Localization and Characterization of a Gene on the ColE3-CA38 Plasmid that Confers Immunity to Colicin E8

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Escherichia coli W3110 cells carrying the ColE3-CA38 plasmid are immune to externally added colicin E8, a newly described member of the E group colicins. By molecular cloning and transposon mutagenesis we localized the colicin E8 immunity gene between the *Eco*RI site (4.0 kb on the restriction map) and the *Pvu*II site (3.68 kb) of the ColE3-CA38 plasmid. This placed the colicin E8 immunity gene between the colicin E3 immunity gene and *lys*, the region which determined mitomycin C sensitivity. Insertion of a transposon into the colicin E3 structural gene prevented the synthesis of active colicin and completely abolished mitomycin C sensitivity, but had no effect on the two immunity genes. In contrast, insertion of a transposon into the colicin E8 immunity gene had no effect upon colicin E3 production or colicin E3 immunity but did abolish mitomycin C sensitivity. The phenotype conferred by plasmids with a transposon inserted into the *lys* region of ColE3-CA38 was dependent upon the site of insertion.

INTRODUCTION

The E colicins are a group of plasmid-coded, antibacterial proteins which use the same receptor (Fredericq, 1949), the gene product of the *Escherichia coli* chromosomal gene *btuB*, to bind to sensitive *E. coli* cells (Di Masi *et al.*, 1973). They are inducible proteins, the synthesis of which is triggered by DNA damage of the host cell (Herschman & Helinski, 1967). This group may be subdivided into colicin E1 to E7 on the basis of immunity tests (Watson *et al.*, 1981). Each E colicin plasmid codes for the production of a specific immunity protein which protects the host cell against colicin of the same type, either during production or if added externally. For example, cells carrying the ColE3-CA38 plasmid are immune to external colicin E3 but not to colicin E1 or E2. *Escherichia coli* K12 strains carrying an E colicin plasmid are also mitomycin C sensitive compared to plasmid-free isogenic strains. This is presumed to be the result of induction of a third plasmid-coded gene which may be involved in cell lysis and subsequent colicin release from the producing cell (Shafferman *et al.*, 1979; Sabik *et al.*, 1983). Since immunity appears to be constitutive, whilst colicin production and mitomycin C sensitivity are inducible by DNA damage (Herschman & Helinski, 1967), the regulatory control of these three genes in response to DNA damage is of considerable interest.

A new member of the E group of colicins, designated colicin E8, has recently been described (Cooper & James, 1984). *Escherichia coli* K12 strains carrying the ColE3-CA38 plasmid exhibit a novel non-reciprocal immunity against colicin E8. Whilst *E. coli* K12(ColE3-CA38) strains are immune to externally added colicin E8, *E. coli* K12(ColE8-J) strains are sensitive to externally added colicin E3. Here we show that the ColE3-CA38 plasmid carries a gene coding for colicin E8 immunity. The localization and characterization of this immunity gene by molecular cloning and transposon mutagenesis provides information on the regulation of the colicin operon. Our data on the regulation of the two immunity genes present on the ColE3-CA38 plasmid are consistent with that suggested for the regulation of the colicin E3 immunity gene derived from the DNA sequence of part of the ColE3-CA38 plasmid (Masaki & Ohta, 1982; Mock *et al.*, 1983).

METHODS

Strains and media. *Escherichia coli* W3110 ColE3-CA38 (PRC595) and *E. coli* W3110 ColE2-P9 (PRC594) were kindly supplied by the Plasmid Reference Centre, Stanford, Ca., USA). *Escherichia coli* W3110 ColE8-J has previously been described (Cooper & James, 1984). *Escherichia coli* RB85 F⁻ *thr leu lacY strA supE44 tonA* and *E. coli* RB308 F⁺ *thr leu thyA deoC lacY* were obtained from R. S. Buxton. The sex factor in the latter strain is the source of the transposon Tn1000 (Buxton & Drury, 1983). The properties and derivation of the plasmids used are described in Fig. 1. *Escherichia coli* JM83 *ara lac pro thi strA* ϕ 80 Δ lacZ M15 was used as the host strain for transformations using the vector pUC8, and was kindly supplied by BRL. *Escherichia coli* W3110 was used as the host strain for transformations using the vector pBR322.

