Nucleotide Sequence of the Immunity and Lysis Region of the ColE9-J Plasmid

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We have determined the nucleotide sequence of a 1500 bp fragment of the ColE9-J plasmid which encodes colicin E9 immunity and colicin E5 immunity and contains two *lys* genes. Open reading frames corresponding to the four genes have been located and their position confirmed by transposon mutagenesis of sub-clones of the ColE9-J plasmid. The *E9imm* gene shows 69% homology at both the nucleotide and the amino acid level to the previously sequenced *E2imm* gene. The *E5imm* gene shows little homology to any other E colicin immunity gene which has been sequenced. The *lys* gene distal to the 3' end of the *E5imm* gene shows considerable sequence homology to all other previously sequenced E colicin *lys* genes. The *lys* gene distal to the 3' end of the *E9imm* gene is identical to the pColE2 and pColE3 *lys* genes for the first 59 nucleotides but encodes a much smaller gene product than any other *lys* gene which has been sequenced. The two *lys* genes sequenced here are exceptions to Shepherd's rule concerning the number of RNY codons in the three possible reading frames.

INTRODUCTION

The E colicins are a group of plasmid-encoded antibacterial proteins which use the same cell-surface receptor, the product of the *Escherichia coli* chromosomal gene *btuB*, to bind to sensitive 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). The killing activity of several of the E colicins has been identified (Konisky, 1982). Each E colicin plasmid codes for the production of a specific immunity protein, which protects colicin-producing cells against colicin of the same immunity type. *E. coli* K12 strains carrying an E colicin plasmid are sensitive to mitomycin C (MC) compared to plasmid-free isogenic strains, due to the induction of a plasmid-coded lysis gene (*lys*) which is involved in colicin release from the producing cell. Transposon mutagenesis of the ColE3-CA38 plasmid has demonstrated that the colicin E3 structural gene (*ceaC*) and the *lys* gene (*celC*) are transcribed from a common SOS promoter located proximal to the colicin E3 gene whilst the ColE3*imm* gene (*ceiC*), which is located between these two genes, has its own promoter (Chak & James, 1984; Jakes & Zinder, 1984). A similar genetic organization has been observed for the colicin E2 operon (Cole *et al.*, 1985).

We have identified a second unrelated immunity gene, to colicin E8 (*ceiH*), located distal to the ColE3*imm* gene and proximal to the *lys* gene *celH* (Chak & James, 1984), the nucleotide sequence of which has subsequently been reported (Lau *et al.*, 1984). Comparison of the nucleotide sequence of the ColE8*imm* gene with that of the ColE2*imm* gene has revealed marked homology, which has led to the suggestion that the ColE3-CA38 plasmid may 'represent a unique case of a plasmid caught in the evolutionary process' (Lau *et al.*, 1984). The subsequent observation of the presence of two immunity genes in the ColE6-CT14 plasmid (Lawrence,

Abbreviations: MC, mitomycin C; ORF, open reading frame; RNY codon, a codon consisting of a purine followed by any base and then by a pyrimidine.

1984) and in the ColE9-J plasmid (Chak & James, 1986) has suggested that the presence of two immunity genes on an E colicin plasmid may be a fairly common occurrence. Our previous characterization of the ColE9-J plasmid was particularly interesting in that as well as the presence of two immunity genes, to colicin E5 and colicin E9, it also showed for the first time with an E colicin plasmid the presence of two functional *lys* genes. Here we present a more detailed analysis of the functional organization of the ColE9 operon and report the nucleotide sequence of a 1500 bp region of the ColE9-J plasmid which includes the two immunity genes and the two *lys* genes.

METHODS

Plasmids. The ColE9-J plasmid (Cooper & James, 1984), and the recombinant plasmids pKC64, pKC65, pKC67 and pKC124 (Chak & James, 1986) have been previously described. Plasmid pRJ261 is the 2.42 kb *Cla*I fragment of pKC64 ligated into the *Cla*I site of pBR322 in the opposite orientation to that found in plasmid pKC64. Plasmids were prepared by a modification of the method of Birnboim & Doly (1979).

Bacterial strains and media. *E. coli* W3110 was used as the host strain for all the recombinant plasmids. Cultures containing recombinant plasmids were grown in LB broth, or on plates of LB agar (Miller, 1972), containing ampicillin (100 µg ml⁻¹).

