ISOLATION AND CHARACTERIZATION OF EXTRACELLULAR PROTEIN EXPORT GENES OF AEROMONAS HYDROPHILA

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ISOLATION AND CHARACTERIZATION OF EXTRACELLULAR PROTEIN EXPORT GENES OF *AEROMONAS HYDROPHILA*

BY

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A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements of the degree of Master of Science

Faculty of Medicine
Memorial University of Newfoundland

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ABSTRACT

Two transposon insertion mutants of *Aeromonas hydrophila* C5.84 and L1.97, which were defective in extracellular protein production, were characterized. Each of the mutations caused pleiotropic effects: the mutants were unable to secrete any of the major proteins normally extracellularly exported by the wild-type strain Ah65 but instead accumulated these proteins in the periplasm. This suggests that the inactivated genes in these two mutants were essential for the general extracellular protein export process. Besides the effects on protein secretion, the mutation in L1.97 also changed the outer membrane structure of the mutant and rendered the cell unusually fragile.

By using the transposon antibiotic resistance genes as selective makers, two wild-type chromosomal fragments (11 kb and 15 kb respectively) corresponding to the transposon insertion regions in L1.97 and C5.84 were isolated. When transferred back into the mutants, the cloned DNA complemented the defects in the corresponding mutants and restored the normal secretory phenotype to the transconjugants. The mutation in C5.84 was also partially complemented by the heterologous 11 kb fragment corresponding to the L1.97 mutation.

By further subcloning, deletion analysis, DNA sequencing and functional complementation studies, an extracellular export gene (*exeE*) located on the 11 kb fragment was identified and characterized. The *exeE* gene potentially encoded a 55 KD polypeptide. The predicted protein product contained a hydrophobic transmembrane segment and was thus classified as an integral membrane protein.

Interestingly, *exeE*, the gene essential for general extracellular protein export in *Aermonas hydrophila*, was found to be highly homologous to *pulE*, a gene involved in the pullulanase secretion in *Klebsiella pneumoniae*. Furthermore, the genes located both upstream and downstream of *exeE*, i.e. *exeD*, *exeF* and *exeG* also shared extensive similarities with *pulD*, *pulF* and *pulG*, suggesting that this cluster of *exe* genes has similar functions as the *pul* genes and are all required for extracellular protein export. These results provided strong evidence that the protein secretion machineries are conserved between two completely different gram-negative bacteria: *Aeromonas hydrophila* and *Klebsiella pneumoniae*.

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INTRODUCTION

1.1 The envelope structure of gram-negative bacteria

Unlike gram-positive bacteria, gram-negative bacteria are surrounded by a complex envelope structure consisting of two hydrophobic membranes separated by a periplasmic compartment that contains (among other things) the peptidoglycan cell wall (Lugtenberg and Van Alphen, 1983). The inner cytoplasmic membrane is a typical plasma bilayer containing phospholipids and an enormous variety of proteins concerned with energy metabolism, biogenesis of the cell surface layers and nutrient transport processes. The periplasm is an aqueous compartment containing a peptidoglycan layer and different enzymes and transport binding proteins. The peptidoglycan molecular network confers shape and rigidity upon the bacterial cell, but is, however, not a barrier to protein translocation across the cell envelope. The outer membrane consists of an asymmetric bilayer of phospholipids: an outer layer containing the lipid A moiety of LPS (lipopolysaccharide), and an inner layer of normal phospholipids. The outer membrane displays a relatively non-fluid nature and the bilayer is quite impermeable to both large and small molecules, thus providing an extra protective

barrier shielding the gram-negative bacterial cells from external attack by enzymes, antibiotics and other harmful reagents.

1.2 Extracellular protein export by gram-negative bacteria

Although the outer membrane constitutes an additional hydrophobic barrier which has to be circumvented, many gram-negative bacteria, such as members of the families *Pseudomonaceae* and *Vibronaceae*, can actively secrete proteins to the extracellular medium (Hirst and Welch, 1988). These secreted proteins display a wide range of functions: including toxins produced by pathogenic bacteria that play key roles in human, veterinary and plant diseases, as well as degradative enzymes that have potential commercial value. Despite the importance of extracellular proteins in bacterial pathogenicity and biopolymer degradation, the process of extracellular protein export by gram-negative bacteria has not received much attention compared to the extensive investigations on protein translocation across the inner membrane of *Escherichia coli*. Therefore, unlike the fairly well characterized *sec* mechinery involved in protein export through the inner membrane, very little is known about the mechanisms underlying extracellular protein secretion across the outer membrane.

According to the limited evidence available and also

based on characteristics of the secreted proteins, the extracellular protein export mechanisms in gram-negative bacteria can be broadly divided into two main pathways, i.e. the N-terminal signal-sequence independent pathway and the extended N-terminal signal-sequence dependent pathway.

1.3 The N-terminal signal-sequence independent secretion pathway

There has been accumulating evidence showing that an increasingly large number of extracellular proteins, which do not have classical N-terminal signal-sequences, are secreted from a variety of gram-negative bacteria by a signal-sequence independent pathway. This pathway is well represented by the extracellular secretion of haemolysin in E. coli (Holland et al., 1990a; 1990b; Blight and Holland, 1990).

1.3.1 Secretion of E. coli haemolysin

Haemolysin has been implicated as an important factor in the virulence of pathogenic E. coli strains (Welch et al., 1981). Several strains of E. coli associated with urinary tract infections in humans and diarrhoea in animals are known to be able to secrete haemolysin into the growth medium. Most haemolysin determinants of E. coli isolated from humans have been shown to reside on the chromosome, while the Hly

determinants expressed by strains isolated from animals, although very similar in structure, are almost always carried on large transmissible plasmids (Muller *et al.*, 1983).

1.3.1.1 Structure of the Hly determinant

By transposon mapping and complementation studies, Goebel and coworkers first demonstrated that the complete Hly determinant of the naturally occuring plasmid pHly152 was encoded on an approximately 8 kb DNA fragment which consisted of four genes; *hlyC*, *hlyA*, *hlyB* and *hlyD* (Noegel *et al.*, 1979). This general organization was later also found in other independently isolated Hly determinants (both chromosomally encoded and plasmid encoded) by several other groups (Felmlee *et al.*, 1985; Mackman and Holland, 1984; Welch *et al.*, 1983) (Figure 1.1).

hlyA is the structural gene for haemolysin. Its product has been identified as a 107 KD highly labile polypeptide. The upstream *hlyC* encodes a 20 KD polypeptide. Its protein product HlyC is located in the cytoplasm and is responsible for post-translational activation of the 107 KD HlyA molecule. HlyC plays no role in the HlyA secretion process. In contrast, the two downstream genes, *hlyB* and *hlyD*, are both absolutely required for the secretion of HlyA. Mutations in either *hlyB* or *hlyD* prevent HlyA extracellular secretion and lead to intracellular accumulation of

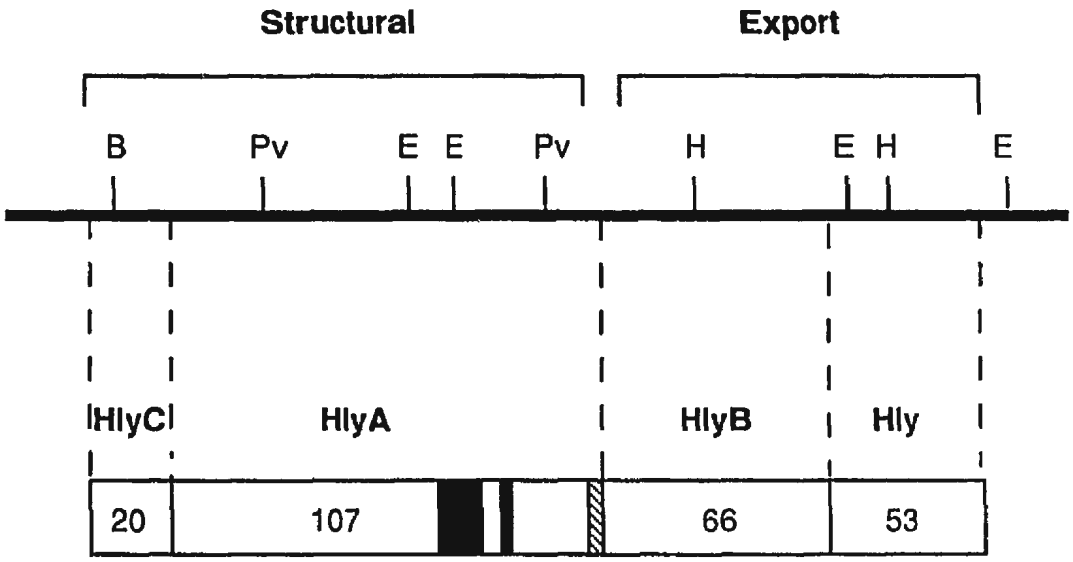


Figure 1.1. HlyA determinant structure. The figure shows the genes and the predicted gene products (not to scale). The numbers are the molecular weights of the gene products (KD). The two solid shading blocks in HlyA represent the 14 glycine-rich nonapeptide repeats. The hatched shading block indicates the position of the HlyA secretion signal. Restriction sites: B: *Bam*HI, E: *Eco*RI, H: *Hind*III, Pv: *Pvu*I. From Holland *et al*. 1990a.

haemolytic activity. Both HlyB and HlyD are located in the inner membrane (Mackman et al., 1985; Noegel et al., 1979; Nicaud et al., 1985).

1.3.1.2 HlyA structure and the identification of a C-terminal secretion signal

In order to analyze the mechanism of HlyA secretion in more detail, the primary structure of the HlyA molecule has been extensively studied to identify sequences or domains within HlyA that are essential for its secretion.

HlyA belongs to the so-called RTX group of toxins, characterized by the presence of several tandem repeats of the glycine-rich nonapeptide consensus GGBGBBXLX. These repeats in HlyA are absolutely required for the haemolytic activity but not essential for recognition and hence secretion by the translocation machinery, since a deletion derivative of HlyA, with 11 out of 14 of these repeats removed, is non-haemolytic but can still be secreted quite efficiently into the medium (Felmlee and Welch, 1988).

Structural analyses, such as those involving the deletion of up to 90% of HlyA (amino acids 1-911), the fusion of heterologous polypeptides to the HlyA C-terminal 218 or 114 amino acids and the deletion of the extreme C-terminal 27 amino acids of HlyA, have indicated the existence of a specific secretion or targeting signal located very close to

the C-terminus of the molecule (Gray et al., 1986; Koronnakis et al., 1989; Mackman et al., 1987). Closer examination of the C-terminal sequence suggested that some secondary structures present within the extreme C-terminal 53 amino acids, including an amphiphilic α-helix, a cluster of charged amino acids and a weakly hydrophobic terminal sequence rich in hydroxylated amino acids might form part of the targeting signal (Holland et al., 1990a; 1990b). From the construction of C-terminal deletion derivatives, sequences essential to secretion have been localized between amino acids 998 and 1016 (Gray et al., 1986; Koronakis et al., 1989). In addition, Koronakis et al. (1989) have shown that deletion of the charged amino acids between 988 and 998 completely blocks the secretion of HlyA, suggesting these amino acids may also play very important roles in the targeting process. However, by joining together normally separated protein segments, internal deletion studies might provide misleading results by recreating particular secondary structures required for targeting, or by causing overall structural variations within the deleted polypeptide that could completely change the targeting signal at the C-terminus of the HlyA molecule. Thus, results from the internal deletion experiments must be interpreted very cautiously. Indeed, initial studies with site-directed mutagenesis performed by Holland et al. (1990a) have showed that at least some individual charged amino acids

are dispensable. Obviously, more experimental evidence is needed to determine whether these charged amino acids are required and, if they are, which charged amino acids are essential for the targeting process.

In summary, it is now clear that amino acids 998-1016 of HlyA play a very important role in its extracellular secretion process. However, other flanking regions that may also be required for the targeting function have not been excluded and the precise structural features within amino acids 998-1016 which are involved in targeting have not yet been established.

1.3.1.3 Localization and membrane organization of HlyB and HlyD

Since both HlyB and HlyD are synthesized at extremely low levels in their natural host, fractionation and localization studies have been all carried out in mini-cells with HlyB or HlyD expressed from various multi-copy plasmids.

After carefully controlled mini-cell fractionation by Sarkosyl separation or equilibrium density gradient procedures, both HlyB and HlyD have been localized primarily to the cytoplasmic membrane (Mackman et al., 1985). However, significant amounts of both HlyB and HlyD were also frequently found in the outer membrane fractions.

Protease treatment of radiolabelled proteins in mini-

cells has shown that HlyB and HlyD were digested only after the outer membrane was made permeable by lysozyme-EDTA treatment, providing further evidence for their inner membrane location (Wang *et al.*, 1991).

The membrane organization of HlyB and HlyD has also been studied, using β-lactamase as a topology probe (Holland *et al.*, 1990b; Wang *et al.*, 1991). The results indicated that HlyD is a monotopic protein, with a short N-terminal domain present in the cytoplasm, whilst HlyB is a polytopic protein. The N-terminal half of the HlyB molecule appears to have at least 6 transmembrane segments and the C-terminal half of the molecule is localised to the cytoplasm.

The C-terminal domain of HlyB shares significant homology with many ATP-binding proteins and it contains two predicted ATP-binding sites, suggesting that this domain is very likely involved in some energy transducing process (Higgins *et al.*, 1986). Detailed genetic and functional studies of HlyB are still at an early stage, but some interesting mutants have been isolated. Two highly conserved residues, Gly^{605} and Gly^{608} in the ATP-binding site have been identified to be essential for HlyB function (Koronakis *et al.*, 1988). Preliminary site-directed mutagenesis of the periplasmic loop most proximal to the cytoplasmic ATP-binding domain and DNA replacement of the N-terminus of the HlyB molecule have also

been carried out, resulting in loss of function in the former case but no effect in the latter, suggesting that the periplasmic loop close to the ATP-binding region may also play a role in the translocation process (Blight and Holland, 1990).

1.3.1.4 Mechanism of HlyA translocation

HlyA is secreted into the extracellular medium at the late exponential phase of growth. As first reported by Felmlee *et al.* (1985), HlyA does not possess a classical N-terminal signal-sequence and the N-terminus is not subjected to any proteolytic processing during its secretion process. HlyA secretion is completely independent of the general *secA*, *secY* pathway. Instead, as indicated before, secretion of HlyA is uniquely dependent on HlyB and HlyD. It is proposed that newly synthesized HlyA molecules are secreted directly to the extracellular medium without a periplasmic intermediate, since no detectable level of HlyA (assayed by haemolytic activity determination, immunoblotting and pulse-chase labeling experiments) can be identified in the periplasmic fraction after careful cell fractionation procedures (Baker *et al.*, 1987; Felmlee and Welch, 1988; Gray *et al.*, 1986; 1989). The fact that active HlyA are accumulated in the cytoplasmic but not periplasmic fraction in cells lacking either the *hlyB* or *hlyD* gene also supported

this view.

All of the above evidence suggests that HlyA is secreted by a novel HlyB and HlyD dependent secretion mechanism. On the basis of the available information, a working model for HlyA secretion has been proposed by Holland and coworkers (Mackman *et al.*, 1986; Holland *et al.*, 1990a; 1990b) (Figure 1.2). This model proposes that HlyB and HlyD form a transenvelope structure spanning both the inner and outer membrane of the bacteria. This membrane-bound translocator can recognize the targeting signal located within the C-terminus of the HlyA molecule and then allow direct translocation of the HlyA polypeptide across both membranes to the extracellular medium.

This model does not exclude the possibility that other *E. coli* membrane proteins are also required for formation of the functional translocator, and indeed, a third membrane protein TolC has recently been indentified by Wandersman and Delepelaire (1990) to be essential for the HlyA secretion process. As a component of the translocator and also as a minor outer membrane protein, TolC's essential role may be to allow a specific interaction between the inner membrane and the outer membrane, since both HlyB and HlyD are mostly located in the inner membrane.

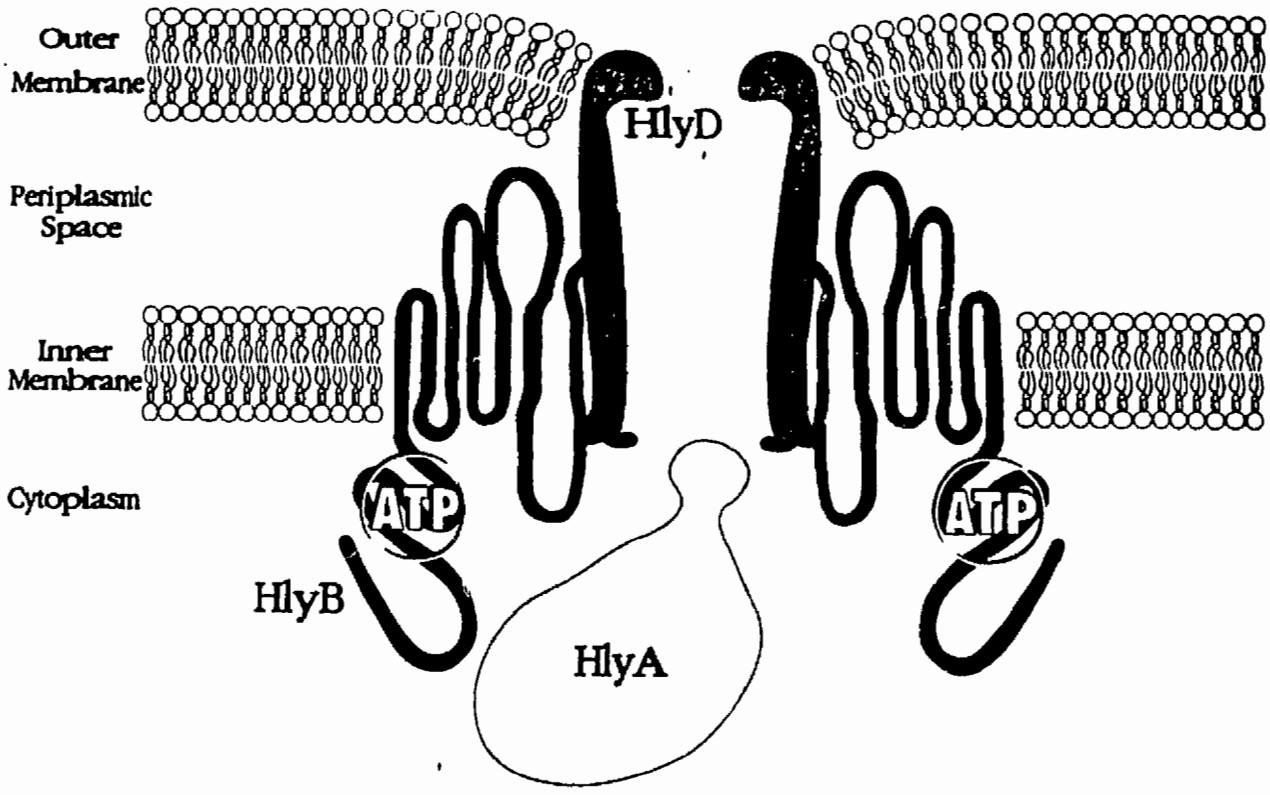


Figure 1.2. A secretion model for HlyA. See text for details. From Holland *et al.*, 1990b.

1.3.2 Secretion of other proteins lacking N-terminal signal-sequences

Extensive studies on different extracellular proteins secreted by a wide variety of gram-negative bacteria have revealed that there is an expanding family of proteins which are secreted by pathway similar to that used by the E. coli haemolysin. Besides HlyA, this family now consists of the haemolysin-like proteins from the genera Proteus, Morganella and Actinobacillus (summarized in Koronakis et al., 1987), the leukotoxin of Pasteurella haemolytica (Lo et al., 1987; Strathdee and Lo, 1989), the bifunctional adenyl cyclase-haemolysin (cyclolysin) of Bordetella pertussis (Glaser et al., 1988), the Erwinia chrysanthemi metalloproteases B and C (Delepelaire and Wandersman, 1989; Delepelaire and Wandersman, 1990; Letoffe et al., 1990), the Serratia marcescens metalloprotease (SM protease) (Letoffe et al., 1991), the Pseudomonas aeruginosa alkaline protease (Guzzo et al., 1991), the Rhizobium leguminosarum NodO protein (Economou et al., 1990) and colicin V and microcin B17 secreted by E. coli (Gilson et al., 1990; Garrido et al., 1988). Although very different in primary structure and function, these proteins do not contain the N-terminal signal-sequences and the components of their secretion machinery show high levels of amino acid sequence similarity. For example, as shown in Figure 1.3, the P. haemolytica

leukotoxin secretion genes lktB and lktD are very similar to the E. coli hlyB and hlyD, with 90.5% and 75.6% identity respectively. Nucleotide sequence analysis of the E. chrysanthemi metalloprotease secretion genes, prtD, prtE and prtF, revealed significant homology with the corresponding HlyA secretion genes hlyB, hlyD and tolC in E. coli (23% for all three genes). prtD and prtE are also homologous to lktB and lktD (28% and 22% respectively). The secretion genes for B. pertussis cyclolysin cyaB, cyaD and cyaE are, again, similar to hlyB, hlyD and prtF (50%, 32% and 20% respectively).

The conserved nature of the signal-sequence independent secretion pathway is demonstrated not only by sequence similarities but also cross-complementation, which has been shown to exist between a number of different secretion systems. By introducing the structural genes of heterogenous extracellular proteins into an E. coli strain expressing the E. chrysanthemi protease secretion genes prtD, prtE and prtF, it has been shown that the E. chrysanthemi protease secretion apparatus can carry out the secretion of at least two proteases produced by other organisms: the P. aeruginosa alkaline protease and the SM protease of S. marcescens. Similarly, the haemolysin secretion apparatus can promote the secretion of several other proteins including the leukotoxin of P. haemolytica, protease B of E. chrysanthemi and the

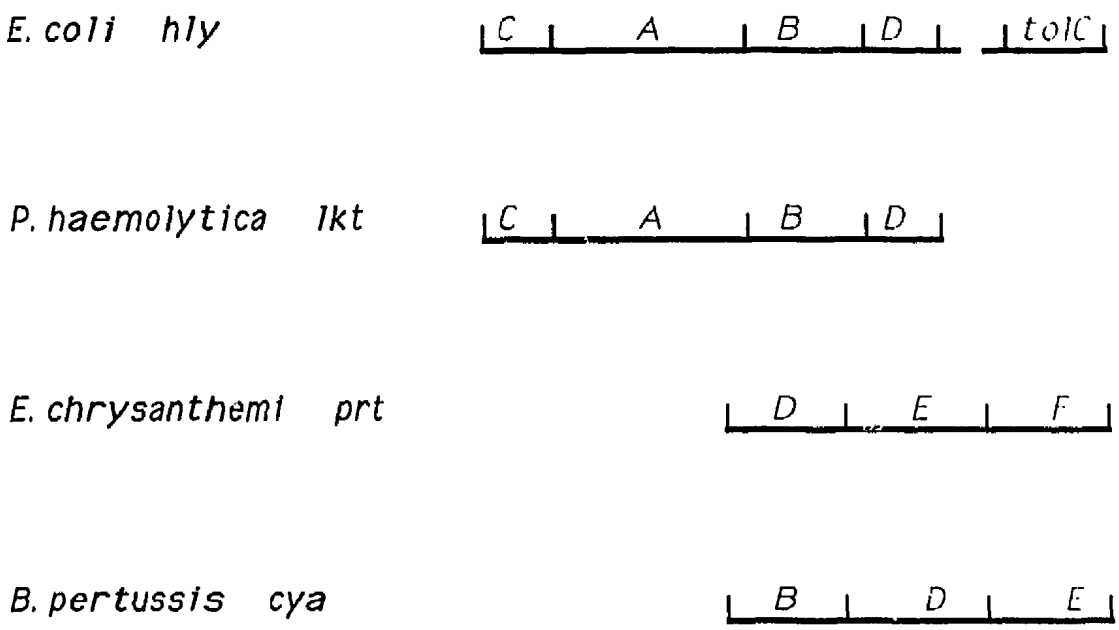


Figure 1.3. Comparison of N-terminal signal-sequence independent protein translocation machineries among different gram-negative bacteria. The secretion genes of four systems are shown (not to scale). See text for details.

alkaline protease of P. aeruginosa.

It appears that these signal-sequence independent secretion mechanisms are all similar, can complement each other more or less efficiently, and are conserved and widespread among many different gram-negative bacteria.

1.3.3 HlyB is a member of a new family of ATP-dependent membrane translocators

In addition to those proteins involved in the protein secretion process of different gram-negative bacteria described above, the HlyB protein has been shown to have many more homologues in both prokaryotes and eukaryotes, which are responsible for the translocation of a very wide range of compounds from large polypeptides to small hydrophobic drugs and probably chloride ions in the case of the CF (cystic fibrosis) protein (Table 1.1). Such proteins are usually characterized by an N-terminal trans-membrane domain and a large cytoplasmic C-terminus with conserved ATP binding sites. The mammalian P-glycoprotein (Mdr, multi-drug resistance) and the cystic fibrosis (CF) proteins are actually tandem duplications of HlyB-like molecules (Gerlach et al., 1986; Riordan et al., 1989). As mentioned, despite their structural similarities, this family of membrane translocators displays a wide range of "substrates" for transport. These different compounds include polypeptides or

Table 1.1 Membrane polypeptides with homologies to the HlyB protein*

System	Protein	Substrate	Reference
E. coli	HlyB	Haemolysin A	Felmlee *et al.* (1985)
E. coli	CvaB	Colicin V	Gilson *et al.* (1990)
P. haemolytica	LktB	Leukotoxin A	Strathdee and Lo (1989)
B. pertussis	CyaB	Cyclolysin	Glaser *et al.* (1988)
E. chrysanthemi	PrtD	Proteases B,C	Guzzo *et al.* (1991)
R. meliloti	NdvA	β-1,2 glucan	Stanfield *et al.* (1988)
A. tumefaciens	ChvA	β-1,2 glucan	Cangelosi *et al.* (1989)
D. melanogaster	White[a]	Pigment	O'Hare *et al.* (1984)
D. melanogaster	Brown[a]	Pigment	Dreesen *et al.* (1988)
Drug-resistant tumor cells	Mdr[b]	Drugs	Gerlach *et al.* (1986)
P. falciparum	Pfmdr[b]	chloroquine	Foote *et al.* (1989)
Human cystic fibrosis	CFTR[b]	Chloride ions	Riordan *et al.* (1989)
S. cerevisiae	Ste6[b]	Pheromone	McGrath and Varshavsky (1989)

* Modified from Holland *et al.* (1990b)

[a] The ATP-binding domain appears at the N-terminus of the molecule in this case. These systems are in fact involved in uptake of pigment precursors.

[b] Tandem duplications of an HlyB-like molecule.

cyclic polysaccharides in a number of bacterial systems, hydrophobic drugs in *Plasmodium falciparum* and man and a peptide pheromone in yeast. Clearly these "substrates" differ enormously in size and structure and such a wide range of specificity implies the presence of specific initial binding or recognition processes. On the other hand, given the relative similarity of the components from different translocators, it is very unlikely that completely different translocation mechanisms will be adopted. Although translocation through some form of specific transport channels have not been excluded, the difference in structures required to transport substrates with such diversity of shape and size, from polypeptides to drugs or even ions, appears to preclude such a common mechanism. An alternative mechanism suitable for the secretion of such varied substrates might involve an initial step of substrate recognition and binding to the membrane domain of the translocator, followed by a translocation step requiring a major conformational change of the translocator molecule which eventually causes a given substrate to move aross the membrane (Holland *et al*. 1990a; 1990b).

1.4 The extended signal-sequence dependent secretion pathway

The majority of extracellular proteins secreted by gram-

negative bacteria are synthesized as precursors with the typical N-terminal signal-sequences which can be recognized by the general *sec* export machinery (Pugsley, 1988; Pugsley et al, 1990a). The signal-sequences initiate and direct the translocation across the inner membrane via the general *sec* machinery. Then, with the help of factors specifically required for translocation across the outer membrane, the proteins are secreted to the extracellular medium. Thus, the extracellular export of these signal-sequence containing proteins may be represented as a two-step process via an extended signal-sequence dependent pathway with a transient stopover in the periplasm. This two-step model is supported by the demonstration of the normal periplasmic secretion intermediates during the extracellular export of heat-labile enterotoxin by *Vibro cholerae* (Hirst and Holmgren, 1987) and of aerolysin by *Aeromonas salmonicida* (Wong *et al.*, 1989). Also in agreement with this two-step secretion pathway, the extracellular proteins normally secreted by wild type bacteria are found to be accumulated in the periplasmic space in secretion defective mutants of several gram-negative bacteria, such as *Pseudomonas aeruginosa* (Lindgren and Wretlind, 1987; Wretlind and Pavlovskis, 1984), *Aeromonas hydrophila* (Howard and Buckley, 1983), a marine *Vibro* species (Ichige *et al.*, 1988), and others. However, the mechanisms involved in this secretion pathway are still not clear and

several extracellular protein secretion systems being examined in different gram-negative bacteria are discussed in more detail in the following sections.

