Genetic analysis of an MDR-like export system: the secretion of colicin V

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The extracellular secretion of the antibacterial toxin colicin V is mediated via a signal sequence independent process which requires the products of two linked genes: *cvaA* and *cvaB*. The nucleotide sequence of *cvaB* reveals that its product is a member of a subfamily of proteins, involved in the export of diverse molecules, found in both eukaryotes and prokaryotes. This group of proteins, here referred to as the 'MDR-like' subfamily, is characterized by the presence of a hydrophobic region followed by a highly conserved ATP binding fold. By constructing fusions between the structural gene for colicin V, *cvaC*, and a gene for alkaline phosphatase, *phoA*, lacking its signal sequence, it was determined that 39 codons in the N-terminus of *cvaC* contained the structural information to allow CvaC–PhoA fusion proteins to be efficiently translocated across the plasma membrane of *Escherichia coli* in a CvaA/CvaB dependent fashion. This result is consistent with the location of point mutations in the *cvaC* gene which yielded export deficient colicin V. The presence of the export signal at the N-terminus of CvaC contrasts with the observed C-terminal location of the export signal for hemolysin, which also utilizes an MDR-like protein for its secretion. It was also found that the CvaA component of the colicin V export system shows amino acid sequence similarities with another component involved in hemolysin export, HlyD. The role of the second component in these systems and the possibility that other members of the MDR-like subfamily will also have corresponding second components are discussed. A third component used in both colicin V and hemolysin extracellular secretion is the *E. coli* host outer membrane protein, TolC.

Key words: MDR-like export/protein secretion/colicin V

Introduction

The ability to localize proteins to different cellular and extracellular compartments is an essential feature of all living cells. Because of their fundamental importance, the processes by which soluble proteins insert into and translocate across membranes have been studied intensively. The best characterized of these is the 'signal sequence dependent' secretory pathway (Randall *et al.*, 1987). Even though signal

sequences can be exchanged among exported proteins (Beckwith and Ferro-Novick, 1986), some proteins which are not normally exported cannot be made to cross a membrane simply by the addition of a signal sequence (Bassford *et al.*, 1979). This suggests that proteins which utilize the signal sequence dependent pathway have other inherent features that are required for their translocation (Lee *et al.*, 1989).

While most exported proteins appear to use the signal sequence secretory pathway, many proteins that are released from cells lack a signal sequence. Several bacteriocins, among them colicins E1 and A and cloacin DF13, are released by 'selective leakage' which follows the activation of a phospholipase by a specific 'lysis' protein (Pugsley, 1984). However, the release of many other proteins which have no signal sequences is accomplished without any cell lysis or leakage. Such proteins include yeast α -factor (Brake *et al.*, 1985), *Escherichia coli* hemolysin (Hartlein *et al.*, 1983; Felmlee *et al.*, 1985a), *Bordetella* cyclolysin (Glaser *et al.*, 1988), *Erwinia* protease (Letoffe *et al.*, 1990) and an *E. coli* peptide antibiotic, microcin B17 (Garrido *et al.*, 1988). The export mechanisms of these proteins have been analyzed, and in each case a dedicated export apparatus was found. This apparatus is composed of one or more proteins, one of which always contains an ~ 200 amino acid putative ATP binding domain. This domain displays a high degree of sequence similarity with a family of proteins involved in diverse transport processes (Higgins *et al.*, 1986).

Within this family, the dedicated protein exporters of hemolysin, cyclolysin, α -factor and *Erwinia* protease are members of a subfamily displaying an even greater degree of similarity in the ATP binding domain and a remarkable conservation of overall size and hydropathy profile. This subfamily is sometimes referred to as the 'MDR-like' subfamily because one of its best known members is the P-glycoprotein or MDR protein, the product of the *mdr1* gene which, when amplified, is responsible for the multi-drug resistance phenotype of tumor cells (Gerlach *et al.*, 1986; Gros *et al.*, 1986). Other members of this subfamily include PFMDR, an MDR homolog found in *Plasmodium falciparum*, also associated with drug resistance (Foote *et al.*, 1989), CFTR, the protein in which mutations leading to cystic fibrosis have been found (Riordan *et al.*, 1989), PMP70, a peroxisomal membrane protein (Kamijo *et al.*, 1990) and two proteins involved in polysaccharide export in bacteria, ChvA (Cangelosi *et al.*, 1989) and NdvA (Stanfield *et al.*, 1988). While the normal physiological roles of MDR, PFMDR, CFTR and PMP70 remain unknown, it is possible that all members of this subfamily are involved in some export process.

Previously we reported that the extracellular export of the small proteinaceous antibacterial toxin, colicin V, requires the action of two specific genes: *cvaA* and *cvaB* (Gilson *et al.*, 1987). Mutations in either of these genes result in the loss of ability to export colicin V extracellularly. To

understand better the process by which this molecule is secreted and the specific roles of *cvaA* and *cvaB* in this process, we have continued our genetic analysis of this system. Here we present the complete nucleotide sequence of the four genes involved in colicin V synthesis, export and immunity. CvaB was found to be a member of the MDR-like subfamily of proteins. CvaA, the second component of the dedicated export system, was found to be similar to HlyD, a second component required for hemolysin secretion (Wagner *et al.*, 1983; Mackman *et al.*, 1985). This suggests that there may be as yet unidentified second components for the other exporters. To characterize the structural features of colicin V which are essential for its recognition by the CvaA/CvaB exporter, we constructed fusions between the colicin V structural gene, *cvaC*, and a *phoA* gene lacking its signal sequence and then studied the chimera's transport. We also isolated mutations in *cvaC* which are defective in export but retain toxin activity.

Results

Organization of the colicin V genes

By genetic complementation analysis, we had previously shown that four genes, *cvaA*, *cvaB*, *cvaC* and *cvi* are necessary for normal production of the toxin colicin V (Gilson *et al.*, 1987). Only mutations in *cvaC* completely abolished colicin V activity. Mutations in *cvaA* and/or *cvaB* led to the blockage of colicin V export from the cell, but colicin V activity could still be detected inside the cell. The *cvi* gene was identified by its ability to confer immunity to colicin V.

To understand better the overall organization of the four colicin V genes, we used mini-MudII(Km,lac) (Castilho *et al.*, 1984) and Tn*phoA* (Manoil and Beckwith, 1985) to generate translational fusions. The complementation results and the fusions indicated the arrangement shown in Figure 1. All of the fusions obtained in *cvaA* and *cvaB* transcribed from left to right (as shown in Figure 1) while all fusions obtained in *cvi* and *cvaC* transcribed in the opposite direction.

Complete nucleotide sequence of the colicin V genes

We sequenced a 4496 bp region of DNA containing all four genes involved in colicin V production, export and immunity (Figure 1). Both strands were sequenced completely and the locations and sizes of the open reading frames corresponded well with the earlier complementation and minicell analysis (Gilson *et al.*, 1987). The genes are encoded in two converging operons, one containing *cvaA* and *cvaB* and the

other containing *cvi* and *cvaC*. There is an intergenic region of 169 bp separating *cvaB* and *cvaC*. The sequence of the entire region and the predicted amino acid sequences of the gene products are shown in Figure 2A and B. To facilitate the display we have arbitrarily split the sequence at the region between *cvaB* and *cvaC* and present it such that all four genes are shown with their sense strand reading from left to right. The predicted amino acid sequences of the gene products have yielded some insights regarding the features of the dedicated export system for colicin V secretion.

