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Analysis of a cloned colicin Ib gene: complete nucleotide sequence and implications for regulation of expression

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ABSTRACT

The complete nucleotide sequence of a 2,971 base pair *Eco*RI fragment carrying the structural gene for colicin Ib has been determined. The length of the gene is 1,881 nucleotides which is predicted to produce a protein of 626 amino acids and of molecular weight 71,364. The structural gene is flanked by likely promoter and terminator signals and in between the promoter and the ribosome binding site is an inverted repeat sequence which resembles other sequences known to bind the LexA protein. Further analysis of the 5' flanking sequences revealed a second region which may act either as a second LexA binding site and/or in the binding of cyclic AMP receptor protein. Comparison of the predicted amino acid sequence of colicin Ib with that of colicins A and E1 reveals localised homology. The implications of these similarities in the proteins and of regulation of the colicin Ib structural gene are discussed.

INTRODUCTION

Colicin Ib is a proteinaceous antibiotic, encoded by the plasmid ColIb, which kills sensitive cells by depolarisation of the cytoplasmic membrane (1). Amongst a population of ColIb$^+$ bacteria only a small proportion produce colicin Ib, as expression of the structural gene (*cib*) is normally repressed (2,3). The *cib* gene is one of several genes including other colicin genes (2,3,4,5,6), genes involved in DNA repair, in mutagenesis and in prophage induction (for review see Little and Mount, ref 7) whose expression is induced as a consequence of damage to DNA resulting in initiation of the so-called "SOS response". Expression of all of these genes is thought to be repressed by the binding of the product of the chromosomal *lexA* gene to an operator sequence that has been termed the SOS box. As a consequence of DNA damage a specific protease activity of the RecA protein is activated and this protease cleaves the bound LexA protein allowing induction of gene expression (7). In addition to control of regulation of expression of colicin genes at this level, physiological studies have suggested that regulation of some colicin genes, including colicin Ib, may also be modulated by catabolite repression (2). DNA sequence analysis of some colicin genes has also identified likely

target sites for the binding of the cAMP receptor protein (5).

A population of ColIb$^+$ bacteria are protected against the toxic effects of the colicin by an immunity protein (8). The colicin Ib immunity protein is coded by a plasmid gene closely linked to *cib* (9), but its mechanism of action is unclear. The immunity protein encoded by ColIb protects bacteria only from the killing effects of colicin Ib and not from any other colicins (10). However, spontaneously occurring mutations to colicin resistance can readily be isolated and these usually lack the *cir* gene product, an outer membrane protein that acts as a receptor for colicins Ia, Ib and V (11).

This communication reports the analysis of a cloned colicin Ib gene, determination of its nucleotide sequence and study of its expression. The DNA fragment which has been cloned encodes a complete functional colicin Ib structural gene, but not the gene for the immunity protein. The colicin Ib gene is 1,881 base pairs (bp) in length and encodes a polypeptide with a predicted molecular weight (M_r) of 71,364. The structural gene is preceded by a classical promoter sequence and by a DNA sequence closely resembling the SOS boxes of several other genes regulated in a manner similar to *cib*. In addition, the sequence analysis revealed a second potential SOS box that is approximately 100 bp 5' of the postulated promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids and phage

Plasmid pLG281 has been previously described (12) and comprises a single *Eco*R1 fragment from ColIb*drd*-1 inserted into the unique *Eco*R1 site in the plasmid vector pBR325 (13). RB308 (F$^+$, *deoC*, *lacY*, *thyA*) was obtained from Roger Burton. DS410 (*minA*, *minB*, *ara*, *xyl*, *mtl*, *azi*, *thi*) (14) was used as the source of minicells. JM103 (15), M13mp8 (16, 17) and BW97 (18) are as described.

General methods

Bacteria were routinely cultivated in Luria broth supplemented with tetracycline (20μg/ml) and ampicillin (50μg/ml) where appropriate. The procedure for the isolation of γ-δ insertions was as described (19, 20). The test systems for colicin production and immunity have been described previously (10).

DNA methodologies

Plasmid DNA was purified either as described by Wilkins *et al.* (21) or on a mini-scale as described by Holmes and Quigley (22).

