Characterization of Three Group A Klebicin Plasmids: Localization of Their E Colicin Immunity Genes

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We have investigated the immunity to E colicins conferred by three group A klebicin plasmids. pP5a, which encodes klebicin A1-P5, like pClo-DF13, confers immunity to colicin E6 on *Escherichia coli* K12, whilst pP5b and pP3, which encode klebicins A2-P5 and A3-P3 respectively, both confer immunity to colicin E3. We have determined the restriction endonuclease and functional maps of the three group A klebicin plasmids. By sub-cloning and transposon mutagenesis we have investigated the relationship between the klebicin immunity and the E colicin immunity conferred by these plasmids. The colicin E6 and the klebicin A1 immunity are encoded by a single gene present on pP5a. The colicin E3 and the klebicin A2 immunity are encoded by a single gene present on pP5b. The colicin E3 and the klebicin A3 immunity are encoded by separate genes present on pP3. Recombinant pML8412, which is derived from the ColE6-CT14 plasmid and encodes colicin E6 immunity, confers klebicin A1-P5 immunity upon *Klebsiella pneumoniae* UNF5023. Recombinant pKC23, which is derived from the ColE3-CA38 plasmid and confers colicin E3 immunity, confers immunity to klebicin A2-P5, but not to klebicin A3-P3.

INTRODUCTION

The E colicins are a group of plasmid-encoded, antibacterial proteins which use the same cell-surface receptor to bind to sensitive *Escherichia coli* cells (Di Masi *et al.*, 1973). This group has been subdivided into colicins E1 to E9 on the basis of immunity tests (Watson *et al.*, 1981; Cooper & James, 1984). Each E colicin plasmid codes for the production of a specific immunity protein, which protects colicin-producing cells from colicin of the same type. *E. coli* K12 strains carrying an E colicin plasmid are sensitive to mitomycin C (MC), and thus differ from plasmid-free isogenic strains, due to the induction of a plasmid-coded lysis gene (*lys*) which is involved in colicin release from the producing cell (Shafferman *et al.*, 1979; Pugsley & Schwartz, 1983; Suit *et al.*, 1983; Chak & James, 1984, 1986; Jakes & Zinder, 1984; Lawrence & James, 1984). Since immunity is constitutively expressed, 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.

The genetic organization of the ColE3-CA38 plasmid has been extensively characterized. The colicin E3 structural gene (*ceaC*) and the *lys* gene (*celC*) 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, has its own promoter (Chak & James, 1984; Jakes & Zinder, 1984; Mock *et al.*, 1984). We have identified a second gene, conferring immunity 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). The ColE8*imm* gene also has its own promoter (Chak & James, 1985). It has been suggested that the ColE3-CA38 plasmid

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Abbreviations: MC, mitomycin C; Xgal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; ORF, open reading frame.

may represent a plasmid 'caught in the act of evolution' (Lau *et al.*, 1984), but the recent findings of further tandem immunity genes, against colicin E8 in the ColE6-CT14 plasmid (Lawrence, 1984) and against colicin E5 in the ColE9-J plasmid (Chak & James, 1986), suggest that the ColE3-CA38 plasmid is far from unique.

Recently we analysed a number of producer strains in the Edmondson & Cooke (1979) klebicin typing panel and described three klebicin A plasmids, pP5a, pP5b and pP3, which encode klebicins A1-P5, A2-P5 and A3-P3 respectively (Cooper & James, 1985). All three of these klebicins use the same cell-surface receptor to kill sensitive cells of *Klebsiella pneumoniae*, but can be differentiated by the absence of cross-immunity. Klebicin A1-P5 belongs to the same immunity group as cloacin DF13 (Oudega *et al.*, 1979). pCloDF13 confers colicin E6 immunity on *E. coli* K12 (Males & Stocker, 1982), but it is not known whether the colicin E6 immunity is encoded by the same gene as that which confers cloacin DF13 immunity. Since *K. pneumoniae* strains are not killed by E colicins, presumably due to the absence of the *btuB* receptor, whilst *E. coli* K12 strains lack the aerobactin receptor (Van Tiel-Menkveld *et al.*, 1981) and are therefore not killed by group A klebicins (Cooper & James, 1985), it is perhaps surprising that the CloDF13 plasmid confers immunity to an E colicin, unless the two immunities are encoded by a single gene. We have investigated the E colicin immunities conferred by the group A klebicin plasmids. We have used gene-cloning and transposon mutagenesis to determine the restriction and functional maps of the group A klebicin plasmids, and to study the relationships between their E colicin and klebicin immunity genes. Similar information has been invaluable in the study of the evolutionary relationships between the E group colicins (Watson *et al.*, 1985; Chak & James, 1986).

