Characterization of the ColE9-J Plasmid and Analysis of its Genetic Organization

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We have determined the restriction and functional map of the ColE9-J plasmid. By sub-cloning and transposon mutagenesis we have shown that the ColE9*imm gene and the ColE5*imm gene present on the ColE9-J plasmid are located on separate EcoRI fragments. Using an expression vector we have demonstrated the presence of two lys genes on the ColE9-J plasmid, both of which are dependent upon the colicin E9 structural gene promoter. Promoter mapping studies imply that the colicin E9 structural gene and the ColE5*imm gene are transcribed in the same direction, but that the ColE9*imm gene is transcribed in the opposite orientation.

INTRODUCTION

The E. coli E. coli K12 strains carrying an E. coli plasmid are sensitive to mitomycin C (MC) compared to plasmid-free isogenic strains, due to the induction of a plasmid-coded lysis gene (lys) which may be involved in colicin release from the producing cell (Shafferman et al., 1979; Pugsley & Schwartz, 1983; Suit et al., 1983; Chak & James, 1984; Jakes & Zinder, 1984; Lawrence & James, 1984). Since immunity is constitutive, whilst colicin production and MC sensitivity are inducible by DNA damage (Herschman & Helinski, 1967), the regulatory control of these three genes is of considerable interest.

Experiments with transposon mutagenesis of the ColE3-CA38 plasmid have demonstrated that the colicin E3 structural gene (ceaeC) and the lys gene (ceC) are transcribed from a common SOS promoter, located proximal to the structural gene, whilst the ColE3*imm gene (ceiC), which is located between these two genes, must have its own promoter (Chak & James, 1984; Jakes & Zinder, 1984). We have identified a second unrelated immunity gene, to colicin E8 (ceiH), located in tandem with the ColE3*imm gene (Chak & James, 1984), the nucleotide sequence of which has subsequently been reported (Lau et al., 1984). Analysis of the nucleotide sequence of this region of the ColE3-CA38 plasmid shows that the colicin structural gene, the ColE3*imm gene, the ColE8*imm gene and the lys gene are all transcribed in the same direction (Chak & James, 1985). We have recently confirmed that the two immunity genes have their own promoters which are independent of the SOS promoter located proximal to the 5' end of the

Abbreviation: MC, mitomycin C.
colicin E3 structural gene (Chak & James, 1985). It has been suggested that the ColE3-CA38 plasmid may be unique in that it is a plasmid ‘caught in the act of evolution’ (Lau et al., 1984). The precise mechanism which has resulted in the presence of the two immunity genes in tandem between the colicin E3 structural gene and the lys gene is unknown, especially as the E8imm gene shows marked homology to the E2imm gene of the ColE2-P9 plasmid but exhibits very considerable sequence divergence from the E13imm gene (Lau et al., 1984). This may well rule out gene duplication of the E3imm gene as the evolutionary origin of the E8imm gene on the ColE3-CA38 plasmid.

The ColE9-J plasmid has previously been reported to encode immunity to both colicin E9 and to colicin E5 (Cooper & James, 1984). Here we show that the ColE9-J plasmid carries two separate immunity genes and two lys genes. Using two recently developed promoter probe vectors (Chak & James, 1985), we have analysed the location and strength of promoters present in the ColE9-J plasmid. Our data suggests that the genetic organization of the two immunity genes found on the ColE9-J plasmid is different to that found in the ColE3-CA38 plasmid in that they are transcribed in opposite directions to each other. A comparison of the genetic organization of a variety of colicin operons may provide an insight into the evolutionary origin of this closely related group of plasmids.

**METHODS**

**Plasmids.** The ColE9-J plasmid has previously been described (Cooper & James, 1984). E. coli W3110(ColE5-099) was obtained from the Plasmid Reference Center, Stanford University, Calif., USA. The promoter-probe vectors pKC86 and pKC87 have a polylinker containing multiple unique cloning sites located proximal to the 5’ end of a promoter-less galactokinase gene (Chak & James, 1985), whilst plasmid pUC8 (Vieira & Messing, 1982) was a kind gift of J. Messing, University of Minnesota, St Paul, USA. The expression vector plasmid pKK223-3 was purchased from Pharmacia, Milton Keynes, UK. This vector is derived from pBR322 and contains the trp-lac operator.
(tac) promoter (de Boer et al., 1983) proximal to a polylinker derived from pUC8. The polylinker sequence is followed by strong transcription terminators to stabilize the vector after induction of the tac promoter by IPTG (Brosius, 1984). Plasmids were prepared by a modification of the method of Birnboim & Doly (1979). The derivation of the recombinant plasmids used in this work is shown in Table 1.