The sequence of the structural gene for colicin V, *cvaC* (Figure 2B), predicts a 103 amino acid residue product with an unusual composition: 17% Gly, 14% Ala and 10% Ser. Thus the colicin V toxin appears very flexible, which might be required of a small polypeptide that must: (i) be extracellularly secreted from the producing cell; (ii) interact with the surface receptor on sensitive cells (Cir) (Davies and Reeves, 1975); (iii) be translocated across the outer membrane into sensitive cells; and (iv) interact with the immunity protein of producer cells. Although colicin V is transported out of the cytoplasm, it does not possess a typical signal peptide. Its hydrophobicity profile (Figure 3) shows a large hydrophobic region in the N-terminal half of the protein. This is unusually long for a signal peptide and moreover it has an uncharacteristic net negative charge at the N-terminus. It is more likely that at least part of this hydrophobic region is involved in the membrane insertion and killing activity in the target cell. There are indications (see below) that the 103 amino acid primary translation product may be processed whereby 12–20 N-terminal residues are removed.

The *cvaB* gene has an open reading frame of 699 codons predicting a protein of 78 213 daltons. The coding region of *cvaB* and *cvaA* overlap. The *cvaB* initiation codon is found within the last two sense codons of *cvaA* and is separated from the *cvaA* termination codon by two bases (see Figure 2A). The hydropathy profile (Figure 4) of the derived amino acid sequence and hydrophobic moment analysis indicate that the protein lacks a signal peptide but does possess up to six potential transmembrane regions. Since many inner membrane proteins lack a signal peptide but have membrane insertion sequences (von Heijne, 1988), we hypothesize that CvaB is an integral inner membrane protein. Homology searches through the GENBANK and NBRF databanks showed that ~200 amino acids at the C-terminal end of CvaB are strikingly similar to analogous domains of several other proteins involved in export processes. These proteins constitute a subfamily within the family of ATP dependent

Fig. 1. Overall genetic arrangement of the colicin V genes required for production, export and immunity. Light arrows indicate the direction of transcription for both operons, and the open boxes represent the open reading frame corresponding to each of the genes. The predicted number of amino acid residues for each gene product is also shown.

transport proteins. The list of members of this subfamily continues to grow rapidly as described in the Introduction.

The 200 amino acid region of similarity is thought to constitute a highly conserved ATP binding domain. The amino acid alignment for this domain from 13 members of the subfamily is shown in Figure 5. Outside of this domain, the amino acid similarity is much less striking. However, the similarity in hydropathy profiles, predicting six trans-membrane regions, remains. The prokaryotic members of this group all have an integral membrane region of ~500 amino acids, followed by the presumed cytoplasmic ATP binding domain. In contrast, the eukaryotic members of the group generally have a duplication of this entire motif. The dissimilarities of the amino acid sequences can be assumed to account for the specificity of their transport.

The *cvaA* gene contains an open reading frame of 414 codons and predicts a product of 47 351 daltons. As shown in Figure 2A, there are also two internal in-frame AUG codons (located to codon positions 157 and 161), both preceded by good ribosome binding sites. The predicted weights for the products from these initiation sites are 29 436 and 28 949 daltons; either would correspond well with the size of a protein observed on SDS-PAGE (see below) and designated CvaA*.

Using minicells we had previously detected two protein products from *cvaA* (Gilson *et al.*, 1987). These were designated CvaA and CvaA*; their respective molecular weights were estimated to be 43 000 and 27 000 daltons. Neither protein appeared in cells harboring transposon insertions in *cvaA*. However, all of the transposon insertions were clustered in the 3' half of the gene. Thus, it was not clear whether the two products result from protein processing or from independent translation initiation events. To distinguish these possibilities, we generated a frameshift mutation early in *cvaA* (pLY17) by filling in the *Bam*HI site shown in Figure 1. In minicell transformants only the CvaA product disappeared (Figure 6) and these cells were unable to export colicin V. These results demonstrated that CvaA is required for colicin V export and that CvaA* is the result of an internal initiation of translation.

The hydrophobicity analysis of the predicted products of *cvaA* indicates that CvaA is largely hydrophilic with the exception of the 39 residues at its N-terminus (Figure 7). This region contains some properties of a typical signal sequence: positive charges at the N-terminus and a hydrophobic core. However, it does not have a leader peptidase cleavage site that follows the established patterns of most transported proteins with signal sequences (von Heijne, 1988). The possibility remains that CvaA has a slightly unusual signal peptide that directs CvaA to the periplasm or outer membrane. Alternatively, it is possible that the N-terminus of CvaA is a transmembrane stretch causing association of CvaA with the inner membrane. The observed molecular weight of CvaA in SDS-PAGE (Laemmli, 1970) is smaller than that predicted, which may indicate a post-translational processing step. CvaA*, in contrast, lacks the possible lipid interacting domain and appears to be a cytoplasmic version of CvaA. However, there is no direct evidence for the localization of either *cvaA* gene product.

CvaA and CvaB appear to form a dedicated two-component system for the export of colicin V. The export of hemolysin, *Bordetella* cyclolysin and *Erwinia* protease also involves two genetically linked components (Hartlein

et al., 1983; Felmlee *et al.*, 1985a; Glaser *et al.*, 1988; Letoffe *et al.*, 1990). In these three cases all the second components also display some amino acid similarities. Therefore we compared the amino acid sequence of CvaA with that of the hemolysin second component, HlyD. We indeed found some amino acid sequence similarity (Figure 8). CvaA and HlyD are 27% identical through 194 amino acids of their central region. Two smaller areas, CvaA 249–270 and HlyD 330–352, and CvaA 307–327 and HlyD 388–408, show 50% identity and include generally rare and evolutionarily conserved amino acids such as Phe, Tyr and Pro. This finding further indicates that many members of the MDR-like subfamily act in conjunction with a second component.

The *cvi* sequence, which predicts a protein of 9104 daltons, is also presented in Figure 2B. The predicted protein contains a hydrophobic region spanning residues 10–32. This hydrophobic N-terminus may mediate interaction with the cytoplasmic membrane. Previous evidence that colicin V kills sensitive cells by disrupting the membrane potential (Yang and Konisky, 1984) is consistent with this model. In this respect, Cvi would be similar to the immunity proteins which have been described for the much larger colicins, colicins E1, A, Ia and Ib, also known to kill by disrupting the membrane potential (Konisky, 1982).

TolC requirement for colicin V secretion

Since both CvaA and CvaB are good candidates for inner membrane proteins, we suspected that a host cell outer membrane protein may be involved in the traverse of colicin V across the *E. coli* outer membrane. Recently, Wandersman and Delepelaire (1990) have found that secretion of hemolysin in *E. coli* is dependent on the presence of TolC, a multi-functional, minor outer membrane protein.

Cells carrying either the high copy pHK11 or native pColV-K30 plasmids are unable to export colicin V when a *Tn5* or *Tn10* insertion occurs in the chromosomal *tolC*. However, the ColV⁺ phenotype can be rescued by transformation with pAX629 (Hiraga *et al.*, 1989), a pACYC184 derivative carrying the *tolC* gene. As with hemolysin, mutations that block production of the major porins, OmpF and OmpC, do not affect the extracellular export of colicin V.

Construction and analysis of *cvaC*–*phoA* fusions

We used *TnphoA* (Manoil and Beckwith, 1985) to generate in-frame *cvaC*–*phoA* gene fusions (see Figure 3). This approach yielded hybrid proteins containing three different amounts of the N-terminus of colicin V linked to an alkaline phosphatase moiety lacking its native signal peptide. CvaC57–PhoA, CvaC68–PhoA and CvaC81–PhoA have 57, 68 and 81 N-terminal residues, respectively, of the colicin V structural gene. Codons number 68 and 81 proved to be hot-spots for *TnphoA* insertion. In order to obtain insertions both earlier and later in the structural gene, we constructed CvaC29–PhoA, CvaC39–PhoA and CvaC97–PhoA fusions by standard *in vitro* techniques. *Bgl*III–*Hpa*II, *Bgl*III–*Pvu*II and *Bgl*III–*Dde*I fragments from the colicin V operon were inserted into a pUC19 derived vector which has a polylinker region upstream of the truncated alkaline phosphatase gene (see Materials and methods).