Enzymes and reagents. Restriction enzymes and T4 DNA ligase were purchased from Anglian Biotechnology, Gibco-BRL or Pharmacia. MC (Sigma) was used as previously described to test the MC sensitivity of cultures containing recombinant plasmids (Chak & James, 1984).

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

Plasmid restriction, ligation, transformation and electrophoresis. Digestion of DNA with restriction endonucleases, ligation of DNA fragments and transformation of competent cells were done as described by Maniatis *et al.* (1982). Electrophoresis of restriction fragments was performed as previously described (Chak & James, 1984).

Transposon mutagenesis. Transposon mutagenesis with Tn1000 was performed as previously described (Chak & James, 1984). Transposon insertion sites were mapped by isolating plasmid DNA and restricting separately with *Eco*RI, *Bam*HI or *Hind*III (Guyer, 1978).

Nucleotide sequencing. The nucleotide sequence of 1500 bp of the 2.42 kb *Cla*I fragment of plasmid pKC124 was determined by the method of Maxam & Gilbert (1980) with the modifications reported by Barker *et al.* (1983). [γ -³²P]ATP was obtained from New England Nuclear. All chemicals used in the DNA sequencing reactions were obtained from the recommended suppliers (Maxam & Gilbert, 1980). Plasmid pKC124 is identical to pKC64 except that the vector was pKC87 (Chak & James, 1985), in which the polylinker sequence facilitates nucleotide sequencing. The nucleotide sequence of the immunity and lysis genes present in this 1500 bp was analysed using a series of computer programs, written for the BBC model B microcomputer by one of us (R. J.), which determines the GC ratio and analyses the distribution of RNY codons in all three possible reading frames.

RESULTS AND DISCUSSION

Functional characterization of the ColE9 operon

A restriction map of the ColE9 operon, with a provisional localization of the relevant genes, has previously been reported (Chak & James, 1986). The 2.42 kb *Cla*I fragment of the ColE9 operon encodes *E9imm*, *E5imm* and two *lys* genes. Cloning of this fragment into the *Cla*I site of pBR322 gave transformants whose phenotype depended upon the orientation of the insert (Fig. 1). In one orientation the recombinant plasmid, pRJ261, encoded *E5imm* alone, whereas in the other orientation the recombinant plasmid, pKC64, encoded both *E5imm* and *E9imm*. This result can be explained in the light of the direction of transcription of the *tet* promoter of pBR322 with respect to the insert, and implies that the *E5imm* gene present in the 2.42 kb *Cla*I fragment must have its own promoter, whilst the *E9imm* gene is dependent upon the *tet* promoter of the vector for its transcription. The two *lys* genes present in the ColE9 operon were both dependent for their transcription on an SOS promoter located proximal to the colicin E9 structural gene (Chak & James, 1986). Although the two *lys* genes were shown to be located on separate *Eco*RI fragments of the ColE9-J plasmid, their position was inferred largely by analogy with that of the *lys* genes of other E colicin plasmids. In an attempt to locate the two *lys* genes more precisely we performed transposon mutagenesis with recombinant plasmid pKC67. The presence of two phenotypically identical *lys* genes on one plasmid causes a problem with nomenclature. Presumably the two *lys* genes should be called *E9lys* (*celI*) and *E5lys* (*celE*), but which is which?

Fig. 1. Sub-cloning and transposon mutagenesis of the ColE9-J plasmid. The restriction fragments present in the plasmids listed are indicated (for clarity the vector sequences are not shown), together with the phenotypes conferred. The *Tn1000* transposon insert sites in plasmids pRJ183 (■) and pRJ185 (□) are indicated. The arrows indicate the direction of transcription of the *tet* promoter in plasmids pKC64 and pRJ261.

In this paper we will refer to the *lys* gene which is proximal to the 5' end of the *E5imm* gene as being the *E9lys* gene, and that which is distal to the 3' end of the *E5imm* gene as being the *E5lys* gene.