METHODS

Bacterial strains and plasmids. The strains of *K. pneumoniae* used in this work are listed in Table 1. *E. coli* JM83 *hsdR* is a restriction-deficient derivative of *E. coli* JM83 Ara⁺ Lac⁻ Pro⁻ Thi⁻ *rpsL* ϕ 80d*lacZ*M15. *E. coli* JM105 Lac⁻ Pro⁻ Thi⁻ *rpsL* F' *traD* *lacI*^q *lacZ*M15 was supplied by Gibco-BRL. Plasmids pP5a, pP5b and pP3 were prepared from strains PC905, PC909 and PC910 respectively (Cooper & James, 1985).

Media. Bacteria were cultured at 37 °C in L broth (Miller, 1972). The chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (Xgal) was included in L agar (L broth containing 1.5%, w/v, agar) at a final concentration of 20 μ g ml⁻¹ to detect recombinant plasmids. Ampicillin (Ap) was included in all media, at a final concentration of 100 μ g ml⁻¹ for the growth of all *E. coli* K12 strains which carried recombinant plasmids, and at a final concentration of 200 μ g ml⁻¹ for all *Klebsiella* strains which carried recombinant plasmids.

Klebicin production and immunity tests. Klebicin production was determined by stab tests (Ozeki *et al.*, 1962), using *K. pneumoniae* UNF5023 as the sensitive indicator strain. Klebicin immunity was determined by overlaying stabs of PC905, PC909 and PC910 with lawns of *K. pneumoniae* UNF5023 carrying the test plasmid. E colicin immunity was determined by streaking cultures of *E. coli* JM83 *hsdR* carrying the test plasmid on LB agar plates

Table 1. *Bacterial strains and plasmids*

	Relevant properties	Source or derivation
<i>K. pneumoniae</i>		
UNF5023	<i>his hsdR rpsL</i>	M. Merrick
PC905	UNF5023(pP5a)	Cooper & James (1985)
PC909	UNF5023(pP5b)	Cooper & James (1985)
PC910	UNF5023(r64-11)(pP3)	Cooper & James (1985)
Plasmid		
pJN73	pClo-DF13::Tn901, cloacin production and immunity, Ap ^R	B. Oudega
pUC8	Ap ^R	J. Messing
pUC18	Ap ^R	J. Messing
pUC19	Ap ^R	J. Messing
pML8412	ColE6 <i>imm</i> , Ap ^R	Lawrence (1984)
pKC23	ColE3 <i>imm</i> , Ap ^R	Chak & James (1984)
pLAX4	ColE3 <i>imm</i> , Ap ^R	Mock <i>et al.</i> (1983)
p3294	<i>Bal</i> 31 deletion mutant of pLAX4, ColE3 ^S	Mock <i>et al.</i> (1983)
pKH3-8	<i>btuB</i> ⁺ , Ap ^R	R. Kadner

spread with *E. coli* extracts prepared as described previously (Chak & James, 1984). Each test was done on at least six isolates from each test culture to determine the phenotype encoded by each recombinant plasmid.

Plasmid isolation, restriction, ligation, transformation and electrophoresis. All plasmids were prepared by a modification of the method of Birnboim & Doly (1979). The methods for restriction, ligation, transformation and electrophoresis of plasmid DNA have been described previously (Chak & James, 1984). *Cla*I linker was obtained from Gibco-BRL and was used as described previously (Maniatis *et al.*, 1982).

Co-transformation. In the absence of a good positive selection for the transformation of the three group A klebicin plasmids into *E. coli*, we used a co-transformation procedure. The native group A klebicin plasmids were transferred into *E. coli* JM83 *hsdR* by transforming with a mixture of pUC8 vector and the native klebicin plasmid in a ratio of 1:10. The relative DNA concentrations of the respective plasmids were estimated by a comparison of their band intensities after agarose gel electrophoresis. Transformants were selected on Ap-containing plates and were then screened for klebicin production.

Transposon mutagenesis. This was done with Tn1000 as described previously (Chak & James, 1984). The insertion sites of Tn1000 were determined by restriction with *Eco*RI, *Bam*HI and *Hind*III (Guyer, 1978).