It has been shown that the alkaline phosphatase moiety

A TTATTGTCATTTAATAAATGACATCTTTTCATCATAAATAAAGACTATTGTTTATAATTTGTTCTCAGCATTATATGATTATTTATCTCTGATAA 100
CTCTCTCATGTTGTATGTTTATATGATTTTCTTGAACATATAATGCAAAATTTTCGATTATTTTCCATCATTATCCAGATAAACAACAACTAATAG 200
TATGCAAGGAGACATTATTGTTTCCCATGATGCTTTAGAAAAAGAAAAATGAAGTGGCAGGGACGGGCAATATTACTTCCCGGAATACCAGCTGTGGT 300
CvAa **ME**TlysTrpGlnGlyArgAlaIleLeuLeuProGlyIleProLeuTrpL
TAATCATGCTGGGAAGCATTGTTTATTACGGCATTCTGATGTTTCATTATTGTTGGTACCTATAGCCCGCTGTTAATGTCAGTGGTGGAGTGCACAA 400
euIleMetLeuGlySerIleValPheIleThrAlaPheLeuMetPheIleIleValGlyThrTyrSerArgArgValAsnValSerGlyGluValThrTh
CTGGCCAAAGAGCTGTCAATATATATTCAGGTGTACAGGGATTTGTTGTCAGGCAGTTTGTTCATGAAGGCAGTTGATAAAAAAGGGATCTGTTTAT 500
rTrpProArgAlaValAsnIleTyrSerGlyValGlnGlyPheValValArgGlnPheValHisGluGlyGlnLeuIleLysLysGlyAspProValTyr
CTGATTGACATCAGTAAAGTACACGCAATGGTATTGTCATGATAATCATCGCCGGGATATAGAAACCAGCTGGTTCGTGGGACAACTATTATTC 600
LeuIleAspIleSerLysSerThrArgAsnGlyIleValThrAspAsnHisArgArgAspIleGluAsnGlnLeuValArgValAspAsnIleIleSerA
GTCTGGAAGAAGTAAAAAATAACGCTAGATACCCCTGGAAAAACAACGTCTGCAATACACAGATGCGTTCGCTCGCTCATCAGACATTATACAGCGTGC 700
rgLeuGluGluSerLysLysIleThrLeuAspThrLeuGluLysGlnArgLeuGlnTyrThrAspAlaPheArgArgSerSerAspIleIleGlnArgAl
AGAGGAGGATAAAAAAATAAGAAAAATATGGAAGTACAGATATCATCAGTCAAAAGGACTGATTAAAGATCAATTAACAAAGTTGCA 800
aGluGluGlyIleLysIle**ME**TLysAsnAsn**ME**TGluAsnTyrArgTyrTyrGlnSerLysGlyLeuIleAsnLysAspGlnLeuThrAsnGlnValAla
TTATATTATCAACAACAAACACCTCTCTCAGTCTGAGCGGACAAATGAACAAATGCCCTGCAGATAACCACTCTGGAGAGTCAGATTACAGACTCAGG 900
LeuTyrTyrGlnGlnAsnAsnLeuLeuSerLeuSerGlnAsnGluGlnAsnAlaLeuGlnIleThrThrLeuGluSerGlnIleGlnThrGlnA
CAGCAGATTTTGATAATCGTATCTATCAGATGGAACGCAACGACTCGAATTGCAGAAAGAACTGGTTAACACTGATGTGGAAGCGAAATCATTATCCG 1000
laAlaAspPheAspAsnArgIleTyrGlnMetGluLeuGlnArgLeuGluLeuGlnLysGluLeuValAsnThrAspValGluGlyIleIleIleAr
GGCGTTGCTGACGGGAAGTTGACTCCCTGAGTGTCTGAGGGCAATGGTCAATACCGGAGACAGCCTTCTGCGAGTTATTCCTGAGAACATTGAA 1100
gAlaLeuSerAspGlyLysValAspSerLeuSerValThrValGlyGlnMetValAsnThrGlyAspSerLeuLeuGlnValIleProGluAsnIleGlu
AACTATTATCTTATCTCTGGGTCGGAATGATGCTGTTCTTATATTTCCGGTGGTACAAAGTGAATATTTCGTTATGAAGCCTTCCCTCAGAAAAAT 1200
AsnTyrTyrLeuIleLeuTrpValProAsnAspAlaValProTyrIleSerAlaGlyAspLysValAsnIleArgTyrGluAlaPheProSerGluLysP
TTGGGAGTCTCTGCTGACGGTAAACATATATCCAGGACTCTCGGTCAACACAGGAATGTTGACCTATAAGGAGCACCTCAAAATACGCGGGTGC 1300
heGluLeuPheSerAlaThrValLysThrIleSerArgThrProAlaSerThrGlnGluMetLeuThrTyrLysGlyAlaProGlnAsnThrProGlyAl
CTCTGTTCCCTGGTAAAGATCATTGCGACGCTGAAAGCAGATAATCAGGTATGACGAAAAATACCTCCCTCTGGAATGGAATGAAAGCGGAAGT 1400
aSerValProTrpTyrLysValIleAlaThrProGluLysGlnIleIleArgTyrAspGluLysTyrLeuProLeuGluAsnGlyMetLysAlaGluSer
ACACTATTTCTGGAAGGCGTATTACAGTGGATGCTTCTCTCTTCTATGACATGAACACAGTGCACAGGACCGATCAATGACTAACAGGAAT 1500
ThrLeuPheLeuGluLysArgArgIleTyrGlnTrpMetLeuSerProPheTyrAspMetLysHisSerAlaThrGlyProIleAsnAsp***
CvAa **ME**TThrAsnArgAsn
TTCAGACAAATATAAATCTGCTTATTGCGCTGGCAACGTCGTTCCGGTTATTCATCAGACGGAGACCGCTGAATGTGGACTGCGCTGCCTAGCAA 1600
PheArgGlnIleIleAsnLeuLeuAspLeuArgTrpGlnArgArgValProValIleHisGlnThrGluThrAlaGluCysGlyLeuAlaCysLeuAlaM
TGATATCGCGTCAATTTGGTAAGAATATTGACCTGATATATCTTCGCGGAAGTTAATCTCTCTGCGCGGTGGAGCAACCTTGCAGGAATCAATGGAAT 1700
eIleCysGlyHisPheGlyLysAsnIleAspLeuIleTurLeuArgArgLysPheAsnLeuSerAlaArgGlyAlaThrLeuAlaGlyIleAsnGlyI
AGCGGAGCACTGGGGATGCCACCGGGCTCTTCACTGGAGTGGATGAACCTCGAGTCTCAAAAGCCGTGTATTCTCCACTGGGATTTCACTCAC 1800
eAlaGluGlnLeuGlyMetAlaThrArgAlaLeuSerLeuGluLeuAspGluLeuArgValLeuLysThrProCysIleLeuHisTrpAspPheSerHis
TTCGTCGCTCTGCTGACGTAAGCGTAACCGTTATGTACTGCATGATCGGCCAGGGCATAAGATATATCAGCCGGGAGGAATGAGCGGATATTTTA 1900
PheValValLeuValSerValLysArgAsnArgTyrValLeuHisAspProAlaArgGlyIleArgTyrIleSerArgGluGluMetSerArgTyrPheT
hArgGlyAlaLeuGluValTrpProGlySerGluPheGlnSerGluThrLeuGlnThrArgIleSerLeuArgSerLeuIleAsnSerIleTyrGlyI 2000
TAAAGAACGCTGGCGAAATTTTCTGCTGTCTGAGTTGAATGAAGCAATCAATCTGCTAATGCCGGTGGGGACACAGCTGGTTATGGATCATGCTATT 2100
eLysArgThrLeuAlaLysIlePheCysLeuSerValValIleGluAlaIleAsnLeuLeuMetProValGlyThrGlnLeuValMetAspHisAlaIle
CCTGCGGGGACAGAGGGCTACTGACGCTAATTTCTGCTGCTCTTATGTTTTATATTACTCAAAGTGCACAGTACGCTGCCGCTGGCTTTCAC 2200
ProAlaGlyHisPheGlyLeuLeuThrLeuIleSerAlaAlaLeuMetPhePheIleLeuLeuLysAlaAlaThrSerThrLeuArgAlaTrpSerSerL
TGGTTATGACACGCTCATATGTACAGTGGCAGTCCGGGCTGTTGATCATCTTCTCAGACTACCGCTGGCGTTTTTGAACCGGAAAAATAGGTGA 2300
euValMetSerThrLeuIleAsnValGlnTrpGlnSerGlyLeuPheAspHisLeuLeuArgLeuProLeuAlaPheGluArgArgLysLeuGlyAs
TATCCAGTCACTTTGACTCCCTTGACACATTGAGGGCCACATTACCACAGTGTGATCGGGTTCAATAGGACAGCATTATGGTTGTCGGTGTTC 2400
pIleGlnSerArgPheAspSerLeuAspThrLeuArgAlaThrPheThrThrSerValIleGlyPheIleMetAspSerIleMetValValGlyValCys
GTGATGATGCTTTATACGGAGGATATCTCACCTGGATAGTCTCTGCTTTACACAAATTTACATTTTATTCAGTCTGGTACATACGGCAATTACCGAC 2500
ValMetMetLeuLeuTyrGlyGlyTyrLeuThrTrpIleValLeuLysPheThrThrIleTyrIlePheIleArgLeuValThrTyrGlyAsnTyrArgG
AGATATCAGAAGATGTCTGTGAGGAGGCGCGTCCGCTCTTATTTATGGAACATTATATGTTATGCCACGGTAAAAATCCAGGGATGTCGG 2600
InIleSerGluGluCysLeuValArgGluAlaArgAlaAlaSerTyrPheMetGluThrLeuTyrGlyIleAlaThrValLysIleGlnGlyMetValGl
AATTCGGGGGCACTGGCTTAATATGAAAATAGATGCGATAAATTCGGGTATTAAGCTAACAGGATGGATTGTCTCTCGGAGGAATAAATACCTTT 2700
ProAlaGlyAlaHisTrpLeuAsnMetLysIleAspAlaIleAsnSerGlyIleLysLeuThrArgMetAspLeuLeuPheGlyGlyIleAsnThrPhe
GTTACCGCTGTGATCAGATTGTAATTTATGGCTGGGAGCAGGCTTGTGATCGATAATCAGATGACAAATAGGAATGTTTGTAGCGTTTAGTCTTTTC 2800
ValThrAlaCysAspGlnIleValIleLeuTrpLeuGlyAlaGlyLeuValIleAspAsnGlnMetThrIleGlyMetPheValAlaPheSerSerPhe
GTGGGAGTTTTTCGAAAGAGTTGCTCTCTGACCACTTTCTCTCAGCTAAGAATAATGAGTCTGCACAAATGAGCGCATTGCAGATATGCATTACA 2900
rgGlyGlnPheSerGluArgValAlaSerLeuThrSerPheLeuLeuGlnLeuArgIleMetSerLeuHisAsnGluArgIleAlaAspIleAlaLeuHi
TGAAAGGAGGAAAGAAACCTGAAATGAAATCGTTGCTGATATGGGCCAATATCCCTGGAACCAATGGTTTAAGCTATCGTTATGACAGTCACTCA 3000
sGluLysGluGluLysLysProGluIleGluIleValAlaAspMetGlyProIleSerLeuGluThrAsnGlyLeuSerTyrArgTyrAspSerGlnSer
GCACCGATTTTCACTGCTCTGAGTTTATCTGAGTCCGGGGGAAAGTGGCTATAACTGGTCTTCCGGTCCGGGAAAAACACATTATGAAGATAC 3100
AlaProIlePheSerAlaLeuSerLeuSerValAlaProGlyGluSerValAlaIleThrGlyAlaSerGlyAlaGlyLysThrThrLeuMetLysValL
TATCTGGACTTTTGAACCTGATACGGGAGGGTACTGATAATGTTATAGATATACGCCAAATTTGAATAAATAATTATCACCAGGTGATAGCTGTGT 3200
euCysGlyLeuPheGluProAspSerGlyArgValLeuIleAsnGlyIleAspIleArgGlnIleGlyIleAsnAsnTyrHisArgMetIleAlaCysVa
TATGACAGGATGACCGGCTATTTTACGGCTCAATTCGTGAAATATCTGTGTTTTGCAGAGGAAATGGATGAAGAGTGGATGGTGAATGTGCCAGAGCA 3300
lMetGlnAspAspArgLeuPheSerGlySerIleArgGluAsnIleCysGlyPheAlaGluGluMetAspGluGluTrpMetValGluCysAlaArgAla
AGTCATATTGATGATGTTATAATGAATAGCCAATGGGATATGAACATTAATAGGTGAACCTGGGGAAGGTCTTTCTGGCGGTCAAAAACAGCGTATAT 3400
SerHisGlnAspValIleMetAsnMetProMetGlyTyrGlnLeuIleGlyGluLeuGlyGluGlyLeuSerGlyGlyGlnLysGlnArgIleP
TTATTGACACGCTTATACCGGAAACAGGAATATTTATTTATGGATGAGGCAACAGTGTCTTGTATTACAGAGTGAACATTTCGTGAATGTTGCCAT 3500
heIleAlaArgAlaLeuTyrArgLysProGlyIleLeuPheMetAspGluAlaThrSerAlaLeuAspSerGluSerGluHisPheValAsnValAlaI
AAAAACATGAATATCACCAGGGTAATTTATGCACACAGAGAAACACGTTGAGAACTGTTGATAGAGTTATTTCTATTAAACCATAGAGGAATACAA 3600
eLysAsnMetAsnIleThrArgValIleIleAlaHisArgGluThrThrLeuArgThrValAspArgValIleSerIle***
CGCTATGAGGAATATTTCTCTGTTAATTTCTCGTATTGCTCAGATATCTGTTGAGGTGGAATGAAGATAGACAAATCCCAAGAAAGAAATATCA 3700

