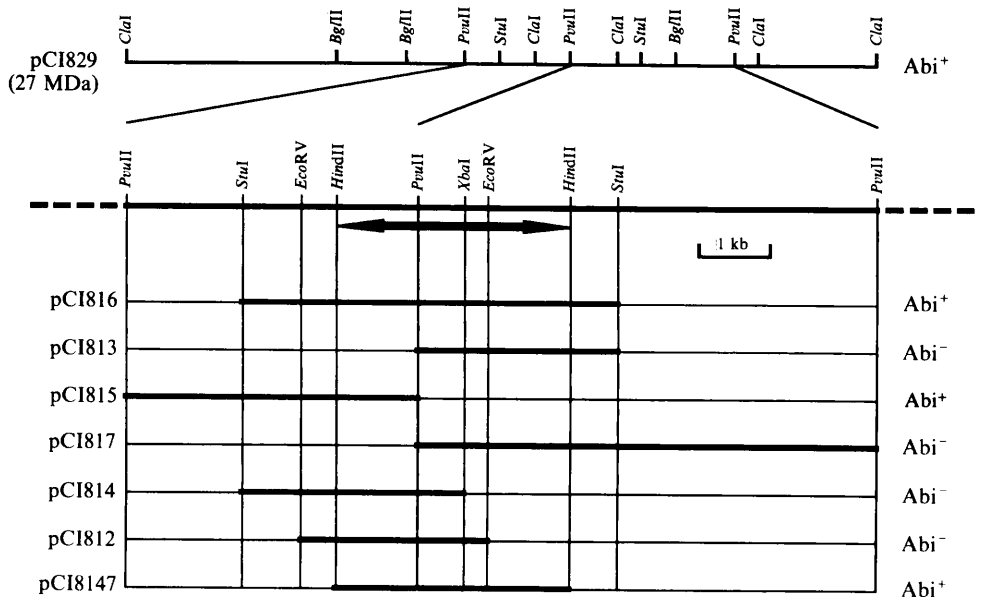

Fig. 3. Restriction map of pCI829. Subclones and their origins are shown underneath. All recombinant plasmids were made using vector pSA3 and insert DNA from either pCI829 or pCI810. Phenotypes (Abi$^+$ or Abi$^-$) are shown on the right. The approximate location of the *abi* gene is indicated by the arrow underneath the line showing the restriction enzymes used in the clonings.

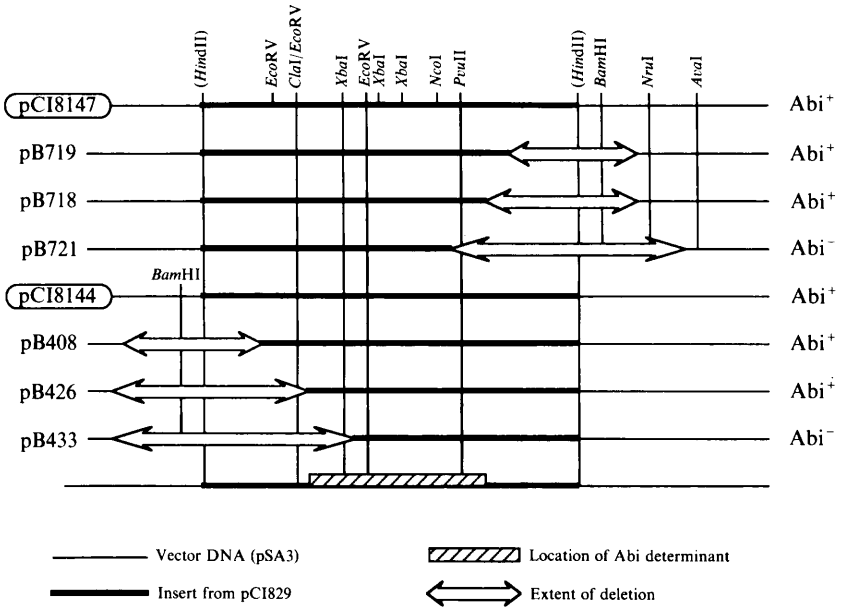

Fig. 4. pSA3 containing the 3·8 kb *Hin*dII insert giving rise to pCI8147 or pCI8144 depending on insert orientation. DNA deleted by nuclease Bal31 from the insert is indicated by the arrows.

no significant reduction in efficiency of plaquing (e.o.p.) with AC816. This contrasts with the observation made in the case of AC002, where phage c2 plaqued with an e.o.p. of $5{\cdot}2 \times 10^{-2}$ (Coffey *et al.*, 1989). These data showed that the level of phage insensitivity conferred by pCI816 was not as strong as that conferred by the original plasmid pCI829.