1.4.1 Pullulanase secretion in Klebsiella pneumoniae

Pullulanase is a lipoprotein which is produced by gram-negative bacteria belonging to the genus Klebsiella (Pugsley et al., 1986). It is initially synthesized as a precursor with a 19-amino acid signal-sequence. It is later modified by fatty acylation to the cysteine residue, which becomes the N-terminus of the mature polypeptide after the cleavage of signal-sequence (D'Enfert et al., 1987; Kornacker and Pugsley, 1989; Michaelis et al., 1985). It is a starch-debranching enzyme that cleaves α(1-6)-linkages in branched starch to release linear dextrins composed of α(1-4)-linked glucose residues. Pullulan is an α(1-6)-linked maltotriose polymer that can be used by Klebsiella as a carbon source. Since this polymer is too large to diffuse across the outer membrane of the bacteria, pullulanase must be located either at the cell surface (with its catalytic site exposed in the medium) or free in the medium, in order to exert its function. In fact, the 117 KD pullulanase polypeptide is initially exported and anchored to the outer membrane (a process which is also called exposition) by the fatty acids attached on the cysteine residue and is subsequently released

into the growth medium at the end of the exponential growth phase (Pugsley et al., 1990b).

Production of pullulanase is induced by growth in the presence of pullulan, maltotriose or maltose and is repressed in the presence of glucose. This property is not found in other enterics including E. coli and it allows Klebsiella alone, among these bacteria, to use pullulan as a carbon source.

Pullulanase is transported to the cell surface in two separate steps. In the first step, pullulanase is translocated across the cytoplasmic membrane by the general signal-sequence dependent export pathway. The signal peptide is processed by lipoprotein signal peptidase and the N-terminal cysteine is fatty-acylated during this step. In the second step, pullulanase is transported to the cell surface by a still poorly understood process which requires the expression of specific secretion genes that are not present in E. coli cells, since the expression of the cloned pullulanase gene pulA in E. coli K12 resulted in the accumulation of correctly processed, fatty acylated pullulanase within the periplasm. The pullulanase molecules were accumulated at the periplasmic side of the E. coli inner membrane and could not translocate across the outer membrane (Michaelis et al., 1985).

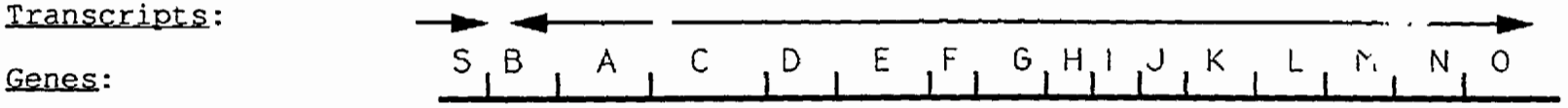
In order to identify specific genes required for

pullulanase translocation across the outer membrane, a plasmid gene bank from strain UNF5023 of *K. pneumoniae* was introduced into *E. coli* and the plasmid-containing cells were grown on pullulan as the sole carbon source to select both pullulanase synthesis and exposition or secretion, on the assumption that the secretion genes required for the second step in pullulanase secretion or exposition may be part of the maltose regulon located close to *pulA* (D'Enfert *et al.*, 1987). In this way, a clone, which carries a 22 kb insert derived from *K. pneumoniae*, was found to be able to expose all of the pullulanase produced to the external face of the outer membrane and to secrete it into the culture medium, indicating that the genes required for the secretion of pullulanase are indeed close to *pulA* and that they can be successfully expressed in *E. coli* cells.

The cloned DNA fragment was later reduced to 19.2 kb without affecting its ability to promote pullulanase exposition and secretion in *E. coli*. By using a combination of many techniques, including DNA sequencing, subcloning, deletion analysis, transposon (Tn*phO* and Tn*tac-2*) mutagenesis, *in vitro/in vivo* protein expression, subcellular fractionation and functional complememtation studies, the structure of this 19.2 kb DNA fragment and the function of the secretion genes located on it has been extentively characterized by Pugsley's group (Pugsley *et al.*, 1990a).

As summarized in Figure 1.4, most of the secretion genes are located in the *pulC-O* operon upstream of *pulA* (D'Enfert *et al.*, 1989; Pugsley and Reyss, 1990; Reyss and Pugsley, 1990). This 13-gene operon is transcribed in the opposite orientation to *pulA* and is co-expressed with *pulA* as part of the maltose regulon, which is induced by the presence of maltose and is co-regulated by MalT, the maltose regulon regulator. Thus, this pullulanase secretion operon is only expressed when *pulA* is transcribed.

The protein products of 10 of the 13 genes in the operon (all except *pulF*, *pulH* and *pulK*) have been identified by at least one of these different techniques: by *in vitro* expression under *lacZp* or other promoter control, by *in vivo* expression in *E. coli* under *lacZp* or T7 gene 10 promotor control, and by immunoblotting. As an alternative approach, fusions between almost every gene in the operon (except *pulE*, *pulF* and *pulI*) and the reporter gene *phoA* (coding for alkaline phosphatase) were made by Th*phoA* mutagenesis. The detection of the hybrid proteins resulting from the transposon insertion and gene fusion confirmed that the genes in the *pulC-O* operon were really expressed *in vivo* and that the predicted translation initiation codons and reading frames were correct. The cellular location of the gene products were determined by subcellular fractionation experiments. In some cases, such as for PulH, PulJ and PulK,



Predicted proteins (KD):	14	19	118	31	71	55	44	15	18	13	22	36	44	18	28	25
Protein identified:																
under lacZp control	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
in minicells	+	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-
T7 promotor control				+	+	+	-	+	-	+	+	-	+	+	+	+
by immunobloting			+		+											
as PhoA hybrid			+	+	+			+	+		+	+	+	+	+	
Location as determined:																
under lacZp control	OM		OM&IM													
by immunoblotting			OM&IM		OM											
T7 promotor control				IM	OM			IM					IM	IM	IM	IM
as PhoA hybrids			IM	IM	P			IM	IM		IM	IM				
Requirement for exposition shown by:																
gene deletion	+	-		+	+		+	+				+				
linker insertion				+	+											
transposon inertion									+	+	+	+	+	+	+	+
Structral features:																
signal peptide	+	-	+	-	+	-										
membrane anchors	0	0	0	0	1	0	>5	1	1	1	1	3	1	1	1	>3
fatty acids	+		+													
predicted location	OM	C	IM	IM	OM/P	C	IM	IM	IM	IM	IM	IM	IM	IM	IM	IM

Figure 1.4. Pullulanase secretion genes of Klebsiella pneumoniae. The figure represents a map of pul DNA region (not to scale) and the characteristics of the pul gene products, as determined by the listed techniques. C: cytoplasm, P: periplasm, IM: inner membrane, OM: outer membrane. From Pugsley *et al.* 1990a.

the subcellular locations of the PhoA hydrids were used as possible indications of the cellular location of PulH, PulJ and PulK themselves.

Although the analysis of the *pulC-O* operon is still not finished (especially with respect to the function of *pulE* and to the direct identification of the *pulF*, *pulH* and *pulK* protein products), the available data demonstrate very clearly that at least 12 of the 13 genes (except *pulE*, since mutations within this gene are not available so far) are essential for pullulanase secretion. Every one of these 12 genes codes for an envelope protein, and among them, only one (PulD) is located in the outer membrane, all the others being located in the inner membrane. Preliminary topology analyses with *phoA* and *lacZ* gene fusions suggest that 8 of the 11 inner membrane proteins have a type II transmembrane protein configuration, that is with the C-terminal part of their sequences located in the periplasm. The PulL protein spans the membrane once via a hydrophobic segment positioned at the central part of the molecule, whereas the PulF and PulO proteins cross the membrane several times (Pugsley *et al.*, 1990a).

Another gene essential for pullulanase secretion, *pulS*, which encodes an outer-membrane lipoprotein, is located downstream of the 3′ end of *pulA* (D'Enfert and Pugsley, 1989). *pulS* is transcribed in the opposite direction to *pulA*

and is separated from *pulA* by another gene *pulB*, which is not involved in the pullulanase secretion process. Unlike *pulA* and all other secretion genes in the *pulC-O* operon, the expression of *pulS* is not induced by maltose and the mutations in the maltose regulator gene *malT* have no effect on *pulS* production. The PulS protein is produced even when pullulanase is not synthesized, suggesting that it may have other functions in addition to its role in pullulanase secretion.

Although there are as many as 14 genes required for pullulanase secretion, these *pul* genes cannot take place of the *sec* genes involved in the general protein export process across the inner membrane. The *pul* genes are only required for and involved in pullulanase translocation across the outer membrane and the functions of *sec* and *pul* genes are completely independent, since it has been demonstrated that at least 6 *sec* genes (*secA*, *secB*, *secD*, *secE*, *secF* and *secY*) are all required for processing of the prepullulanase signal peptide (Pugsley *et al.*, 1991a) and that the translocation process across the two membranes can be successfully uncoupled by first allowing pullulanase to translocate across the cytoplasmic membrane via the signal-sequence dependent general export pathway, and then inducing the *pulC-O* operon to carry out the translocation across the outer membrane (Pugsley *et al.*, 1991b).

Based on these results, a model for pullulanase exposition and secretion has been proposed by Pugsley *et al.* (Mackman *et al.*, 1986; Pugsley *et al.*,1990a; 1991b), which is illustrated in Figure 1.5. This model proposes that pullulanase secretion involves three distinct steps, the first of which is carried out completely by the signal-sequence dependent general export pathway while the second step requires specific secretion factors encoded by the *pul* genes. The final step in the secretion pathway is the spontaneous release of what appear to be pullulanase micelles (held together by the fatty-acid chains present on pullulanase molecules) from the cell surface.

Although the *K. pneumoniae* pullulanase secretion system has been reconstituted successfully in *E. coli* by co-expressing the cloned *pul* genes, it does not exclude the possibility that other unidentified genes, which may be present in both *E. coli* and *K. pneumoniae*, are also required for pullulanase secretion, since the reconstitution approach being used can not identify such genes. Indeed, two Tn*10* insertions in the K21 chromosome of *K. plantolytica* that prevented pullulanase secretion were apparently located outside the *pul* gene complex (Kornacker *et al.*, 1989).

1.4.2 Protein secretion in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a pathogen which is able to

secrete a wide variety of proteins involved in pathogenesis both in animals and plants. These proteins include the signal-sequence containing elastase, phospholipase, lipase, alkaline phosphatase and exotoxin A, as well as the alkaline protease which does not have a signal-sequence (Lazdunski *et al.*, 1990).

Genetic analysis has led to the identification of two secretion pathways: (1) the signal-sequence independent pathway specific for alkaline protease (Guzzo *et al.*, 1991), (2) an extended signal-sequence dependent pathway, defined by *xcp* (extracellular protein-deficient) mutations, which mediates secretion of most extracellular proteins (Lazdunski *et al.*, 1990).

The *xcp* mutants were initially isolated as elastase/protease deficient colonies on elastin/skim-milk agar plates, after treatment of *P. aeruginosa* with the chemical mutagen ethylmethane sulfonate or via transposon insertion (Wretlind *et al.*, 1977; Lindgren and Wretlind, 1987). By conjugation and transduction analysis, the *xcp* mutations were mapped at three different locations on the chromosome (0′, 55′ and 65′) (Wretlind and Pavlovskis, 1984). Up to now, 6 *xcp* mutations (*xcp-5, 51, 52, 53, 54, 55*), which are located in two distinct loci, have been mapped in the 55′ region of the chromosome. Only 2 *xcp* mutations have been located to the other parts of the chromosome, *xcp-1* at 0′ and

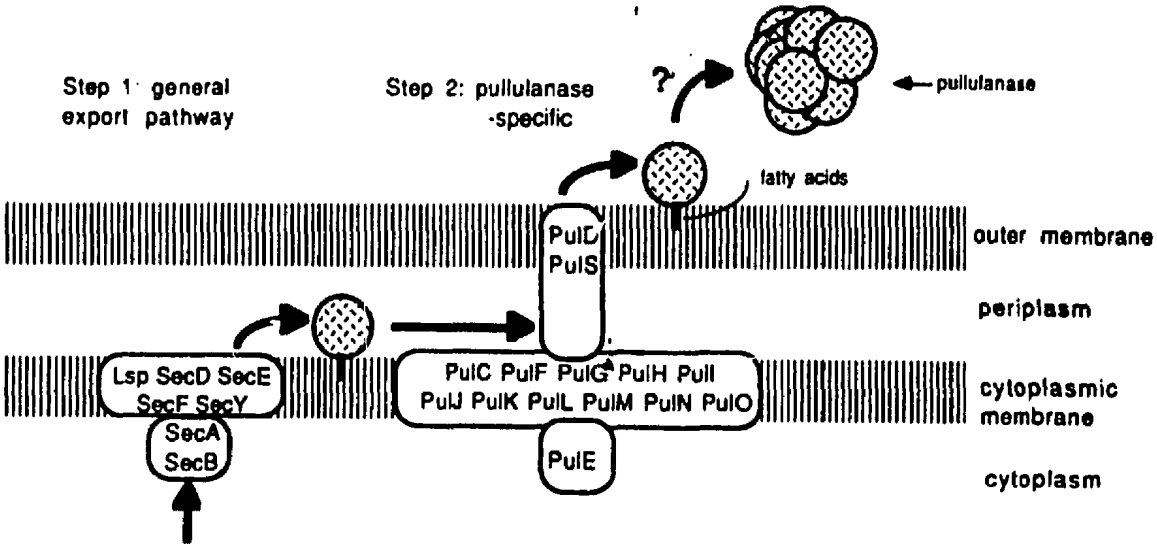


Figure 1.5. A secretion model for PulA. The figure shows the location of proteins required for pullulanase secretion and the predicted secretion intermediate between steps 1 and 2. Extracellular pullulanase exists as protein micelles due to the presence of fatty acids. From Pugsley *et al.*, 1991b.

xcp-6 at 65′.

Although the mutations in each of these *xcp* mutants are apparently different, they all display a similar phenotype, causing pleiotropic intracellular accumulation of extracellular proteins, including exotoxin A, lipase, alkaline phosphatase, phospholipase and elastase. As expected, the secretion of alkaline protease is not affected by any of the *xcp* mutations because it is secreted by a different signal-sequence independent pathway as described earlier. Since the extracellular proteins accumulate in the periplasm in *xcp* mutants, it is believed that the *xcp* genes are required for translocation across the outer membrane after the extracellular proteins have reached the periplasm in a signal-sequence dependent manner (Lazdunski *et al.*, 1990).

A 40 kb chromosomal fragment that complements the 6 mutations at 55′ region (*xcp-5, 51, 52, 53, 54, 55*) has been isolated from a *Pseudomonas aeruginosa* PAO genomic DNA bank (Filloux et al., 1989). By deletion analysis and transposon insertions, all the *xcp* mutations have been located in a 9 kb *Eco*RI DNA fragment. The mutations are found to be clustered in two separate loci which are independently expressed. DNA sequence analysis on one of the two loci revealed the existence of two genes, *xcpY* and *xcpZ* (Filloux *et al.*, 1990). Mutation *xcp-51* is ascribed to the *xcpY* gene and mutations

xcp-5 and *xcp-52* to the *xcpZ* gene, confirming that both genes are essential for extracellular protein secretion. *xcpY* encodes a 41 KD polypeptide, *xcpZ* encodes a 19 KD protein and both proteins have been shown to reside in the inner membrane in their natrual host *P. aeruginosa* PAO.

Similarly, another secretion gene *xcpA*, corresponding to mutation *xcp-1*, has been isolated and characterized from the 0′ chromosome region (Bally *et al.*, 1991). It encodes a highly hydrophobic 32 KD protein and, like *XcpY* and *XcpZ*, XcpA is also located in the inner membrane of *P. aeruginosa* PAO.

More interestingly, all of these identified secretion genes *xcpY*, *xcpZ* and *xcpA* share very significant homologies with the *pulL*, *pulM* and *pulO* genes that are required for the specific secretion of pullulanase in *Klebsiella pneumoniae*. The subcellular locations of these gene products are also the same, i.e. they are all located in the inner membrane of the cell envelope. The overall identity at the amino acid level is 30% for XcpY and PulL, 25% for XcpZ and PulM, and 48% for XcpA and PulO. This high degree of homology between XcpY, XcpZ, XcpA and PulL, PulM, PulO suggests that these proteins have been evolutionarily conserved due to functional similarities in these two secretion systems. These results provided the first evidence indicating the existence a signal-sequence dependent mechanism for extracellular protein

secretion common to two unrelated gram-negative bacteria: *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

1.4.3 Protein secretion in *Erwinia chrysanthemi*

Erwinia chrysanthemi is a plant pathogen which can secrete a large number of plant cell wall degrading enzymes. Similar to *Psedomonas aeruginosa*, it has two distinct secretion pathways: (1) the signal-sequence independent pathway specific for the proteases B and C (Delepelaire and Wandersman, 1989; 1990) and (2) an extended signal-sequence dependent pathway responsible for most of the plant cell wall-degrading polysacchridases, including at least four isoenzymes of pectate lyase, exo-poly-α-D-galacturonosidase, pectin methylesterase and two cellulases. The second pathway is characterized by *out* genes since the *out* mutants cannot secrete any of these degradative enzymes beyond the periplasm. The *out* mutants also display severely reduced virulence, indicating that the secreted extracellular proteins play very important roles in the pathogenesis of this bacterium (Murata et *al.*, 1990).

Recently, the *out* genes from *E. chrysanthemi* have been cloned in a cosmid by complementing several transposon-induced *out* mutations (He *et al.*, 1991). Restriction mapping and transposon mutagenesis of the cosmid showed that all the *out* genes were located in a 12 kb chromosomal DNA fragment.

This 12 kb fragment could complement all existing *out* mutations in *E. chrysanthemi* EC16, and allowed *E. coli* strains to successfully secrete the extracellular pectic enzymes expressed from cloned *E. chrysanthemi* genes. DNA sequencing of a 2.4 kb *Eco*RI fragment within this 12 kb fragment revealed four genes, *outH*, *outI*, *outJ*, and *outK*, arranged colinearly in a operon-like structure and showed extensive homology (75%, 62%, 61.6% and 54.5% respectively) with the *Klebsiella pneumoniae* secretion genes *pulH*, *pulI*, *pulJ*, and *pulK*.

These results again suggest that the signal-sequence dependent secretion pathways in different gram-negative bacteria share a conserved translocation apparatus. However, *K. pneumoniae* cells could not secrete the pectate lyase expressed from the cloned *E. chrysanthemi pelE* gene, and the *E. chrysanthemi out* system failed to secrete an extracellular pectate lyase encoded by a gene from a closely related plant pathogen, *Erwinia carotovara spp. carotovora*, indicating that the *out* secretion system in *E. chrysanthemi* possessed a translocation mechanism homologous to the *pul* system of *K. pneumoniae* but evolved completely different protein recognition capacities.

1.4.4 Protein secretion in *Aeromonas hydrophila*

Species of the genus *Aeromonas* are water-borne

microorganisms that have been implicated as causative agents of clinical infections (Davis *et al.*, 1978). *Aeromonas hydrophila* has received particular attention since it was associated with infections in soft tissue, wounds and blood (Hussain Quadri *et al.*, 1976; Ketover *et al.*, 1973; Pillips *et al.*, 1974).

Aeromonas hydrophila secretes a wide range of extracellular exzymes, including aerolysin (a haemolysin) (Chakraborty *et al.*, 1986; Howard and Buckley, 1986), proteases (Lueng and Stevenson, 1988), amylase (Gobius and Pemberton, 1988), acyltransferase (Thornton *et al.*, 1988) and an enterotoxin (Chakraborty *et al.*, 1984). Several of them, such as aerolysin, protease and enterotoxin, have been implicated as virulence factors in the pathogenesis of this organism.

The secretion of aerolysin has been extensively studied. Aerolysin is synthesized as a precursor with a 23 amino acid N-terminal signal-sequence (Howard and Buckley, 1986). Results from pulse-labeling experiments showed that the translocation across the inner membrane and processing of the signal peptide occured cotranslationally, then, after a short delay (about 4 min), the processed aerolysin was translocated across the outer membrane by a unknown mechanism and appeared in the extracellular medium (Howard and Buckley, 1985a). Aerolysin is first secreted by the bacteria in a higher-

molecular-weight inactive form. The proaerolysin, after being released into the medium, is activated by proteolytic cleavage catalyzed by a protease, which is also secreted by the bacteria (trypsin treatment in vitro can also activate the protoxin efficiently). This activation process involves the cleavage of a 2.5 KD peptide from the carboxyl terminus of the protein (Howard and Buckley, 1985b). The initial step in aerolysin secretion, i.e. the translocation across inner membrane, is signal-sequence dependent and can be comlpetely blocked by the presence of CCCP (Carbonyl Cyanide m-Chlorophenylhydrazone), probably by uncoupling an energy-dependent process required for translocation of nascent chains across the inner membrane. The mechanisms involved in the subsequent translocation process across the outer membrane are still largely unknown. It is clear, however, that this process depends upon additional gene products since aerolysin translocation across the outer membrane is impaired in several previously isolated chemically induced secretion mutants (Howard and Buckley, 1983). Although efficiently processed and translocated across the inner membrane, aerolysin cannot be translocated through the outer membrane and is accumulated in the periplasm of these mutants. The same outer membrane translocation mechanism used by aerolysin is also shared by several other proteins, since the chemically induced mutations have pleiotropic effects on

protein secretion and caused the periplasmic accumulation of other normally secreted extracellular proteins, including protease and glycerophospholipid:cholesterol acyltransferase. Furthermore, the same mechanisms also appear to be shared by other members of the family Vibronaceae, since aerolysin expressed from the cloned Aeromonas hydrophila gene can be efficiently secreted by Aeromonas salmonicida (Wong et al., 1989), and can also be exported extracellularly by wild-type cells but accumulated periplasmicly in pleiotropic secretion mutants of a marine Vibro strain (Wong et al., 1990).

1.5 Purpose of this Dissertation

Despite all the above mentioned evidence suggesting the existence of additional factors essential for extracellular protein export in Aeromonas hydrophila, no component of this system has yet been identified, isolated or characterized. In order to identify some of the components of this extracellular export machinary, seven isogenic, single-hit transposon insertion mutants of Aeromonas hydrophila, which are defective in the production of extracellular proteins, have been isolated in this laboratory. Preliminary analysis of these mutants showed that the structural gene of aerolysin (aerA) in one of the seven mutants (I2.66) was interrupted by Tn5-751, the transposon used, so no aerolysin is produced in

this mutant. Aerolysin is synthesized in reduced amounts by another mutant (E2.86) but is secreted normally. L1.24, L2.48 and L3.33 formed small colonies on agar plates and grew in liquid cultures with a doubling time of more than twice that of the wild-type strain. These three mutants thus appeared to be impaired in some aspects of general metabolism rather than specifically in extracellular protein export. The remaining two mutants (C5.84 and L1.97) grew at the same rate as did the wild-type strain, and they appeared to be the most interesting mutants with respect to the extracellular secretion process, since they were defective in extracellular production of not only aerolysin, as shown in Figure 1.6, but also protease and amylase.

The purpose of this dissertation is to further characterize the two mutants C5.84 and L1.97, to define, isolate and characterize the extragenic factors/genes involved in the protein secretion process and to obtain some insight into the protein translocation machinery used by this gram-negative bacterium.

In this study, by using the transposon antibiotic resistance genes as markers, two wild type chromosomal fragments corresponding to those regions affected by the transposon in the mutants were cloned. One of these fragments was further analyzed by DNA sequencing and a secretion gene required for A. hydrophila extracellular

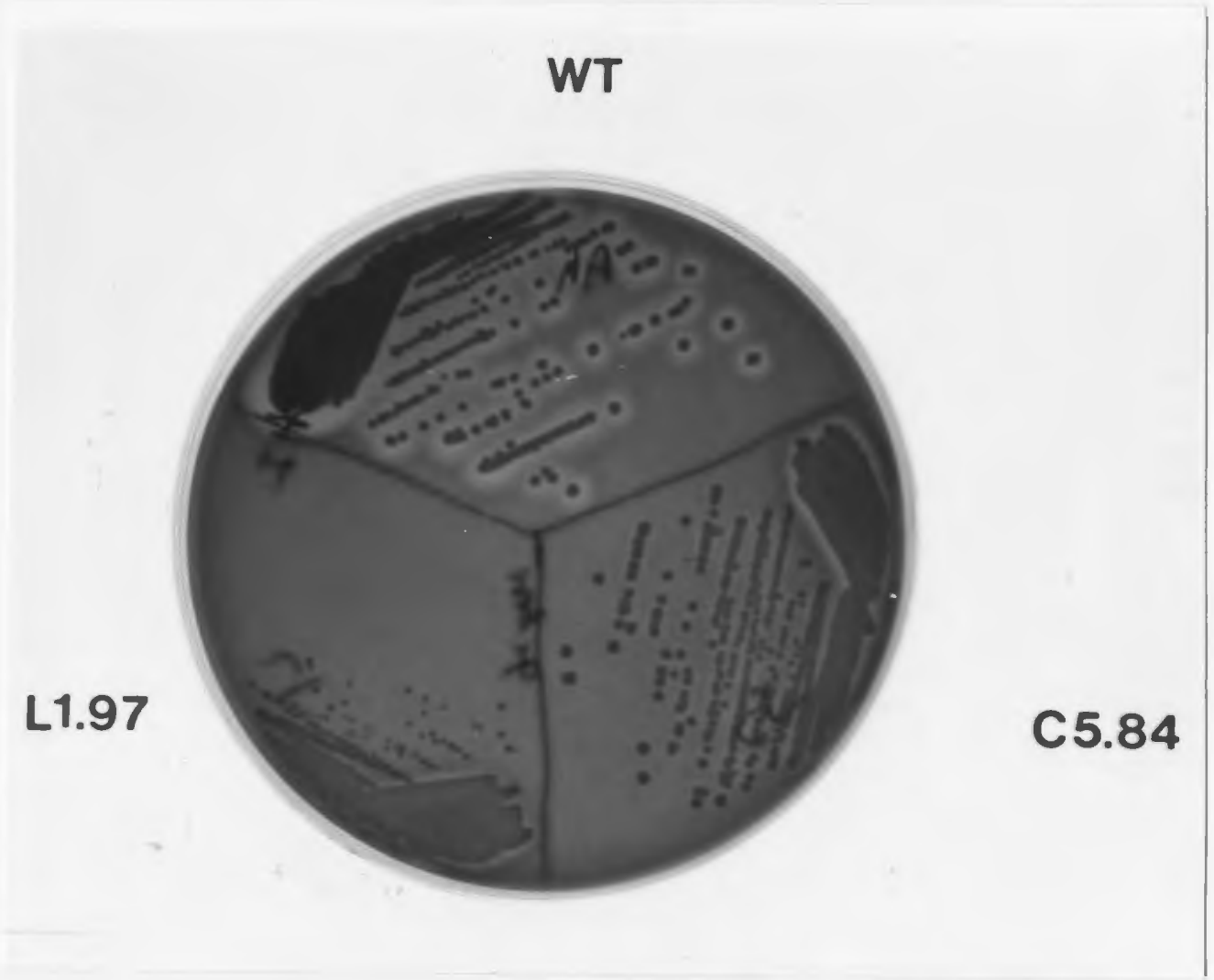


Figure 1.6. Phenotype of Ah65, C5.84 and L1.97. Cells are streaked on a BHI-blood plate. A clear zone surrounding the wild-type cell is easily seen because of the hemolytic activity of secreted aerolysin. Since C5.84 and L1.97 cells cannot secrete aerolysin, they display non-hemolytic phenotye on the plate.

secretion identified and characterized.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

Strains and plasmids are listed in Table 2.1.

A. hydrophila was grown routinely at 30^{0}C in brain heart infusion (BHI) or 0.4% glycerol M9 minimal salts medium (MM) (Miller, 1972), or in a chemically defined minimal medium (DMM) (Riddle *et al.*, 1981).

E. coli strains were grc..n at 37^{0}C in BHI or LB medium (Miller, 1972). Solid medium used for the detection of extracellular activity of aerolysin was BHI agar containing 5.0% human erythrocytes.

Ampicillin was used at 50 μg/ml, kanamycin sulfate was used at 50 μg/ml, tetracyclin was used at 10 μg/ml, chloramphenicol was used at 10 μg/ml, trimethoprim was used at 250 μg/ml, nalidixic acid was used at 10 μg/ml, and streptomycin sulfate was used at 100 μg/ml.