Restriction endonucleases and other DNA modifying enzymes were obtained

from Bethesda Research Laboratories and were used according to the manufacturers instructions.

Minicells and SDS polyacrylamide gel electrophoresis

Minicells were purified from DS410 harbouring the appropriate plasmid and labelled with ^{35}S-methionine (1000 Ci/mmol; Amersham International, PLC) as described (21). Conditions for gel electrophoresis (23) and fluorography (24) have been described.

Shotgun cloning and dideoxy sequencing

Randomly sheared fragments of the *Eco*RI insert in pLG281 were generated using DNAse1 as described by Anderson (25). Dideoxy sequencing was carried out as described by Sanger *et al.*(26).

RESULTS

Restriction map and phenotypic properties of pLG281

A map of the cutting sites for a number of different enzymes has been determined and the results are summarised in Fig. 1. The cleavage sites for *Pst*I, *Sph*I, *Cla*I, and *Hind*III which lie in the cloned DNA were located by performing double digests with either *Bam*HI or *Eco*RI. Plasmid pLG281, as well as conferring on *E. coli* ampicillin (Ap^R) and tetracycline (Tc^R) resistance (vector determinants) also directs the synthesis of colicin Ib. *E. coli* W3110 harboring this plasmid were not killed by colicins Ia, Ib, or V, but were sensitive to colicin B (12). The relationships between this pattern of colicin resistance and true colicin immunity will be discussed below.

Localisation of the coding sequence for colicin Ib on pLG281

The *cib* gene carried on pLG281 was located with respect to the restriction map by the isolation and mapping of γ-δ insertions that abolished colicin Ib production. Plasmid pLG281 was introduced by transformation into strain RB308

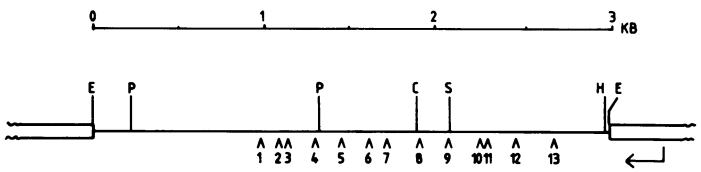


Figure 1 Restriction map of the insert in pLG281. E, P, C, S and H mark the cutting sites for the restriction endonucleases *Eco*RI, *Pst*I, *Cla*I, *Sph*I and *Hind*III respectively. The numbered arrows denote the position of the γ-δ insertions in pLG281 that abolish colicin Ib production. The arrow shows the origin and direction of transcription of the vector gene for chloramphenicol transacetylase.

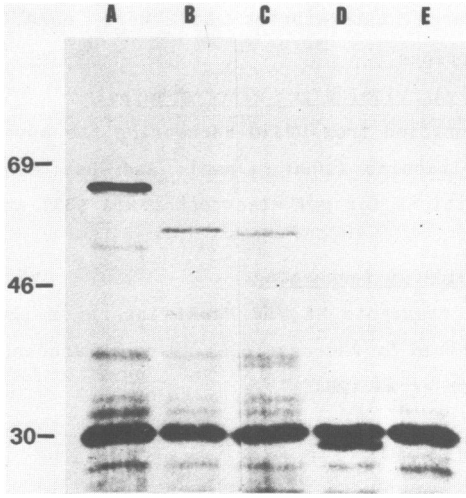


Figure 2 Minicell analysis of pLG281 and selected γ-δ insertion mutants. Tracks contained extracts of minicells from DS410 harbouring pLG281 (lane A) and insertion mutants 11 (lane B), 10 (lane C), 5 (lane D) and 2 (lane E). In the left margin can be seen the migration positions of molecular weight standards expressed in KDa.

which carries the F plasmid, a transformant was mated with BW97 and Tc^R, Ap^R transformants were selected. These arose by transposition of the γ-δ element from the F plasmid to pLG281 resulting in the formation of a cointegrate plasmid which, following transmission to the recipient, is resolved generating a γ-δ insertion into the recombinant plasmid (19). Insertion mutants were screened for their inability to produce colicin Ib. Plasmid DNA from these was partially purified and the site of γ-δ insertion mapped by cleavage with *SalI*. The sites of the insertions that inactivated colicin production are shown in Fig. 1 and indicated that a minimum size for the colicin Ib structural gene was about 1.6 kb. The limits of the *cib* gene as defined by this procedure were 1.0 and 2.6 co-ordinates on the map (see Fig 1).

