Cloning and characterization of the ColE7 plasmid

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The 6.2 kb ColE7-K317 plasmid was mapped and the DNA fragments of the colicin E7 operon subcloned into pUC18 and pUC19. The size of the functional colicin E7 operon deduced by subcloning was 2.3 kb. The colicin E7 gene product was purified by carboxymethylcellulose chromatography. Both colicin E7 and E9 were demonstrated to exhibit a non-specific DNAase-type activity by *in vitro* biological assay. The molecular mass of colicin E7 was 61 kDa, as determined by SDS-PAGE. From DNA sequence data, the estimated sizes of the E7 immunity protein and the E7 lysis protein were 9926 Da and 4847 Da, respectively. Comparison of restriction maps and DNA sequence data suggests that ColE7 and ColE2 are more closely related than other E colicin plasmids.

Introduction

The E-group colicins are a group of plasmid-borne bacteriocins (Fredericq, 1949) which exhibit antibiotic-like inhibition of bacterial growth. The activity spectrum of colicins is confined to closely related organisms such as *Escherichia*, *Salmonella* and *Shigella* strains (the *Enterobacteriaceae*). The E-group colicins have been subdivided into colicins E1 to E9 on the basis of immunity tests (Watson *et al.*, 1981; Cooper & James, 1984). The modes of action of some E-group colicins are well documented (Konisky, 1982). Colicin E1 exhibits an ionophore effect on the membrane of target cells (Tokuda & Konisky, 1979), colicin E3 is an RNAase (Bowman *et al.*, 1971), and colicins E2 and E8 are DNAases (Schaller & Nomura, 1976; Toba *et al.*, 1988).

The production of E colicins is controlled and regulated by a set of genes on the plasmid forming an 'SOS' response operon which includes structural (*cea*), immunity (*cei*) and lysis/release (*cel*) genes. The production of colicin is inducible by DNA damage of the host cell, which can be induced by mitomycin C or UV irradiation (Herschman & Helinski, 1967). The synthesis of the immunity gene protein appears to be constitutive (Jakes *et al.*, 1974). The lysis gene is promoterless (Chak & James, 1985), its transcription relying upon transcriptional readthrough from the promoter of the colicin

structural gene (Chak & James, 1985). In previous studies of the ColE3-CA38 and the ColE9-J plasmids, a second unrelated immunity gene, encoding immunity to colicin E8 (Chak & James, 1984) and E5 (Chak & James, 1986), and located distal to the E3-*cei* and E9-*cei* genes, respectively, was identified.

In this report, we present the genetic organization of the ColE7 plasmid and the DNA sequence of E7-*cei* and E7-*cel*, and verify the mode of action of colicin E7 by an *in vitro* biological assay.

Methods

Bacterial strains, plasmids and media. *Escherichia coli* W3110 (ColE7-K317), *E. coli* (ColE8-J) and *E. coli* (ColE9-J) were from the collection of Dr R. James. Plasmids pUC18 and pUC19 (Yanisch-Perron *et al.*, 1985) were used as cloning vectors. *E. coli* W3110 was used as a standard strain for colicin production and immunity tests. *E. coli* JM101 and HB101 were used as cloning hosts. A pair of promoter-probe vectors, pKC86 and pKC87 (Chak & James, 1985), were used for promoter activity tests of the recombinant plasmids. *E. coli* JC3272 (*his* *lys* *trp* *lacY* *galK* *strA*) was used as the host for the promoter-probe vectors. Details of the construction of recombinant plasmids and derivatives are shown in Table 1.

Bacteria were cultured at 37 °C in L-broth (Miller, 1972). For selection of recombinant clones, ampicillin was included in L-agar at a final concentration of 100 µg ml⁻¹. The chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and isopropyl β-D-thiogalactoside (IPTG) were added to the plates at a final concentration of 20 µg ml⁻¹ to select pUC18 or pUC19 recombinants.

Enzymes and reagents. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Labs, Boehringer Mannheim, Promega Corporation or Toyobo Co. Mitomycin C (MC) was obtained from Sigma.

Abbreviation: MC, mitomycin C.

The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number M57540.