Fig. 2. The complete nucleotide sequence of the colicin V operons. The numbers on the right margins refer to the nucleotide position. **A.** The nucleotide sequence and predicted amino acid sequence of the *cvaA* *cvaB* operon. Potential start sites for CvaA, CvaA* and CvaB are marked in bold letters. Potential transmembrane domains in CvaB are boxed (Eisenberg, 1984). The highly conserved putative ATP binding sites in CvaB are underlined (Walker *et al.*, 1982). The *Bam*HI site within *cvaA* is shown. **B.** The nucleotide sequence and predicted amino acid sequence of the *cvi* *cvaC* operon. Potential start sites for *cvi* and *cvaC* are marked in bold letters. The *Bgl*III site used in constructing some of the *cvaC*–*phoA* fusions is shown.

of chimeric proteins is active only when translocated across the inner membrane and out of the cytoplasm (Boyd *et al.*, 1987a). Thus alkaline phosphatase activity can be used as a measurement of export. Using the chromogenic indicator X-P on solid growth medium, we determined that all of the CvaC–PhoA fusion proteins yield active proteins when in the presence of CvaA and CvaB. These results suggest that 29 N-terminal residues of colicin V are sufficient to promote translocation of alkaline phosphatase. When either CvaA or CvaB was absent or mutated, the hybrid-containing cells displayed decreased levels of alkaline phosphatase activity. Thus, the translocation of the PhoA moiety in the hybrid proteins is dependent on the presence of CvaA and CvaB.