Electrophoresis of klebicins. Cultures of *K. pneumoniae* UNF5023 carrying appropriate plasmids were grown in L broth to an OD₅₅₀ of 0.2 before the addition of MC (0.5 µg ml⁻¹) to half of each culture to induce the synthesis of klebicins. After 2 h further incubation, 1 ml of each culture was removed and the cells were recovered by centrifugation. The cell pellet was resuspended in 100 µl electrophoresis sample buffer, heated to 100 °C for 5 min and then centrifuged again. Portions (25 µl) of the total cell proteins were then analysed by SDS-PAGE (Laemmli, 1970) in 10% (w/v) gels using standard proteins as molecular mass markers. This experiment was repeated twice with identical results.

Hybridization. Specific fragments to be used as hybridization probes were isolated from low-melting-point agarose and labelled with [³⁵S]CTP (Amersham) using a Multiprime kit (Amersham), following the supplier's instructions. Restricted plasmids were tested for their hybridization with the probes after Southern blotting onto nitrocellulose filters. Hybridization was done at 68 °C for 16 h in the absence of formamide as described by Maniatis *et al.* (1982).

RESULTS AND DISCUSSION

E. coli colicin immunities conferred by A klebicin plasmids

The native group A klebicin plasmids were transferred to *E. coli* JM83 *hsdR* by co-transformation with pUC8. Representative Ap^R colonies which carried pUC8, with either pP5a, pP5b, pP3 or no plasmid, were then tested for their immunity to *E. coli* colicins (Table 2). *E. coli* JM83 *hsdR* which had been transformed with pJN73, a CloDF13 plasmid which carries Tn901 inserted in a non-essential region of the plasmid (Andreoli *et al.*, 1978), was included as a control. The results clearly show that pP5a, like CloDF13, confers colicin E6 immunity, whilst the pP5b and the pP3 plasmids confer colicin E3 immunity. In order to study the relationship between the respective klebicin and colicin immunity phenotypes conferred by the group A klebicin plasmids we used molecular cloning techniques and transposon mutagenesis.

Cloning of the klebicin A1-P5 plasmid

Preliminary restriction mapping of native pP5a indicated considerable similarity with pCloDF13 (data not shown). We cloned *Sal*I-restricted pP5a DNA into the *Sal*I site of vector pUC8 and then transformed *E. coli* JM83 *hsdR* to Ap resistance. The white transformant colonies on Xgal/Ap plates were tested for klebicin production. Recombinant pRJ119 was isolated from a klebicin-encoding transformant but this plasmid, which contained the whole 9.8 kb pP5a plasmid, was unstable. We have been unable to construct a stable recombinant plasmid which produces klebicin A1-P5. Restriction of pP5a DNA with *Bam*HI yields two fragments of 8.3 kb and 1.5 kb. Ligation of *Bam*HI-restricted pP5a DNA with *Bam*HI-restricted pUC8, and subsequent transformation of *E. coli* JM83 *hsdR* to Ap^R and colicin E6 immunity, yielded transformant colonies which carried pRJ118. On testing, this recombinant plasmid did not encode klebicin production. Restriction of pRJ118 with *Bam*HI yielded fragments of 8.3 kb and 2.7 kb. These results indicate that the klebicin A1-P5 structural gene is at least partially located within the 1.5 kb *Bam*HI fragment of pP5a. A similar location has previously been reported for the cloacin DF13 structural gene on the Clo-DF13 plasmid (Hakkaart *et al.*, 1981). A restriction map of pRJ118 is shown in Fig. 1. pRJ118 was transformed into *K. pneumoniae*

Fig. 1. Restriction map and sub-clones of pP5a. The restriction fragments present in the plasmids listed are indicated (for clarity the vector sequences are not shown) together with the phenotypes conferred. The arrows indicate the direction of transcription through the insert fragment initiated from the vector *lac* promoter.

Table 2. *E. coli* colicin immunities conferred by group A *klebsiella* plasmids

The plasmid present in the indicator strain is shown in parentheses. +, indicator is sensitive to the colicin extract; -, indicator is insensitive to the colicin extract.

Indicator strain	Sensitivity to colicins							
	E2	E3	E4	E5	E6	E7	E8	E9
JM83(pJN73)	+	+	+	+	-	+	+	+
JM83(pP5a)	+	+	+	+	-	+	+	+
JM83(pP5b)	+	-	+	+	+	+	+	+
JM83(pP3)	+	-	+	+	+	+	+	+
JM83	+	+	+	+	+	+	+	+

UNF5023 and a representative colony was tested to confirm that klebsiella A1-P5 immunity was encoded by this plasmid. *K. pneumoniae* cultures carrying pRJ118 were, however, sensitive to klebsiella A2-P5 and A3-P3.