Table 1. Derivation of recombinant plasmids

Plasmid	Derivation
pHK101	The whole 6.2 kb ColE7-K317 linearized fragment cloned into the <i>EcoRI</i> site of pUC18
pHK202	Same as pHK101, but with the insert in the opposite orientation
pHK001	The 5.0 kb <i>EcoRI</i> - <i>Bgl</i> II fragment of ColE7 cloned into the <i>EcoRI</i> - <i>Bam</i> HI sites of pUC18
pHK14	The 1.6 kb <i>Sau</i> 3A fragment of pHK101 deleted
pHK25	The 0.75 kb <i>Sau</i> 3A fragment of ColE7 cloned into the <i>Bam</i> HI site of pUC18
pHK80	The 0.65 kb <i>Sau</i> 3A fragment of ColE7 cloned into the <i>Bam</i> HI site of pUC19
pHK80-R	As pHK80, but with the insert in the opposite orientation
pHK80-P10	The 0.32 kb <i>Dra</i> I- <i>Sau</i> 3A fragment of pHK80 deleted
pHK80-E4	The 0.34 kb <i>Sau</i> 3A- <i>Dra</i> I fragment of pHK80 deleted
pSE4	The linearized 6.2 kb <i>EcoRI</i> fragment of ColE7 was partially restricted with <i>Dra</i> I, and the resulting restriction fragments were then cloned into the <i>EcoRI</i> - <i>Sma</i> I sites of pUC18; the 1.3 kb <i>Dra</i> I- <i>EcoRI</i> fragment is present in this recombinant
pSE5	Subcloning method as for pSE4; the 1.8 kb <i>Dra</i> I- <i>EcoRI</i> fragment is present in this recombinant
pSE3	Subcloning method as for pSE4; the 2.9 kb <i>Dra</i> I- <i>EcoRI</i> fragment is present in this recombinant
pSB57	pHK001 was linearized by <i>Bgl</i> II, then partially restricted with <i>Dra</i> I. After self-ligation, three deletion mutants were obtained; a 4.2 kb <i>Dra</i> I- <i>Bgl</i> II fragment was deleted in pSB57
pSB64	Subcloning method as for pSB57; a 3.2 kb <i>Dra</i> I- <i>Bgl</i> II fragment was deleted from recombinant pHK001
pSB58	Subcloning method as for pSB57; a 2.4 kb <i>Dra</i> I- <i>Bgl</i> II fragment should have been deleted from recombinant pHK001, but the deletion was slightly shorter than expected
ppPV-57	The 2.1 kb <i>Bgl</i> II- <i>Dra</i> I fragment from pBS57 was cloned into the <i>Bam</i> HI- <i>Sma</i> I site of pKC86
ppPV-57R	As ppPV-57, but the 2.1 kb <i>Bgl</i> II- <i>Dra</i> I fragment was cloned into pKC87
ppPV-EH1	A 0.85 kb fragment, containing the 5' terminus of ColE7, from pHK14 was released by restriction with <i>Xba</i> I and <i>Hinc</i> II, then this fragment was subcloned into the <i>Xba</i> I- <i>Sma</i> I site of pKC86
ppPV-EH1R	As ppPV-EH1, but the 0.85 kb fragment was cloned into pKC87
ppPV80	The 0.65 kb <i>Sau</i> 3A fragment was released from pHK80 by restriction with <i>Hind</i> III and <i>Eco</i> RI, and this fragment was subcloned into the <i>Hind</i> III- <i>Eco</i> RI site of pKC86
ppPV80R	As ppPV80, but the 0.65 kb fragment was cloned into pKC87
ppPV80-P10	The 0.32 kb <i>Dra</i> I- <i>Sau</i> 3A fragment was released from pHK80-P10 by restriction with <i>Hind</i> III and <i>Eco</i> RI, and the fragment was subcloned into the <i>Hind</i> III- <i>Eco</i> RI site of pKC86
ppPV80-P10R	As ppPV80-P10, but the fragment was cloned into pKC87
ppPV80-E4	The 0.34 kb <i>Sau</i> 3A- <i>Dra</i> I fragment from pHK80-E4 was released by restriction with <i>Hind</i> III and <i>Eco</i> RI, and the fragment was subcloned into the <i>Hind</i> III- <i>Eco</i> RI site of pKC86
ppPV80-E4R	As ppPV80-E4, but the fragment was cloned into pKC87

Nucleotide sequencing. The nucleotide sequence of the *cei* and *cel* genes of the ColE7 plasmid was determined by the method of Sanger *et al.* (1977). The double-stranded DNA sequencing method was that described by Chen & Seeburg (1985). Modified T7 DNA polymerase (Tabor & Richardson, 1987) was used for polymerization of nucleotides in our nucleotide sequence determination. [α - 35 S]dATP was obtained from Amersham. The 17-mer forward and reverse pUC/M13 sequencing primers were purchased from Promega Biological Research Products. PC/Gene, a DNA sequence analysis program from Genetech, was used for the analysis of the DNA sequences.

