ISOLATION AND CHARACTERIZATION OF AN OPERON, exeAB, CONTAINING TWO GENES WHICH ARE REQUIRED FOR EXTRACELLULAR PROTEIN SECRETION IN Aeromonas hydrophila

RAVINDRA V JAHAGIRDAR

ISOLATION AND CHARACTERIZATION OF AN OPERON, *exeAB*, CONTAINING TWO GENES WHICH ARE REQUIRED FOR EXTRACELLULAR PROTEIN SECRETION IN *Aeromonas hydrophila*

by

Ravindra V Jahagirdar

A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Master of Science in Medicine

Faculty of Medicine
Memorial University of Newfoundland

September 1994

St. John's Newfoundland

ISBN 0-612-01870-9

DEDICATION

This thesis is dedicated

to my beloved parents,

Vithalrao and Vijayalaxmi

ABSTRACT

Aeromonas hydrophila secretes many proteins into the extracellular milieu. Strain C5.84 is a Tn5-751 insertion mutant of this bacterium which is unable to secrete extracellular proteins, instead accumulating them in the periplasm (Jiang and Howard, 1991). A 3.5 kb *Bgl* II fragment which complements the mutation in C5.84 was isolated from the chromosome of the wild type strain and sequenced. Computer analysis of this fragment revealed an operon-like structure with two complete genes, *exeA* and *exeB*, a promoter 5' to the *exeA* gene and a 13 base pair inverted repeat immediately 3' to the *exeB* gene. Although the transposon had inserted in *exeA*, provision of a wild type copy of this gene alone *in trans* did not restore competence for export to C5.84. Complementation required the presence of both *exeA* and *exeB*, and marker exchange mutagenesis further established the requirement for both gene products for extracellular secretion. In addition, immunoblots with an anti-ExeB serum demonstrated that the Tn5-751 insertion in *exeA* had a polar effect on the expression of *exeB*.

In vivo and *in vitro* expression studies confirmed that there is a promoter 5' to *exeA* and that *exeA* and *exeB* form an operon. Analysis of the deduced amino acid sequence of ExeA indicated that it is a hydrophilic 60 kDa protein with a consensus ATP-binding site. ExeB is a 25 kDa basic protein which shares limited homology with PulB, a protein of unknown function associated with the maltose regulon of *Klebsiella oxytoca*, and OutB, a protein which has been shown to be required for efficient secretion in *Erwinia chrysanthemi*. The

hydropathy analysis of these proteins and preliminary localization studies suggested that both ExeA and ExeB may be anchored to the inner membrane.

These results demonstrate the requirement of a second operon encoding a putative ATP-binding protein in the secretion of extracellular proteins from Gram-negative bacteria, and further suggest that the cytoplasmic compartment may play a greater role in protein translocation across the outer membrane from the periplasm than previously thought.

ACKNOWLEDGEMENTS

I would like to thank my family and my best friend, my wife for their continual support and understanding during the course of this thesis.

Special thanks are due to my supervisor, Dr. S. Peter Howard, for his guidance, advice and assistance during the course of my research, for his critical review of this thesis and for his patience and understanding.

Thanks to Dr. Jon Church for critically reviewing this thesis. Thanks to Dr. Martin Mulligan and Dr. Laura Gillespie for critically reviewing this thesis and for their suggestions.

I would also like to thank Heather Meiklejohn for taking pictures and my lab colleagues for their encouragement.

I would like to acknowledge the School of Graduate Studies for financial assistance in the form a Graduate Scholarship.

This research was supported by a grant from the Medical Research Council of Canada.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

1.1 Envelope structure of Gram-negative bacteria

A continuous exchange of molecules takes place between the bacterial cytoplasm and the extracellular milieu, which includes processes such as the uptake of nutrients and the secretion of proteins. The bacterial cell envelope separates the intracellular compartment from the external medium. The cell envelope of Gram-negative bacteria consists of three layers, the inner or cytoplasmic membrane, the outer membrane, and the periplasm which lies between these two lipid bilayers and contains the peptidoglycan layer. The inner membrane is a typical plasma membrane containing a range of biosynthetic and transport proteins, and phospholipids. The periplasm is an aqueous compartment containing enzymes and transport proteins. An asymmetric bilayer of phospholipids, proteins and lipopolysaccharides (LPS) make up the outer membrane. The outer membrane is a rather inert structure which acts as a molecular sieve, permitting the passage of hydrophilic molecules in a size dependent manner. It acts as a protective barrier against the attack of enzymes and antibiotics, and prevents the entry of a wide range of compounds (Nikaido and Vaara, 1987).

1.2 Protein secretion in Gram-negative bacteria

In contrast to proteins secreted by Gram-positive bacteria, proteins secreted by Gram-negative bacteria have to cross an additional lipid bilayer, the

outer membrane. Many Gram-negative bacteria such as *Escherichia coli* (Noegel *et al.*, 1979), *Pasteurella haemolytica* (Strathdee and Lo, 1989), *Bordetella pertussis* (Glaser *et al.*, 1988), *Neisseria gonorrheae* (Pohlner *et al.*, 1987), *Klebsiella oxytoca* (Pugsley, 1986; 1993a; Pugsley *et al.*, 1990b), *Vibrio cholerae* (Hirst *et al.*, 1984; Overbye *et al.*, 1993), *Pseudomonas aeruginosa* (Lory *et al.*, 1983; Bally *et al.*, 1992; Akrim *et al.*, 1993), *Erwinia chrysanthemi* (Lindeberg and Collmer, 1992), *Erwinia carotovora* (Reeves *et al.*, 1993), *Xanthomonas campestris* (Dums *et al.*, 1991; Hu *et al.*, 1992) and *Aeromonas hydrophila* (Jiang and Howard, 1991; 1992; Howard *et al.*, 1993) actively secrete proteins to the extracellular milieu. Bacterial secretory proteins include a wide range of degradative enzymes, toxins and virulence factors which play significant roles in veterinary, plant and human diseases, and also certain enzymes that have potential value in the biotechnology industry. Considerable interest has been generated recently in the targeting processes that direct these proteins to the extracellular medium. Although these proteins are synthesized in the cytoplasm, their functional location is outside of this compartment. Therefore it is of interest to study how these hydrophilic proteins are translocated across the hydrophobic core of the two separate lipid bilayers of the Gram-negative cell wall. A number of different mechanisms exist for protein translocation in Gram-negative bacteria. Two very distinct modes of extracellular secretion which have been studied in some detail are: the signal sequence independent pathway as exemplified by the α-hemolysin of *E. coli*, and the signal sequence dependent or general secretion pathway as exemplified by pullulanase of *K. oxytoca*.

1.3 Signal sequence independent secretion pathway

Studies on extracellular protein secretion have revealed that many Gram-negative bacteria utilize the signal sequence independent secretion pathway. In this pathway, extracellular proteins (exoproteins) are secreted without typical amino terminal signal sequences and appear to be exported across both the inner and outer membranes in a single step, possibly through membrane adhesion sites, thereby bypassing the periplasm. The common features shared by the proteins secreted in the signal peptide independent pathway are; (a) The proteins are secreted without the involvement of N-terminal signal sequences, (b) The secretion system is composed of two inner membrane proteins, of which one has a conserved ATP-binding motif, plus one outer membrane protein and (c) A secretion signal containing a glycine-rich repeated motif close to the C-terminus of the protein (Ghigo and Wandersman, 1992). The α-hemolysin produced by hemolytic *E. coli* strains is the best characterized protein of this secretion pathway.

1.3.1. α-hemolysin secretion by *Escherichia coli*

E. coli α-hemolysin is encoded by the hemolysin secretion determinants present on transmissible plasmids in certain strains of animal origin as well as on the chromosome of some strains isolated from humans (Muller *et al.*, 1983). These *E. coli* strains secrete a 110 kDa cytotoxin (α-hemolysin) which is implicated in the pathogenesis of diseases including diarrhea among newborn animals, intestinal infections of pigs and poultry, and urinary tract and extra-intestinal infections of humans (Mackman and Holland, 1984).

The α-hemolysin of *E. coli* was the first protein identified as belonging to the RTX (*r*epeats in *to*x*in*) family that contain tandem nine amino acid repeats of the sequence GGBGBBXLX called the glycine-rich repeat. Noegel *et al.* (1979), using transposon mutagenesis, complementation and recombination studies identified the location of cistrons on the transmissible plasmid pHly152, which are essential for hemolysis. The production of active α-hemolysin requires at least two cistrons *hlyA* and *hlyC* and its secretion is controlled by $hlyB_a$ and $hlyB_b$ (Noegel *et al.*, 1979; Wagner *et al.*, 1983). Sequencing of an 8 kb chromosomal region from strain J96 also led to the identification of four cistrons which coincided with those reported by Noegel *et al.* (1979), and a general transcriptional organization model for the *E. coli* α-hemolysin was proposed (Felmlee *et al.*, 1985b). The polypeptides encoded by *hlyC*, *hlyA*, *hlyB*, and *hlyD* are 20 kDa, 110 kDa, 80 kDa, and 50 kDa respectively and all lack typical N-terminal signal sequences (Mackman *et al.*, 1985; Nicaud *et al.*, 1985; Felmlee *et al.*, 1985b). The four *hly* genes dictate the synthesis and export of the cell-free hemolysin HlyA, and are contiguous and co-transcribed from a promoter 5' to *hlyC* (Koronakis and Hughes, 1988). The α-hemolysin is encoded by the structural gene *hlyA* in an inactive form, and is converted to an active form in the cytoplasm by specific fatty acylation by the HlyC protein (Nicaud, *et al.*, 1985; Issartel *et al.*, 1991). In mutants lacking *hlyB* and or *hlyD*, or which have deletions in *hlyA* that fail to secrete the hemolysin, no periplasmic activity was detected, suggesting that the secretion across the two membranes is tightly coupled (Hirst and Welch, 1988).

The extent of N-terminal processing of the *hlyA* gene product was defined by amino acid sequencing and comparison with the predicted amino acid

sequence based on DNA sequencing. It was found that the first nine N-terminal residues were the same except for the absence of the formylated methionine. This confirmed the absence of N-terminal signal sequence processing by signal peptide cleavage (Felmlee *et al.*, 1985a). α-hemolysin secretion is independent of *secA* which is normally required for signal sequence dependent secretion (see later). This was demonstrated by efficient secretion of α-hemolysin in *secA*^{ts} mutants (Mackman *et al.*, 1987). In addition, Nicaud *et al.* (1985), demonstrated by transposon mutagenesis and complementation analysis that α-hemolysin is efficiently secreted in the absence of *hlyC*, which is necessary only for its activation, suggesting the necessity of only *hlyB* and *hlyD* for its secretion (Nicaud *et al.*, 1985). Therefore, it was proposed that HlyB and HlyD may form a specific secretion complex, which spans the inner and outer membrane and may bind to HlyA to promote its translocation.

1.3.1.2 Secretion signal of α-hemolysin

Studies involving a truncated derivative of the HlyA determinant (pLG574) containing the last 27 amino acids of the C-terminus (Gray *et al.*, 1986), a chimeric OmpF-HlyA protein containing the last 113 residues of HlyA and also C-terminal truncated HlyA derivatives generated by Tn5 insertion mutagenesis localized the α-hemolysin secretion signal close to its C-terminal end. In addition, the C-terminal secretion signal alone was also shown to allow secretion of heterologous proteins in a HlyB/HlyD dependent manner (Mackman *et al.*, 1987). This was subsequently confirmed by demonstrating the export of hybrid proteins generated by fusing various C-terminal portions of the hemolysin to the

cytoplasmic protein chloramphenicol acetyltransferase, β-galactosidase, or mammalian prochymosin (Kenny *et al.*, 1991). The absence of the cytoplasmic marker malate dehydrogenase (MDH), and the periplasmic marker β-lactamase in the medium following the induction and secretion of the hybrid proteins, confirmed that α-hemolysin was released neither by lysis nor by preferential release of portions of the outer membrane or the periplasmic compartment (Mackman *et al.*, 1987). Another proof of true secretion was complete degradation of the proteins present in culture supernatants by protease treatment, and the presence of the chimeric protein in the supernatant fractions after high speed centrifugation.

Comparison of the C-terminal 103 amino acids of homologous *hlyA* genes cloned from the genera *Proteus* and *Morgenella* identified a conserved potential amphiphilic α-helix at residues 973-990 followed by a cluster of charged residues at residues 991-996, and weakly hydrophobic sequences rich in hydroxylated residues very close to the C-terminus at residues 999-1024 (Koronakis *et al.*, 1989). An inframe deletion plasmid encoding only the C-terminal 53 amino acids directed HlyB-HlyD dependent export similar to that of the wild type, whereas plasmids encoding only the C-terminal 38 amino acids could export less than 10% of the α-hemolysin the fragment produced, indicating the requirement for the amphiphilic α-helix for export. Deletion of the cluster of charged residues also eliminated the HlyA export. These studies led to the conclusion that residues 970 to 1017 at the C-terminal end of HlyA are essential for its secretion (Koronakis *et al.*, 1989). By extensive mutational analysis, the role of the last 48 amino acids of the C-terminal region of the *E. coli* α-hemolysin was confirmed as the targeting signal for the extracellular secretion (Stanley *et al.*, 1991).

1.3.1.3 Membrane topology and functional domains of HlyB

Using the β-lactamase topology vector pJBS633, a HlyB topology map was generated (Holland *et al.*, 1990; Wang *et al.*, 1991). HlyB consists of eight transmembrane domains, plus four periplasmic loops and three cytoplasmic loops, with a short hydrophobic N-terminal domain, and a large C-terminal domain containing a highly conserved ATP-binding region found in many bacterial transport proteins. HlyB thus belongs to the family of ABC (ATP-binding cassette) proteins involved in transport across membranes (Higgins *et al.*, 1986). *In vitro* studies on a homologous protein, purified mammalian P-glycoprotein, which mediates translocation of drugs, demonstrated that the protein was able to hydrolyze ATP. Therefore, it was postulated that HlyB which is homologous to the P-glycoprotein, hydrolyzes ATP during translocation of HlyA and acts as a membrane translocator (Blight and Holland, 1990). In addition, amino acid substitutions in the nucleotide binding site of HlyB either reduced or completely eliminated HlyA secretion, suggesting the requirement for this ATP-binding site in α-hemolysin secretion (Koronakis *et al.*, 1988).

1.3.1.4 HlyD structure and function

A topological model for HlyD was proposed based on the results of gene fusions of *lacZ* with the first 180 base pairs of *hlyD* and of alkaline phosphatase (*phoA*) between nucleotides 262 and 1405 of *hlyD*. The gene fusion data implied that HlyD is anchored to the inner membrane by a single transmembrane segment located between amino acids 60 and 80 of the protein, while the residual remaining 400 amino acids are located in a periplasmic domain rich in α-

helix forming sequences (Shulein *et al.*, 1992). In the absence of HlyD, hemolysin was shown to accumulate in the cytoplasm. Based on localization and protease digestion experiments, it was concluded that HlyB and HlyD form a transmembrane complex spanning both membranes (Wang, *et al.*, 1991). On the basis of the demonstration that the minor outer membrane protein TolC is required for extracellular export of HlyA (see below), a model was proposed where TolC forms a pore in the outer membrane which is opened or modified by the presence of a transporter consisting of HlyB and HlyD in the inner membrane (Wandersman and Letoffe, 1993). The amphipathic domain present in the C-terminus of HlyA which is involved in α-hemolysin secretion, may serve as a signal to the translocation apparatus HlyB-HlyD. This domain is believed to spontaneously insert HlyA into the inner membrane in an orientation such that the HlyB-HlyD complex can translocate the remaining polypeptide across both membranes (Lory, 1992). There is no direct evidence as to the locations of HlyB and HlyD in wild type cells because of a low level of expression and the absence of specific antibodies against these proteins. The location of HlyB and HlyD is inferred to be inner membrane based on sequence data, the fusion proteins discussed above, and the fractionation of radiolabeled proteins produced in mini or maxi cells (Mackman *et al.*, 1985; Wang *et al.*, 1991).

1.3.1.5 Requirement for TolC

In addition to HlyB and HlyD, the requirement for the minor outer membrane protein TolC for α-hemolysin secretion has been demonstrated (Wandersman and Delepelaire, 1990). A plasmid carrying the chromosomal

DNA fragment encoding TolC restored the hemolytic phenotype when introduced into a *tolC*::Tn5 insertion mutant and a spontaneous *tolC* mutant. The specificity of the requirement of TolC for α-hemolysin secretion was established when a plasmid carrying protease B and C determinants of *Er. chrysanthemi* was introduced into *E. coli* wild type and *tolC*::Tn5 insertion mutant strains. The culture supernatants from both strains were analyzed by western blotting. The hemolytic phenotype was observed only in the wild type strain but not in the insertion mutant, whereas the protease secretion which was observed in the insertion mutant was due to an analogous function of TolC in *Er. chrysanthemi*, suggesting that the plasmid carrying protease B and C determinants of *Er. chrysanthemi* also carries an counterpart of TolC. Based on *hlyA-lacZ* operon fusion studies, it was also shown that the *tolC* mutation does not effect *hlyA* transcription, indicating a specific effect on α-hemolysin secretion by TolC (Wandersman and Delepelaire, 1990). Therefore, secretion of HlyA into the extracellular medium is governed by three accessory genes. Two of these genes, *hlyB* and *hlyD* are linked to the structural gene *hlyA*, while TolC is the product of an unlinked gene, and might allow the interaction of the HlyB and HlyD complex with the outer membrane.

1.3.1.6 Role of lipopolysaccharides (LPS) in α-hemolysin secretion

The vancomycin resistant *E. coli* non-hemolytic chromosomal mutants VANR23 and VANR33 carrying the α-hemolytic determinant identified the loci *rfaH/hlyT* (Bailey *et al.*, 1992), involved with hemolysin secretion, LPS assembly, and sex pilus production, and *galU* (Bachmann, 1990), which affects colicin E_2

resistance, LPS biosynthesis and detergent sensitivity (Fukasawa *et al.*, 1963). Western blots of membrane fractions of VAN23 and VAN33 with anti-TolC showed the presence of very low amounts of TolC in the envelope fractions, suggestive of a defect in the insertion of TolC in the envelope. The fact that hemolysin secretion as well as LPS biosynthesis are affected in *rfaH* and *galU* mutants indicated that the secretion defect is due to incomplete LPS assembly. In these mutants, the polysaccharide core is missing all the sugars beyond the heptose region. It was hypothesized that this defect in LPS might prevent the insertion of TolC in the outer membrane (Wandersman and Letoffe, 1993).

1.3.2 Signal sequence independent secretion pathway in other bacteria

A large number of proteins are secreted by a signal sequence independent pathway similar to that used by the α-hemolysin of *E. coli*. This family consists of the cytotoxins from *Proteus vulgaris*, *Proteus mirabilis*, *Morgenella morganii* (Koronakis *et al.*, 1987), the metalloproteases B and C of *Er. chrysanthemi* (Delepelaire and Wandersman, 1990; Letoffe *et al.*, 1990), the metalloprotease of *S. marcescens* (Letoffe *et al.*, 1991), the alkaline protease of *Ps. aeruginosa* (Guzzo *et al.*, 1991a), the bifunctional adenylate-cyclase hemolysin, cyclolysin, of *Bo. pertussis* (Glaser *et al.*, 1988), the leucotoxin of *Pa. haemolytica* (Strathdee and Lo, 1989), the *Rhizobium leguminosarum* NodO protein (Economou *et al.*, 1990) and the colicin V and microcin B17 secreted by *E. coli* (Gilso *et al.*, 1990; Garrido *et al.*, 1988) suggesting that this family of secretion proteins are widely distributed among the Gram-negative bacteria.

Although these proteins are often very distinct in their structure and

function, the components of their secretion machineries show a high level of amino acid sequence similarity. For example, the *lktB* and *lktD* leucotoxin export genes of *Pa. haemolytica* have 90.5% and 75.6% identity to *E. coli hlyB* and *hlyD* respectively (Strathdee and Lo, 1989). Sequence analysis of the *Er. chrysanthemi* metalloprotease secretion genes *prtD, prtE* and *prtF* revealed significant homology to the HlyA secretion genes *hlyB, hlyD* and *tolC* of *E. coli*, suggesting that *E. coli* α-hemolysin and *Er. chrysanthemi* proteases share a similar mechanism of secretion (Letoffe *ei al.*, 1990; Ghigo and Wandersman, 1992). A similar situation exists with the alkaline protease secretion genes *aprD, aprE* and *aprF* of *Ps. aeruginosa* (Guzzo *et al.*, 1991b), the adenylate-cyclase genes *cyaA, cyaB* and *cyaD* of *Bo. pertussis* (Glaser *et al.*, 1988) and with the protease secretion genes *prtD* and *prtE* of *S. marcescens* (Letoffe *et al.*, 1993). When a plasmid containing *prtD* and *prtE* genes of *S. marcescens* was introduced into a *tol*$^+$ and a *tolC*::Tn10 insertion mutant of *E.coli*, protease was secreted only in the wild type strain and not in the insertion mutant. This suggested that a third component, homologous to TolC, is also required for protease secretion in *S. marcescens*, however this has not been identified (Letoffe *et al.*, 1993).

It has also been demonstrated that a fusion protein carrying an N-terminal fragment of *cyaA* of *Bo. pertussis* with the C-terminal secretion signal of the protease PrtB of *Er. chrysanthemi* containing conserved glycine-rich repeats was secreted in *E. coli*, whereas without any of these repeats it was not, indicating the involvement of the glycine-rich repeat domain of the metalloprotease in secretion as had been shown for the α-hemolysin of *E. coli* (Letoffe and Wandersman, 1992). Trans-complementation studies have also

demonstrated that the signal sequence independent secretion pathway in Gram-negative bacteria is well conserved in terms of its function as well as its sequence, at least in some cases. For example, *Er. chrysanthemi* efficiently secreted the alkaline protease AprA of *Ps. aeruginosa* and protease of *S. marcescens* but not HlyA of *E. coli*, whereas the Hly secretion factors in *E. coli* secreted the proteases PrtB and PrtA of *Er. chrysanthemi*, AprA of *Ps. aeruginosa* and leucotoxin of *Pa. haemolytica* (Delepalaire and Wandersman, 1990; Guzzo *et al.*, 1991a). In addition, it was shown that when a plasmid containing the *cyaA* gene of *Bo. pertussis* was transformed into *E. coli* strains containing the *hly* operon, the adenylate-cyclase protein was secreted, suggesting that the *hly* secretion factors can substitute for the corresponding *Bo. pertussis* components (Masure *et al.*, 1990). It was also demonstrated that a hybrid secretion apparatus consisting of *S. marcescens* PrtD and PrtE in *E. coli* could promote the secretion of protease B of *Er. chrysanthemi* but only in a tolC$^+$ strain (Letoffe *et al.*, 1993).

1.4 Other specialized secretion systems in Gram-negative bacteria

Certain classes of proteins, including the IgA protease of *N. gonorrheae* (Pohlner *et al.*, 1987), the IgA protease of *Haemophilus influenzae* (Grundy *et al.*, 1987) and the serine protease of *S. marcescens* (Yanagida *et al.*, 1986), are secreted via an outer membrane-anchored intermediate. These proteases are secreted across the cytoplasmic membrane via the signal sequence dependent general export pathway, but unlike other proteins do not seem to require additional secretion factors. The C-terminal β-domain of the protease precursor

integrates into the outer membrane thus providing an essential transport function. The protease is then released by autoproteolytic cleavage from the outer membrane. Since the information for secretion resides within the secreted protein itself, they are also called "autosecreted" proteins (Klauser *et al.*, 1990; Pugsley, 1993a).

Another less well understood secretion system is the secretion of virulence plasmid encoded proteins called Yops (*Yersinia* outer proteins) by the animal pathogen *Yersinia enterocolitica*. Yops are secreted without signal sequences, and are targeted to the extracellular environment by a Sec independent system, but their secretion is dependent on the gene products of 13 genes, *yscA-M*, situated at the *virC* locus of the virulence plasmid (Michiels *et al.*, 1991; Woestyn *et al.*, 1994). Although it is not yet known whether or not these proteins enter the periplasm, it is clear that they follow a novel secretion pathway. A number of other bacteria have been shown to use genes homologous to the *yop* genes for the secretion of protein factors, including *Shigella flexneri* (Allaoui *et al.*, 1993) and *H. influenzae* (Tomb *et al.*, 1991).

1.5 Two step signal sequence dependent or general secretion pathway (GSP)

The most prevalent pathway of extracellular protein secretion is a two step process, which appears to involve translocation first across the inner membrane and then in a separate step, translocation across the outer membrane with a transient stopover in the periplasm. This was first suggested by studies in which a periplasmic intermediate was observed in the extracellular secretion of aerolysin by *A. hydrophila* (Howard and Buckley, 1983) and of heat labile

enterotoxin of *V. cholera* (Hirst and Holmgren, 1987).

Proteins secreted by this pathway have an N-terminal signal sequence which usually consists of 20-25 amino acid residues. The signal sequence has three domains, a positively charged N-terminal domain, a central highly hydrophobic uncharged core and a more polar C-terminal domain containing the signal peptidase cleavage site (Pugsley, 1987; Von Heijne, 1984). This sequence directs the protein to the inner membrane and facilitates its translocation across the membrane, following which the signal sequence is proteolytically cleaved from the protein by the membrane enzyme signal peptidase (Pugsley, 1987; Pugsley *et al.*, 1990).

Translocation of proteins across the inner membrane has been characterized by numerous genetic and biochemical studies and comprises a complex secretion machinery termed the general export pathway (GEP). This pathway consists of the translocase SecA, SecY, SecE, SecD and SecF proteins and the chaperone SecB (Schatz and Beckwith, 1990; Salmond and Reeves, 1993).

The second step of the signal sequence dependent pathway has been best characterized for pullulanase secretion in *K. oxytoca* and has also been identified in a growing number of other families of Gram-negative bacteria (see later). Recently, much interest has been generated in studies of secretion across the outer membrane by this pathway and it has been shown to involve a multitude of additional protein factors comprising a secretion machinery known as the general secretion pathway (GSP) (Pugsley *et al.*, 1990; Pugsley, 1993a).

1.5.1 Pullulanase secretion by *Klebsiella oxytoca*

Pullulanase, a starch debranching enzyme cleaves glucose polymers at α-(1-6) glucosidic linkages to release linear dextrins composed of α-(1-4) linked glucose residues. Pullulan, an α-(1-6) linked glucose maltotriose polymer, is used as a carbon source by *Klebsiella* (Michaelis *et al.*, 1985). *pulA*, the structural gene for pullulanase was cloned from the chromosome of *K. oxytoca* and transferred into *E. coli* K12 to study the regulation and export of gene products involved in maltose metabolism. The *pulA* structural gene was localized by Tn5 mutagenesis and by *in vitro* constructed deletions (Michaelis *et al.*, 1985; Chapon and Raibaud, 1985).