To quantitate these observations, colorimetric assays of alkaline phosphatase activity were performed on strains harboring the *cvaC*–*phoA* fusions in the presence or absence of *cvaA* and *cvaB*. The results, shown in Table I, indicate that while the 29 N-terminal amino acids are sufficient for recognition by CvaB/CvaA, the increase in alkaline phosphatase activity is only 2-fold. In contrast, the fusion at amino acid number 39 shows a 32-fold increase in enzymatic activity in the presence of CvaB and CvaA. Therefore, amino acids 30–38 contain information affecting the efficiency of recognition and/or export of the protein. All of the fusions containing 39 or more residues show a significant increase (from 7- to 32-fold) in alkaline phosphatase activity when in the presence of CvaA and CvaB. Both CvaA and CvaB are required for translocation of the chimeric proteins.

Using anti-PhoA antibodies, we examined the mobility of the chimeric proteins with SDS–PAGE using Western analysis (Figure 9). In cells lacking CvaA and CvaB, a larger chimeric protein was observed. One possible reason for this mobility difference could be the removal of some residues from the N-terminus of these hybrid proteins. This suggests that CvaA and CvaB, in addition to playing a role in transport, could be involved in processing the chimeric proteins. The observed decrease in the amount of the chimeric proteins when in the absence of CvaA and CvaB is likely the result of degradation due to improper localization. Similar results were obtained with strains lacking only CvaA or CvaB (results not shown).

Fig. 3. Hydrophobicity plot of the predicted CvaC product (Kyte and Doolittle, 1982). The arrows indicate the location of residues after which PhoA fusions were generated. The light arrow marks the position of the CvaC29–PhoA fusion which was only poorly exported in the presence of CvaA/CvaB. The positions of two glycine residues, which when mutated led to a defect in colicin V export, are also shown.

Fig. 4. Hydrophobicity plot of the predicted CvaB product.

In contrast to the extracellular secretion of colicin V, the CvaC–PhoA hybrid proteins do not result in alkaline phosphatase activity in the extracellular medium. This indicates that, while the colicin exporter can translocate the

CvaB	LSLSVAPGESVAITGASGAGKTTLMKVLGCLFE...PDSGRVLINGIDIRQIGINNYHRMIACVMQDDRLFGSIRENICGFA...EEMDEEW....MVE
HlyB	IN--IKQ--VIG-V-R--S--S--T-LIQRFYI...EN-Q--D-H-LALADP-WLR-QVG-V-L--NV-LNR--ID--SLAN...PG-SV-K...VIY
NdvA	V-FKAKA-QTI--V-PT-----VNL-QRVH...KH-QI--D-V--ATVTRKSLR-S--T-F--AG-MNR--G--RLGR...DASLDE....VMA
Mdr-hn	-N-K-QS-QT--LV-N--C--S-TVQLMQR-YD...TE-M-SVD-Q--T-NVRFLREI-GV-S-EPV--ATT-A--RYGR...NVMTde....IEK
Mdr-hc	--E-KK-QTL-LV-S--C--S-VVQL-ERFYD...LA-K--LD-KE-KRLNVQWLAHLGI-S-EPI--DC--A--AYGD...NSRVVSQee...I-R
Mdr-mn	-N-K-KS-QT--LV-N--C--S-TVQLMQR-YD...LE-V-S-D-Q--T-NVRYLREI-GV-S-EPV--ATT-A--RYGR...DVTMde....IEK
Mdr-mc	--E-KK-QTL-LV-S--C--S-VVQL-ERFYD...MA-S-FLD-KE-K-LNVQWLAHLGI-S-EPI--DC--A--AYGD...NSRAVShee...I-R
CFTR-n	INFKIER-QLL-VA-ST-----S-LMMIM-EL...SE-KIK.HSGR.....SFCs-FSWIMP-T-K--IFGV...YDEYR....YRS
CFTR-c	I-F-IS--QR-GLL-RT-S--S--LSAFLR-LN...TEGEIQI.D-VSWDS-TLQQWRKAFGVIP-KVFI--TF-K-LDPYE...QWS-Q....IWK
STE6-n	V--NFSA-QFTF-V-K--S--S--SNL-LRFYD...GYN-SIS--HN-QT-DQKLLIEN-TV-E-RCT--NDTL-K--LLGS...TDSVRNadc[*]IKD
STE6-c	MMDFMFC-QTLG-I-E--T--S--VLL-TK-YN...CEV-KIK-D-T-VNDWNLTLRKE-SV-E-KPL--N-T--D-LTYGL...QDEIL....IEM
PFMDR-n	--FTLKE-KTY-FV-E--C--S-IL-LIER-YD...TE-DIIV-DSHNLKDINLKW-SKIG-VSQ-P-LFSNSIK-NIKYS[**]QTIKSDS...V-D
PFMDR-c	--FTCDSSKTT--V-ET--S--S-F-NL-LRFYD[+]NNN-EI-LDD-N-CDYNLRDLRLNLSI-S-EPM--NM--Y--KFGR...DATL-D....VKR

CvaB	CARASHIDVIMNPMGYETLIGELGELSGGQKQRIFIARALYRKPGILFMDEATSALDSESEHFVNVAIKN...MNITRVIIAHRETTLRVDRVISI
HlyB	A-KLAGA--F-SELRE--N-IV--Q-A-----R--A-----VNN-K--IF-----Y--VIMRNMHKic..KGR-VI-----LS-VKNA--I-VM
NdvA	A-E-AAAS-F-EDRLN--D-VV--R-NR-----ER--VA-----ILKNAP--VL-----V-T-AR-KD--DALr..KDR-TF-----LS-V-EA-L--FM
Mdr-hn	AVKEANAY-F--KL-HKFD--V--R-AQ-----A-----V-N-K--LL-----T--AV-Q--LDKar..KGR-TIV-----LS-V-NA-VIAGF
Mdr-hc	A-KEAN--AF-ESL-NK-S-KV-DK-TQ-----A-----V-Q-H--LL-----T--KV-QE-LDKar..EGR-CIV-----LS-IQNA-LIVVF
Mdr-mn	AVKEANAY-F--KL-HQFD--V--R-AQ-----A-----V-N-K--LL-----T--AV-QA-LDKar..EGR-TIV-----LS-V-NA-VIAGP
Mdr-mc	A-KEAN--QF-DSL-DK-N-RV-DK-TQ-----A-----V-Q-H--LL-----T--KV-QE-LDKar..EGR-CIV-----LS-IQNA-LIVV
CFTR-n	VIK-CQLEED-SKFAEKDNIVL--G-IT-----RA--SL--V-KDADLYLL-SPFGY--VLT-KEIFESCVCk1..-ANKTR-LVTSKMEHLKKADK-L
CFTR-c	V-DEVGLRS--EQF-GKLDVFLVDG-CV--H-H--LMCL--SVLS-AK--LL--PSAH--PVTYQIIRRTL-Qaf..ADC-VILCE--IEAMLEQQFLV
STE6-n	ACQMALLDLF-LDL-D-L--TG-VT-----Q-VA-----FI-DTP--L--V--IVHRNLLMK--RHwr..KKG-TI-LT-ELSQIESD-YLYLM
STE6-c	YDALKYVGHDFVSSPQGLDTRIDTTL-----A-LC-----L-SK--IL--C-----V-SSII-EIV-Kgp..PALLTMV-T-S-QMM-SCNSIAVL
PFMDR-n	VSKVL--FVSSL-DK-D--V-SNASK-----S--IM-N-K--IL-----S-NK--YL-QKT-N-lkgnE-RITY-----LS-I-YANTIFVL
PFMDR-c	VSKFAA-DEF-ESL-NK-D-NV-PY-KS-----A-----L-E-K--LL-----S--N--KLIEKT-VDikdKADK-IIT--IASIKRS-KIVVF

Fig. 5. Alignment of the amino acid sequence of the ATP binding domain of the MDR-like subfamily of export proteins. Dashes represent amino acids identical to those present in CvaB. Dots represent gaps in the sequence, except when marked with brackets; in this case, the number within the brackets represents the number of residues in that molecule which do not align. Designations: CvaB, colicin V export, this paper; HlyB, hemolysin export (Felmlee *et al.*, 1985b); NdvA, polysaccharide export in *Rhizobium* (Stanfield *et al.*, 1988); MDR, multiple drug resistance phenotype, h, human (Chen *et al.*, 1986); m, mouse (Gros *et al.*, 1986); n, N-terminal; c, C-terminal; CFTR, cystic fibrosis transport regulator (Riordan *et al.*, 1989); STE6, yeast a-factor secretion (Kuchler *et al.*, 1989); PFMDR, drug resistance phenotype in *P.falciparum* (Foote *et al.*, 1989).