In an attempt to localize the klebsiella A1-P5 immunity gene and the colicin E6 immunity gene, we constructed a series of sub-clones of pRJ118 (Fig. 1). The phenotypes conferred by recombinant plasmids pRJ150 and pRJ153 suggest that the colicin E6 and the klebsiella A1 immunity genes are located in the 2.2 kb *SmaI*-*BamHI* fragment of pP5a. This was confirmed by deletion of the 2.1 kb *BamHI*-*SmaI* fragment of pRJ150 by restriction with *BamHI* and *SmaI* and subsequent religation. The resulting plasmid, pRJ262, conferred immunity to colicin E6 upon *E. coli* JM105. During the construction of pRJ262 from pRJ150, the orientation of the insert fragment is reversed with respect to the vector *lac* promoter. This required the use of *E. coli* JM105 as a host strain for transformation in order to repress the *lac* promoter of the vector, since we had previously observed that the 2.2 kb *SmaI*-*BamHI* fragment could not be cloned in

E. coli JM83 when the vector promoter was transcribing this fragment in this orientation. pRJ262 conferred an IPTG^S phenotype upon *E. coli* JM105, presumably, by analogy with the genetic organization of cloacin DF13 and other group E colicins, due to constitutive expression of the *lys* gene present in this DNA fragment. Upon transformation into *K. pneumoniae* UNF5023, pRJ262 conferred klebicin A1 immunity. For these immunity tests, *K. pneumoniae* UNF5023(pRJ262) cultures were grown in L broth supplemented with glucose to reduce the expression of the vector *lac* promoter. In the absence of glucose, growth of these cultures was poor and Ap^S segregants were readily observed.

The precise localization of the colicin E6 *imm* gene was determined by deletion of the 300 bp *Cla*I fragment of pRJ262, from the single *Cla*I site in the insert fragment to a *Cla*I site in the vector, introduced by inserting a *Cla*I linker into the *Hinc*II site of the vector. The resulting plasmid pRJ263 did not confer E6 immunity upon *E. coli* JM105, although it still exhibited the IPTG^S phenotype, indicating that the vector promoter was still transcribing through the insert fragment. When transformed into *K. pneumoniae* UNF5023, again in the presence of glucose, pRJ263 did not confer klebicin A1 immunity. This result is in agreement with the localization of the cloacin DF13 immunity gene (Hakkaart *et al.*, 1981) and confirms that the colicin E6 immunity and the klebicin A1 immunity genes are identical.

Cloning of the klebicin A2-P5 plasmid

Restriction mapping of the native 7.5 kb pP5b plasmid indicated the presence of a single *Bgl*II site (data not shown). We cloned *Bgl*II-restricted pP5b DNA into the *Bam*HI site of vector pUC8 and then transformed *E. coli* JM83 *hsdR* to Ap^R. Plasmid pRJ132 was isolated from a recombinant clone which produced klebicin A2-P5 and conferred immunity to colicin E3. After transfer to *K. pneumoniae* UNF5023 by transformation, plasmid pRJ132 conferred klebicin A2-P5 immunity. Restriction mapping confirmed that this plasmid consisted of the whole pP5b plasmid cloned into the *Bam*HI site of the vector. In an attempt to locate the klebicin A2-P5 structural gene and the two immunity genes, we constructed a series of sub-clones of plasmid pRJ132 (Fig. 2). The phenotype conferred by plasmid pRJ155 localizes the three genes of interest to the 4.3 kb *Xho*I-*Bgl*II fragment of pP5b. Klebicin production was inactivated by deletion of the 1.5 kb *Bam*HI fragment of pRJ155. The resulting plasmid, pRJ159, still conferred klebicin A2-P5 and colicin E3 immunity. The phenotypes conferred by plasmids pRJ164 and pRJ244 localize the two immunity genes to the 600 bp *Sph*I-*Bam*HI fragment of pP5b. *Tn1000* mutagenesis of pRJ155 confirmed that the two immunity genes must have a separate promoter(s) to the klebicin structural gene as four independent transposon inserts located at 1.5 kb, 1.7 kb, 1.9 kb and 2.4 kb all inactivated the klebicin structural gene but did not affect either immunity phenotype (Fig. 2). This identifies a minimum region coding for the klebicin A2-P5 gene of 900 bp, sufficient to code for a protein of 33 kDa. Using plasmid pRJ164, all *Tn1000* inserts which inactivated the colicin E3 immunity phenotype also inactivated klebicin A2-P5 immunity. The site of insertion of three of these inserts in pRJ164 is shown in Fig. 2. In a modified *Tn1000* mutagenesis experiment we transformed *K. pneumoniae* UNF5023 with plasmid DNA prepared from 36 randomly selected transconjugant *E. coli* colonies. Single, representative transformant colonies were grown up in LB containing Ap and then tested for sensitivity to klebicin A2-P5. The transposon insertion sites of six klebicin A2 sensitive transformants were all located in the right-hand 350 bp of the 950 bp *Xho*I-*Bam*HI fragment of pRJ164. After transformation of these six plasmids into *E. coli* JM83 *hsdR*, all of the resulting transformants were colicin E3 sensitive. This result strongly suggests that the colicin E3 *imm* gene and the klebicin A2 *imm* gene are identical.