Sequence analysis of the 5' end of *pulA* revealed that the first 19 amino acids of pullulanase resemble a lipoprotein signal peptide (Chapon and Raibaud, 1985). The N-terminal sequence of pullulanase bears strong similarities to the precursors of known lipoproteins and PulA was subsequently shown to be a lipoprotein using the antibiotic globomycin. Globomycin inhibits pro-lipoprotein signal peptidase II causing the precursor form to accumulate within the membranes. The precursor form was then chased into the mature form after the removal of globomycin (Pugsley *et al.*, 1986). The last four residues (Leu^{16}-Leu-Ser-Gly^{19}) of the pullulanase signal peptide and the first residue of the mature pullulanase (Cys^{20}) are typical of those found in bacterial lipoproteins. These lipoproteins are exported via the general export pathway and are cleaved by lipoprotein signal peptidase during which the (Cys^{20}) is modified to glyceryl-cysteine and eventually fatty acylated (Murooka and Ikeda, 1989). This was demonstrated by site directed mutagenesis of *pulA* replacing the (Cys^{20}) with (Ser^{20}). The wild type pullulanase was modified with glyceryl-lipids but the

mutant was not. The precursor form of the mutant pullulanase was cleaved between residues (Ser^{13}) and (Leu^{14}), six amino acids upstream of the normal lipid modified cleavage site, and this cleavage was resistant to the antibiotic globomycin (Murooka and Ikeda, 1989). Specific labeling of the pullulanase protein by [^{3}H] glycerol and [^{3}H] palmitate demonstrated that pullulanase contains covalently linked fatty acids (Pugsley *et al.*, 1986). Since the processing of signal peptides takes place co-translationally during translocation across the inner membrane, it is unlikely that this lipoprotein signal sequence could play a role in subsequent translocation of protein across the outer membrane (Pugsley, 1987; Pugsley *et al.*, 1990).

Pullulanase is a 117 kDa protein which is exposed to the cell surface prior to its release into the extracellular medium (d'Enfert *et al.*, 1987b; Michelis *et al.*, 1985). When the *pulA* gene from strain ATCC 15050 was expressed in *E. coli* K12, pullulanase was not exposed to the cell surface or released into the medium. In a search for additional genes required for pullulanase secretion, a plasmid gene bank from *K. oxytoca* UNF 5023 which synthesized and exported pullulanase was introduced into *E. coli* which was selected for growth on pullulan (Kornacker and Pugsley, 1989). A 18.8 kb chromosomal fragment thus isolated was shown to carry the structural gene *pulA* and all other genes needed for cell surface localization (d'Enfert *et al.*, 1987b). Tn10 insertions adjacent to and on either side of *pulA* were defective in pullulanase synthesis or secretion suggesting that in addition to *pulA*, more genes were required for pullulanase synthesis and secretion (d'Enfert *et al.*, 1987b). It was shown that the secretion genes located 5' to *pulA* are transcribed divergently to form a part of the maltose regulon under the control of the *malX* promoter, whereas *pulB*, located 3' to *pulA*

was co-transcribed with it under maltose regulation (Kornacker *et al.*, 1989). The final gene, *pulS*, is 3' to *pulAB*, although it is oriented towards this operon and is not under maltose regulation (d'Enfert and Pugsley, 1989).

The secretion signal required for translocation across the outer membrane was shown to be located at the N-terminus of the pre-pullulanase, since a hybrid protein containing 656 N-terminal residues of pre-pullulanase fused to the mature alkaline phosphatase was partially localized to the cell surface by immuno fluorescence tests with anti-pullulanase serum (Kornacker and Pugsley, 1989).

Extensive work on the 18.8 kb DNA fragment of *K. oxytoca* containing the *pul* structural genes by various techniques such as DNA sequencing, complementation studies, TnPho and Tntac-1 mutagenesis, subcellular localization, western blots, and *in vitro* and *in vivo* expression was done and the secretion genes carried on this fragment were characterized (Pugsley *et al.*, 1990, d'Enfert *et al.*, 1987b). Most of the secretion genes were located in the *pulC* operon (now *pulC-O*) 5' and divergent to *pulA.* DNA sequencing of the 5' end of the *pulC* operon revealed two complete genes, which were designated *pulC* and *pulD.* The *pulC* gene (formerly *malX*) encodes a 31 kDa protein and *pulD* encodes a 71 kDa protein with a typical signal sequence. *pulC* and *pulD* were shown to be required for pullulanase secretion and exposition which was abolished by Tn5 insertion mutants (d'Enfert *et al.*, 1989). By TnphoA mutagenesis, subcellular fractionation and immunoblotting, PulC and PulD were localized to the inner and outer membranes respectively (d'Enfert *et al.*, 1989). A 2.5 kb DNA fragment downstream of *pulA* that abolished pullulanase exposition was sequenced to reveal three open reading frames which were designated as

pulS, *pulB* and *ORFY* (d'Enfert and Pugsley, 1989). *pul*S encodes a 12 kDa mature protein, contains an N-terminal signal sequence, and is a lipoprotein. Subcellular fractionation of [^{3}H] palmitate labeled cells localized PulS to the outer membrane fractions. *pulB* was shown to be not required for pullulanase secretion as pullulanase was secreted and exposed in its absence (d'Enfert and Pugsley, 1989).

Four *pul* genes *pulL*, *pulM*, *pulN* and *pulO*, whose molecular weights are 44 kDa, 18 kDa, 27 kDa and 24 kDa, respectively, are located at the 3' end of the *pulC* operon (Pugsley and Reyss, 1990). The region in the center of the *pulC* operon codes for five genes, *pulG*, *pulH*, *pulI*, *pulJ* and *pulK* whose molecular weights are 15 kDa, 18 kDa, 13 kDa, 22 kDa and 36 kDa respectively (Reyss and Pugsley, 1990). Nucleotide sequencing of the last fragment of the 18.8 kb DNA revealed the extreme 3' end of *pulD*, the extreme 5' end of *pulG* and two complete genes designated as *pulE* and *pulF* which encode 55 kDa and 44 kDa proteins respectively (Possot *et al.*, 1992). By Tntac-1 mutagenesis and deletion analysis it was shown that all the aforementioned *pul* genes in the complete *pulC-O* operon are essential for pullulanase secretion.

PulE was shown to have a consensus sequence that shared homology with many known ATP-binding proteins belonging to the membrane associated ABC protein group (also called traffic ATPases) (Pugsley, 1993a; Hobbs and Mattick, 1993). Bacterial ABC proteins involved in the transport of amino acids or sugar transport were shown to interact with integral membrane components of the corresponding permeases (Higgins, 1992). Therefore, PulE might interact with an integral membrane component of the pullulanase secretion machinery (Possot *et al.*, 1992). Site directed mutagenesis of the glycine-rich consensus

sequence GXXGXGKT/S or Walker box A (Walker *et al.*, 1982) of PulE revealed that the consensus sequence was necessary for pullulanase secretion (Possot and Pugsley, 1994). PulE and PulF demonstrate a considerable homology throughout their entire length with proteins involved in secretion, pilin assembly, conjugation and transformation competence in different bacteria as discussed below.

The protein products of the other *pul* genes were identified by *in vitro* expression under the control of *lacZ* promoter, *in vivo* expression under the control of the *lacZ* or T7 promoter, immunoblotting and production in minicells (Pugsley *et al.*, 1990; Possot *et al.*, 1992). The cellular locations of these gene products were identified by subcellular fractionation. Of the 13 *pulC-O* genes essential for pullulanase secretion, 12 encode envelope proteins and appear to be located in the inner membrane, while PulD is located in the outer membrane. PulE, containing the ATP-binding site, was originally reported to be cytoplasmic but later found to be tightly associated with the inner membrane (Possot *et al.*, 1992; Possot and Pugsley, 1994). Positions of potential membrane spanning hydrophobic segments and the positions of TnphoA insertions which encoded active Pul-PhoA fusions in these proteins, suggested that most of them span the inner membrane once with the C-terminal being exposed in the periplasm (Reyss and Pugsley, 1990) except PulF, which spans the membrane three times (Possot *et al.*, 1992) and PulO, which spans the inner membrane several times (Pugsley and Ryess, 1990).

Although 14 *pul* genes (*pulC-O* plus *pulS*) are required for pullulanase secretion, all of them appear to act after the *sec* gene products, since mutations in any of the six *sec* genes block processing of the pre-pullulanase signal peptide

(Pugsley *et al.*, 1991a). Thus, translocation of pullulanase across the cytoplasmic membrane is dependent on six *sec* genes, *secA*, *secB*, *secD*, *secE*, *secF* and *secY* (Schatz and Beckwith, 1990), whereas the *pul* secretion genes are required for the translocation of the pullulanase across the outer membrane (Pugsley *et al.*, 1991a). The secretion of pullulanase by *E. coli* in two distinct steps was demonstrated by Pugsley *et al.* (1991b). They uncoupled the two steps by allowing the enzyme to translocate across the cytoplasmic membrane via the signal sequence and *sec* gene dependent general export pathway, followed by induction of the *pulC-O* operon for translocation to the cell surface (Pugsley *et al.*, 1991b).

To summarize, so far 16 complete *pul* genes have been identified that are present on an 18.8 kb fragment of the *K. oxytoca* chromosome, which contains the genes required for secretion of pullulanase in *E. coli*. The structural gene *pulA* is followed by *pulB*, which has no known function in pullulanase secretion, and both form an operon. Upstream of *pulA* is the 13 gene *pulC-O* operon, all of which are essential for pullulanase secretion, and downstream of *pulAB* is *pulS*, which is also required for secretion. Thus, the 14 *pul* gene products, plus six Sec proteins together with lipoprotein signal peptidase and the glyceryl and fatty acyltransferase enzymes required for lipoprotein modification are known to be involved in pullulanase secretion and modification (Pugsley, 1993a).

1.5.2 Type IV pili and extracellular secretion

An interesting feature of the components of the general secretory pathway is that they share similarity with some of the determinants of type IV pilus biogenesis. Many Gram-negative bacteria such as *N. gonorrheae*, *Moraxella*

bovis, *Bacteroides nodosus*, and *Ps. aeruginosa* express type IV pili (Hultgren and Normark, 1991). These pilins have a conserved amino terminal hydrophobic domain with an amino terminal phenylalanine that is methylated upon processing and secretion. They have a shorter leader sequence than the typical signal sequence. The cleavage sites are well conserved in type IV pilins, except for the major pre-pilin of *V. cholerae* (TcpA), which is preceded by a long hydrophilic segment containing several charged residues (Hultgren and Normark, 1991).

Type IV pre-pilin peptidase was first characterized by Lory and colleagues. They showed that the product of the *pilD* gene of *Ps. aeruginosa* is a peptidase which can cleave and methylate the type IV pre-pilin *in vitro* (Nunn and Lory, 1991). Bacteria that do not have type IV pili have also been shown to contain the gene coding for these peptidases (Pugsley, 1993a). These peptidases cleave a number of pre-pilin-like precursor proteins that are part of the general secretory pathway and most have >50% sequence identity with PilD of *Pseudomonas*. Thus the PulG, PulH, PulI and PulJ proteins share homology at the amino terminus with precursors of type IV pilins and are processed by PulO, which is functionally and structurally related to the peptidase PilD. PulO and PilD have 52% identity (Nunn and Lory, 1991). It was also shown that the pre-pilin peptidase activity of PulO could correctly process the product of the *pilE*1 type IV pilin structural gene from *N. gonorrhea* cloned in *E. coli* (Dupuy *et al.*, 1992). PulO processes pre-pulG at the consensus type IV pre-pilin peptidase cleavage site, and the processed PulG has a methylated N-terminal phenylalanine residue.

In another connection between secretion and pilin assembly, PulE was shown to have 43% identity with the PilB protein of *Ps. aeruginosa* encoded by the *pilBCD* operon. The *pilBCD* operon encodes the PilD pre-pilin peptidase, and

is involved in both processing and assembly of type IV pilin monomers (Nunn *et al.*, 1990). PulE also has 29% identity with PilT, a protein that is required for twitching motility which involves type IV pili, and also may be involved in pilus disassembly (Bradley, 1980; Whitchurch *et al.*, 1991). PulF is homologous over it's entire length with PilC (Whitchurch *et al.*, 1991). Similarities to those discussed above have also been found between the *pul* genes and the Tcp pilus assembly genes of *V. cholerae* (Kaufmann *et al.*, 1991; 1993). The role of these proteins in extracellular secretion is not yet completely understood. Based on these homologies, the hypothesis proposed was that the PilB, PilC and PilD homologues of *K. oxytoca*, PulE, PulF and PulO, may be involved in the assembly of the type IV pre-pilin like proteins PulG, PulH, PulI and PulJ into the cell envelope. These proteins might connect the inner and outer membranes with a pilus like structure that facilitates extracellular secretion (Pugsley, 1993a). In a recent study however, no evidence was found for such a multiprotein complex in *K. oxytoca* (Pugsley and Possot, 1993).

1.5.3 Signal sequence dependent secretion in *Pseudomonas aeruginosa*

Exoproteins of *Pseudomonas* are synthesized as precursors with N-terminal signal sequences that are quite long, but otherwise are similar to the signal sequences of *E. coli* (Pritchard and Vasil, 1986; Bitler *et al.*, 1991). Pleiotropic export mutants deficient in extracellular secretion of proteins like exotoxin, alkaline phosphatase, elastase, staphylolytic enzyme and phospholipase were isolated by treating the bacterium with ethyl methane sulfonate (Wretlind and Pavlovskis, 1984). By genetic analysis the mutants were

mapped and many of the *xcp* genes were found to be clustered at 40 minute (previously the 55 minute region) on the new chromosome map (Wretlind and Pavlovskis, 1984). The mutation in the *xcp*-1 (later designated as *xcpA*) locus was genetically and enzymatically characterized, and it was shown that the exoenzymes accumulate in the periplasm of this mutant (Lindgren and Wretlind, 1987). By deletion and subcloning analysis, the *xcpA* gene was isolated from the 0 minute region of the chromosome and was found to encode a 26 kDa inner membrane protein (Bally *et al.*, 1989). The sequence of the *xcpA* gene was determined and it was found to be identical to the *pilD* gene for pre-pilin peptidase as discussed above, and also homologous to the *pulO* gene of *K. oxytoca*. This indicated that in this bacteria the pre-pilin peptidase is specifically required not only for pili biogenesis, but also for secretion of extracellular proteins (Bally *et al.*, 1991; Nunn *et al.*, 1990).

A 40 kb DNA fragment from the 40 minute region of the chromosome of *Ps. aeruginosa* PAO1 strain was cloned and shown to contain the loci identified by six *xcp* mutations, *xcp* 5, 51, 52, 53, 54 and 55 (Filloux *et al.*, 1991). By transposon mutagenesis and restriction mapping, a 9 kb chromosomal DNA was shown to be required for *xcp* expression. Transduction analysis suggested that two different loci exist for protein secretion in this region that are expressed independently (Filloux *et al.*, 1989). Sequencing of a 2.5 kb DNA fragment revealed that it contained two open reading frames, *xcpY* and *xcpZ*. The *xcp* 51 mutation was assigned to the *xcpY* gene and the *xcp* 5 and *xcp* 52 mutations to *xcpZ*. *xcpY* and *xcpZ* were found to be highly homologous to *pulL* and *pulM* with 35% and 30% identity respectively. *xcpY* and *xcpZ* encode proteins of 41 kDa and 19 kDa respectively which were localized to the inner membrane (Filloux *et*

al., 1990).

Sequencing of the region upstream of *xcpY* revealed seven additional genes required for protein secretion and were designated *xcpR* to *xcpX* (Bally *et al.*,1992). XcpR is probably cytoplasmic membrane associated, has a consensus ATP-binding site and is highly homologous to the PulE of *K. oxytoca* and to PilB (Tommassen *et al.*, 1992). Replacement of the glycine residue with serine in the ATP-binding site of XcpR and PilB prevented exotoxin secretion and assembly of pili, and overexpression of the mutant *xcpR* led to the periplasmic accumulation of extracellular enzymes indicating a requirement for ATP-binding for both of these processes (Turner *et al.*, 1993). XcpS and XcpX are also inner membrane proteins and are highly homologous to PulF and PulK of *K. oxytoca*. XcpT, XcpU, XcpV, XcpW, and XcpP are homologous to PulG, PulH, PulI, PulJ, and PulC of *K. oxytoca* respectively. The only protein synthesized with a classical N-terminal signal sequence is XcpQ, which is homologous to PulD, an outer membrane protein in *K. oxytoca*. No homologues for PulN and PulS have been found in *Ps. aeruginosa* (Tommassen *et al.*, 1992).

In total, cloning and sequencing of the 40 minute region of the *Ps. aeruginosa* PAO chromosome revealed 11 *xcp* genes located in two divergently transcribed operons, which correspond to *pulC-M* genes of *K. oxytoca*. As discussed previously, the *pulO* homologue *xcpA/pilD*, is found in the *pilBCD* operon. Mutations in the *pilD* gene were shown to result in pleiotropic defects in extracellular secretion as well as an inability to assemble pili (Strom *et al.*, 1991). Nunn and Lory (1992) identified *xcpT, xcpU, xcpV* and *xvpW* as genes that have PilD-dependent cleavage sites for pre-pilin peptidase and demonstrated by site directed mutagenesis that these genes were also required for protein secretion.

The N-termini of XcpT, XcpU, XcpV, and XcpW are homologous to that of type IV pilin subunits, just as for PulG-J of *K. oxytoca*. XcpA/PilD is the pre-pilin leader peptidase which cleaves the leader peptide of these proteins. XcpR has 42% identity to PilB and XcpS is 31% identical to PilC (Bally *et al.*, 1992). Recently, a new locus required for fimbrial assembly was identified by transposon mutagenesis and was designated *pilQ*. PilQ is a 77 kDa protein with a characteristic N-terminal signal sequence which is homologous to XcpQ and to PulD of *K. oxytoca*. It is thus clear that a close relationship exists between the mechanism of transport of macromolecules across the surface of the bacterial cell and biogenesis of type IV pili which remain attached to the surface (Martin *et al.*, 1993).

1.5.4 Signal sequence dependent secretion in *Erwinia chrysanthemi*

The phytopathogenic bacterium *Erwinia chrysanthemi* secretes a host of plant cell-wall degrading enzymes such as pectate lyases, cellulases, exo-poly-α-D-galacturonidases, pectin methylesterases and proteases which are responsible for soft rot disease of many plant species. Mutants in the secretion of both pectinases and cellulases were isolated by transposon mutagenesis or by selecting strains that did not grow on polygalacturonate (PGA), or those which did not produce haloes in a pectate lyase detection test. Mutants defective in enzyme secretion were designated *out*$^-$ and were severely reduced in virulence (Ji *et al.*, 1989; Wandersman *et al.*, 1990).

Cosmids complementing several transposon induced *out* mutations were isolated and the *out* genes were shown to be clustered on a 12 kb chromosomal

DNA region. Sequencing of a 2.4 kb DNA fragment within this region revealed four colinearly arranged genes sharing significant homology to the *pulH*, *pulI*, *pulJ*, and *pulK* genes of *K. oxytoca*, and were designated as *outH*, *outI*, *outJ*, and *outK* respectively. The homology was 75% for *outH* and *pulH*, 62% for *outI* and *pulI*, 61.6% for *outJ* and *pulJ* and 54.5% for *outK* and *pulK* (He *et al.*, 1991). Eight additional *out* genes were identified when a 3.2 kb downstream fragment and a 7.2 kb upstream fragment were sequenced. These eight genes shared significant homology with the other *pul* genes of the *pulC-O* operon. The amino acid identity between individual Out proteins and their Pul homologues ranges from 34% to 77%. *outO* is transcribed separately from the *outC* through *outM* genes, and there is no homologue to *pulN*, as was also found in *Ps. aeruginosa.* (Lindberg and Collmer, 1992).

OutS has 37% identity with PulS and the C-terminal part of OutB has 53% identity with PulB protein of *K. oxytoca*. Mutants in OutB retain 30% of the pectate lyases produced in the periplasm suggesting that they are affected slightly for secretion (Condemine *et al.*, 1992). Although *outT* is required for secretion, it does not have homology to any of the *pul* genes. Despite the fact that the organization of the *out* genes of *Er. chrysanthemi* and the *pul* genes of *K. oxytoca* are very much identical, the secretion systems of these two organisms are not compatible since the two bacteria are incapable of exporting each others' extracellular proteins (Condemine *et al.*, 1992). Recently, Reeves *et al.* (1993), isolated and characterized 13 *out* genes designated *outC-O* from *Er. carotovora* subspecies *carotovora* that are homologous to the *pul* genes of *K. oxytoca*.

The celiulase endogluconase (EGZ) of *Er. chrysanthemi* contains a disulfide bond in the C-terminal cellulose binding domain between residues Cys-

325 and Cys-382. Recently it has been demonstrated that secretion of EGZ was arrested immediately after the addition of dithiothreitol to *Er. chrysanthemi* cells and that the protein accumulated in the periplasm. Site directed mutagenesis of Cys residues involved in disulfide bond formation also resulted in EGZ accumulation in the periplasm, suggesting that periplasmic disulfide bond formation is a requisite step in providing a secretion competent, pre-folded EGZ (Isabelle *et al.*, 1994).

In addition to the secretion systems of *Ps. aeruginosa* and *Er. chrysanthemi* discussed above, homology to the *pul* genes of *K. oxytoca* (Pugsley, 1993a) were found with the *exe* genes of *A. hydrophila* (see below), the *eps* genes of *V. cholerae* (Overbye *et al.*, 1993), and the *xps* genes of *X. campestris* pv *campestris* (Hu *et al.*, 1992). This suggests that the general secretion pathway is well conserved among the Gram-negative bacteria.

1.5.5 Protein secretion in *Aeromonas hydrophila*

A. hydrophila, a facultatively anaerobic Gram-negative rod which belongs to the Order *Pseudomonadales* and Family *Vibrionaceae* (Bergey's Manual of Systematic Bacteriology, 1984) is an opportunistic pathogen of man (Graevenitz and Mensch, 1968), animals (Wohlgemuth *et al.*, 1972), reptiles (McCoy and Seidler, 1973) and fish (Boulanger *et al.*, 1977). Some of the pathological features associated with *Aeromonas* infections are local edema, tissue necrosis, septicemia, and mortality (Ljungh and Wadstrom, 1983). *A. hydrophila* secretes a host of toxic and degradative proteins into the extracellular milieu (Howard and Buckley, 1983) such as the hemolysin aerolysin (Chakraborty *et al.*, 1986;

Howard and Buckley, 1986), protease (Lueng and Stevenson, 1988), amylase (Gobius and Pemberton, 1988), acyltransferase (Thornton *et al.*, 1988) and an enterotoxin (Chakraborty *et al.*, 1984). These toxins have in some cases been implicated in the pathogenesis of this organism (Chakraborty, *et al.*, 1987). As discussed above for a number of other Gram-negative bacteria, the exoproteins of *A. hydrophila* must be translocated across both the inner and outer membranes in order to reach the extracellular milieu.

Studies done on the secretion of aerolysin began with the demonstration that aerolysin is a pore forming water soluble protein (Howard and Buckley, 1982). Aerolysin is produced as a propolypeptide (Howard and Buckley, 1985b) which may protect the producing bacteria from the mature toxin during its transit, since the toxin can insert directly into any lipid layer (Buckley, 1992). Pro-aerolysin is secreted as a precursor with a 23 amino acid signal sequence (pre-proaerolysin) which directs transport across the cytoplasmic membrane during which it is cleaved from the protein (Howard and Buckley, 1985a). This translocation step was shown to be completely inhibited by the energy uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Howard and Buckley, 1983). The pro-aerolysin is then secreted across the outer membrane and once free of the cells is activated by proteolytic removal of 43 amino acids from the C-terminus (Howard and Buckley, 1985a; Buckley, 1992).

Mutants in which protease and aerolysin were synthesized but could not be exported were isolated by treating *A. hydrophila* with ethyl methane sulfonate (Howard and Buckley, 1983). Cold osmotic shock treatment of the mutants revealed that the proteins were accumulating in the periplasmic space, thereby indicating the periplasm as part of the normal route for protein export and also

that a specific mechanism for extracellular secretion common to different proteins exists in *A. hydrophila* (Howard and Buckley, 1983). Likewise, the aerolysin structural gene (*aer*) was cloned into *A. salmonicida*, which was shown to secrete the protein (Wong *et al.*, 1989) but aerolysin could not be secreted by pleiotropic export mutants of a marine *Vibrio* species (Wong *et al.*, 1990; Ichige *et al.*, 1988), suggesting that the members of the family *Vibrionaceae* share a common pathway for extracellular secretion. Studies with the proton ionophore CCCP indicated that a proton motive force is required for the translocation of pro-aerolysin across the outer membrane as well as the inner membrane (Wong and Buckley, 1989). The authors proposed that the energy requirement could involve a coupling to the inner-membrane proton gradient, or could involve a gradient across the outer membrane itself (Wong and Buckley, 1989).

In studies aimed at identifying the components of the extracellular secretion pathway, Tn5-751 mutagenesis was used to isolate mutants that synthesized but did not secrete any of the major extracellular proteins, and instead accumulated them in the periplasm. In one of the mutants, L1.97, the transposon insertion also led to a marked reduction in the amounts of the outer membrane protein II and rendered the cells osmotically fragile (Jiang and Howard, 1991). The wild type chromosomal fragment that complemented the mutation in L1.97 was isolated and sequenced (Jiang and Howard, 1991; 1992). Nucleotide sequence analysis of a 13 kb DNA fragment revealed 12 genes, *exeC-N*, which are highly homologous to the *pul* genes of the *pulC-O* operon of *K. oxytoca* (Jiang and Howard, 1992; Howard *et al.*, 1993). The amino acid identity between Exe proteins and their Pul homologues is 63% for E, 49% for F, 71% for G, 27% for H, 50% for I, 43% for J, 38% for K, 29% for L, 26% for M and

29% for N. ExeE was also shown to be homologous to the PilB (Nunn *et al.*, 1990) and PilT protein (Whitchurch *et al.*, 1990), required for type IV pilin assembly and twitching motility in *Ps. aeruginosa*, the ComG protein required for DNA uptake in *Bacillus subtilis* (Albano *et al.*, 1989) and the VirB11 protein, involved in DNA transfer in *Agrobacterium tumefaciens* (Ward *et al.*, 1988). Like PulE, ExeE also has a consensus nucleotide binding site and is believed to transduce the energy derived from ATP hydrolysis to other Exe proteins in the membranes which are responsible for translocation of proteins out of the cell (Jiang and Howard, 1992). ExeG, ExeH, ExeI, ExeJ and ExeK have a consensus cleavage sequence for the pre-pilin peptidase, and ExeG was shown to be processed in *E. coli* cells which were also expressing the XcpA/PilD pre-pilin peptidase gene (Howard *et al.*, 1993). The pre-pilin peptidases PulO of *K. oxytoca* and OutO of *Er. chrysanthemi* are encoded by the last ORFs of *pul* and *out* operons, whereas the *exe* operon ends with N. Therefore, like *Ps. aeruginosa* the pre-pilin peptidase gene of *A. hydrophila* may be located elsewhere on the chromosome. Marker exchange mutagenesis of *exeC*, *exeE*, and *exeK* demonstrated that mutants for any one of these genes were unable to secrete the exoproteins and also had decreased quantities of OmpII, confirming the link between a functioning *exeC-N* operon and the outer membrane assembly (Howard *et al.*, 1993).

To summarize, most of the extracellular proteins of *A. hydrophila* are secreted by the two step general secretion pathway, which is well conserved among the Gram-negative bacteria. The proteins are presumably first translocated across the inner membrane by *sec* gene products similar to those of *E. coli*, while the 12 *exe* genes of the *exeC-N* operon plus a pre-pilin peptidase

are required for the translocation of the proteins across the outer membrane. In addition, however, another locus has been shown to be involved in extracellular secretion in this bacterium, as discussed below, and this locus is the subject of the research described in this thesis.

