Molecular Cloning and Purification of Klebicin B

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A novel klebicin, klebicin B, produced by an isolate of *Klebsiella pneumoniae* has been identified. It is encoded by a 5.5 kb plasmid, pKlebB-K17/80, which is mobilized into *K. pneumoniae* UNF5023 by a large plasmid found in the same strain. The 5.5 kb plasmid has been cloned into the high-copy-number vector pUC19 and the restriction map of the resulting recombinant plasmid pRJ180 has been determined. Using sub-cloning and transposon mutagenesis, the klebicin B structural gene, the klebicin B immunity gene and the mitomycin C (MC) sensitivity gene (*lys*) present on pRJ180 have been localized. Transposon inserts which inactivated klebicin production also abolished lysis protein production encoded by pRJ180, but did not affect klebicin B immunity. Using SDS-PAGE an MC-induced polypeptide of 85 kDa was observed in cultures of *K. pneumoniae* UNF5023(pRJ180). This polypeptide was absent in cultures carrying plasmid pRJ180 with a Tn1000 insert which inactivated klebicin production. Analysis of the polypeptides present in the medium of *Escherichia coli* JM83 *hsdR*(pRJ180) or *K. pneumoniae* UNF5023(pRJ180) indicated that the 85 kDa polypeptide is specifically secreted from the producing cell. Klebicin B has been purified, using gel filtration, from a cell-free extract of *K. pneumoniae* UNF5023(pRJ180) which had been induced with MC. After boiling in sample buffer the purified klebicin B gave rise to two peptides on SDS-PAGE, one of 85 kDa and the other of 11 kDa. Klebicin B-resistant mutants of *K. pneumoniae* UNF5023 were sensitive to klebicin A, colicin B and colicin D.

INTRODUCTION

Klebsiella species are common causes of hospital-acquired infections of the lower respiratory tract, urinary tract, surgical wounds and blood (Cross *et al.*, 1983). The mortality rates reported for *Klebsiella* bacteraemia and pneumonia range from 25% to 50% (Cryz *et al.*, 1986). These serious consequences of infection by *Klebsiella* indicate the need for detailed epidemiological analysis of such hospital-acquired infections, but this is limited by the absence of suitable typing schemes. At present the only feasible methods involve capsular serotyping, which involves 77 different antisera (Ørskov & Ørskov, 1984), bacteriophage typing (Gaston *et al.*, 1987) and klebicin (bacteriocin) typing.

Bacteriocins are plasmid-encoded, protein antibiotics which are produced by a wide variety of bacterial species. The most extensively studied bacteriocins are the colicins, produced by strains of *Escherichia coli*. The colicins are classified into groups based upon their target receptor on the sensitive cell, e.g. the E group colicins all use the *btuB* gene product of *E. coli* as a receptor on the cell surface of sensitive cells. E colicin plasmids also encode an immunity protein which protects the producing cell from the lethal effect of the colicin. In most of the E colicins which have been studied, the immunity protein forms a complex with the colicin soon after synthesis (Pugsley & Oudega, 1987). *E. coli* K12 strains carrying ColE plasmids are also sensitive to mitomycin C (MC) compared to plasmid-free isogenic strains (Herschman & Helinski, 1967). This is the

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

result of induction by MC of a third plasmid-coded lysis gene which is involved in the release of the colicin/immunity protein complex from the producing cell (Shafferman *et al.*, 1979; Pugsley & Schwartz, 1983; Suit *et al.*, 1983; Chak & James, 1984).