Preparation of colicin extracts, and colicin production and immunity tests. These were performed as previously described (Chak & James, 1984).

Colicin titre assay. The preparation of colicin extracts for titre assay was as previously described (Chak & James, 1984). Colicin titres were determined by the method of Reeves (1965) with some modifications. Two-fold serial dilutions of colicin extract were made from 2^0 to 2^{35} in L-broth, containing the appropriate antibiotic, in a microtitre dish. Then 10 μ l of each diluted colicin extract was spotted on an L-agar plate which was overlaid with 3 ml soft L-agar containing 100 μ l of the indicator culture (*E. coli* W3110). Plates were incubated overnight at 37 °C. The clear or turbid zones resulting from the inhibition of cell growth by the diluted colicin extracts were recorded. The number of clear or turbid zones was plotted against the reciprocal of the corresponding dilution factor. Cell survival followed a sigmoidal-like curve as the colicin dilution increased. The colicin titre was defined as the reciprocal of the dilution that killed 50% of the cells as measured from the survival curve (Reeves, 1965).

Promoter activity test. Two promoter-probe vectors, pKC86 and pKC87 (Chak & James, 1985), were used to detect the promoter strength of the cloned fragment present in recombinant plasmids. The galactokinase assay was as previously described (McKenny *et al.*, 1981), with some modifications: in the induction experiments, cultures containing recombinant plasmids were grown to an OD₆₅₀ of 0.4 before the addition of MC (0.5 μ g ml⁻¹); and 5 μ l, instead of 20 μ l, of galactokinase extract was used.

Plasmid isolation, restriction, ligation and transformation. Plasmids were prepared by the method of Birnboim (1983). Digestion of DNA with restriction endonucleases, ligation of DNA fragments and transformation of competent cells were carried out as described by Maniatis *et al.* (1982).

Electrophoresis of DNA and purification of fragments. Electrophoresis of DNA fragments was performed as previously described (Chak & James, 1984). Specific fragments were recovered from gels using the method of Dretzen *et al.* (1981).

Purification of colicins. The purification of colicins, using carboxymethylcellulose chromatography after acidic precipitation, was as described by Pugsley & Oudega (1987). The crude extracts of colicin E7 and colicin E9 were prepared from 5 litres of culture medium as previously described (Chak & James, 1984). Cell debris was removed by centrifugation and the crude colicin was then precipitated by ammonium sulphate (60% saturation). The precipitate was resuspended in 50 ml 10 mM-potassium phosphate buffer (pH 7.0) and dialysed overnight against 5 litres of the same buffer. The colicin solution was centrifuged at 20000 *g* for 10 min and the supernatant fraction was applied to a carboxymethylcellulose column. The column was rinsed for several hours with the same buffer until all unbound material was eluted. The colicin fraction was then eluted with a linear salt gradient of 1.5 bed volumes from 0 to 400 mM-NaCl in phosphate buffer (pH 7.0). Fractions were monitored for protein, by measuring A₂₈₀, and for colicin activity. The fractions containing the highest

concentration of colicin were pooled and dialysed overnight against several changes of distilled water at 4 °C. Finally, purified colicin was lyophilized and stored at -20 °C. The purity of the colicin preparation was checked by SDS-PAGE.

In vitro biological activity assay of purified colicins. Approximately 2 µg of CsCl-purified pUC18 DNA was dissolved in reaction buffer (20 mM-Tris/HCl pH 8.0, 80 mM-NaCl, 10 mM-MgSO₄). After the addition of 1 µg purified colicin to the reaction mixture, sterile distilled water was added to a final volume of 40 µl. The reaction mixture was then equally distributed into four tubes, which were incubated at 37 °C. After 0, 30, 60 and 90 min, 1 µl 250 mM-EDTA was added to one tube. After all samples had been collected, the plasmid DNA was analysed by 0.8% agarose gel electrophoresis. The mixture in the control tube was the same as all other sampling tubes except that colicin was not included.

Results

Restriction endonuclease mapping and cloning of the ColE7 plasmid

After restriction endonuclease digestion of the ColE7-K317 plasmid with *Dra*I, three fragments, of 4.6, 1.1 and 0.5 kb, were obtained (data not shown). The size of ColE7-K317 was therefore estimated to be around 6.2 kb. Unique *Eco*RI, *Bgl*II, and *Cla*I restriction sites were located in the 4.6 kb *Dra*I fragment by double digests (Fig. 1). Three *Hind*II sites were also mapped on the plasmid (Fig. 1). No sites were found for *Bam*HI, *Hind*III, *Pst*I, *Sma*I or *Sac*I.