PhoA moiety from the cytoplasm, it cannot completely release the hybrids from the cell. To distinguish whether the chimeric proteins are free in the periplasm or are associated with the inner or outer membranes with the PhoA moiety extending into the periplasm or growth medium, cells containing the fusion proteins and the transport components were fractionated (see Materials and methods). The fusion proteins were localized by both alkaline phosphatase activity and with anti-PhoA antibodies. The results indicate that the hybrid proteins are associated with the inner membrane. Given that the alkaline phosphatase is active, this moiety is most likely facing the periplasm.

Mutations in CvaC that affect colicin V export

The results from the CvaC-PhoA fusions indicate that the first 39 codons of *cvaC* contain sufficient structural information to translocate CvaC-PhoA fusions efficiently across the cytoplasmic membrane. To determine which structural features of the CvaC sequence are recognized by the CvaA/CvaB exporter, we isolated mutations in *cvaC* that result in an inability to export the toxin without the loss of colicin V activity. We screened for mutations which resulted in intracellular colicin V activity. A plasmid containing the *cvl cvaC* operon was mutagenized *in vitro* with hydroxylamine (Hashimoto and Sekiguchi, 1976); this DNA was used to transform a strain harboring a compatible plasmid containing the *cvaA cvaB* operon. Transformants were screened for their inability to produce extracellular colicin V. Of 1000 transformants screened, 36 proved to be phenotypically ColV⁻. These isolates were then analyzed further by assaying cell lysates for colicin V activity. Eight isolates yielded colicin V activity upon lysis. Plasmids from these strains were isolated and the *cvaC* genes sequenced. Three different alleles were isolated, one of which was obtained three times. The changes were Gly14 to Asn, Gly38 to Arg and Gly14 to Asp. The first two mutations were

Fig. 6. Analysis of the protein products of the *cvaA* gene. Autoradiogram of an SDS-PAGE gel of minicells harboring the following plasmids: lane 1, pHK11; lane 2, pHK11-8 (*cvaA::Tn5*); lane 3, pLY17. The arrows on the left indicate CvaA and CvaA*, which had been identified previously (Gilson *et al.*, 1987). Both bands disappear in lane 2, while CvaA* remains in lane 3. The additional bands appearing in lane 2 are products of genes present in Tn5.

slightly leaky such that a small amount of colicin V was secreted. In contrast, the Gly14 to Asp change yielded a complete export defect. These results are consistent with the fusion results, suggesting in both cases that export determinants of *cvaC* are located in the first 29 codons of the gene, and that some important export signals are located between residues 29 and 39.

Discussion

We have shown here that extracellular export of the antibacterial toxin colicin V is mediated by a signal sequence independent pathway. This export system involves the products of two accessory genes, *cvaA* and *cvaB*. CvaA and CvaB, as well as the colicin V substrate, are plasmid encoded and comprise a dedicated export system which has several features in common with other transport systems. The most

Fig. 7. Hydrophobicity plot of the predicted CvaA product. The arrow indicates the location of the internal start site(s) which give rise to CvaA*.

CvaA	IQRAEEGKIMKNNMEMYRYQSKGLINKDQLTNQVALYQQQNNLLSLSGQNEQNALQITTTLESQ
HlyD	INRYENVSRVRSRLDDFRSLHLKQIAKHAHVLEQENKYVEAANELRVYKSQLEQIESEILSAKEE
CvaA	IQTQAADFNRQY...MELQRLQK.EL.VNTDVEGEIIIRALSDGKVDLSV.TVGQMVNTGD
HlyD	YQLVTQLFKNEILDKLRQTDSIELLTLEKNEERQASVIRAPVSGKVQQLKVHTEGGVVTAE
CvaA	SLQVPIPENIENYLLILWVPNDVFIYSAGDKVNIRYEAFFSEKFGQFSATVKTISRTPASTQEM.L
HlyD	TLMVIVPED.DTLEVLTALVQNKDIGRINVGNQAIKVEAFPYTRYGYLVGKVRNINLDAIEDQKLGL

Fig. 8. Alignment of the central region of the predicted sequences of CvaA (amino acids 147–338) and HlyD (amino acids 223–420) (Felmlee *et al.*, 1985b). Dots in the sequence represent gaps. Vertical bars represent identities while stars represent conservative substitutions.

striking of these features is the finding that the CvaB protein is a member of the growing MDR-like subfamily of proteins. Surprisingly, the molecules which are believed to be transported by MDR-like proteins are extremely diverse. They include lipophilic drugs (Chen *et al.*, 1986), chloride ions (Riordan *et al.*, 1989), polysaccharides (Stanfield *et al.*, 1988; Cangelosi *et al.*, 1989), peptides (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989) and proteins (Felmlee *et al.*, 1985b; Letoffe *et al.*, 1990). It is possible that the conserved structural features present in the MDR-like molecules simply reflect special energetic requirements for extracellular export of any molecule. Alternatively, some of the molecules mentioned above may not be the physiological substrates for translocation. It is clear that lipophilic drugs are not natural substrates for the MDR proteins, nor has NdvA or ChvA been demonstrated to mediate polysaccharide transport directly. It is possible that most of the MDR-like exporters indeed translocate protein or peptides. Why cannot these proteins utilize the signal sequence dependent pathway? It is known that some cytoplasmic proteins contain sequences which poison signal sequence dependent export (Lee *et al.*, 1989). If such sequences are present in a molecule destined to be exported, this molecule may require a different export system. In addition, as was proposed by Kuchler *et al.* (1989), and demonstrated by the peroxisomal MDR-like protein, PMP70 (Kamijo *et al.*, 1990), localization to specific compartments, such as the extracellular medium in Gram-negative bacteria or the vacuole in yeast, may be facilitated by dedicated systems.