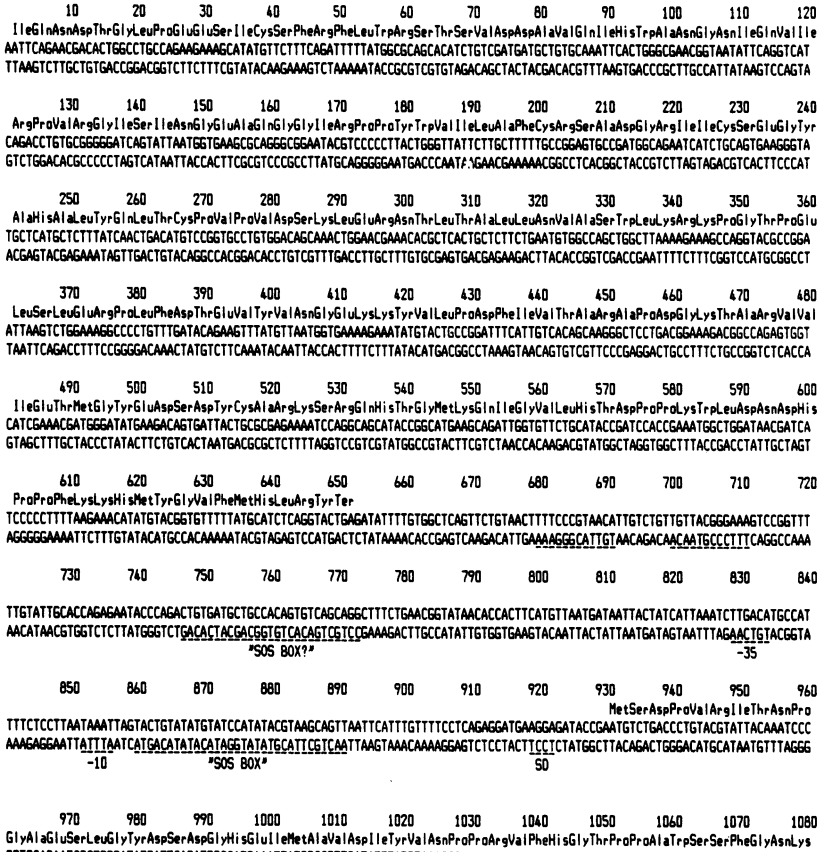
Polypeptides made in minicells by pLG281 and ς-δ insertions.

Plasmids such as pBR325 and its derivatives readily segregate into chromosome-free minicells providing a convenient system for the identification of plasmid-encoded polypeptides. Plasmid pLG281 and selected γ-δ insertions were introduced into the minicell-producing strain DS410, minicells were purified and their proteins were labelled with ^{35}S-methionine. Labelled polypeptides were analysed by polyacrylamide gel electrophoresis (PAGE) and autoradiography. The results (Fig. 2) demonstrate that γ-δ insertions that

abolish the ability of pLG281 to direct the synthesis of colicin Ib prevent the production of a polypeptide with an apparent molecular weight (M_r) of about 64,000 daltons. The reported figure for purified colicin Ib is 78,000 daltons (27). Mutant plasmids carrying insertions 11, 10 and 5 encode novel polypeptides of M_r 56,000, 55,000 and 28,000 respectively which are assumed to be truncated versions of colicin Ib. This would suggest that the *cib* gene is transcribed from left to right on the map presented in Fig. 1, the colicin promoter presumably lying between the left hand *Eco*RI site and the site of the γ-δ insertion number 1. This interpretation was confirmed by the DNA sequence analysis presented below.