1.6 Purpose of this dissertation

During earlier studies on the mechanism of extracellular secretion in *A. hydrophila*, Tn5-751 insertion mutants defective in export of extracellular proteins were isolated. These proteins accumulated in the periplasmic space, and one of these mutants, L1.97, led to the isolation of the *exeC-N* locus (Jiang and Howard, 1991). In the same mutagenesis experiment, another mutant, C5.84 was isolated which, like L1.97, produces all of the extracellular proteins assayed and accumulates them in the periplasmic space. Southern hybridization experiments demonstrated that the transposon had inserted into a different locus in this mutant and not in the *exeC-N* operon. In addition, these mutants differed in possessing an outer membrane protein profile similar to that of the wild type (Jiang and Howard 1991). These results thus suggested that the mutation in C5.84 had affected an aspect or a step in extracellular secretion which is separate from that controlled by the *exeC-N* operon. The purpose of the research described here was to characterize this C5.84 mutant, and to define, isolate, and characterize the gene(s) required for extracellular protein secretion identified by this locus.

To this end, a wild-type 3.5 kb *Bgl* II chromosomal fragment from *A. hydrophila* which complemented the mutation in C5.84 was isolated and

sequenced. It was also demonstrated that this locus consists of an operon composed of two genes, *exeA* and *exeB*, each of which is required for extracellular protein secretion.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 2.1.

The wild type strain used in this study was *A. hydrophila* Ah65, and all other strains were derived from it. Routinely, bacteria were grown in brain heart infusion (BHI) media (Miller, 1972) or in a chemically defined minimal medium (Riddle *et al.*, 1981) at 30^0C.

E. coli strains XL1-Blue (Stratagene), S17-1, *E.coli* BL21(DE3) (Novagen) were grown at 37^0C in Luria Bertani [LB] or M9 medium (Miller, 1972) supplemented with 0.5% methionine assay medium (Difco). For detection of extracellular secretion of aerolysin, BHI agar containing 5% (v/v) human blood was used.

Antibiotics used were: Ampicillin at 100 µg/ml, chloramphenicol at 5 µg/ml, gentamycin at 30 µg/ml, kanamycin at 50 µg/ml, nalidixic acid at 10 µg/ml, rifampicin at 200 µg/ml, streptomycin at 100 µg/ml and tetracycline at 10 µg/ml.

The plasmids pBluescript II SK+ and pBluescript II KS+ were used for subcloning and the wide host range vectors used were pMMB67EHcam, pMMB67HEcam (Jiang and Howard, 1991), pMMB207 and pMMB208 (Morales *et al.*, 1991).

Table 2.1 Strains and plasmids

Strain/plasmid	Genotype/phenotype	Source/Reference
A. hydrophila		
Ah65	Wild type	
C5.84	See the text	Jiang and Howard (1991)
AA41	See the text	This laboratory
AB69	See the text	This laboratory
E. coli		
XL1-Blue	*rec*A1 *end*A1 *gyr*A96 *lac*⁻ *thi hsd*R17 (F′*pro*AB *lacI*^{q} *lacz*M15 Tn10)	Stratagene
MM294	F⁻ *hsd* R17 ($r_k^-m_k^-$) Sup E44 *thi*⁻ 1 *end*A1	Hanahan (1983)
S17-1	*rec*A⁻ *thi pro hsd*	Simon *et al.* (1983)
BL21(DE3)	F⁻ *ompT* $r_B^-m_B^-$ (DE3)	Novagen
Plasmids		
pBluescript II SK/KS+	Ap^r	Stratagene
pRK2013	Kn^r	Figurski and Helinski (1979)
pMMB67EH/HEcam	Cm^r	Jiang and Howard (1991)
pMMB207/208	Cm^r	Morales, *et al.* (1991)
pET9	Ap^r	Novagen
pUC4K	Kn^r	Vieira and Messing (1982)
pSUP202	Ap^{r}Cm^{r}Tc^r	Simon *et al.* (1983)
pJQ200SK	Ap^{r}Gn^r	Quandt and Hynes (1993)
pJB4	See the text	This study
pRJ3.1-4.1	See the text	This study
pRJ12.1-13.1	See the text	This study
pRJ24.1-26.3	See the text	This study
pRJ30.1-31.1	See the text	This study

Table 2.1 Strains and plasmids

Strain/plasmid	Genotype/phenotype	Source/Reference
Plasmids		
pRJ60.1	See the text	This study
pRJ39.1-40.1	See the text	This study
pSRJ5.1-5.2	See the text	This study
pKRJ18.1-20.2	See the text	This study
pSRJ19.1-21.1	See the text	This study
pKRJ46.1	See the text	This study
pKRJ50.2-50.3	See the text	This study
pJRJ52.1	See the text	This study
pSRJ57.1	See the text	This study
pKRJ58.1	See the text	This study
pERJ63.1	See the text	This study

2.2 DNA preparation, manipulation and analyses

Small scale plasmid DNA preparation was done by the alkaline lysis protocol (Birnboim and Doly, 1979), or the Magic miniprep protocol (Promega) and large scale plasmid DNA preparation was done by cesium chloride (CsCl) ethidium bromide density gradient centrifugation (Sambrook *et al.*, 1989). Routine DNA analysis such as restriction mapping, blunting of overhanging single-stranded ends using the Klenow fragment of *E. coli* DNA polymerase or T4 DNA polymerase, T4 DNA ligation, vector dephosphorylation using Calf intestinal alkaline phosphatase and agarose gel electrophoresis were essentially performed as described (Sambrook *et al.*, 1989). Restriction fragments were purified from agarose gels using a Geneclean II Kit (BIO 101 Inc). Plasmid DNA ligation mixtures were transformed by electroporation into *E. coli* strains XL1-Blue and BL21(DE3) cells and selected by plating on media containing the appropriate antibiotics. Wide host range plasmids were introduced into *A. hydrophila* by conjugation using the helper plasmid pRK2013 (Figurski and Helinski, 1979) from *E. coli* MM294 or S17-1 cells.

A. hydrophila chromosomal DNA was isolated as described (Murray and Thompson, 1980) with slight modifications. 1.5 ml of saturated cultures were spun down and the cell pellet was resuspended in 517 µl of TE. The cells were lysed by incubating with lysozyme at 0.2 mg/ml for 15 minutes at 37^0C, followed by a further incubation at 37^0C for 1 hour after adding Sodium dodecyl sulphate (SDS) at 0.5% and proteinase K at 0.1 mg/ml. The proteins and cell debris were precipitated by adding NaCl at 0.7 M and the DNA was recovered by phenol-chloroform extraction and precipitation with 0.6 vol. of isopropanol. After a 70%

ethanol wash the chromosomal DNA was incubated in 100 µl of TE containing 0.05 mg/ml RNAse for 1 hour at 37^{0}C. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation and resuspended in 100 µl of TE.

2.3 *Preparation of nested deletion clones*

The 3.5 kb *Bgl* II fragment was cloned into the *Bam* HI site of the pBluescript II SK+ vector. Two sets of unidirectional nested deletion clones were obtained starting from either end of the 3.5 kb *Bgl* II fragment using exonuclease III and mung bean nuclease (Henikoff, 1987). The deletions from the 5' end of the 3.5 kb *Bgl* II fragment were done in the plasmid pSRJ5.2 and for the 3' end using plasmid pSRJ5.1. Plasmids pSRJ5.2 and pSRJ5.1 were digested with *Xho*I and the 5' overhang was protected by filling with deoxythio d-NTP derivatives using the Klenow fragment of *E. coli* DNA polymerase I. The protection was confirmed by incubating 0.2 µg of the filled in DNA with 20 units of Exonuclease III for 15 minutes at 37^{0}C followed by electrophoresis on a 1% agarose gel. The *Eco*RI site between the insert and the 3' protected *Xho*I restriction site was used for the second digestion to prepare the fragments for the unidirectional exonuclease III digestion. The reactions were carried out at 30^{0}C, and samples were withdrawn every 30 seconds. The DNA was then treated with Mung bean nuclease to create blunt ends, ligated with T4 DNA ligase, and transformed into the *E. coli* XL1-Blue cells.

2.4 Polymerase chain reaction (PCR)

In addition to restriction analysis of small scale plasmid DNA preparations, PCR was used to characterize the clones in the pBluescript II SK/KS+ vector. The deletion derivatives were screened by PCR amplification of the insert DNA directly using bacterial cells from the transformation plates (Gurpreet *et al.*, 1989). For PCR, the bacterial colony was first suspended in 25 µl of sterile distilled water, vortexed and heated to 99^{0}C for 5 minutes to lyse the bacteria and denature the plasmids. Thirty cycles of denaturation (1 minute at 94^{0}C), annealing (1 minute at 50^{0}C), and elongation (3 minutes at 72^{0}C) followed by a 15 minute extended cycle at 73.5^{0}C were carried out in a 50 µl reaction mixture with buffer containing 100 mM Tris-HCl (pH8.8), 1% Triton X-100, 1.0 mM $MgCl_2$, dNTPs (at a final concentration of 200 µM for each dNTP), 20 pmol each of oligonucleotide HO2 (5' GTAATACGACTCACTATAGGGCGAA 3'), and oligonucleotide HO3 (5' AGCGGATAACAATTTCACACAGGA 3') and 0.5 U of Taq DNA polymerase (Promega). The PCR mix was overlaid with a drop of mineral oil to prevent condensation. The amplified product was visualized by running 5 µl of the reaction mixture on a 1% agarose gel.

2.5 Determination of the nucleotide sequence

The appropriate deletion clones were superinfected with VCSM13 helper phage for single-strand DNA template isolation (Vieira and Messing, 1987). Sequencing was performed by the di-deoxy chain termination method of Sanger *et al.* (1977) using α-[^{35}S]dATP as the sequencing label. Sequencing reactions were performed using T7 DNA polymerase (Sequenase, U. S. Biochemical

Corp). Ambiguities in the sequence were resolved by using dITP in place of dGTP in the sequencing reaction mix.

The sequencing reaction products were resolved on 6% polyacrylamide-8M urea sequencing gels. The sequencing gels were run at 2000V, fixed in 5% methanol and 5% acetic acid in water, vacuum dried at 80^{0}C on filter paper, exposed to X-ray film and visualized by autoradiography.

2.6 *In vitro* Transcription-Translation Reactions

Polypeptides encoded by the plasmids pSRJ5.1, pSRJ5.2, pKRJ18.1, pSRJ19.1, pKRJ20.2, pSRJ21.1, pKRJ46.1, pSRJ57.1, pKRJ58.1, pKRJ50.2 and pKRJ50.3 were identified by using a S30 cell free extract of *E. coli* in a coupled transcription-translation system as described by Zubay (1980). [^{35}S]-methionine was used as the labeling reagent. Reactions were carried out as per standard conditions recommended by the supplier (Promega). CsCl purified supercoiled plasmid DNA or plasmids purified by the Magic mini prep protocol (Promega) were used as the templates and the translated products were run on a 12% SDS-PAGE gel and visualized by autoradiography.

2.7 Enzyme assays

Aerolysin activity was measured as described by Howard and Buckley (1985) in micro titre plates by two fold serial dilution of 0.1 ml samples in 0.1% bovine serum albumin (BSA). Equal volumes of 0.8% (v/v) human erythrocytes

in phosphate buffered saline (PBS) were added to each well and incubated at 37^{0}C for 1 hour. Trypsin was used to activate the samples prior to assay at 5 µg/ml for 5 minutes at room temperature. The activity of the samples is measured as the inverse of the highest dilution of the original samples in which 100% erythrocyte lysis has occurred after 1 hour of incubation at 37^{0}C.

Protease activity was measured by the method of Twining (1984) using resorufin-conjugated casein (Boehringer Mannheim) as the substrate. Each assay mixture contained 50 µl of 0.4% (w/v) casein in distilled water, 50 µl of 200 mM Tris-HCl (pH7.8), 200 mM $CaCl_2$ and 100 µl of the sample. The mixture was incubated at 37^{0}C for 1 hour and the reaction was stopped by incubating the mixture at 37^{0}C for 10 minutes after adding 480 µl of 5% (w/v) Trichloroacetic acid. After centrifuging at 14,000 rpm for 5 minutes, 400 µl of the supernatant was mixed with 600 µl of 500 mM Tris-HCl (pH 8.8) and the A_{574} nm was recorded. A change in the optical density at 574 nm of 0.01/h at 37^{0}C is defined as one unit.

2.8 Marker exchange mutagenesis

Marker exchange mutagenesis was performed using pSUP202 (Simon *et al.*, 1983) for *exeA* and pJQ200SK (Quandt and Hynes, 1993) for *exeB* as the mobilizing plasmids. In either case, a kanamycin resistance cassette from pUC4K (Viera and Messing, 1982) was first introduced into the region of subclone pSRJ5.1 to be exchanged and the fragment was then ligated into pSUP202 or pJQ200SK. After transforming the resulting clones into *E. coli* S17-1, they were conjugated into Ah65 with selection for kanamycin and further

screened for the chloramphenicol and ampicillin resistance markers of the plasmid in the case of pSUP202. For pJQ200SK derivatives, the transconjugants were selected on 5% sucrose and kanamycin and further screened for the gentamycin resistance marker of the plasmid.

2.9 Southern hybridization

Equal amounts of *Sma*I restricted chromosomal DNA were electrophoresed on 0.6% agarose gels at 25 volts for 14 hours. After staining with ethidium bromide for 10 minutes, the DNA was depurinated in 0.25N HCl for 15 minutes and rinsed twice in distilled water (Sambrook *et al.*, 1989). The DNA was then transferred onto a positively charged nylon membrane (Boehringer Mannheim) using a vacuum blotter (Biorad) for 90 minutes at 5 inches Hg pressure. The transfer solution was 0.5 N NaOH, 0.6 N NaCl. DNA fixation, prehybridization, hybridization, washing, development, and detection was carried out as described (Holtke *et al.*, 1992). The 3.5 kb *Bgl* II wild type fragment and the 1.2 kb fragment containing the *aph* gene of the plasmid pUC4K were used as probes. The probes were non-radioactively labeled with digoxigenin-11-dUTP using a random primer method (Holtke *et al.*, 1992). An anti-digoxigenin antibody-alkaline phosphatase conjugate and the substrate Lumiphos (Boehringer Mannheim) was used to detect the probes.

2.10 Preparation of mRNA

mRNA was isolated by a rapid isolation protocol as described (Summers, 1970) with slight modifications. A 10 ml culture was grown in BHI media to mid-log phase and the cells were harvested by centrifuging at 10,000 rpm for 5 minutes at 4^0C. The cell pellet was suspended in 10 ml of protoplasting buffer (15 mM Tris-HCl, (pH8.0), 0.45 M sucrose, 8 mM EDTA) and incubated on ice for 5 minutes. The protoplasts were collected by centrifugation at 6000 rpm for 5 minutes at 4^0C, resuspended in 0.5 ml lysis buffer (10 mM Tris-HCl (pH8.0), 1 mM sodium citrate, 1.5% SDS) and further incubated for 15 minutes at 37^0C after addition of 15 µl of Diethyl pyrocarbonate (DEPC) to inhibit RNAse. The samples were then incubated on ice for 10 minutes after adding 250 µl of saturated NaCl to precipitate the detergent, protein, and chromosomal DNA. After centrifugation for 5 minutes at 14,000 rpm the mRNA supernatant was obtained and recovered by ethanol precipitation, redissolved in 100 µl of DEPC treated water, and stored at -70^0C.

2.11 Northern analysis of mRNA

Equal amounts of mRNA samples were electrophoresed on a 1.2% agarose/2.2M formaldehyde gel at 15 volts for 15 hours and the gel was stained with ethidium bromide for 10 minutes and photographed (Rosen *et al.*, 1990). The gel was then soaked extensively in distilled water to reduce the background as described by Engler-Blum *et al.* (1993). Prior to transfer, the gel was soaked in 0.05 N NaOH for 15 minutes and equilibrated in 20 X SSC for 45 minutes. The mRNA was transferred by capillary action onto a positively charged nylon

membrane (Sambrook *et al.*, 1989). A 7% SDS buffer was used for prehybridization and hybridization was done at 42^{0}C (Church and Gilbert, 1984). mRNA fixation, washing, development and detection were carried out as described (Holtke *et al.*, 1992). A 2.5 kb fragment of pKRJ52.1 containing only the *exeA* and *exeB* genes was used to prepare a RNA probe using T7 RNA polymerase and was non-radioactively labeled with digoxigenin-11-UTP. The blot was incubated with an anti-digoxigenin antibody-alkaline-phosphatase conjugate and the substrate Lumiphos (Boehringer Mannheim) was used to detect the antibody bound to the probe.

2.12 Expression and localization of ExeA and ExeB

A 2.8 kb *Eco*RI-*Hin*DIII fragment from the plasmid pKRJ58.1 (Figure 3.1) (containing both *exeA* and *exeB*) was filled in with the Klenow fragment of DNA polymerase I and cloned into the filled in *Bam* HI site downstream of the T7 bacteriophage promoter of the pET9 expression plasmid to obtain the clone pERJ63.1. Plasmid pERJ63.1 was then transformed into *E. coli* BL21(DE3) cells by electroporation. These cells carry a chromosomal copy of the T7 RNA polymerase gene which is under the control of the inducible *lacUV5* promoter.

2.13 Pulse labeling and Cell fractionation

E. coli BL21(DE3) cells containing pERJ63.1 or pET9 were grown in 10 ml of M9 methionine assay medium with 50 μg/ml of kanamycin, induced at 0.6 O.D. 600_{nm} with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 30 minutes,

and 200 μg/ml of rifampicin was added. After a further 15 minute incubation, 100 μl of the culture was pulsed for 5 minute with 100 μCi/ml [^{35}S]-methionine. The pulsed sample was then added to the rest of the culture (7 ml), which acted as a carrier. The cell pellet obtained from the carrier culture was resuspended in 250μl of 25% sucrose made in 10mM Hepes, 5mM EDTA pH7.4. A cell lysate was then prepared using lysozyme at 0.5mg/ml (made in 10mM Hepes, 5mM EDTA pH7.4), and envelope and cytoplasmic fractions were obtained by treatment with lysozyme and EDTA as described previously (Jiang and Howard, 1991). The envelopes were extracted with 0.5% Sodium lauroyl sarcosinate (SLS) to solubilize the inner membrane proteins (Fillip *et al.*, 1973) which were then recovered in the supernatant fraction after centrifugation at 100,000 Xg for 1 hour to pellet the outer membrane vesicles. All fractions were then electrophoresed on SDS-PAGE gels and visualized by autoradiography.

E. coli BL21(DE3) cells containing the plasmid pERJ63.1 or pET9 were also grown in LB with 50 μg/ml kanamycin, induced at 0.6 O.D. 600_{nm} with 0.4 mM IPTG and the cells were harvested after a 3 hour incubation. A cell lysate was then prepared and the fractions were obtained as described above and then electrophoresed on SDS-PAGE gels and stained with Coomassie blue for visualization.

2.14 Gel electrophoresis and immunoblots

SDS-PAGE was carried out on 10 or 12% acrylamide gels (Laemmli, 1990). For immunoblots, the proteins were transferred onto nitrocellulose (Towbin *et al.*, 1979) and reacted with an anti-aerolysin monoclonal antibody or

with an anti-ExeB serum (Howard, S. P., unpublished). The secondary antibody used was alkaline phosphatase conjugated goat anti-mouse or goat anti-rabbit antibody and the blots were developed using the chromogenic substrates BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) and NBT (Nitro Blue Tetrazolium).

Chapter 3

RESULTS

3.1 Mapping and subcloning of the 15 kb *Eco*RI *Aeromonas hydrophila* chromosomal fragment which complements the mutation in C5.84

C5.84 was isolated as a Tn5-751 insertion mutant of *A. hydrophila* which is unable to secrete any extracellular proteins (Jiang and Howard, 1991). A 15 kb *Eco*RI fragment was isolated from the wild type genome that complemented the mutation in C5.84 (Jiang and Howard, 1991). As an initial analysis of this fragment (in pJB4) it was partially mapped (Fig 3.1). A 6.6 kb *Xho* I-*Sal* I fragment which contained the insertion site of the transposon in the mutant C5.84 was cloned into the pBluescript II SK/KS+ and then transferred into the wide host range vector pMMB67EH/HEcam in both orientations with respect to the *tac* promoter of the vector to obtain clones pRJ3.1 and pRJ4.1 respectively (see Fig. 3.1). Both of these plasmids complemented the mutation when conjugated into the wild type Ah65 from the wide host range plasmids using pRK2013 as the conjugative helper plasmid. The phenotypic evidence of complementation was observed as a halo of hemolysis on blood agar plates that was similar to that of the wild type.

Further subcloning was done to specifically identify the region of this fragment involved in extracellular secretion. Thus, a 3.5 kb *Bgl* II fragment corresponding to the transposon insertion site in the mutant C5.84 was cloned into pBluescript II SK+ and then transferred into the wide host range vector pMMB67EH/HEcam in both orientations with respect to the *tac* promoter of the

vector, giving rise to pRJ12.1 and pRJ13.1 respectively (see Fig. 3.1). When these plasmids were conjugated into C5.84, the secretory phenotype was restored but the zone of hemolysis was smaller around C5.84(pRJ13.1) as compared to C5.84(pRJ12.1) and the wild type. This suggested that sequences present on this fragment were required for restoring the wild-type secretory phenotype. To demonstrate that these transconjugants secreted extracellular proteins, aerolysin titres were conducted on the supernatants after growing these transconjugants in BHI broth (Table 3.1). Both C5.84(pRJ12.1) and C5.84(pRJ13.1) had titres similar to that of the wild type, although C5.84(pRJ13.1) had a lower titre than C5.84(pRJ12.1), whereas C5.84 had a very low titre.

3.2 Analysis of the synthesis and extracellular export of aerolysin by the transconjugants

As a further measure of complementation, Ah65 as well as C5.84 and the transconjugants C5.84(pRJ12.1) and C5.84(pRJ13.1) were grown in BHI broth and subjected to western blot analysis. Samples of cells and culture supernatants were collected, electrophoresed on a 10% SDS-PAGE gel, transferred onto nitrocellulose by the method of Towbin *et al.*, (1979) and immunoblotted with an anti-aerolysin monoclonal antibody. Immunoblots showed that most of the aerolysin was exported into the supernatant of C5.84(pRJ12.1) and C5.84(pRJ13.1) like that of the wild type (Fig. 3.2, lane 3S, lane 4S and lane 1S respectively), and in C5.84 most of the aerolysin remained within the cells (Fig. 3.2, lane 2S).

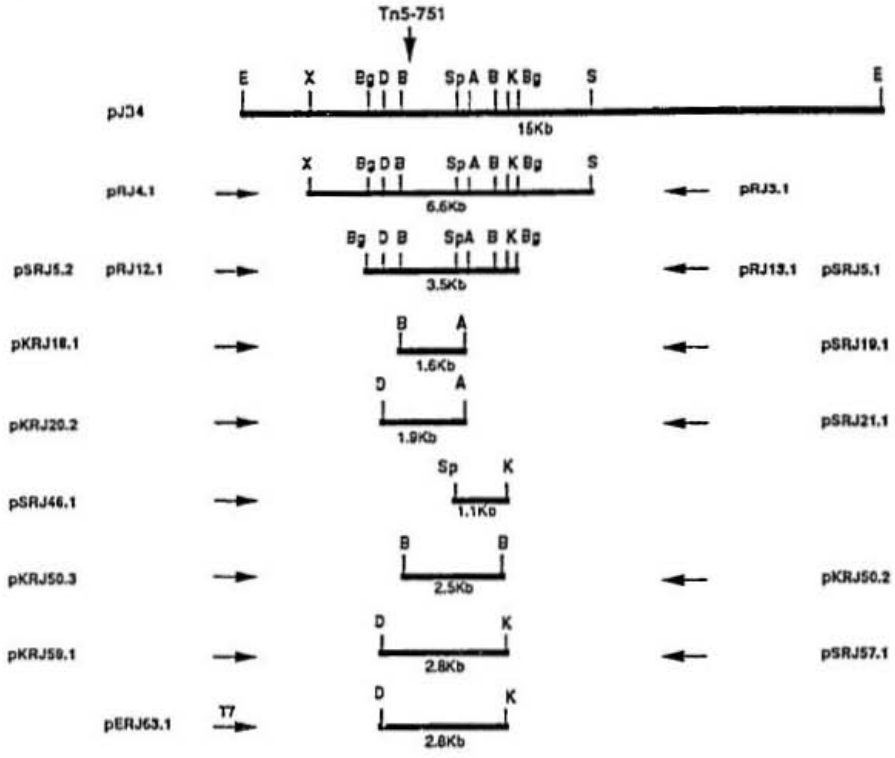


Figure 3.1. Subcloning of a region complementing C5.84 from pJB4. The Ah65 fragment in pJB4 and the fragments subcloned from it are shown. The vertical arrow indicates the site of Tn-751 insertion in C5.84 and the horizontal arrows indicate the direction of the vector promoter for each clone. All pRJ clones were constructed in wide host range vectors and used in the complementation studies, while the pKRJ and pSRJ clones were constructed in pKSII+ and pSKII+ respectively and used in the *in vitro* transcription-translation reactions. Clone pERJ63.1 was used in the study of expression of proteins using a T7 promoter system. A, *Acc*I; B, *Bst* XI; Bg, *Bgl*I; D, *Dra*III; E, *Eco*RI; K, *Kpn*I; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. Note that for clarity, not all sites are shown for all all of the subclones.

Table 3.1 Aerolysin titres of Ah65, C5.84, C5.84(pRJ12.1) and C5.84(pRJ13.1).

Supernatant fraction	Aerolysin (HU)
Ah65	256
C5.84	8
C5.84(pRJ12.1)	256
C5.84(pRJ13.1)	128

Hemolytic activities are given as the titres, expressed as hemolytic units (HU).

The results of the complementation assays and the immunoblots collectively indicated that the 3.5 kb *Bgl* II fragment had gene(s) necessary for extracellular protein secretion which had been inactivated by transposon insertion in C5.84. The fact that complementation was observed when the fragment was cloned in either orientation with respect to the *tac* promoter of the vector also suggested that a promoter for the complementing gene(s) was present on the fragment. Since C5.84(pRJ12.1) had a higher titre than C5.84(pRJ13.1) these results also suggested that the direction of transcription is from left to right as the fragment is drawn in Figure 3.1.

3.3 DNA sequencing and sequence analysis

To determine the structure of the gene(s) involved in extracellular protein secretion, the 3.5 kb *Bgl* II fragment which complemented the mutation was cloned into pBluescript II SK+ and sequenced using the exonuclease III/ mung bean nuclease method of Henikoff (1987). Both strands of the fragment were sequenced by creating a set of nested deletions starting from either end of the fragment. The complete sequence of the 3537 base pairs is shown in Figure 3.3. The nucleotide sequence reported here has been submitted to the EMBL and GenBank databases under accession number X81473.

Computer analysis of the sequence revealed that this fragment had a number of open reading frames (ORFs), as shown in Figure 3.4. In the direction of transcription as suggested by the complementation studies, two complete ORFs and a partial ORF, ORF1, ORF2, and ORF3 are present, whereas in the opposite orientation three ORFs, ORF4, ORF5 and ORF6 are present.

