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

AIDAN G. COFFEY, GERALD F. FITZGERALD* and CHARLES DALY

Food Microbiology Department, University College, Cork, Ireland

(Received 22 June 1990; revised 18 January 1991; accepted 20 February 1991)

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 promoter 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 Tn5 mutagenesis and deletion analysis (Hill et al., 1989a, 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 similar 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.
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. The antibiotics erythromycin (Em), chloramphenicol (Cm), tetracycline (Tc) and ampicillin (Ap) were added to selective media for strains of E. coli, respectively. For lactococci, Em was added at 2 µg ml⁻¹.

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 BamHI 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 7% 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.

Table 1. Bacterial strains and bacteriophages

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sensitivity to phage 712*</th>
<th>Plasmid content</th>
<th>Origin/derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis subsp. lactis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363Sm</td>
<td>S†</td>
<td>None</td>
<td>Sm¹ plasmid-free derivative of L. lactis subsp. lactis 712 (Gasson, 1983)</td>
</tr>
<tr>
<td>AC002</td>
<td>I</td>
<td>pCI829</td>
<td>Lac⁴ derivative of L. lactis subsp. lactis AC001 (Coffey et al., 1989)</td>
</tr>
<tr>
<td>AC816</td>
<td>I</td>
<td>pCI816</td>
<td></td>
</tr>
<tr>
<td>AC817</td>
<td>S</td>
<td>pCI817</td>
<td></td>
</tr>
<tr>
<td>AC815</td>
<td>S</td>
<td>pCI815</td>
<td></td>
</tr>
<tr>
<td>AC813</td>
<td>S</td>
<td>pCI813</td>
<td></td>
</tr>
<tr>
<td>AC814</td>
<td>S</td>
<td>pCI814</td>
<td></td>
</tr>
<tr>
<td>AC812</td>
<td>S</td>
<td>pCI812</td>
<td></td>
</tr>
<tr>
<td>AC8147</td>
<td>I</td>
<td>pCI8147</td>
<td></td>
</tr>
<tr>
<td>AC8144</td>
<td>I</td>
<td>pCI8144</td>
<td></td>
</tr>
<tr>
<td>MM1</td>
<td>I</td>
<td>pMM1</td>
<td></td>
</tr>
<tr>
<td>AC719</td>
<td>I</td>
<td>pB719</td>
<td></td>
</tr>
<tr>
<td>AC718</td>
<td>I</td>
<td>pB718</td>
<td></td>
</tr>
<tr>
<td>AC721</td>
<td>S</td>
<td>pB721</td>
<td></td>
</tr>
<tr>
<td>AC408</td>
<td>I</td>
<td>pB408</td>
<td></td>
</tr>
<tr>
<td>AC426</td>
<td>I</td>
<td>pB426</td>
<td></td>
</tr>
<tr>
<td>AC433</td>
<td>S</td>
<td>pB433</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB11(pSA3)</td>
<td>–</td>
<td>pSA3</td>
<td>Dao &amp; Ferretti (1985)</td>
</tr>
<tr>
<td>HB101</td>
<td>–</td>
<td>None</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>TG1</td>
<td>–</td>
<td>pUC18/19 clones</td>
<td>Courtesy of W. deVos, NIZO, Ede, The Netherlands</td>
</tr>
<tr>
<td>AC810</td>
<td>–</td>
<td>pCI810</td>
<td>Deletion derivative of pCI816 (this study)</td>
</tr>
<tr>
<td>AC8147</td>
<td>–</td>
<td>pCI8147</td>
<td>Deletion derivatives of pCI8144 (this study)</td>
</tr>
<tr>
<td>AC8144</td>
<td>–</td>
<td>pCI8144</td>
<td></td>
</tr>
</tbody>
</table>

* S. sensitive; I. insensitive.
† MG1363Sm also sensitive to phages c2 and m13.
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 immunosassay using an antibody conjugate and subsequent enzymecatalysed 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 StuI, fragments of approximately 37 and 6.2 kb were generated. These fragments were mixed with pSA3 which had been linearized with NruI 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 10^6 and 10^7 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
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 $5.2 \times 10^{-2}$ (Coffey et al., 1989). These data showed that the level of phage insensitivity conferred by pC1816 was not as strong as that conferred by the original plasmid pC1829.

**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 *AvaI* [pSA3 is composed of pACYC184 with a
Fig. 5. Nucleotide sequence of the \( abt \) region of pC1829. The putative promoter 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 AvaI site (Diao & Ferretti, 1985). The resulting 10.2 kb plasmid, designated pCI810, is equivalent to pACYC184 containing the 6.2 kb StuI 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 MG1363Sm 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 HindII 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 PvuII 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 PvuII 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 ClaI 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 738 kDa (Fig. 5). The ORF was preceded by a putative promoter 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 promoters (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 BclI 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 BclI or EcoRV 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 similar to pCI829 has been described (Hill et al., 1989b, 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 BclI 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 StuI or the 3.8 kb HindII 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 \(5.2 \times 10^{-2}\) 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 pGKBl7 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 BclI 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.

This work was supported by the European Community Biotechnology Action Programme (contract no. BAP-0008-IRL). We acknowledge the suggestion by T. R. Klaenhammer to use vector pSA3 in the initial cloning experiments.

**References**