The complete nucleotide sequence of the colicin Ib gene and its control elements

The nucleotide sequence of the *Eco*RI fragment cloned in pBR325 to yield pLG281 was determined by the shotgun cloning and sequencing strategy (25). DNA from both strands was sequenced at least two times from independent phage isolates except for one short region outside the coding region for colicin Ib (see below), where the unambiguous sequence of one strand only was determined. The complete DNA sequence of the cloned *Eco*RI fragment is presented in Fig. 3. The total length of the cloned *Eco*RI fragment is 2971 bp and a search for open reading frames in the sequence revealed several substantial possible coding sequences. Only one of these was of sufficient length to be the coding sequence for colicin Ib (Fig.3). Several lines of evidence point to this being the gene for colicin Ib. Firstly, the position of the section of DNA coding for this large polypeptide matches the limits of the structural gene for colicin Ib as determined by γ-δ insertion into pLG281 (see Fig.1). Secondly, the predicted size of the protein, 71,364 daltons, is between the figure of 64,000 obtained by minicell analysis (Fig. 2) and of 78,000 obtained from gel electrophoresis (27). Thirdly, the amino acid content of the polypeptide predicted from the nucleotide sequence closely resembles that determined for the purified colicin Ib protein as determined by Konisky (27, table 1). Finally the predicted amino acid sequence of the polypeptide reveals that the N-terminal residue is serine (assuming clipping of the methionine) which has been shown to be the case for colicin Ib (27). Analysis of the DNA sequences flanking the putative gene reveals the expected control sequences for the initiation of transcription (28) and translation (29, 30), and a TGA termination codon followed by an inverted repeat sequence which is probably also involved in the termination of transcription (31) (see Fig. 3). Between the putative promoter sequences and the ribosome binding site is an inverted