**Bacterial strains and media.** *E. coli* W3110(ColE5-099) and *E. coli* W3110(ColE9-J) were used to prepare extracts of colicin E5 and colicin E9 respectively (Chak & James, 1984). *E. coli* JC3272 (His- Lys+ Trp- lacY galK rpsL) was used as the host in all transformations using promoter probe vectors: *E. coli* JM83 (Ara- Lac+ Pro- Thi+ rpsL g80d lacZM15) was the host for transformations with pUC8 and *E. coli* JM105 (Lac- Pro- Thi- endA sbcB1.5 hspR4 rpsL traD36 proAB+ lacI+ lacZM15) was the host for transformations with pKK223-3. For selection of recombinant plasmids carrying promoter fragments we used LB agar (Miller, 1972) containing ampicillin, supplemented with thiamine and glycerol. 

**Enzymes and reagents.** Restriction enzymes and T4 DNA ligase were purchased from Anglian Biotechnology, Colchester, UK; BRL, Cambridge, UK; Pharmacia, Milton Keynes, UK; or Boehringer Mannheim, Lewes, UK. MC (Sigma) was used as previously described to test the MC sensitivity of cultures containing recombinant plasmids (Chak & James, 1984; Lawrence & James, 1984).

**Colicin production and immunity tests.** Colicin production and immunity testing were as previously described (Cooper & James, 1984).

**Plasmid restriction, ligation, transformation and electrophoresis.** Digestion of DNA with restriction endonucleases, ligation of DNA fragments, filling-in of sticky-ends, and transformation of competent cells were done as previously described (Maniatis et al., 1982). Electrophoresis of DNA fragments was done as previously described (Chak & James, 1984). Specific fragments were recovered from gels using the method of Dretzen et al. (1981).

**Galactokinase assay.** The activity of promoters present in recombinant plasmids derived from pKK86 or pKK87 was determined by a previously described method (McKenney et al., 1981; Brandsma et al., 1983) using [14C]galactose (Amersham). In the induction experiments with MC, cultures containing recombinant plasmids were grown to an OD600 of 0.4 before the addition of MC (0.5 μg ml⁻¹). Duplicate 1 ml samples were removed after various times of incubation in the presence of MC for galactokinase assay.

**Transposon mutagenesis.** Transposon mutagenesis with Tn1000 was done as previously described (Chak & James, 1984). Transposon insertion sites were located by isolating plasmid DNA and restricting separately with ampicillin, supplemented with thiamine and glycerol.

**RESULTS AND DISCUSSION**

**Restriction mapping of the ColE9-J plasmid**

An EcoRI digest of the native ColE9-J plasmid yielded fragments of 3.45 kb, 3.42 kb and 0.8 kb. A partial EcoRI digest of the ColE9-J plasmid and subsequent ligation into the EcoRI site of the vector pUC8 yielded recombinant plasmid pKC56 which carries the whole 7.67 kb ColE9-J plasmid. Recombinant plasmids were constructed which contained the 3.45 kb EcoRI fragment (pKC65), the 3.42 kb EcoRI fragment (pKC66), or the 4 kb HincII fragment (pKC67) of the native ColE9-J plasmid cloned into pUC8. These plasmids were analysed by restriction mapping and the data combined with that obtained from restriction mapping of the native ColE9-J plasmid (Fig. 1).

**Location of the ColE9 structural gene**

The phenotypes conferred by plasmids pKC65 and pKC67 locate the ColE9 structural gene on the 2.76 kb HincII−EcoRI fragment of the ColE9-J plasmid. The insertion sites of transposon Tn1000 which inactivate colicin production in plasmids pKC65 and pKC67 further localize the ColE9 structural gene (Fig. 1).