Bacteria were cultured at 37 °C in LB broth (Miller, 1972). For selection of recombinant clones, antibiotics were included in LB agar (LB broth containing 1.5%, w/v, agar) at the following final concentrations; ampicillin, 50 μ g ml⁻¹; tetracycline, 10 μ g ml⁻¹; kanamycin, 25 μ g ml⁻¹; streptomycin 200 μ g ml⁻¹. The chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) was used to select pUC8 recombinants and was included in LB agar at a final concentration of 20 μ g ml⁻¹.

Preparation of colicin extracts. Colicin E2, E3 and E8 extracts were prepared from cultures of *E. coli* W3110 E2-P9 (PRC594), *E. coli* W3110 E3-CA38 (PRC595) and *E. coli* W3110 ColE8-J, respectively. Overnight cultures were diluted in 50 ml LB and grown to an OD₅₅₀ of 0.2 before addition of mitomycin C (0.5 μ g ml⁻¹). After 4 h incubation, chloroform was added and incubation continued for 15 min at 37 °C. Any remaining cells were removed by centrifugation at 10000 g for 15 min. The aqueous phase was then removed and stored in portions at -20 °C. LB agar plates containing colicin extracts were prepared by spreading 0.2 ml of the appropriate extract over the surface of the plate.

Colicin production and immunity tests. Colicin production was determined by stab tests (Kennedy, 1971), using *E. coli* W3110 as the sensitive indicator strain. Colicin E8 immunity was scored as resistance to colicin E8 but sensitivity to colicin E2 or E3. Similarly, colicin E3 immunity was scored as resistance to colicin E3 but sensitivity to colicin E2 and E8. This was tested by streaking a loopful of a broth culture onto LB agar plates containing extracts of colicin E2, E3 or E8.

Colicin titres. Colicin titres were determined by a modification of the method of Reeves (1965) developed by G. M. P. Lawrence.

Plasmid isolation. For screening of recombinants, plasmids were prepared by the method of Hopwood *et al.* (1983). For endonuclease restriction and transformation, plasmids were prepared by a modification of the method of Birnboim & Doly (1979).

Two plasmid vectors were used for the isolation of recombinant clones, pBR322, which is 4.36 kb in size and confers ampicillin and tetracycline resistance (Sutcliffe, 1978), and pUC8, which is 2.7 kb in size and carries single cloning sites for a number of restriction endonucleases within a 'polylinker' inserted close to the N-terminus of a fragment of the β -galactosidase gene (Vieira & Messing, 1982). Insertion of recombinant DNA in the polylinker prevents a complementation with the defective *lacZ* gene carried by *E. coli* JM83.

Restriction, ligation and transformation. Digestion of DNA with restriction endonucleases, ligation of DNA fragments and transformation of competent cells were carried out as previously described (Maniatis *et al.*, 1982).

Electrophoresis of DNA. The sizes of DNA fragments were analysed by gel electrophoresis in 0.8% or 1.0% (w/v) agarose. Tris/acetate or Tris/borate buffers were used depending upon the sizes of the fragments (Maniatis *et al.*, 1982). Lambda DNA digested with *Hind*III or *Pst*I was used as molecular weight markers. Fragment sizes were computed from a least-squares fit of the mobility of the molecular weight markers using a program developed by one of us (R.J.) on a BBC microcomputer model B.

Mitomycin C sensitivity. For screening the mitomycin C sensitivity of large numbers of recombinants we devised a microtitre assay in which 10 μ l volumes of cultures were added to 190 μ l pre-warmed LB, containing mitomycin C (0.5 μ g ml⁻¹), in a microtitre dish. The initial optical density values of all 96 wells in a tray were then read using a Titertek Multiscan MC plate reader at 540 nm. The microtitre plate was then gently shaken at 37 °C and the optical density values were read at hourly intervals for 6 h and then again after 24 h. Control cultures of *E. coli* W3110 reached stationary phase at optical density values of about 0.5, whilst mitomycin-sensitive cultures lysed at an optical density value of about 0.2. Using this method it was feasible to screen several hundred recombinant colonies in one day. Cultures which exhibited a delayed lysis in the microtitre assay were studied further in the shaken flask assay. Overnight cultures were diluted 50-fold into 50 ml LB in a 100 ml flask and were incubated with shaking at 37 °C until the OD₅₅₀ reached 0.3 to 0.4. At this time 20 ml of the culture was transferred to a second flask to which mitomycin C (0.5 μ g ml⁻¹) was added. The optical density readings of both flasks were taken at intervals up to 6 h.