The unique *Eco*RI site of the ColE7 plasmid was cleaved and the linearized DNA fragment was ligated into the *Eco*RI site of the cloning vector pUC18. Two different orientations of the insert were obtained in recombinant plasmids pHK101 and pHK201, respectively (Fig. 2).

Subcloning and localization of the colicin operon of the ColE7 plasmid

In order to generate more subclones of the ColE7 plasmid, we adopted the site-specific deletion method by partially digesting the ColE7 plasmid with *Dra*I and then completely digesting the DNA with *Eco*RI or *Bgl*II. The resulting fragments were cloned into the *Eco*RI/*Sma*I or *Bam*HI/*Sma*I sites respectively, of pUC18. The resulting recombinant plasmids are shown in Fig. 2. The new recombinant plasmids generated by the deletion of *Dra*I fragments, namely pSE3, pSE4, pSE5, pSB57 and pSB64, were obtained as expected (Fig. 2). Recombinant plasmid pSB58 (Fig. 2) was shorter than expected; the reason for this is not known. Three more subclones, pHK14, pHK25 and pHK80, were obtained by ligating the partially restricted *Sau*3A fragments of the ColE7 plasmid into the *Bam*HI site of pUC18 (Fig. 2).

Fig. 1. Circular restriction map of the ColE7-K317 plasmid. The unique *Eco*RI site was used as 0 kb of the map; the *Cla*I and *Bgl*II sites were also unique. The boxes represent the region of the *cea*, *cei* and *cel* genes of the colicin E7 operon. Restriction sites for *Bam*HI, *Hind*III, *Pst*I, *Sma*I and *Sac*I were not found in this plasmid.

Recombinant pHK001 was obtained by subcloning the large *Eco*RI-*Bgl*II fragment of the ColE7 plasmid into the *Eco*RI/*Bam*HI sites of pUC18.

The results of the phenotypic testing of cultures carrying these recombinant plasmids for colicin production and immunity are shown in Fig. 2. Cultures harbouring pHK14 or pHK001 were immune to colicin E7 as well as producing colicin E7. On plates overlaid with *E. coli* W3110, *E. coli*(pHK001) produced a larger clear zone than *E. coli*(pHK14). This suggested that the *Sau*3A fragment deleted from pHK14 may affect colicin production. *E. coli*(pHK80) was immune to colicin E7 (Fig. 2). This showed that the gene encoding the immunity protein is completely located on this *Sau*3A fragment. In order to locate the immunity protein gene more precisely, pHK80 was restricted with *Eco*RI and *Pst*I to isolate the 0.65 kb *Sau*3A fragment (Fig. 3) from the recombinant plasmid. The purified fragment was then cleaved with *Dra*I and subcloned into either the *Eco*RI/*Sma*I or the *Pst*I/*Sma*I sites of pUC18, to give the recombinant plasmids pHK80-P10 and pHK80-E4 (Fig. 3). *E. coli*(pHK80-P10) was immune to colicin E7, but *E. coli*(pHK80-E4) was not. This demonstrated that the immunity protein gene of the ColE7 plasmid was on the 0.34 kb fragment of the recombinant pHK80-P10. This result has been confirmed by DNA sequencing of this region (see Fig. 5).

In previous studies (Chak & James, 1985), the *cel* gene of other E colicin plasmids was localized in tandem with the *cei* gene. However, *E. coli*(pHK80) did not exhibit cell lysis even when the *lac* promoter of pUC18 was induced by IPTG (data not shown). We speculated that

Fig. 2. Localization and characterization of the colicin E7 operon. Details of the construction of these subclones are described in the text and in Table 1. The thicker line at the top represents the 6.2 kb linearized restriction map of the ColE7 plasmid. The arrows in parentheses show the orientation of the promoter of the cloning vector, pUC18. S3A, *Sau3A*; BII, *Bgl*II; DI, *Dra*I; EI, *Eco*RI. Imm and Col stand for immunity and colicin production phenotypes, respectively. The symbol + indicates the subclones which confer immunity to colicin E7, or produce colicin in the production test (+ + indicates a strong effect), whilst the symbol - represents a negative result in these two *in vivo* tests.