2.2 Southern blotting and colony hybridization

To facilitate efficient transfer of high molecular weight DNA in Southern blotting, DNA was partially depurinated in

Table 2.1 Strains and plasmids

Strain or plasmid	Genotype/phenotype	Source/reference
A. hydrophila		
Ah65	Wild-type	
C5.84	See the text	This laboratory
L1.97	See the text	This laboratory
E. coli		
HB101	F⁻*hsd*S20($r_B^-m_B^-$) *ara*14λ⁻*gal*K2*lac*YI *pro*A2*rsp*L2*sup*E44 *rec*A13*xyl*-5*mtl*-1	BRL
MM294	F⁻*hsd*R17($r_K^-m_K^-$) *sup*E44*thi*-1*end*A1	Hanahan (1983)
XL1-Blue	*rec*A1*end*A1*gyr*A96 *lac*⁻*thihsd*R17 (F′*pro*AB*lac*I^q *lac*ZΔM15Tn*10*)	Stratagene
Plasmids		
pBR322	Ap^r Tc^r	Bolivar *et al.* (1977)
pBR328	Ap^r Tc^r Cm^r	Soberon *et al.* (1980)
pBluescript II KS$^+$/SK$^+$	Ap^r	Stragene
pVK101	Tc^r Km^r	Knauf and Nester (1982)
pRK2013	Km^r	Figurski and Helinski (1979)
pMMB67HE/EH	Ap^r	Furste *et al.* (1986)
pMMB67HE.tet	See the text	This study
pMMB67HE/EH.cam	See the text	This study
pJB1-pJB4	See the text	This study
pJB5-pJB6	See the text	This study
pJB7.3-pJB7.4	See the text	This study
pMJB8.1 pMJB8.2 pMJB9	See the text	This study
pMJB10-15	See the text	This study

0.25 N HCl for 10 min after electrophoresis. Following depurination, the gel was briefly rinsed in distilled water. Then, the DNA was denatured by a solution of 0.5 N NaOH, 1.0 M NaCl for 2 X 15 min, and subsequently was neutralized in 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl 2 X 15 min. Then, the DNA was transferred from the agarose gel to nitrocellulose paper as described by Southern (1975). Colony hybridization was carried out by transferring bacterial colonies from a master plate to a nitrocellulose filter (Grunstein and Hogness, 1975). The colonies on the filter were first treated with 10% SDS to reduce diffusion by laying the filter, with the colonies facing up, onto a sheet of Whatman 3MM paper soaked with 10% SDS for 10 min. The colonies were lysed by laying onto Whatman 3MM paper soaked with 0.5 N NaOH, 1.5 M NaCl for 15 min, neutralized by 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl for 15 min, and the liberated DNA was fixed to the filter by baking in a 80^{0}C vaccum oven for 30 min.

Non-radioactive DNA labeling and detection systems from both Bethesda Research laboratories (using biotin-labeled probes prepared by a nick-translation reaction) and Boehringer Mannheim (using digoxigenin-labeled probes prepared by a random-priming reaction) were used. The prehybridization, hybridization, washing and detection procedures were carried out under the standard stringent

conditions recommended by the manufacturers.

2.3 DNA preparation and manipulation

Aeromonas hydrophila chromosomal DNA was isolated by the method of Priefer et al. (1984). Cells collected from 5 ml overnight cultures were first resuspended in 10 ml 1 M NaCl and treated at 0^0C by shaking the mixture gently for 1 hour. Then the cells were pelleted and resuspended in 10 ml cold 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 150 mM NaCl, pelleted again and resuspended in 2.5 ml of the same solution. The cells were then lysed by first incubating with 0.5 ml of 2 mg/ml lysozyme dissolved in TE (10mM Tris-HCl, pH8.0, 1 mM EDTA) at 37^0C for 15 min, followed by adding 0.6 ml 50 µg/ml protease K in 10% SDS and incubated at 37^0C for another 60 min. The liberated chromosomal DNA was purified by gentle phenol, phenol:chloroform and chloroform extractions followed by ethanol precipitation. After washing the precipitated DNA with 70% ethanol, the chromosomal DNA was dissolved in 2 ml TE containing 10 µg/ml RNAse.

Small scale preparation of plasmid DNA from E. coli cells was performed using the alkaline lysis protocol (Birnboim and Doly, 1979). Large-scale preparation was carried out by essentially the same alkaline lysis procedure followed by purification by CsCl-ethidium bromide density gradient

centrifugation, as described by Maniatis *et al.* (1982).

Standard methods for analysis of DNA, such as restriction endonuclease analysis, T4 DNA ligation, the blunting of overhanging single-stranded ends by the Klenow fragment of *E. coli* DNA polymerase or T4 DNA polymerase, vector dephosphorylation using bacterial alkaline phosphatase and calf intestinal alkaline phosphatase, and agarose gel electrophoresis were performed as described by Maniatis *et al.* (1982). Plasmid DNA and ligation mixtures were transformed into *E. coli* cells by the $CaCl_2$ method and the transformants were selected by plating on agar plates containing the appropriate antibiotics (Cohen *et al.*, 1972). Wide-host-range plasmids, such as pVK101 (Knauf and Nester, 1982), pMMB67EH/HE (Furste *et al.*, 1986), pMMB67HE.tet, pMMB67EH/HE.cam and their derivatives were transferred into *Aeromonas hydrophila* cells by triparental mating, using pRK2013 (Figurski and Helinski, 1979) as the mobilizing system.

2.4 Cell fractionation

Aeromonas hydrophila cells were fractionated by the method of Willis *et al.* (1974) with slight modifications. Freshly grown culture was first conditioned by mixing with

1/29 volume of 1.0 M Tris-HCl, pH 7.3, 1.0 M NaCl solution at room temperature. The cells were then collected by centrifugation and immediately resuspended into an equal volumn of 33 mM Tris-HCl, pH 7.3, 2.0 mM EDTA, 20% (w/v) sucrose solution, and incubated at room temperature for 10 min. The plasmolysed cells were separated from the supernatant (which was kept as the wash fluid) by centrifugation and osmotically shocked by adding an equal volume of ice-cold water in the presence 1.0 mM $MgCl_2$. After another centrifugation, the shocked cell pellet was ruptured by treatment with 0.35 mg/ml lysozyme in the presence of 10 mM EDTA followed by rapid dilution into cold water. The cytoplasmic fraction was collected after pelleting of the cell membranes by centrifugation at 35,000 rpm in a Beckman 60Ti rotor for 1 hour. Cell envelopes were then resuspended in 20 mM Tris-HCl, pH 8.0. The outer membrane samples were prepared as described by Filip et al. (1973), using 0.5% sodium lauroyl sarcosinate to solubilize the inner membrane.

When necessary, culture supernatants were concentrated by precipitation with 15% trichloroacetic acid and centrifugation for 30 min at 15,000 X g. The pellets were washed with 90% acetone and resuspended directly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (Maniatis et al., 1982).

2.5 Enzyme assays

Aerolysin activity was measured in microtiter plates by two-fold serial dilutions of 0.1 ml samples in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (Howard and Buckley, 1985b). An equal volume (0.1 ml) of washed human erythrocytes (0.8% v/v in PBS) were added to each well, and the plates were incubated at 37^{0}C for 1 hour. All samples were activated with 5 μg/ml of trypsin for 5 min at room temperature before serial dilution. The activity of the samples was expressed as the inverse of the highest dilution of the original sample at which 100% erythrocyte lysis was observed after incubation.

β-lactamase activity was assayed with the chromogenic substrate PADAC (Howard and Buckley, 1985a). Each assay contained 0.9 ml of 25 μM PADAC in 20 mM Tris-HCl, pH 8.0 and 0.1 ml of sample, and the decrease in absorbance at 571 nm was monitored. Units were defined as nmoles of substrate hydrolyzed per minute at 25^{0}C. Lactate dehydrogenase activity was assayed with sodium pyruvate as the substrate (Stambaugh and Post, 1966), and glutamate dehydrogenase was assayed with α-ketoglutarate as the substrate (Halpern and Lupo, 1965). Units for both were nmoles of substrate catalyzed per minute at 25^{0}C.

Protease activity was assayed with azocoll as the substrate (Chavira et al., 1984). Each assay mixture

contained 500 μl of 20 mg/ml azocoll in 0.5 mM $CaCl_2$, 10 mM Na_2HPO_4 (PH7.2) and an appropriate amount of sample in a final volume of 900 μl. The mixture was incubated at 37°C for 1 hour with vigorous shaking, and the reaction was stopped by placing the samples into ice. After centrifugation at 5,000 X g for 5 min at 4°C, 500 μl of the supernatant of the reaction mixture was mixed with 500 μl of 1 N NaOH, and the OD_{520} was recorded. Units were defined as the change in the optical density at 520 nm per hour. Amylase activity was assayed with starch azure as the substrate (Rinderknecht et al., 1967). Each assay mixture contained 400 μl of 5% starch azure in 20 mM Na_2HPO_4, pH 7.0, 50 mM NaCl and an appropriate amount of sample in a final volume of 1 ml. The mixture was incubated at 37°C for 1 hour with vigorous shaking, and the reaction was stopped by adding 100 μl of 2.5 M sodium acetate. After centrifugation, 500 μl of the supernatant was mixed with 500 μl of H_2O, and the OD_{595} was recorded. Units were defined as the change in the optical density at 595 nm per hour.

2.6 The construction of wide-host-range vectors

Several derivatives of the wide-host-range vector pMMB67HE or pMMB67EH were constructed by the strategies

depicted in Figure 2.1. The *Eco*R1-*Ava*I fragment containing the *tet* gene from pBR322 was inserted into the unique *Pvu*I site in pMMB67HE. The insertion inactivated the β-lactamase gene but instead confered tetracylcine resistance to cells containing the new plasmid. Similarly, the small *Aha*III-*Pvu*I fragment in pMMB67HE/EH was exchanged with the *Bcl*I-*Pvu*I fragment from pBR328 containing the whole *cat* (chloramphenicol acyltransferase) gene and part of the β-lactamase gene, to give rise to pMMB67HE/EH.cam, which confered both ampicillin and chloramphenicol resistance.

2.7 Preparation of nested deletion derivatives

The DNA fragment to be deleted for subsequent sequencing (the 4.1 kb *Kpn*I fragment) was cloned into the *Sma*I site of the pBluescript II phagmids SK^+ or KS^+. The orientation of this fragment was different with respect to the *tac* promotor in the two vectors.

By using the Exo/Mung DNA sequencing system (Stratagene), two sets of unidirectional nested deletion clones, starting from both ends of this 4.1 kb fragment, were constructed according to the procedure recommended by the manufacturer. The deletion from the 5′ end of the 4.1 kb fragment was carried out in the SK^+ vector. The DNA was first digested by

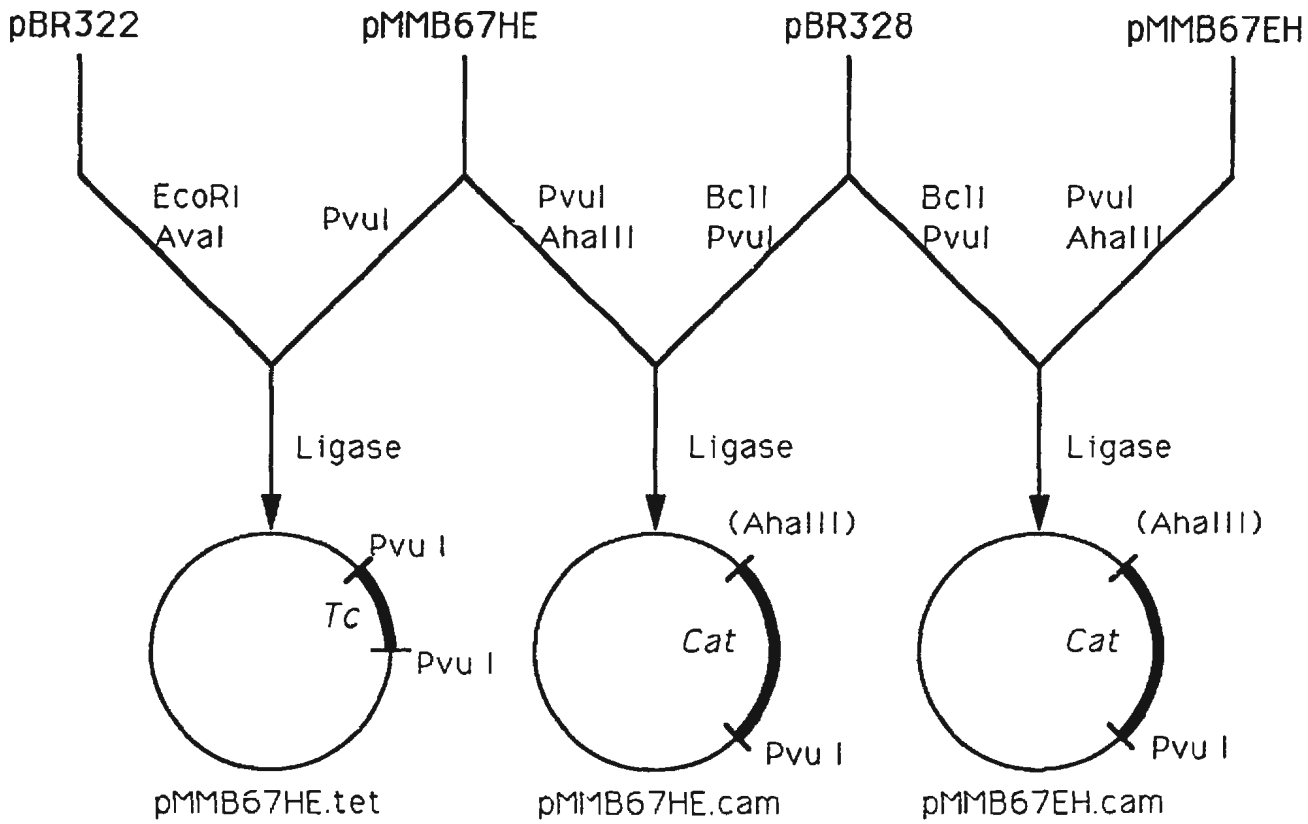


Figure 2.1. Construction of new wide-host-range vectors. The figure shows schematically the cloning strategy for the vector construction. The black-shaded fragments represent the *Tc* gene and *Cat* gene from pBR322 and pBR328, respectively. The restriction sites shown in parentheses are destroyed during the cloning procedure.

KpnI, which produced a unique 3' overhang to protect the vector from Exonuclease III deletion, and then cut by EcoRI, which gave a unique 5' overhang that lay between the insert and the KpnI site. Deletion from the 3' end of the 4.1 kb fragment was performed in the KS^+ vector. The DNA was first digested with NotI, and the resulting 5' overhang was filled with deoxy-thioderivatives using Klenow to protect against Exonuclease III digestion. Then the DNA was cut by XbaI, which lay between the protected NotI site and the insert.

Deletion from the un-protected 5' overhang sites (the EcoRI site in SK^+ vector and the XbaI site in KS^+ vector) was performed by treating the double digested DNA with Exonuclease III at room temperature, so that a portion of the insert was made single stranded by the enzyme, followed by Mung Bean Nuclease digestion to create blunt ends. Then the deleted DNA was circularized by T4 DNA ligase and transformed into E. coli XL1-Blue cells.

The Exo/Mung deletion transformants were screened by restriction analysis of minipreps or, alternatively by the polymerase chain reaction (PCR).

2.8 Polymerase chain reaction (PCR)

Besides the conventional methods (e.g. miniprep followed

by restriction analysis), PCR was used to characterize the structure of recombinant clones in pBluescript vectors by amplifying the insert DNA directly from bacterial cells on a transformation plate.

The amplification was performed essentially according to Gurpreet et al. (1989). The bacterial colony was first suspended in 50 μl H_2O, and heated to 94^{0}C for 10 min to lyse the cells and denature the plasmids. Then, thirty PCR cycles (1 min @94^{0}C, 1 min @50^{0}C, and 2 min @72^{0}C) were carried out in a 100 μl reaction with 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, dNTPs (final concentration of 200 μM for each nucleotide triphosphate), 20 pmoles of M13 forward sequencing primer (5'-GTAAAACGACGGCCAGT-3'), 20 pmoles M13 reverse primer (5'-AACAGCTATGACCATG-3') and 0.5 unit of Taq DNA polymerase (Promega). The PCR product was visualized by running 10 μl of the reaction mixture on an agarose gel.

In some cases, single-stranded insert DNA was prepared by asymmetric PCR. Supercoiled plasmid or symmetric PCR product was subjected to amplification with 0.5 unit of Taq DNA polymerase as described by Gyllensten and Erlich (1988). The primer ratio was 50 pmole:1 pmole, and the amplification was performed for 35 cycles (1min @94^{0}C, 1 min @50^{0}C and 2 min @72^{0}C).

2.9 Determination of nucleotide sequence

Single-stranded DNA template was either prepared from the nested deletion clones in pBluescript vector by asymmetric PCR, or by superinfection with helper phage VSM13 as described by Katayama (1990).

DNA sequencing was done by the dideoxynucleotide chain termination method by using [α-^{35}S]dATP as the sequencing label (Sanger *et al.*, 1977). In all cases the sequencing reactions were performed with T7 DNA polymerase (Sequenase, U.S. Biochemical Corp.). To resolve band compressions, the nucleotide dITP was used instead of dGTP in the sequencing reaction.

The sequencing reaction products were resolved on 6% polyacrylamide-8 M urea sequencing gels. The sequencing gels were run at 2000 V, fixed in 10% methanol, 10% acetic acid in water, vaccum dried at 80^{0}C on filter paper, exposed to X-OMAT AR (Eastman Kokak Co.) film and visualized by autoradiography.

2.10 Gel electrophoresis and immunoblots

SDS-PAGE was carried out on 10 or 12% acrylamide gels (Laemmli, 1970). For immunoblots, the proteins were transferred to nitrocellulose paper after electrophoresis,

reacted with an anti-aerolysin monoclonal antibody (unpublished results), and developed with alkaline phosphatase-conjugated goat anti-mouse antibodies, using BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) and NBT (4-Nitro blue tetrazolium chloride) as substrates (Towbin *et al.*, 1979).

2.11 *In vitro* transcription and translation

An *E. coli* S30 coupled transcription translation system, a cell free extract originally described by Zubay and coworkers (Zubay, 1980), was used to identify the polypeptides encoded by plasmids pMJB8.1, pMJB8.2, pJB7.3 and pJB7.4. The reaction was carried out under the standard condition recommended by the supplier (Promega), using [^{35}S]-methionine as the labelling reagent. CsCl purified supercoiled plasmid was used as the template and the translated products were analysed by SDS-PAGE. Following the electrophoresis, labeled protein bands in the gel were visualized by autoradiography.

Chapter 3

RESULTS

3.1 Analysis of the synthesis and extracellular export of aerolysin and other proteins by the mutants

To differentiate between aerolysin synthesis defects and aerolysin export defects caused by transposon insertion, wild-type strain Ah65 as well as mutants C5.84 and L1.97 were grown in broth. Samples of cells and culture supernatants were collected and immunoblotted with an anti-aerolysin monoclonal antibody (Figure 3.1). Each of the two mutants produced as much aerolysin as did the wild-type but failed to export it into the supernatant. The immunoblot also showed that aerolysin was accumulated within the mutants as 54.0 KD proaerolysin (not the signal-sequence containing preproaerolysin), which is normally digested to the 51.5 KD active toxin after extracellular secretion. However, if the mutant cells were manipulated, as, for example, during osmotic shock of the subcellular fractionation procedure, this protoxin was found to have been converted to the mature form to various degrees when samples were later electrophoresed and immunoblotted (compare Figure 3.1 and Figure 3.10).

As mentioned before, besides aerolysin, *Aeromonas*

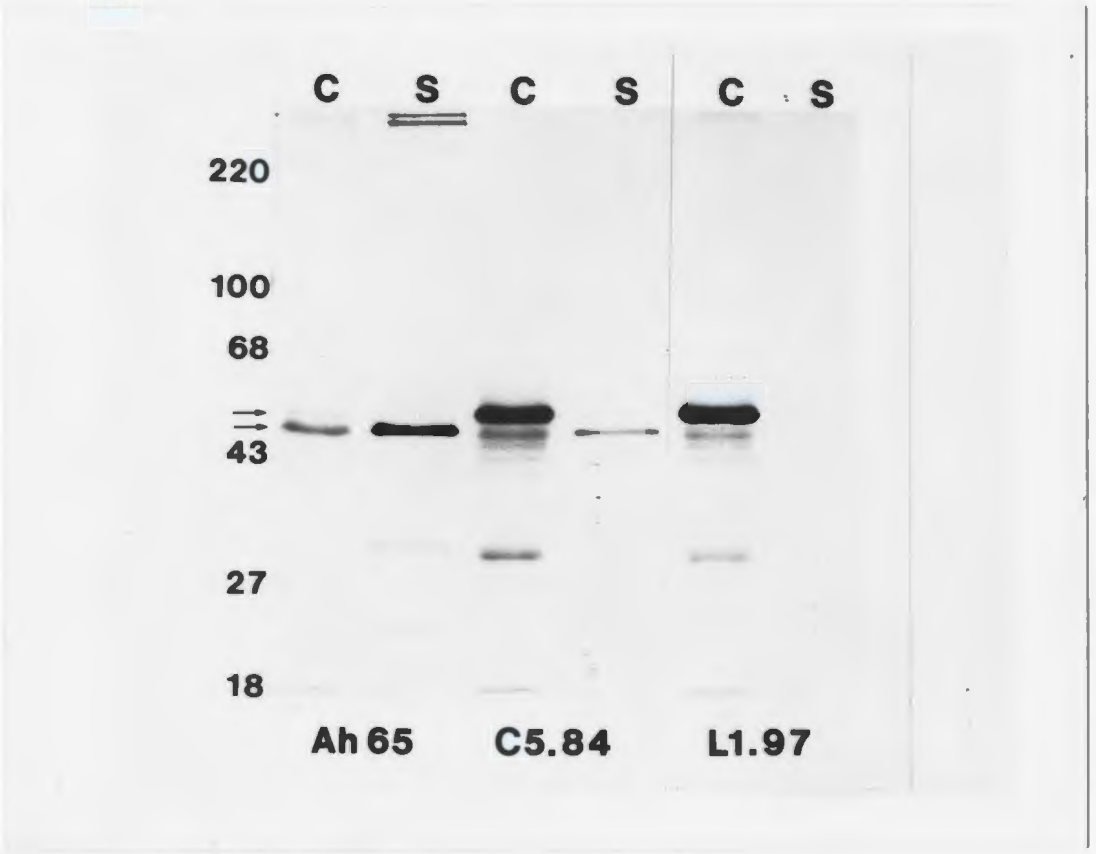


Figure 3.1. Immunoblot analysis of aerolysin in wild-type Ah65 and mutant C5.84 and L1.97 strains. Samples of cells (lane C) and culture supernatants (lane S) representing equal volumes of original cultures (5 μl) were electrophoresed, transferred to nitrocellulose and immunoblotted with an anti-aerolysin antibody. The upper and lower arrows show the positions of proaerolysin and aerolysin, respectively. Numbers at left are M_rs in thousands.

hydrophila can secrete a variety of other extracellular proteins into the growth medium. Thus, in addition to specifically examining synthesis and cellular location of aerolysin in the mutants by immunoblotting, concentrated culture supernatants of the wild-type cells and the mutants were analyzed by SDS-PAGE. The proteins secreted to minimal media by the bacteria are shown in Figure 3.2. Essentially all of the proteins normally present in the supernatant of wild-type cells were missing in either C5.84 or L1.97. This indicated that the mutations caused by the transposon-insertion in these two mutants have pleiotropic effects on the protein secretion process, preventing the export of all the normally secreted proteins.

3.2 Periplasmic accumulation of extracellular proteins by C5.84 and L1.97 cells

To determine the subcellular location within the mutant cells at which the normally secreted extracellular proteins were accumulated, cells were grown to saturation (stationary growth phase) in BHI medium, harvested, and fractionated as described in materials and methods. Samples of the shocked cells, periplasm, plasmolysis wash fluid and culture supernatant were assayed for aerolysin, protease and amylase activity. As controls to monitor the fractionation

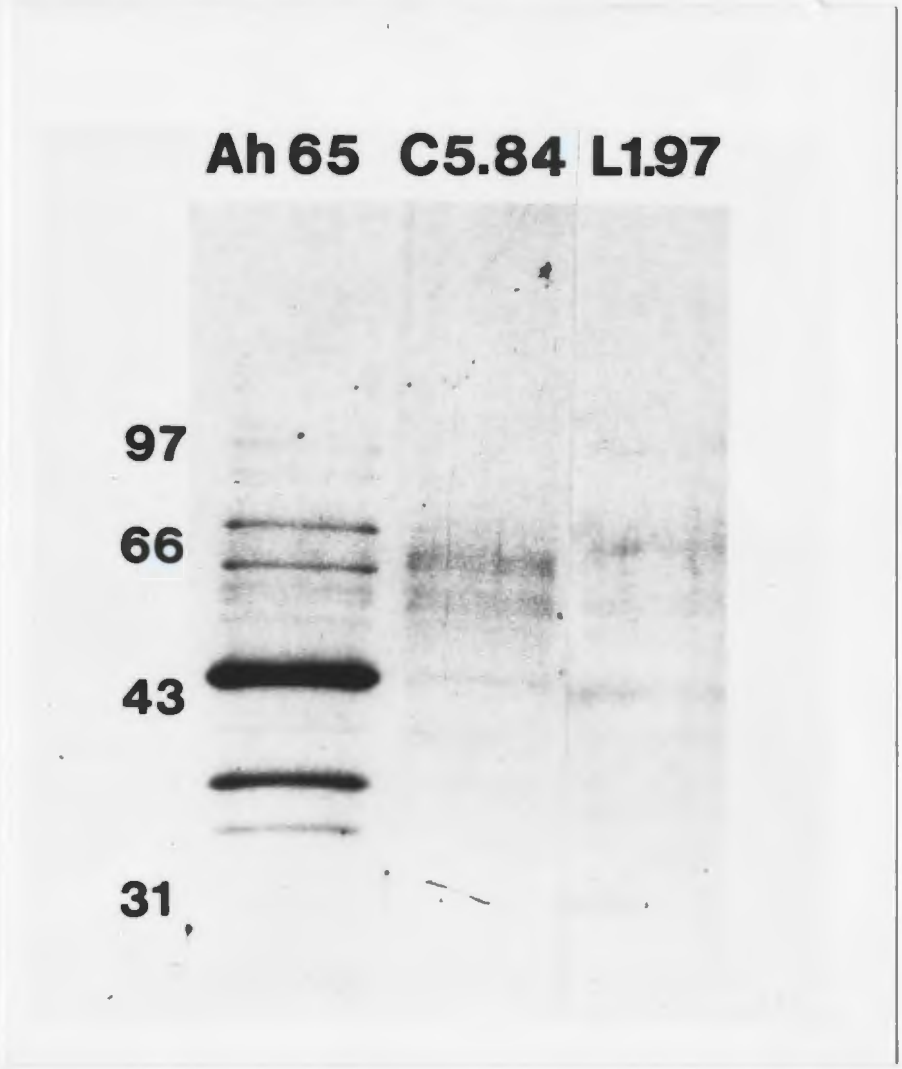


Figure 3.2. SDS-PAGE of extracellular proteins secreted by wild-type and mutant strains in M9 minimal medium culture supernatant. A 10% gel of 10X-concentrated culture supernatants is shown. The gel is stained with Coomassie blue R250. The M_rs of the standards are shown on the left in thousands.

procedure, activities of the intracellular enzyme lactate dehydrogenase (LDH) and the periplasmic enzyme β-lactamase were also measured.

As shown in Table 3.1, aerolysin, protease and amylase activities were mostly found in the supernatant fraction of the wild-type cells, indicating that they are secreted efficiently by these bacteria. For the C5.84 mutant, the largest amount of each of the three activities was detected in the periplasmic fraction. However, this mutant is somewhat leaky, since significant amounts of the activities were also found in the supernatant. In cultures harvested in the log growth phase, as shown in Table 3.2, the extent of the leakage was greatly reduced.

In contrast, the L1.97 mutant displayed a much tighter non-secretory phenotype: only a very small amount of each activity was found in the supernatant fraction (regardless of the growth phase). The pattern of accumulation of the activities was also quite different for the L1.97 mutant. Again, the majority of each of the activities was found to be accumulated in the periplasmic fraction. In this case, however, the majority of the cytoplasmic marker LDH was also released into the periplasm during osmotic shock, whereas very little of it was released during the same procedure for wild-type or C5.84 cells. As shown in Table 3.3, the release of the LDH occurred during osmotic shock regardless of the

Table 3.1 Enzyme activities in subcellular fractions of the mutants grown in BHI

Strain and fraction	Activity of[a]: (%)				
	Aerolysin	Protease	Amylase	LDH[b]	β-lactamase
Ah65					
Shocked-cell	2	0.0(0)	9.8(9)	55.1(96)	0.76(10)
Periplasm	2	0.3(1)	0.0(0)	0.9(2)	6.28(84)
Wash	0	4.2(8)	1.7(2)	1.4(2)	0.32(4)
Supernatant	256	50.1(92)	95.7(89)	0.0(0)	0.13(2)
C5.84					
Shocked-cell	32	5.0(4)	15.4(24)	52.0(96)	0.38(5)
Periplasm	64	87.1(75)	24.3(38)	1.7(3)	6.94(89)
Wash	0	3.9(3)	1.5(3)	0.0(0)	0.17(2)
Supernatant	32	20.0(17)	22.3(35)	0.5(1)	0.30(4)
L1.97					
Shocked-cell	16	10.8(37)	18.0(48)	18.4(29)	0.38(5)
Periplasm	64	14.5(49)	17.5(47)	40.3(64)	6.93(86)
Wash	0	2.4(8)	1.3(4)	2.2(3)	0.36(4)
Supernatant	0	1.7(6)	0.3(1)	2.2(4)	0.38(5)

[a] Aerolysin activities are expressed as titre. All other enzyme activities are expressed as units per milliliter of original culture. Numbers in parentheses are percentages of the total activity obtained from sample.
[b] Lactate dehydrogenase.