Several klebicin typing schemes have been proposed for the epidemiological investigation of *Klebsiella* infections (Slopek & Maresz-Babczynsyn, 1967; Hall, 1971; Buffenmeyer *et al.*, 1976; Heddel & Mitchell, 1978; Edmondson & Cooke, 1979; Bauernfeind *et al.*, 1981; Israil, 1981), but none has received universal acceptance. Each scheme involves testing isolates for resistance or sensitivity to the bacteriocins (klebicins) produced by a set of producer strains. The choice of producer strains, different in most of the schemes, is empirical, and in some schemes MC is used to increase the titre of the klebicin. The klebicins used are uncharacterized as regards their number, titre and type, whilst the interpretation of the results is subjective, especially the 'weak sensitive' response. The use of stable, high-titre preparations of receptor-specific klebicins will markedly increase the reliability and therefore the applicability of klebicin typing in clinical microbiology (Cooper & James, 1985; James *et al.*, 1987). In continuation of the attempt to develop a panel of high-titre, receptor-specific klebicins, here the identification, characterization and molecular cloning of klebicin B is described.

METHODS

Bacterial strains and plasmids. The strains of *Klebsiella pneumoniae* and *Escherichia coli* used in this work are listed in Table 1; the derivation of recombinant plasmids is shown in Table 2.

Media. Bacteria were cultured at 37 °C in L broth (Miller, 1972). The chromogenic substrate Xgal was included in L agar (L broth containing 1.5%, w/v, agar) at a final concentration of 20 µg ml⁻¹ to detect recombinant plasmids. Ampicillin was included in all media, at a final concentration of 100 µg ml⁻¹ for the growth of all *E. coli* K12 strains which carried recombinant plasmids, and at a final concentration of 200 µg ml⁻¹ for all *K. pneumoniae* 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 klebicin-producing strains with lawns of *K. pneumoniae* UNF5023 carrying the test plasmid. Each test was performed on at least six isolates from each test culture to determine the phenotype conferred by each recombinant plasmid.

Plasmid isolation, restriction, ligation, transformation and electrophoresis. All plasmids were prepared by the method of Birnboim & Doly (1979). The methods for restriction, ligation, transformation and electrophoresis of plasmid DNA have been previously described (Chak & James, 1984). Phosphorylated linkers (Pharmacia) were inserted into plasmids using the method of Maniatis *et al.* (1982).

Conjugation. Conjugation between klebicinogenic donors and *K. pneumoniae* UNF5023 was done as previously described (Cooper & James, 1985).

Transposon mutagenesis. Transposon mutagenesis with Tn1000 was done as previously described (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, 1.5 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. Samples (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. The supernate from the 1.5 ml culture was used to prepare samples of extracellular proteins by the method described in Pugsley & Oudega (1987). This experiment was repeated twice with identical results.

Purification of klebicin B. Klebicin B was purified from a 1 litre culture of *K. pneumoniae* UNF5023(pRJ180) after induction with MC for 2 h. The culture was then centrifuged at 10000 g for 15 min and the supernate recovered. Solid ammonium sulphate was added slowly, with stirring, to a final concentration of 60% saturation and the supernate was then left at 4 °C for 30 min. The precipitated proteins were recovered by centrifugation at 10000 g for 15 min and resuspended in 50 ml 50 mM-phosphate buffer, pH 7.0, before dialysis against 5 litres of the same buffer for 16 h. The retentate was then titrated on soft agar lawns of UNF5023 and analysed by gel filtration on a Superose 6 column using an FPLC system (LKB). Column fractions were concentrated by precipitation with 2 vols 100% ethanol at 4 °C and were resuspended in 50 mM-phosphate buffer, pH 7.0.