Fig. 3. Identification of the DNA fragment of the colicin E7 operon responsible for immunity and cell lysis. Detailed cloning methods are described in the text and in Table 1. Symbols and abbreviations are the same as in Fig. 2; Lys, test for IPTG induction of cell lysis (NT, not tested).

the 0.65 kb fragment of recombinant pHK80 was in the wrong orientation with respect to the *lac* promoter. In order to prove this, the 0.65 kb insert from recombinant pHK80 was released by digestion with both *Eco*RI and *Hind*III, and the fragment was ligated into the *Eco*RI and *Hind*III sites of pUC19. To avoid the occurrence of cell lysis, no IPTG was added to the selective plate for

screening of recombinant plasmids. As judged by the size of the insert, recombinant pHK80-R (Fig. 3) was identified as carrying the 0.65 kb DNA fragment from pHK80. When the *lac* promoter of *E. coli* JM101- (pHK80-R) was induced by IPTG, cell lysis started 60 min after induction. This result demonstrated that the 0.65 kb fragment contained genes for both the immunity

Fig. 4. Subcloning of the DNA fragments from the colicin E7 operon into promoter-probe vectors pKC86 and pKC87. The orientation of the fragments with respect to the galactokinase gene of the promoter-probe vector is indicated by the direction of the arrows; fragments with arrows pointing to the right are located in the same orientation with respect to the *galK* gene, whilst those with arrows pointing to the left are in the reverse orientation with respect to the *galK* gene. The approximate locations of the *cea*, *cei* and *cel* genes are shown, together with the restriction map of the relevant portion of the ColE7 plasmid.

protein and the lysis protein. The position of the *cel* gene was confirmed by DNA sequencing of this fragment (see Fig. 5).

Assay of colicin titre from *E. coli* harbouring recombinant plasmids

E. coli(pHK001) produced a much larger clear zone (7.5 mm in diameter) on a plate seeded with the indicator strain, *E. coli* W3110, than did *E. coli* harbouring the recombinant plasmid pHK14 (2 mm) (Fig. 2). To measure the amount of colicin produced from these two *E. coli* strains, a quantitative assay of colicin was performed (see Methods).

From the survival curve (data not shown), the titre of colicin produced by *E. coli*(pHK001) was $2^{17.5}$ (equal to 1.9×10^5), which was in agreement with the titre assay of colicin E3 produced from *E. coli*(pKC11) (Chak & James, 1984) using similar assay conditions. However, due to the very low colicin production of *E. coli*(pHK14), the titre of the colicin produced by this *E. coli* strain could not be determined by this assay method. The formation of a very small clear zone by this strain in the stab assay suggested that this strain only produces the basal level of colicin, with or without MC induction.

Promoter activity test of the colicin E7 operon

The DNA fragments which might contain promoters along the colicin E7 operon were cloned into the promoter-probe vectors pKC86 and pKC87 (Fig. 4). Without MC induction, the promoter activity of recombinant plasmid pPPV80-P10 was 38 galactokinase units (Fig. 4). However, no promoter activity was detected from the recombinant plasmid pPPV80-E4. This implied that the former recombinant contains the *cei* promoter,

whereas the latter recombinant, which contains the *cel* gene, does not have its own promoter. Furthermore, no promoter activity could be detected from the recombinant pPPV80, even though *E. coli* harbouring this plasmid is immune to colicin E7. The reason for this will be discussed later. Under the same conditions, the promoter activity of the recombinants pPPV-57 and pPPV-EH1 was 25 and 22 galactokinase units, respectively (Fig. 4). This indicated that, without induction, the promoter activity of the *cei* gene is stronger than that of the *cea* gene.

When *E. coli* harbouring recombinant pPPV-57 was induced with MC at $0.5 \mu\text{g ml}^{-1}$, the promoter activity was increased from the basal level (20–30 units) to over 400 units within 2 h (data not shown). In contrast, the promoter activity of recombinant pPPV-EH1 under MC induction was the same as the basal activity. This implied that the DNA fragment which was deleted from the upstream region of the colicin operon in recombinant pPPV-EH1 may be important for the 'SOS' response.

Nucleotide sequence of the *cei* and *cel* genes of the ColE7 plasmid

The recombinant plasmids pHK80-P10 and pHK80-E4 (Fig. 3) were used to determine the nucleotide sequence of the *cei* and *cel* genes of the ColE7 plasmid. A sequence of 659 bp, which included *cei* and *cel*, and a part of the 3' end of the colicin E7 gene, was determined (Fig. 5). The sizes of the *cei* and *cel* genes determined by the ORFs were 261 bp and 141 bp, respectively. The sequence data further confirmed the finding that the *cei* and *cel* genes were located in plasmids pHK80-P10 and pHK80-E4, respectively (Fig. 3). From the sequence data, it was noticed that the last three codons (AAA, CAG and GGC) of *cei* in the plasmid pHK80-P10 were not

cea gene

'-10'