After *Tn1000* mutagenesis of plasmid pKC67 several transconjugant colonies were observed which exhibited small zones when tested for colicin E9 production. We had observed a similar phenomenon in earlier work with the ColE3-CA38 (Chak & James, 1984) and the ColE8-J plasmids (Lawrence & James, 1984) and had demonstrated that transposon inserts which showed this phenotype were located in the *lys* genes of these plasmids. The *Tn1000* insert sites in pKC67 were mapped in six of these transconjugants and were found to be located in a single cluster in a region between 400 and 300 bp to the left of the *EcoRI* site in plasmid pKC67. This group of transposon inserts, characterized by plasmid pRJ183 (Fig. 1), exhibited immunity to both colicin E5 and E9. A second group of four interesting transconjugants, characterized by plasmid pRJ185, were identified due to their loss of immunity to colicin E5. Their *Tn1000* insert sites were located in a region within between 100 and 350 bp to the right of the *EcoRI* site of pKC67. *E. coli* W3110(pRJ185) was as sensitive to MC as was *E. coli* W3110(pKC67), whilst *E. coli* W3110(pRJ183) was MC resistant (Fig. 2). These results confirm the presence of the *E9lys* gene located in the right hand 400 bp of the *EcoRI* fragment of pKC65. In previous experiments with *Tn1000* all inserts distal to the SOS promoter and proximal to a *lys* gene have prevented expression of the *lys* gene (Chak & James, 1984, 1986). Since the *Tn1000* insert in pRJ185 was proximal to the *E5lys* gene but did not affect MC sensitivity this suggests a dominant role of the *E9lys* gene in MC sensitivity.

Fig. 2. MC sensitivity phenotypes conferred by plasmid pKC67 carrying Tn1000 inserts. At time zero, MC ($0.5 \mu\text{g ml}^{-1}$) was added to exponentially growing cultures of *E. coli* W3110 carrying plasmid pBR322 (□), pKC67 (■), pRJ183 (●) or pRJ185 (○). This experiment was repeated three times, and in each case the results were comparable to those shown. For the sake of clarity the control curves, which were essentially identical for all four cultures, are omitted.

Nucleotide sequencing

The 2.42 kb *Cla*I fragment of the ColE9-J plasmid encodes colicin E9 immunity and colicin E5 immunity and also contains the genetic information for two *lys* genes. Starting from the left-hand *Cla*I site we have sequenced 1500 bp of DNA (Fig. 3). From our previous sub-cloning experiments, the first 720 bp of this sequence, from the *Cla*I site to the *Eco*RI site, must encode the *E9imm* gene and the *E9lys* gene, whilst the sequence from the *Eco*RI site to the *Pvu*I site must encode the *E5imm* gene and the *E5lys* gene. Very few open reading frames (ORFs) of any significant size were found in this 1500 bp sequence.

The 720 bp *Cla*I–*Eco*RI fragment. A large ORF (bases 10 to 270) encodes a protein of 86 amino acids. Since a transposon insert located 600 bp proximal to the *Eco*RI site inactivates colicin E9 immunity (Chak & James, 1986), we believe that this ORF is the *E9imm* gene. The absence of a promoter proximal to the coding region of the *E9imm* gene is also in agreement with our findings that transcription of this immunity gene is dependent upon the *tet* promoter when the 2.42 kb *Cla*I fragment is cloned into pBR322. A comparison of the nucleotide sequence of the presumptive *E9imm* gene with the three *E* colicin immunity genes which have previously been sequenced reveals a marked homology between the *E9imm* gene and the *E2imm* gene (Fig. 4). The degree of homology (69% at both the nucleotide and the amino acid level) is even higher than that previously reported for the *E2imm* and the *E8imm* genes (Lau *et al.*, 1984). In contrast, the homology between the *E9imm* gene and the *E3imm* gene is 37% at the nucleotide level and only 7% at the amino acid level (data not shown).

Several ORFs are located distal to the 3' end of the *E9imm* gene: bases 274 to 399, 295 to 399, 299 to 412 and 332 to 412. Our Tn1000 mutagenesis data are consistent with any of these ORFs encoding the *E9lys* gene but, based on a comparison with the nucleotide sequence of other *lys* genes (Toba *et al.*, 1986), we believe that the latter ORF corresponds to the *E9lys* gene. This presumptive *E9lys* gene, unlike the other three ORFs, is also preceded by a potential ribosome-binding site sequence (5'-AGGAG) starting at base 321 (Shine & Dalgarno, 1974). As has been pointed out by Cole *et al.* (1985) and by Toba *et al.* (1986), there is considerable sequence homology between the *lys* genes encoded by the ColE2-P9, ColE3-CA38, CloDF13 and the ColE1 plasmids; indeed the nucleotide sequences of the pColE2 and the pColE3 *lys* genes are identical. These two lysis proteins consist of 47 amino acids and have an M_r of 4854. The first 59 nucleotides, and the first 19 amino acids, of the presumptive *E9lys* gene are identical to those