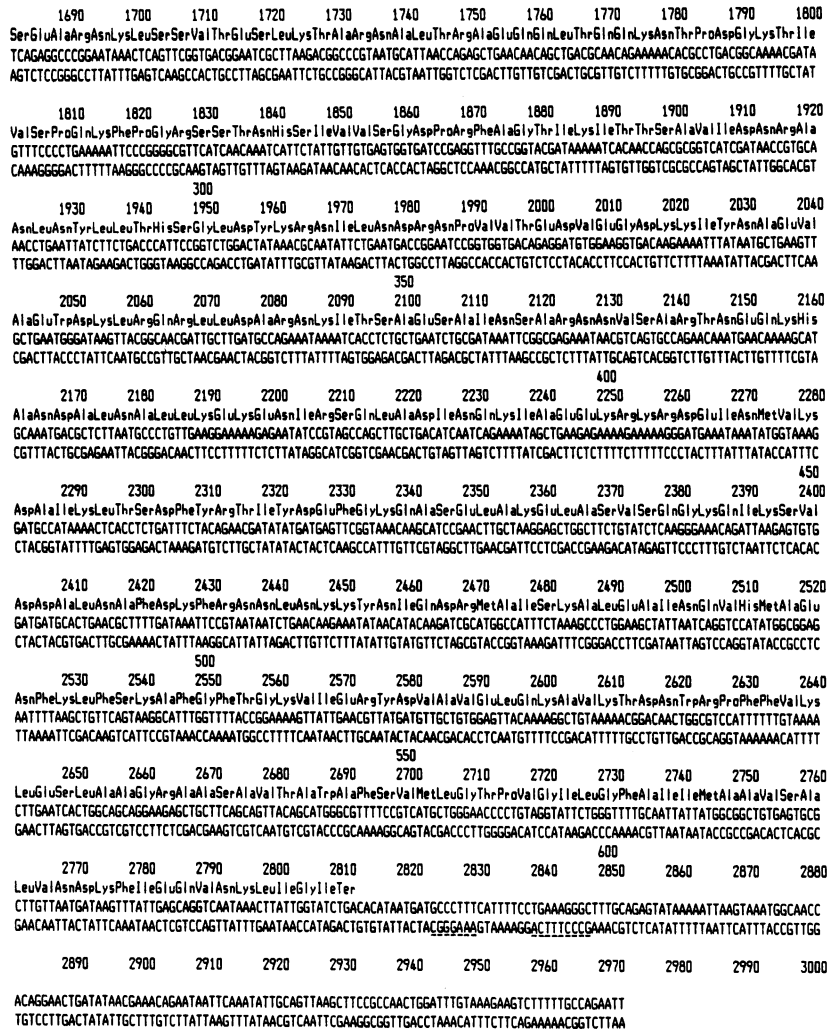


Figure 3 The complete nucleotide sequence of the *Eco*RI fragment cloned in pBR325 to give pLG281. The nucleotides are numbered to correlate with Fig. 1. Amino acid numbering starts with the methionine and is shown below the nucleotide sequence. The predicted polypeptide sequence of the open reading frame at the 5' end of the *Eco*RI fragment is also shown, the amino acids are not numbered. Inverted repeats which are probably involved in termination of transcription of the two genes are shown by arrows. The SOS box, Pribnow box (-10) and RNA polymerase recognition site (-35) for colicin Ib are underlined. SD is the potential ribosome binding site and the second region which may bind LexA protein or be implicated in catabolite repression is also marked (SOS box?).

repeat sequence that bears strong similarities to known LexA protein binding sites and that are thought to be involved in the regulation of the expression of genes modulated by the SOS response. Figure 4a shows a comparison of the sequence of the presumed colicin Ib SOS box with those of other sequenced SOS boxes. It is interesting to note that to the 5' side of the proposed promoter is a region that contains three sequences with CTG inversely repeated at their ends, that may have the potential to bind LexA protein (see Fig. 4a and b). In addition to bearing certain similarities to the SOS box consensus sequence, this region also has a sequence that also resembles the consensus sequence for the cyclic AMP repressor protein (CRP) binding site (Fig 4b, ref 32).

In addition to the region of pLG281 encoding the structural gene for colicin Ib, there are several other substantial open reading frames. One of these is shown on figure 3. This large open reading frame may encode the 216 C-terminal amino acids of a gene transcribed in the same direction as colicin

a)

Colicin A	TACTGTATATAAACACATGTGAATATATACAGTT (6)
Colicin Ib 1	TACTGTATATGTATCCATATACGTAAGCAGTT
Colicin E1	TGCTGTATATAAAACCAGTG (33)
Cloacin DF13	TACTGTGTATATATACAGTA (5)
sulA	TACTGTACATCCATACAGTA (37)
recA	TACTGTATGAGCATACAGAT (39)
lexA 1	TGCTGTATATACTCACAGCA (40,41)
lexA 2	AACTGTATATACACCCAGGG (40,41)
uvrA	TACTGTATATTCATTCAGGT (42)
uvrB	AACTGTTTTTTTATCCAGTA (38)
uvrD	ATCTGTATATATACCCAGCT (43)
Consensus	TACTGTATATACATACAGTt
	t cc a

b)

Consensus CRP binding site	AANTGTGANNTNNNNCANATTNNNN	(32)
Colicin Ib 2	GACTGTGATGCTGCCACAGTGTCAGCAGGC	
	----------	11bp
	----------	9bp
	----------	12bp

Figure 4 a) A comparison of the nucleotide sequences of known operators involved in LexA binding. Colicin Ib 1 is the SOS box marked in Fig. 3. The SOS boxes are aligned with respect to the 5' CTG (underlined in each case). The 3' CAG is also underlined (see text for further details). A consensus sequence has also been listed and has been derived from all the SOS boxes sequenced to date. References are given the right of each sequence.

b) The sequence of the second region 5' of the *cib* gene (marked SOS box? in Fig 3) which may be involved in LexA binding. The three repeats which have CTG inverted repeats are underlined and the number of bases between the inverted repeats are given to the right. The CRP binding site consensus sequence is also shown (32) for comparison with this region.

Ib, the promoter region and N-terminal coding sequences not being carried on the *Eco*RI fragment cloned. There are other open reading frames on the fragment but of more dubious significance for one or other of the following reasons. Firstly, we have found no evidence of polypeptides other than colicin Ib produced by pLG281 (Fig. 2). Secondly, several of the large open reading frames have no recognisable regulatory sequences flanking them. Finally, the amino acid composition of some are highly abnormal.

DISCUSSION

The complete nucleotide sequence of a 2971bp *Eco*RI-generated fragment that carries the colicin Ib structural gene has been determined. Examination of the sequence (Fig.3) reveals several large open reading frames, only one of which has the characteristics expected of the colicin Ib structural gene. This would encode a polypeptide with the same amino acid composition as colicin Ib (Table 1) with a predicted M_r of 71,364.