Table 3.2 C5.84 fractionation at different growth phases

Growth phase	Activity of[a]:			
	Aerolysin	Protease	Amylase	β-lactamase
Log growth phase				
cell-bound	128	96%	84%	97%
supernatant	0	4%	16%	3%
Stationary phase				
cell-bound	96	83%	65%	96%
supernatant	32	17%	35%	4%

[a] Aerolysin activities are expressed as titres. All other enzyme activities are expressed as the percentages of the total sample activity recovered.

Table 3.3 LDH activities in L1.97 fractionated at different growth phases[a]

Growth phase	Fraction			
	Shocked-cell	Periplasm	Wash	Supernatant
Log growth phase	30%	70%	0%	0%
Stationary phase	29%	64%	3%	4%

[a] Activities are all expressed as the percentages of the total activity recovered.

growth-phase at which the L1.97 cells were harvested.

In attempts to prevent this apparent L1.97 cell lysis during the osmotic shock procedure and to determine the cellular location of the accumulated extracellular proteins unambiguously, similar fractionation experiments were carried out on L1.97 cells grown in minimal medium (Table 3.4). Under these conditions, the cells did not release the cytoplasmic marker glutamate dehydrogenase (GDH) in the osmotic shock procedure and most of the GDH activities could only be detected in the cytoplasmic fraction. The aerolysin, amylase and protease activities, however, were still fractionated along with the periplasmic marker β-lactamase in the osmotic shock fluid, indicating that they are accumulated in the periplasm of L1.97.

These results demonstrated that although the cells of each of the two mutants displayed distinct phenotypes and fractionated quite differently in BHI, the extracellular proteins were all accumulated in the periplasm.

3.3 Analysis of the envelope and outer membrane proteins of the mutants

The results obtained from the fractionation experiments on BHI-grown L1.97 mutant cells indicated clearly that the mutation caused by transposon insertion had rendered these

Table 3.4 Enzyme activities in subcellular fractions of L1.97 grown in M9 Minimal Medium

Fraction	Activity of[a]: (%)				
	Aerolysin	Protease	Amylase	GDH[b]	β-lactamase
Shocked-cell	8	16.9(21)	10.9(23)	8.6(92)	0.09(7)
Periplasm	16	56.5(70)	31.9(66)	0.3(3)	1.01(77)
Wash	0	4.9(6)	5.2(11)	0.3(3)	0.11(9)
Supernatant	0	2.6(3)	0.0(0)	0.2(2)	0.09(7)

[a] Aerolysin activities are expressed as titre. All other enzyme activities are expressed as units/ml of original culture. Numbers in parentheses are percentages of the total activity obtained from sample.
[b] Glutamate dehydrogenase.

mutant cells fragile. To investigate whether there was a structural basis for this fragility, cell envelope and outer membrane samples were prepared and analysed by SDS-PAGE (Figure 3.3). In contrast to C5.84, whose envelope and outer membrane protein contents were essentially the same as that of wild-type cells, the protein profiles of L1.97 cell-envelope and outer membrane were markedly different. A 43 KD protein, which is one of the most abundant proteins of the wild-type cell outer membrane and probably functions as a porin, was almost completely depleted from the outer membrane of L1.97 mutant. The L1.97 outer membrane also contained far less of a major 29 KD protein and of a minor 45 KD protein but more of an abundant protein of 23 KD. Interestingly, the same alterations were also observed in L1.97 membranes prepared from the more stable minimal medium-grown cells (Figure 3.4). It should also be noted that although dramatic changes in the amounts of these major proteins were easily observed, small amounts of each protein could still be detected in L1.97 membranes.

3.4 Cloning of the wild-type chromosomal fragments corresponding to the transposon insertion regions in the mutants

Taking advantage of the feature that the kanamycin and

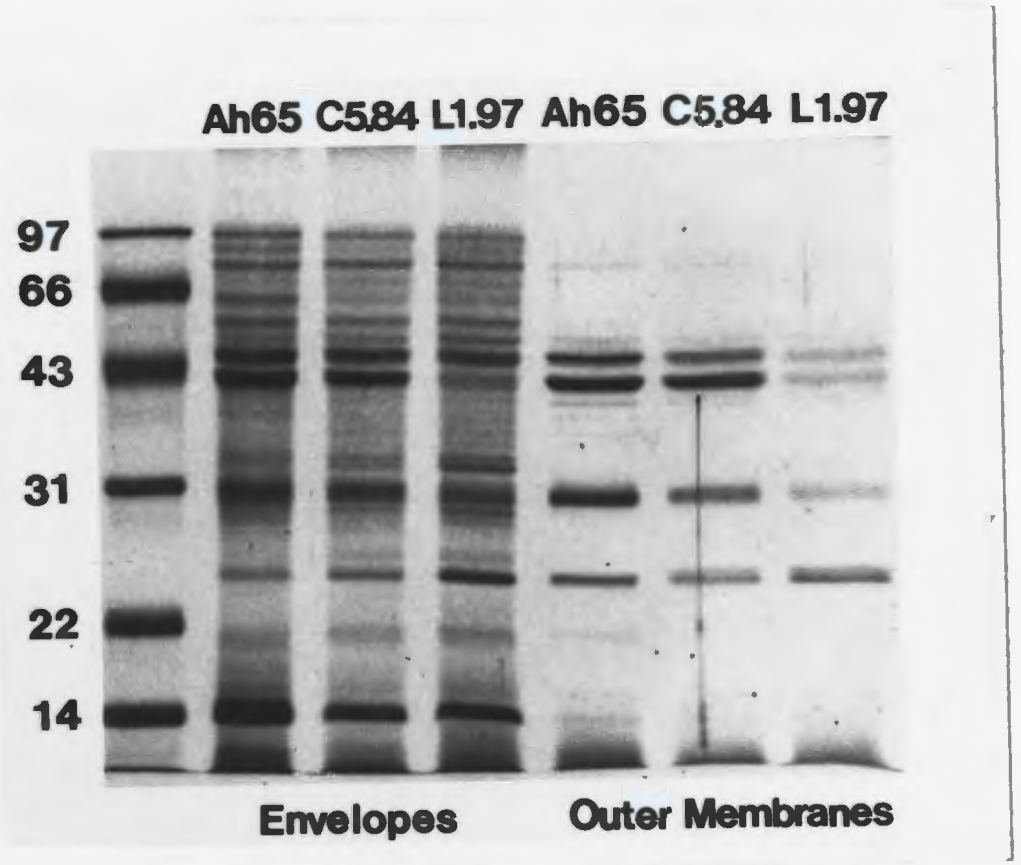


Figure 3.3. SDS-PAGE of membrane proteins of Ah65, C5.84 and L1.97 grown in BHI. Envelope and outer membrane samples were electrophoresed and the gel was stained with Coomassie blue R250. Samples derived from equivalent amounts of cultures (approximately 10^8 viable cells) were applied to each lane. The M_rs of the standards are shown on the left in thousands.

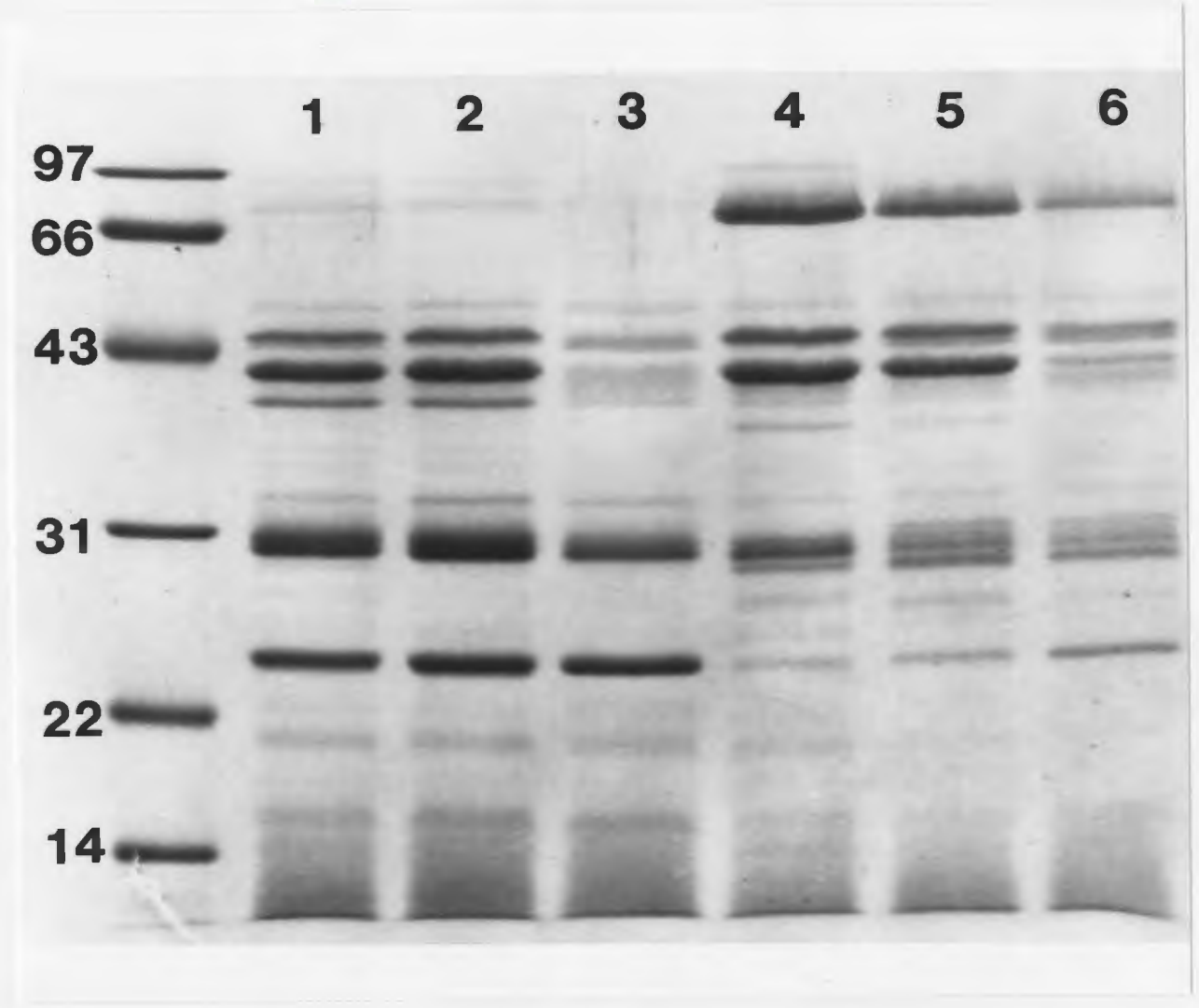


Figure 3.4. SDS-PAGE of outer membrane proteins of Ah65 (lanes 1, 4), C5.84 (lanes 2, 5) and L1.97 (lanes 3, 6) grown in different media. Outer membrane samples prepared from BHI (Lanes 1-3) and DMM (lanes 4-6) were electrophoresed and the gel was stained with Coomassie blue R250. Samples derived from equivalent amounts of cultures were applied to each lane. The M_rs of the standards are shown on the left in thousands.

trimethoprim antibiotic resistance genes encoded by Tn*5-751* are separated by a unique *Eco*RI restriction site, chromosomal DNA bordering the transposon insertion sites for the two mutants was first isolated by the strategy showed in Figure 3.5. Chromosomal DNA from the mutants was first digested to completion by *Eco*RI. Aliquots of the digested DNA were analysed on agarose gels and the fragments containing the transposon sequences were identified by Southern blotting using pME9, which contains the whole Tn*5-751* transposon, as the probe. Then, a scaled-up preparative agarose gel was run and a small piece of agarose containing the transposon-containing DNA fragment was cut out from the gel. The DNA was recovered by electroelution followed by ethanol precipitation and subsequently ligated into the *Eco*RI site of the vector pBR322. The recombinants were plated on LB agar plates containing ampicillin plus trimethoprim and ampicillin plus kanamycin, to select the antibiotic marker carried by the Tn*5-751* transposon. From the L1.97 genome, clone pJB1, containing the Tp^r gene from the transposon Tn*5-751* plus a 2.7 kb fragment of adjacent *Aeromonas hydrophila* chromosomal DNA was isolated; while pJB2, containing a 4.0 kb chromosomal DNA fragment and the Km^r gene was obtained from the C5.84 genome.

Based on the information obtained from partial

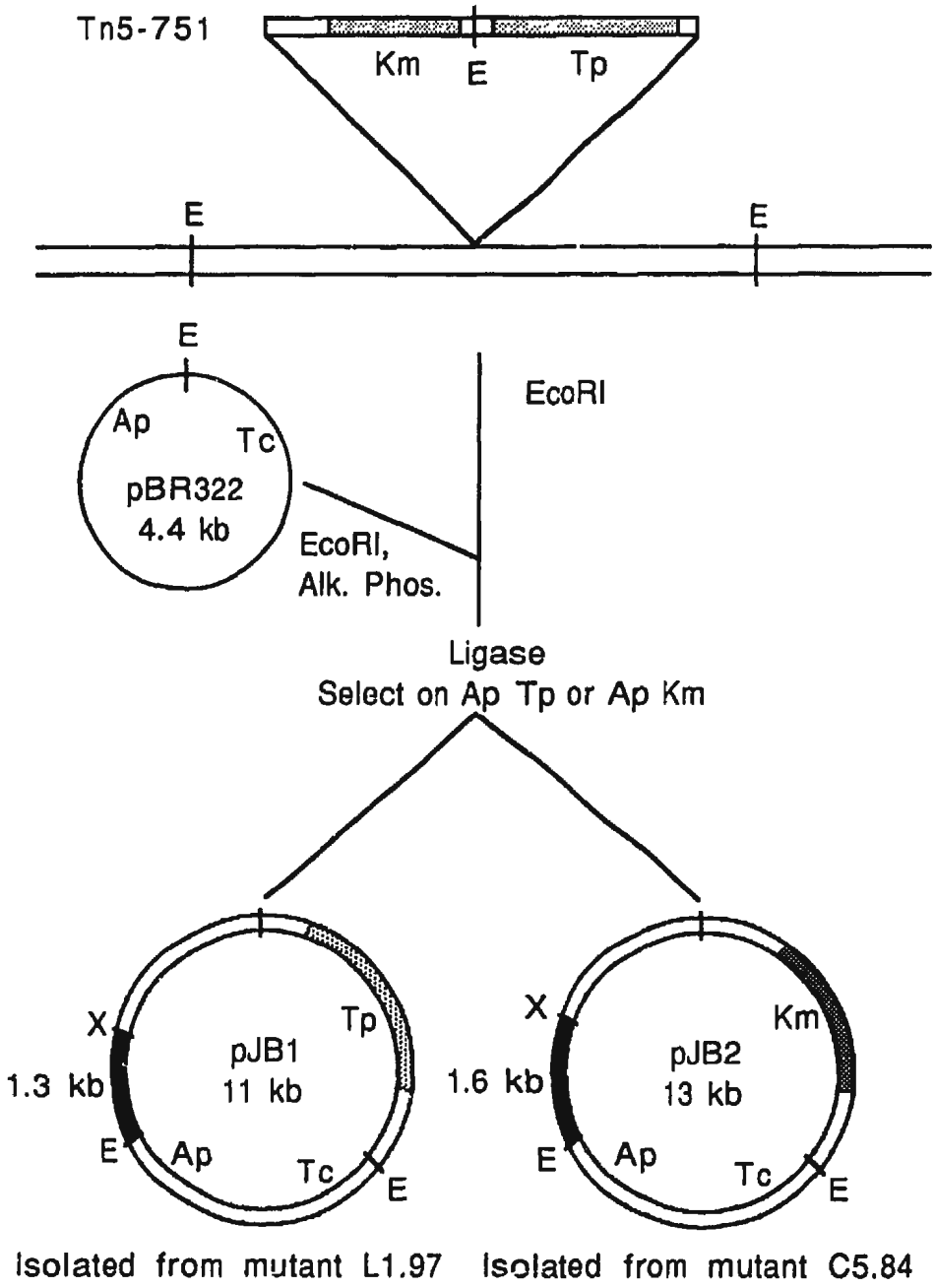


Figure 3.5. Cloning of the transposon insertion sites in L1.97 and C5.84. E, *Eco*RI, X, *Xho*I, Alk.Phos., alkaline phosphatase used to dephosphorylate the vector.

restriction analysis on the inserts in pJB1 and pJB2, a 1.3 kb XhoI-EcoRI chromosomal fragment and a 1.6 kb EcoRI-XhoI chromosomal fragment, from L1.97 and C5.84 respectively, were isolated and biotin-labeled (Figure 3.6). They were used as Aeromonas hydrophila chromosome-specific probes to isolate the corresponding unmutated regions from the wild-type genome, by screening transformant colonies after the ligation of wild type chromosomal DNA into vector pBR322 (Figure 3.7). In this way, clone pJB3, which contains an 11 kb insert of wild type Aeromonas hydrophila chromosomal DNA corresponding to the transposon insertion region in L1.97, and clone pJB4, with a 15 kb chromosomal DNA fragment corresponding to the insertion site in C5.84, were isolated. The inserts were then transfered into the wide-host-range plasmid pVK101, yielding pJB5 and pJB6 respectively, to allow transfer of the fragments back into Aeromonas hydrophila cells.

3.5 Southern blot analysis of L1.97 and C5.84 with the cloned chromosomal fragments

Southern blotting analysis was performed to verify the relationship between the isolated clones and the orignal transposon insertion regions in the mutants. EcoRI digested chromosomal DNA from Ah65, C5.84 and L1.97 were hybridized with digoxigenin-labeled probes derived either from the

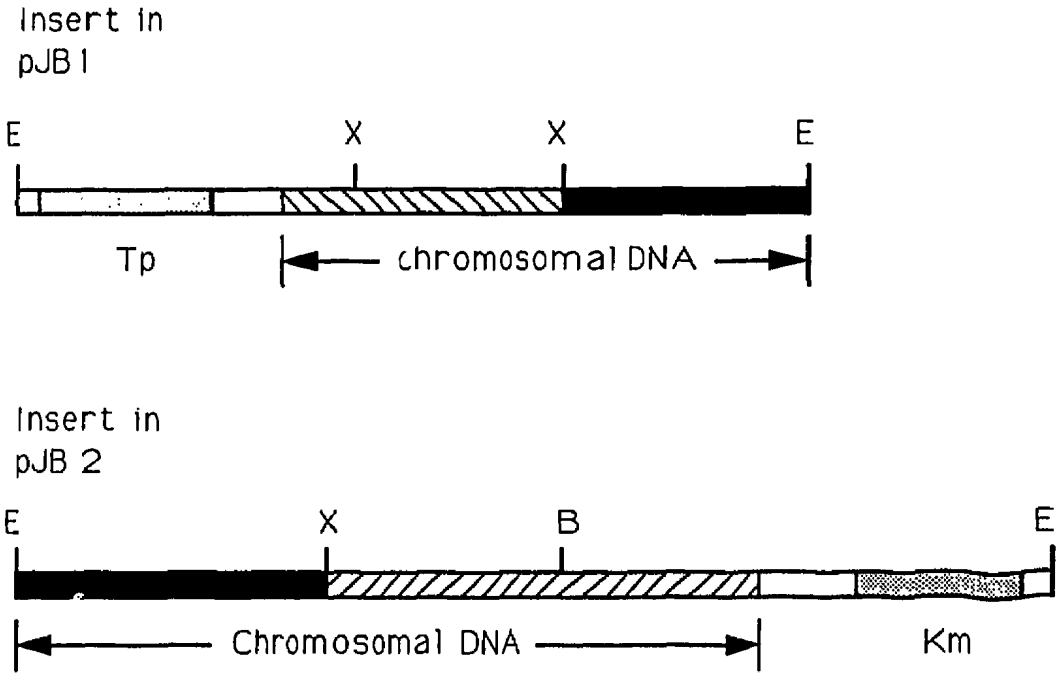


Figure 3.6. Partial restriction maps of inserts in pJB1 and pJB2. The antibiotic resistance genes from Tn5-751 are shown. The black-shaded region in each of the inserts represents the chromosome-specific probe later used in colony hybridization experiment to isolate the un-mutated DNA fragment from wild-type genome. E, *Eco*RI; B, *Bgl*II; X, *Xho*I.

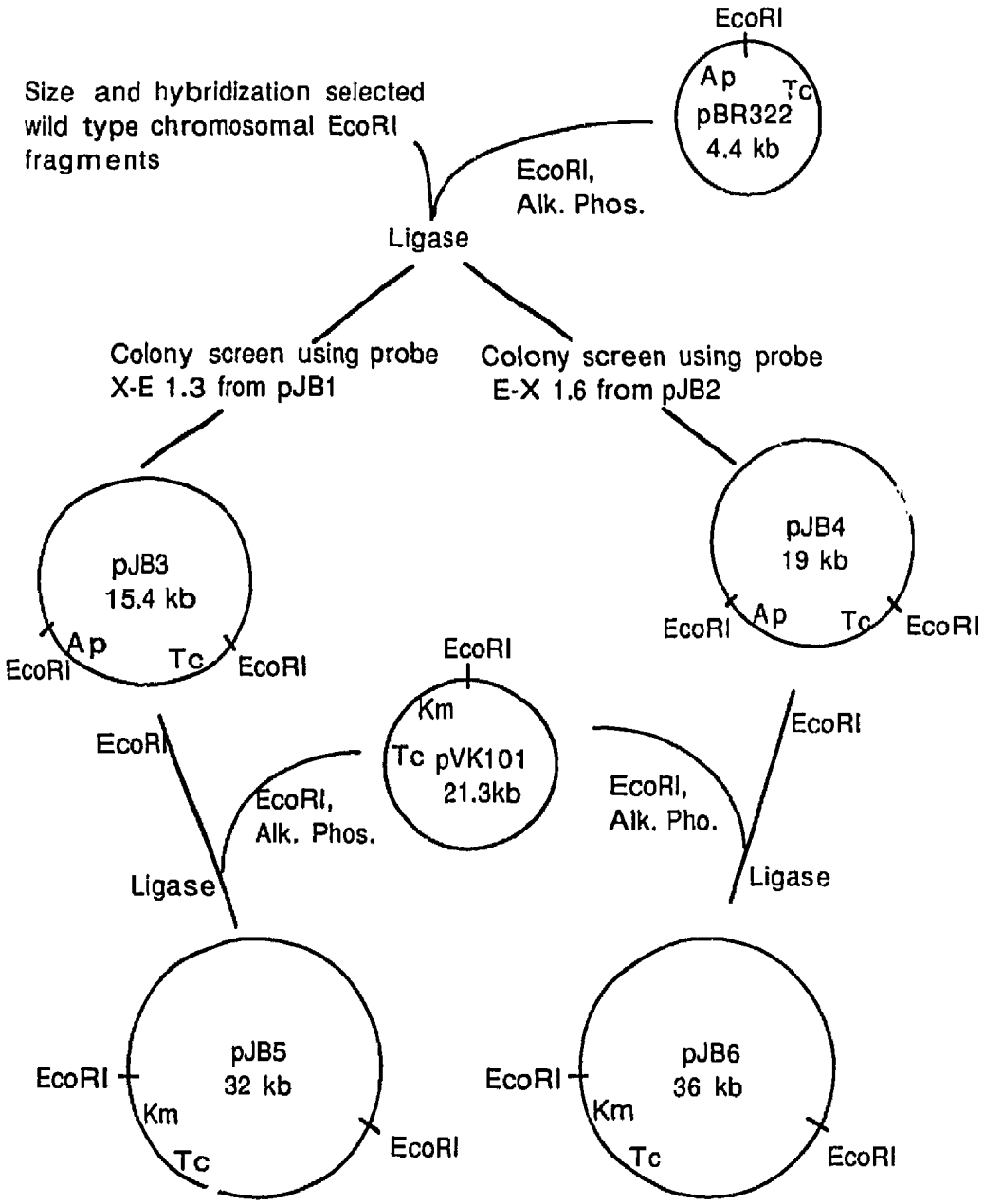


Figure 3.7. Cloning of the extracellular export genes from wild-type genome. The figure shows the strategy used to clone the un-mutated chromosomal regions corresponding to the Tn5-751 insertion sites in the mutants. The origins of probes X-E 1.3 and E-X 1.6 are shown in Figure 3.6. Alk. Phos., alkaline phosphatase.

chromosomal fragments adjacent to the transposon sequences in plasmids pJB1 and pJB2 or from the entire inserts in plasmids pJB3 and pJB4. The region corresponding to each of the probes as well as the site of transposon insertion in the mutants is shown in Figure 3.8. As shown in the Southern blot (Figure 3.9), probe X-E 1.3, the sequence adjacent to the insertion site in L1.97, hybridized to an 11 kb *Eco*RI fragment in both Ah65 and C5.84 cells, but hybridized to a 7.2 kb fragment in L1.97 cells, indicating that the 11kb wild-type chromosomal *Eco*RI fragment was interrupted by the transposon in the L1.97 mutant but not in C5.84. The E-E 13 probe from pJB3 represents this entire *Eco*RI fragment, it hybridized again to the 11 kb fragment in Ah65 and C5.84 cells but hybridized to two fragments, i.e. both sides of the interrupted fragment, in the L1.97 cells. A similar situation existed for the clones corresponding to the C5.84 mutation: probes derived from the insertion site of C5.84 hybridized to a 15 kb *Eco*RI fragment in the Ah65 and L1.97 cells but to the interrupted fragments in C5.84 cells.

These results demonstrated that the cloned inserts in pJB3 and pJB4 indeed represented the regions of the wild-type chromosome into which the transposon had been inserted in L1.97 and C5.84. The results also confirmed that the transposon had been inserted into different regions of the chromosome in the two mutants since the Southern blot

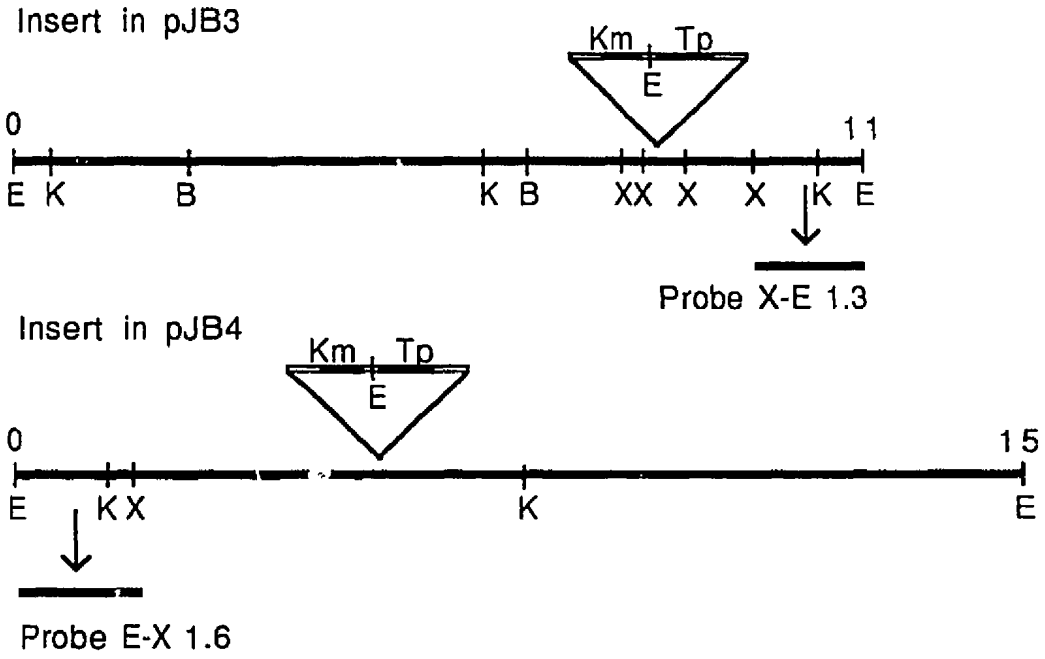


Figure 3.8. Partial restriction maps of the isolated chromosomal fragments. The predicted transposon insertion sites and probes used for subsequent Southern blotting are shown. E, *Eco*RI; K, *Kpn*I; B, *Bgl*II; X, *Xho*I.