Cloning of the klebicin A3-P3 plasmid

Restriction mapping of the native 12.5 kb pP3 plasmid indicated the presence of two *Hind*III sites, yielding fragments of 7.0 and 5.5 kb. We cloned *Hind*III-restricted pP3 DNA into the *Hind*III site of pUC18 and transformed *E. coli* JM83 *hsdR* to Ap^R and colicin E3^R. pRJ157 was isolated from a recombinant clone which produced klebicin A3-P3 and was immune to colicin E3. After transfer to *K. pneumoniae* UNF5023, this plasmid conferred immunity to klebicin A3-

Fig. 2. Restriction map and sub-clones of pP5b. The restriction fragments present in the plasmids listed are indicated together with the phenotypes conferred. *Tn1000* insertion sites which inactivate the klebicin A2-P5 gene of pRJ155 or the E3imm gene of pRJ164 are indicated by triangles on the restriction map of the relevant plasmid. The minimum sizes of the klebicin and the A2imm gene, determined from the *Tn1000* data, are indicated by boxes.

Fig. 3. Restriction map and sub-clones of pP3. The restriction fragments present in the plasmids listed are indicated together with the phenotypes conferred. *Tn1000* insertion sites which inactivate the klebicin A3-P3 gene of pRJ157 or the E3imm gene of pRJ246 are indicated by triangles. The minimum sizes of the klebicin and the E3imm genes, determined from the *Tn1000* data, are indicated by boxes.

Table 3. *Klebicin immunities conferred by E colicin immunity genes*

The plasmid present in the indicator strain is shown in parentheses. +, a zone of inhibition was present around the producing stab; -, no zone of inhibition.

Indicator strain	Sensitivity to klebicins		
	A1-P5	A2-P5	A3-P3
UNF5023(pML8412)	-	+	+
UNF5023(pKC23)	+	-	+
UNF5023(pLAX4)	+	-	+
UNF5023(p3294)	+	+	+
UNF5023	+	+	+

P3, but not to klebicin A1-P5 or to klebicin A2-P5. Restriction mapping confirmed that pRJ157 consisted of the 7 kb *Hind*III fragment of pP3 cloned into the *Hind*III site of pUC18. In an attempt to localize the klebicin A3-P3 structural gene and the two immunity genes we constructed a series of sub-clones of pRJ157 (Fig. 3). The phenotype conferred by plasmids pRJ246 and pRJ250 indicates that the *E3imm* gene must lie in the 1.35 kb *Eco*RI-*Pst*I fragment of pRJ246. The phenotype conferred by pRJ253 shows that the *E3imm* gene is distinct from the klebicin A3imm gene and that the *E3imm* gene is cut by *Eco*RI. *Tn1000* inserts located at 1.9 kb, 2.6 kb, 2.95 kb and 3.15 kb in pRJ157 inactivated klebicin A3-P3 production (Fig. 3). This identifies a region of 1.25 kb as the minimum size of the klebicin A3-P3 structural gene, sufficient to encode a protein of 46 kDa. The *Tn1000* inserts located at 2.6 kb, 2.95 kb and 3.15 kb did not affect klebicin A3 or colicin E3 immunity; however, the insert at 1.9 kb abolished klebicin A3 immunity but not colicin E3 immunity. This suggests that the klebicin A3 immunity gene promoter is located between 1.9 kb and 2.6 kb. This is in agreement with the phenotype conferred by pRJ246, in which the klebicin A3 immunity gene promoter has presumably been deleted. Transposon inserts located at 3.8 kb and 4.3 kb in pRJ246 inactivated the *E3imm* gene. Although we have no direct evidence, by analogy with the genetic organization of other bacteriocins it may be reasonable to assume that the A3imm gene is located between the klebicin structural gene and the *E3imm* gene.