Subcloning of abi

A closer estimation of the location of the *abi* determinant was achieved by carrying out a number of subcloning experiments. Before mapping the 6·2 kb insert in pSA3 the Gram-positive part of the vector was eliminated using *Ava*I [pSA3 is composed of pACYC184 with a

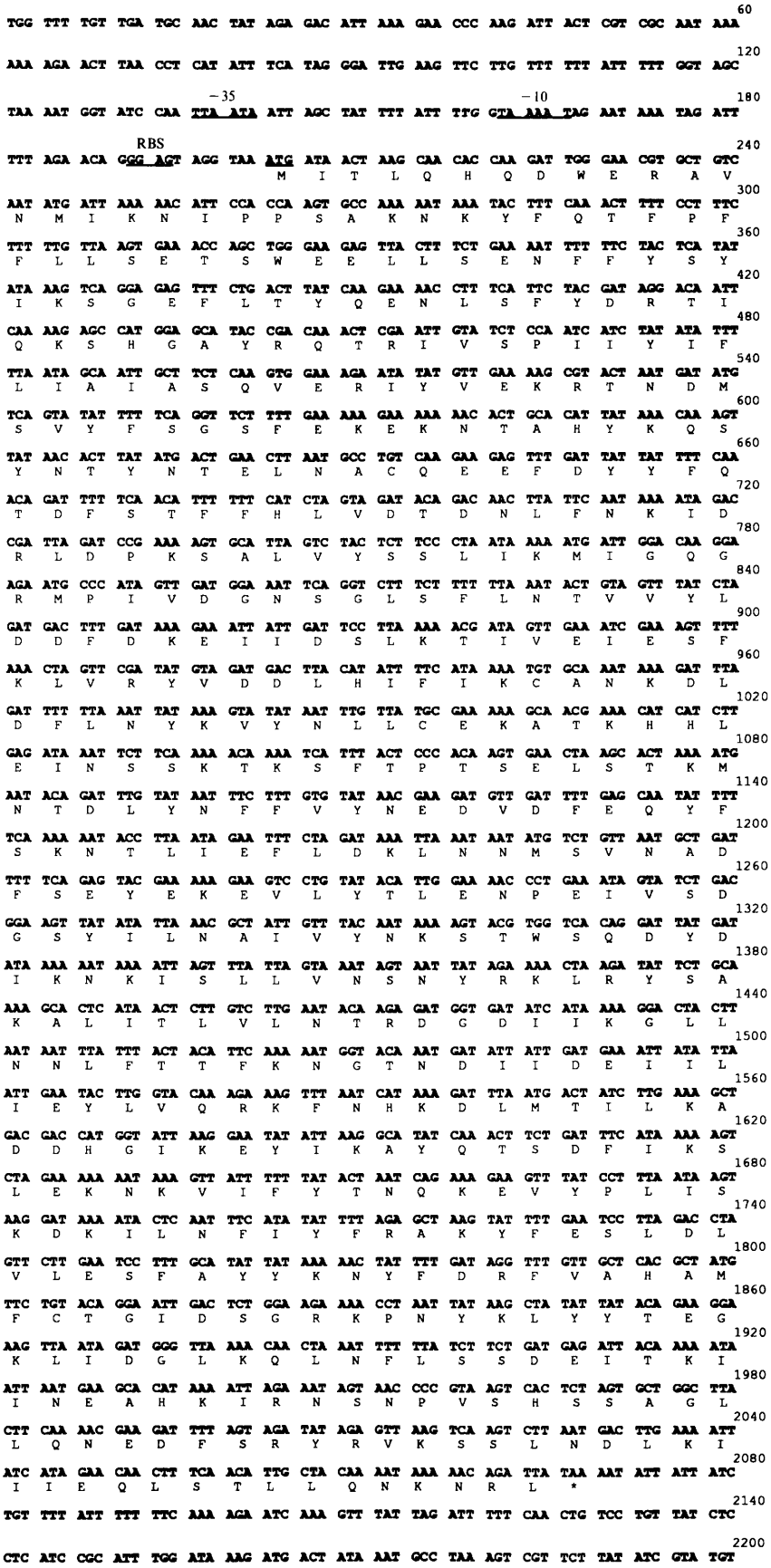

Fig. 5. Nucleotide sequence of the *abi* region of pCI829. The putative promotor regions, −10 and −35, and the putative ribosome-binding site (RBS) are underlined. The deduced amino acid sequence is denoted by the single-letter code.

fragment of the streptococcal plasmid pGB305 inserted into the *Ava*I site (Dao & Ferretti, 1985)]. The resulting 10·2 kb plasmid, designated pCI810, is equivalent to pACYC184 containing the 6·2 kb *Stu*I fragment (Fig. 2). Various fragments within or overlapping the 6·2 kb insert of pCI810 were subcloned into pSA3 and resulting recombinant plasmids were transformed into MG-1363Sm and assessed for their ability to mediate a reduction in plaque size of phage c2. MG1363Sm containing pCI8147, which is pSA3 containing a 3·8 kb *Hin*dII fragment, exhibited the same level of phage insensitivity as a strain containing pCI816 (Fig. 3). When the 3·8 kb fragment was present in the opposite orientation (pCI8144) the phenotype was unchanged (Fig. 4).

Bal31 deletion analysis of pCI8147 and pCI8144

The exact location of the *abi* determinant was identified using nuclease Bal31. In the case of pCI8147 it was observed that when deletions included the *Pvu*II site the resulting plasmid reverted to an Abi$^-$ phenotype. Examination of one deletion derivative, pB718 (Fig. 4), indicated that up to 0·2 kb outside the *Pvu*II site was part of the *abi* determinant. Using the plasmid with the insert in the reverse orientation (pCI8144) for deletion analysis it was observed that the *abi* gene did not extend to the *Cla*I site (Fig. 4, pB426). The remaining 2·0 kb of insert DNA must therefore be essential for expression of Abi.