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MetGluLeuLysHisSerIleSerAspTyrThrGluAlaGluPheLeuGlnLeuValThrThrIle
GTAAGTAAATGGAACCTGAAGCATAGCATTAGTGATTATACAGAAGCTGAATTTTACAACCTGTAACAACAATT
. . . . . 75

CysAsnAlaAsnThrSerSerGluGluGluLeuValLysLeuValThrHisPheGluGluMetThrGluHisPro
TGTAATGCGAACACTTCCAGTGAAGAAGAACTGGTTAAATTGGTTACACACTTTGAGGAAATGACTGAGCACCCCT
. . . . . 150

SerGlySerAspLeuIleTyrTyrProLysGluGlyAspAspAspSerProSerGlyIleValAsnThrValLys
AGTGGTAGTGATTTAATATATTACCCAAAAGAAGGTGATGATGACTCACCTTCAGGTATTGTAAACACAGTAAAA
. . . . . 225

GlnTrpArgAlaAlaAsnGlyLysSerGlyPheLysGlnGly***
CAATGGCGAGCCGCTAACGOTAAAGTCAGGATTAAACAGGGCTAAAAATATGAGTGCCGGTTGTTTAAGGATGAAT
. . . . . 300

T1 MetLysLysIleThrGlyIleIleLeuLeuLeuAlaValIle
GGCTGGCATTCTTTTACAAACAAGGAGTCGTTATGAAAAAATAACAGGGATTATTTTATTGCTTCTTGCAGTCAT
. . . . . 375

IleLeuSerAlaTrpGlySerLysProLysThr***
TATTCTGTCTGCATGGGGTTCTAAGCCGAAAACCTAGAAAAATCCGTAACCAAAGCCAGTAATTGACAGATTTCG
. . . . . 450

ATGACGTTGAATAGGTGACGGGTATGTGACGAAATCTGATGCAGAAATCGTTGTTTCAGTGACAGTCACTCAAT
. . . . . 525

CGGTCGTTTATGTGACAACCCACGCGGTTACTGGTCGCGGAAAAATCCAGGTTTCCGGCCTGGAACCCGAAATG
. . . . . 600

ATCCAGCAACAGTGTTATGGTTCTCCTGGTAAATATGTTGTTGTCATGATCGTACTGGTGAGGTTACTCAGATTA
. . . . . 675
EcoRI
GTGATAAGACAGATCCGGGTTGGGTGGACGATTTCGAGAATTCAATGGGGAATAAAAAATGACCAATAAATTATTT
. . . . . 750

-35 -10 MetLysLeuSerPro
GAACATACGGTGTATATGATAGTGGTGATGCCTTTTTTGAATTAAGGAAATGCTTCTATGAAGTTATCACCA
. . . . . 825

LysAlaAlaIleGluValCysAsnGluAlaAlaLysLysGlyLeuTrpIleLeuGlyIleAspGlyGlyHisTrp
AAAGCTGCAATAGAAGTTTGTAATGAAGCAGCGAAAAAGGCTTATGGATTTTGGGCATTGATGGTGGGCATTGG
. . . . . 900

LeuAsnProGlyPheArgIleAspSerPheAlaSerTrpThrTyrAspMetProGluGluTyrLysSerLysThr
CTGAATCCTGGATTTCAGATAGATAGTTTCAGCATCATGGACATATGATATGCCGGAGGAATACAAATCAAAACC
. . . . . 975

ProGluAsnAsnArgLeuAlaIleGluAsnIleLysAspAspIleGluAsnGlyTyrThrAlaPheIleIleThr
CCTGAAAATAATAGATTGGCTATTGAAAATATTAAAGATGATATTGAGAATGGATACACTGCTTTCATTATCAGC
. . . . . 1050

LeuLysMet***
TTAAAGATGTAAATAGTGTATAGAATTTTATGTTTCATGGATGATTTCACCTTTGGATTTCAGGTTTTTATGG
. . . . . 1125

MetLysLysIleThrGlyIleIleLeuLeuLeuAlaAla
ATGAACGCCTGAGTTTCATATATACAAGGAACAGTATGAAAAAATAACAGGGATTATTTTATTGCTTCTTGCAGC
. . . . . 1200