Table 1. *Bacterial strains*

Strain	Relevant properties	Source or derivation
<i>K. pneumoniae</i>		
K17/80	Prototrophic strain	P. Mortimer (PHLS, Colindale)
UNF5023	<i>his hsdR rpsL</i>	M. Merrick (University of Sussex)
PC902	UNF5023(pCloDF13)	Cooper & James (1985)
PC905	UNF5023(pP5a)	Cooper & James (1985)
PC909	UNF5023(pP5b)	Cooper & James (1985)
PC910	UNF5023(R64-11)(pP3)	Cooper & James (1985)
RJ102	UNF5023 CloDF13 ^R	This paper
RJ105	UNF5023 KlebB ^R	This paper
<i>E. coli</i>		
W3110	Prototrophic strain	Pugsley & Oudega (1987)
JM83 <i>hsdR</i>	Ara ⁻ Lac ⁻ Pro ⁻ Thi ⁻ <i>rpsL hsdR</i> ϕ 80 <i>dlac</i> ZM15	This laboratory
RJ103	W3110(pColB)	Pugsley & Oudega (1987)
RJ104	W3110(pColD)	Pugsley & Oudega (1987)
RJ106	JM83 <i>hsdR</i> (pUC18)	Vieira & Messing (1982)
RJ107	JM83 <i>hsdR</i> (pUC19)	Vieira & Messing (1982)

Table 2. *Derivation of recombinant plasmids*

Plasmid	Derivation
pRJ180	The whole pKleB-K17/80 plasmid cloned into the <i>Hind</i> III site of pUC19
pRJ271	The 4.5 kb <i>Hind</i> III– <i>Sal</i> I fragment of pKleB-K17/80 cloned into the <i>Sal</i> I– <i>Hind</i> III site of pUC18
pRJ72	The 1.0 kb <i>Pst</i> I fragment of pRJ271 deleted
pRJ277	The 4.5 kb <i>Hind</i> III– <i>Sal</i> I fragment of pKleB-K17/80 cloned into the <i>Hind</i> III– <i>Sal</i> I site of pUC19
pRJ278	The 2.8 kb <i>Bam</i> HI– <i>Sal</i> I fragment of pRJ277 deleted
pRJ279	The 1.05 kb <i>Sac</i> I– <i>Bam</i> HI fragment of pRJ278 deleted
pRJ281	Insertion of an <i>Eco</i> RI linker into the <i>Eco</i> RV site of pRJ279
pRJ282	Insertion of an <i>Hind</i> III linker into the <i>Eco</i> RV site of pRJ279
pRJ283	Deletion of the 0.65 kb <i>Eco</i> RI fragment of pRJ281
pRJ284	Deletion of the 1.05 kb <i>Hind</i> III fragment of pRJ282
pRJ285	Insertion of an <i>Hind</i> III linker into the <i>Eco</i> RV site of pRJ277
pRJ286	Deletion of the 1.05 kb <i>Hind</i> III fragment of pRJ285

RESULTS AND DISCUSSION

Identification of 'novel' klebicins

In an attempt to identify strains producing 'novel' klebicins, strains from klebicin-producing panels described by others and from collections of clinical isolates were screened. Strains which produced a zone of inhibition against *K. pneumoniae* UNF5023 were then tested as producers against *K. pneumoniae* RJ102. This mutant strain was isolated as a spontaneous cloacin DF13-resistant mutant of UNF5023. It is also resistant to klebicins A1, A2 and A3, and is therefore a presumptive receptor mutant for the group A klebicins. Strains which produced a zone of inhibition against RJ102 were then tested as indicators against *E. coli* strains producing colicin B and colicin D, colicins which are also active against *Klebsiella* (P. C. Cooper, personal communication). Strains which were sensitive to these two colicins were then classed as presumptive 'novel' klebicin-producing strains and were subjected to further analysis. One such strain was K17/80 from a collection of *Klebsiella* clinical isolates collected by P. Mortimer, Coventry, UK. The klebicin produced by this strain was tentatively assigned to the group B klebicins.