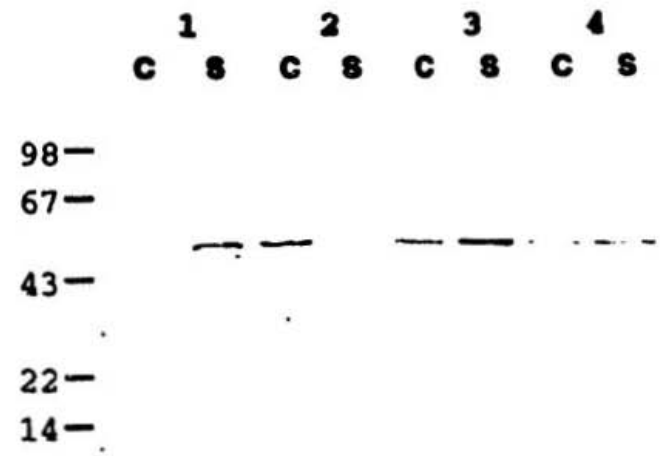
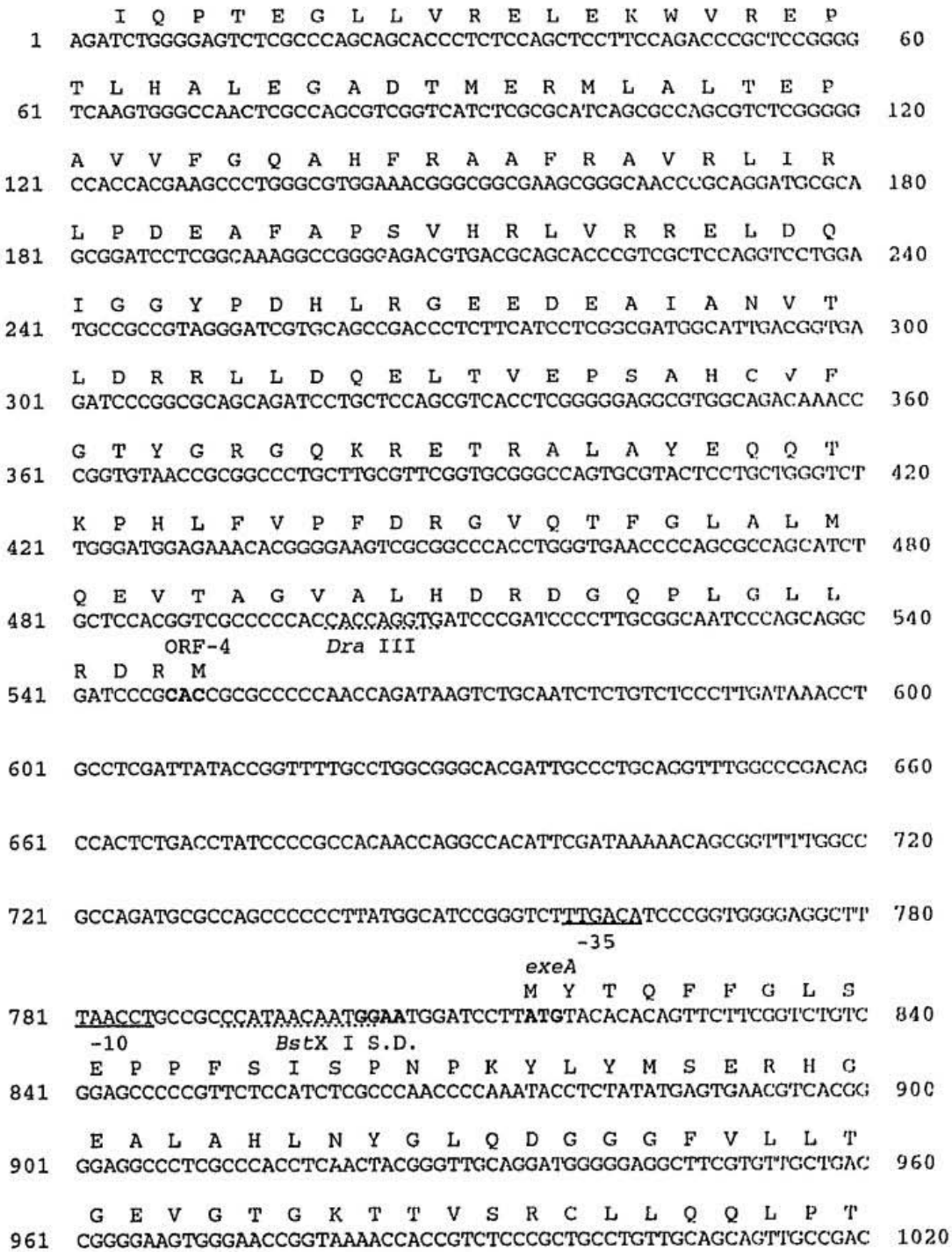
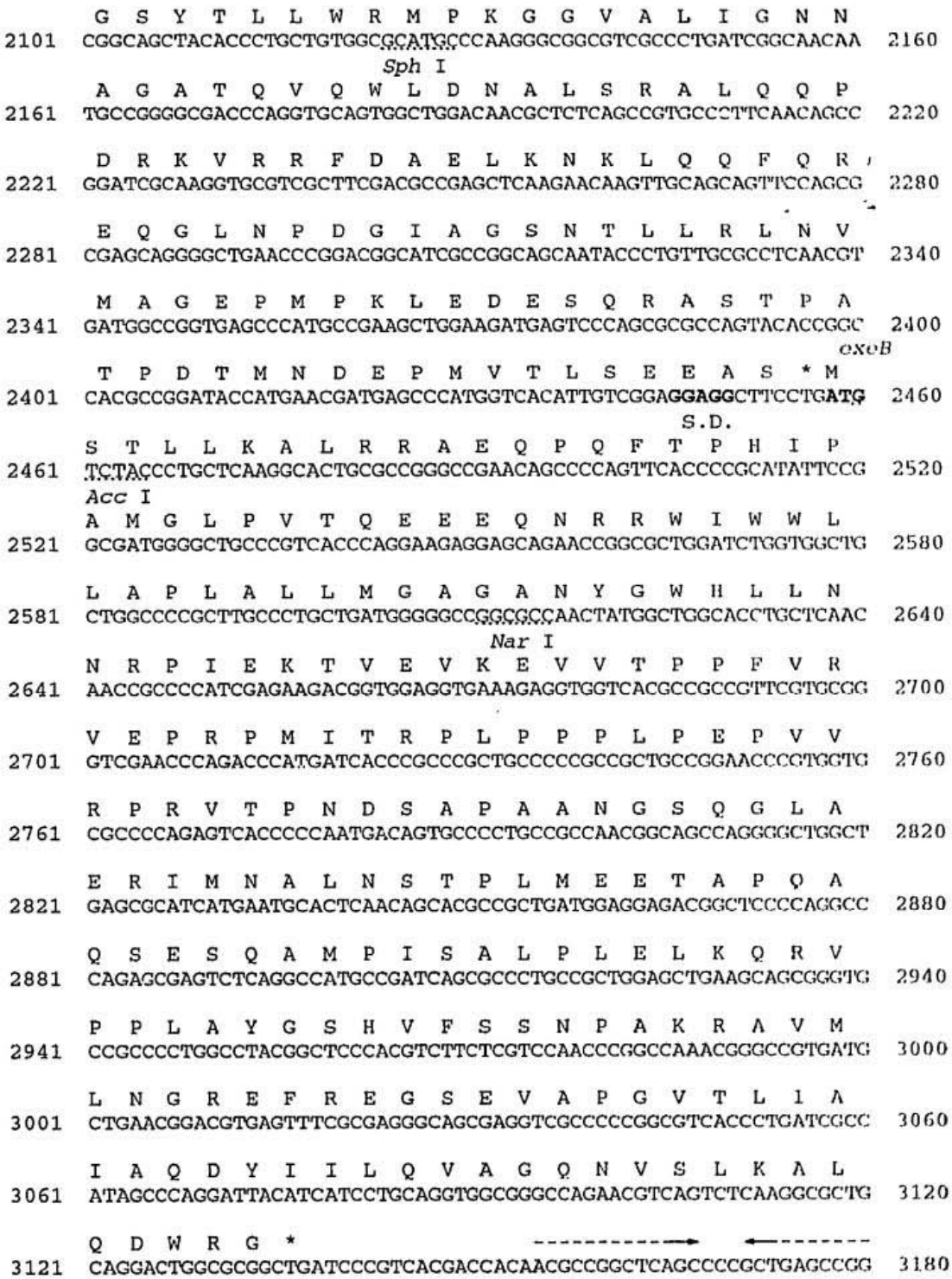


Figure 3.2. Immunoblot analysis of aerolysin secretion by the transconjugants C5.84(pRJ12.1) and C5.84(pRJ13.1). Cell samples (lanes C) and culture supernatants (lanes S) representing equal volumes of freshly grown BHI cultures were electrophoresed, transferred to nitrocellulose, and immunoblotted with an anti-aerolysin monoclonal antibody. Ah65 (lane 1); C5.84 (lane 2); C5.84(pRJ12.1) (lane 3) and C5.84(pRJ13.1) (lane 4). The upper and lower bands in the supernatant fraction of lane 1 are proaerolysin and aerolysin respectively. Molecular masses of standards are given on the left in kDa.


I Q P T E G L L V R E L E K W V R E P
1 AGATCTGGGGAGTCTCGCCCAGCAGCACCCTCTCCAGCTCCTTCCAGACCCGCTCCGGGG 60
T L H A L E G A D T M E R M L A L T E P
61 TCAAGTGGGCCAACTCGCCACCGTCGGTCATCTCGCGCATCAGCGCCAGCGTCTCGGGGG 120
A V V F G Q A H F R A A F R A V R L I R
121 CCACCACGAAGCCCTGGGCGTGGAAACGGGCGGCGAAGCGGGCAACCCGCAGGATGCGCA 180
L P D E A F A P S V H R L V R R E L D Q
181 GCGGATCCTCGGCAAAGGCCGGGCAGACGTGACGCAGCACCCGTCGCTCCAGGTCCTGGA 240
I G G Y P D H L R G E E D E A I A N V T
241 TGCCGCCGTAGGGATCGTGCAGCCGACCCTCTTCATCCTCGGCGATGGCATTGACGGTGA 300
L D R R L L D Q E L T V E P S A H C V F
301 GATCCCGGCGCAGCAGATCCTGCTCCAGCGTCACCTCGGGGGAGGCGTGGCAGACAAACC 360
G T Y G R G Q K R E T R A L A Y E Q Q T
361 CGGTGTAACCGCGGCCCTGCTTGCGTTCGGTGCGGGCCAGTGCGTACTCCTGCTGGGTCT 420
K P H L F V P F D R G V Q T F G L A L M
421 TGGGATGGAGAAACACGGGGAAGTCGCGGCCCACCTGGGTGAACCCCAGCGCCAGCATCT 480
Q E V T A G V A L H D R D G Q P L G L L
481 GCTCCACGGTCGCCCCCACCACCAGGTGATCCCGATCCCCTTGCGGCAATCCCAGCAGGC 540
ORF-4 Dra III
R D R M
541 GATCCCGCACCGCGCCCCCAACCAGATAAGTCTGCAATCTCTGTCTCCCTTGATAAACCT 600
601 GCCTCGATTATACCGGTTTTGCCTGGCGGGCACGATTGCCCTGCAGGTTTGGCCCGACAG 660
661 CCACTCTGACCTATCCCCGCCACAACCAGGCCACATTCGATAAAAACAGCGGTTTTGGCC 720
721 GCCAGATGCGCCAGCCCCCCTTATGGCATCCGGGTCTTTGACATCCCGGTGGGGAGGCTT 780
-35
exeA
M Y T Q F F G L S
781 TAACCTGCCGCCCATAACAATGGAATGGATCCTTATGTACACACAGTTCTTCGGTCTGTC 840
-10 BstX I S.D.
E P P F S I S P N P K Y L Y M S E R H G
841 GGAGCCCCCGTTCTCCATCTCGCCCAACCCCAAATACCTCTATATGAGTGAACGTCACGG 900
E A L A H L N Y G L Q D G G G F V L L T
901 GGAGGCCCTCGCCCACCTCAACTACGGGTTGCAGGATGGGGGAGGCTTCGTGTTGCTGAC 960
G E V G T G K T T V S R C L L Q Q L P T
961 CGGGGAAGTGGGAACCGGTAAAACCACCGTCTCCCGCTGCCTGTTGCAGCAGTTGCCGAC 1020

```
          E  T  E  I  A  Y  I  L  N  P  S  L  T  E  R  D  L  L  A  A
1021  CGAGACCGAGATCGCCTACATCCTCAATCCATCCCTGACCGAGCGTGACCTGCTGGCGGC  1080

          I  C  D  E  F  Q  L  P  Y  D  K  D  A  G  L  K  L  L  F  D
1081  CATCTGCGACGAGTTCCAGCTGCCGTACGACAAGGACGCCGGTCTCAAGCTGCTGTTCGA  1140

          L  I  R  D  H  L  L  A  N  L  A  A  G  K  R  S  V  V  L  V
1141  TCTCATTCGCGACCACCTGCTGGCCAATCTGGCGGCAGGCAAGCGCAGCGTGGTGCTGGT  1200

          D  E  A  Q  H  L  L  P  G  V  L  E  Q  L  R  L  L  T  N  L
1201  GGATGAGGCCCAGCACCTGCTGCCCGGTGTGCTGGAGCAACTGCGCCTGCTCACCAACCT  1260

          E  T  D  E  K  K  L  L  Q  V  V  L  I  G  Q  P  E  L  Q  Q
1261  GGAAACCGACGAGAAGAAGCTGCTGCAAGTGGTGCTCATCGGTCAGCCCGAGCTGCAACA  1320

          M  L  R  Q  P  L  L  R  Q  L  A  Q  R  I  T  A  R  Y  H  L
1321  GATGCTGCGCCAGCCCCTGCTGCGCCAGCTGGCCCAGCGCATCACGGCCCGTTATCACCT  1380

          L  P  L  S  H  Q  D  V  D  A  Y  V  R  F  R  L  Q  V  A  G
1381  GCTGCCGCTTTCGCATCAGGACGTGGATGCCTATGTGCGCTTCCGGCTGCAGGTCGCGGG  1440

          C  V  Q  P  I  F  T  P  K  A  L  Q  T  L  H  R  L  S  G  G
1441  CTGCGTCCAGCCCATCTTTACCCCCAAGGCACTTCAGACCCTGCACCGCCTCTCCGGCGG  1500

          I  P  R  L  I  N  L  I  C  D  R  A  L  I  A  A  F  A  R  G
1501  CATTCCCCGCCTCATCAACCTCATCTGCGATCGCGCCCTGATCGCCGCCTTCGCCCGTGG  1560

          S  H  K  I  V  H  G  D  I  S  L  A  A  Y  E  V  S  G  I  R
1561  CAGCCACAAGATAGTGCACGGCGACATCAGCCTGGCGGCCTATGAGGTGAGCGGCATTCG  1620

          D  E  G  T  W  Q  S  G  L  M  V  A  L  A  G  A  L  L  V  A
1621  CGACGAAGGCACCTGGCAGTCCGGCCTCATGGTCGCCCTGGCGGGGGGCCTTGCTGGTCGC  1680

          T  G  W  W  G  W  Q  F  F  G  F  F  P  E  R  P  V  I  K  V
1681  CACCGGCTGGTGGGGCTGGCAGTTCTTCGGCTTCTTCCCCGAACGCCCCGTGATCAAGGT  1740

          E  V  P  V  K  V  D  D  T  P  E  Q  Q  E  Q  L  T  R  A  I
1741  GGAGGTGCCGGTCAAGGTGGACGACACCCCGGAGCAGCAGGAGCAGCTGACCCGCGCCAT  1800

          N  Q  A  L  E  P  D  S  A  M  Q  N  L  Y  K  V  W  G  Y  Q
1801  CAATCAGGCGCTGGAGCCGGATAGCGCCATGCAGAATCTCTACAAGGTGTGGGGCTACCA  1860

          T  E  L  E  E  A  T  C  D  N  A  P  R  A  G  L  R  C  Q  E
1861  GACAGAGCTGGAGGAGGCGACCTGCGACAATGCACCGCGCGCCGGCTTGCGCTGCCAGGA  1920

          G  D  A  S  L  A  E  L  Q  A  L  Q  H  P  A  L  I  S  L  T
1921  GGGCGACGCCTCGCTGGCGGAGCTGCAGGCCCTGCAGCACCCGGCCCTCATCAGCCTGAC  1980

          D  E  T  G  G  I  Y  Y  A  T  L  V  N  L  G  P  D  K  A  N
1981  CGACGAGACGGGCGGCATCTATTACGCAACCCTGGTGAATCTCGGGCCGGACAAGGCCAA  2040

          L  L  I  G  N  Q  S  W  Q  V  D  R  Q  W  L  S  D  F  W  G
2041  CCTGCTCATCGGCAACCAGAGCTGGCAGGTGGACAGGCAGTGGCTGTCCGACTTCTGGGG  2100
```


G S Y T L L W R M P K G G V A L I G N N
2101 CGGCAGCTACACCCTGCTGTGGCGCATGCCCAAGGGCGGCGTCGCCCTGATCGGCAACAA 2160
Sph I
A G A T Q V Q W L D N A L S R A L Q Q P
2161 TGCCGGGGCGACCCAGGTGCAGTGGCTGGACAACGCTCTCAGCCGTGCCCTTCAACAGCC 2220
D R K V R R F D A E L K N K L Q Q F Q R
2221 GGATCGCAAGGTGCGTCGCTTCGACGCCGAGCTCAAGAACAAGTTGCAGCAGTTCCAGCG 2280
E Q G L N P D G I A G S N T L L R L N V
2281 CGAGCAGGGGCTGAACCCGGACGGCATCGCCGGCAGCAATACCCTGTTGCGCCTCAACGT 2340
M A G E P M P K L E D E S Q R A S T P A
2341 GATGGCCGGTGAGCCCATGCCGAAGCTGGAAGATGAGTCCCAGCGCGCCAGTACACCGGC 2400
exoB
T P D T M N D E P M V T L S E E A S * M
2401 CACGCCGGATACCATGAACGATGAGCCCATGGTCACATTGTCGGAGGAGGCTTCCTGATG 2460
S.D.
S T L L K A L R R A E Q P Q F T P H I P
2461 TCTACCCTGCTCAAGGCACTGCGCCGGGCCGAACAGCCCCAGTTCACCCCGCATATTCCG 2520
Acc I
A M G L P V T Q E E E Q N R R W I W W L
2521 GCGATGGGGCTGCCCGTCACCCAGGAAGAGGAGCAGAACCGGCGCTGGATCTGGTGGCTG 2580
L A P L A L L M G A G A N Y G W H L L N
2581 CTGGCCCCGCTTGCCCTGCTGATGGGGGCCGGCGCCAACTATGGCTGGCACCTGCTCAAC 2640
Nar I
N R P I E K T V E V K E V V T P P F V R
2641 AACCGCCCCATCGAGAAGACGGTGGAGGTGAAAGAGGTGGTCACGCCGCCGTTCGTGCGG 2700
V E P R P M I T R P L P P P L P E P V V
2701 GTCGAACCCAGACCCATGATCACCCGCCCGCTGCCCCCGCCGCTGCCGGAACCCGTGGTG 2760
R P R V T P N D S A P A A N G S Q G L A
2761 CGCCCCAGAGTCACCCCCAATGACAGTGCCCCTGCCGCCAACGGCAGCCAGGGGCTGGCT 2820
E R I M N A L N S T P L M E E T A P Q A
2821 GAGCGCATCATGAATGCACTCAACAGCACGCCGCTGATGGAGGAGACGGCTCCCCAGGCC 2880
Q S E S Q A M P I S A L P L E L K Q R V
2881 CAGAGCGAGTCTCAGGCCATGCCGATCAGCGCCCTGCCGCTGGAGCTGAAGCAGCGGGTG 2940
P P L A Y G S H V F S S N P A K R A V M
2941 CCGCCCCTGGCCTACGGCTCCCACGTCTTCTCGTCCAACCCGGCCAAACGGGCCGTGATG 3000
L N G R E F R E G S E V A P G V T L I A
3001 CTGAACGGACGTGAGTTTCGCGAGGGCAGCGAGGTCGCCCCCGGCGTCACCCTGATCGCC 3060
I A Q D Y I I L Q V A G Q N V S L K A L
3061 ATAGCCCAGGATTACATCATCCTGCAGGTGGCGGGCCAGAACGTCAGTCTCAAGGCGCTG 3120
Q D W R G *
3121 CAGGACTGGCGCGGCTGATCCCGTCACGACCACAACGCCGGCTCAGCCCCGCTGAGCCGG 3180

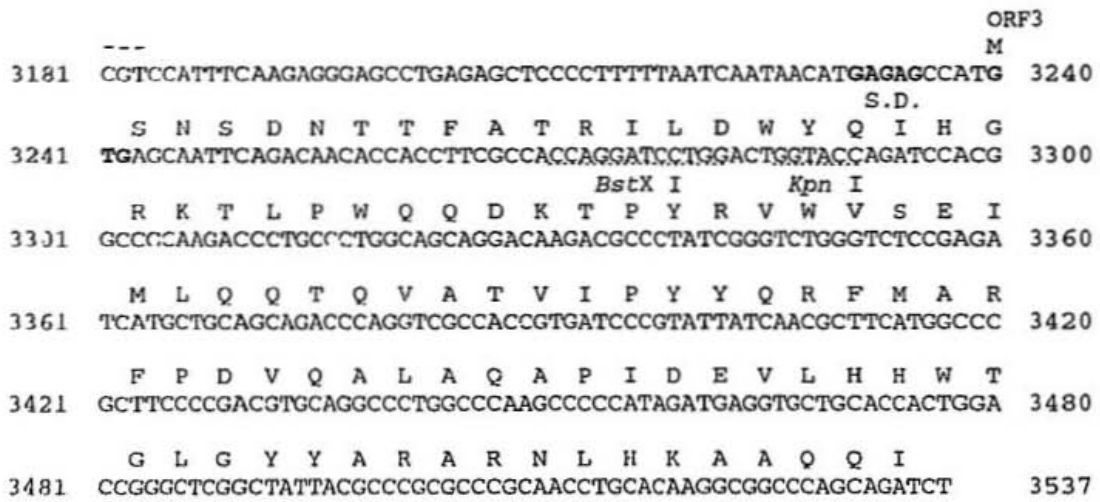


Figure 3.3. Sequence of the 3.5 kb *Bgl* II fragment. The non-coding strand is presented along with its translation. Potential ribosome binding sites and start codons are bolded. Putative -35 and -10 promoter sequences upstream of *exeA* are underlined. The 13 base pair inverted repeat is overlined with arrows. Stop codons are denoted by an asterisk and the restriction endonuclease cleavage sites used in the construction of the subclones described in the text are dotted.

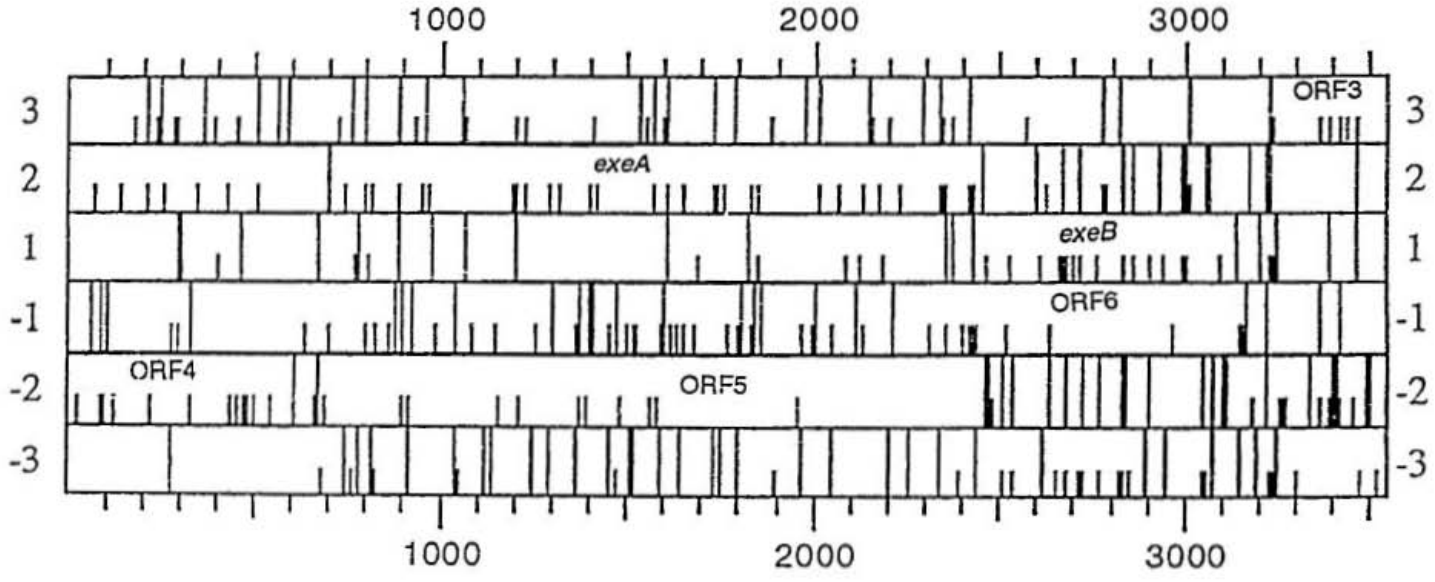


Figure 3.4. Computer analysis of the sequenced 3.5 kb *Bgl* II fragment for open reading frames. ORFs *exeA*, *exeB*, ORF3, ORF4, ORF5, and ORF6 are indicated. The small vertical lines represent AUG and GUG codons, while the large vertical lines indicate a stop codon.

Based on sequence homology with previously identified genes of *E. coli* (see later) the partial ORF extending beyond the 3' end of the fragment as shown in Fig. 3.5 was judged to be a gene and was designated ORF3, while the ORF extending past the 5' end of the fragment in the opposite direction was designated ORF4. ORF3 is thus in the same direction as ORF1 and ORF2, and ORF4 is in the opposite orientation (Fig. 3.5). Since ORF5 and ORF6 would be transcribed in the opposite direction of transcription with respect to the orientation which complemented C5.84, no further investigation was carried out on them. ORF1(hereafter *exeA*) starts with an ATG codon at nucleotide position 743 with several inframe start codons and stops at nucleotide position 2456 with a TGA codon. The first and second ATG codons are not preceded by sequences resembling ribosome binding sites (AGGAGGT) (Shine and Dalgarno, 1974). The third ATG codon, however, which is at nucleotide 815, is preceded by a putative ribosome binding site GGA at nucleotide 806 about nine bases upstream of the start codon. This ORF would encode a 547 amino acid protein of 60,170 Da. ORF2 (hereafter called *exeB*) starts with an ATG codon at nucleotide position 2458 which overlaps with the stop codon of *exeA,* and ends at nucleotide position 3136 with a TGA codon. *exeB* is also preceded by a putative ribosome binding site, GGAGG, at nucleotide 2450 7 bases upstream of the start codon. Thus *exeB* would encode for a 226 amino acid protein of 24,860 Da. Immediately following the stop codon of *exeB* is a 13 base pair inverted repeat at nucleotide 3155 (Fig. 3.3). ORF3 starts with a GTG codon at nucleotide position 3240 and extends beyond the 3' end of the sequenced fragment. ORF4 also starts with a GTG codon at nucleotide position 551, and extends beyond the 5' end of the sequenced fragment.