IleIleLeuAlaAlaCysGlnAlaAsnTyrIleArgAspValGlnGlyGlyThrValSerProSerSerSerAla
CATTATCTTGTGTCATGTTCAGGCAAACTATATCCGCGATGTTTCAGGGGGGACTGTATCCCGTCATCCTCAGC
. . . . . 1275

GluLeuThrGlyLeuAlaThrGln*** T2
TGAACCTGACCGGATTAGCAACGCAGTAACCCGAAATCCTCTTTGACAAAAACAAAGCGTGCAGGCTGATTCTGA
. . . . . 1350
T3
TCGCGCTTTTTTTTGAATGTCAAAAAGTTCCATGTGGGAGATGGGATCTAAATCCCTGTGCAGAACCTTCCA
. . . . . 1425
PvuI
TCCGGTGGGAAAAAACTGTGCATTTTGACCTGTTTCGCGATTTCGAAACGGTTCGAAACCGATCCGCGCATCGTTTCG
. . . . . 1500

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Fig. 3. Nucleotide sequence of 1500 bp of the 2.42 kb *Cla*I fragment of plasmid pKC124. The deduced primary sequence of the products of *E9imm*, *E5imm* and the two *lys* genes are shown above the nucleotide sequence. The presumptive promoter sequences (-35 and -10) located proximal to the 5' end of the *E5imm* gene, and the transcription terminators, T1, T2 and T3, are indicated, as are the restriction sites for *Eco*RI and *Pvu*I.

Met	Glu	Leu	Lys	His	Ser	Ile	Ser	Asp	Tyr	Thr	Glu	Ala	Glu	Phe	Leu	Gln	E9imm
ATG	GAA	CTG	AAG	CAT	AGC	ATT	AGT	GAT	TAT	ACA	GAA	GCT	GAA	TTT	TTA	CAA	
***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	E2imm
																	Glu
G**	**G	**A	**A	A*A	**T	***	G**	***	**C	**T	***	A*C	***	**C	AA*	A**	E8imm
				Lys			Gly					Thr			Lys	Lys	
Leu	Val	Thr	Thr	Ile	Cys	Asn	Ala	Asn	Thr	Ser	Ser	Glu	Glu	Glu	Leu	Val	E9imm
CTT	GTA	ACA	ACA	ATT	TGT	AAT	GCG	AAC	ACT	TCC	AGT	GAA	GAA	GAA	CTG	GTT	
T**	***	A*	A*	**A	***	*GA	C*T	G*A	GG*	G*T	*C*	***	**G	**T	GAC	AA*	E2imm
Phe		Lys	Lys			Arg		Glu	Gly	Ala	Thr			Asp	Asp	Asn	
T**	A*T	GA*	GAC	**C	ATC	***	TGT	G*A	GG*	GAT		***	A**	A**	*A*	*A*	E8imm
Phe	Ile	Glu	Lys		Ile		Cys	Glu	Gly	Asp			Lys	Lys	Glu	Asp	
Lys	Leu	Val	Thr	His	Phe	Glu	Glu	Met	Thr	Glu	His	Pro	Ser	Gly	Ser	Asp	E9imm
AAA	TTG	GTT	ACA	CAC	TTT	GAG	GAA	ATG	ACT	CAG	CAC	CCT	AGT	GGT	AGT	GAT	
***	**A	**G	*G*	G*G	***	***	CG*	T*A	***	***	***	**A	GA*	***	TCA	***	E2imm
			Arg	Glu			Arg	Leu					Asp				
G*T	AAC	C*C	GAG	T*T	***	ATA	A*T	G*T	***	***	**T	***	***	***	TC*	***	E8imm
Asp	Asn	Leu	Glu	Tyr		Ile	Asn	Val									
Leu	Ile	Tyr	Tyr	Pro	Lys	Glu	Gly	Asp	Asp	Asp	Ser	Pro	Ser	Gly	Ile	Val	E9imm
TTA	ATA	TAT	TAC	CCA	AAA	GAA	GGT	GAT	GAT	GAC	TCA	CCT	TCA	GGT	ATT	GTA	
C*G	**T	***	**T	**T	CGC	**T	*AC	AGG	**A	**T	AGT	***	GAA	**G	***	GTC	E2imm
					Arg	Asp	Asp	Arg	Glu				Glu				
C*G	**T	***	***	***	G**	*GT	AA*	A**	***	*GT	AGC	***	GAA	***	G**	A*T	E8imm
					Glu	Gly	Asn	Asn		Gly			Glu		Val	Ile	
Asn	Thr	Val	Lys	Gln	Trp	Arg	Ala	Ala	Asn	Gly	Lys	Ser	Gly	Phe	Lys	Gln	E9imm
AAC	ACA	GTA	AAA	CAA	TGG	CGA	GCC	GCT	AAC	GGT	AAG	TCA	GGA	TTT	AAA	CAG	
G	GA*	A*T	*	G**	***	***	**T	***	***	***	***	***	***	***	***	***	E2imm
Lys	Glu	Ile		Glu													
A	GAG	A*T	*	G**	***	C**	***	***	***	***	***	***	***	***	***	***	E8imm
Lys	Glu	Ile	Glu														
Gly																	
GGC																	E9imm
***																	E2imm
***																	E8imm