**Location of the ColE9imm and the ColE5imm genes**

The phenotypes conferred by plasmids pKC65 and pKC66 clearly demonstrate that the colicin E9 and colicin E5 immunity conferred by the ColE9-J plasmid are encoded by separate genes, which are located on different EcoRI fragments. In order to locate the position of the two genes...
Immunity genes more precisely, further recombinants were constructed (Fig. 1). The phenotypes conferred by pKC75 and pKC78 locate the ColE9\textit{imm} gene (\textit{cei}1) on a 0.78 kb \textit{CfaI–EcoRI} fragment and the ColE5\textit{imm} gene (\textit{cei}E) on a 0.66 kb \textit{EcoRI–PvuII} fragment of the ColE9-J plasmid. The location of the ColE5\textit{imm} gene and the ColE9\textit{imm} gene was also confirmed by transposon mutagenesis of plasmid pKC66 and plasmid pKC129 (Fig. 1). \textit{Tn}1000 inserts located 550 bp or 700 bp from the left-hand \textit{EcoRI} site of plasmid pKC66 had no affect upon colicin E5 immunity, whereas inserts located 200 bp or 350 bp from the left-hand \textit{EcoRI} site inactivated colicin E5 immunity. In the case of plasmid pKC129, in which the absence of a functional colicin E9 structural gene allows the isolation of \textit{Tn}1000 inserts which inactivate the E9\textit{imm} gene, an insert located 600 bp from the right-hand \textit{EcoRI} site inactivated colicin E9 immunity, whereas inserts located 650 bp or 750 bp from the left-hand \textit{EcoRI} site did not.

\textit{Comparison with the ColE5-099 plasmid}

The ColE5-099 plasmid is 6.7 kb in size and consists of \textit{EcoRI} fragments of 3.57 kb, 2.48 kb and 0.7 kb. After partial \textit{EcoRI} digestion we cloned the whole ColE5-099 plasmid into the \textit{EcoRI} site of pBR322. The resulting plasmid pKC19 encoded colicin E5 production, MC sensitivity and immunity to colicin E5. After purification of the two large \textit{EcoRI} fragments of the ColE5-099 plasmid from agarose gels we attempted to subclone them into the \textit{EcoRI} site of pBR322. Plasmid pKC33 consists of the 3.57 kb \textit{EcoRI} fragment of the ColE5-099 plasmid cloned into the \textit{EcoRI} site of pBR322 and encodes colicin E5 immunity alone. We were unable to isolate any recombinant plasmid which contained the 2.48 kb \textit{EcoRI} fragment alone. We found a similar result with the ColE8-J plasmid (Lawrence & James, 1984), and believe that this is due to the lethality of recombinant plasmids which encode the colicin structural gene without the homologous immunity gene being present on the same fragment. Our restriction map of the ColE5-099 plasmid (data not shown) is consistent with that of Watson et al. (1985), and our subcloning has allowed us to localize the E5\textit{imm} gene to a separate \textit{EcoRI} restriction fragment to that of the colicin structural gene.
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Location of the MC sensitivity gene(s)

The MC sensitivity conferred by plasmid pKC65 (Fig. 2) is surprising in comparison with the results previously obtained in analysis of the ColE3-CA38 plasmid, which also contains two immunity genes (Chak & James, 1984). The ColE9-J plasmid appears to encode colicin E9 production, colicin E9 immunity and MC sensitivity on a single EcoRI fragment. The phenotype conferred by recombinant plasmid pKC129 confirmed that the MC sensitivity of plasmid pKC65 is due to the presence of a lys gene, and not due to the induction of colicin E9 synthesis itself, as the deletion of the 0.64 kb ClaI fragment of pKC65 abolished colicin E9 production, but had no effect upon colicin E9 immunity, or on MC sensitivity (Fig. 1). Transposon Tn1000 inserts which inactivated colicin E9 production also abolished MC sensitivity (data not shown), a result which has previously been found with ColE1 (Suit et al., 1983), ColE2-P9 (Pugsley & Schwartz, 1983), ColE3-CA38 (Chak & James, 1984; Jakes & Zinder, 1984) and ColE8-J (Lawrence & James, 1984) plasmids.