GATCAGCCAAAATGGTGGTGTCTATGATATGGATAACATCAGCGTGGTAACACCTAAAA

cea stop RBS *cea* start

GGCATATTGATATTCACCGAGGTAAATAAT ATG GAA CTG GAA AAT AGT ATT

AGT GAT TAC ACA GAG GCT GAG TTT GTT CAA CTT CTT AAG GAA ATT

GAA AAA GAG AAT GAA GCT GCA ACT GAT GAT GTG TTA GAT GTG TTA

CTC GAA CAC TTT GTA AAA ATT ACT GAG CAT CCA GAT GGA ACG GAT

CTG ATT TAT TAT CCT AGT GAT AAT AGA GAC GAT AGC CCC GAA GGG

ATT GTC AAG GAA ATT AAA GAA TGG CGA GCT GCT AAC GGT AAG CCA

DnaI *cea* stop IR

GGA TTT AAA CAG GGC TGA AATATGAATTCGGTTGTTTAAGGATGAATGACTG

cel start

GCATTCTTTCACAACAAGGAGTCGTT ATG AAA AAA ATA ACA GGG ATT ATT

TTA TTG CTT CTT GCA GCC ATT ATT CTT GCT GCA TGT CAG GCA AAC

TAT ATC CGT GAT GTT CAG GGC GGG ACA GTA TCA CCG TCG TCA ACT

GCT GAA CTG ACC GGA GTG GAA ACG CAG TAA CCTGCAATCCTCATTGAC

cel stop IR

AAGAACAAGCGTGTGTCAGGCTGATTCTGACGCGCTTTTTTGTGTAAGTGCCACAAA

AATTCATGTGGGAGATGGGATC

Fig. 5. Nucleotide sequence of the *cei* and *cel* genes of the colicin E7 operon. The start and stop codons of the *cei* and *cel* gene are indicated. The putative stop signal of the colicin E7 structural gene, the ribosome-binding site (RBS) and the putative '-10' region are also indicated. The arrows represent the inverted repeat (IR) sequences located distal to the *cei* and *cel* genes.

E7 *imm* MELENSISDYTEAEFVQLKEIEKE 25
 E2 *imm* ***KH*****LEFV*K*CRA
 E8 *imm* *****N*****T*KKIIED*INC
 E9 *imm* ***K*****L**VTT*CNA

E7 *imm* NEAATDDVLDVLLEHFVKITEHPDG 50
 E2 *imm* *G***EEDDNK*VRE*ERL*****
 E8 *imm* EGDEKKQDDN ***ISV*****S
 E9 *imm* * TSSEELVK*VT**EEM*****S

E7 *imm* TDLIYPSDNRRDSSPEGIVKEIKEW 75
 E2 *imm* S*****R*D*E*****
 E8 *imm* S*****EG*N*G***AVI*****
 E9 *imm* S*****KEGD****S***NTV*Q*

E7 *imm* RAANGKPGFKQG 87
 E2 *imm* *****S*****
 E8 *imm* *****S*****
 E9 *imm* *****S*****

Fig. 6. Homology between E colicin immunity proteins. The deduced amino acid sequence of the E7 immunity protein is shown. Where the amino acids of the E2, E8 and E9 immunity proteins are identical to E7 immunity protein, this is indicated by asterisks. Only the amino acids which differ from the E7 immunity protein sequence are shown. Padding spaces are included to optimize the alignment.

included in this clone (Table 1, Fig. 5). However, this plasmid still confers immunity to colicin E7. This implied that deletion of these three codons does not affect the function of the E7 immunity protein.

A putative '-10' sequence, 'TATGAT', was found 67 bp upstream of the *cei* ORF, but no '-35' sequence was found. In other experiments, it was confirmed that this recombinant plasmid conferred immunity to colicin E7 (Fig. 3) and exhibited promoter activity (Fig. 4). It is

E7 *lys* MKKITGIILLLLAAIILAACQANYI 25
 E2 *lys* *****V***S*****
 E5 *lys* *****W*****
 E8 *lys* *****V*****
 E9 *lys* *****V***S*WGSKPK

E7 *lys* RDVQGGTVSPSSTAELTGVTETQ 47
 E2 *lys* *****V***LA**
 E5 *lys* H*****S*****LA**
 E8 *lys* *****V***LA**
 E9 *lys* T

Fig. 7. Homology between E colicin lysis gene products. The amino acid sequences of the E2, E5, E8 and E9 lysis proteins are shown when they differ from that of the E7 lysis protein. Identical amino acids are indicated by asterisks.

not known whether the *cei* gene uses a rare '-35' sequence which differs from the consensus sequence (Rosenberg & Court, 1979), or whether the '-35' sequence is not necessary for the expression of the *cei* gene. Two inverted repeat sequences were found immediately after the translational stop signal (TGA) of *cei* and that (TAA) of *cel* (Fig. 5). These two inverted repeat sequences must be the terminators of the respective genes. The sequence data support the finding that no promoter activity is located in plasmid pPPV80 (Fig. 4).