Fig. 4. Homology between E colicin immunity genes. The nucleotide and amino acid sequence of the E9imm gene is shown in full. Where the nucleotide sequence of the E2imm and E8imm genes is identical to that of the E9imm gene, this is indicated by an asterisk. The amino acid sequence of the E2imm and E8imm genes is only shown when it differs from that of the E9imm gene.

Met	Lys	Lys	Ile	Thr	Gly	Ile	Ile	Leu	Leu	Leu	Leu	Ala	Val	Ile	Ile	Leu	
ATG	AAA	AAA	ATA	ACA	GGG	ATT	ATT	TTA	TTG	CTT	CTT	GCA	GTC	ATT	ATT	CTG	E2/lys
***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	E5/lys
													Ala				
***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	E9/lys
Ser	Ala	Cys	Gln	Ala	Asn	Tyr	Ile	Arg	Asp	Val	Gln	Gly	Gly	Thr	Val	Ser	
TCT	GCA	TGT	CAG	GCA	AAC	TAT	ATC	CGG	GAT	GTT	CAG	GGC	GGG	ACC	GTA	TCT	E2/lys
G**	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	E5/lys
Ala																	
***	***	**G	GGT	T*T	**G	CCG	*AA	ACC	TAG								E9/lys
	Trp	Gly	Ser	Lys	Pro	Lys	Thr										
Pro	Ser	Ser	Thr	Ala	Glu	Val	Thr	Gly	Leu	Ala	Thr	Gln					
CCG	TCA	TCA	ACA	GCT	GAA	GTG	ACC	GGA	TTA	GCA	ACG	CAG	TAA				E2/lys
***	***	**C	T**	***	***	C**	***	***	***	***	***	***	***				E5/lys
	Ser					Leu											

Fig. 5. Homology between E colicin lysis genes. The nucleotide and amino acid sequence of the E2/lys gene is shown in full. Where the nucleotide sequence of the E5/lys (the *lys* gene located distal to the 3' end of the E5imm gene on the ColE9-J plasmid) and E9/lys genes is identical to that of the E2/lys gene, this is indicated by an asterisk. The amino acid sequence of the E5/lys and E9/lys genes is only shown when it differs from that of the E2/lys gene. The presumptive cleavage site of the signal sequences of the E2/lys and E5/lys genes is indicated by an arrow.

found in the ColE2 and ColE3 *lys* genes, but the remainder of the presumptive E9/lys gene is very different, consisting of only 26 amino acids in total (Fig. 5).

The non-coding region of DNA located distal to the 3' end of the E9imm gene and proximal to the 5' end of the E9lys gene is virtually identical (58 of 60 bases) to a similar region located between the 3' end of the E2imm gene and the 5' end of the *lys* gene found on the ColE2-P9 plasmid, and to a region located between the 3' end of the E8imm gene and the 5' end of the *lys* gene found on the ColE3-CA38 plasmid. As has been pointed out for this region of the ColE2-P9 plasmid (Cole *et al.*, 1985) and of the ColE3-CA38 plasmid (Lau *et al.*, 1984), the sequence from base 269 can be folded into a hairpin loop which terminates at base 314 with the sequence 5'-TTCTTT. This conformation resembles a transcription terminator (Rosenberg & Court, 1979), which may play a role in the regulation of transcription of the *lys* gene.