Klebicin immunity conferred by E colicin plasmids

In view of the E colicin immunity conferred by the A klebicin plasmids and the probability, in the case of the pP5a and the pP5b plasmids, that the colicin and klebicin immunity genes are identical, we investigated the klebicin immunity conferred by recombinant plasmids which carry defined regions of E colicin plasmids. Recombinant pML8412 consists of the 1.2 kb *Cla*I-*Eco*RI fragment of pColE6-CT14 cloned into pBR322 restricted with *Cla*I and *Eco*RI (Lawrence, 1984). This plasmid confers colicin E6 immunity on *E. coli* K12 and klebicin A1-P5 immunity on *K. pneumoniae* UNF5023 (Table 3), and thus supports our findings that these two immunity proteins are encoded by a single gene. pKC23 consists of the 1.0 kb *Eco*RI-*Cla*I fragment of pColE3-CA38 cloned into pBR322 restricted with *Eco*RI and *Cla*I (Chak & James, 1984). This plasmid confers colicin E3 immunity on *E. coli* K12 and klebicin A2-P5 immunity on *K. pneumoniae* UNF5023; however, pKC23 does not confer immunity to klebicin A3-P3. Inspection of the nucleotide sequence of the 1 kb *Cla*I-*Eco*RI fragment of pColE3-CA38 has shown the presence of a second open reading frame (ORF) at the 3' end of the colicin E3 structural gene. This ORF directs the synthesis of an 11 kDa protein *in vitro* (Mock *et al.*, 1983). To exclude the possibility that it is this ORF which encodes the klebicin A2 immunity protein we examined the phenotype conferred by p3294, which is a *Bal*31 deletion from the *Eco*RI site of pLAX4, a plasmid identical to pKC23 (Mock *et al.*, 1983, 1984). p3294, which has lost the *E3imm* gene as a result of the *Bal*31 deletion and still directs the synthesis of the 11 kDa protein *in vitro* (Mock *et al.*, 1983), does not confer immunity to klebicin A2-P5 (Table 3). These results strongly suggest that the *E3imm* gene of pColE3-CA38 and the *klebA2imm* gene of pP5b are capable of conferring immunity to both colicin E3 and klebicin A2-P5. Since immunity is very specific, this implies that the killing activities of the two bacteriocins are very similar, if not

Fig. 4 SDS-PAGE of klebicins. The migration of the molecular mass markers used and their molecular masses (kDa) are indicated by lines on the left-hand side. The band corresponding to the klebicins is indicated by an arrow. Total cell proteins were prepared from cultures of *K. pneumoniae* UNF5023 carrying pRJ157 (lanes 1 and 2); pRJ157::Tn1000 (lanes 3 and 4); pRJ155 (lanes 5 and 6); pRJ155::Tn1000 (lanes 7 and 8); pJN73 (lanes 9 and 10); and no plasmid (lanes 11 and 12). In the left-hand lane of each pair the culture was grown in the presence of MC ($0.5 \mu\text{g ml}^{-1}$); in the right-hand lane of each pair the culture was grown in the absence of MC.

identical. Nucleotide sequencing of the klebicin A2-P5 structural gene and the immunity gene would allow a detailed comparison with that published previously for colicin E3 (Mock *et al.*, 1983; Masaki & Ohta, 1985). Recombinant plasmids which carried the colicin E5, colicin E8 or colicin E9 immunity gene did not confer any immunity to the group A klebicins (data not shown).

Transfer of the btuB gene to Klebsiella

K. pneumoniae strains are insensitive to E colicins, presumably because they lack the *btuB* receptor. The *btuB* gene of *E. coli* K12 has been cloned in pBR322, yielding the recombinant pKH3-8 (Heller *et al.*, 1985). When *K. pneumoniae* UNF5023 was transformed with pKH3-8 DNA all Ap^R transformants tested, unlike *K. pneumoniae* UNF5023 itself, were sensitive to colicins E3, E6 and E8. They were also sensitive to phage BF23, which uses the *btuB* gene

product of *E. coli* as a receptor (Buxton, 1971). This implies that, with the exception of the *btuB* receptor, *K. pneumoniae* cells contain all the machinery for the uptake of E colicins and BF23 bacteriophages.