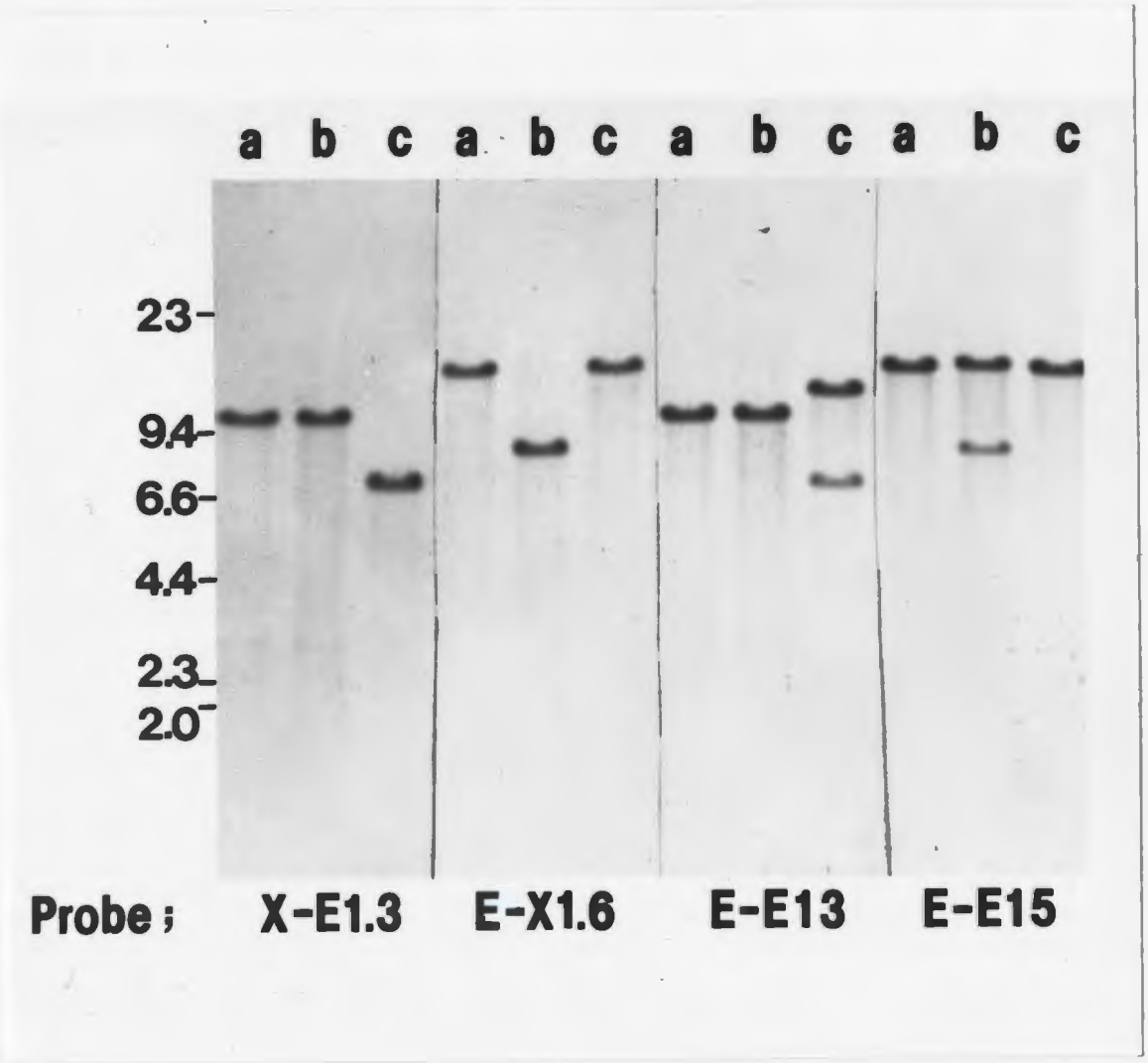


Figure 3.9. Southern blot analysis of Ah65, C5.84 and L1.97 chromosomal DNA. Approximately 3 μg of EcoRI-digested chromosomal DNA from Ah65 (lane a), C5.84 (lane b) and L1.97 (lane c) were electrophoresed, transferred to nitrocellulose and hybridized with each of the four probes (see Figure 3.8) indicated. DNA size standards are shown on the left in kb.

patterns were completely different.

3.6 Complementation of the mutants by the cloned wild-type chromosomal fragments

The wide-host-range plasmids pJB5 (containing the region corresponding to the transposon insertion site in L1.97) and pJB6 (containing the region corresponding to the transposon insertion site in C5.84) were transferred into the mutants, with the help of the conjugative helper plasmid pRK2013. To examine the aerolysin distribution directly in the transconjugants, samples from different cellular fractions of the transconjugant cells were immunoblotted with the anti-aerolysin monoclonal antibody (Figure 3.10). Both the 54 KD proaerolysin and 51.4 KD active aerolysin forms could be seen in the various fractions. As can be seen from the immunoblots, plasmid pJB5 complemented the mutation in L1.97 cells, so that the transconjugants exported most of the aerolysin to the supernatant, as did the wild-type cells. The pJB6 was unable to complement the mutation in L1.97, therefore most of the aerolysin was still accumulated in the periplasmic fraction of L1.97(pJB6). As expected, pJB6 complemented C5.84 successfully: C5.84(pJB6) cells, like the wild-type cells, secreted most of the aerolysin into the supernatant (Figure 3.10). In addition, however, C5.84 cells

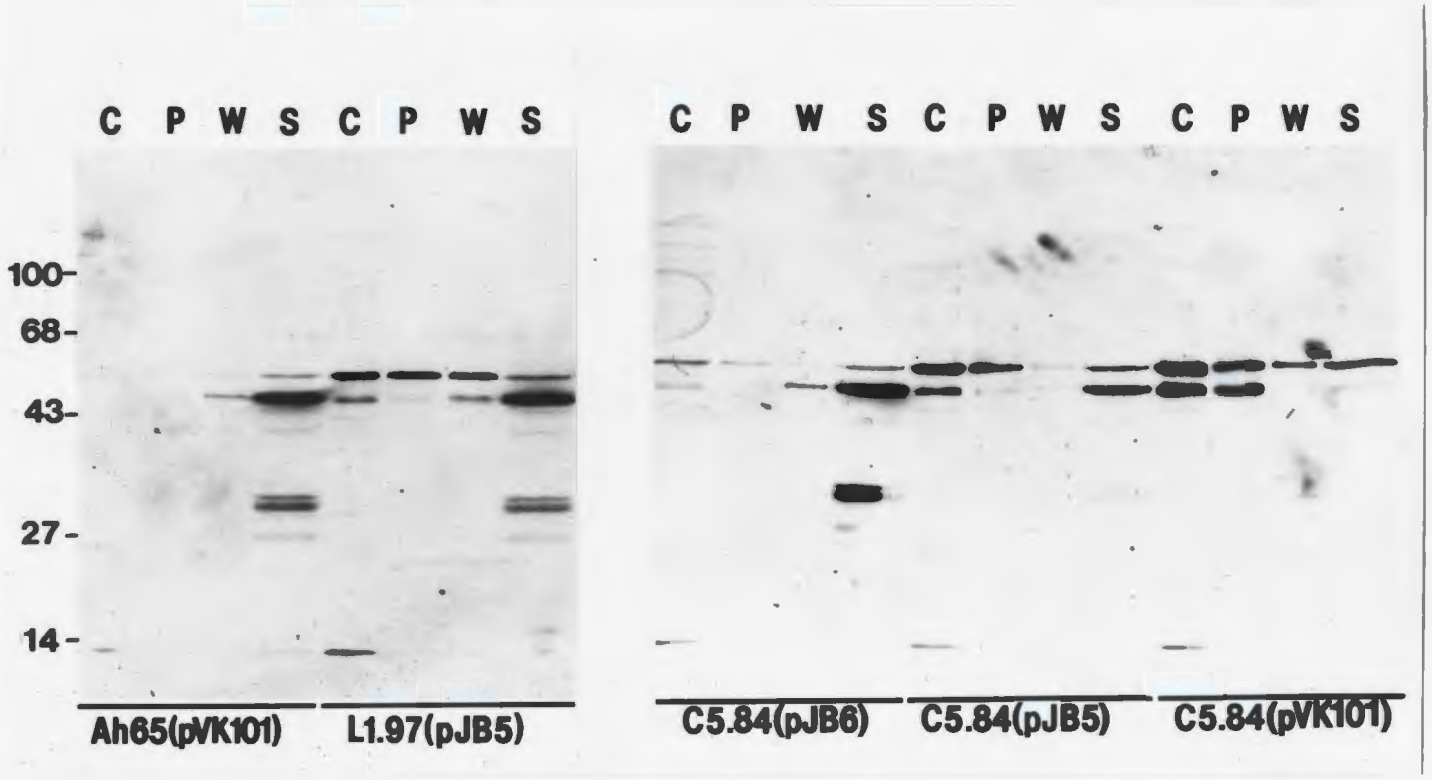


Figure 3.10. Immunoblot analysis of aerolysin in the transconjugants. Different transconjugants were fractionated and each of the subcellular fractions was immunoblotted against an anti-aerolysin antibody. C, shocked-cell; P, periplasm; W, wash fluid; S, supernatant. Each lane represents an equal amount (5μl) of original culture. Numbers at left are M_rs in thousands. Refer to Figure 3.1 for comparison with the L1.97 profile.

carrying pJB5, which contains the insert corresponding to the transposon insertion region in L1.97 mutant, also exported considerably more aerolysin than did C5.84(pVK101).

Further enzyme activity assays confirmed the complementation results for aerolysin obtained by immunoblotting and extended them to the protease and amylase activities (Table 3.5). Both L1.97(pJB5) and C5.84(pJB6) showed the wild-type secretion phenotype, secreting the aerolysin, protease and amylase efficiently into the supernatant. The pJB5 also partially restored the C5.84 mutant's capability for extracellular export for all three proteins. The fact that most of the activity of the periplasmic enzyme β-lactamase was still found in the periplasmic fraction of all the transconjugants demonstrated clearly that the complementation by pJB5 or pJB6 was specific, and was not due to any artifact such as leakage of the accumulated proteins resulting from the presence of multiple copies of the genes carried by these plasmids.

As another examination of the specific complementation of L1.97, outer membrane samples from L1.97 cells containing plasmid pJB5, pJB6 or the vector pVK101 were prepared and analysed by SDS-PAGE. The Coomassie blue-stained gel shown in Figure 3.11 showed clearly that pJB5 restored the wild-type protein profile to the outer membrane of this mutant.

Table 3.5 Complementation of L1.97 and C5.84 with pJB5 and pJB6

Strain and fraction	Activity[a](%)			
	Aerolysin	Protease	Amylase	β-lactamase
Ah65(pVK101)				
Shocked-cell	2	0.25(3)	1.5(13)	0.8(7)
Periplasm	2	0.37(4)	0.7(1)	8.2(80)
Wash	0	0.68(8)	1.8(15)	0.4(4)
Supernatant	128	7.14(85)	8.5(72)	0.4(4)
L1.97(pJB5)				
Shocked-cell	2	0.25(3)	0.7(8)	0.7(10)
Periplasm	2	0.30(3)	0.0(0)	4.0(59)
Wash	0	0.69(7)	0.9(11)	1.3(19)
Supernatant	256	8.16(87)	6.7(81)	0.8(12)
C5.84(pJB6)				
Shocked-cell	4	0.36(4)	1.2(9)	0.4(4)
Periplasm	8	0.82(8)	2.0(14)	8.5(75)
Wash	0	0.60(6)	1.9(14)	1.3(12)
Supernatant	512	8.43(83)	8.2(62)	1.1(10)
C5.84(pJB5)				
Shocked-cell	4	0.56(6)	1.2(10)	0.6(6)
Periplasm	8	1.10(12)	1.6(14)	6.6(69)
Wash	0	0.73(8)	1.3(12)	1.3(14)
Supernatant	128	6.81(74)	7.1(63)	1.0(11)

[a] Aerolysin activities are expressed as titre. All other enzyme activities are expressed as units/ml of original culture. Numbers in parentheses are percentages of the total activity obtained from the sample.

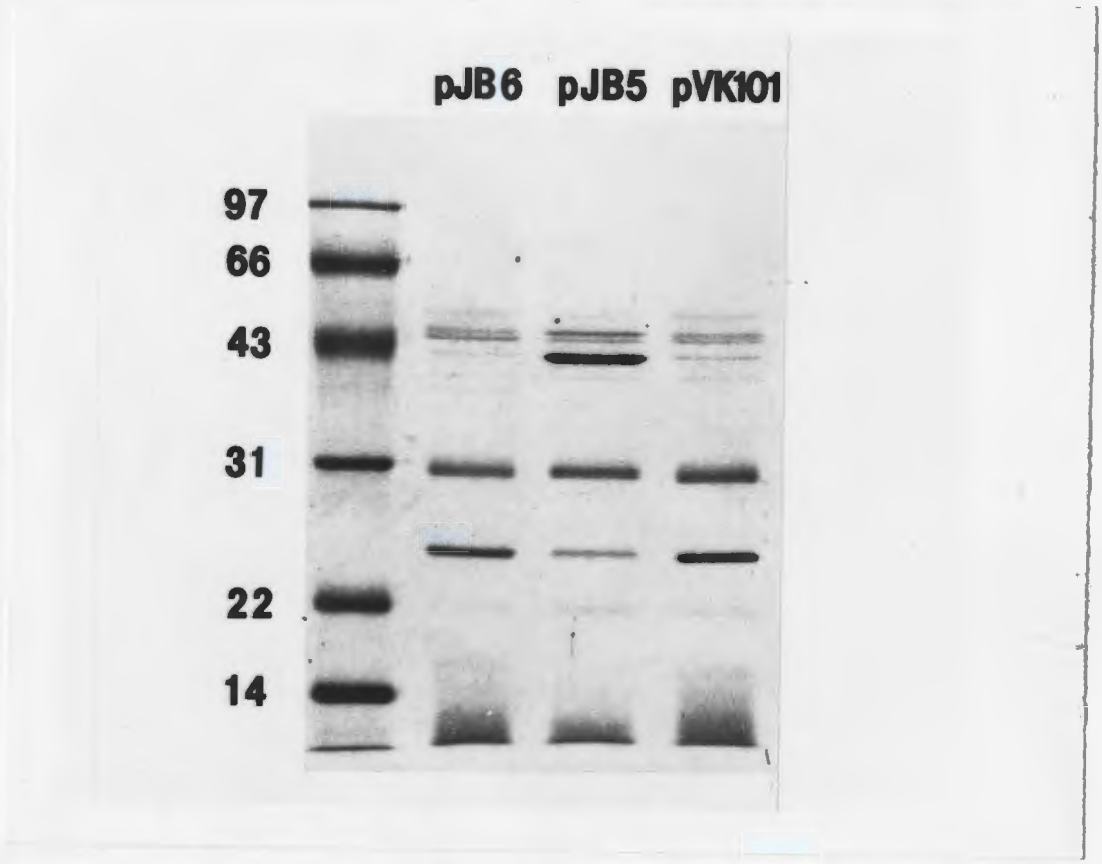


Figure 3.11. Outer membrane protein profiles of L1.97 transconjugants. Outer membrane samples prepared from L1.97 cells containing the plasmids indicated were electrophoresed and the gel was stained with Commassie blue. Samples derived from equivalent amounts of cultures were applied to each lane. The M_rs of the standards are shown on the left in thousands. Refer to Figure 3.3 for comparison with the Ah65 profile.

3.7 Mapping and subcloning of the extracellular export (*exe*) gene located on pJB3

As a first attempt in the analysis of the *exe* gene carried on pJB3, a partial restriction map of the 11 kb insert was constructed (Figure 3.12) and the *Bgl*II-*Eco*RI fragment containing the transposon insertion region was subcloned in pVK101, to give rise to pJB9. When transferred into L1.97 by conjugation, this construct failed to complement the mutation, thus, the phenotype of the transconjugants remained non-hemolytic on blood agar. This suggested that some DNA on the left of the *Bgl*II site, as depicted in Figure 3.12, was required for the secretion function. This was later confirmed by the fact that, when the 4.1 kb *Kpn*I fragment was subcloned into the *Kpn*I site of the pMMB67HE.tet vector to give rise to pMJB8.1 and introduced into L1.97, it complemented the mutation and restored the hemolytic phenotype. However, complementation was not observed when the same 4.1 kb *Kpn*I fragment was cloned in the opposite orientation with respect to the *tac* promotor carried by the vector, indicating that the transcription of the *exe* gene(s) on this fragment required the presence of a heterologous promotor.

To investigate the transcription of this *exe* gene in more detail, the transconjugants were grown in liquid growth media with or without the presence of IPTG and the aerolysin

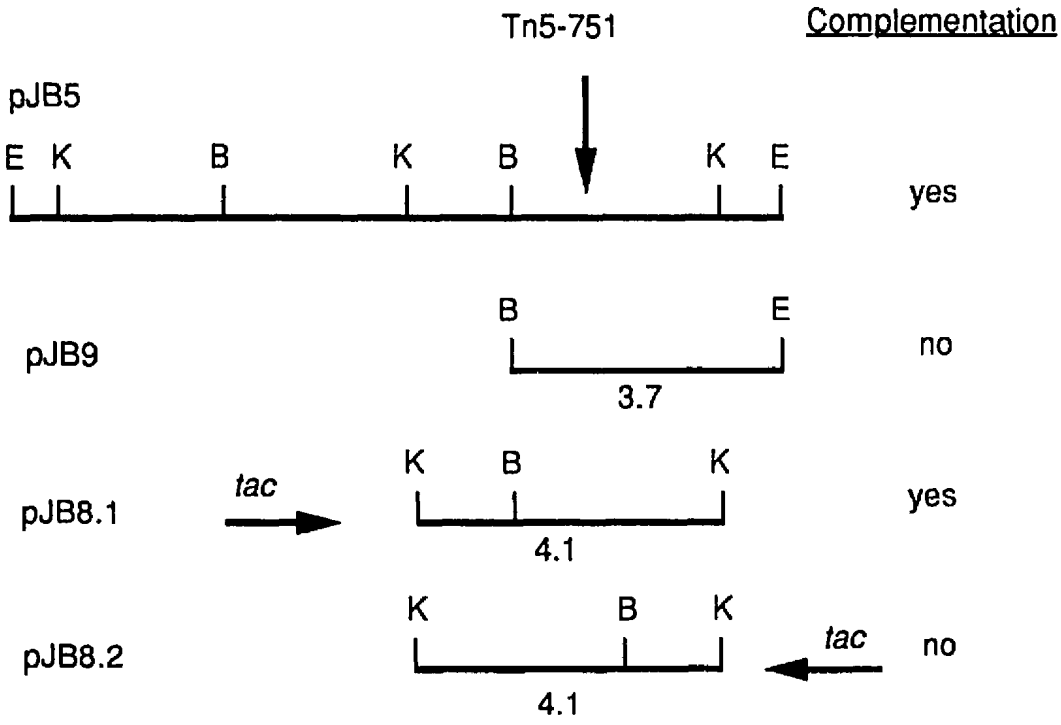


Figure 3.12. Subcloning of the *exe* gene on the 11 kb fragment. The vertical arrow indicates the predicted transposon insertion site. Horizontal arrows indicate the tac promotor transcription direction. Restriction sites: E, *Eco*RI; K, *Kpn*I; B, *Bgl*II.

activities secreted into the supernatant were assayed. The results in Table 3.6 showed clearly that complementation could be achieved only when the transcription of the *exe* gene in pMJB8.1 was initiated by inducing the *tac* promotor with IPTG. The small amount of aerolysin activity present in the supernatant of L1.97(pMJB8.1) without IPTG could be ascribed to the partial induction of the *tac* promotor by inducers present in the BHI medium. This was later confirmed by the results obtained from L1.97(pMJB8.1) grown in minimal medium, in which no aerolysin activity was detected in the absence of IPTG (Table 3.7).

These results demonstrated that the 4.1 kb *Kpn*I fragment contained the promotorless structural *exe* gene involved in the extracellular protein export process and that its transcription direction is from left to right as shown in Figure 3.12.

3.8 DNA sequencing

In order to define the *exe* gene more precisely, the sequence of both DNA strands of the 4.1 kb *Kpn*I fragment that complemented the mutation of L1.97 was determined and the complete sequence is shown in Figure 3.13.

Computer analysis of the entire sequence showed that this fragment had an operon-like structure with four large open

Table 3.6 Extracellular aerolysin activities of transconjugants grown in BHI

Transconjugants		Activity[a]
Ah65(mmb67HE.tet)	- IPTG	64
	+ IPTG	64
L1.97(mmb67HE.tet)	- IPTG	0
	+ IPTG	0
L1.97(pMJB8.1)	- IPTG	32
	+ IPTG	256
L1.97(pMJB8.2)	- IPTG	0
	+ IPTG	0

[a] Aerolysin activities are expressed as titres.

```
     ----  'ORF 1
   1 CACTACGGGTCTTGCCAAGCTGGCTGAGAATTTTAACGGCATGGCGGCCGGTTTCTATCA   60
      ThrThrGlyLeuAlaLysLeuAlaGluAsnPheAsnGlyMetAlaAlaGlyPheTyrGl

  61 AGGTAACTGGGCCATGTTGGTGACGGCACTGTCGACCAACACCAAGAGCGATATTCTCTC   120
     nGlyAsnTrpAlaMetLeuValThrAlaLeuSerThrAsnThrLysSerAspIleLeuSe

 121 CACCCCGAGTATCGTCACCATGGATAACAAGGAAGCCTCCTTCAACGTCGGTCAGGAAGT   180
     rThrProSerIleValThrMetAspAsnLysGluAlaSer'heAsnValGlyGlnGluVa

 181 GCCGGTGCAGACGGGCACCCAGAACTCCACCTCCGCTGACACCACCTTTAGCACCATAGA   240
     lProValGlnThrGlyThrGlnAsnSerThrSerGlyAspThrThrPheSerThrIleGl

 241 GCGCAAGACTGTGGGCACCAAGCTGGTGGTGACGCCGCAGATCAACGAAGGGGACTCGGT   300
     uArgLysThrValGlyThrLysLeuValValThrProGlnIleAsnGluGlyAspSerVa

 301 GCTGCTGACCATAGAGCAGGAGGTCTCCAGCGTGGGCAAACAAGCGACAGGTACGGACGG   360
     lLeuLeuThrIleGluGlnGluValSerSerValGlyLysGlnAlaThrGlyThrAspGl

 361 TCTGGGCCCCACCTTCGATACCCGTACAGTCAAGAACGCCGTGCTGGTGAAAAGCGGTGA   420
     yLeuGlyProThrPheAspThrArgThrValLysAsnAlaValLeuValLysSerGlyGl

 421 GACTGTGGTGCTGGGTGGCCTGATGGATGAGCAGACCAAGGAAGAGGTTTCCAAGGTGCC   480
     uThrValValLeuGlyGlyLeuMetAspGluGlnThrLysGluGluValSerLysValPr

 481 GCTGCTCGGGGATATCCCTGTACTCGGCTATCTGTTCCGTTCCACTTCCAATAACACTTC   540
     oLeuLeuGlyAspIleProValLeuGlyTyrLeuPheArgSerThrSerAsnAsnThrSe

 541 CAAGCGCAACCTGATGGTCTTTATCCGGCCCACCATATTGCGGGATGCAAACGTCTACTC   600
     rLysArgAsnLeuMetValPheIleArgProThrIleLeuArgAspAlaAsnValTyrSe

 601 AGGCATTTCCAGCAACAAGTACACCCTCTTCCGTGCTCAACAGCTAGATGCTGTTGCTCA   660
     rGlyIleSerSerAsnLysTyrThrLeuPheArgAlaGlnGlnLeuAspAlaValAlaGl

 661 AGAAGGATATGCCACCTCACCGGATCGTCAGGTGCTGCCTGAATATGGCCAGGATGTGAC   720
     nGluGlyTyrAlaThrSerProAspArgGlnValLeuProGluTyrGlyGlnAspValTh

 721 CATGTCTCCCGAGGCGCAGAAGCAGATCGAGCTGATGAAAACACACCAGCAGGCGACGGC   780
     rMetSerProGluAlaGlnLysGlnIleGluLeuMetLysThrHisGlnGlnAlaThrAl
                                        ----  ORF 2
 781 CGACGGAGTGCAACCATTCGTTCAGGGTAACAAGTAATGGCTGCGTACCAGCTTGATGAC   840
     aAspGlyValGlnProPheValGlnGlyAsnLys* MetAlaAlaTyrGlnLeuAspAsp

 841 ACGAGTCTGCCTGCAGCCCTGCCTGAGCTGCCGTTCGCCTTTGCCCGCAACTTCGGTGTC   900
     ThrSerLeuProAlaAlaLeuProGluLeuProPheAlaPheAlaArgAsnPheGlyVal

 901 GTTCTGACCGAGCGGCAGGGGACCCCCCTGCTGCTGTGCCGTCCTGGGGTGGCGCCGCAA   960
     ValLeuThrGluArgGlnGlyThrProLeuLeuLeuCysArgProGlyValAlaProGln

 961 ACCCTGCTGGAGGTACGGCGAGTGGCGGGCTGCGCCTTCGAGGTGGAGCAGCTCGGCAGT   1020
     ThrLeuLeuGluValArgArgValAlaGlyCysAlaPheGluValGluGlnLeuGlySer

1021 GACGAGTTTGAAGAGCTGCTGATGGCGCACTACCAGCGCGACTCCTCCGAGGCGCGCCAG   1080
     AspGluPheGluGluLeuLeuMetAlaHisTyrGlnArgAspSerSerGluAlaArgGln
```

Table 3.7 Extracellular aerolysin activities[a] of transconjugant L1.97(pMJB8.1) grown in DMM

	Growth phase	
	Log phase	Stationary phase
- IPTG	0	0
+ IPTG	32	64

[a] Aerolysin activities expressed in titres.

BglII

1081 CTGATGGAAGATCTCGGCAACGAGATGGACTTCTTCGCCCTGGCGGAAGAGCTGCCGCAA 1140
LeuMetGluAspLeuGlyAsnGluMetAspPhePheAlaLeuAlaGluGluLeuProGln

1141 AGCGAGGATCTGCTGGACGCCGATGACGACGCCCCCATCATTCGCCTCATCAATGCCATG 1200
SerGluAspLeuLeuAspAlaAspAspAspAlaProIleIleArgLeuIleAsnAlaMet

1201 TTGAGCGAGGCGATAAAAGAGGAGGCGTCTGACATCCATATCGAGACCTTCGAGCGGGTG 1260
LeuSerGluAlaIleLysGluGluAlaSerAspIleHisIleGluThrPheGluArgVal

1261 CTGGTCATTCGTTTCCGCATCGATGGCGTGCTGCGGGAGATACTGCGCCCCCATCGCAAG 1320
LeuValIleArgPheArgIleAspGlyValLeuArgGluIleLeuArgProHisArgLys

1321 CTGGCCTCCCTGCTGGTGTCGCGCATCAAGGTCATGTCGCGGATGGACATAGCCGAGAAG 1380
LeuAlaSerLeuLeuValSerArgIleLysValMetSerArgMetAspIleAlaGluLys

1381 CGGGTGCCCCAGGATGGCCGCATCTCGCTGCGTATCGGTGGCCGGGCGGTGGACGTGCGG 1440
ArgValProGlnAspGlyArgIleSerLeuArgIleGlyGlyArgAlaValAspValArg

1441 GTCTCCACCATGCCGTCCAGCTACGGCGAGCGGGTGGTATTGCGTCTGCTCGACAAGAAC 1500
ValSerThrMetProSerSerTyrGlyGluArgValValLeuArgLeuLeuAspLysAsn

1501 AACGTGCGCCTCGAGCTCAAGCAGCTCGGCATGACGCTGGCCAACCGCAACATCATCAGC 1560
AsnValArgLeuGluLeuLysGlnLeuGlyMetThrLeuAlaAsnArgAsnIleIleSer

1561 GAACTCATTCGCAAGCCCCACGGCATCATATTGGTGACGGGCCCGACCGGTTCGGGCAAG 1620
GluLeuIleArgLysProHisGlyIleIleLeuValThrGlyProThrGlySerGlyLys

1621 TCCACCACCCTGTATGCGGCGCTCTCCGAGATCAACTCGCGGGATCGCAACATTCTCACG 1680
SerThrThrLeuTyrAlaAlaLeuSerGluIleAsnSerArgAspArgAsnIleLeuThr

1681 GTGGAAGACCCTATCGAATATGACCTGGAGGGTGTGGGTCAGACTCAGGTCAACACCAAG 1740
ValGluAspProIleGluTyrAspLeuGluGlyValGlyGlnThrGlnValAsnThrLys

1741 GTGGACATGACCTTCGCCCGCGGCCTGCGCGCCATTCTGCGTCAGGATCCGGATGTGGTG 1800
ValAspMetThrPheAlaArgGlyLeuArgAlaIleLeuArgGlnAspProAspValVal

1801 ATGGTGGGGGAAATTCGCGACCTCGAGACCGCCCAGATAGCGGTGCAGGCGTCGCTGACC 1860
MetValGlyGluIleArgAspLeuGluThrAlaGlnIleAlaValGlnAlaSerLeuThr

1861 GGTCACCTGGTGATGTCGACCCTGCACACCAACACCGCCATAGGTGCCATCACCCGGATG 1920
GlyHisLeuValMetSerThrLeuHisThrAsnThrAlaIleGlyAlaIleThrArgMet

1921 CGGCACATGGCGATAGAGCCTTTCCTGCTCTCCTCGTCCCTGTTGGCGGTTCTGGCCCAG 1980
ArgAspMetGlyIleGluProPheLeuLeuSerSerSerLeuLeuAlaValLeuAlaGln

1981 CGGCTGGTGCGGACCCTCTGCCCGGATTGCCGCGCCCCGCGCCCCATCACGGAGCAGGAG 2040
ArgLeuValArgThrLeuCysProAspCysArgAlaProArgProIleThrGluGlnGlu

2041 CGTCTGGCCATGGGCATGGAACTGGCGCCGGATCAGCAGGTGTGGCGGCCGGTGGGCTGC 2100
ArgLeuAlaMetGlyMetGluLeuAlaProAspGlnGlnValTrpArgProValGlyCys

2101 GAGCAGTGCAACCACACCGGCTATCGCGGCCGGACCGGCATTCACGAGCTGGTGGTGATC 2160
GluGlnCysAsnHisThrGlyTyrArgGlyArgThrGlyIleHisGluLeuValValIle

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2161  GACGAGGCGGTGCGCGAAGCGATCCACAGCGCCAGCGGCGAGCTGGCCATCGAGCGGCTG  2220
      AspGluAlaValArgGluAlaIleHisSerAlaSerGlyGluLeuAlaIleGluArgLeu