Nucleotide sequence analysis of the abi region

The nucleotide sequence of the DNA from the region implicated in Abi by Bal31 deletion analysis was determined. Examination of the sequence indicated the presence of a potential open reading frame (ORF) of 1887 base pairs beginning with an ATG codon at position 202 and ending at a TAA codon at position 2086 and which was capable of encoding a peptide of 73·8 kDa (Fig. 5). The ORF was preceded by a putative promotor sequence and ribosome-binding site which exhibited similarity to consensus *E. coli* and *Bacillus subtilis* transcription/translation signals manifested by a -35 region in which four of the six nucleotides conformed to the canonical sequence and a -10 region where five of the six conformed. In addition, the -10 region was preceded by a TG sequence frequently observed in lactococcal promotors (van der Vossen *et al.*, 1987).

Hybridization against pMM1

A number of plasmids which are phenotypically similar to pCI829 have been identified in various laboratories worldwide. Consequently, it is of interest to determine the degree of homology which may exist between their *abi* determinants. Hence the relationship between pCI829 and the previously described phage insensitivity plasmid pCI750 (Baumgartner *et al.*, 1986; Coffey *et al.*, 1989) was examined. The recombinant plasmid pMM1 is composed of the vector plasmid pGB301 and 1·8 and 13·9 kb *Bcl*I fragments from pCI750. The 13·9 kb fragment has been indicated by Steele *et al.* (1989) to contain the *abi* determinant. The relatedness between the pCI829- and pCI750-derived *abi* loci was examined in this study by hybridization analysis in which pMM1 digested with either *Bcl*I or *Eco*RV was probed with two pCI829-derived intergenic *abi* DNA segments. Hybridization was only observed in lanes containing control digests of pCI816 and no homology was detected with any of the pMM1-derived DNA fragments (data not shown).