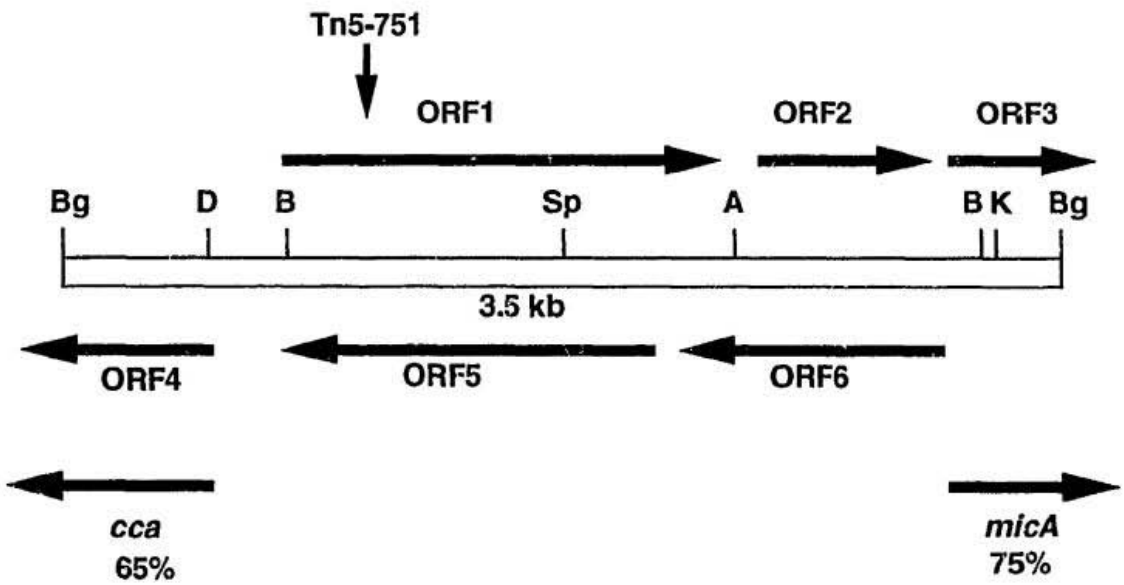


Figure 3. 5. ORF map of the sequenced 3.5 kb *Bgl* II fragment. Horizontal arrows represent ORFs and the vertical arrow indicates the site of Tn5-751 insertion in C5.84. ORF1 (*exeA*), ORF2 (*exeB*), ORF3, ORF4, ORF5 and ORF6 are shown. ORF3 and ORF4 which are homologous to *micA* and *cca*, are also indicated with their % nulceotide identity. A, *Acc*I; B, *Bst*XI; Bg, *Bgl* II; D, *Dra*III; K, *Kpn*I, Sp, *Sph*I.

3.4 Comparison of the 3.5 kb *Bgl* II sequence with other DNA sequences

The Fasta and Blast analysis programs were used for a similarity search of the GenBank database with the sequenced 3.5 kb *Bgl* II fragment and its translations (Pearson and Lipman, 1988; Altschul *et al.*, 1990). No significant matches were found between *exeA* and any known genes, however, two proteins were found which had low levels of sequence similarity with ExeB. These were PulB from *K. oxytoca* (d'Enfert and Pugsley, 1989), and OutB from *Er. chrysanthemi* (Condemine *et al.*, 1992). An alignment of these three proteins is shown in Figure 3.6.

Significant homology was also found between ORF3 and the *E. coli micA* gene, and between ORF4 and the *E. coli cca* gene. The *micA* gene encodes the A/G specific adenine glycosylase which specifically recognizes and repairs A/G mismatches in a methylation independent pathway (Tsai-wu *et al.*, 1991). *micA* has a nucleotide identity of 71% with the sequenced portion of ORF3 as shown in Figure 3.5. The *cca* gene encodes for tRNA nucleotidyl-transferase, which incorporates AMP and CMP residues into the acceptor stem of tRNAs (Cudny *et al.*, 1986). The *cca* gene has a nucleotide sequence identity of 65% with the sequenced portion of ORF4.

```
           1                                                  50
     ExeB  MSTLLKALRR AEQPQFTPHI PAMGLPVTQE EEQNRRWIWW LLAPLALLMG
     PulB  .......... .......... .......... .......... ..........
     OutB  .......... .......... .......... .......... ..........
Consensus  ---------- ---------- ---------- ---------- ----------
           51                                                 100
     ExeB  AGANYGWHLL NNRPIEKTVE VKEVVTPPFV RVEPRPMITR PLPPPLPEPV
     PulB  .......... .MLVRPQEPY PQSEPPAAVG RMVQIPYVTV PLYAALLIAL
     OutB  .......... .......... .......... .......... .....MVPAS
Consensus  ---------- ---------- ---------- r----p--t- pl---l--a-

           101                                                150
     ExeB  VRPRVTPNDS APAANGSQGL AERIMNALNS TPLMEETAPQ AQSESQAMPI
     PulB  GWFGGEQWRN KPEPQPMRQS VAHAALVPLN QPAVKAAVAP VNAGPEIQAE
     OutB  TAVTADENHP GSLHAAAENS ATQTAASGSQ TSASSQEATP DTKPAKLVTG
Consensus  -------n-- -p-------s a---a----- tpa----a-p ----------

           151                                                200
     ExeB  SALPLELKQR VPPLAYGSHV FSSNPAKRAV MLNGREFREG SEVAPGVTLI
     PulB  PEIAID.EDN LPPLRYSAHV YASLADKRSI VLNGQSWKEG DSPLANLVIE
     OutB  WQTA.K.PGE LPYIAFSAHV YTSAPDKRSV TLNGERYREG DSPYQGLVIE
Consensus  ---a------ lPplaysaHV y-S-pdKRsv -LNG---rEG dsp--glvie

           201                                                250
     ExeB  AIAQDYIILQ VAGQNVSLKA LQDWRG.... .......... ..........
     PulB  QIQQDLTVFS FNGKTFTLAA LDDWPGGAIE ESPQAE.... ..........
     OutB  QIEQDMVIFS FNGEPFILDS LQDWPGGKPG DDAAQGNEQE PTSKPEQTVR
Consensus  qI-QD--ifs fnG--f-L-a LqDWpGg--- ---------- ----------

           251
     ExeB  ....
     PulB  ....
     OutB  TTKK
Consensus  ----
```

Figure 3.6. Alignment of the predicted amino acid sequence of ExeB with the amino acid sequence of PulB and OutB. Dots indicate gaps inserted to optimize the alignment. A consensus sequence is presented using upper case letters when all three proteins share an amino acid and lower case when two proteins share an amino acid.

3.5 Polypeptides encoded by the 3.5 kb *Bgl* II fragment

To substantiate the presence of the genes predicted to be present on the basis of DNA sequencing, pSRJ5.2 and pSRJ5.1, which contain the 3.5 kb *Bgl* II fragment in the PBluescript II SK+ vector in both orientations (see Fig. 3.1) were expressed in an *in vitro* transcription-translation system (Zubay, 1980). [^{35}S]-methionine was used to label the translated proteins as described in Materials and Methods. The samples were run on SDS-PAGE gels and visualized by autoradiography. Polypeptides whose apparent molecular weights were 60 kDa, 31.5 kDa, 30 kDa, 29 kDa, 27 kDa and 22 kDa, were present in the pSRJ5.2 sample (Fig. 3.7, lane 5). When the *Bgl* II fragment was in the opposite orientation with respect to the *lac* promoter of the vector (in pSRJ5.1), polypeptides whose apparent molecular weights were 60 kDa, 33 kDa, 32 kDa, 31.5 kDa, 29 kDa, and 14 kDa were identified (Fig. 3.7, lane 6). A comparison of these proteins with those produced by the vector alone (Fig. 3.7, lane 7) indicated that the 31.5 kDa protein was β-lactamase, whereas the others were encoded by the insert.

Further subcloning of pSRJ5.2 was done followed by expression *in vitro* in order to identify the individual polypeptides. Construct pKRJ18.1 (*Bst*XI-*Acc*I fragment, see Fig. 3.1) which contains nucleotides 800 to 2462 of the sequence produced the 60 kDa polypeptide which was expressed very weakly (Fig. 3.7, lane 1), whereas construct pSRJ19.1 (Fig. 3.1), which had the same fragment in the opposite orientation with respect to the *lac* promoter of the vector, did not produce this polypeptide (Fig. 3.7, lane 2). Inclusion of the 300 bases upstream of the *Bst*XI site, in construct pKRJ20.2 (*Dra* III-*Acc* I fragment, see Fig. 3.1), also produced the 60 kDa protein (Fig. 3.7, lane 3), even when the fragment was

in the opposite orientation (in pSRJ21.1) with respect to the *lac* promoter of the vector (Fig. 3.7, lane 4). These results suggested the presence of a promoter in the region between nucleotides 500 to 800 (*Dra*III-*Bst*XI) of the 3.5 kb *Bgl* II fragment. Construct pKRJ46.1 (*Sph* I-*Kpn* I fragment, see Fig. 3.1) which contains only the *exeB* ORF and 334 base pairs 5' to the putative start codon of *exeB*, produced the 29 kDa polypeptide (Fig. 3.8, lane 2). This protein must migrate anomalously on SDS-PAGE, since as noted earlier, its calculated molecular mass is 24,860 Da. It also migrated with an apparent molecular weight of 29 kDa when synthesized *in vivo* under the control of a T7 RNA polymerase promoter (see Fig. 3.23 and 3.24).

Constructs pKRJ58.1 and pSRJ57.1 contained the *Dra* III-*Kpn* I fragment encompassing the complete *exeA* and *exeB* ORFs plus the 300 bp upstream of the putative *exeA* start, in both orientations. Both of these constructs produced the 60 kDa and 29 kDa proteins (Fig. 3.8, lanes 3 and 4). In contrast, construct pKRJ50.3 (*Bst*XI-*Bst*XI fragment, Fig. 3.1) containing both the ORFs but beginning just 15 nucleotides 5' to the start codon of *exeA*, produced both the 60 kDa and 29 kDa proteins (Fig. 3.8, lane 5), whereas the same fragment in the opposite orientation (pKRJ50.2, Fig. 3.1) produced neither protein (Fig.3.8, lane 6). These results allowed us to conclude that ORFs *exeA* and *exeB* produce the expected proteins, and that a promoter functional in *E. coli* lies in the 300 base pairs between the *Dra*III and *Bst*XI sites 5' to *exeA* (see Fig. 3.16).

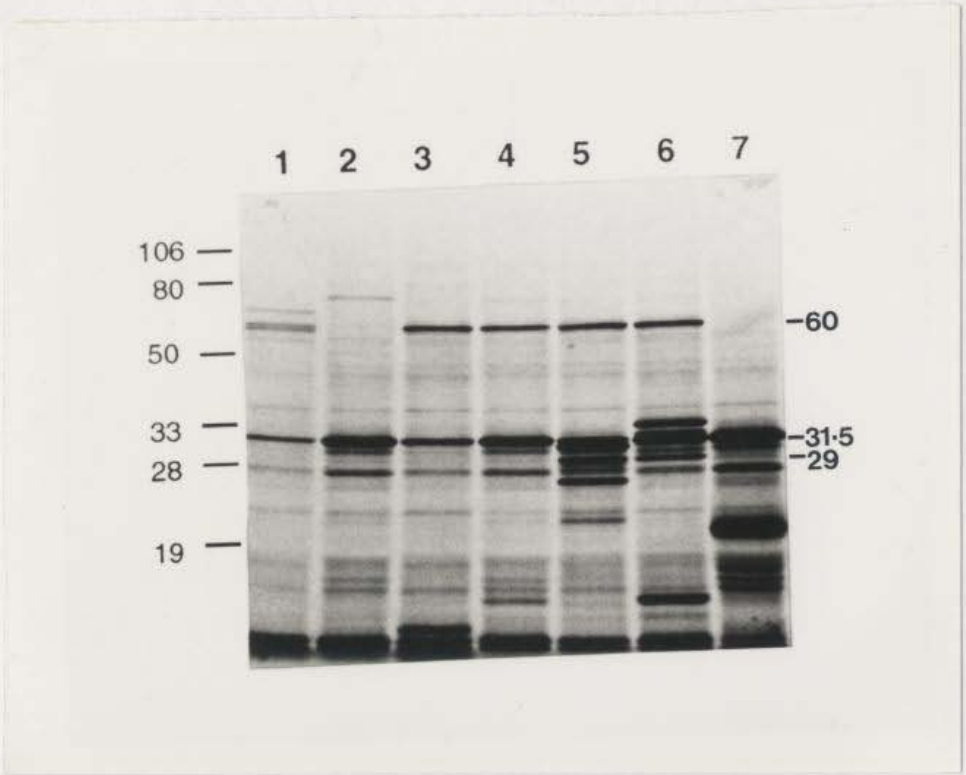


Fig. 3.7. *In vitro* transcription-translation reactions of the 3.5 kb *Bgl* II fragment and its subclones. An autoradiograph of a 12% SDS-PAGE gel of the [^{35}S]-methionine labeled proteins produced in subclones pKRJ18.1 (lane 1), pSRJ19.1 (lane 2), pKRJ20.2 (lane 3), pSRJ21.1 (lane 4), pSRJ5.2 (lane 5), pSRJ5.1 (lane 6), and pBluescript II KS+ (lane 7) is shown. Proteins discussed in the text are identified on the right with their molecular weights (in kDa) and the molecular mass of radiolabeled protein standards (in kDa) are shown on the left.

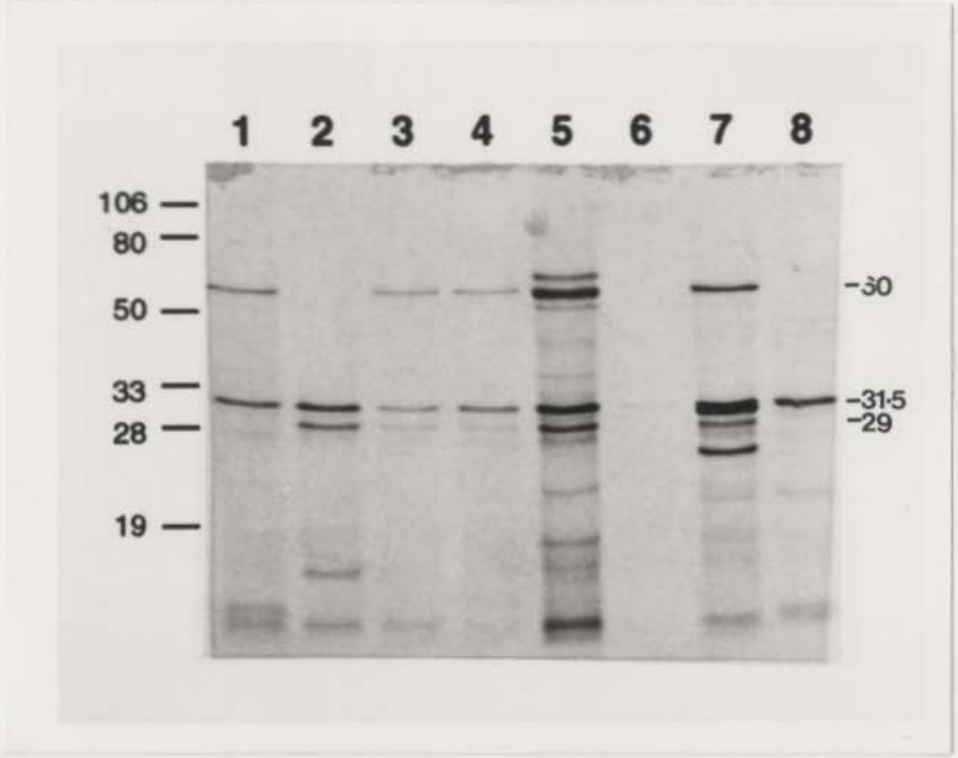


Figure 3.8. *In vitro* transcription translation reactions of the *exeA* and *exeB* genes. An autoradiograph of a 12% SDS-PAGE gel of the [^{35}S]-methionine labeled proteins produced in subclones pKRJ20.2 (lane 1), pKRJ46.1 (lane 2), pKRJ58.1 (lane 3), pSRJ57.1 (lane 4), pKRJ50.3 (lane 5), pKRJ50.2 (lane 6), pSRJ5.2 (lane 7) and pBluescript II SK+ (lane 8) is shown. Proteins discussed in the text are identified on the right with their molecular weights (in kDa) and the molecular mass of radiolabeled protein standards (in kDa) are shown on the left.

3.6 Complementation analysis of the 3.5 kb *Bgl* II fragment

The site of transposon insertion in C5.84 was mapped to a location about 250 bases downstream of the *Bst* XI site in the *exeA* ORF (Fig. 3.9) as deduced from restriction analysis of pJB2 (Jiang and Howard, 1991) which contains a fragment of the transposon and the adjacent chromosomal DNA cloned from C5.84. Therefore, it was very likely that the *exeA* ORF represented the coding region for the gene interrupted by the transposon and hence a 1.6 kb *Bst* XI-*Acc* I fragment which contained only *exeA* ORF was cloned from pSRJ19.1 (see Fig. 3.1) into pMMB207 and pMMB208 in either orientation to give rise to pRJ24.2 and pRJ30.1 respectively (Fig. 3.10). These constructs were conjugated into C5.84 and the transconjugants were then streaked on to a BHI blood agar plate to examine their hemolytic phenotype. No zones of hemolysis were observed around these colonies, indicating that this fragment was unable to complement the mutation whether or not it was placed under the control of the *tac* promoter of the vector.

Another construct, containing the *Dra* III-*Acc* I fragment from pSRJ21.1 (see Fig. 3.1) which contained the 300 bases upstream of *exeA* as well as *exeA* was similarly subcloned into pMMB207 and pMMB208 in both orientations (pRJ25.3 and pRJ26.3, Fig. 3.10) and conjugated into C5.84. Both of these constructs also failed to complement the mutation. Aerolysin titres were also conducted on these transconjugants after growth in BHI media. The aerolysin titres of these transconjugants were similar to that of the mutant C5.84 (Table 3. 2), confirming that *exeA* alone cannot complement the mutation. The *Bst*XI-*Acc*I fragment was therefore extended to include the *exeB* ORF (*Bst*XI-*Bst*XI fragment) and cloned in both orientations to give rise to constructs pRJ31.1 and

pRJ60.1 (Fig. 3.10). When pRJ31.1 was conjugated into C5.84, it complemented the mutation and restored the wild-type secretory phenotype, whereas pRJ60.1, containing the same fragment in opposite orientation with respect to the *tac* promoter of the vector, did not. Aerolysin titres and immunoblot analysis were performed on the culture supernatants of transconjugants C5.84(pRJ31.1) and C5.84(pRJ60.1) as well as the wild type and the mutant to demonstrate the secretory activity. For the immunoblot, the samples were run on a 10% SDS PAGE gel, transferred to nitrocellulose and immunoblotted with an anti-aerolysin monoclonal antibody. The results showed that C5.84(pRJ31.1) exported most of the aerolysin into the supernatant, similar to wild type, whereas in C5.84(pRJ60.1) most of the aerolysin remained within the cells (Fig 3.11 compare lanes 3C and 3S with 4C and 4S). The aerolysin titre of the culture supernatants of C5.84(pRJ31.1) was similar to that of the wild type whereas the titre of C5.84(pRJ60.1) was similar to that of the mutant C5.84 (Table 3.3).

The *Dra* III-*Kpn* I fragment, which contained both *exeA* and *exeB* ORFs plus the 300 bases upstream of the *exeA* ORF, was also cloned in pMMB207 and pMMB208 in both orientations to give rise to constructs pRJ39.1 and pRJ40.1 respectively (Fig. 3.10). These were conjugated into the mutant and assayed for complementation. The aerolysin titres of both of the transconjugants, C5.84(pRJ39.1) and C5.84(pRJ40.1) were similar to the wild type titres (Table 3.4), indicating that the fragment was able to complement independently of the vector promoter. Finally, to rule out the possibility that only the second ORF, *exeB*, was required to complement the mutation, a *Sph* I-*Kpn* I fragment which contained *exeB* alone was also cloned in pMMB67EHcam and pMMB67HEcam in both orientations (pRJ10.1 and pRJ11.1, see Fig. 3.10) and conjugated into C5.84.

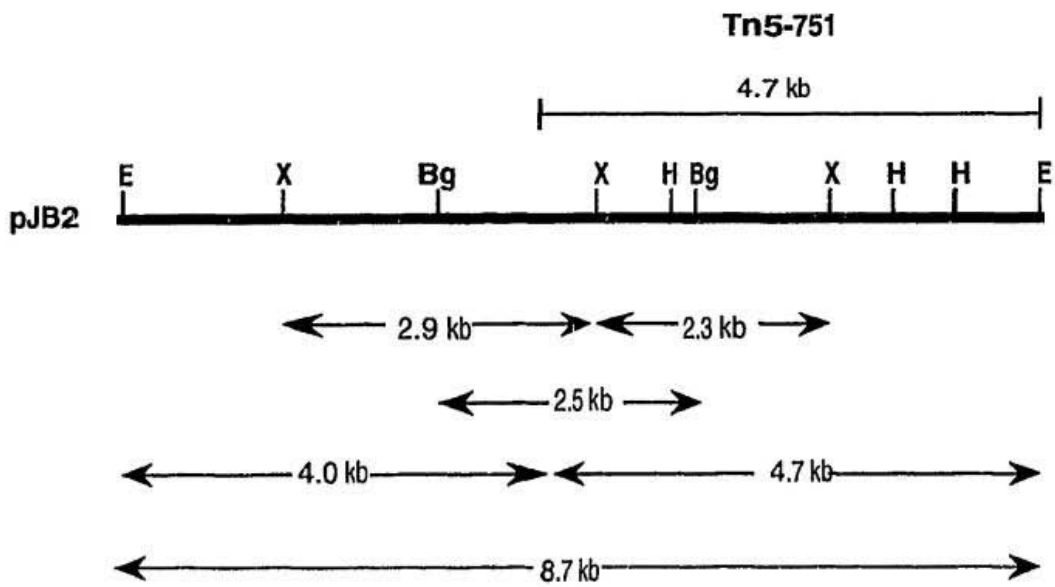


Figure 3.9. Restriction endonuclease mapping of Tn5-751 insertion in C5.84. Clone pJB2 contains 4.0 kb of chromosomal DNA and 4.7 kb of Tn5-751, isolated from the C5.84 genome (Jiang and Howard,1991). The fragment sizes obtained by restriction digests are shown. From these and the published map of Tn5-751 (Rella, *et al.*, 1985), the Tn insertion site was calculated to be about 1.0 kb downstream of the *Bgl* II site of the chromosomal DNA, which is about 250 bases 3' to the *Bst* XI site of the *exeA* ORF (see text for details). Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; X, *Xho* I.

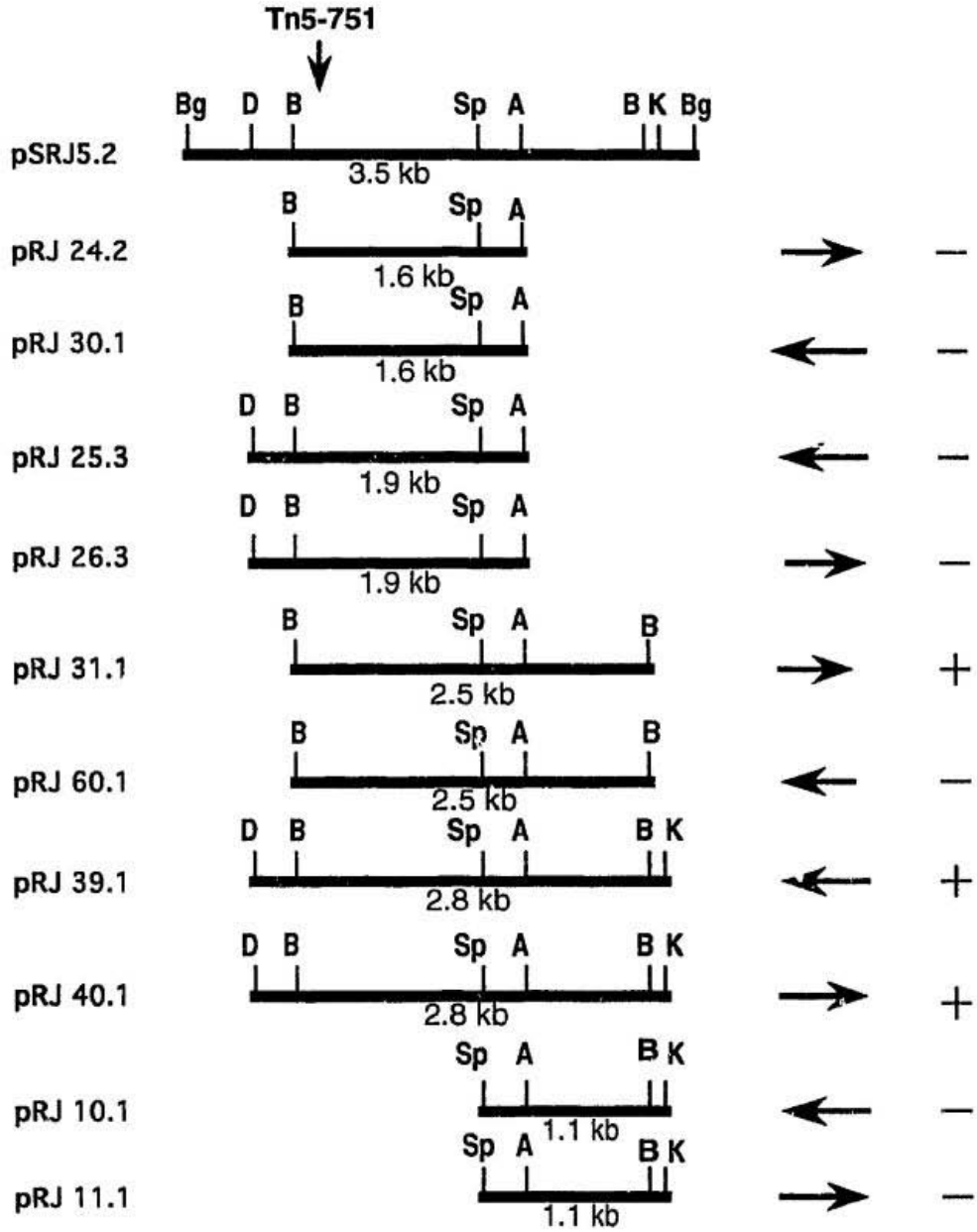


Figure 3.10. Subcloning and complementation analysis of the 3.5 kb *Bgl* II fragment complementing the mutation in C5.84. The vertical arrow indicates the site of Tn5-751 insertion in C5.84 and the horizontal arrows indicate the direction of the *tac* promoter. Constructs pRJ are in broad host range vectors pMMB207/208 and construct pSRJ5.2 is in pBluescript SK II + vector. +/- indicate complementation and no complementation respectively. A, *Acc*I; B, *Bst*XI; Bg, *Bgl*II; D, *Dra*III; K, *Kpn*I; Sp, *Sph*I.

Table 3.2 Aerolysin titres of the wild-type, mutant and transconjugants containing only *exeA* alone and *exeB* alone.

Supernatant fraction		Aerolysin (HU)
Ah65		128
C5.84		8
C5.84(pRJ24.2)	*exeA* alone	8
C5.84(pRJ30.1)		8
C5.84(pRJ25.3)		8
C5.84(pRJ26.3)		8
C5.84(pRJ11.1)	*exeB* alone	8
C5.84(pRJ10.1)		8

Hemolytic activities are given as the titres, expressed as hemolytic units (HU).