2221  ATCCGGGACCATACCCCCAGCATACGCCGCGACGGCATCGACAAGGTGCTCAAGGGGCAG  2280
      IleArgAspHisThrProSerIleArgArgAspGlyIleAspLysValLeuLysGlyGln

2281  ACCAGCCTCGAAGAGGTGCTGCGCGTGACCCGGGAAGATTGATGTTTGGTCAGGCAAGCG  2340
      ThrSerLeuGluGluValLeuArgValThrArgGluAsp*

2341  ATTTGAATCGGGATAACGAGACGTCACAGTGCTAGGGCAGAGGAGCAAGAGTATTGTCAG  2400
                                                    ----  ORF 3
2401  CATTTGAATACAAAGCCCTCGACAGCAAGGGGCGCCAGAAGCAGGGGGTGACCGAGGGTG  2460
                                                    ValThrGluGlyA

2461  ACTCGGCCCGTCAGGTGCGCCAGCAACTGCGTGAGCAGGGGCTGACCCCGCTCGAGGTGA  2520
      spSerAlaArgGlnValArgGlnGlnLeuArgGluGlnGlyLeuThrProLeuGluValA

2521  ACGAGACCACAGAGAAGGCAAAGCGGGAAGCGAATCGCTTCGTGCTGTTTCGCCGTGGCG  2580
      snGluThrThrGluLysAlaLysArgGluAlaAsnArgPheValLeuPheArgArgGlyA

2581  CCTCCACCTCAGAGCTGGCCCTCATCACCCGTCAGCTGGCGACCCTGGTGGGCGCAGGGC  2640
      laSerThrSerGluLeuAlaLeuIleThrArgGlnLeuAlaThrLeuValGlyAlaGlyL

2641  TGACCATCGAAGAGGCGCTGCGGGCGGTGGCCGAGCAGTGCGAGAAGGCCCATCTGCGCA  2700
      euThrIleGluGluAlaLeuArgAlaValAlaGluGlnCysGluLysAlaHisLeuArgS

2701  GCCTGGTGGCGACGGTACGCAGCAAGGTGGTAGAAGGTTACTCGCTGGCCGATTCGCTGG  2760
      erLeuValAlaThrValArgSerLysValValGluGlyTyrSerLeuAlaAspSerLeuG

2761  GGGCCTTCCCTCACGTGTTCGATCAGCTGTTTCGCTCCATGGTGGCGGCCGGTGAAAAAT  2820
      lyAlaPheProHisValPheAspGlnLeuPheArgSerMetValAlaAlaGlyGluLysS

2821  CGGGCCATCTGGAGAAGGTGCTGAACCGGCTCGCCGACTACACGGAGCAGCGCCAGCACA  2880
      erGlyHisLeuGluLysValLeuAsnArgLeuAlaAspTyrThrGluGlnArgGlnHisM

2881  TGCGCACCAAGCTGCTGCAGGCGATGATCTACCCCATAGTCCTCACTCTGGTGGCGGTCG  2940
      etArgThrLysLeuLeuGlnAlaMetIleTyrProIleValLeuThrLeuValAlaValG

2941  GGGTCATCTCGATCCTGCTCACTGCAGTCGTGCCCAAGGTAGTTGCCCAGTTTGAACACA  3000
      lyValIleSerIleLeuLeuThrAlaValVa'ProLysValValAlaGlnPheGluHisM

3001  TGGGCCAGCAACTGCCTGCGACGACCCGTTTCCTCATCGGCACCAGCGAGCTGATGCAGC  3060
      etGlyGlnGlnLeuProAlaThrThrArgPheLeuIleGlyThrSerGluL   etGlnH

3061  ATTACGGCCTCTGGTTCCTGCTGCTGCTGTTTATCGGTGGTTTTGTCTGGCGCTGGTGGC  3120
      isTyrGlyLeuTrpPheLeuLeuLeuPheIleGlyGlyPheValTrpArgTrpTrpL

3121  TGACCGACGAGAAGCGTCGCCGTCACTGGCATCAGGTGGTGCTGCGCCTGCCGGTCATAG  3180
      euThrAspGluLysArgArgArgHisTrpHisGlnValValLeuArgLeuProValIleG

3181  GCCGGGTCAGCCGTGGTCTCAACACGGCCCGCTTCGCCCGCACCCTCAGCATACTCAACG  3240
      lyArgValSerArgGlyLeuAsnThrAlaArgPheAlaArgThrLeuSerIleLeuAsnA
```

```
3241  CCAGCGCCGTCCCCCTGCTGGAGGGGATGAAGATTGCCGGCGAGGTGCTCTCCAATGACT  3300
      laSerAlaValProLeuLeuGluGlyMetLysIleAlaGlyGluValLeuSerAsnAspP

3301  TCGCCCGTACTCGCATCGGTGAGGCCACCGAACGGGTGCGGGAGGGGACCAGCCTGCGCA  3360
      heAlaArgThrArgIleGlyGluAlaThrGluArgValArgGluGlyThrSerLeuArgL

3361  AGGCACTGGATGAAACCAAGATTTTCCCGCCCATGATGCTGCACATGATCGCCTCCGGCG  3420
      ysAlaLeuAspGluThrLysIlePheProProMetMetLeuHisMetIleAlaSerGlyG

3421  AGCAGAGCGGCGAGCTCGACAGCATGCTGGAGCGGGCGGCTGACAACCAGGACAGGGAAT  3480
      luGlnSerGlyGluLeuAspSerMetLeuGluArgAlaAlaAspAsnGlnAspArgGluP

3481  TTGAAACCCAGGTGAATATCGCCCTCGGCGTGTTCGAGCCCCTGCTGGTGGTCTCCATGG  3540
      heGluThrGlnValAsnIleAlaLeuGlyValPheGluProLeuLeuValValSerMetA

3541  CCGGGGTCGTGCTGTTTATCGTGATGTCGATCCTGCAGCCGATCCTCGAGCTCAACAACA  3600
      laGlyValValLeuPheIleValMetSerIleLeuGlnProIleLeuGluLeuAsnAsnM

3601  TGGTGAATTTATGATGTTGGCGTGAGGGTGACTTCGGTCGATCCTGCCGTCAATTCGCTC  3660
      etValAsnLeu*

3661  GAACTCAATAATATGGTCAATCTCTGATGGCCCGCGCCATCTCATTTCGTTAGGGAGTAA  3720
         ----  ORF 4'
3721  GTCATGCAAAAGCGTCGTCAATCCGGTTTTACCCTGCTGGAAGTCATGGTGGTCATAGTG  3780
         MetGlnLysArgArgGlnSerGlyPheThrLeuLeuGluValMetValValIleVal

3781  ATCCTCGGGATCCTGGCCAGCCTGGTGGTGCCCAACCTGATGGGCAACAAGGAGAAGGCT  3840
      IleLeuGlyIleLeuAlaSerLeuValValProAsnLeuMetGlyAsnLysGluLysAla

3841  GACCAGCAGAAAGCCGTCTCCGACATAGTGGCGCTGGAAAATGCCCTCGACATGTACAAG  3900
      AspGlnGlnLysAlaValSerAspIleValAlaLeuGluAsnAlaLeuAspMetTyrLys

3901  CTGGACAACAACCGTTACCCGACCACGGAGCAAGGCCTGGATGCGCTGGTCAACAAGCCG  3960
      LeuAspAsnAsnArgTyrProThrThrGluGlnGlyLeuAspAlaLeuValAsnLysPro

3961  ACCGCCGCCCCCGAGCCGCGCAGCTACCGTGACGGCGGCTACATCAAGCGCCTGCCGCAG  4020
      ThrAlaAlaProGluProArgSerTyrArgAspGlyGlyTyrIleLysArgLeuProGln

4021  GATCCCTGGGGCAACCCGTACCAGATGCTGAGCCCGGGCCAGTTTGGCAAGATCGACATC  4080
      AspProTrpGlyAsnProTyrGlnMetLeuSerProGlyGlnPheGlyLysIleAspIle

4081  TTCTCCATGGGGCTGGATGGCGAAGCCG  4108
      PheSerMetGlyLeuAspGlyGluAla
```

Figure 3.13. DNA sequence of the 4.1 kb KpnI fragment and the deduced amino acid sequence of the gene products. The start codon for each open reading frame (ORF) is highlighted in bold. Proposed ribosome binding sites,as well as the *Bgl*II site in ORF2, are underlined. There are two possible initiation sites for ORF 2. See text for details.

reading frames (ORF1,2,3 and 4) in the direction of the transcription of the exe gene (Figure 3.14).

ORF1, which is a partial open reading frame, stops at the TAA codon at position 814. ORF2 has two potential in-frame ATG start codons at positions 817 and 1042, and ends at 2322, potentially coding for proteins of 55,882 Daltons or 47,707 Daltons. The first ATG is preceded by a potential ribosome binding site AGGG 10 bp upstream and the second ATG also has a potential ribosome binding site GAAGAG 7 bp upstream. Since it was clear that the transposon in L1.97 was inserted in this open reading frame, deduced from restriction analysis, the complete ORF2 very likely represented the coding region for the exe gene. ORF3 has three in-frame ATG start codons at 2799, 2880 and 2904, it also has an in-frame GTG start codon at 2448. As can be seen in Figure 3.13, only the GTG codon at 2448 is preceded by a good potential ribosome binding site, AGAAG at -6 position. This open reading frame stops at 3611. Starting from the GTG codon, it codes for a potential product of 43,431 Daltons. At position 3724, ORF4 starts with an ATG codon, which is again preceded by a ribosome binding site AGGGAG at -7, and it extends beyond the end of the 4.1 kb KpnI fragment, coding for a truncated polypeptide of 14,182 Daltons.

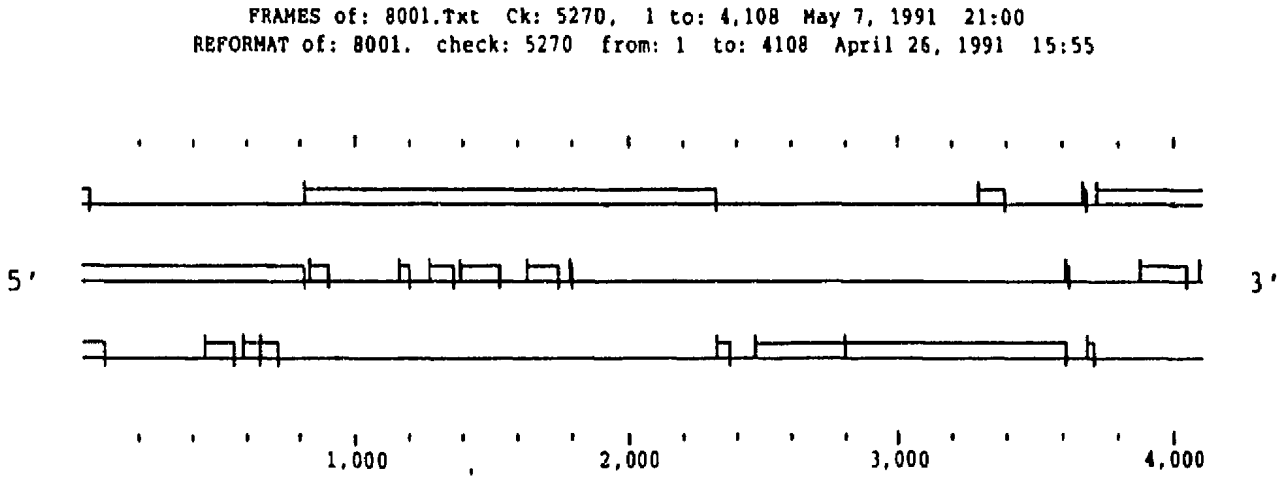


Figure 3.14. Open reading frame analysis of the sequenced 4.1 kb *KpnI* fragment. The computer analysis was carried out using the GCG (Genetics Computer Group, Inc) sequence analysis software package. Four large open reading frames (ORFs) are identified. The third is started from a GTG codon, instead of the regular ATG codon.

3.9 Expression of polypeptides encoded by the 4.1 kb *Kpn*I fragment

The full length 4.1 kb *Kpn*I DNA fragment and the complete 11 kb *Eco*RI fragment were first transferred into pBluescript vectors in both orientations and expressed in an *in vitro* transcription and translation system, as decribed in materials and methods, to visualize the gene products encoded by these sequences. The plasmid-encoded polypeptides were labeled with [^{35}S]methionine and analysed by SDS-PAGE and autoradiograghy. As shown in Figure 3.15, the 4.1 kb *Kpn*I fragment expressed four easily detected products in the direction of the transcription of the *exe* gene. The apparent molecular weight of the top band is about 50 KD, and it is very likely the product of the *exe* gene coded by ORF2, starting from either the first or second ATG initiation codon. Further experiments are definitely required to determine the real start codon for this polypeptide, but for the time being, the first ATG condon is assumed to be the start. The bottom band shows a MW of 16.5 KD and most probably represents a hybrid protein encoded by ORF4 and downstream vector sequence which provides an in-frame stop codon, which would have a deduced molelular weight of 14,547 Daltons. The middle two bands have apparent molecular weights of 39 KD and 36 KD. Since the 36 KD product is

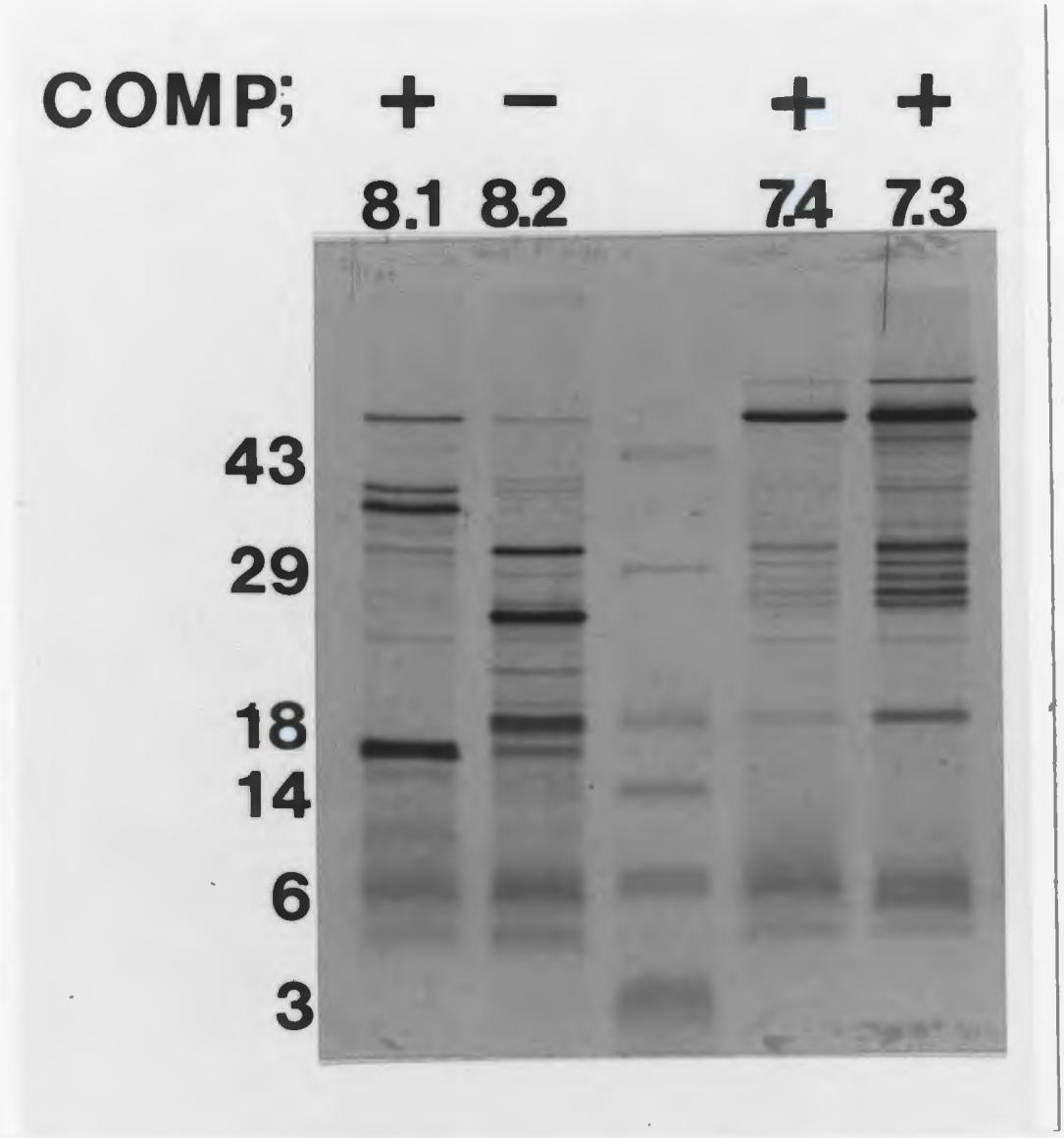


Figure 3.15. In vitro expression analysis of the 4.1 kb KpnI fragment. Polypeptides encoded by differnt plasmids were expressed in vitro and analysed by SDS-PAGE and visualized by autoradiograpy. COMP, complementation; 8.1, pJB8.1; 8.2, pJB8.2, 7.4, pJB7.4; 7.3, pJB7.3. pJB8.1 and pJB8.2 contain the 4.1 kb KpnI fragment, whereas pJB7.3 and pJB7.4 contain the 11 kb EcoRI fragment as pJB3 does. The direction of the in vitro expression for pJB8.1 and pJB7.3 was the same as that of the exe gene. pJB8.2 and pJB7.4 were expressed in the opposite direction. The middle lane contains radioactive MW standards (BRL).

completly absent in the 2nd lane from left, the same 4.1 kb KpnI fragment expressed in the opposite orientation, it is deduced to be the product of a fusion protein between the vector based β-galactosidase gene upstream of the insert and the ORF1. The 39KD polypeptide is assumed to the product of ORF3 starting from the GTG codon. Since the protein encoded by ORF3 would be very hydrophobic, the apparent molecular weight calculated from the SDS-PAGE gel (39 KD) may result from aberrant migration of the 43 KD protein predicted from the DNA sequence.

3.10 Further deletion analysis and complementation studies on the 4.1 kb KpnI fragment

From the restriction analysis data, it is clear that the transposon insertion site in L1.97 is approximately 2.7 kb away from the right hand end of the 11 kb EcoRI fragment as shown in the figure, and this indicated clearly that an exe gene is located in the ORF2 of the 4.1 kb fragment. To define the boundaries of the exe gene more precisely in vivo, further deletion analysis and functional complementation studies were performed.

First, a pJB8.1 deletion derivative, which contains only the KpnI-SacI fragment, was made (pMJB12 in Figure 3.16). This construct apparently interrupted the integrity of ORF2, since it did not complement the mutation when transfered back

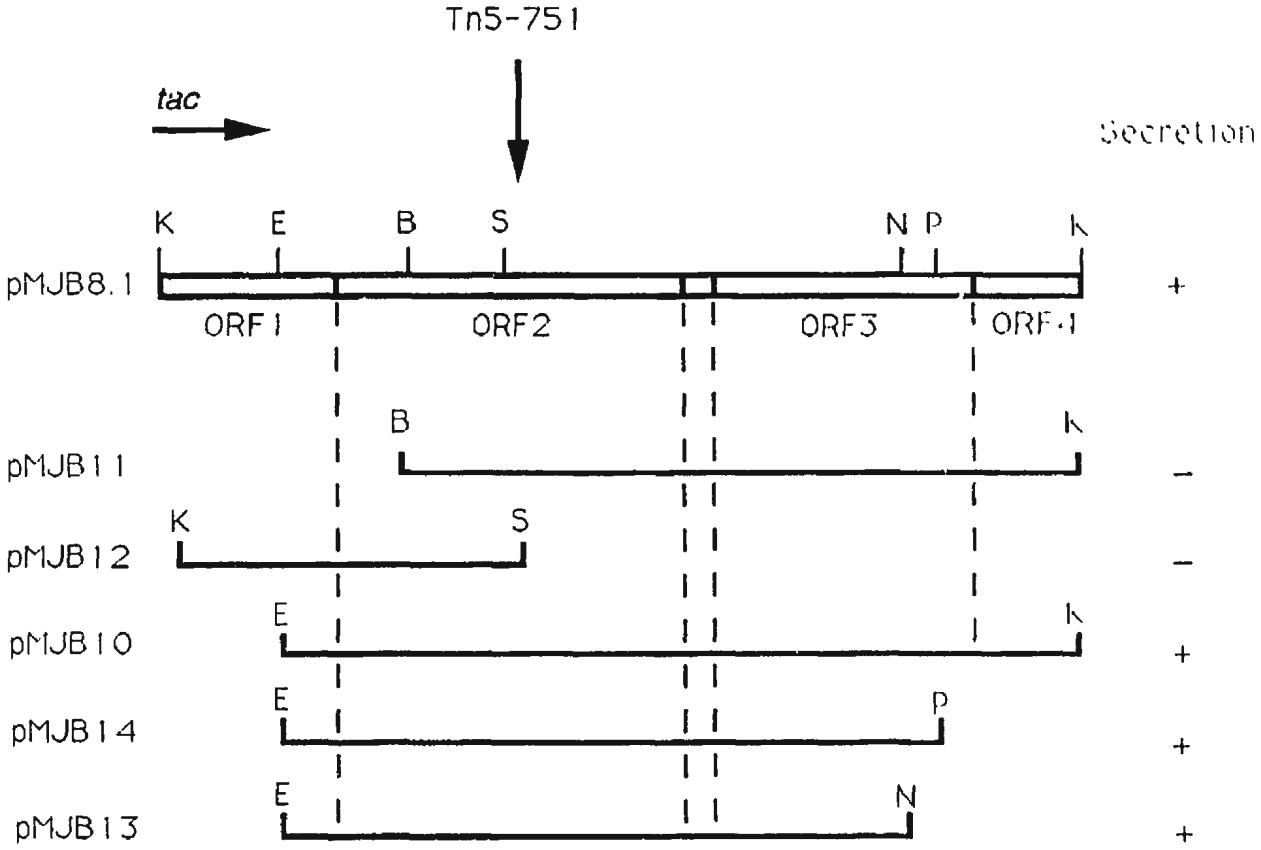


Figure 3.16. Deletion analysis and complementation of the 4.1 kb *Kpn*I fragment.

Part A: Construction of the deletion derivatives. Restriction sites: K, *Kpn*I; E, *Eco*RV; B, *Bgl*II; S, *Sac*I; N, *Nae*I; P, *Sph*I. The vertical bars represent the start and stop codons of the ORFs.

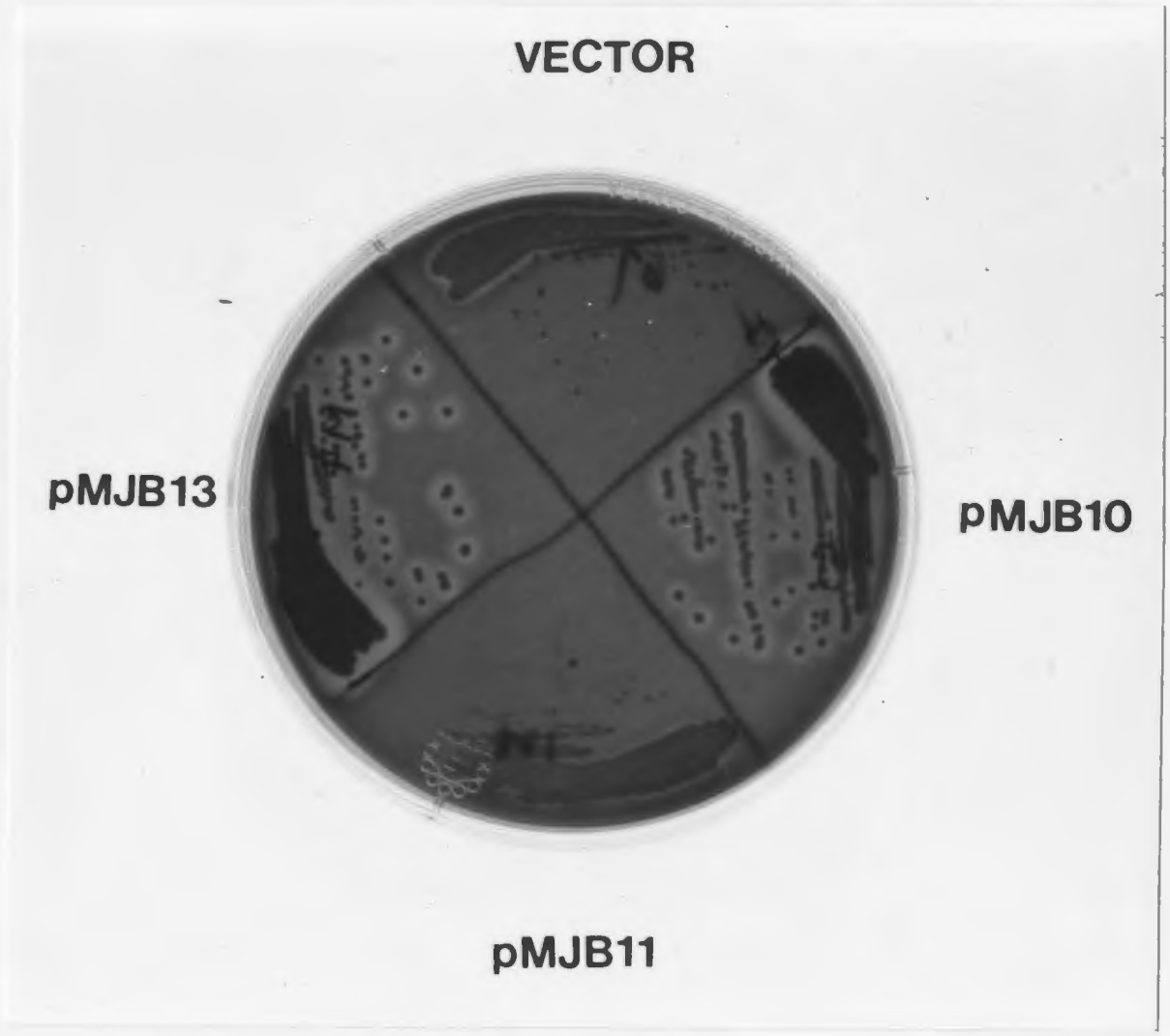


Figure 3.16. Deletion analysis and complementation of the 4.1 kb *KpnI* fragment.

Part B: Phenotype of L1.97 transconjugants containing different deletion derivatives. Cells are streaked on a BHI-blood plate.

into L1.97 mutant. Then, a new series of deletion derivatives of the 4.1 kb KpnI fragment, including fragments of EcoRV-KpnI, EcoRV-SphI, EcoRV-NaeI and BglII-KpnI, were constructed behind the tac promotor in a new vector, pMMB67HE.cam, which offered more unique sites in the multiple cloning site. After being introduced into L1.97 mutant cells, the transconjugants carrying different constructs were plated on blood agar plates containing IPTG (to induce the tac promotor) to test whether or not a particular construct could complement the mutation in L1.97, i.e. to restore the hemolytic phenotype. To summarize the results shown in Figure 3.16, only those fragments carrying the complete ORF2 could complement the mutation. The constructs containing only part of the ORF2, such as pMJB11 and pMJB12, were unable to complement. The fact that pMJB13, which carried only one complete open reading frame, ORF2, could complement equally well as pMJB8.1, which contained the entire 4.1 kb KpnI fragment, also demonstrated that ORF2 itself was sufficient to restore the wild type phenotype of the mutant. These results further confirmed that ORF2 indeed codes for the extracellular export (exe) gene which was inactivated by transposon insertion in mutant L1.97.

3.11 Comparision of the exe gene of Aeromonas hydrophila with other DNA sequences in the Genbank data bank

In order to obtain some functional insights about this exe gene of Aeromonas hydrophila, a homology search was carried out using the computer DNA homology search program FASTA. Three significant matches were found: the pulE gene (69.0% identity in 1482 nt overlap), which is involved in pullulanase secretion in Klebsiella pneumoniae UNF5023 (Kornacker and Pugsley, 1989); the pilB gene (58.2% identity in 1270 nt overlap), which is involved in the biogenesis of Pseuodomonas aeruginosa pili (Nunn et al., 1990); and ORF1 of the comG operon (52.5% identity in 753 nt overlap) which is required for competence for DNA uptake in Bacillus subtilis (Albano et al., 1989) (Figure 3.17).

More interestingly, the other three ORFs in the 4.1 kb KpnI fragment also share extensive similarity to the Klebsiella pneumoniae UNF5023 pullulanase secretion genes pulD, pulF and pulG respectively (Figure 3.18). Because of the apparently similar functions and the colinear gene organization, the corresponding genes of Aeromonas hydrophila are designated as exeD, exeE, exeF and exeG. The overall nucleotide similarity was 71.6% for sequenced fragment of exeD and pulD, 69.0% for exeE and pulE, 67.5% for exeF and pulF, and 73.4% for the sequenced fragment of exeG and pulG. The amino acid similarity between the predicted Exe and Pul

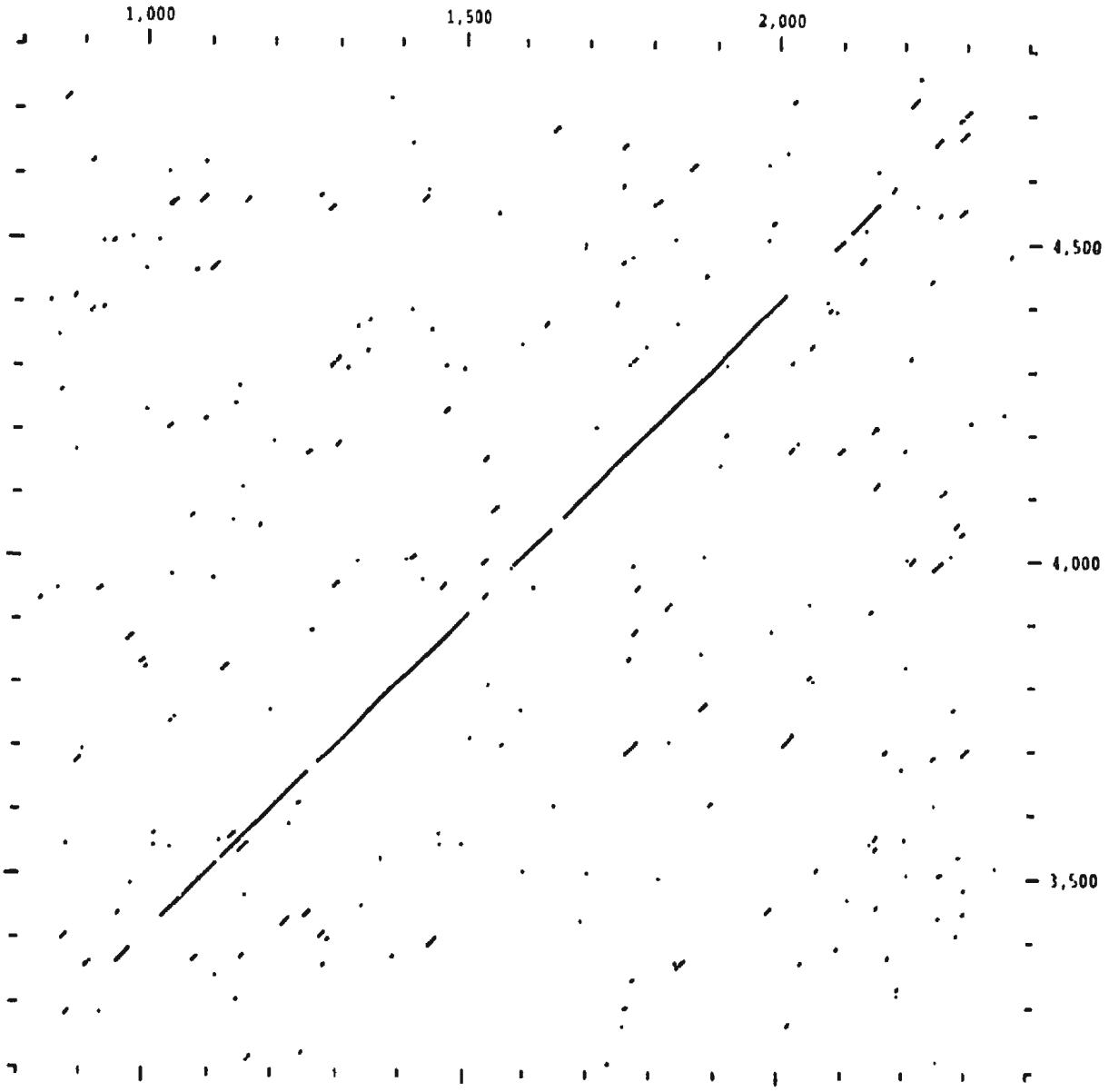


Figure 3.17. DNA sequence comparision of ORF2 with *pulE*, *pilB* and the ORF1 of *comG*.

Part A: ORF2 vs. *pulE*. The dot-plot was performed by using the GCG (Genetics Computer Group, Inc) sequence analysis software package. A searching window of 21 nucleotides was used and the stringency was 14. The horizontal axis represents the DNA sequence of ORF2, the vertical axis represents sequence of *pulE*.

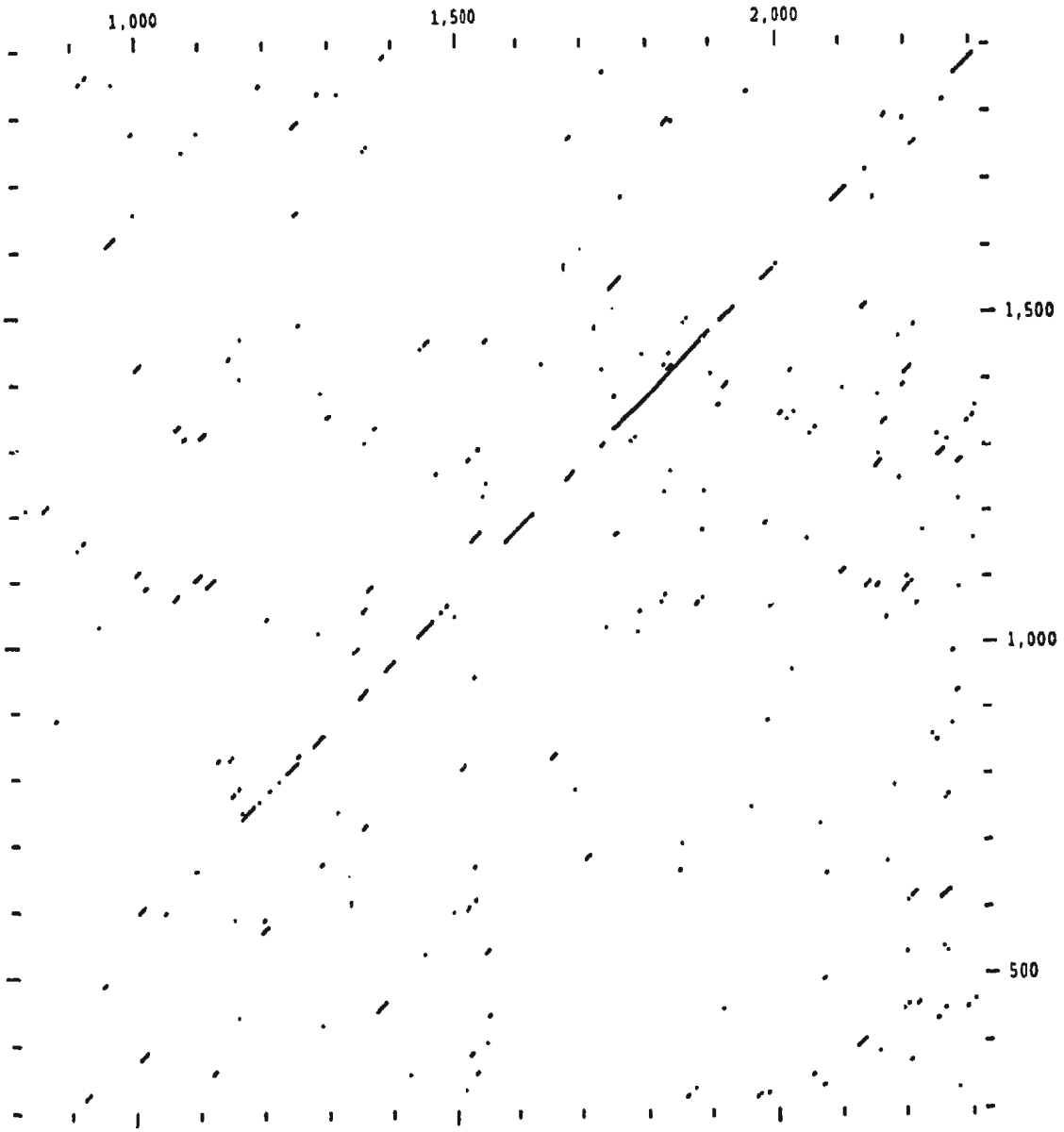


Figure 3.17. DNA sequence comparision of ORF2 with *pulE*, *pilB* and the ORF1 of *comG*.

Part B: ORF2 vs. *pilB*. The dot-plot was performed by using the GCG (Genetics Computer Group, Inc) sequence analysis software package. A searching window of 21 nucleotides was used and the stringency was 14. The horizontal axis represents the DNA sequence of ORF2, the vertical axis represents sequence of *pilB*.

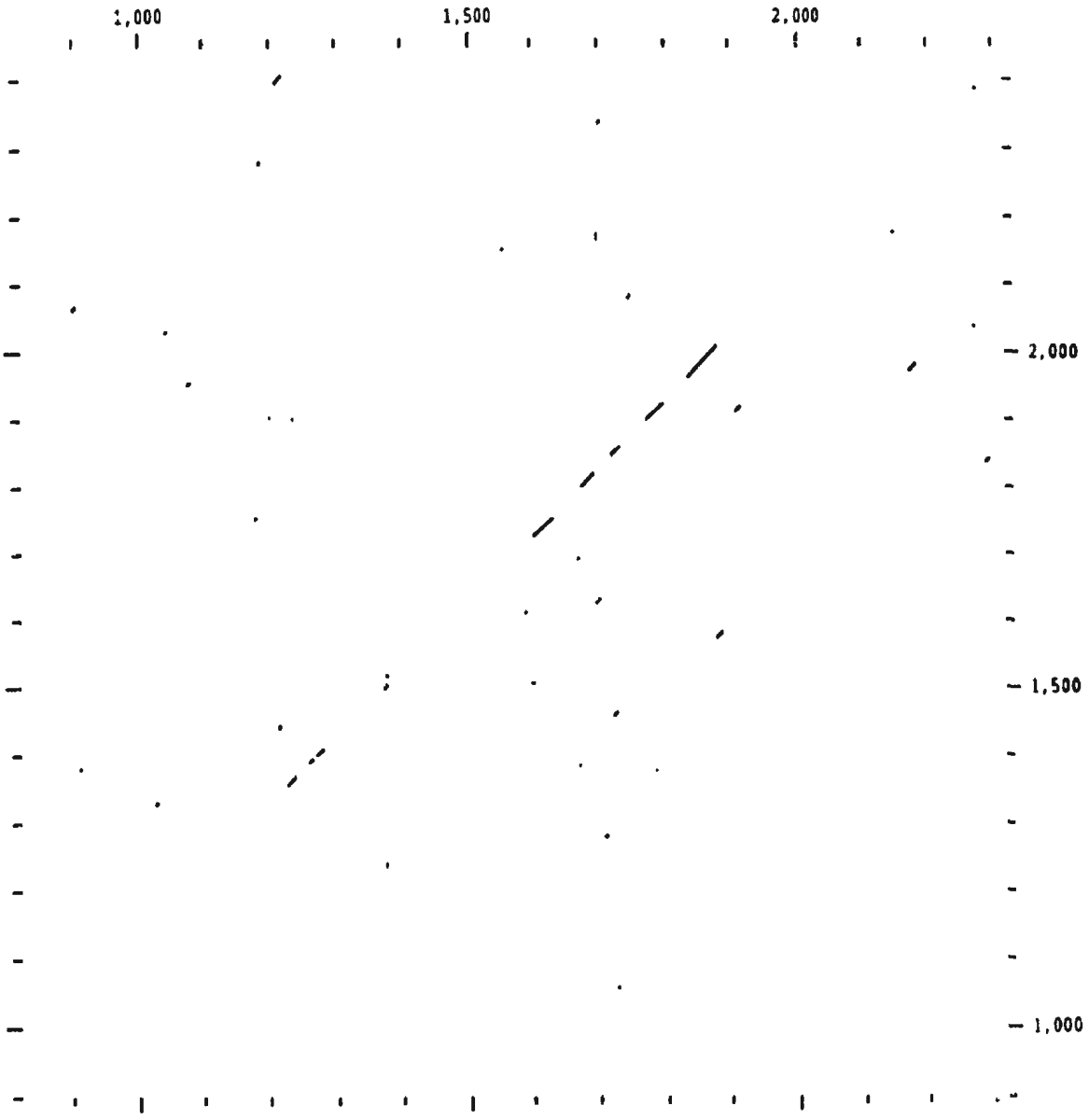


Figure 3.17. DNA sequence comparision of ORF2 with *pulE*, *pilB* and the ORF1 of *comG*.
Part C: ORF2 vs. *comG*. The dot-plot was performed by using the GCG (Genetics Computer Group, Inc) sequence analysis software package. A searching window of 21 nucleotides was used and the stringency was 14. The horizontal axis represents the DNA sequence of ORF2, the vertical axis represents sequence of the ORF1 of *comG*.

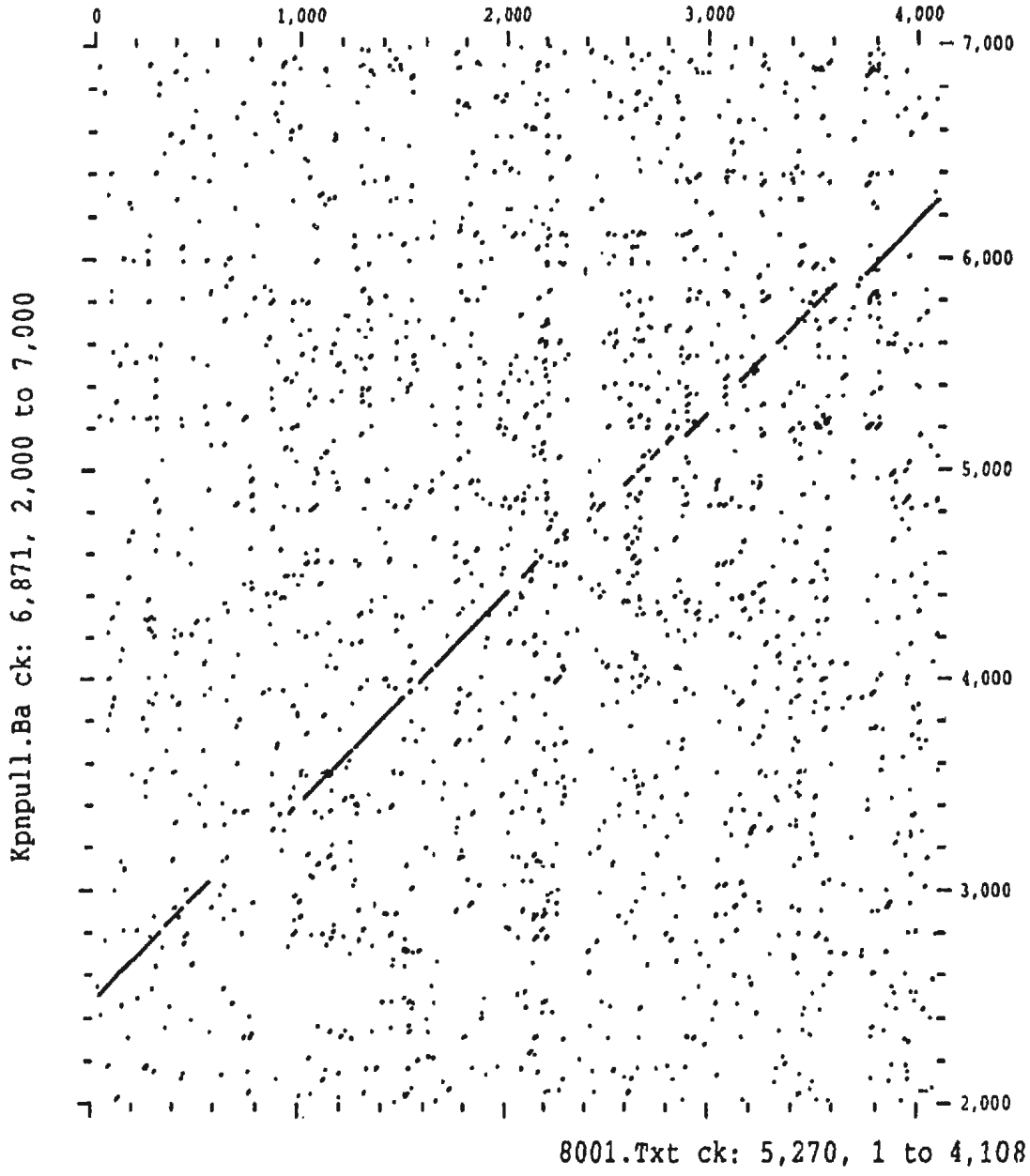


Figure 3.18. DNA sequence comparision between the entire 4.1 kb fragment and part of the *pulC-O* operon. The dot-plots was performed by using the GCG (Genetics Computer Group, Inc) sequence analysis software package. A searching window of 21 nucleotides was used and the stringency was 14. The horizontal axis represents the DNA sequence of the 4.1 kb fragment, the vertical axis represents the corresponding part of *pulC-O* operon.

proteins was also very extensive, as shown in Figure 3.19.

3.12 Hydrophobicity and possible membrane association of the predicted *exe* gene products

Hydropathy plot analysis was performed, using the method described by Kyte and Doolittle (1982), to identify possible transmembrane segments present in the Exe proteins deduced from the DNA sequences (Figure 3.20).

The ExeE protein (deduced from the larger potential open reading frame starting from position 817 of the KpnI 4.1 kb fragment) appears to be quite hydrophilic with an overall average hydropathy of -1.35 (the grand average of hydropathy for soluble proteins is -0.4) (Kyte and doolittle, 1982). However, by the method of Klein *et al.* (1985), a putative hydrophobic transmembrane segment between residues 371 to 387 was identified, thus the ExeE protein was classified as an integral membrane protein with a P:I (peripheral:integral) odds of 0.53.

In contrast, the ExeF protein (deduced from the open reading frame starting from the GTG codon) is largely hydrophobic with an overall average hydrophobicity of 0.96. It has three putative transmembrane segments at residues 148-182, 205-226 and 351-383. The P:I odds for these three