Discussion

The conjugative plasmid pCI829 mediates temperature-independent phage insensitivity by an abortive infection mechanism which confers complete resistance to small isometric-headed phages and partial insensitivity to prolate phages (Coffey *et al.*, 1989). The phage insensitivity associated with pCI829 appears to be similar to many other abortive phage infection systems described previously (Sanders, 1988).

Recently, the cloning of a number of these Abi systems which appear to be mechanistically similiar to pCI829 has been described (Hill *et al.*, 1989*b*, McKay *et al.*, 1989) and since these have been introduced into the closely related plasmid-free *L. lactis* subsp. *lactis* strains MG1363 or LM0230, it is possible to perform a comparative analysis of the effect of the cloned DNA on the phage sensitivity of the host to the 712/c2 group of phages. Approximately 2·0 kb of DNA was essential to confer the phage insensitivity phenotype of pCI829. Examination of the nucleotide sequence of this region did not indicate the presence of any strongly hydrophobic domains, suggesting that the pCI829-associated *abi* encodes what would appear to be a cytosolic protein.

In the case of pCI750 the phage insensitivity determinants were cloned on a 13·9 kb *Bcl*I fragment using the streptococcal vector pGB301, giving rise to the recombinant plasmid pMM1 (Steele *et al.*, 1989). Analysis of the physical map of this 13·9 kb region showed that it did not have any restriction sites in common with the Abi-encoding region of pCI829, suggesting that they are physically unrelated (unpublished data). The same is true for the Abi system cloned from pKR223 (Laible *et al.*, 1987). However, comparison of the nucleotide sequence of the pCI829-associated Abi to that of *hsp* from

pTR2030 (Hill *et al.*, 1990) indicates that these two genes are identical. This is interesting, as it shows that this gene may be widely disseminated both geographically and also in different host backgrounds, as strains ME2 and UC811 have distinct plasmid profiles (Klaenhammer & Sanozky, 1985; Coffey *et al.*, 1989). Neither the *abi* gene nor the predicted protein product showed any homology to any other existing sequence data in the GenBank database.

The phage insensitivity phenotypes exhibited by lactococcal transformants harbouring plasmids containing either the 6·2 kb *Stu*I or the 3·8 kb *Hin*dII fragment or plasmids treated with Bal31 were similar to those observed with a strain containing pCI829: insensitivity against small isometric phages was total (no plaques) and there was a reduction in plaque size for prolate phages, although this reduction was not as marked as that observed with a strain harbouring the complete pCI829 plasmid. It is interesting to note also that the reduction in e.o.p. mediated by pCI829 ($5{\cdot}2 \times 10^{-2}$) was not evident in the case of the recombinant plasmids pCI816 or pCI8147. This slightly lower level of expression by the latter plasmids may be due to the fact that the vector used has a lower copy number than pCI829. A decreased level of expression by *abi* determinants when cloned from their original plasmid has also been observed in the case of pCI750 (unpublished data). A marked difference in the level of expression between pKR223 and pGKB17 has not been reported (McKay *et al.*, 1989): both plasmids reduced the plaque size of phage c2 from 2·3 mm to pinpoint. Because of the minute size of these plaques it was not possible to compare the e.o.p. values for strains containing either of these plasmids (Laible *et al.*, 1987; McKay *et al.*, 1989). However, the drastic reduction in plaque size mediated by either of these plasmids contrasts with our findings for pCI816.

It is probable that many of the Abi systems reported by different laboratories are mediated by closely related genetic determinants. Indeed, a recent report by Steele *et al.* (1989) suggested that a degree of homology existed among a number of these plasmids. However, not all of the plasmids encoding this type of phage resistance showed homology to the rather large 13·9 kb probe which contained the determinants for reduced bacteriophage sensitivity (Rbs) from pCI750. In our laboratory no homology was detected when 1·0 kb and 0·3 kb fragments from within the *abi* region of pCI829 were probed against the 13·9 kb *Bcl*I fragment of pMM1, demonstrating that these two plasmids encode physically distinct but phenotypically similar mechanisms of phage insensitivity.

This report highlights some of the recent advances made in the genetic analysis of such a commercially significant trait as phage insensitivity. However, a continued research effort is needed to improve understanding of the interactions between phage and host at the molecular level. An increased knowledge in this area will maximize the potential to eliminate the phage problem in dairy fermentations.

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