Characterization of the klebicin-encoding plasmid of K17/80

Electrophoresis of plasmid DNA prepared from *K. pneumoniae* K17/80 revealed the presence of a large plasmid of > 80 kb and a smaller plasmid of 5.5 kb (data not shown). Using a partially purified, cell-free extract prepared from MC-induced cultures of K17/80 as a selection agent, K17/80 was conjugated with UNF5023. Klebicin B-immune, streptomycin-resistant transconju-

Fig. 1. Restriction map and subclones of pKleB-K17/80. 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. Tn1000 insertion sites which inactivate the klebicin B gene of pRJ180 are indicated by triangles on the restriction map.

gants were screened for the production of klebicin B. All of the transconjugant colonies which produced klebicin contained both the large and the small plasmid, suggesting that the small plasmid may encode klebicin B. It appears that the large plasmid present in K17/80 is capable of mobilizing the small klebicin-encoding plasmid. A plasmid preparation from K17/80 was used to transform UNF5023 to klebicin B immunity. All colonies which produced klebicin B contained the 5.5 kb plasmid. This plasmid was designated as pKleB-K17/80.

Restriction mapping of plasmid pKleB-K17/80

Restriction of the 5.5 kb plasmid pKleB-K17/80 with *Bam*HI, *Hind*III, or *Sal*I yielded single fragments of 5.5 kb which were ligated into pUC19 restricted with the appropriate enzyme. After transformation of *E. coli* JM83 *hsdR*, clones carrying recombinant plasmids were then screened for klebicin B production. A recombinant plasmid, pRJ180, which encoded klebicin B was obtained from the *Hind*III clones. Restriction of pRJ180 with *Hind*III confirmed the presence of the 2.7 kb vector fragment and a 5.5 kb insert fragment derived from pKleB-K17/80. Using pRJ180 a restriction map of the pKleB-K17/80 plasmid was obtained (Fig. 1). Colonies of UNF5023(pRJ180) expressed klebicin B production, klebicin B immunity and lysis protein production (Fig. 2). Klebicin B immunity was tested using stabs of PC909 and UNF5023(pRJ180). All klebicin B-immune strains were still sensitive to the klebicin A2 produced by PC909. Also the MC sensitivity encoded by pRJ180 was expressed in *E. coli* JM83 *hsdR* (data not shown).

Transposon mutagenesis of pRJ180

In an attempt to localize the klebicin, immunity protein and lysis genes present on pRJ180 and to investigate their genetic organization, transposon mutagenesis with Tn1000 was performed. Tn1000 inserts which mapped at 1.9 kb, 3.5 kb and 3.7 kb from the left-hand

Fig. 2. MC-inducible cell lysis conferred by pRJ180. Cultures of *K. pneumoniae* UNF5023 carrying pUC19 (○, □) or pRJ180 (●, ■) were grown in L broth containing ampicillin. At time zero, MC ($0.5 \mu\text{g ml}^{-1}$) was added to half of each culture, as indicated by the square symbols. This experiment was repeated three times, and in each case the results were comparable to those shown.

*Hind*III site of pRJ180 inactivated klebicin B production (Fig. 1) and lysis, but did not affect klebicin B immunity. In many other bacteriocinogenic plasmids cell lysis is dependent upon transcription from the DNA-damage inducible promoter located proximal to the 5' end of the bacteriocin structural gene, and is abolished by transposon inserts which inactivate the bacteriocin structural gene (Chak & James, 1986). Bacteriocin immunity protein genes often have their own promoters which are independent of the promoter of the bacteriocin structural gene (Chak & James, 1985, 1986). Our results with the klebicin B recombinant plasmid pRJ180 are consistent with this functional organization.