```
PULD - LIIANVIRSFSLTLLIFAALLFRPAAAEEFSASFKGTDIQEFINTVSKNL -50

PULD - NKTVIIDPSVRGTITVRSYDMLNEEQYYQFFLSVLDVYGFAVINMNNGVL -100

PULD - KVVRSKDAKTAAVPVASDAAPGIGDEVVTRVVPLTNVAARDLAPLLRQLN -150

PULD - DNAGVGSVVHYEPSNVLLMTGRAAVIKRLLTIVERVDNAGDRSVVTVPLS -200

PULD - WASAADVVKLVTELNKDTSKSALPGSMVANVVADERTNAVLVSGEPNSRQ -250

PULD - RIIAMIKQLDRQQATQGNTKVIYLKYAKASDLVEVLTGISSTMQSEKQAA -300

PULD - KPVAALDKNIIIKAHGQTNALIVTAAPDVMNDLERVIAQLDIRRPQVLVE -350

PULD - AIIAEVQDADGLNLGIQWANKNAGMTQFTNSGLPISTAIAGANQYNKDGT -400

EXED -  TTGLAKLAENFNGMAAGFYQGNWAMLVTALSTNTKSDILSTPSIVTMDN -49
        .. ::     :::.:::::::::::::::::. :: :::.:::::::.::
PULD - VSSSLASALSSFNGIAAGFYQGNWAMLLTALSSSTKNDILATPSIVTLDN -450

EXED - KEASFNVGQEVPVQTGTQNSTSGDTTFSTIERKTVGTKLVVTPQINEGDS -99
        ::.:::::::::: ::.: .::::  : :.:::::: :: : ::::::::
PULD - MEATFNVGQEVPVLTGSQ-TTSGDNIFNTVERKTVGIKLKVKPQINEGDS -499

EXED - VLLTIEQEVSSVGKQATGTDGL-GPTFDTRTVKNAVLVKSGETVVLGGLM -148
       ::: :::::::::   :. :    : :: :::: ::::: :::::::.::::.
PULD - VLLEIEQEVSSVADAASSTSSDLGATFNTRTVNNAVLVGSGETVVVGGLL -549

EXED - DEQTKEEVSKVPLLGDIPVLGYLFRSTSNNTSKRNLMVFIRPTILRDANV -198
       :    .   ::::::::::.: ::::::   :::::::.::::::..::
PULD - DKSVSDTADKVPLLGDIPVIGALFRSTSKKVSKRNLMLFIRPTVIRDRDE -599

EXED - YSGISSNKYTLFRAQQLDAVAQEGYATSPDRQVLPEYGQDVTMSPEAQKQ -248
       :   ::  :: :  :      :   .    .:  :    : .
PULD - YRQASSGQYTAFNDAQSKQRGKENNDAMLNQDLLEIYPRQDTAA------ -643

EXED - IELMKTHQQATADGVQPFVQGNK -271
              : .:      . ::
PULD - ------FRQVSAAIDAFNLGGNL -660
```

Figure 3.19 Amino acid sequence alignments between the predicted Exe proteins and the Pul proteins.

Part A: ExeD vs. PulD. The ":" indicates that two aligned residues are identical, the "." shows that two aligned residues are similar. Amino acids considered to be similar are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W. See text for details.

```
EXEE - MAAYQLDDTSLPAA--LPELPFAFARNFGVVLTERQGTPLLLCRPGVAPQ -48
       :.         :::   : ::: .::   :.:     .  . :    :::
PULE - MT------ --PAAERRPLLPFGYARAHSVMLLSSGESCEVFCLAVTAPQ -41

EXEE - TLLEVRRVAGCAFEVEQLGSDEFEELLMAHYQRDSSEARQLMEDLGNEMD -98
       .::: ::::   : .: :  . :: ::.  :::::.::: .: :.::::.:
PULE - ALLEARRVAAMPFRLERLEEEAFEKLLVLSYQRDSAEARRMMADIGNELD -91

EXEE - FFALAEELPQSEDLLDADDDAPIIRLINAMLSEAIKEEASDIHIETFERV -148
         ..::::::: ..:::::.:::::::::::::::::.::::: :::::::::.::
PULE - LYTLAEELPDTDDLLDSEDDAPIIRLINAMLTEAIKEKASDIHIETYERH -141

EXEE - LVIRFRIDGVLREILRPHRKLASLLVSRIKVMSRMDIAEKRVPQDGRISL -198
       : ::::.:::::::::: :.:::.::.:::::. .:::::::.:::::..:
PULE - LQIRFRVDGVLREILRPQRRLAALLISRIKVMASLDIAEKRIPQDGRMAL -191

EXEE - RIGGRAVDVRVSTMPSSYGERVVLRLLDKNNVRLELKQLGMTLANRNIIS -248
       ::::::::::::::.:::::::::::::::::::: : :.:  :::: :    .
PULE - RIGGRAVDVRVSTLPSSYGERVVLRLLDKNSVNLDLLTLGMTPALLRQVD -241

EXEE - ELIRKPHGIILVTGPTGSGKSTTLYAALSEINSRDRNILTVEDPIEYDLE -298
        :: .::::.:::::::::::::::::::: . .:.::::.:.:::::.::
PULE - GLIARPHGIVLVTGPTGSGKSTTLYAALSRLDARERNIMTIEDPIEYELE -291

EXEE - GVGQTQVNTKVDMTFARGLRAILRQDPDVVMVGEIRDLETAQIAVQASLT -348
       :.:::::::.:::::::::::::::::::::::.:::::: ::::::::::::
PULE - GIGQTQVNAKVDMTFARGLRAILRQDPDVVLVGEIRDGETAQIAVQASLT -341

EXEE - GHLVMSTLHTNTAIGAITRMRDMGIEPFLLSSSLLAVLAQRLVRTLCPDC -398
       ::::.:::::::.:.:::.:. :::.:::::::.:::::..:::::: ::: :
PULE - GHLVLSTLHTNSALGAISRLQDMGVEPFLLSTSLLAVMSQRLVRRLCPHC -391

EXEE - RAPRPITEQERLAMG----MELAPDQQVWRPVGCEQCNHTGYRGRTGIHE -444
       :       ::         ::.::   .: : :: : ::::::::::
PULE - R------QQEPANADTAHQMEIAPGTALWQPRGCAECGFTGYRGRTGIHE -435

EXEE - LVVIDEAVREAIHSASGELAIERLIRDHTPSIRRDGIDKVLKGQTSLEEV -494
       :...:. :: ::: :...  .  ..:: : .: : : ::  ::
PULE - LLLVDDRVRMAIHRGENEVTLIQQLGTDYMTLRRAGREKALAGITSWQEV -485

EXEE - LRVTRED      -501
       ::::
PULE - LRVTEQPIAEAC -497
```

Figure 3.19 Amino acid sequence alignments between the predicted Exe proteins and the Pul proteins.

Part B: ExeE vs. PulE. The ":" indicates that two aligned residues are identical, the "." shows that two aligned residues are similar. The predicted transmembrane segments are underlined. See text for details.

```
EXEF -                      VTEGDSARQVRQQLREQGLTPLEVNETTEKAK -32
                            :   :::: :: ::: :   :.. ..   .
PULF - MALFRYQALDEQGKPRRGVQQADSARHARQLLREKGWLALDIDPAAGGGR -50

EXEF - REANRFVLFRRGASTSELALITRQLATLVGAGLTIEEALRAVAEQCEKAH -82
           ::.   : .:. .:::.:::::::: : . .: :: ::: : ::
PULF - --PSRFM---RRTSARDLALVTRQLATLVAAAIPLEKALDAVAQQSEKPQ -95

EXEF - LRSLVATVRSKVVEGYSLADSLGAFPHVFDQLFRSMVAAGEKSGHLEKVL -132
       :..:.: :: ::.:: :::...   : :: :. .:::::: :::
PULF - LKTLIAGVRGKVLEGHSLAEAMRGHPGCFDALYCAMVAAGEASGH----- -140

EXEF - NRLADYTEQRQHMRTKLLQAMIYPIVLTLVAVGVISILLTAVVPKVVAQF -182
                          .:::::::::::::::: :: :::..:::::: ::
PULF - ---------------RLLQAMIYPIVLTLVAVSVIVILLSTVVPKVVEQF -175

EXEF - EHMGQQLPATTRFLIGTSELMQHYGLWFLLLLFIGGFVWRWWLTDEKRRR -232
        :. : :: .:: :.  :...   : : :: . .   . :. :    .:
PULF - IHLKQALPFSTRLLMAMSDMLRAAGPWLLLAILLLILLLRYLLRQPAKRL -225

EXEF - HWHQVVLRLPVIGRVSRGLNTARFARTLSILNASAVPLLEGMKIAGEVLS -282
        :: ..:::::.::::.: .:.:::.:::::::::::::::::  :.:. .:::
PULF - AWHRLLLRLPLIGRVARSVNSARYARTLSILNASAVPLLLAMRISADVLS -275

EXEF - NDFARTRIGEATERVREGTSLRKALDETKIFPPMMLHMIASGEQSGELDS -332
       : .:.  .  :.. :::: :: .::. : .:::::  :.:::: :::: :
PULF - NAWAKRQLEAASDAVREGVSLHRALEMTQLFPPMMRYMVASGERSGELNS -325

EXEF - MLERAADNQDREFETQVNIALGVFEPLLVVSMAGVVLFIVMSILQPILEL -382
       ::::::::::::.  .:...:: .:::::::.:::.:::::..:::::: :
PULF - MLERAADNQDRDLSAQIQLALSLFEPLLVVAMAGMVLFIVLAILQPILQL -375

EXEF - NNMVNL -388
       : .. .
PULF - NTLMSM -381

EXEG - MQKRRQSGFTLLEVMVVIVILGILASLVVPNLMGNKEKADQQKAVSDIVA -50
       ::  :: ::::::.:::::::.:::::::::::::::::: :: :::.::
PULG - MQ--RQRGFTLLEIMVVIVILGVLASLVVPNLMGNKEKADRQKVVSDLVA -48

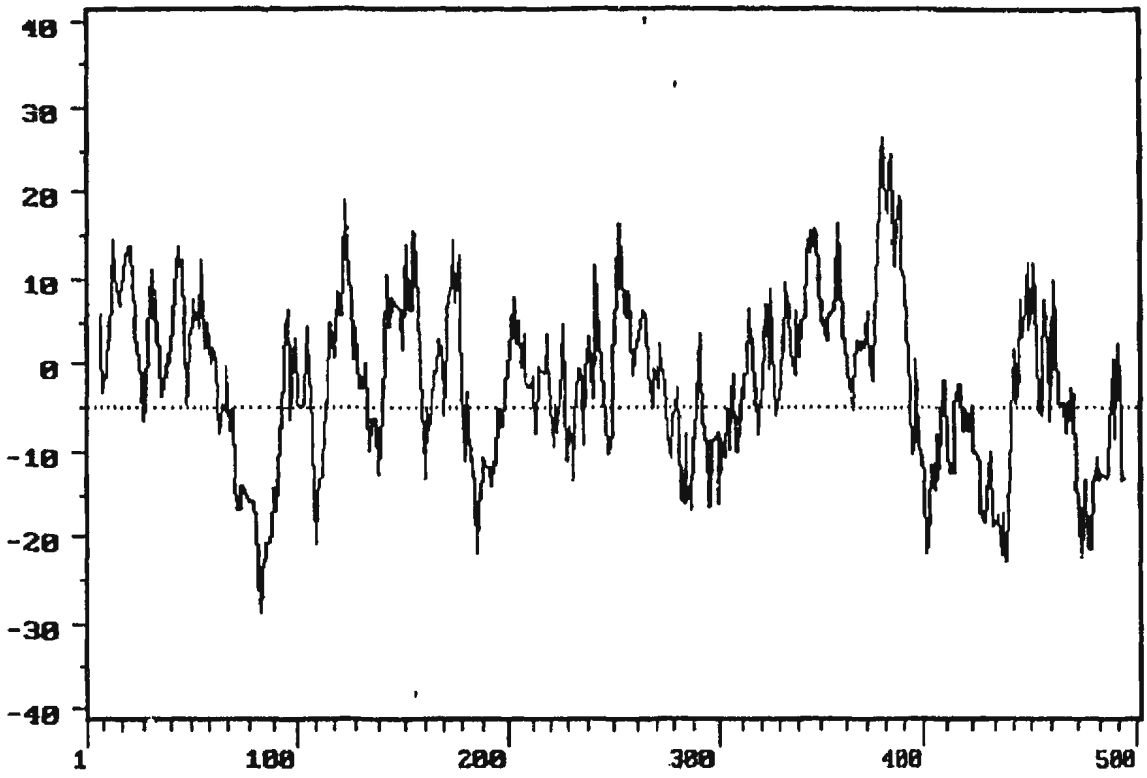
EXEG - LENALDMYKLDNNRYPTTEQGLDALVNKPTAAPEPRSYRDGGYIKRLPQD -100
       :: ::::::::: :::::::::  :::  :.: :  : : .::::.:::::
PULG - LEGALDMYKLDNSRYPTTEQGLQALVSAPSAEPHARNYPEGGYIRRLPQD -98