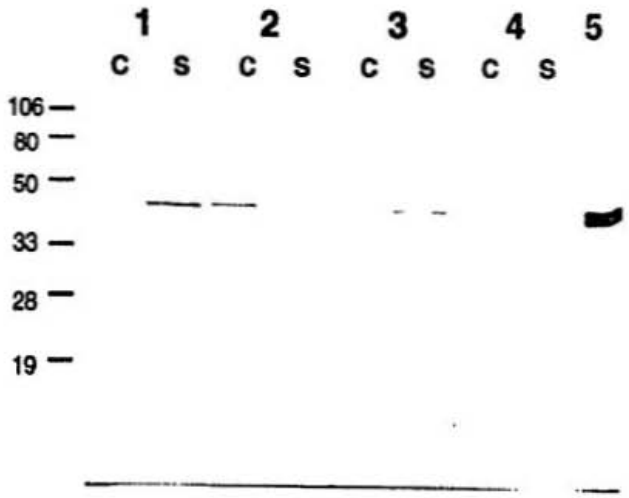


Figure 3.11. Immunoblot analysis of aerolysin secretion by C5.84(pRJ31.1) and C5.84(pRJ60.1). Cell samples (lanes C) and culture supernatants (lanes S) representing equal volumes of freshly grown BHI cultures were electrophoresed, transferred to nitrocellulose, and immunoblotted with an anti-aerolysin monoclonal antibody. Ah65 (lane 1); C5.84 (lane 2); C5.84(pRJ31.1) (lane 3); C5.84(pRJ60.1) (lane 4) and purified aerolysin (lane 5). The upper and lower bands in lane 5 are proaerolysin and aerolysin respectively. Molecular masses of standards are given on the left in kDa.

Table 3.3 Aerolysin titres of the transconjugants containing both *exeA* and *exeB* without the putative promoter.

Supernatant fraction	Aerolysin (HU)
Ah65	256
C5.84	8
C5.84(pRJ31.1)	128
C5.84(pRJ60.1)	8

Hemolytic activities are given as the titres, expressed as hemolytic units (HU).

Table 3.4 Aerolysin titres of the transconjugants containing both *exeA* and *exeB* with the putative promoter.

Supernatant fraction	Aerolysin (HU)
Ah65	128
C5.84	8
C5.84(pRJ39.1)	128
C5.84(pRJ40.1)	128

Hemolytic activities are given as the titres, expressed as hemolytic units (HU).

Aerolysin titres conducted on these two transconjugants grown in BHI media indicated that the plasmids were unable to complement the mutant (Table 3.2).

3.7 Marker exchange mutagenesis of *exeA* and *exeB*

To corroborate that both *exeA* and *exeB* genes are required for extracellular protein secretion, both of the genes were individually inactivated by insertion of a kanamycin cassette. Insertion of a kanamycin cassette in *exeA* essentially recreated the original transposon insertion mutation, and *exeB* was inactivated by kanamycin insertion as shown in Figure 3.12. Each inactivated gene was subcloned into a suicide vector, pSUP202 for *exeA* and pJQ200SK for *exeB*. The resulting clones were then conjugated into Ah65 for exchange with the wild type *exe* genes and mutants in which an exchange had resulted were obtained as described in Chapter 2. AA41 and AB69 are the mutant strains of *A. hydrophila* in which *exeA* and *exeB* respectively have been inactivated through the insertion of the kanamycin drug resistence cassette.

In each case, the structure of the mutated *exe* gene was confirmed by performing a Southern blot on a *Sma* I chromosomal digest of the selected exconjugants. As shown in figure 3.13, the 3.5 kb *Bgl* II insert probe hybridized to a 5.2 kb *Sma* I fragment in Ah65 (lane 1), 3.6 and 2.8 kb *Sma* I fragments in AA41 (lane 2) and 4.2 and 2.2 kb *Sma* I fragments in AB69 (lane 3). The total of both the bands in each mutant is 6.4 kb, the 5.2 kb of wild type plus the 1.2 kb of the kanamycin cassette used for insertion mutagenesis, indicating that mutants AA41 and AB69 were correct in their structure.

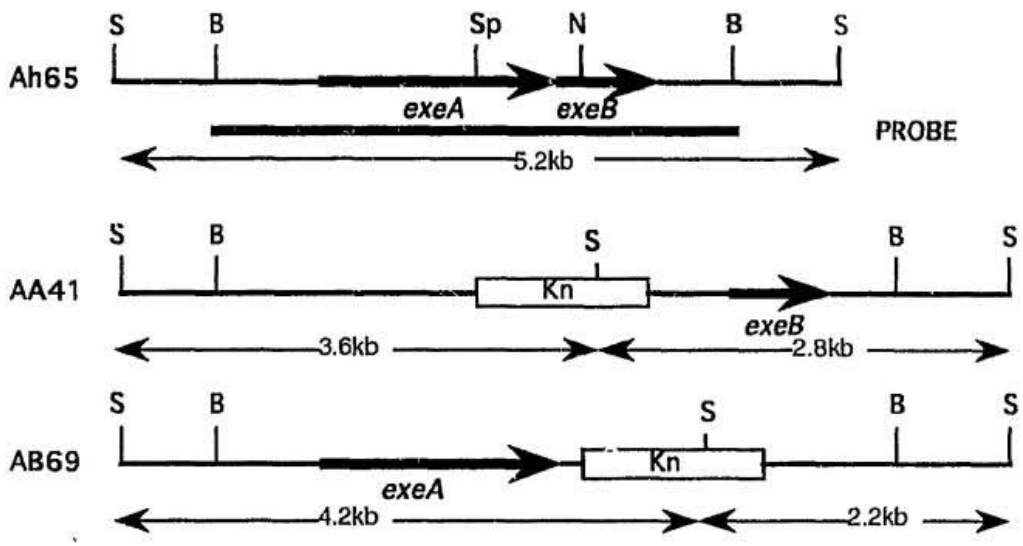


Figure 3.12. Physical maps of Ah65, *exeA* mutant AA41 and *exeB* mutant AB69. S, *Sma*I; B, *Bgl*II; Sp, *Sph*I; N, *Nar*I. Kn-kanamycin drug resistence cassette.

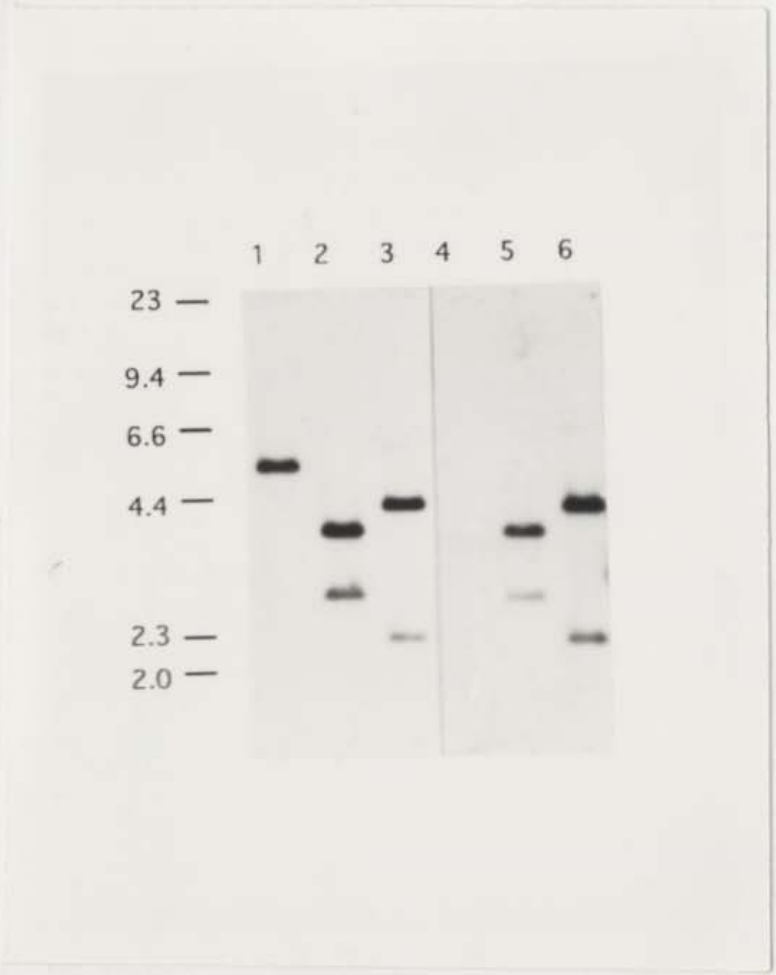


Figure 3.13. Fluorographs of the Southern blot analysis of Ah65, *exeA* mutant AA41 and *exeB* mutant AB69. Genomic DNAs were digested to completion with *Sma* I (lanes 1 to 6). Lanes 1 to 3 were probed with the 3.5 kb *Bgl* II insert from pSRJ5.2 and Lanes 4 to 6 were probed with the *aph* gene from pUC4K. Lanes 1 and 4, Ah65; Lanes 2 and 5, AA41; Lanes 3 and 6, AB69.

In order to confirm that the difference in the size of the bands was due to insertion of the kanamycin cassette, the *aph* gene was also used as a probe. As shown in figure 3.13, the *aph* gene probe did not hybridize to the wild type, whereas in AA41 and AB69 the same fragments as noted above were observed indicating that the alteration in the structure of both of these mutants were in fact due to the insertion of the kanamycin cassette.

The phenotype of the marker exchange mutants on BHI blood plates was similar to the original mutant C5.84, in which the normal halo of hemolysis was absent. As a specific measure of secretion, each of the mutants AA41 and AB69 as well as the wild type and C5.84 were grown in BHI broth culture, and cell and supernatant fractions were immunoblotted with an anti-aerolysin monoclonal antibody (Fig. 3.14). Both of the marker exchange mutants retained aerolysin within the cells, as did the transposon insertion mutant C5.84 (Fig. 3.14, lanes 2C, 3C and 4C), whereas for the wild type, the aerolysin was secreted extracellularly (Fig. 3.14, lane 1S). The cell and supernatant fractions from these cultures were also assayed for aerolysin and protease activities. As can be seen in Table 3.5, both of the mutants AA41 and AB69 displayed the same inability to secrete the protease as did the original mutant C5.84, and the aerolysin also fractionated with the cell contents for all three of the mutants. These results further confirmed that both *exe* genes are essential for extracellular protein secretion in *A. hydrophila*.

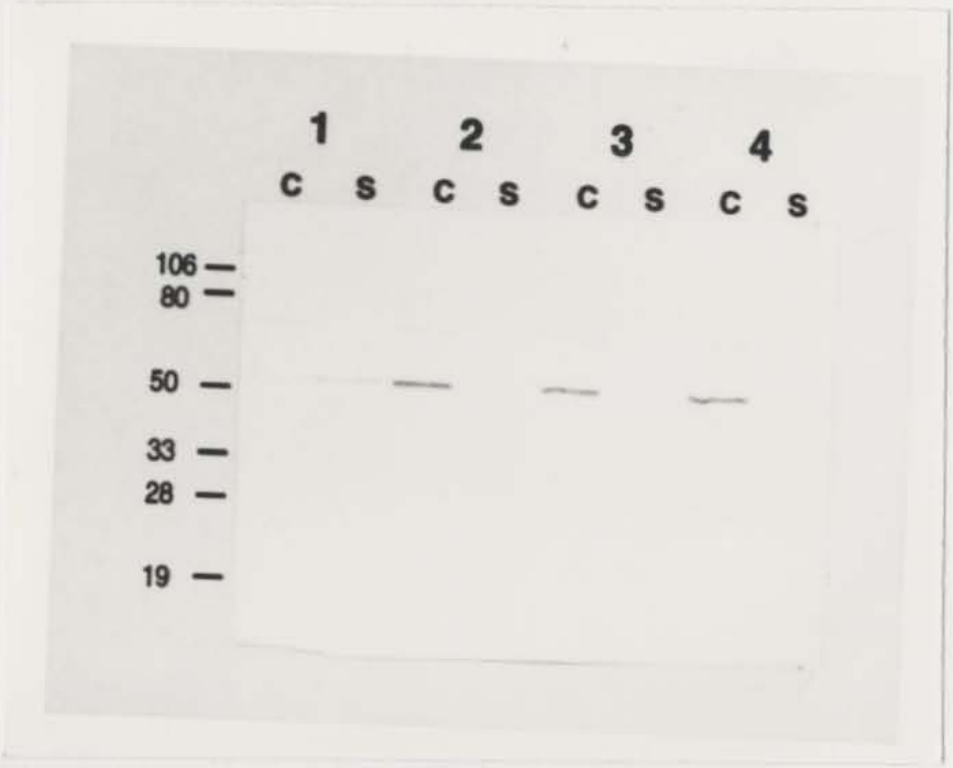


Figure 3.14. Immunoblot analysis of aerolysin secretion in marker exchange mutants. Cell samples (lanes C) and culture supernatants (lanes S) representing equal volumes of freshly grown BHI cultures were electrophoresed, transferred to nitrocellulose, and immunoblotted with an anti-aerolysin monoclonal antibody. Ah65 (lane 1); C5.84 (lane 2); AA41 (lane 3) and AB69 (lane 4). Molecular masses of standards are given on the left in kDa.

TABLE 3.5 Enzyme activities in Ah65, C5.84, AA41 and AB69.

Fraction	Aerolysin in HU	Protease in U/ml (%)
Ah65		
Supernatant	128	199 (76%)
Cells	8	64 (24%)
C5.84		
Supernatant	8	106 (18%)
Cells	128	488 (82%)
AA41		
Supernatant	8	93 (15%)
Cells	128	527 (85%)
AB69		
Supernatant	0	14 (3%)
Cells	128	618 (97%)

Protease activities are given as U/ml of the original culture, while hemolytic activities are given as the titres, expressed as hemolytic units (HU).

3.8 Immunoblots on cell fractions of the marker exchange mutants AA41 and AB69 with anti-ExeB serum

The complementation studies suggested that the transposon insertion in the *exeA* gene had resulted in a polarity effect on the expression of the downstream *exeB* gene, since both *exeA* and *exeB* were required to complement C5.84 for secretion. To substantiate this, the marker exchange mutants AA41 and AB69 along with the wild type strain and C5.84 were grown in BHI broth and cell samples were electrophoresed and immunoblotted with an anti-ExeB serum (Howard, S. P., unpublished) (Fig. 3.15). The 29 kDa band representing *exeB* was observed in the wild type (Fig. 3.15, lane 1) but was missing in the original transposon insertion mutant C5.84, and also in both of the marker exchange mutants AA41 and AB69 (Fig. 3.15, lanes 2, 3, and 4). The absence of the ExeB protein in the marker exchange mutant AB69 confirmed the inactivation of *exeB* by the insertion of kanamycin cassette, whereas its absence in C5.84 and AA41 confirmed that the insertional inactivation of *exeA* also resulted in a polar effect on *exeB* expression. As discussed earlier in section 3.5, complementation studies also showed that *exeB* alone is not sufficient to restore protein secretion in C5.84 indicating that both genes are required for secretion.

3.9 Analysis of mRNA

The complementation analysis and the *in vitro* transcription-translation reactions both indicated that there is a promoter upstream of *exeA*, between the *Dra* III and *Bst* XI sites.

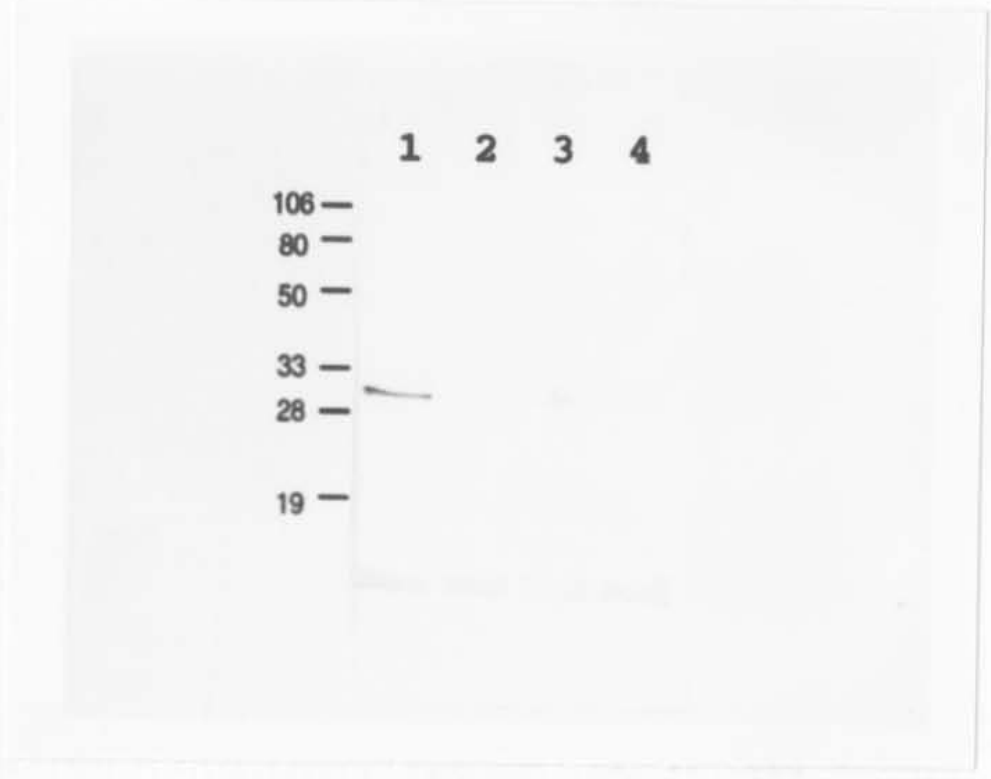


Figure 3.15. Immunoblot analysis of cell fractions of the marker exchange mutants with anti-ExeB serum. Equal volumes of freshly grown BHI cultures were electrophoresed, transferred to nitrocellulose, and immunoblotted with an anti-ExeB serum. Ah65 (lane 1); C5.84 (lane 2); AA41 (lane 3) and AB69 (lane 4). Molecular masses of standards are given on the left in kDa.

```
500                                                          540
CACCAGGTGA   TCCCGATCCC   ATTGCGGCAA   TCCCAGCAGG
  DraIII
541                                                          580
CGATCCCGCA   CCGCGCCCCC   AACCAGATAA   GTCTGCAATC
581                                                          620
TCTGTCTCCC   TTGATAAACC   TGCCTCGATT   ATACCGGTTT
621                                                          660
TGCCTGGCGG   GCACGATTGC   CCTGCAGGTT   TGGCCCGACA
661                                                          700
GCCACTCTGA   CCTATCCCCG   CCACAACCAG   GCCACATTCG
701                                                          740
ATAAAAACAG   CGGTTTTGGC   CGCCAGATGC   GCCAGCCCCC
741                                                          780
CTTATGGCAT   CCGGGTCTTT   GACATCCCGG   TGGGGAGGCT
781                      -35                                 820
TTAACCTGCC   GCCCATAACA   ATGGAATGGA   TCCTTATGTA
  -10          BstXI                    S.D.
```

CONSENSUS PROMOTER:

-35 region	16-18 bps	-10 region
TTGACA		TATAAT

Figure 3.16. Putative promoter sequence between *Dra*III and *Bst*XI site 5' of *exeA*. The -35 and -10 sequences are boxed. The putative ATG initiation codon is underlined, as is a possible Shine Dalgarno (S.D.) ribosome binding site. The consensus promoter sequence of *E. coli* is also shown.

The complementation and immunoblot analysis also strongly suggested that *exeA* and *exeB* form an operon, both members of which are required for secretion in *A. hydrophila*. If the putative promoter sequence between the *Dra* III and *Bst* XI sites as shown in Fig. 3.16 is used to initiate a transcript which terminates in the vicinity of the 13 base pair inverted repeat which follows *exeB* as shown in Figure 3.17, an mRNA of about 2378 bases would result. In order to determine whether or not an *exe* specific message of this size was produced, mRNA was isolated from mid-log phase cells of wild type and C5.84, and a northern blot was performed. The blot was hybridized to an RNA probe obtained from the 2.5 kb *Bst* XI-*Bst* XI fragment. The results in Figure 3.18 show that the wild type cells indeed contained a message of approximately 2500 nucleotides which was lacking in the mutant C5.84 used as a negative control. These results are consistent with the co-expression of *exeA* and *exeB* as an operon.

3.10 Structural features of the predicted *exe* gene products

The amino acid sequences of ExeA and ExeB were deduced from the nucleotide sequence and analyzed by computer. Hydropathy analysis was done on ExeA (Figure 3.19) and ExeB (Figure 3.20) by the method of Kyte and Doolittle (1982). The average hydrophobicity for the product of *exeA* is -1.9; it has no sequence resembling an amino terminal signal sequence, and it is quite hydrophilic. ExeA has one very hydrophobic segment, however, in the region of residues 276 and 295 (Fig. 3.21), which would be classified as transmembrane using the algorithm of Kyte and Doolittle (1982). ExeA has a calculated isoelectric point of 5.06.

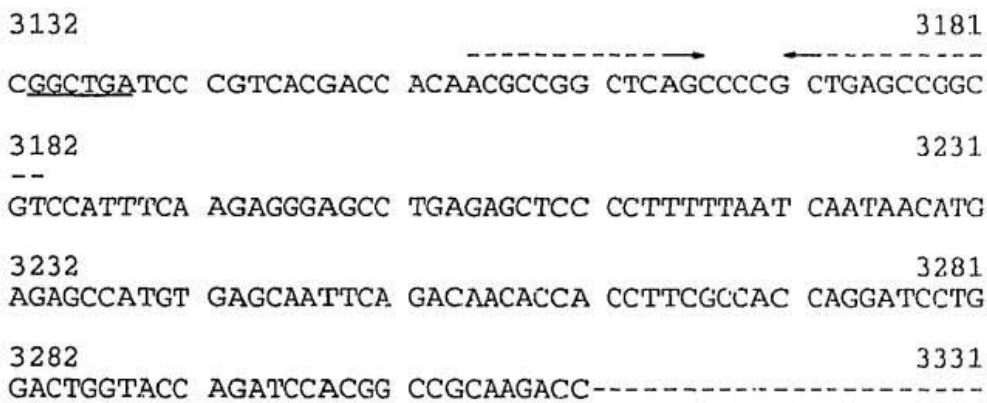


Figure 3.17. Potential terminator structure following *exeB*. The 13 base pair inverted repeat is overlined with arrows. The free energy for melting of the potential terminator is $\Delta G^{0'}$ = -30.60 kcal/mole (Zuker and Stiegler, 1981). The region of sequence underlined shows the last codon and the termination codon of *exeB*.

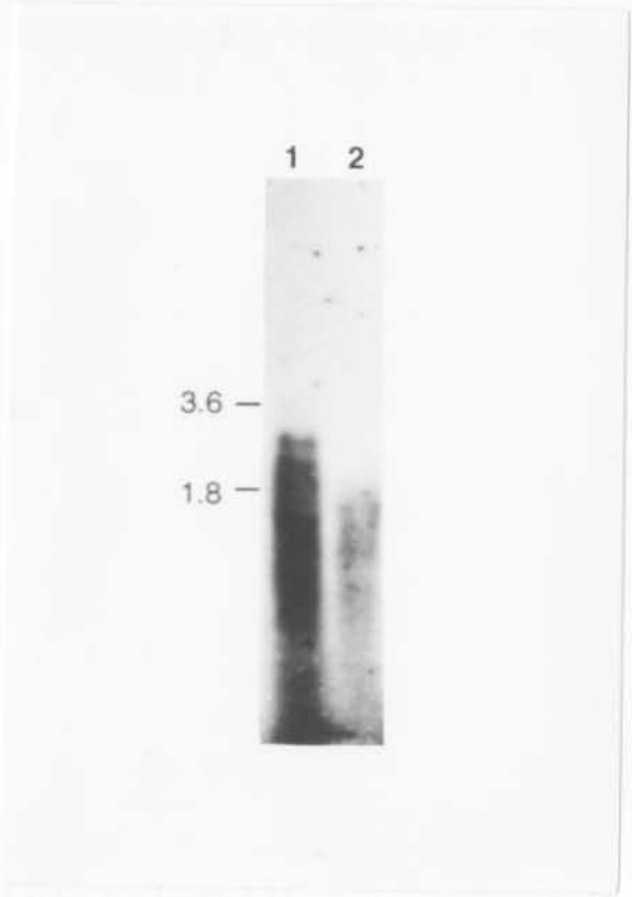


Figure 3.18. Northern analysis of *exeAB* mRNA. A northern blot of Ah65 (Lane 1) and C5.84 (Lane 2) mRNA was prepared and hybridized with an *exeAB* RNA probe. Equal amounts of mRNA were applied to each lane. The position of the 23S and 16S ribosomal RNA bands is shown on the left.

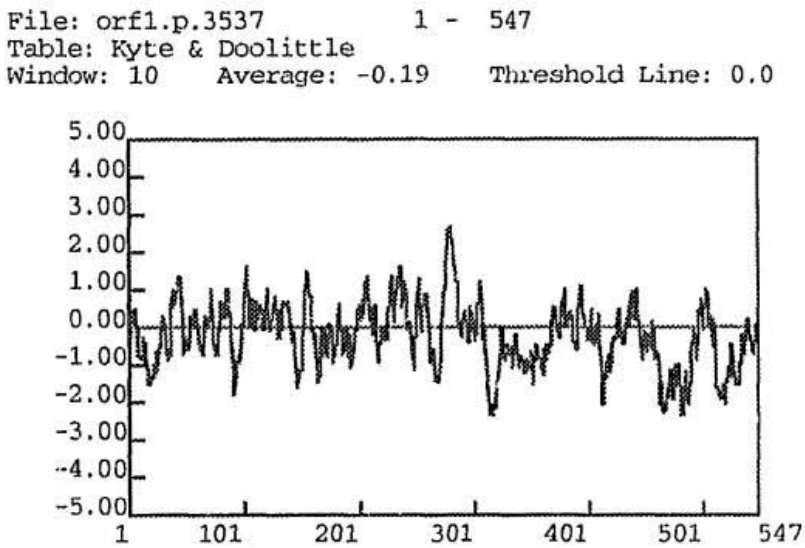


Figure 3.19. Hydropathy profile of ExeA by the method of Kyte and Dolittle. A highly hydrophobic segment is located between the residues 276 and 295.

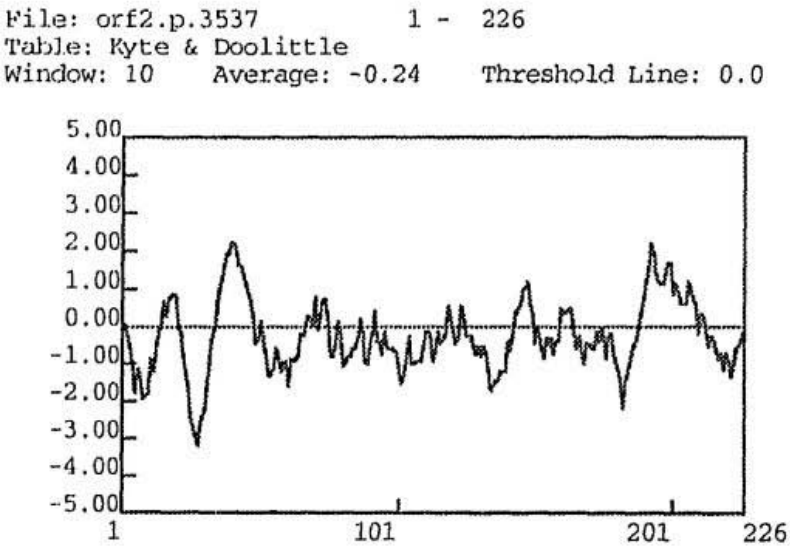


Figure 3.20. Hydropathy profile of ExeB by the method of Kyte and Doolittle. Two hydrophobic segments are located at residues 37-53 and 193-218.