Comparison of the deduced amino acid sequences of the E7 immunity protein and lysis/release protein with those of other immunity and lysis/release proteins (Fig. 6, Fig. 7), showed that the E7 immunity protein is more closely related to the E2 immunity protein than to the other immunity proteins examined. Their amino acid and DNA sequences have a higher degree of similarity

Fig. 8. SDS-PAGE of colicins E7 and E9. The colicins were purified by carboxymethylcellulose column chromatography (see Methods). The concentration of the purified colicins was adjusted to $1 \mu\text{g } \mu\text{l}^{-1}$. Lanes 1, 4 and 7 contain molecular mass markers. Lanes 2 and 3 were loaded with $1 \mu\text{l}$ and $5 \mu\text{l}$ of purified colicin E7, respectively. Lanes 5 and 6 were loaded with $1 \mu\text{l}$ and $5 \mu\text{l}$ of purified colicin E9, respectively.

Table 2. Similarity between *E. coli* colicin immunity and lysis genes and proteins

PC/Gene, a DNA analysis program from Genetech, was used for the comparison analysis. The amino acid sequences of the E7 immunity protein and E7 lysis protein were deduced from the DNA sequences. The other DNA and amino acid sequences are from: E2, Cole *et al.* (1985); E8, Uchimura & Lau (1987); E9, James *et al.* (1987); E5, Curtis *et al.* (1989).

	Similarity (%) with other E colicin immunity genes and proteins			
	E2	E8	E9	
E7 immunity gene	78	73	69	
E7 immunity protein	67	62	61	
	Similarity (%) with other E colicin lysis genes and proteins			
	E2	E5	E8	E9
E7 lysis gene	92	92	92	81
E7 lysis protein	89	89	92	74

(67% and 78%, respectively) than the other immunity proteins or genes (Table 2). *E7-cel* is evidently related to *E8-cel*, as both the amino acid and DNA sequences show 92% homology with each other (Table 2). The molecular mass of the E7 immunity protein deduced from the DNA sequence (Fig. 6) is 9926 Da, which agrees well with the

Fig. 9. *In vitro* biological activity assay of colicins E7 and E9. Colicins E7 and E9 were incubated with pUC18 DNA for 0, 30, 60 and 90 min, as indicated. C, Negative controls, which contained no colicin and were incubated at 37°C for 90 min. The central lane contains *Hind*III-digested λ DNA.

Fig. 10. Evolutionary relationships of the colicin E7 plasmid to some selected E colicin plasmids. The linearized restriction map of ColE7 (this work) was used as a reference. The restriction maps of ColE3-CA38, ColE5-099, ColE8-J and ColE9-J were reported by Chak & James (1986); that of ColE2-CA42 was reported by Watson *et al.* (1985). The differences and similarities of the restriction maps of the plasmids are shown. The lines above certain of the plasmids represent the hypothetical insertion of a DNA fragment in the corresponding positions of these plasmids. The boxes represent the colicin operon, comprising the *cea*, *cei* and *cel* genes. The acquired extra *cei* and *cel* genes are shown for individual plasmids where appropriate. PvI, *PvuI*; HII, *HincII*; EI, *EcoRI*; CI, *ClaI*; SI, *SmaI*; PstI, *PstI*; KI, *KpnI*.

molecular mass (9.4 kDa) of this protein determined by SDS-PAGE. The deduced molecular mass of the E7 lysis protein (Fig. 7) is 4872 kDa.

Purification and *in vitro* activity of colicins

The purity and molecular masses of purified preparations of colicins E7 and E9 were checked by SDS-PAGE (Fig. 8). The molecular mass of colicin E7 and its immunity protein were 61 kDa and 9.4 kDa, respectively (the deduced molecular mass from DNA sequence is 9.9 kDa). The sizes of colicin E9 and its immunity protein were 62 kDa and 10.1 kDa, respectively.

In the *in vitro* activity tests for DNAase activity, the amount of the linear form of plasmid DNA increased, accompanied by a decrease in the amount of the

covalently closed circular and open circular forms, during the first 60 min incubation at 37 °C (Fig. 9). Eventually, most of the plasmid DNA turned into very small fragments forming a smear of DNA visualized by agarose gel electrophoresis. This implied that both colicins were non-specific DNA endonucleases. In the control incubation no change was observed in the plasmid DNA.