Transposon mutagenesis. Transposon mutagenesis of plasmids pKC10, pKC11 and pKC22 carried out using the method of Tommassen *et al.* (1982). Cultures of *E. coli* W3110 carrying the appropriate plasmid were grown up overnight in LB supplemented with ampicillin and maltose (0.2%, w/v). The cells were collected by centrifugation and concentrated threefold in 0.9% (w/v) NaCl, 20 mM-MgCl₂. After 45 min incubation at 37 °C, 10 μ l of phage

lambda b221 c1857 *rex::Tn5* was added to 0.1 ml volumes of cells at a multiplicity of infection of 10. The mixtures were incubated at 30 °C for 30 min then diluted 100-fold in LB containing ampicillin and grown overnight at 30 °C. Subsequently the cultures were diluted 100-fold in LB containing ampicillin, kanamycin and sodium citrate (0.1%, w/v) and grown overnight at 30 °C. Plasmid DNA was isolated from the overnight culture and was used to transform *E. coli* W3110, selecting for Amp^R Kan^R transformants. Colonies were picked and grown overnight in 200 µl LB containing ampicillin and kanamycin in microtitre dish wells. To maintain selection pressure for the presence of Tn5 we routinely included kanamycin in all growth media. Tn1000 transposon mutagenesis was carried out by transforming plasmid pKC10, pKC11 or pKC22 into *E. coli* RB308 which is F⁺. These donor strains were then grown overnight in LB containing ampicillin whilst the recipient strain *E. coli* RB85 was grown in LB. After diluting 50-fold in LB, the cultures were grown with gentle shaking at 37 °C for 2 to 3 h, at which time 0.1 ml donor culture was mixed with 1.0 ml recipient culture and incubated without shaking for 1 h at 37 °C. The mating mixture was then diluted into 4 ml LB containing streptomycin and incubation continued with vigorous aeration for 2 h. The culture was then diluted and plated on LB agar containing streptomycin and ampicillin to select for mobilization of pKC10, pKC11 or pKC22.

Transposon insertion sites were located by isolating plasmid DNA and restricting separately with a group of restriction endonucleases. For Tn5 inserts, *Bam*HI, *Hind*III and *Sal*I were employed to analyse the location and the orientation of the insertions (Jorgensen *et al.*, 1979), while for Tn1000, *Eco*RI alone was usually sufficient to determine the location and orientation (Guyer, 1978).

RESULTS

Analysis of recombinant plasmids

A partial *Eco*RI digest of the ColE3-CA38 plasmid and subsequent ligation into the *Eco*RI site of pBR322 yielded recombinant plasmid pKC11 which carried the whole 7.2 kb ColE3-CA38 plasmid. A restriction map of plasmid pKC11 was obtained after single and double digests with a range of restriction endonucleases. The restriction sites in the ColE3-CA38 portion of plasmid pKC11 are shown in Fig. 1. Transformants of *E. coli* W3110 carrying plasmid pKC11 were immune to colicin E3 and colicin E8. In contrast, plasmid pKC10, which carried the 3.12 kb *Eco*RI fragment of ColE3-CA38 ligated into pBR322, conferred immunity to colicin E3 but not to colicin E8. *Escherichia coli* W3110, when transformed with plasmids pKC13 or pKC14, in which *Xho*I-restricted ColE3-CA38 DNA was ligated into the *Sal*I site of pBR322 in either orientation, were immune to colicin E3 but were sensitive to colicin E8. The recombinant plasmid pKC25, in which the 2.76 kb *Sma*I-*Pvu*II fragment of ColE3-CA38 was ligated into the *Hinc*II site of pUC8, conferred immunity to both colicin E3 and E8. The structures of these recombinant plasmids placed the colicin E8 immunity gene between the *Pvu*II site (3.68 kb on the restriction map and the *Eco*RI site (4.08 kb) of the ColE3-CA38 plasmid, and indicated that it was cut by *Xho*I.

Transposon mutagenesis

The transformants resulting from Tn5 mutagenesis and the transconjugants from Tn1000 mutagenesis were screened for the insertional inactivation of colicin production, colicin E8 immunity and mitomycin C sensitivity as described above. With Tn5, the frequency of inserts which inactivated colicin production, colicin E8 immunity or mitomycin C sensitivity was 21%. For Tn1000, the frequency of transconjugants in which the transposon had inactivated one of the three phenotypic characters was 12%. The phenotypes conferred by the mutant plasmids used in this work are shown in Table 1. We were unable to isolate a transposon insert which abolished colicin E3 immunity, even with plasmid pKC22 which does not produce active colicin E3.