The presence of a second MC sensitivity gene located on the same EcoRI fragment as the ColE5imm gene was suggested by experiments which showed that the MC sensitivity conferred by plasmids pKC67 or pKC68 was markedly greater than that conferred by plasmid pKC65, but, surprisingly, not than that conferred by pKC56 (Fig. 2). In order to test this hypothesis directly, recombinant plasmids were constructed containing regions of the 3.42 kb EcoRI fragment, which encoded colicin E5 immunity and the presumptive second lys gene, under the control of an inducible promoter. The expression vector pKK223-3 contains a group of unique restriction sites located distal to a tac promoter and proximal to strong terminator sequences (Fig. 3). The 0.66 kb EcoRI–PvuII fragment and the 1.31 kb EcoRI–HincII fragment of the ColE9-J plasmid were cloned into pKK233-3 which had been restricted with EcoRI and SmaI, yielding plasmids pKC148 and pKC149 respectively. The presence of these plasmids conferred an IPTG-sensitive phenotype to E. coli JM105 as compared to the plasmid-free isogenic strain (Fig. 4).

Promoter mapping of the ColE9-J plasmid

Fragments of the ColE9-J plasmid were cloned into the promoter-probe vectors pKC86 and pKC87 (Table 1). The cloned fragments, their orientation with respect to the promoter-less galactokinase gene and the galactokinase level as a judge of the strength of any promoter present are shown in Fig. 5. The DNA-damage inducible SOS promoter is located in the 1.37 kb HincII–ClaI fragment present in recombinant plasmid pKC142. This promoter, which transcribes
Fig. 3. Restriction map of plasmid pKK223-3. The polylinker region of the plasmid is shown in an enlarged form for clarity. The SalI site which is present in the polylinker cannot be used for cloning due to the presence of a SalI site elsewhere in the vector.

Fig. 4. IPTG induction of cell lysis. At time zero IPTG (1 mM) was added to exponentially growing cultures of *E. coli* JM105 carrying no plasmid (●), pKK223-3 (○), pKC148 (■) and pKC149 (▲). The results shown are typical of a series of three experiments.

towards the *ClaI* restriction site, is relatively weak in the absence of DNA damage, a result which was reported previously for the SOS promoter of the ColE3-CA38 plasmid (Chak & James, 1985). In the presence of MC however, this promoter is strongly induced (Fig. 6). This result implies that the *lys* gene present in plasmid pKC65, which is dependent upon the SOS promoter for induction, is also transcribed in the same orientation. The reproducible difference between the promoter activity of recombinant plasmids pKC142 and pKC135 also agrees with earlier findings with the SOS promoter of the ColE3-CA38 plasmid and is presumably due to the presence of terminator sequences proximal to the *lys* gene which prevent cell lysis until full induction of the SOS promoter occurs. The absence of MC induction of galactokinase in plasmid pKC131, even though MC-induced cell lysis does occur (Fig. 6), is suggestive of a strong terminator located in the 0.64 kb *PvuII*-HincII fragment of ColE9-J.

The results shown in Fig. 5 clearly locate a strong promoter in the fragment present in recombinant pKC143. As with pKC75 (Fig. 1), this recombinant plasmid encodes colicin E9 immunity and, by analogy with other *E. coli* plasmids, a *lys* gene, although this must be transcribed in the opposite orientation with respect to the 5' end of the *galK* gene. This implies that the direction of transcription of the ColE9*imm* gene is in the opposite orientation to that of
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Fig. 5. Promoter activity of sub-clones of the ColE9-J plasmid. The restriction fragments present in the plasmids listed are indicated (for clarity, vector sequences are not shown), together with the phenotypes conferred and the galactokinase level. The orientation of the fragments with respect to the galK gene is indicated by the arrowheads; fragments with arrowheads pointing to the right are located in the same orientation as the galactokinase gene. The approximate positions of the relevant genes are indicated under the restriction map of the ColE9-J plasmid.