The MDR-like proteins share the greatest similarity in the 200 amino acids surrounding an ATP binding domain. CvaB and MDR are 44% identical at the amino acid level within this region. Outside this region all members of the subfamily

Table I. Alkaline phosphatase assays of CvaC–PhoA fusions

Fusion protein	<i>cvaA</i> <i>cvaB</i> genotype ^a	AP units ^b	Fold-increase ^c
Tn <i>phoA</i> fusions			
CvaC57–PhoA	A ⁺ B ⁺	2206	38 ×
	A [−] B [−]	58	
	A ⁺ B [−]	66	
	A [−] B ⁺	148	
CvaC68–PhoA	A ⁺ B ⁺	373	7 ×
	A [−] B [−]	52	
	A ⁺ B [−]	54	
	A [−] B ⁺	76	
CvaC81–PhoA	A ⁺ B ⁺	336	8 ×
	A [−] B [−]	44	
	A ⁺ B [−]	63	
	A [−] B ⁺	94	
pBoneIB fusions			
CvaC29–PhoA	A ⁺ B ⁺	65	2 ×
	A [−] B [−]	30	
	A ⁺ B [−]	26	
	A [−] B ⁺	28	
CvaC39–PhoA	A ⁺ B ⁺	450	32 ×
	A [−] B [−]	14	
	A ⁺ B [−]	19	
	A [−] B ⁺	54	
CvaC97–PhoA	A ⁺ B ⁺	105	8 ×
	A [−] B [−]	14	
	A ⁺ B [−]	16	
	A [−] B ⁺	17	

^aThe complementing plasmids used for the TnpH fusions were: *cvaA*⁺ *cvaB*⁺, pLY11; *cvaA*[−] *cvaB*[−], pBR322; *cvaA*[−] *cvaB*⁺, pLY17; *cvaA*⁺ *cvaB*[−], pLY16. The complementing plasmids used for the pBoneIB fusions were: *cvaA*⁺ *cvaB*⁺, pHK22-6; *cvaA*[−] *cvaB*[−], pLY26; *cvaA*[−] *cvaB*⁺, pHK22-8; *cvaA*⁺ *cvaB*[−], pHK22-4.

^bAverage of two independent assays, each done in duplicate.

^cDetermined by dividing the A⁺B⁺ by the A[−]B[−] value.

Fig. 9. Western analysis of CvaC–PhoA fusions. Total cell extracts were analyzed by SDS–PAGE. Electrophoresis and blotting were performed as described in Materials and Methods. Lanes 1, CvaC81–PhoA CvaA[−]CvaB[−]; 2, CvaC81–PhoA CvaA⁺CvaB⁺; 3, CvaC57–PhoA CvaA[−]CvaB[−]; 4, CvaC57–PhoA CvaA⁺CvaB⁺. The positions of migration of mol. wt standards, along with their size in kd, are shown in the left margin.

share a general monomer size and hydrophathy profile which suggests that each is an integral membrane protein. For each member it is possible to predict six potential transmembrane helices. However, these predictions should still be considered tentative. There is still no direct evidence about the membrane topology of these proteins. Current algorithms for detection of membrane spanning segments are based on

a small number of transmembrane proteins whose three-dimensional structures have been determined. These algorithms are not applicable for all proteins. Only α -helices are currently considered, overlooking β -sheets that might span the membrane. Even among the α -helices, it can be difficult to distinguish membrane spanning regions from globular hydrophobic regions (Eisenberg, 1984; Jennings, 1989). These algorithms will be refined when more experimental evidence on proteins, showing exactly which segments are within the membrane, has accumulated. Meanwhile a powerful genetic approach to membrane protein topology has been the generation of protein fusions (Manoil and Beckwith, 1986; Boyd *et al.*, 1987b). This approach is currently in use in our laboratory to study the membrane topology of CvaB.

The region of CvaC that is recognized by its transport system was identified by making chimeric proteins with alkaline phosphatase. Fusions containing at least 29 N-terminal residues of CvaC displayed appreciable alkaline phosphatase activity, but only when synthesized in the presence of CvaA and CvaB. This result was particularly surprising given the well documented observations that the secretion signal for hemolysin is located in the C-terminal region of the molecule (Mackman *et al.*, 1987; Koronakis *et al.*, 1989). When the N-terminal region of hemolysin was fused to alkaline phosphatase some phosphatase activity was detected. However, the export was shown to be extremely inefficient and independent of the hemolysin export genes (Erb *et al.*, 1987). The finding that different MDR-like exporters recognize different export signals, located in different regions of the exported protein, showing no apparent structural homology, further indicates the functional diversity found in this group of proteins.

Alkaline phosphatase has been fused to a number of different proteins that are released independent of a signal sequence (Pugsley and Cole, 1986). To our knowledge the results presented here represent the first case in which the alkaline phosphatase has been successfully translocated by an otherwise dedicated export system. The results demonstrate that the N-terminal 29 amino acids of CvaC are sufficient to be recognized by CvaA and CvaB since the CvaC29-PhoA fusion gives a 2-fold increase in alkaline phosphatase activity in the presence of the *cvaA* and *cvaB* genes. However, it appears that the residues between amino acid numbers 29 and 39 are important for efficient transport. These findings concur with the Gly38 to Arg38 and Gly14 to Asp14 mutations; the first greatly decreases the extracellular export of colicin V and the second abolishes detectable export.

The electrophoretic mobility of the fusion proteins is increased when CvaA and CvaB are present. This suggests that a processing step is associated with the translocation of colicin V. The relative change in mobility would correlate with the removal of 12–20 residues from the N-terminal of the CvaC moiety. This again contrasts with the export of hemolysin, in which the C-terminal export signal is preserved in the final product (Gray *et al.*, 1986).

The active chimeric CvaC-PhoA proteins are localized to the periplasm with an inner membrane association. Since the chimeras are transported to the periplasm, we conclude that initial recognition occurs. The inability of CvaA and CvaB to export the chimeric proteins into the medium may be due to the relatively large size of the PhoA moiety. It

is also possible that translocation across the outer membrane may need other signals or properties present in CvaC. The inner membrane association of the periplasmic hybrid may be due to the N-terminal hydrophobic region of CvaC guiding the chimeric protein to the membrane, which is considered the target of colicin V.

The role of CvaB, like that of other members of the group, may be to provide the specific pore for colicin V translocation, as well as the energy for the process from ATP hydrolysis. CvaA, and similarly HlyD, may either form an integral part of the pore or maintain the substrates in an export-competent conformation and deliver them to the CvaB pore. Several proteins, collectively referred to as chaperonins, have been shown to maintain the conformation of proteins to be exported via the signal sequence dependent pathway (Ellis, 1987). The predominantly low hydropathy profiles of HlyD and CvaA are consistent with their possible roles as chaperonins. One significant difference between CvaA and HlyD is that CvaA has an in-frame internal start site that produces a second smaller protein, CvaA*, lacking the hydrophobic N-terminus predicted for CvaA. By creating a mutation early in *cvaA* that does not affect the production of CvaA*, we have shown that CvaA is necessary for extracellular secretion of colicin V. If CvaA has a chaperonin function, it may be required both in the cytoplasm and periplasm. It is possible that CvaA is periplasmic while CvaA* is cytoplasmic. Hemolysin has been shown not to have a periplasmic phase in its transport pathway (Gray *et al.*, 1986); accordingly, HlyD does not have a very hydrophobic region or signal peptide, nor does it have an obvious internal start site for production of a secondary HlyD protein. Despite this divergence in the evolution of the colicin V and hemolysin transport pathways, we feel the conservation of their function and amino acid sequences suggest common features important in signal sequence independent export systems. Future experiments that focus on these similarities will be critical to understanding non-signal sequence export systems. These similarities might also be helpful in identifying second components in other systems. For example, Foote *et al.* (1990) recently proposed that chloroquine resistance in *P.falciparum* might be a multigenic phenotype, dependent not only on an amplification of a mutant allele of *pfmdr1* but also on a mutation in a second, as yet unidentified gene. This gene might be similar in sequence and function to *cvaA*.

Recently, a third component, TolC, was found by Wandersman and Delepelaire (1990) to be necessary for hemolysin secretion. We found this same *E.coli* outer membrane protein to be necessary for colicin V extracellular export. An analogous component was found to be linked to genes for production of *Erwinia chrysanthemi* protease B and *Bordetella pertussis* cyclolysin (Wandersman and Delepelaire, 1990). This third component may be specialized for signal sequence independent, extracellular secretion in Gram-negative bacteria.