(a) *Localization of the colicin E8 immunity region.* With plasmid pKC11 we obtained three independent transpositions of Tn1000 that inactivated colicin E8 immunity (Fig. 2a). In an attempt to increase the frequency of transposition into this relatively small target region we repeated the transposition using plasmid pKC22 which lacked the 2.1 kb *Cla*I fragment of pKC11. Three more transposons in the colicin E8 immunity region were isolated with this plasmid (Fig. 2b). Using pKC11 we did not obtain any Tn5 transpositions into the colicin E8 immunity region but four independent inserts in this region were obtained with plasmid pKC22

Fig. 1. Restriction map of the ColE3-CA38 plasmid and of fragments of ColE3-CA38 cloned into pBR322 or pUC8. The restriction fragments present in the plasmids listed at the left are indicated, together with the phenotypes conferred and the size of the recombinant plasmid. Plasmid pKC14 is identical to pKC13 except that the ColE3-CA38 *XhoI* fragment is in the opposite orientation.

Fig. 2. (a) Insertion sites of Tn1000 in plasmid pKC11 which inactivate colicin E8 immunity. Only the relevant part of the restriction map of pKC11 is shown. (b) Insertion sites of Tn1000 (above the line) and Tn5 (below the line) in plasmid pKC22 which inactivate colicin E8 immunity. For Tn5 the arrow shows the orientation of the transposon.

Table 1. Phenotypes of plasmids carrying transposon inserts

Phenotypes conferred by plasmids with transposon inserts which inactivate colicin production, colicin E8 immunity or mitomycin C sensitivity. Partial mitomycin C sensitivity is recorded as +/–.

Plasmid	Phenotype			
	Col	ImmE3	ImmE8	Lys
pKC10	+	+	–	–
pKC10::Tn5-106*	–	+	–	–
pKC11	+	+	+	+
pKC11::Tn1000-167†	+	+	–	–
pKC11::Tn1000-168‡	–	+	+	–
pKC11::Tn5-85§	–	+	+	–
pKC11::Tn1000-166¶	+	+	+	+/–
pKC22	–	+	+	–
pKC22::Tn1000-185¶¶	–	+	–	–

* Transposon inserts Tn5-107, 111, 113, 120 and 122 confer the same phenotype.

† Transposon inserts Tn1000-173 and 177 confer the same phenotype.

‡ Transposon inserts Tn1000-170, 171, 182 and 184 confer the same phenotype.

§ Transposon inserts Tn5-89, 90, 92 and 96 confer the same phenotype.

¶ Transposon inserts Tn1000-172, Tn5-87 and 91 confer the same phenotype.

¶¶ Transposon inserts Tn1000-186, 187 and Tn5-138, 142 and 146 confer the same phenotype.

(Fig. 2b). The above data confirm the localization of the colicin E8 immunity gene of the ColE3-CA38 plasmid to a 320 bp site which includes the XhoI and EcoRI restriction sites.

(b) *Localization of the colicin E3 structural gene.* The phenotype conferred by plasmid pKC10 localized the colicin E3 structural gene on the 3.12 kb EcoRI fragment of ColE3-CA38. With pKC11 we obtained five independent transpositions of Tn1000 and of Tn5 which prevented the synthesis of active colicin E3. The map positions and orientations of these insertions are shown in Fig. 3(a). A further six independent transpositions of Tn5 which inactivated colicin E3

Fig. 3. (a) Insertion sites of Tn1000 and Tn5 in plasmid pKC11 which inactivate colicin E3 production. (b) Insertion sites of Tn5 in plasmid pKC10 which inactivate colicin E3 production.

production were obtained with pKC10 (Fig. 3b). The sites of insertion of some of this latter group were significantly different to those obtained in pKC11, two of them being located between the *SmaI* and *HincII* restriction sites.