EXEG - PWGNPYQMLSPGQFGKIDIFSMGLDGEA              -128
       :::  ::.::::: : .::::.: ::
PULG - PWGSDYQLLSPGQHGQVDIFSLGPDGVPESNDDIGNWTIGKK -140
```

Figure 3.19 Amino acid sequence alignments between the predicted Exe proteins and the Pul proteins.

Part C: ExeF vs. PulF and ExeG vs. PulG.

Total number of amino acids is: 501.

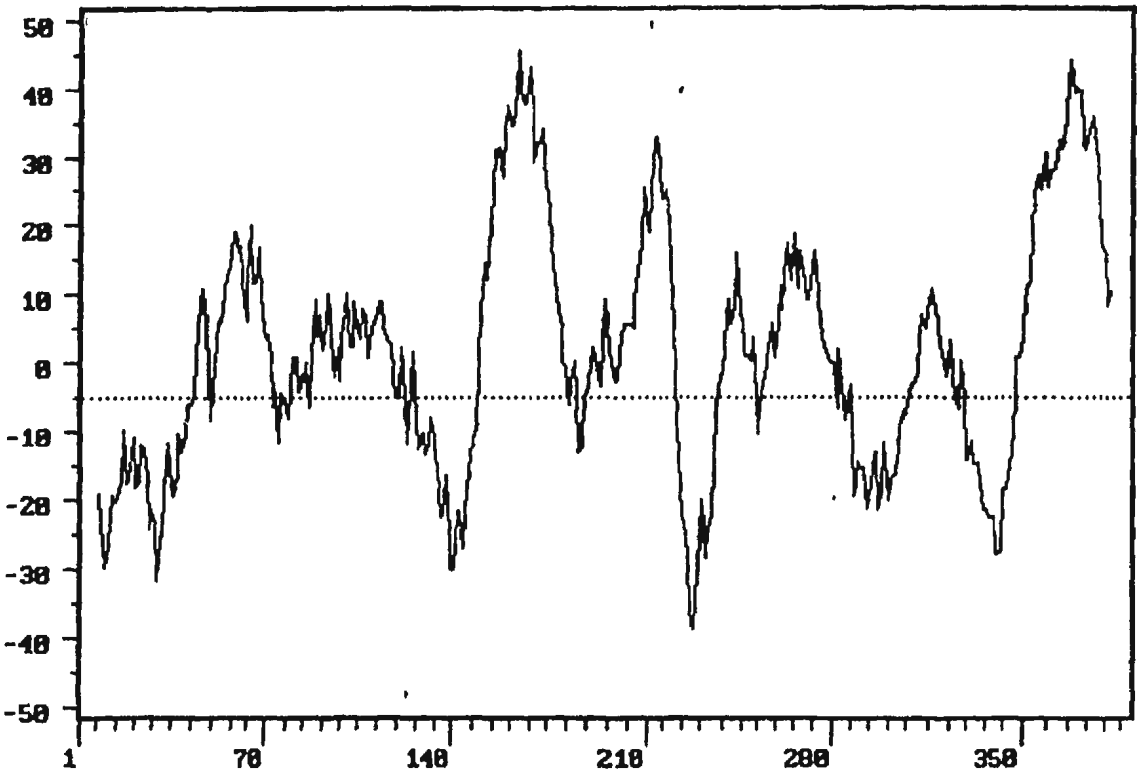


Hydropathic index of EXEE from amino acid 1 to amino acid 501.
Computed using an interval of 15 amino acids. (GRAVY = -1.35).

Figure 3.20. Hydropathy profiles of the predicted Exe proteins.

Part A: ExeE. The hydropathy index was computed by the method of Kyte and Doolittle (1982) and was plotted in this figure. GRAVY: grand averages of hydropathy. See text for details.

Total number of amino acids is: 388.

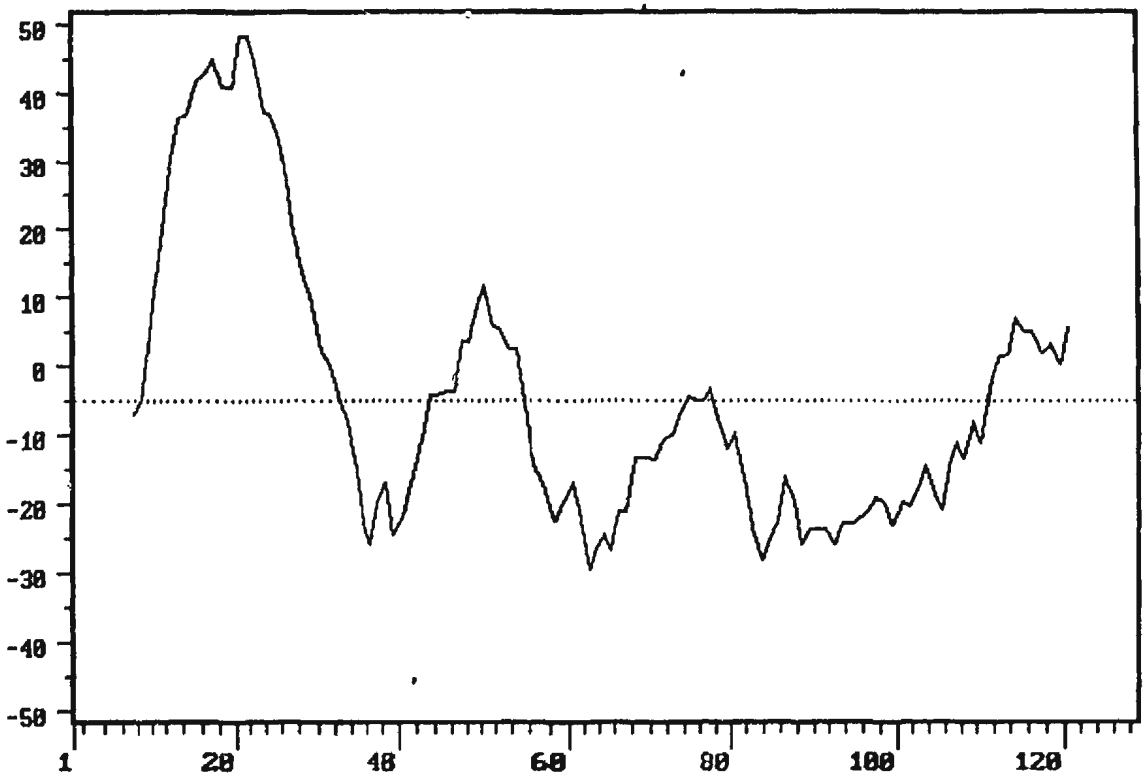


Hydropathic index of EXEF from amino acid 1 to amino acid 388.
Computed using an interval of 15 amino acids. (GRAVY = .96).

Figure 3.20. Hydropathy profiles of the predicted Exe proteins.

Part B: ExeF. The hydropathy index was computed by the method of Kyte and Doolittle (1982) and was plotted in this figure. GRAVY: grand averages of hydropathy. See text for details.

Total number of amino acids is: 128.



Hydropathic index of EXEG from amino acid 1 to amino acid 128.
Computed using an interval of 15 amino acids. (GRAVY = -3.01).

Figure 3.20. Hydropathy profiles of the predicted Exe proteins.

Part C: ExeG. The hydropathy index was computed by the method of Kyte and Doolittle (1982) and was plotted in this figure. GRAVY: grand averages of hydropathy. See text for details.

segments are 3.04×10^{-5}, 0.40 and 1.52×10^{-6}, respectively. Thus by the Klein algorithm, ExeF is classified as an integral membrane protein with multiple transmembrane domains.

The ExeG protein is also considered as an integral membrane protein, since the hydropathic profile of the truncated ExeG protein deduced from the partial *exeG* gene sequence revealed a putative transmembrane segment from residues 5-35, which has a P:I odds of 2.79×10^{-6}.

Since no typical signal-sequence can be identified at the N-terminus of any of these predicted Exe proteins, ExeE, ExeF and ExeG are very likely integrated into the inner membrane of the *Aeromonas hydrophila* cell envelope.

The hydropathic profiles of these predicted Exe proteins are very similar to those of the corresponding Pul proteins (data not shown). This is not surprising, since the primary sequences between them are highly homologous.

Chapter 4

DISCUSSION

In this study, two extracellular protein export-defective mutants of *Aeromonas hydrophila*, C5.84 and L1.97, in each of which a single copy of Tn*5-751* is inserted into the chromosome, have been biochemically characterized. The wild type chromosomal regions, into which the transposon has been inserted in the two mutants, have been cloned and one of them has been further analysed by subcloning and DNA sequencing.

Each of these two mutations causes pleiotropic effects on extracellular protein export; both of the mutants are unable to secrete any of the major extracellular proteins normally extracellularly exported by the wild-type cells, indicating that the genes mutated by the transposon insertion are essential for general protein translocation across the cell envelope.

4.1 Extracellular protein export in *Aeromonas hydrophila* follows a two-step, signal-sequence dependent pathway

Previous experiments showed that at least two of the normally secreted extracellular proteins, i.e. aerolysin and amylase, were initially synthesized as precursors which

contained N-terminal signal-sequences that were remove by signal peptidase after translocation across the inner membrane (Gobius and Pemberton, 1988; Howard and Buckley, 1986). In the mutants C5.84 and L1.97, aerolysin and amylase, as well as protease, were all accumulated in the periplasmic space and could be released during osmotic shock. The immunoblot analysis also showed that the aerolysin was accumulated in the mutants as the processed form without the signal-sequence, indicating that aerolysin could translocate across the cytoplasmic membrane successfully in the mutants. These results suggested that the secretion of aerolysin and amylase, and perhaps all the other extracellualar proteins not secreted by the mutants, follow a two step, signal-sequence dependent pathway. The signal sequence first directs and faciliates the transfer of the protein across the inner membrane via the general sec machinery. Then in the second step which requires additional factors such as the exe gene products that were inactivated in C5.84 and L1.97, the proteins are somehow recognized among periplasmic proteins, translocated across the outer membrane and finally released into the extracellular medium.

4.2 The mutations in the two extracellular export-defective mutants are distinct but may be related to each other

Although C5.84 and L1.97 are both defective in the extracellular protein export process, they are very different mutants since they display different non-secretory phenotypes. C5.84 is more leaky than L1.97, and L1.97 possesses an altered outer membrane structure and is very fragile when fractionated in rich media. In addition, Southern blotting analysis showed unambiguously that the mutations in the two mutants are located in different chromosomal loci. Even if the two cloned *Eco*RI fragments in pJB3 and pJB4 were adjacent to each other in the chromosome, the two transposon insertion sites would still be at least 6.7 kb apart, a distance much longer than the average size of a prokaryotic gene. All the above mentioned evidence indicates that the mutations caused by transposon insertion in C5.84 and L1.97 are distinct, and that these two mutated genes are most likely involved in different stages or aspects of the extracellular protein export process.

Taking this fact into consideration, the finding that the 11 kb fragment complementing the mutation in L1.97 can also at least partially complement the mutation in C5.84 was quite unexpected. This result may suggest that the two genes or their gene products interact with each other in some way during the protein export process. One possibility suggested

by recent studies is that these gene products are involved in transducing energy from the inner membrane to the outer membrane for the protein translocation process, since proton motive force was found to be required for protein translocation across the outer membrane of *Aeromonas salmonicida*, which possesses a extracellular protein export pathway very similar to that of *Aeromonas hydrophila* (Wong and Buckley, 1989). Since the study of these genes required for protein export in *Aeromonas hydrophila* is still at a very early stage, further experiments and more evidence are certainly required to investigate this possibility. However, if these gene products were indeed involved in some aspects of a general energy transduction process required for extracellular export, it certainly would explain the pleiotropic nature of the mutations.

4.3 L1.97 has an altered outer membrane structure

Besides its effects on extracellular protein export, the transposon insertion mutation in L1.97 also markedly alters the outer membrane protein profile and renders the mutant very fragile when grown in BHI. The fragility most likely results from the structural changes in the outer membrane rather than from the accumulation of extracellular proteins in the periplasm, since mutant C5.84, which accumulates the

same proteins in the periplasm, is apparently not fragile. These complex phenotypic changes caused by a single mutation might be explained by at least two possible hypotheses. One of these is that the transposon mutation somehow interferes with the general biogenesis of the outer membrane and the resulting altered architecture prevents the passage of extracellular proteins across the membrane. Another possibility is that the mutated gene in L1.97 is essential for a common step in the targeting and localization of both the extracellular proteins and the integral outer membrane proteins. The latter possibility is perhaps more likely since an analogous situation has been reported. Recent studies have shown that TolC, which is essential for normal production of the outer membrane porin OmpF, is also required for hemolysin export by *E. coli* cells (Wandersman and Delepelaire, 1990). If a similar coupling of envelope processes takes place in *Aeromonas hydrophila*, it could explain why L1.97 cells are defective in both extracellular protein export and in the synthesis of the two outer membrane proteins (which are also remarkably diminished in the envelope samples (Figure 3.3) and whole cell extracts (data not shown)).

4.4 *Aeromonas hydrophila exe* genes are homologous to *Klebsiella pneumoniae pul* genes

Since the mutation in L1.97 has such dramatic effects, its secretion gene was chosen for furthur analysis in this study.

The DNA sequence analysis has provided some very interesting results. The sequence of the 4.1 kb *Kpn*I fragment, which contains the *exe* gene mutated in L1.97, revealed a cluster of 4 colinearly arranged genes sharing substantial similarties with the *Klebsiella pneumonia* genes *pulD*, *pulE*, *pulF* and *pulG*. These *pul* genes are part of the *pulC-O* operon which has been shown to be required for specific secretion of pullulanase. *pulD* encodes an outer membrane protein, *pulF* and *pulG* encode inner membrane proteins, and these three proteins are all essential for the pullulanase secretion, since deletion or interruption of any of them resulted in the intracellular accumulation of pullulanase (Pugsley *et al.*, 1990a).

pulE is the least well characterezed gene in the whole *pulC-O* operon. The only information available about *pulE* is its DNA sequence and its protein product identified by *in vitro* and *in vivo* expression. Its DNA sequence revealed that it encodes a 55 KD protein. The predicted protein product was assumed to be located in the cytoplasm since no putative transmembrane segment could be identified. Whether or not

pulE is required for pullulanase secretion is still uncertain, since no mutations in this gene have been obtained, thus no direct evidence about its function is available at present.

In contrast, this study showed unambiguously that the pulE homologue in Aeromonas hydrophila, exeE, is absolutely required for extracellular protein export since the transposon insertion in this gene caused pleiotropic defects in extracellular protein secretion. Unlike PulE, the product of exeE, which is also a 55KD protein as predicted from the DNA sequence, was predicted to be an integral membrane protein and is probably anchored in the inner membrane by the single hydrophobic segment between residues 271-287. However, one must be cautious in such interpretations, since the P:I odds value of this protein is only 0.53.

Similar to PulF, the product of exeF is a highly hydrophobic protein. It possesses at least 3 putative membrane-spanning segments and is predicted to be an inner-membrane protein. Interestingly, the two highly hydrophobic transmembrane regions (the first and the third) are extremely homologous to the corresponding regions in PulF, with 82.8% and 75% identity respectively (compared to the 55.6% identity over the entire protein). In contrast, the second, less hydrophobic transmembrane region is very poorly conserved, with only 28.6% identity compared to the

corresponding PulF region. Since the second membrane-spanning segment is followed immediately by a highly hydrophilic loop, which is also highly heterologous, this region (including the transmembrane segment and the following hydrophilic loop) might be involved in some recognition function for the secreted proteins which could be completely different in the two systems. On the other hand, the two highly homologous transmembrane domains in ExeF and PulF are more likely to be involved in certain critical and highly conserved translocation functions, rather than merely playing structural roles to maintain membrane configurations as suggested for the transmembrane domains in XcpA and PulO (Bally *et al.*, 1991).

The truncated ExeG deduced from the DNA sequence is extremely homologous to PulF, with 75.8% overall identity and 82.8% similarity. Each protein has a highly hydrophobic segment at the N-terminus and the same subcellular location (inner membrane) is assigned to both of them.

At this stage, whether or not the other three *exe* genes, *exeD*, *exeF* and *exeG*, are also required for protein secretion is not clear. However, the high degree of similarities between them and the *pulD*, *pulF* and *pulG*, as well as the same gene organization suggests that these genes have been subjected to selection pressures resulting from functional similarities in these two secretion systems. Further

experiments are needed to answer this question and to identify the cellular location and the topology of the protein products of these exe genes.

The limited sequencing data which are presently available at the downstream region of exeF, i.e. the 3' end region of the 11 kb fragment in pJB3, reveals the existence of an open reading frame which is homologous to pulI (data not shown). Therefore, it seems likely that this region of chromosome contains a cluster of genes which are all homologous to the pul genes and that are involved in the protein secretion process (Figure 4.1). The cluster of genes located on the 11kb fragment contains the genes exeD, exeE, exeF, exeG and exeI, and very probably also contains genes like exeC upstream of exeD and exeH between exeG and exeI. However, whether the downstream region contains the remaining genes homologous to pulJ-O, or whether the mutated exe gene in C5.84 is related to one of these pul genes remain to be determined.

4.5 Extracellular protein export mechanisms among gram-negative bacteria are conserved

Until recently, it was believed that every protein or group of proteins secreted by gram-negative bacteria employed its own specific secretion machinery, since the secretion

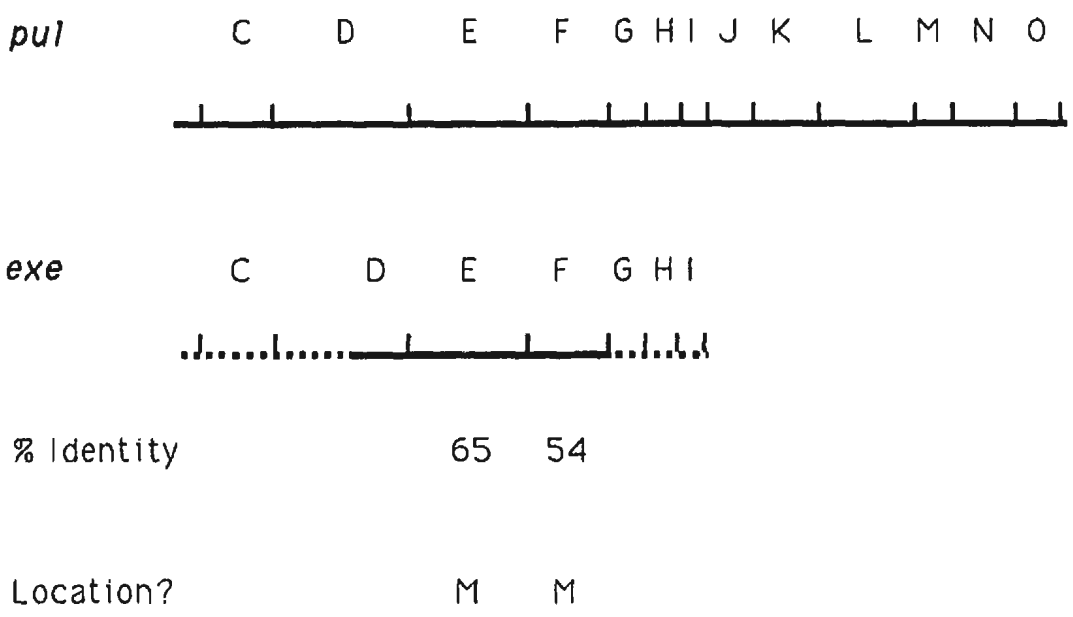


Figure 4.1. Extracellular export genes of *A. hydrophila* and pullulanase secretion genes of *K. pneumoniae*. The solid line represents the sequenced DNA region and the dashed line indicates the unsequenced region. The figure also shows the overall amino acid sequence identity between secretion factors of the two systems. The predicted cellular location of ExeE and ExeF is also indicated.

process used by various gram-negative bacteria appeared very different. For example, the pullulanase secreted by Klebsiella pneumonia has a lipoprotein signal peptide and is anchored on the external face of the outer membrane by the fatty-acyl chains attached to the N-terminal cysteine residue before being released into the medium. In contrast, proteins such as aerolysin secreted by Aeromonas hydrophila have signal peptides typical of those processed by signal peptidase I and are rapidly released to the medium.

In this study, however, the finding that these two bacteria have homologous secretion genes suggests that although the function and structure of the extracellular proteins secreted are completely different, the extracellular protein precursors are processed by different signal peptidases, and the secreted proteins differ in their covalent modification, release kinetics and association with the outer membrane, the secretion pathways employ a conserved translocation apparatus.

Recent studies on the extracellular protein export systems of other gram-negative bacteria have shown that similar secretion pathways are also shared by Pseudomonas aeruginosa and Erwinia chrysanthemi. The xcpX, xcpY and xcpA genes in P. aeruginosa are homologous to pulL, pulM and pulO, while the outH, outI, outJ and outK genes in E. chrysanthemi are homologous to pulH, pulI, pulJ and pulK (Figure 4.2).

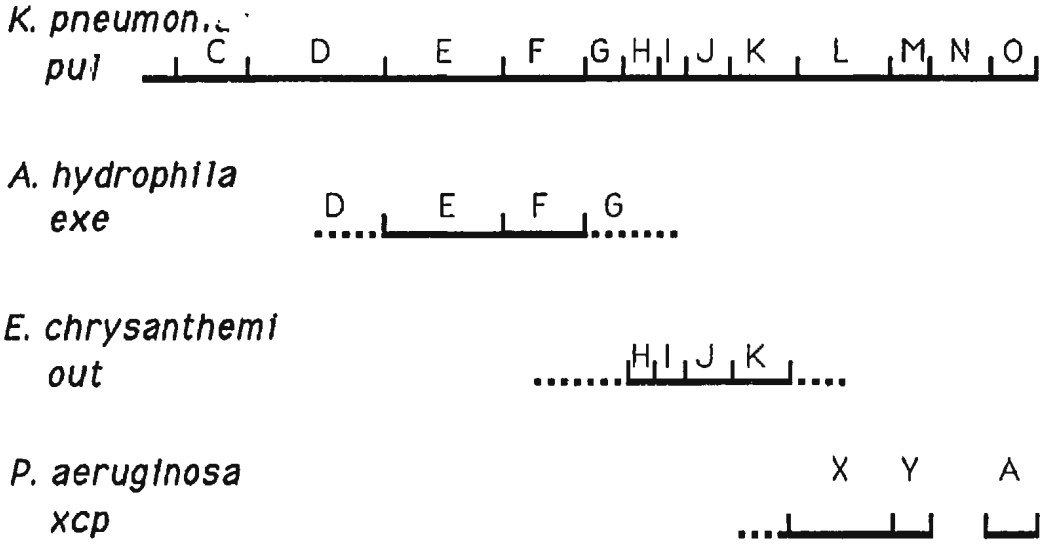


Figure 4.2. Comparision of the signal-sequence dependent secretion machineries among different gram-negative bacteria. The protein secretion genes of four different systems are shown (not to scale). See text for details.

4.6 Possible roles of the Pul-like factors

Whereas it is clear that the pul related genes are required for protein translocation across the outer membrane of gram-negative bacteria, it is not yet known how they exert their functions. It has been proposed that the pul secretion factors form a cell envelope spanning complex that is responsible for the translocation of proteins from the periplasm across the outer membrane to the extracellular growth medium (Pugsley et al., 1990a).

XcpA, which is required for protein secretion in Pseudomonas aerugiosa and homologous to PulO, appeares to be identical to the recently described PilD, with only two amino acid changes in the reported sequences: Thr-18 and Asn-30 of XcpA to Ala residues in PilD (Bally et al., 1991; Nunn et al., 1990). PilD protein is an endopeptidase with a substrate specificity for proteins with special leader sequences typically found on prepilin or related proteins, which includes precusors of other type IV methylphenylalanine pilins (Nunn and Stephen, 1991). These leader sequences are composed of six or seven positively charged residues and thus are completely different from the typical signal-sequences responsible for directing protein export across the inner membrane.

PilD mutants are unable to process prepilin to the mature form and are also deficient in secretion of a number of

extracellular enzymes, which are instead accumulated in the periplasmic space (Strom et al., 1991). These proteins include exotoxin A, phospholipase C, alkaline phosphatase and elastase. None of these are synthesized with leader sequences similar to that of the pilin subunit. Moreover, for one of these typical signal-sequence containing proteins, the periplasmic form has been shown to be the mature form, with its signal-sequence successfully processed. Therefore, the effect of PilD on the protein secretion process is apparently indirect. PilD may act on components of a secretion apparatus for these proteins, rather than on the secreted proteins themselves.

Pilin itself can not be part of a protein-secretion apparatus, since the mutations within the pilin structural gene do not interfere with protein secretion. Therefore, it is very likely that certain structural components required for proper assembly of the translocation apparatus contain the newly identified leader peptides and need processing by the PilD/XcpA proteolytic activity. In support of this idea, regions within proteins PulG, PulI, PulJ and ExeG are found to share significant amino acid homologies in the area surrounding the leader peptide cleavage site of the prepilin of Pseudomonas aeruginosa (Figure 4.3).

Considering that exeE is very homologous to pilB, which is required for the pili assembly in Pseudomonas aeruginosa,

Prepilins of:	
P.aeruginosa	MetLys AlaGlnLysGly PheThrLeuIleGluLeuMetIleValVal-
M.bovis	MetAsn AlaGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
N.gonorrhoeae	MetAsnThrLeuGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
B.nodosus	MetLysSerLeuGlnLysGly PheThrLeuIleGluLeuMetIleValIle-
V.cholerae	Met-(21aa)--GlnGluGly MetThrLeuLeuGluValIleIleValLeu-
K.pneumoniae	
PulG	MetGlnArgGlnArgGly PheThrLeuLeuGluIleMetValValIle-
PulI	MetLysLysGlnSerGly MetThrLeuIleGluValMetValAlaLeu-
PulJ	MetIleArgArgSerSerGly PheThrLeuValGluMetLeuLeuAlaLue-
A.hydrophila	
ExeG	MetGlnLysArgArgGlnSerGly PheThrLeuLeuGluValMetValValIle-
Consensus	-Gly Phe or Met ThrLeu*Pho*Glu-(*Pho*16-18)-

Figure 4.3 Comparison of the amino acid sequences of general secretion factors with the N-terminus of type IV pilins. The cleavage site of the prepilin is indicated by the arrow. *Pho* represents any hydrophobic residue. Modified from Nunn and Lory, 1991.

mutants; (3) gene-specific mutagenesis of *exeD*, *exeF*, *exeG* and other *exe* genes, to see if they are essential for extracellular protein export, by first inactivating the cloned genes *in vitro* and then incorporating the mutation into the wild-type chromosome by homologous recombination; (4) examination of the similarities among different gram-negative bacteria protein secretion systems by cross-complementation studies, or by introducing cloned extracellular proteins of one bacterium into another bacterium to see if the heterologous protein can be secreted.

that *pulO* is similar to *pilD*, and that *xcpA* is identical to *pilD*, it is certainly possible that some of the components required for pili assembly are also involved in the general extracellular protein export process as suggested by Bally *et al.* (1991) and Strom *et al.* (1991). This hypothesis is very reasonable, since the pilin subunits have to be translocated across both the inner and outer membranes before being assembled. It is also possible that some pilin-like proteins form part of the general extracellular protein translocation apparatus. Thus, it will be very interesting to study pilin assembly in the *A. hydrophila* extracellular protein export mutants, to see if this process is also affected by the *exe* gene mutations.

Currently, the *in vivo* expression of the *exeE* gene in *E. coli* cells and the subcellular location of ExeE protein as well as the DNA sequence of the chromosomal fragment downstream from the 11 kb fragment cloned in pJB3 are being studied in this laboratory. Other experiments for future research should include: (1) the subcloning and DNA sequencing of the *exe* gene(s) mutated in C5.84 and *in vivo* identification and localization of its protein product; (2) determination of the relationship between these transposon insertion mutants and the previously isolated chemically-induced mutants by complementation analysis, i.e. by introducing various cloned *exe* genes into the chemical

Chapter 5

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