Analysis of the nucleotide sequence proximal to the 5' end of the E9lys gene did not reveal any promoter-like sequences, consistent with our earlier finding that significant expression of the *lys* gene is dependent upon the SOS promoter (Chak & James, 1986). Experiments with promoter probe vectors had demonstrated the activity of a promoter in the *Cla*I-*Eco*RI fragment which transcribed towards the *Cla*I site (Chak & James, 1986). In the absence of any indications of any other gene being encoded by this fragment, it was suggested that this may represent the E9imm gene promoter. This proposal meant that the transcription of the E9imm gene was in the opposite orientation to that of the *lys* gene present in the 640 bp *Cla*I-*Eco*RI fragment (Chak & James, 1986). The nucleotide sequencing data reported here do not support this conclusion; however, they clearly show the presence of a complementary sequence to the consensus -10 promoter sequence TATAAT (Rosenberg & Court, 1979) commencing at base 40, and a complementary sequence to a -35 promoter sequence TTGTAA (Rosenberg & Court, 1979) commencing at base 60 (Fig. 3). The activity of this presumptive promoter transcribing in the

opposite orientation to the *E9imm* and *lys* genes would explain our earlier findings. A possible ORF transcribed by this promoter commences at base 29. Nucleotide sequencing of clones containing DNA sequences upstream of the *Cla*I site will be required to determine whether this ORF translates into a protein of any significant size and whether it overlaps the colicin E9 coding sequence.

The 760 bp EcoRI-PvuI fragment. There are two ORFs in the 760 bp *Eco*RI-*Pvu*I fragment of the ColE9 plasmid which could encode the *E5imm* gene. They both terminate at base 1062 and commence from base 733, or from base 811. Both ORFs are consistent with our observation that insertion of Tn1000 between 100 and 350 bp to the right of the *Eco*RI site of pKC67 inactivates the *E5imm* gene. In view of the presence of a reasonable -10 (TTATAT) promoter sequence at base 769, and the fact that the *E5imm* gene has its own promoter, we conclude that the ORF from base 811 to base 1062 encodes the *E5imm* gene. A search for a -35 promoter sequence did not reveal any strong candidates, with the possible exception of the TTGAAC sequence starting at base 749. Although the distance between this presumptive -35 sequence and the -10 promoter sequence is only 8 bp compared with the optimal spacer length of 17 bp (Stefano & Gralla, 1982), a similar value was observed for the presumptive *E8imm* gene promoter found on the ColE3-CA38 plasmid (Lau *et al.*, 1984). The presumptive *E5imm* gene encodes a protein of 83 amino acids which shows little homology to any of the E colicin immunity proteins that have been sequenced. The AT-rich region of DNA downstream of the presumptive *E5imm* gene can be folded into a hairpin structure as was previously reported for the region downstream of the *E8imm* gene (Lau *et al.*, 1984).

The ORF which encodes the *E5lys* gene present in this fragment is presumably located between bases 1160 and 1303 (Fig. 3). This ORF when translated would encode a protein of 47 amino acids, showing remarkable homology to the *lys* genes which have previously been sequenced (Fig. 5). The nucleotide sequence proximal to the 3' end of the *lys* gene is identical to that found downstream of the 3' end of the *lys* gene on the ColE2-P9 plasmid and includes a classic rho-independent transcription terminator (Cole *et al.*, 1985). We have not been able to isolate any Tn1000 inserts in this gene, presumably due to the absence of any resulting phenotypic alteration.

RNY analysis

It was reported by Morlon *et al.* (1983), and by Cole *et al.* (1985), that E colicin genes are quite unusual in that they contain a high proportion of 'rare' codons. Our data from pKC124 are consistent with this observation, but if an analysis of the nucleotide sequences of immunity and lysis genes is made by the method of Shepherd (1981), then some interesting differences are observed. In an investigation of the distribution of RNY codons in a variety of genes, Shepherd observed that the correct reading frame of the genes contains a higher number of RNY codons than the other two possible reading frames. A relatively small number of exceptions to this rule have been reported (Shepherd, 1984). It is therefore perhaps surprising that all the E colicin *lys* genes which have been sequenced, including that encoded by the CloDF13 plasmid, do not obey Shepherd's rule (Table 1). In contrast, the ColA *lys* gene and the *S* lysis gene of phage λ do obey Shepherd's rule. The lysis gene (*E*) of phage ϕ X174 was previously reported to also be an exception to Shepherd's rule (Shepherd, 1984). The *S* gene of λ , which has been reported to be functionally equivalent to the colicin E1 *lys* gene (Suit *et al.*, 1983), is at least twice the size of any of the E colicin *lys* genes. All of the E colicin immunity genes follow Shepherd's rule, although the *E1imm* gene is rather unusual in having a similar number of RNY codons in all three reading frames. The *E1imm* gene also has a very low GC ratio when compared to all the other E colicin genes. The ColAimm gene, however, is also an exception to Shepherd's rule.