Sub-cloning of pRJ180

Deletion of the 1 kb *Hind*III–*Sal*I fragment of pRJ180, yielding pRJ271, had no effect upon klebicin production, immunity or lysis protein production. This localizes the klebicin B structural gene, the klebicin B immunity gene and the lysis gene to the 4.5 kb *Sal*I–*Hind*III fragment of pKleB-K17/80 (Fig. 1). Deletion of the 1.1 kb *Pst*I fragment of pRJ271, yielding pRJ272, abolished klebicin production but did not affect klebicin B immunity, or lysis. This localizes at least part of the klebicin B structural gene, but not its promoter, to this *Pst*I fragment, a result which is consistent with the transposon mutagenesis data. Deletion of the 2.8 kb *Bam*HI–*Sal*I fragment of pRJ277, resulting in pRJ278, abolished klebicin production and cell lysis, but did not affect klebicin immunity. Further deletion of the 1.05 kb *Sac*I–*Bam*HI fragment of pRJ278 yielded pRJ279, which still conferred klebicin immunity. In order to further localize the klebicin immunity gene we inserted linkers which contain a restriction site for *Eco*RI or *Hind*III into the blunt-ended *Eco*RV site of pRJ279. The resulting plasmids, pRJ281 and pRJ282 respectively, were then restricted with *Eco*RI or *Hind*III in order to delete the 650 bp *Eco*RV–*Sac*I fragment, or the 1.05 kb *Hind*III–*Eco*RV fragment, respectively. The phenotypes conferred by the resulting plasmids pRJ283 and pRJ284 (Fig. 1) conclusively localized the klebicin B immunity gene to the 650 bp *Sac*I–*Eco*RV fragment of pRJ180.

The *lys* gene of pRJ180 was localized after insertion of *Hind*III linker into the *Eco*RV site of pRJ277. The resulting plasmid, pRJ285, was then restricted with *Hind*III to delete the 1.05 kb *Eco*RV–*Hind*III fragment. The resulting recombinant plasmid, pRJ286, conferred klebicin production and immunity but not lysis (data not shown). This localized the *lys* gene to the 1.05 kb *Eco*RV–*Hind*III fragment of pRJ180 and is consistent with our findings that this fragment can only be cloned downstream of the strong *lac* promoter, present in pUC vectors, in the orientation found in pRJ283. This strongly suggests that the direction of transcription of the *lys* gene is from left to right in Fig. 1. This orientation of transcription is also consistent with our identification of an MC-inducible promoter in the 900 bp *Sal*I–*Pst*I fragment of pRJ180, which also transcribes from left to right (data not shown). The genetic organization of the klebicin B operon is thus similar to that of most *E. coli* colicin plasmids, with the bacteriocin gene and the *lys* gene being transcribed in the same orientation from the SOS promoter localized proximal to the 5' end of the bacteriocin gene. The immunity gene is localized between the klebicin gene and the *lys* gene and probably has its own promoter.

Purification of klebicin B

SDS-PAGE of total cell proteins of *K. pneumoniae* UNF5023 carrying pRJ180 demonstrated the presence of an MC-inducible protein of 85 kDa. This protein was identified as klebicin B on the basis that *Tn1000* inserts in pRJ180, which inactivated the klebicin B gene, resulted in the absence of this band (Fig. 3a). A protein of this size would require a coding capacity of 2.3 kb, which indicates that the *Tn1000* inserts in pRJ180 which inactivate klebicin B production delimit some 80% of the gene (Fig. 1). No low-molecular-mass polypeptide corresponding to the immunity protein was clearly visible in these gels. Analysis of the proteins in the medium of MC-induced cultures of *E. coli* JM83 *hsdR*(pRJ180) or *K. pneumoniae* UNF5023(pRJ180) indicated that the klebicin is specifically secreted from the producing cell (Fig. 3b). This observation was used as the first stage of a protein purification procedure for klebicin B. Gel filtration of the partially purified klebicin B revealed the presence of two major protein peaks (data not shown). The first had an apparent molecular mass of 87 kDa and, unlike the second peak, contained active klebicin B. On SDS-PAGE the 87 kDa peak gave rise to two bands of 85 kDa and 11 kDa, which suggested that klebicin B, like other bacteriocins, is secreted as a complex of the klebicin and its associated immunity protein. Klebicin B is the largest bacteriocin to be reported with the exception of colicin D (Pugsley & Oudega, 1987).