Homology between klebicin A plasmids

A comparison of the restriction maps of the three klebicin A plasmids reveals few common sites, with the exception of the 1.5 kb *Bam*HI fragment of pP5a and pP5b, both of which include at least part of the coding sequence of their respective klebicin structural genes. This is in contrast to the considerable restriction site homology observed between members of the E group of colicin plasmids (Watson *et al.*, 1985; Chak & James, 1986), and prevents any alignment of their restriction maps based upon common restriction sites. The molecular masses of klebicins A2-P5 and A3-P3 appear to be almost identical to that of cloacin DF13 as judged by SDS-PAGE (Fig. 4). The identification of the bands corresponding to klebicins A2-P5 and A3-P3 was assisted by a comparison of the total cell proteins of cultures of *K. pneumoniae* UNF5023 carrying klebicin-encoding plasmids, after growth in the presence and absence of MC. The bands which corresponded to the presumptive klebicins A2-P5 and A3-P3 were missing from total cell proteins derived from cultures carrying plasmids pRJ155 and pRJ157 with Tn1000 inserts which inactivate the respective klebicin structural genes.

In order to investigate further the homology between klebicins A2-P5 and A3-P3 we did some preliminary experiments with a hybridization probe consisting of the 1.5 kb *Bam*HI fragment of pRJ155. This fragment, which must encode a considerable part of the klebicin A2-P5 structural gene but does not include the *E3imm* gene (Fig. 2), hybridized to the 5.7 kb *Bam*HI fragment of pRJ157 but did not hybridize with pUC18 or pUC19 (data not shown). The 5.7 kb *Bam*HI fragment of pRJ157, which consists of the 3 kb *Hind*III-*Bam*HI fragment of pRJ157 plus the 2.7 kb vector (Fig. 3), includes a significant part of the klebicin A3-P3 structural gene. The 1.5 kb *Bam*HI probe also hybridized with both *Pst*I fragments of pRJ157. These results imply considerable homology between the klebicin A2-P5 and the klebicin A3-P3 structural genes. A detailed comparison of the nucleotide sequences of these three plasmids will be invaluable in elucidating their evolutionary relatedness. It will be particularly interesting to compare the nucleotide sequence of the *E3imm* gene of pP3 with that of the *klebA3-P3imm* gene of the same plasmid to look for signs of divergent evolution after a gene duplication event. An understanding of the genetic organization and regulation of the three klebicin operons will also be of value in obtaining high titre extracts of these klebicins for use in a klebicin typing scheme (Cooper & James, 1985).

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REFERENCES

- ANDREOLI, P. M., OVERBEEKE, N., VELTKAMP, E., EMBDEN, J. D. A. & NIJKAMP, H. J. J. (1978). Genetic map of the bacteriocinogenic plasmid CloDF13 derived by insertion of the transposon Tn901. *Molecular and General Genetics* **144**, 159-170.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening plasmid DNA. *Nucleic Acids Research* **7**, 1513-1523.
- BUXTON, R. S. (1971). Genetic analysis of *Escherichia coli* K12 mutants resistant to bacteriophage BF23 and the E-group colicins. *Molecular and General Genetics* **113**, 154-156.
- CHAK, K.-F. & JAMES, R. (1984). Localization and characterization of a gene on the ColE3-CA38 plasmid that confers immunity to colicin E8. *Journal of General Microbiology* **130**, 701-710.
- CHAK, K.-F. & JAMES, R. (1985). Analysis of the promoters for the two immunity genes present in the ColE3-CA38 plasmid using two new promoter probe vectors. *Nucleic Acids Research* **13**, 2519-2531.
- CHAK, K.-F. & JAMES, R. (1986). Characterization of the ColE9-J plasmid and analysis of its genetic organization. *Journal of General Microbiology* **132**, 61-71.
- COOPER, P. C. & JAMES, R. (1984). Two new E colicins, E8 and E9, produced by a strain of *Escherichia coli*. *Journal of General Microbiology* **130**, 209-215.
- COOPER, P. C. & JAMES, R. (1985). Three immunity types of klebicins which use the cloacin DF13 receptor of *Klebsiella pneumoniae*. *Journal of General Microbiology* **131**, 2313-2318.