(c) *Localization of the mitomycin C sensitivity region.* A comparison of the mitomycin C sensitivity encoded by a number of recombinant plasmids is shown in Fig. 4. Plasmid pKC11 conferred a considerably enhanced mitomycin C sensitivity compared to plasmid pKC10 in *E. coli* W3110. We obtained two independent insertions of Tn5 and of Tn1000 which significantly reduced the mitomycin C sensitivity (in the microtitre assay) associated with the presence of plasmid pKC11 in *E. coli* W3110. These insertions mapped in a 200 bp region which included the *PvuI* and the *PvuII* restriction sites (Fig. 5). The mitomycin C sensitivities of two of these strains are shown in Fig. 4 and were intermediate compared to *E. coli* W3110(pKC10) and *E. coli* W3110(pKC11). The presence of a transposon in the mitomycin C sensitivity region of plasmid pKC11 had a dramatic effect upon the release of colicin from mitomycin C induced cells (Table 2). A comparison of the data for plasmids pKC10 and pKC11 shows a dramatic change in the ratio of intracellular and extracellular colicin 3 h after mitomycin C induction. The total colicin titre was also consistently lower in cultures carrying plasmid pKC10 as compared to pKC11. Plasmid pKC11::Tn5-87 gave essentially the same results as plasmid pKC10, whilst the results with pKC11::Tn5-91 were similar to those with plasmid pKC11::Tn5-87 at 1 or 3 h after induction, but changed dramatically at 5 h.

Regulation of the colicin E3 operon

Our results with transposon mutagenesis clearly showed that inactivation of the colicin E3 structural gene completely abolished mitomycin sensitivity (Table 1), without affecting colicin E3 or colicin E8 immunity. Similarly, insertion of a transposon into the colicin E8 immunity gene also abolished mitomycin sensitivity, but did not affect colicin E3 immunity or colicin E3 production. In contrast, a transposon inserted into the mitomycin C sensitivity gene did not affect colicin E3 or colicin E8 immunity, or colicin E3 production. These results suggest that the

Fig. 4. Effect of mitomycin C on growth of *E. coli* W3110 carrying plasmids pBR322 (□), pKC10 (○), pKC11 (●), pKC11::Tn5-87 (▲) and pKC11::Tn5-91 (△). For the sake of clarity the control curves, which were essentially identical for all five cultures, are omitted from the Figure.

Fig. 5. Insertion sites of Tn1000 and Tn5 in plasmid pKC11 which reduce mitomycin C sensitivity.

Table 2. Colicin titres in mitomycin C induced cultures

Colicin titres of *E. coli* W3110 carrying the plasmid indicated at 1, 3 and 5 h after mitomycin C induction.

Plasmid	1 h		3 h		5 h	
	Total titre	Percentage intracellular	Total titre	Percentage intracellular	Total titre	Percentage intracellular
pKC11	1.8×10^3	88.9	1.3×10^5	0.4	1.3×10^5	2.5
pKC10	1.4×10^2	93.6	4.2×10^3	98.2	3.3×10^4	99.5
pKC11::Tn5-87	1.2×10^3	93.5	5.8×10^4	99.6	1.6×10^5	98.7
pKC11::Tn5-91	8.6×10^2	91.3	8.3×10^4	98.6	7.9×10^4	21.1

two immunity genes have their own promoter(s). Digestion of plasmid pKC11::Tn5-96, which has the transposon inserted 110 bp to the right of the *KpnI* site, with *SaII* and re-ligation removes a 3.32 kb *SaII* fragment from within Tn5 to the *SaII* site in the vector. The resulting plasmid pKC32 still conferred immunity both to colicin E3 and E8.

DISCUSSION

We have demonstrated, by subcloning fragments of plasmid ColE3-CA38, that the non-reciprocal immunity of *E. coli* W3110 carrying this plasmid against a colicin E8 producing strain (Cooper & James, 1984) is due to the presence of a colicin E8 immunity gene. Whilst a similar non-reciprocal immunity between *E. coli* W3110 carrying the ColE7-plasmid and colicin E2 producing strains has previously been reported (Males & Stocker, 1980), that was the result of the presence of a second plasmid conferring colicin E2 immunity but not production, along with the ColE7 plasmid (Watson *et al.*, 1981). This is the first example amongst the E group colicin plasmids of two immunity genes being found on the same plasmid. The colicin E8 immunity gene has been localized to a 320 bp region of the ColE3-CA38 plasmid. A piece of DNA this size could code for a protein of approximately 12 500 Dal. This coding capacity is consistent with the molecular weight of 9500 reported for the colicin E3 immunity protein (Sidikaro & Nomura, 1974), together with a promoter or any regulatory sequences. The colicin E8 immunity gene of the ColE8-J plasmid is found in an identical location to that in ColE3-CA38, being cut by both *Xho*I and *Eco*RI restriction enzymes (G. M. P. Lawrence & R. James, unpublished).