Fig. 6. Kinetics of induction of galactokinase. The galactokinase level (○) of cultures of E. coli JC3272 carrying the plasmids indicated are shown along with the OD₅₅₀ (●) of the cultures from which samples were removed. MC was added at time 0. The results of a typical experiment are shown; each experiment was repeated at least twice.
the colicin E9 structural gene and of the lys gene. A weak promoter is present in the 1.31 kb EcoR1–HincII fragment present in recombinant pKC136. This recombinant plasmid encodes colicin E5 immunity, as well as the second SOS inducible lys gene. Again, in the absence of an SOS promoter this lys gene will not be induced; this implies that the direction of transcription of the ColE5imm gene is the same as that of the colicin E9 structural gene. The presence of a strong terminator in the 0.64 kb PvuII–HincII fragment of the ColE9-J plasmid may result in an underestimate of the strength of the E5imm promoter in plasmid pKC136. Neither of the presumptive immunity gene promoters is inducible by MC (Fig. 6).

By a comparison of the data for plasmids pKC123 and pKC108 (Fig. 9), this experiment has identified a promoter in the 1.87 kb ClaI–EcoR1 fragment of pKC108. Plasmids containing this region are incompatible with the native ColE9-J plasmid but are compatible with the native ColE8-J plasmid (F. Hawkins & P. C. Cooper, personal communication). It remains to be determined whether the promoter identified in this region has any role in plasmid incompatibility.

**Comparison with other E. coli plasmids**

Our results demonstrate that the ColE9-J plasmid, like the ColE3-CA38 plasmid, has two immunity genes. Since the ColE6-CT14 plasmid also carries an extra E8imm gene (Lawrence, 1984), the proposal that the ColE3-CA38 plasmid may represent a unique case of a plasmid caught in the act of evolution (Lau et al., 1984) now needs revising. The genetic organization of the two immunity genes of the ColE9-J plasmid is however very different to that found with ColE3-CA38. The only other E. coli plasmid in which the immunity gene is transcribed in the opposite orientation to the respective colicin structural gene is ColE1 (Suit et al., 1983). Colicin E9 can be distinguished from colicin E1 by the insensitivity of tolA and tolD mutants to the former but not to the latter. Antisera raised against colicin E3 also protects sensitive E. coli cells
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to all E colicins, except colicin E1 (Cooper & James, 1984). One interesting problem which results from the SOS promoter and the E9imm gene promoter being transcribed in opposite orientations is the manner in which the synthesis of colicin E9 and its homologous immunity protein is co-ordinated upon SOS induction so as to avoid death of the producing cell caused by excess colicin E9. No evidence of MC-inducibility of the E9imm gene promoter was found (Fig. 6); however it should be borne in mind that inducibility of the promoter of this gene can be assayed only in the absence of the colicin E9 structural gene. This promoter in vitro appears to be intermediate in strength between those of the E3imm gene and the E8imm gene found on the ColE3-CA38 plasmid (Chak & James, 1985), so it is unlikely to result in the constitutive synthesis of sufficient excess colicin E9 immunity protein, irrespective of the rate of synthesis of the colicin E9 gene product. The transcriptional and translational control of the E9imm gene is under investigation to determine whether or not the rate of synthesis of the colicin E9 immunity protein is induced by MC by an unknown regulatory mechanism. It is interesting, however, that the colicin E9 gene product. The transcriptional and translational control of the E9imm gene is under investigation to determine whether or not the rate of synthesis of the colicin E9 immunity protein is induced by MC by an unknown regulatory mechanism. It is interesting, however, that the strength of the colicin E9 SOS promoter when fully induced (Fig. 6) is less than 50% of that found for the colicin E3 SOS promoter present on the ColE3-CA38 plasmid (Chak & James, 1985). The novel functional and genetic organization of the ColE9-J plasmid is illustrated in Fig. 7.

As has been pointed out by Watson et al. (1985), by including hypothetical deletions in the case of the ColE5-099 and the ColE8-J plasmids, and a 4 kb insertion in the case of ColE6-CT14, it is possible to align the restriction and functional maps of these plasmids with that of ColE3-CA38. The deletion implied by Watson et al. (1985), but not stated in their restriction map of the ColE5-099 plasmid, would include the EcoRI site which separates the colicin E5 structural gene and the E5imm gene. Our data does not support this hypothesis. A comparison of the restriction and functional maps of the ColE5-099 and the ColE9-J plasmids reveals considerable similarities. They are the only E colicin plasmids which have a functional immunity gene on a separate EcoRI fragment from the colicin structural gene. Further analysis of the DNA sequence of these two plasmids will be invaluable in our understanding of the evolutionary origin of the E colicin plasmids.

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REFERENCES