- DI MASI, R. D., WHITE, J. C., SCHNAITMAN, C. A. & BRADBEER, C. (1973). Transport of vitamin B12 in *E. coli*: common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope. *Journal of Bacteriology*, **115**, 566–573.
- EDMONDSON, A. S. & COOKE, E. M. (1979). The development and assessment of a bacteriocin typing method for *Klebsiella*. *Journal of Hygiene* **82**, 207–223.
- GUYER, M. S. (1978). The $\gamma\delta$ sequence of F is an insertion sequence. *Journal of Molecular Biology* **126**, 347–365.
- HAKKAART, M. J. J., VELTKAMP, E. & NIJKAMP, H. J. J. (1981). Protein H encoded by plasmid CloDF13 involved in lysis of the bacterial host. *Molecular and General Genetics* **183**, 318–325.
- HELLER, K., MANN, B. J. & KADNER, R. J. (1985). Cloning and expression of the gene for the vitamin B12 receptor protein in the outer membrane of *Escherichia coli*. *Journal of Bacteriology* **161**, 896–903.
- HERSCHMAN, H. R. & HELINSKI, D. R. (1967). Comparative study of the events associated with colicin induction. *Journal of Bacteriology* **94**, 691–699.
- JAKES, K. S. & ZINDER, N. D. (1984). Plasmid ColE3 specifies a lysis protein. *Journal of Bacteriology* **157**, 582–590.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAU, P. C. K., ROWSOME, R. W., WATSON, R. J. & VISENTIN, L. P. (1984). The immunity genes of colicin E2 and colicin E8 are closely related. *Bioscience Reports* **4**, 565–572.
- LAWRENCE, G. M. P. (1984). *Characterization of the ColE8-J plasmid*. PhD thesis, University of East Anglia.
- LAWRENCE, G. M. P. & JAMES, R. (1984). Characterization of the ColE8-J plasmid, a new member of the group E colicin plasmids. *Gene* **29**, 145–155.
- MALES, B. M. & STOCKER, B. A. D. (1982). Colicins E4, E5, E6 and A and properties of *btuB*⁺ colicinogenic transconjugants. *Journal of General Microbiology* **128**, 95–106.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MASAKI, H. & OHTA, T. (1985). Colicin E3 and its immunity genes. *Journal of Molecular Biology* **182**, 217–227.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MOCK, M., MIYADA, C. G. & GUNSALUS, R. P. (1983). Nucleotide sequence for the catalytic domain of colicin E3 and its immunity protein. Evidence for a third gene overlapping colicin. *Nucleic Acids Research* **11**, 3547–3557.
- MOCK, M., MIYADA, C. G. & COLLIER, R. J. (1984). Genetic analysis of the functional relationship between colicin E3 and its immunity protein. *Journal of Bacteriology* **159**, 658–662.
- OUDEGA, B., OLDENZIEL-WERNER, W. J. M., KLAASEN-BOOR, P., REZEE, A., GLA, J. & DE GRAAF, F. K. (1979). Purification and characterization of cloacin DF13 receptor from *Enterobacter cloacae* and its inactivation with cloacin DF13 *in vitro*. *Journal of Bacteriology* **138**, 7–16.
- OZEKI, H., STOCKER, B. A. D. & SMITH, S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *Journal of General Microbiology* **28**, 671–687.
- PUGSLEY, A. P. & SCHWARTZ, M. (1983). A genetic approach to the study of mitomycin induced lysis of *Escherichia coli* K12 strains which produce colicin E2. *Molecular and General Genetics* **190**, 366–372.
- SHAFFERMAN, A., FLASHNER, Y. & COHEN, S. (1979). ColE1 DNA sequences interacting in *cis*, essential for mitomycin C induced lethality. *Molecular and General Genetics* **176**, 139–146.
- SUIT, J. L., FAN, M.-L. J., SABIK, J. F., LABARRE, R. & LURIA, S. E. (1983). Alternative forms of lethality in mitomycin C-induced bacteria carrying ColE1 plasmids. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 579–583.
- VAN TIEL-MENKVELD, G. J., OUDEGA, B., KEMPERS, O. & DE GRAAF, F. K. (1981). The possible involvement of the cloacin DF13 receptor protein in the hydroxamate-mediated uptake of iron by *Enterobacter cloacae* and *Escherichia coli* (ColV). *FEMS Microbiology Letters* **12**, 373–380.
- WATSON, R., ROWSOME, W., TSAO, J. & VISENTIN, L. P. (1981). Identification and characterization of Col plasmids from classical colicin E-producing strains. *Journal of Bacteriology* **147**, 569–577.
- WATSON, R. J., VERNET, T. & VISENTIN, L. P. (1985). Relationships of the Col plasmids E2, E3, E4, E5, E6 and E7: restriction mapping and colicin gene fusions. *Plasmid* **13**, 205–210.