Klebicin B-resistant mutants

A purified klebicin B preparation was used to select klebicin B-resistant mutants of UNF5023. Twelve klebicin B-resistant mutant colonies were then grown up in LB and tested as indicators against stabs of PC902, PC909, PC910, RJ103, RJ104 and UNF5023(pRJ180). In all twelve the results obtained were identical to those shown in Table 3 for the representative mutant strain RJ105. Klebicin B-resistant mutants of UNF5023 are sensitive to all klebicin A producers as well as to colicin B and colicin D. By analogy with other bacteriocin-resistant mutants selected in this way, it is probable that the klebicin B-resistant mutants of UNF5023 which were selected are receptor mutants. The mutant strain RJ105 is therefore a specific indicator strain for klebicin B producers.

Evolutionary relationships between bacteriocins

Detailed characterization of the cloacin DF13 and the colicin E3 operons has indicated sequence homology between the C-terminal killing activity fragments of these two bacteriocins, although the N-terminal receptor binding fragments are very different (Mock *et al.*, 1983). A further relationship between pCloDF13 and the ColE plasmids is indicated by the observation that pCloDF13 confers colicin E6 immunity (Males & Stocker, 1982). Two other members of the group A klebicin plasmids, to which pCloDF13 also belongs, confer colicin E3 immunity. Using subcloning and transposon mutagenesis, it has been shown that in one case the colicin E3 immunity and the klebicin immunity are determined by the same gene, whilst in the other they are determined by separate genes (James *et al.*, 1987). These examples of relationships are

Fig. 3. SDS-PAGE of klebicin B. The positions of molecular mass markers are indicated to the left of the photographs. The band corresponding to the klebicins is indicated by an arrow. (a) Total cell proteins of *K. pneumoniae* UNF5023 carrying no plasmid (lanes 1 and 2), carrying pRJ180 (lanes 3 and 4), carrying pRJ180::Tn1000-1 (lanes 5 and 6), and carrying pRJ180::Tn1000-3 (lanes 7 and 8). (b) Extracellular proteins of *E. coli* JM83 *hsdR* carrying no plasmid (lanes 1 and 2), and carrying pRJ180 (lanes 3 and 4); extracellular proteins of *K. pneumoniae* UNF5023 carrying no plasmid (lanes 5 and 6), and carrying pRJ180 (lanes 7 and 8); and total cell proteins of *K. pneumoniae* UNF5023 carrying pRJ180 (lanes 9 and 10). In the right-hand lane of each pair the culture was grown in the presence of MC ($0.5 \mu\text{g mg}^{-1}$); in the left-hand lane of each pair the culture was grown in the absence of MC.

Table 3. *Sensitivity and resistance of RJ105 to bacteriocins*

The plasmid present in the indicator strain is given in parentheses.

Indicator strain	Reaction to bacteriocin*					
	Cloacin DF13	Klebicin A2	Klebicin A3	Colicin B	Colicin D	Klebicin B
UNF5023	+	+	+	+	+	+
UNF5023(pCloDF13)	—	+	+	+	+	+
UNF5023(pP5b)	+	—	+	+	+	+
UNF5023(pP3)	+	+	—	+	+	+
RJ102	—	—	—	+	+	+
RJ105	+	+	+	+	+	—

* +, Clear zone of inhibition; —, no zone of inhibition.

particularly interesting in that klebicins, or cloacin DF13, do not kill *E. coli*, whilst the E colicins do not kill *K. pneumoniae*; this presumably reflects a divergence in the receptor-binding region of the klebicins/colicins. The detailed mechanisms of the evolution of these bacteriocin genes can only be speculated upon until we have much more detailed information, including nucleotide sequence data, on a large number of related bacteriocins. Based upon the limited information provided by the restriction map of pRJ180, there is no restriction site homology with any other bacteriocin operon which has been characterized. The identification and characterization of 'novel' klebicins may therefore be of value in these basic studies as well as for our aim of developing a rational klebicin typing scheme.

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