A consensus nucleotide binding site [G] X (4) GK [T] (Walker *et al.*, 1982) was revealed at residues 50-57 (Fig. 3.21), when a search of the EMBO protein sequence motif database was done using the prosite algorithm of Fuchs (1991). The average hydrophobicity for the product of *exeB* was -2.4; it does not have an obvious signal sequence either, and it is quite basic, with a calculated isoelectric point of 9.58. It contains a highly hydrophobic stretch of 17 amino acids between residues 37 and 53, and another region between residues 193 and 218 which is quite hydrophobic (but contains an Asp as residue 205) as shown in Figure 3.22. The presence of the ATP-binding site in ExeA and the hydrophilic profile of both ExeA and ExeB suggested that these proteins would be either cytoplasmic or at best anchored to the inner membrane by a single hydrophobic hairpin.

3.11 Expression and localization of ExeA and ExeB

As a first attempt to determine the localization of ExeA and ExeB, both *exeA* and *exeB* were cloned into a pET expression vector as described by Studier *et al.* (1990). In this system, target genes are placed under the control of strong bacteriophage T7 promoter, and a chromosomal copy of the gene for the T7 RNA polymerase is then induced with IPTG (Studier *et al.*, 1990). *E. coli* BL21(DE3) cells containing pERJ63.1 (Fig. 3.1) and pET9 as a vector control were grown in M9 methionine assay medium and pulse labeled with [^{35}S]-methionine.

```
1   M Y T Q F F G L S E P P F S I S P N P K Y L Y M S E R H G E   30
31  A L A H L N Y G L Q D G G G F V L L T G E V G T G K T T V S   60
61  R C L L Q Q L P T E T E I A Y I L N P S L T E R D L L A A I   90
91  C D E F Q L P Y D K D A G L K L L F D L I R D H L L A N L A  120
121 A G K R S V V L V D E A Q H L L P G V L E Q L R L L T N L E  150
151 T D E K K L L Q V V L I G Q P E L Q Q M L R Q P L L R Q L A  180
181 Q R I T A R Y H L L P L S H Q D V D A Y V R F R L Q V A G C  210
211 V Q P I F T P K A L Q T L H R L S G G I P R L I N L I C D R  240
241 A L I A A F A R G S H K I V H G D I S L A A Y E V S G I R D  270
271 E G T W Q S G L M V A L A G A L L V A T G W W G W Q F F G F  300
301 F P E R P V I K V E V P V K V D D T P E Q Q E Q L T R A I N  330
331 Q A L E P D S A M Q N L Y K V W G Y Q T E L E E A T C D N A  360
361 P R A G L R C Q E G D A S L A E L Q A L Q H P A L I S L T D  390
391 E T G G I Y Y A T L V N L G P D K A N L L I G N Q S W Q V D  420
421 R Q W L S D F W G G S Y T L L W R M P K G G V A L I G N N A  450
451 G A T Q V Q W L D N A L S R A L Q Q P D R K V R R F D A E L  480
481 K N K L Q Q F Q R E Q G L N P D G I A G S N T L L R L N V M  510
511 A G E P M P K L E D E S Q R A S T P A T P D T M N D E P M V  540
541 T L S E E A S----------------------------------------------  570
```

Consensus ATP-binding site

A/G X X X X G K S/T

Figure 3.21. Deduced amino acid sequence of ExeA. The putative ATP-binding site in *exeA* between residues 50 and 57 is underlined and the consensus ATP-binding site (Walker *et al.*, 1982) is shown. The hydrophobic segment located at residues 275-296 is doubly underlined.

```
1    M S T L L K A L R R A E Q P Q F T P H I P A M G L P V T Q E   30
31   E E Q N R R W I W W L L A P L A L L M G A G A N Y G W H L L   60
61   N N R P I E K T V E V K E V V T P P F V R V E P R P M I T R   90
91   P L P P P L P E P V V R P R V T P N D S A P A A N G S Q G L  120
121  A E R I M N A L N S T P L M E E T A P Q A Q S E S Q A M P I  150
151  S A L P L E L K Q R V P P L A Y G S H V F S S N P A K R A V  180
181  M L N G R E F R E G S E V A P G V T L I A I A Q D Y I I L Q  210
211  V A G Q N V S L K A L Q D W R G---------------------------  240
```

Figure 3.22. Deduced amino acid sequence of ExeB. The hydrophobic segments located at residues 37-53 and 193-218 are doubly underlined.

Proteins corresponding to both ExeA and ExeB were produced in the *E. coli* BL21(DE3)(pERJ63.1) cells following induction, and each of the proteins appeared to be present in both cytoplasmic and cell envelope fractions after ultra-centrifugation of cell lysates (Fig. 3.23, lanes 3 and 5). When the envelope fraction was extracted using sodium lauroyl sarcosinate to solubilize the inner membrane, both proteins were found in the solubilized (inner membrane) fraction (Fig. 3.23, lane 7). These cells were also grown in LB medium and induced with IPTG for 3 hours and visualized on Coomassie blue stained gels for analysis of proteins produced following induction. Again each of the proteins was easily observed, and in this case essentially all of each of the two proteins fractionated with the cell envelopes (Fig. 3.24, lane 3). Both proteins were also completely extracted when 0.5% SLS was used to solubilize the inner membrane of envelopes isolated from cell extracts of these cultures (Fig. 3.24, lane 4).

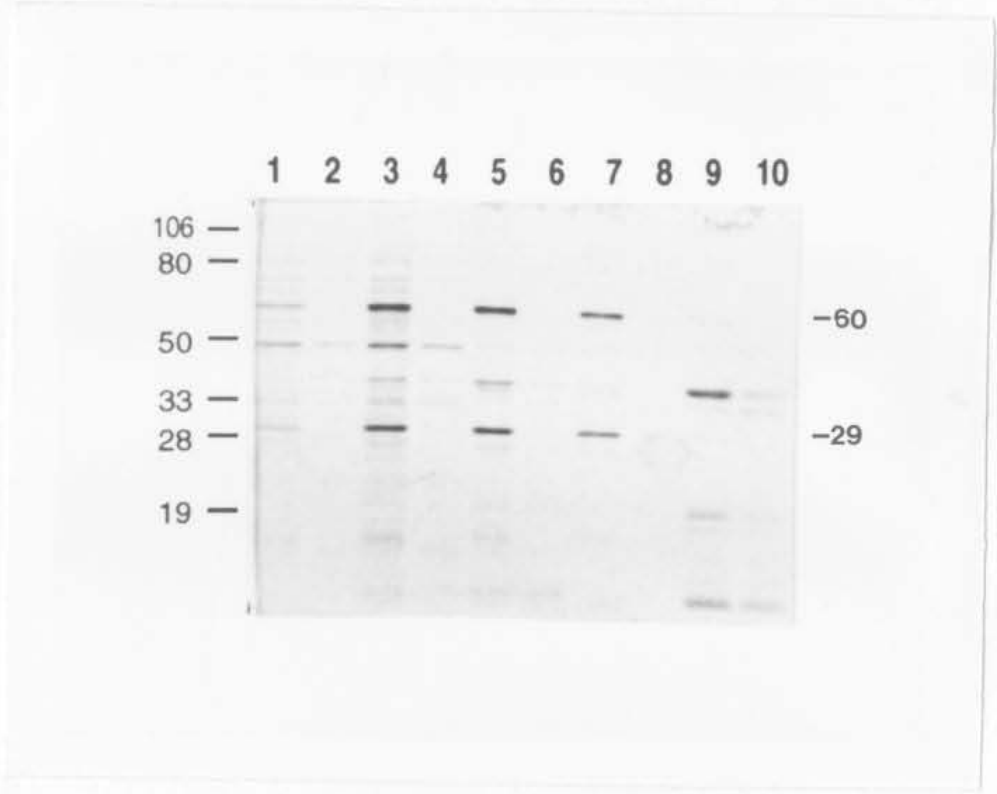


FIG. 3.23. Autoradiograph of membrane fractions of [^{35}S]-methionine labeled *E. coli* BL21(DE3) cells containing pERJ63.1 and pET9. *E. coli* BL21(DE3) cells containing pERJ63.1 (lanes 1, 3, 5, 7, and 9) and pET9 (lanes 2, 4, 6, 8, and 10) were induced as described in Materials and Methods, and labeled with 100 μCi of [^{35}S]-methionine per ml for 5 minutes. The 60 kDa and 29 kDa of ExeA and ExeB proteins respectively are given on the right and the position of molecular mass standards are given on the left. Lanes 1-2, lysate; Lanes 3-4, cytoplasm; Lanes 5-6, envelope; Lanes 7-8, inner membrane; Lanes 9-10, outer membrane.

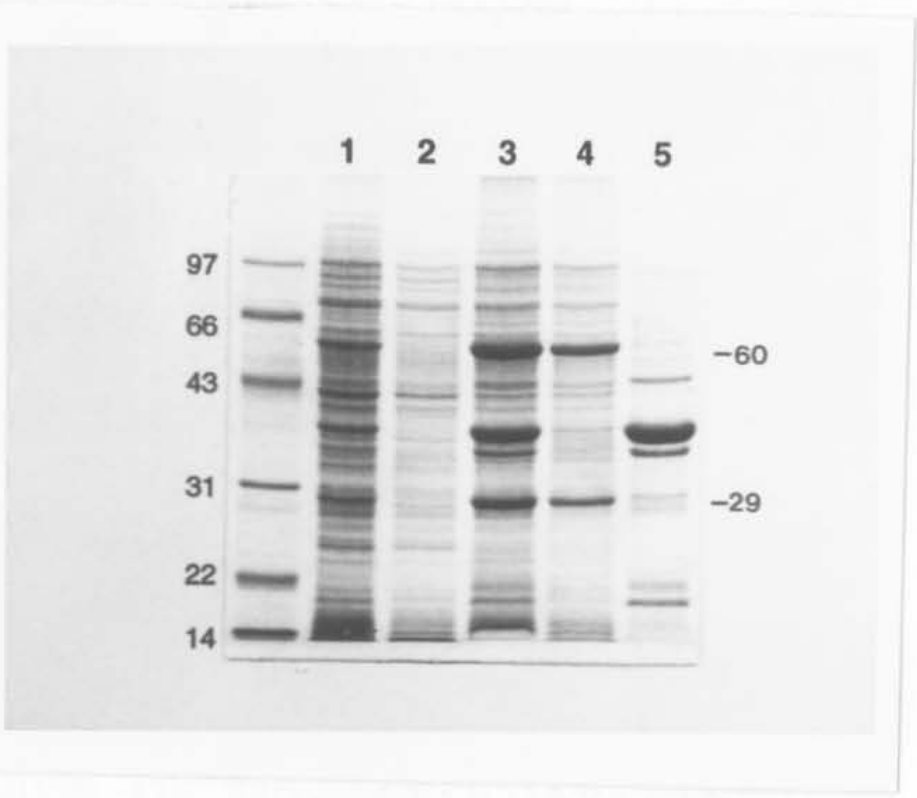


Figure 3.24. Coomassie blue stained SDS-PAGE gel of fractions of *E. coli* BL21(DE3) cells containing pERJ63.1. Lane 1, whole cells; Lane 2, cytoplasm; Lane 3, envelopes; Lane 4, inner membrane; Lane 5, outer membrane. The prominent bands are the 60 kDa and 29 kDa of ExeA and ExeB proteins respectively and are included on the right. Equal amounts of the fractionated culture were applied to each lane. The position of molecular mass standards are given on the left.

Chapter 4

DISCUSSION

C5.84 is an extracellular secretion-deficient Tn5-751 insertion mutant which accumulates exoproteins in the periplasmic space (Jiang and Howard, 1991). In this study, a 3.5 kb *Bgl* II chromosomal fragment complementing this mutation was isolated, sequenced and analyzed. Complementation and subcloning studies, analysis of *in vitro* and *in vivo* expression, and marker exchange mutagenesis, indicate that this locus consists of an operon composed of two genes, each of which is required for extracellular protein secretion.

4.1 Extracellular protein export in *Aeromonas hydrophila* appears to follow the general secretion pathway (GSP), an extension of the Sec pathway

A. hydrophila, like many other Gram-negative bacteria, secretes a wide range of extracellular proteins. These exoproteins are translocated across the outer membrane in a two step, signal peptide dependent pathway, as demonstrated for aerolysin (Howard and Buckley, 1983; 1985a). Aerolysin and amylase were found to be secreted as precursors containing an N-terminal signal sequence, which is cleaved by the signal peptidase after translocation across the inner membrane (Howard and Buckley, 1986; Gobius and Pemberton, 1988). These results indicate that at least until the transit across the inner membrane is completed, these proteins follow a pathway similar or identical to the Sec

pathway of *E. coli* (Schatz and Beckwith, 1990). The information present in the signal sequence is not sufficient, however, for release of the proteins from the cell; additional secretory factors are required for translocation across the outer membrane. It is now evident that the second step requires the products of a large operon, the prototype of which is the *pul* operon of *K. oxytoca* (Pugsley *et al*, 1990).

The transposon insertion mutants L1.97 and C5.84 were both isolated as mutants defective in protein secretion, and it was shown by Southern hybridization that the transposon had inserted into different locations in the two strains (Jiang and Howard, 1991). The locus identified by the mutation in L1.97 contains 12 genes, forming the *exeC-N* operon, which was shown to be homologous to the *pul* operon of *K. oxytoca* (Jiang and Howard, 1992; Howard *et al*, 1993). Until this study however, there had been no further information about the locus identified by C5.84 or its relationship with other identified extracellular secretion genes.

4.2 *exeA* and *exeB* form an operon and both are required for extracellular protein secretion

As revealed by restriction mapping, the transposon had inserted close to the *BstX*I site in *exeA* of C5.84. The provision of *exeA* alone did not complement the mutation, however, nor did *exeB* alone. Only when both *exeA* and *exeB* were supplied together was the secretory phenotype restored to C5.84. Marker exchange mutagenesis of *exeA* and *exeB* confirmed the requirement for both of the genes for extracellular protein secretion in *A. hydrophila*.

The requirement for both genes for the complementation of C5.84 not only indicated that both were required for secretion, but also that the insertion of Tn5-751 into *exeA* was causing a polar effect on the expression of *exeB*. In addition, the immunoblots of C5.84 and the marker exchange mutants AA41 and AB69 with anti-ExeB serum (Fig. 3.15) demonstrated the failure of all of these mutants to produce the 29 kDa ExeB protein. The ExeB protein should have been expressed in both C5.84 and the *exeA* marker exchange mutant AA41, unless *exeB* is coexpressed with *exeA*. The overlap between the start codon of *exeB* and the stop codon of *exeA*, the presence of a 2.5 kb *exe* specific message as demonstrated by northern analysis and the demonstration that there is a promoter upstream of *exeA* (see below) all provide further evidence that *exeAB* form an operon.

4.3 A promoter functional in both *Escherichia coli* and *Aeromonas hydrophila* is present between the *Dra*III and *Bst*XI sites of the sequenced fragment

The fact that the 3.5 kb *Bgl* II fragment was able to complement C5.84 when it was cloned in both orientations in the wide host range vector pMMB207 and pMMB208 demonstrated that the *exe* genes were under the control of their own promoter on this fragment. This ability to complement independently of the vector promoter was also demonstrated for pKRJ58.1 and pSRJ57.1, containing the *Dra*III-*Kpn*I fragment. For the *Bst*XI-*Bst*XI fragment, however, complementation was only observed when it was cloned in the orientation of pKRJ58.1, and not in the opposite orientation, as in pSRJ57.1. This demonstrated that in *A. hydrophila*, a promoter for the complementing genes is

present in the 300 bases between the *Dra*III and the *Bst*XI site immediately upstream of the *exeA* gene (see Fig. 3.16). This same promoter also seems to be functional in *E. coli*, since the *Dra* III-*Kpn* I fragment also produced ExeA and ExeB in the *in vitro* transcription-translation reactions no matter what its orientation with respect to the vector promoter (see Fig. 3.8, lane 3 and 4), whereas the *Bst*XI-*Bst*XI fragment produced the proteins only when the *exe* genes were oriented in the same direction as the promoter present on the vector (see Fig. 3.8, lane 5 and 6). The analysis of the sequenced 3.5 kb *Bgl* II fragment revealed a putative -10 region promoter sequence which has the hexamer TAACCT and a putative -35 region promoter sequence with the hexamer TTGACA, 5' to *exeA* (Fig. 3.16). The sequence of this putative promoter is therefore in perfect agreement with the consensus *E. coli* -35 region TTGACA, whereas the -10 sequence shows homology with only 3 of the 6 nucleotides of the consensus *E. coli* -10 sequence TATAAT. It should also be noted, however, that the 3 homologous nucleotides are in fact the most conserved bases of the consensus *E. coli* -10 sequence (Hawley and McClure, 1983; Lisser and Margalit, 1993). The putative -35 and -10 promoter regions are separated by 17 nucleotides and therefore conform with the spacing of 17 +/- 1 nucleotides found in the consensus *E. coli* promoter (McClure, 1985).

4.4 Localization of ExeA and ExeB

The protein encoded by *exe*A is hydrophilic and does not have any obvious signal sequences, but does contain a single hydrophobic stretch which might possibly anchor the protein to the cytoplasmic membrane. The protein

encoded by *exeB* also lacks signal sequences and is hydrophilic, but it contains two hydrophobic segments which might span the inner membrane. The results of the T7 overexpression experiments indicated that both ExeA and ExeB were localized to the cytoplasmic membrane (Fig. 3.23 and 3.24). These results must be viewed cautiously however, since they were obtained in the heterologous host *E. coli* and their overproduction in this system might lead to aberrant localization. On the basis of its structure, PulB was predicted to be cytoplasmic (Pugsley, *et al.*, 1990a), however, localization studies for either PulB or OutB have not been reported. The amino terminal region of ExeB, which is not present in PulB and OutB (see Fig. 3.6), contains the most hydrophobic of the possible transmembrane regions. This fact, in addition to the apparent differences in function of these three proteins (see below) makes it quite possible that these proteins may not even be found in the same compartment of their respective cells.

4.5 Substantial divergence exists between ExeB of *Aeromonas hydrophila* and PulB of *Klebsiella oxytoca* and OutB of *Erwinia chrysanthemi*

ExeB has limited homology with OutB (Condemine *et al.*, 1992) and PulB (d'Enfert and Pugsley, 1989), two proteins which have been reported to play little or no role in secretion in *Er. chrysanthemi* and *K. oxytoca*. *pulB* mutants had no effect on the export of pullulanase. *pulB* is a component of the *K. oxytoca* maltose regulon and is co-transcribed with the pullulanase structural gene *pulA* but is neither required for *pullulanase* secretion nor its synthesis (d'Enfert and Pugsley, 1989). The function of *pulB* is not known. *outB* is one of the genes of

the *out* gene cluster involved in the translocation of pectate lyases and cellulases across the outer membrane of *Er. chrysanthemi*. *outB* mutants secreted 70% of the normal amount of pectinases produced by wild type *Er. chrysanthemi*. *outB* is transcribed independently of the other *out* genes, however, and is not part of the *kdgR* regulon which controls their expression (Condemine *et al.*, 1992).

The non secretory phenotype of the original transposon insertion mutant C5.84 (Jiang and Howard, 1991) and that of the marker exchange mutants produced here demonstrate that both ExeA and ExeB are absolutely required for extracellular secretion in *A. hydrophila*. This suggests a substantial divergence in the function of this gene product between *A. hydrophila* and the other two bacteria. This divergence in function may account for the low levels of homology observed between these proteins (19.5% between ExeB and PulB and 19.7% between ExeB and OutB; see Fig. 3.6) and also for the considerable variation in the size of these proteins, with PulB of 174 amino acids and OutB of 157 amino acids compared to 226 for ExeB. Another difference is the association of *exeB* in an operon with *exeA*. Sequence information for the regions adjacent to *pulB* and *outB* has been reported, and indicates that ExeA homologues are not present, at least in an operon with the ExeB homologues (d'Enfert and Pugsley, 1989; Condemine *et al.*, 1992).

4.6 Extracellular protein secretion in *Aeromonas hydrophila* requires a second protein which may bind and hydrolyze ATP

Although it is clear that the *exeC-N* operon and operons homologous to it in other Gram-negative bacteria are involved in extracellular secretion, it is not

clear how they function. In particular, as of yet there is no specific information regarding the source of energy for the transfer of the exoproteins across the outer membrane, despite the evidence that a proton motive force is required for protein translocation across the outer membrane of *A. salmonicida* (Wong and Buckley, 1989). PulE, the homologue of ExeE, is the most hydrophilic of the *pul* gene products, and contains an ATP-binding site. Recently it was demonstrated to be essential for protein secretion (Possot and Pugsley, 1994; Turner *et al.*, 1993). ATPase and autophosphorylation activities were also demonstrated for the *A. tumefaciens* conjugation factor VirB-11 (Christie *et al.*, 1991), which is homologous to ExeE. However, on the basis of the homologies between type IV pilins and some of the proteins of the *pulC-O* operon, and between PulE and PilB as discussed in the introduction, it is now generally assumed that PulE and its homologues are involved in the assembly of the secretion apparatus rather than its continuing function. One possibility is therefore that ExeA, which also contains a consensus ATP-binding site, provides the continuing source of energy for extracellular export, through the hydrolysis of ATP. Another possibility is that ExeA may maintain the exoproteins in a translocation competent state and derive the energy for this by binding to and hydrolyzing ATP. The absence of recognizable signal sequences in the ExeA and ExeB proteins, their largely hydrophilic character, and the presence of the consensus ATP-binding site in ExeA all suggest that the proteins function on the cytoplasmic side of the inner membrane. It is therefore possible that the recognition step for the extracellular export of exotoxins begins in the cytoplasm.

4.7 In the *exeAB* mutants, the ExeC-N apparatus is most likely assembled but not functional

Although the evidence shown here demonstrates that both *exeA* and *exeB* are required for extracellular secretion, little is known about their role in this process. Two possibilities compatible with phosphorylation events that may be catalyzed by ExeA are; (1) involvement of these proteins in the regulation of other *exe(C-N)* genes that make up the terminal segments of the general secretion pathway, and (2) direct involvement of these proteins in the secretion process. Indeed, the fact that a 11 kb fragment which complements the mutation in L1.97 strain also partially complemented the mutation in C5.84, suggested the two were possibly related in a regulatory manner (Jiang and Howard, 1991). One can argue against this first possibility based on the observations that the mutations in the two *exe* operons have very distinct phenotypes. Strain L1.97, which was obtained by transposon insertion into *exeE*, and three marker exchange mutants in *exeC*, *exeE* and *exeK*, all resulted in an outer membrane with a reduction in the major porins, and in addition rendered the cells osmotically fragile (Jiang and Howard, 1991; Howard *et al.*, 1993). In contrast, the Tn5-751 insertion mutant into *exeA*, or the marker exchange mutants of *exeA* and *exeB* created in this study, had no effect on the outer membrane protein composition nor rendered the cells osmotically fragile. This makes it unlikely that the mutations in the *exeAB* genes are in fact simply preventing the expression of the *exeC-N* operon. With respect to a direct role for ExeA and ExeB in secretion, the differences in the mutant phenotypes would also suggest that these proteins may not be involved in the assembly of the ExeC-N secretion apparatus, since if this was true, the membrane effects typical of *exeC-N* mutations would be expected.

It is therefore most likely that in the *exeAB* mutants, the ExeC-N apparatus is assembled but not functional, either because the exoproteins cannot move through the apparatus, or because they do not reach it in the absence of ExeAB. Since, as discussed above, ExeA and B probably function in the cytoplasmic compartment, an attractive hypothesis would be that these proteins guide exoproteins to functional extracellular secretion ports in the envelope.

4.8 Future research

Future research goals should include; (1) localization of both ExeA and ExeB within the cell by producing antibodies and immunoblotting fractionated cells, (2) investigation of the requirement for the putative ATP-binding site of ExeA in protein translocation by site specific mutagenesis of the ATP-binding site, followed by incorporation of the mutations into the wild type by homologous recombination, (3) study the individual functions of these genes by using a system that does not show polarity effects, such as inframe deletions, and (4) examination of the interaction between these two proteins and between these and exoproteins such as aerolysin or the previously identified *exeC-N* gene products. These experiments would include cross linking and co-stabilization studies, and further mutant isolation.

Chapter 5

LITERATURE CITED

Akrim, M., Bally, M., Ball, G., Tommassen, J., Teerink, H., Filloux, A., and Lazdunski, A. (1993). Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of *xcp* gene expression. Mol. Microbiol. 10, 431-443.

Albano, M., Breitling, R., and Dubnau, D. A. (1989). Nucleotide sequence and genetic organization of the *Bacillus subtilis comG* operon. J. Bacteriol. 171, 5386-5404.

Allaoui, A. P., Sansonetti, P., and Parsot, C. (1993). MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasins. Mol. Microbiol. 7, 59-68.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Bachmann, B. J. (1990). Linkage map of *Escherichia coli* K-12. Edition 8. Microbiol. Rev. 54, 103-197.

Bally, M., Wretlind, B., and Lazdunski, A. (1989). Protein secretion in *Pseudomonas aeruginosa*: Molecular cloning and characterization of the *xcp*-1 gene. J. Bacteriol. 171, 4342-4348.

Bally, M., Ball, G., Badere, A., and Lazdunski, A. (1991). Protein secretion in *Pseudomonas aeruginosa*: the *xcp*A gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function protein PulO. J. Bacteriol. 173, 479-486.

Bally, M., Filloux, A., Akrim, M., Ball, M., Lazdunski, A., and Tommassen, J. (1992). Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. 6, 1121-1131.

Bailey, M. J. A., Koronakis, V., Schmol, T., and Hughes, C. (1992). *Escherichia coli* HlyT protein, a transcriptional activator of hemolysin synthesis and secretion, is encoded by the *rfaH* (*sfrB*) locus required for expression of sex factor and lipopolysaccharides genes. Mol. Microbiol. 6, 1003-1012.

Bergey's Manual of Systematic Bacteriology (1984). Williams and Wilkins,

Baltimore. MD. USA.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513-1523.

Bitler, W., Marugg, J. D., deWeger, L. A., Tommassen, J., and Weisbeek, P. J. (1991). The ferric pseudobactin receptor PupA of *Pseudomonas putida* WCS358: homology to TonB dependent *E. coli* receptors and specificity of the protein. Mol. Microbiol. 5, 647-655.

Blight, M. A., and Holland, I. B. (1990). Structure and function of hemolysin B, P-glycoprotein and other members of a novel family of membrane translocators. Mol. Microbiol. 4, 873-880.

Boulanger, Y., Lallier, R., and Cousineau, G. (1977). Isolation of enterotoxigenic *Aeromonas* from fish. Can. J. Microbiol. 23, 1161-1164.

Bradley, D. E. (1980). A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. Can. J. Microbiol. 26, 146-154.