Materials and methods

Media and antibiotics

TB (10 g tryptone/8 g NaCl/l) was used as both liquid and solid growth medium for all experiments except for the mini-cell and λ Tn ϕ A transduction experiments in which M63-glucose and LB were used (Miller, 1972). Except where noted, antibiotics were used at the following final concentrations (μ g/ml): ampicillin, 150; chloramphenicol, 20; kanamycin, 50. Growth

media of ColV-PhoA alkaline phosphatase assays and localizations also contained 0.1 mM 2,2'-dipyridyl (Sigma). This iron chelator gives increased expression of both colicin V operons (Chehade and Braun, 1988; L. Gilson and R. Kolter, unpublished data).

Plasmids and bacterial strains

pHK11 and pHK22 were described in earlier work (Gilson *et al.*, 1987). They were derived from pBR322 and pACYC184, respectively and contain a *HindIII*-*SaII* fragment with both colicin V operons and 2–3 kb upstream of each promoter. pHK11-1 is *cvaC*⁺ via a Tn5 insertion in pHK11. Tn5 insertions in pHK22 generated *cvaA*⁺, *cvaB*⁺ and *cvaC*⁺ mutants designated pHK22-8, pHK22-4, and pHK22-6, respectively (Gilson *et al.*, 1987). pLY11 is a derivative of pHK11-1 with a *SaII* deletion from a site within Tn5 to a site in the vector. This *SaII* deletion removes *cvi* and most of *cvaC*. pLY16 is a similar *SaII* deletion in pHK11-6 which removes *cvi*, *cvaC* and half of *cvaB*. pLY26, a *HindIII* deletion between sites in the Tn5 of pHK22-6 and the vector, lacks *cvaA*, *cvaB* and half of *cvaC*. pLY17 is a derivative of pHK11 and contains a filled-in *BamHI* site within *cvaA*. pLY21 is a derivative of pHK22 with an *EcoRI* deletion that removes *cvaA* and most of *cvaB*. pAX629, provided by C. Wandersman, is a pACYC184 derivative carrying *tolC* (Hiraga *et al.*, 1989). pColV-K30::Tn10 was supplied by V. de Lorenzo. pBoneI is a derivative of pBoneI (generously supplied by M. Ehrmann and J. Beckwith). pBoneI is a pUC19 derived vector containing a polylinker immediately upstream from the *phoA* reading frame; it was derived from pPHO7 (Gutierrez and Devedjian, 1989). We inserted five base pairs by filling in a *BstEII* site to create in pBoneI a termination codon for the protein fusions because in pBoneI the *phoA* gene is fused in-frame to *lacZ* α . The final *phoA* gene in this construct contains 18 codons beyond the normal termination of *phoA*. KS300, supplied by K. Strauch and J. Beckwith, is MC1000 *recA1* Δ *phoA*-*pvull*. Strain GC7442 is F⁺ *his* *trpE* *tolC*::Tn5 and pop4160 is MC4100 *tolC*::Tn10; both were provided by C. Wandersman. W3110 Δ *lacU169**ma2ompR101*, was used as the *OmpF*⁺ strain.

Manipulation and sequencing of DNA

Plasmid DNA preparations, restriction enzyme digestions and other routine DNA manipulations were performed using standard procedures (Ausubel *et al.*, 1989). DNA was sequenced by the chain termination method (Sanger *et al.*, 1977). The colicin V operons were sequenced from M13 subclones and nested deletions that were generated using the Cyclone System from IBI (Dale *et al.*, 1985). Parts of the operons and all of the *cvaC*-*phoA* fusions were sequenced with double stranded techniques adapted from Bartlett *et al.* (1986) and using Sequenase reagents from USB.

Minicells

Strain P678-54T⁺ (Adler *et al.*, 1967) was transformed with pHK11, pHK11-6 and pLY17. Minicells were grown in M63-glucose and isolated as described (Meagher *et al.*, 1977). The minicells were labeled for 10 min with [³⁵S]Met and labeled proteins were separated using 12.5% SDS-PAGE (Laemmli, 1970) at 130 V.

Generation of ColV-PhoA protein fusions and assaying of alkaline phosphatase activity

CvaC-PhoA fusions at CvaC amino acids 57, 68 and 81 were generated using λ TnphoA as described (Boyd *et al.*, 1987b). In 10 independent transductions, $\sim 10^8$ p.f.u.s were used to infect 10⁹ KS300/pLY21 cells growing in LB broth containing 0.2% maltose. After 20 min at 23°C, 50-fold excess of fresh LB was added and the cells were incubated at 37°C for 150 min. At this time chloramphenicol (80 μ g/ml) and kanamycin (100 μ g/ml) were added and the cells were incubated overnight at 37°C. Plasmid DNA was isolated and used to transform KS300/pLY11. Transformants were then plated on TB plates containing Cm, Amp, Kan and the chromogenic indicator of alkaline phosphatase activity, 5-bromo-4-chloro-3-indolyl phosphate (X-P). Clones which were blue and deficient in colicin V secretion were analyzed. CvaC-PhoA fusions at CvaC amino acids 29, 39 and 97 were made *in vitro* by inserting *BglIII*-*HpaII*, *BglIII*-*PvuII* and *BglIII*-*DdeI* fragments of the colicin V operon into the polylinker of pBoneI. To assay alkaline phosphatase activity, cells were grown in TB with Cm, Amp and Kan to stationary phase, then diluted 10-fold and grown in TB with antibiotics and dipyridyl at 37°C for 3 h. Assays measured the rate of *p*-nitrophenyl phosphate hydrolysis by permeabilized cells as described (Michaelis *et al.*, 1983). Absolute units of activity show variation between experiments. We believe this is due to sensitive iron regulation of the *cvaAB* operon. The examples used were the results of two independent sets of assays showing full induction.

Cell fractionation and Western blots

Initial testing of the periplasmic fractions was performed by resuspending cells grown to late log in TB with antibiotics in 1/10th vol cold 18% sucrose/0.1 M Tris pH 8.0 then adding EDTA pH 8.0 to 1 mM and lysozyme to 100 μ g/ml. After standing on ice for 15 min the spheroplasts were pelleted and the supernatant was tested as the periplasmic fraction. Membrane fractionations were carried out exactly as described (Osborn and Munson, 1974). Twenty-three fractions of 0.5 ml each were collected from 30–55% sucrose gradients. To determine the inner membrane fractions, NADH oxidase assays were done in a total volume of 1 ml using 0.1 ml of the fraction sample in 0.24 mM NADH, 10 mM Tris pH 7.5 and 0.2 mM DTT. The change in OD 340 at 22°C was measured over 2 min. For the Western blots, 0.2 ml of each fraction were pelleted at 55 000 r.p.m. in a Beckman Type 65 rotor for 2 h, resuspended in Laemmli loading buffer and half the sample loaded onto a 10% SDS-PAGE gel (Laemmli, 1970). The Western blot was later probed with *OmpA* and *PhoA* antibodies generously supplied by P.C. Tai and J. Beckwith, respectively. Western blots of 10% SDS-PAGE gels were performed according to the protocol of the Vectastain Kit from Vector Laboratories Inc.

Generation of hydroxylamine mutants and assays for colicin V production

Plasmid pLY21 was treated with 0.4 M hydroxylamine for 36 h at 37°C as described (Hashimoto and Sekiguchi, 1976). The treatment yielded a 90% drop in transformation frequency. The mutagenized DNA was used to transform KS300 pLY11, selecting on TB plates containing Amp and Cam. Transformants were individually assayed for extracellular colicin V production as described (Frick *et al.*, 1981). Those strains unable to make colicin V were then grown and lysed with lysozyme/EDTA/detergent (Clewell and Helinski, 1969). The lysates were tested for colicin V activity by spotting lysate supernatants on a lawn of sensitive cells and noting any inhibition of growth as described (Mayr-Harting *et al.*, 1972). Sequencing of the mutant templates was accomplished using Sequenase (Tabor and Richardson, 1987) and a synthetic oligonucleotide primer complementary to a region within *cvi*.

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