Expression of the colicin Ib gene is controlled at the level of transcription by the *lexA* gene product (2). Examination of the sequences preceding the coding sequence for colicin Ib (Fig 3) reveals an inverted repeat which may be the operator sequence for the colicin Ib gene, involved in binding the *lexA* gene product, since it closely resembles other elements that

Table 1. Amino acid composition of colicin Ib

Amino acid residue	Predicted from nucleotide sequence	Reported (27)
Lysine	50	60
Histidine	7	9
Arginine	46	50
Cysteine	0	0
Aspartic acid	43	⎫92
Asparagine	43	⎭
Threonine	34	33
Serine	41	30
Glutamic acid	46	⎫84
Glutamine	26	⎭
Proline	15	21
Glycine	30	38
Alanine	75	87
Valine	32	31
Methionine	9	8
Isoleucine	39	44
Leucine	51	59
Tyrosine	13	16
Phenylalanine	20	23
Tryptophan	6	8

have been shown to bind LexA protein (Fig.4a). The sequence is longer than that found in some other systems in which the consensus is CTG (N_{10}) CAG, but it is shorter than that identified for colicin A (6). However when both the colicin A and the colicin Ib SOS boxes are aligned with others identified to date in such a way that the 5' CTGs are coincident, a CAT aligns with the 3' CAG found in all other SOS boxes (Fig 4a). The sequences which are in between the CTG repeats in the SOS boxes are also conserved (Fig 4a) and both the colicin A and the colicin Ib sequences are similar. We cannot yet rule out the possibility that the SOS boxes for these two colicins are longer than the others identified to date, which are all CTG (N_{10}) CAG, but they may be the same length and have a variant CTG (N_{10}) CAT sequence. The position of the SOS box in relation to the other control regions for the *cib* gene is also consistent with a function as an operator sequence. Further examination of the nucleotide sequence at the 5' side of the colicin Ib structural gene reveals the existence of a second region that may also have the capacity to bind the *lexA* gene product (Fig.4b). In this region there are three potential LexA binding regions which resemble the consensus sequence for the SOS box, although the spacing between the CTG repeats is not the same as the consensus (Fig 4a and b). To our knowledge there is no other published instance of such organisation although in most cases the sequence data does not extend as far to the 5' of the structural gene as that determined for colicin Ib. We are currently examining the entire 5' region to determine which of the sequences function as control elements. This second putative LexA binding region, approximately 70 bp upstream from the -35 element of the postulated promoter for *cib*, also bears some similarities to the DNA sequences that bind the cAMP repressor protein (CRP, ref 32). Physiological experiments (2) have indicated that the *cib* gene may be subject to catabolite repression and it is possible that we may have identified the site responsible for CRP binding, although we have no genetic evidence to support this idea.

Recent genetic data (9) indicates that the *Eco*RI fragment cloned in pLG281 does not carry the gene for the colicin Ib immunity protein and all our data are in aggreement with this. This raises the question of how the *cib* gene was cloned without its associated immunity protein gene in a host sensitive to colicin Ib. One possibility is that there may be a considerable delay in the expression of the *cib* gene in newly transformed colicin-sensitive hosts. Consistent with this notion is the finding (unpublished data) that pLG281 can be introduced into Cir^+ or Cir^- bacteria with equal efficiencies. Accumulation of host chromosomal *cir* mutations may be essential for the longer term

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                  ▽▽▽▽ ↓↓▽↓▽ ▽  ↓     ↓↓  ↓↓↓ ↓ ↓↓
COLICIN Ib 98     AGKRLSAAIA AREKDENTLKTLRAGNADAA  127
COLICIN E1 234    AGKRNELAQASAKYKELDELVKKLSPRANDP  264
COLICIN A  223    AGKRVEAAQA AINTAQLN VNNLSGAVSAA  251


                  ▽▽↓  ▽▽▽            ↓ ▽ ↓↓ ↓↓↓    ↓↓↓   ↓  ↓↓ ↓↓ ↓
COLICIN Ib 442    ARQKIAEE            KRKRDEINMV    KDAIKLTSDFYRTIYD
COLICIN E1 324    ARVHEAEE            NLKKAQNNLLNSQIKDAVDATVSFYQTLTE
COLICIN A  371    ARQRQAEEAERQRQAMEVAEKAK DERELLEKTSELIAGMGDKIGEHLED