Discussion

The ColE7 plasmid is 6.2 kb in size, and it encodes a 61 kDa colicin (Fig. 8). The molecular masses of the E7 immunity protein and the E7 lysis protein, deduced from the DNA sequence data, were 9926 and 4872 Da,

respectively. Purified colicins E7 and E9 exhibited DNA endonuclease activity.

The production of colicin by *E. coli*(pHK001) was much higher than that by *E. coli*(pHK14), suggesting that the 1.5 kb *Sau*3A fragment which was deleted from upstream of the colicin E7 structural gene in pHK14 may be important for the control of colicin production. This observation was supported by the *in vivo* promoter activity tests of these recombinant plasmids.

It is not known why the production of colicin by *E. coli*(pPPV-EH1) is not induced by MC. However, the promoter activity of this recombinant plasmid is at a basal level, which was very similar to the basal level activity of recombinant plasmid pPPV-57. It is not known whether the 'SOS' box, or any other regulatory region, has been deleted in pPPV-EH1. The DNA sequence of this region may help to solve this problem. *E. coli*(pHK80) was immune to colicin E7, but did not cause cell lysis even after IPTG induction. However, when the 0.65 kb insert of pHK80 was placed in the same orientation with respect to *lac* promoter control, cell lysis occurred 60 min after IPTG induction. This result was similar to previous findings indicating that the immunity gene has its own promoter and that expression of this promoter is constitutive, whereas the *cel* gene is promoterless (Chak & James, 1985). The expression of the *cel* gene is dependent upon transcriptional read-through from the colicin promoter. This genetic organization is also supported by the *in vivo* promoter activity tests and the DNA sequence of the *cei* and *cel* regions reported in this paper. Although the *cel* gene is located distal to the *cei* gene, transcriptional readthrough from the *cei* promoter alone is not sufficient to result in significant expression of the *cel* gene. This finding is slightly different from results obtained in studies of the expression of E2-*cei* (Masaki *et al.*, 1985) and E3-*cei* (Masaki & Ohta, 1985), which showed that the presence of an internal promoter of the *cei* gene was questionable. The *Bam*HI fragment which was used for the promoter activity test of the E2-*cei*E2 gene by Masaki *et al.* (1985) contained the termination sequence located distal to the *cei* gene, so the promoter activity of the *cei* gene could not be detected properly. The E3-*cei* gene (298 bp *Sau*3A fragment) containing only 42 nucleotides upstream of the initiation codon conferred partial immunity to colicin E3 (Masaki & Ohta, 1985). This finding may be due to the fact that the putative Pribnow box located at nucleotides -67 to -61 of the initiation start site of the gene E7-*cei* (this paper) was not included in this *Sau*3A fragment. The question of whether or not *cei* genes contain their own promoters may be answered by cloning various DNA fragments containing *cei* genes from all ColE plasmids into a promoter-probe vector system.

The organization of the ColE7 plasmid and the

possible evolutionary relationship of some ColE plasmids is illustrated in Fig. 10. A comparison of the restriction and functional maps of most of the ColE plasmids reveals considerable similarities. In most of the ColE plasmids there is a DNA fragment (roughly 1 kb in length) located proximal to the 5' end of the colicin E structural gene. ColE2 and ColE7 are the only two plasmids which lack this fragment (Fig. 10); these plasmids are about 1 kb shorter than the other ColE plasmids. This suggests that the upstream sequences of the colicin E2 and colicin E7 structural genes may differ from those of all other ColE plasmids. It is interesting that deletion of a 1.5 kb *Sau*3A fragment upstream of the colicin E7 structural gene caused a reduction in colicin production (see Results). The ColE2 and ColE7 plasmids are also the only ColE plasmids in which there is a *Hinc*II site in the structural gene (Fig. 1; Watson *et al.*, 1985). It seems reasonable to predict that the structural genes of colicin E2 and colicin E7 are more closely related to each other than to the other E colicin genes.

The sequence data support the idea that the E2 and E7 *cei* genes are more closely related than other E colicin *cei* genes. The ColE7 plasmid has a unique *Eco*RI site at 1.0 kb and an internal *Hinc*II site in the *cel* gene. The significance of these two characteristics with respect to the position of the ColE7 plasmid in the evolutionary pathway of the ColE plasmids is not known. Further study of the ColE7 plasmid should increase our understanding of the evolutionary origin of the ColE plasmids.

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