The minimum size of the colicin E3 structural gene, which may include promoter and regulatory sequences, is defined by the insertion site of pKC11::Tn5-96, at 110 bp to the right of the single *Kpn*I site, and the insertion site of pKC10::Tn5-111, at 7 bp to the left of the single *Hinc*II site, to be 2.03 kb. A piece of DNA this size has a coding capacity for a protein of approximately 75 000 Dal, which is somewhat larger than the reported size of colicin E3 (Jakes & Zinder, 1974). The C-terminal sequence of the colicin E3 structural gene has recently been published (Masaki & Ohta, 1982; Mock *et al.*, 1983), and is consistent with our data. The location of the promoter and the N-terminus of the structural gene are as yet unreported. We do not know if the isolation of plasmid pKC10 with transposition sites between the *Sma*I and *Hinc*II sites, significantly further to the right than any transposition sites obtained in plasmid pKC11, is related to the reduced target size of the insert in recombinant plasmid pKC10, or whether the absence of the 3.47 kb *Eco*RI fragment, which includes the mitomycin C sensitivity region, has a more direct effect on the transposon mutants of the structural gene which are viable.

Insertion of a transposon into the mitomycin C region of the ColE3-CA38 plasmid only partially abolished the mitomycin C sensitivity (Fig. 5). In contrast, the absence of this region in recombinant plasmid pKC10 completely abolished mitomycin C sensitivity (Fig. 4), as does insertion of a transposon into the colicin E3 structural gene, or into the colicin E8 immunity gene (Table 1). The physiological role of this region of E colicin plasmids is open to debate. Sabik *et al.* (1983) have identified a region of the ColE1 plasmid, which they call *kil*, which appears to be required for cell lysis and colicin release after mitomycin C induction. The insertion of a transposon into the *kil* region of ColE1 abolished mitomycin C sensitivity and colicin release from cells. Watson & Visentin (1982), using zone sizes produced by strains carrying recombinant plasmids of ColE3-CA38 lacking the *Pvu*I to *Eco*RI region, which includes our 200 bp DNA sequence, have designated this region as *hic*, for high colicin production on solid media. Our results on the total colicin titre of cultures carrying plasmids pKC10 and pKC11 are consistent with some role of this region in colicin production (Table 2); however, we have observed no difference in the sizes of the zones of inhibition produced by *E. coli* W3110 carrying either pKC10, pKC11, pKC11::Tn5-87, or pKC11::Tn5-91 after chloroform lysis of the producing stab (K.-F. Chak, unpublished). We are continuing our study of this region of ColE3-CA38 in order to determine the number of gene products and their physiological role in colicin induction and release.

The genetic regulation of colicin E3 production and immunity, colicin E8 immunity and mitomycin sensitivity may be partially inferred from our data on the phenotypes conferred by plasmids carrying transposon insertions (Table 1). The colicin E3 structural gene, or its regulatory sequences, presumably begins in the 100 bp *Sma*I to *Hinc*II fragment of ColE3-CA38 and transcribes towards the two immunity genes and the mitomycin C sensitivity gene. The two immunity genes must have their own promoter(s), whilst the mitomycin C sensitivity gene is transcribed from the structural gene promoter. Masaki & Ohta (1982) and Mock *et al.* (1983)

claim that the colicin E3 immunity gene is transcribed in the same direction as the colicin E3 structural gene, with a possible promoter site for the immunity gene located in the colicin structural gene. An active promoter within the colicin E3 structural gene is supported by the phenotype of *E. coli* W3110(pKC11::Tn5-96), which has the transposon inserted 110 bp to the right of the *KpnI* site. Although this transposon is oriented such that its own promoter could transcribe through into the colicin E3 immunity gene (Berg *et al.*, 1980), we think that this is unlikely because removal of the Tn5 promoter by digestion of pKC11::Tn5-96 with *SalI* does not abolish colicin E3 or colicin E8 immunity. The location of the promoters for the two immunity genes along with their regulation independently of the flanking colicin E3 structural gene and the mitomycin C sensitivity region of the ColE3-CA38 plasmid remains an unanswered question. The presence of two immunity genes on an E colicin plasmid perhaps reflects an intermediate state in the evolution of colicin plasmids. Our findings mean that some caution must be used in classifying E colicins on the basis of their activity against reference cultures. Unless the panel of indicator strains carry recombinant plasmids containing single colicin immunity genes there remains possible ambiguity in the identification of E colicins.

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