          ↓↓↓  ↓↓  ▽ ↓↓↓  ↓↓▽▽ ▽ ↓↓↓↓▽↓↓↓↓ ▽   ↓↓↓↓▽ ↓↓↓▽▽↓▽↓ ↓▽↓  ↓
Ib        EFGKQASELAKELASVSQGKQIKSVDDALNAFDKFRNNLNKKYNIQDRMAISKALEAI
E1        KYGEKYSKMAQELADKSKGKKIGNVNEALAAFEKYKDVLNKKFSKADRDAIFNALASV
A         KYKAIAKDIADNIKNF QGKTIRSFDDAMASLNKITANPAMKINKADRDALVNAWKHV

             ↓↓▽  ↓  ↓↓▽↓↓↓ ↓↓ ▽   ↓↓▽     ↓   ▽↓↓▽ ▽↓↓↓ ↓▽↓ ↓↓   ↓▽
Ib        NQVHMAENFKLFSKAFGFTGKVIERYDVAVELQKAVKTDNWRPFFVKLESLAAGRAAS
E1        KYDDWAKHLDQFAKYLKITGHVSFGYDVVSDILKIKDTGDWKPLFLTLEKKAADAGVS
A         DAQDMANKLGNLSKAFKVADVVMKVEKVREKSIEGYETGNWGPLMLEVESWVLSGIAS

          ▽ ↓  ▽▽  ↓        ▽↓↓ ↓↓ ▽↓▽ ▽  ↓↓↓       ▽▽↓      ▽↓ ↓↓↓
Ib        AVTAWAFSVML       GTPVGILGFA IIMAAVSALV NDKFIEQV NKLIGI    626
E1        YVVALLFSLLA       GTTLGIWGIA IVTGILCSYI  DKNKLNTINEVLGI    522
A         SVALGIFSATLGAYALSLGVPAIAVGIAGILLAAVVGALIDDKFADALNNEIIRPAH  592
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Figure 5 Homologous amino acid sequences in colicins Ib, E1 and A. Perfectly homologous amino acids are marked with a triangle and amino acids homologous between two of the three colicins are indicated by an arrowhead.

viability of the bacteria and this would account for the observed pattern of resistance to killing of W3110(pLG281) by colicins Ib, Ia and V (12).

In common with other colicins whose amino acid sequence is known, the hydrophobicity profile of colicin Ib (data not shown) reveals a strongly hydrophobic region near the C-terminus. In the case of colicins E1 and A (33, 34) this region has been postulated to mediate membrane depolarisation. Since these two colicins and colicin Ib all act to depolarise the bacterial cytoplasmic membrane it is tempting to speculate that that the hydrophobic domain at the C-terminus of the colicin Ib polypeptide also mediates membrane depolarisation. Our comparison of the amino acid sequences of colicins E1, A and Ib has extended considerably the observations of Morlon *et al.*(34) in which short regions of colicins E1 and A were found to be homologous. Our analysis (Fig. 5) shows extensive homology between the C-terminal regions of the three colicins extending over at least 200 amino acid residues. The functional significance of these findings is unclear at present, but would seem to suggest divergence of the three genes from a common ancestral gene.

The bacterial surface receptor for colicins E1 and A is the BtuB polypeptide that is involved in uptake of vitamin B_{12} (35). Both colicins share a common amino acid sequence that is present in a peptide fragment that

binds to the BtuB receptor (34, 35). Surprisingly a very similar amino acid sequence is present in the colicin Ib protein (Fig. 5). However the cell surface receptor for colicin Ib is the *cir* product and the BtuB receptor is not known to play a role in colicin Ib uptake.

We have been unable to find significant homology between the N-terminal region of colicin Ib and the other two colicins, the most noticeable of which is an absence of the concentration of glycine residues as described for colicins A and E1 (34). We have also found no significant homology in the amino acid sequences of colicin Ib and cloacin DF13 (36). Further information on both the DNA and the polypeptide sequences of other colicins should prove valuable in identifying conserved polypeptides and hopefully in correlating their structure with their biological properties.

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