It has been proposed that an RNY codon message could have been the basis of a primitive, comma-free, genetic code which has since evolved by the acquisition of the ability to use start and stop codons to delimit genes (Shepherd, 1981). One consequence of an RNY message is that a frameshift which results in the highest number of RNY codons being found in reading frame 3, when the correct reading frame is frame 1, will result in a higher than average number of

Table 1. RNY codon analysis

Gene	No. of codons	GC ratio (mol %)	Total RNY	RNY codons		
				RF1	RF2	RF3
E1 <i>lys</i>	45	46.4	29	10	7	12
E2 <i>lys</i>	48	43.05	36	13	4	19
E3 <i>lys</i>	48	43.05	36	13	4	19
E5 <i>lys</i>	47	44.4	36	14	5	17
E9 <i>lys</i>	26	35.8	18	7	2	9
ColA <i>lys</i>	51	43.6	39	25	3	11
Clo <i>lys</i>	49	46.7	34	13	5	16
λ S	107	48.1	82	41	17	24
ϕ X174 E	91	47.8	61	14	12	35
E1 <i>imm</i>	113	28.6	72	25	23	24
E2 <i>imm</i>	86	39.8	69	30	22	17
E3 <i>imm</i>	85	33.7	69	33	23	13
E5 <i>imm</i>	83	35.7	62	28	12	22
E8 <i>imm</i>	85	35.7	69	38	22	9
E9 <i>imm</i>	86	37.1	69	29	21	19
ColA <i>imm</i>	179	34.1	138	51	23	64
Clo <i>imm</i>	85	34.5	68	32	20	16

hydrophobic residues in the protein structure (Shepherd, 1984). It will be of interest to see how many other proteins which interact with membranes, like the E colicin *lys* genes, the ColA*imm* gene, and less so the ColE1*imm* gene, will be exceptions to Shepherd's rule.

Evolutionary relationships between *E. coli* colicin operons

Our sequence data support a very close evolutionary relationship between the E9*imm* gene, the E2*imm* gene, and the E8*imm* gene present on the ColE3-CA38 plasmid. This predicts that the amino acid sequence of the three colicin structural genes will also be very similar (thus far only the colicin E2 gene has been sequenced: Cole *et al.*, 1985). Changes in the nucleotide sequence of an ancestral colicin gene, tied closely with parallel changes in the homologous immunity protein gene, have presumably resulted in the evolution of a group of related bacteriocins which are only inactivated by their homologous immunity proteins. A comparison of the differences in amino acid sequence of these three colicins and their immunity proteins may be a particularly valuable system for the study of molecular recognition between proteins.

One particularly interesting conclusion from our sequencing and transposon mutagenesis data is that a functional E colicin *lys* gene need only be 26 amino acids long. The primary function of an E colicin *lys* gene product is not fully understood, although it is believed to interact in some way with the enzyme phospholipase A to achieve colicin secretion from the host cell (Pugsley & Schwartz, 1984). MC-induced cell lysis, as observed in the laboratory, may reflect an extreme level of induction of colicin operons and therefore may have little real relevance to the process of normal colicin secretion by a colicinogenic *E. coli* cell. The introduction of an artificial ochre stop codon 9 amino acids from the C-terminus of the pColE2 and the pColE3 *lys* genes did not significantly reduce the MC sensitivity conferred on *E. coli* by the modified *lys* genes (Toba *et al.*, 1986). It has been suggested that the N-terminal 19 amino acids of the pColE2 and pColE3 *lys* genes are signal sequences which direct the protein to the correct membrane location before cleavage (Cole *et al.*, 1985). This leaves 19 amino acids of these two *lys* gene products to interact with phospholipase A and result in colicin release from the cell. Our data show that the E9*lys* gene encodes a protein of only 26 amino acids and that it lacks the cleavage site present in the pColE2 and pColE3 *lys* genes. This 'truncated' *lys* gene is obviously still capable of promoting cell lysis (Fig. 2), and we are investigating its ability to promote colicin secretion. Site-directed mutagenesis of the E9*lys* gene and the synthesis of oligonucleotides which encode mutant forms of the E9*lys* gene will be invaluable in furthering our understanding of the processes of colicin secretion and cell lysis.

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