Buckley, J. T. (1992). Crossing three membranes, channel formation by aerolysin. FEBS. 307, 30-33.

Chakraborty, T., Montenegro, M. A., Sanyal, S. C., Helmuth, R., Bulling, E., and Timmis, K. N. (1984). Cloning of enterotoxin gene from *Aeromonas hydrophila* provides conclusive evidence of a cytotoxic enterotoxin. Infect. Immun. 46, 435-441.

Chakraborty, T., Huhle, B., Bergbauer, H., and Goebel, W. (1986). Cloning, expression and mapping of *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. J. Bacteriol. 167, 363-373.

Chakraborty, T., Huhle, B., Hof, H., Bergbauer, H., and Goebel, W. (1987). Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*-associated systemic infections. Infect. Immun. 55, 2274-2280.

Chapon, C., and Raibaud, O. (1985). Structure of two divergent promoters located infront of the gene encoding pullulanase in *Klebsiella pneumonaie*. J. Bacteriol. 164, 639-645.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA 81, 1991-1995.

Condemine, G., Dorel, C., Hugouvieux-Cotte-Patta, N., and Robert-Baudouy, J. (1992). Some of the *out* genes involved in the secretion of pectate lyases in

Erwinia chrysanthemi are regulated by *kdgR*. Mol. Microbiol. 6, 3199-3211.

Cudny, H., Lupski, J. R., Godson, G. N., Deutscher, M. P. (1984). Cloning, sequencing, and species relatedness of the *Escherichia coli cca* gene encoding the enzyme tRNA nucleotidyl-transferase. J. Biol. Chem. 261, 6444-6449.

Darveau, R. P., MacIntyre, S., Buckley, J. T., and Hancock, R. (1988). Purification and reconstitution in lipid bilayer membranes of an outer membrane, pore-forming protein of *Aeromonas salmonicida*. J. Bacteriol. 156, 1006-1011.

Delepelaire, P., and Wandersman, C. (1990). Protein secretion in gram negative bacteria: The extracellular metalloprotease B. from *Erwinia chrysanthemi* contains a C-terminal secretion signal analogous to that of *Escherichia coli* α-hemolysin. J. Biol. Chem. 265, 17118-17125.

d'Enfert, C., Chapon, C., and Pugsley, A. P. (1987a). Export and secretion of the lipoprotein pullulanase by *Klebsiella pneumonaie*. Mol. Microbiol. 1, 107-116.

d'Enfert, C., Reyter, A., and Pugsley, A. P. (1987b). Cloning and expression in *E. coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. EMBO J. 6, 3531-3538.

d'Enfert, C., and Pugsley, A. P. (1989). *Klebsiella pneumoniae pul*S gene encodes an outer membrane lipoprotein required for pullullanase secretion. J. Bacteriol. 171, 3673-3679.

d'Enfert, C., Reyss, I., Wandersman, C., and Pugsley, A. P. (1989). Protein secretion by gram negative bacteria. Characterization of two membrane proteins required for pullulanase secretion by *E. coli* K12. J. Biol. Chem. 264, 17462-17468.

Dums, F., Dow, J. M., and Daniels, M. J. (1991). Structural characterization of protein secretion genes of the bacterial phytopathogen *Xanthomonas campestris* pathovar *campestris*: relatedness to secretion systems of other gram negative bacteria. Mol. Gen. Genet. 229, 357-364.

Dupuy, B., Taha, M. K., Passot, O., Marchal, C., and Pugsley, A. P. (1992). PulO, a component of the pullulanase secretion pathway of *Klebsiella*

oxytoca, correctly and efficiently processes gonococcol type IV prepilin in *E. coli*. Mol. Microbiol. 6, 1887-1894.

Economou, A., Hamilton,W. D. O., Johnston, A. W. B., and Downie, J. A. (1990). The *Rhizobium* nodulation gene *nodO* encodes a Ca^{2+}-binding protein that is exported without N-terminal cleavage and is homologous to hemolysin and related proteins. EMBO J. 9, 349-354.

Engler-Blum, G., Meier, M., Frank, J., and Muller, G. A. (1993). Reduction of background problems in nonradioactive northern and southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. Anal. Biochem. 210, 235-244.

Felmlee, T., Pellet, S., Lee, E-Y., and Welch, R. A. (1985a). *Escherichia coli* hemolysin is released extracellular without cleavage of a signal peptide. J. Bacteriol. 163, 88-93.

Felmlee, T., Pellet, S., Lee, E-Y., and Welch, R. A. (1985b). Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. 163, 94-105.

Figurski, D. H., and Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 79, 1648-1652.

Filip, C., Fletcher, G., Wulff, J. L., and Earhart, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauroyl sarcosinate. J. Bacteriol. 115, 717-722.

Filloux, A., Bally, M., Murgier, M., Wretlind, B., Lazdunski, A. (1989). Cloning of *xcp* genes located at the 55 min region of the chromosome and involved in protein secretion in *Pseudomonas aeruginosa*. Mol. Microbiol. 3, 261-265.

Filloux, A., Bally, M., Ball, G., Akrim, M., Tommassen, J., and Lazdunski, A. (1990). Protein secretion in gram negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. EMBO J. 9, 4323-4329.

Fuchs, R. (1991). A protein pattern database. Trends Biochem. Sci. 16, 290.

Fukasawa, T., Jokura, K., and Kurahashi, K. (1963). Mutations in *Escherichia coli* that affect uridine diphosphate glucose pyrophosphorylase activity and galactose fermentation. Biochem Biophiys Acta. 74, 608-620.

Garrido, M. C., Herrero, M., Kolter, R., and Moreno, F. (1988). The export of

DNA replication inhibitor Microcin B17 provides immunity for the host cell. EMBO J. 7, 1853-1862.

Ghigo, J. M., and Wandersman, C. (1992). Cloning, nucleotide sequence and characterization of the gene encoding the *Erwinia chrysanthemi* B374 PrtA metalloprotease secreted via a C-terminal secretion signal. Mol. Gen. Genet. 236, 135-144.

Gilson, L., Mahanty, H. K., and Kolter, R. (1990). Genetic analysis of an MDR-like export system: the secretion of colicin V. EMBO J. 9, 3875-3884.

Glaser, P., Sakamato, H., Ullmann, A., and Danchun, A. (1988). Secretion of cyclolysin, the calmodulin sensitive adenylate cyclase-hemolysin bifunctional protein of *Bordetella pertussis*. EMBO J. 7, 3997-4004.

Gobius, K. S., and Pemberton, J. M. (1988). Molecular cloning, characterization and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. J. Bacteriol. 170, 1325-1330.

Graevenitz, V. A., and Mensch, A. H. (1968). The genus *Aeromonas* in human bacteriology. Report of 30 cases and review of the literature. N. Engl. J. Med. 278, 245-249.

Gray, L., Mackman, N., Nicaud, J. N., and Holland, I. B. (1986). The carboxy terminal region of hemolysin 2001 is required for secretion of the toxin from *E.coli*. Mol. Gen. Genet. 205, 127-133.

Groot, A., Filloux, A., Tommassen, J. (1991). Conservation of *xcp* genes, involved in the two-step protein secretion process, in different *Pseudonomas* species and other gram negative bacteria. Mol. Gen. Genet. 229, 278-284.

Grundy, F. J., Plaut, A., and Wright, A. (1987). *Haemophilus infuenzae* immunoglobulin A1 protease genes: cloning by plasmid integration-excision, comparative analyses, and localization of secretion determinants. J. Bacteriol. 169, 4442-4450.

Gurpreet, S. S., Precup, J. W., and Kline, B. C. (1989). Rapid one step characterization of recombinant vectors by direct analysis of transformed *Escherichia coli* colonies. BioTechniques 7, 689-690.

Guzzo, J., Pages, J. M., Duong, F., Lazdunski, A., and Murgier, M. (1991a). *Pseudomonas aeruginosa* alkaline protease: evidence for secretion genes and study of secretion mechanism. J. Bacteriol. 173, 5290-5297.

Guzzo, J., Duong, F., Wandersman, C., Murgier, M., and Lazdunski, A. (1991b). The secretion genes of *Pseudomonas aeruginosa* alkaline protease are functionaly similar to those of *Erwinia chrysanthemi* proteases and *E.coli* α-haemolysin. Mol. Microbiol. 5, 447-453.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557-580.

Hawley, D. K., and McClure, W. R. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. 11, 2237-2255.

He, S. Y., Lindberg, M., Chatterjee, A. K., and Collmer, A. (1991). Cloned *Erwinia chrysanthemi out* genes enable *E. coli* to selectively secrete a diverse family of heterologous proteins to it's milieu. Proc. Natl. Acad. Sci. USA 88, 1079-1083.

Henikoff, S. (1987). Construction of unidirectional deletions using exonuclease III. Methods Enzymol. 155, 156-165.

Higgins, C. F., Hiles, I. D., Salmond, G. P., Gill, D. R., Downie, J. A., Evans, I. J., Holland I. B., Gray, L., Buckel, S. D., Bell, A. W., and Hermodson, M. A. (1986). A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature 323, 448-450.

Hirst. T. R., Sanchez, J., Kaper, J. B., Hardy, S. J. S., and Holmgren, J. (1984). Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harbouring plasmids that encode heat-labile enterotoxins of *Escherichia coli.* Proc. Natl. Acad. Sci. USA 81, 7752-7756.

Hirst, T. R., and Holmgren, J. (1987). Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from *Vibrio cholerae.* J. Bacteriol. 169, 1037-1045.

Hirst, T. R., and Welch, R. A. (1988). Mechanisms for secretion of extracellular proteins by gram-negative bacteria. Trends Biochem. Sci. 13, 265-269..

Hobbs, M., and Mattick, J. S. (1993). Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein secretion apparatus: general system for the formation of surface-associated protein complexes. Mol. Microbiol. 10, 233-243.

Holland, I. B., Kenny, B., and Blight, M. (1990). Hemolysin secretion from *E. coli.* Biochim. 72, 131-141.

Holtke, H. J., Sagner, G., Kessler, C., and Schmitz, G. (1992). Sensitive chemiluminescent detection of digoxigenin-labeled nucleic acids: a fast and simple protocol and its applications. Biotechniques. 12, 104-113.

Howard, S. P., and Buckley, J. T. (1982). Membrane glycoprotein receptor and hole forming properties of a cytolytic protein toxin. 21, 1662-1667.

Howard, S. P., and Buckley, J. T. (1983). Intracellular accumulation of extracellular proteins by pleiotropic export mutants of *Aeromonas hydrophila*. J. Bacteriol. 154, 413-418.

Howard, S. P., and Buckley, J. T. (1985 a). Activation of the hole forming toxin aerolysin by extracellular processing. J. Bacteriol. 163, 336-340.

Howard, S. P., and Buckley, J. T. (1985 b). Protein export by gram negative bacterium: Production of aerolysin by *Aeromonas hydrophila*. J. Bacteriol. 161, 1118-1124.

Howard, S. P., and Buckley, J. T. (1986). Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila*. Mol. Gen. Genet. 204, 289-295.

Howard, S. P., Critch, J., and Bedi, A. (1993). Isolation and analysis of *Aeromonas hydrophila* genes which are required for extracellular secretion. J. Bacteriol. 173, 1241-1249.

Hu, Nein-Tai., Hung, Mung-Ni., Chiou, Shu-Jiaw., Tang, F. Chaing, Dong-ching., Huang, Hui-Yuan., and Wu, Chen-Yeu. (1992). Cloning and characterization of a gene required for the secretion of extracellular enzymes across the outer membrane by *Xanthomonas campestris* pv *Campestris*. J. Bacteriol. 174, 2679-2687.

Hultgren, S. J., and Normark, S. (1991). Chaperone assisted assembly and molecular architecture of adhesive pili. Annu. Rev. Microbiol. 45, 383-415.

Ichige, A., Oishi, K., and Mizushima, S. (1988). Isolation and characterization of mutants of a marine *Vibrio* strain that are defective in the production of extracellular proteins. J. Bacteriol. 170, 3537-3542.

Isabelle, B.-G., Brun, E., Py, B., Chippaux and Barras, F. (1994). Periplasmic disulphide formation is essential for cellulase by the plant pathogen *Erwinia crysanthemi*. Mol. Microbiol. 11, 545-553.

Issartel, J. P., Koronakis, V., and Hughes, C. (1991). Activation of *E. coli*

prohemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. Nature 351, 759-761.

Ji, J., Hugovieux, Cotte-Pattat, N., and Robert-Baudouy, J. (1989). Molecular cloning of the *outJ* gene invoved in pectate lyase by *Erwinia chrysanthemi*. Mol. Microbiol. 3, 285-293.

Jiang, B., and Howard, S. P. (1991). Mutagenesis and isolation of *Aeromonas hydrophila* genes which are required for extracellular secretion. J. Bacteriol. 173, 1241-1249.

Jiang, B., and Howard, S. P. (1992). The *aeromonas hydrophila exeE* gene, required both for protein secretion and normal outer membrane biogenesis, is a membrane of a general secretion pathway. Mol. Microbiol. 6, 1351-1361.

Kaufman, M. R., Seyer, J. M, and Taylor, R. K. (1991). Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram negative bacteria. Genes Dev. 5, 1834-1846.

Kaufman, M. R., Shaw, C. E., Jones, I. D, and Taylor, R. K. (1993). Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated pilus: analogies to other virulance factor secretoty systems. Gene 126, 43-49.

Kenny, B., Haigh, R., and Holland, I. B. (1991). Analysis of hemolysin transport process through the secretion from *Escherichia coli* of PCM, CAT or β-galactosidase fused to the HlyC-terminal signal domain. Mol. Microbiol. 5, 2557-2568.

Klauser, T., Pohlner, J., and Meyer, T. F. (1990). Extracellular transport of cholera toxin B subunit using IgA protease β-domain; conformation-dependent outer membrane translocation. EMBO J. 9, 1991-1999.

Koronakis, V., Cross, M., Senior, B., Koronakis, E., and Huges, C. (1987). The Secreted hemolysins of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the α-haemolysin of *Escherichia coli*. J. Bacteriol. 169, 1509-1515.

Koronakis, V., and Hughes, C. (1988). Identification of the promoters directing in vivo expression of hemolysin genes in *Proteus vulgaris* and *Escherichia coli*. Mol. Gen. Genet. 213, 99-104.

Koronakis, V., Koronakis, E., and Hughes, C. (1988). Comparison of the

hemolysin secretion protein HlyB from *Proteus vulgaris* and *Escherichia coli*; Site directed mutagenesis causing impairment of export function. Mol. Gen. Genet. 213, 551-555.

Koronakis, V., Koronakis, E., and Hughes, C. (1989). Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. EMBO J. 8, 595-605.

Kornacker, M. G., and Pugsley, A. P. (1989). Molecular characterization of *pulA* and it's product, pullulanase, a secreted enzyme of *Klebsiella pneumoniae* UNF5023. Mol. Microbiol. 4, 73-85.

Kornacker, M. G., Boyd, A., Pugsley, A. P., and Plastow, G. (1989). A new regulatory locus of the maltose regulon in *Klebsiella pneumoniae* strain K21 identified by the pullulanase secretion mutants. J. Gen. Microbiol. 135, 397-408.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1990). Protease secretion by *Erwinia chrysanthemi*: the specific functions are analogous to those of *E. coli* α-hemolysin. EMBO J. 9, 1375-1382.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1991). Cloning and Expression in *E.coli* of the *Serratia marcescens* metalloprotease gene: secretion of the protease from *E.coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. J. Bacteriol. 173, 2160-2166.

Letoffe, S., and Wandersman, C. (1992). Secretion of CyaA-PrtB and HlyA-PrtB fusion proteins in *E.coli*: Involvement of the Glycine rich repeat domain of *Erwinia chrysanthemi* Protease B. J. Bacteriol. 174, 4920-4927.

Letoffe, S., Ghigo, J. M., and Wandersman, C. (1993). Identification of two components of the *Serratia marcescens* metalloprotease transporter; Protease SM secretion in *E.coli* is TolC dependent. J. Bacteriol. 175, 7321-7328.

Lindberg, M., and Collmer, A. (1992). Analysis of eight *out* genes in a cluster required for pectic enzyme secretion by *Erwinia chrysanthemi* : sequence comparison with secretion genes from other gram-negative bacteria. J.

Bacteriol. 174, 7385-7397.

Lindgren, V., and Wretlind, B. (1987). Characterization of a *Pseudomonas aeruginosa* Tn insertion mutant with defective release of exoenzymes. J. Gen. Microbiol. 133, 675-681.

Lisser, S., and Margalit, H. (1993). Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids. Res. 21, 1507-1516.

Ljungh, A., and Wadstrom, T. (1983). Toxins of *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. J. Toxicol. Toxin. Rev. 1, 257-307.

Lory, S., Tai, P. C., and Davis, B. D. (1983). Mechanism of excretion of proteins by Gram-negative bacteria: *Pseudomonas aeruginosa* exotoxin A. J. Bacteriol. 156, 695-702.

Lory, S. (1992). Determinants of extracellular protein secretion in gram negative bacteria. J. Bacteriol. 174, 3423-3428.

Lueng, K. Y., and Stevenson, R. M. (1988). Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. J. Gen. Microbiol. 133, 675-681.

Mackman, N., and Holland, B. (1984). Functional characterization of a cloned haemolysin determinant from *Escherichia coli* of human origin, encoding information for the secretion of a 107 kDa polypeptide. Mol. Gen. Genet. 196, 129-134.

Mackman, N., Nicaud, J. M., Gray, L., and Holland, I. B. (1985). Genetic and functional organization of the *Escherichia coli* hemolysin determinant 2001. Mol. Gen. Genet. 201, 529-536.

Mackman, N., Baker, K., Gray, L., Haigh, R., Nicaud, J. M. and Holland, I. B. (1987). Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. EMBO J. 6, 2835-2841.

McClure, W. R. (1985). Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. 54, 171-204.

McCoy, R. H., and Seidler, R. J. (1973). Potential pathogens in the environment; isolation, enumeration and identification of seven genera of intestinal bacteria associated with small green pet turtles. Appl. Microbiol. 25, 534-538.

Masure, H. R., Au, D. C., Gross, M. K., Donovan, M. G., and Strom, D. R.

(1990). Secretion of the *Bordetella pertussis* adenylate cyclase from *E. coli* containing the haemolysin operon. Biochemistry. 29, 140-145.

Martin, P. R., Hobbs, M., Free, P. D., Jeske, Y., and Mattick, J. S. (1993). Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbrae in *Pseudomonas aeruginosa*. Mol. Microbiol. 9, 857-868.

Michaelis, S., Chapon, C., d'Enfert, C., Pugsley, A. P., and Schwartz, M. (1985). Characterization and expression of the structural gene for pullulanse, a maltose inducible secreted protein of *Klebsiella pneumoniae*. J. Bacteriol. 164, 633-638.

Michiels, T., Vanooteghem, J-C., DE Rouvroit, C. L., China, B., Gustin, A., Boudry, P., and Cornelis, G. R. (1991). Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J. Bacteriol. 173, 4994-5009.

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor. N. Y.

Morales, V., Backman, A., and Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 97, 39-47.

Murooka, Y., and Ikeda, R. M. (1989). Biosynthesis and secretion of pullulanase, a lipoprotein from *Klebsiella aerugenes*. J. Biol. Chem. 264, 17524-17531.

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high-molecular-weight DNA. Nucleic Acids Res. 8, 4321-4325.

Muller, D., Hughes, C., Goebel, W. (1983). Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli*. J. Bacteriol. 153, 846-851.

Nicaud. J. M., Mackman, N., Gray, L., and Holland, I. B. (1985). Characterization of HlyC and mechanism of activation and secretion of hemolysin from *Escherichia coli* 2001. FEBS Lett. 187, 339-344.

Nikaido, H., and Vaara, M. (1987). Outer membrane, pp. 9-22. In Neidhardt, F. C., Ingraham, J. L., Low, B. K., Magasanik, B., Schaechter, M., and Umbarger, E. H. (ed.), *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology. American society for microbiology. Washington, DC.

Noegel, A., Rdest, U., Springer, W., and Goebel, W. (1979). Plasmid cistrons controlling synthesis and excretion of the exotoxin α-hemolysin of *Escherichia coli*. Mol. Gen. Genet. 175, 343-350.

Nunn, D., Bergman, S., and Lory, S. (1990). Products of three accessory genes, *pilB*, *pilC* and *pilD* are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. 172, 2911-2919.

Nunn, D., and Lory, S. (1991). Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc. Natl. Acad. Sci. USA 88, 3281-3285.

Nunn, D. N., and Lory, S. (1992). Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. USA 89, 47-51.

Overbye, L. J., Sandkvist, M., and Bagdasarian, M. (1993). Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. Gene 132, 101-106.

Pearson, W. R., and Lipman, D. J. (1988). Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85, 2444-2448.

Pohlner, J., Halter, R., Beyreuther, J., and Meyer, T. F. (1987). Gene structure and extracellular secretion of *Neisseria gonorrheae* IgA protease. Nature 325, 458-462.

Possot, O., d'Enfert, C., Reyss, I., and Pugsley, A. P. (1992). Pullulanase secretion in *E. coli* K12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. Mol. Microbiol. 6, 95-105.

Pritchard, A. E., and Vasil, M. L. (1986). Nucleotide sequence and expression of a phosphate-regulated gene encoding a secreted hemolysin of *Pseudomonas aeruginosa*. J. Bacteriol. 167, 291-298.

Pugsley, A. P. (1987). Protein secretion across the outer membrane of Gram-negative bacteria, pp. 607-652. In R. C. Das and P. W. Robbins (ed.), Protein transfer and organelle biogenesis. Academic press, Inc., Orlando, Fla.

Pugsley, A. P. (1993a). The complete general secretory pathway in gram-negative bacteria. Microbol. Rev. 57, 50-108.

Pugsley, A. P. (1993b). Processing and methylation of *Pul*G, a pilin like component of the general secretory pathway of Klebsiella oxytoca. Mol.

Microbiol. 9, 295-308.

Pugsley, A. P., Chapon, C., and Schwartz, M. (1986). Extracellular pullulanase of *Klebsiella pneumoniae* is a lipoprotein. J. Bacteriol. 166, 1083-1088.

Pugsley, A. P., and Reyss, I. (1990). Five genes at the 3' end of the *Klebsiella pneumoniae* pulC operon are required for pullulanase secretion. Mol. Microbiol 4, 365-379.

Pugsley, A. P., dEnfert, C., Reyss, I., and Kornacker, M. G. (1990). Genetics of extracellular protein secretion by gram negative bacteria. Annu. Rev. Genet. 24, 67-90.

Pugsley, A. P., Kornacker, M. G., and Poquet, I. (1991a). The general secretion pathway is directly required for extracellular pullulanase secretion in *E. coli*. Mol. Microbiol. 5, 343-352.

Pugsley, A. P., Poquet, I., and Kornacker, M. G. (1991b). Two distinct steps in pullulanase secretion by *E. coli* K12. Mol. Microbiol. 5, 865-873.

Pugsley, A. P, and Dupuy, B. (1992). An enzyme with type IV prepilin peptidase activity is required to process components of the general extracellular protein secretion pathway of *Klebsiella oxytoca*. Mol. Micro. 6, 751-760.

Pugsley, A. P., and Possot, O. (1993). The general secretory pathway of *Klebsiella oxytoca*: no evidence for relocalization or assembly of pilin-like PulG protein into a multiprotein complex. Mol. Micro. 10, 665-674.

Quandt, J., and Hynes, M. F. (1993). Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene 127, 15-21.

Reeves, P. J., Whitcombe, D., Wharam, S., Allison, G., Bunce, N., Barallon, R., Douglas, P., Mulholland, V., Stevens, S., Walker, D., and Salmond, G. P. C. (1993). Molecular cloning and characterization of 13 *out* genes from *Erwinia carotovora* subspecies *carotovora*: genes encoding members of a general secretion pathway (GSP) widespread in gram negative bacteria. Mol. Microbiol. 8, 443-456.

Rella, M., Mercenier, A., and Haas, D. (1985). Transposon insertion mutagenesis of *Pseudomonas aeruginosa* witha Tn5 derivative: application to physical mapping of the arc cluster. Gene 33, 293-303.

Reyss, I., and Pugsley, A. P. (1990). Five additional genes in the pulC-O operon of the gram negative bacterium *Klebsiella oxytoca* UNF 5023 which are

required for pullulanase secretion. Mol. Gen. Genet. 222, 176-184.

Riddle, L. M., Graham, T. E., Amborski, R. L., and Hugh-Jones. M. (1981). Production of hemolysin and protease by *Aeromonas hydrophila* in a chemically defined medium. International Symposium on Fish Biologics: Serodiagnostics and Vaccines. Dev. Biol. Stand. 49, 125-133.

Rosen, K. M., Lamperti, E. D., and Villa-Komaroff, L. (1990). Optimizing the northern blot. Biotechniques. 8, 398-403.

Salmond,G. P. C., and Reeves, P. J. (1993). Membrane traffic wardens and protein secretion in Gram-negative bacteria. Trends Biochem. Sci. 18, 7-12.

Sambrook, J. Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Schatz, P. J., and Beckwith, J. (1990). Genetic analysis of protein export in *Escherichia coli*. Annu. Rev. Genet. 24, 215-248.

Shine, J., and Dalgarno, L. (1974). The 3' terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to non-sense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71, 1342-1346.

Shulein, R., Gentschev, I., Mollenkopf, H. J., and Goebel, W. (1992). A topological model for the hemolysin translocator protein HlyD. Mol. Gen. Genet. 234, 155-163.

Simon, R., Priefer, U., and Puhler, A. (1983). A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. BioTechnology 1, 784-791.

Stanley, P., Koronakis, V., and Hughes, C. (1991). Mutational analysis supports a role for multiple structural features in the C-terminal secretion signal of *Escherichia coli* hemolysin. Mol. Microbiol. 5, 2391-2401.

Strathdee, C. A., and Lo, R. Y. C. (1989). Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* leucotoxin determinant. J. Bacteriol. 171, 916-928.

Strom, M. S., Nunn, D., and Lory, S. (1991). Multiple roles of the pilus biogenesis protein PilD: invovement of PilD in excretion of enzymes from

Pseudomonas aeruginosa. J. Bacteriol. 173, 1175-1180.

Studier, F., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60-89.

Thornton, J., Howard, S. P., and Buckley, J. T. (1988). Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferase and other lipases. Biochem. Biophys. Acta. 959, 153-159.

Tomb, J-F., El-Hajj, H., and Smith, H. O. (1991). Nucleotide sequence of a cluster of genes involved in the transformation of *Haemophilus influenzae* Rd. Gene 104, 1-10.

Tommassen, J., Filloux, A., Bally, M., Murgier, M., and Lazdunski, A. (1992). Protein secretion in *Pseudomonas aeruginosa*. FEMS. Microbiol. Rev. 73-90.

Towbin, H., Stachelin, and J. Gordon. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Tsai-wu, J., Radicella, J. P., and Lu, A. (1991). Nucleotide sequence of the *Escherichia coli micA* gene required for A/G specific mismatch repair: identity of MicA and MutY. J. Bacteriol. 173, 1902-1910.

Turner, L. R., Lara, J. C., Nunn, D. N., and Lory, S. (1993). Mutations in the consensus ATP binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J. Bacteriol. 175, 4962-4969.

Twining, S. S. (1984). Fluorescein isothiocynate-labeled casein assay for proteolytic enzymes. Anal. Biochem. 143, 30-34.

Vieira, J., and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Methods Enzymol. 153, 3-11.

Walker, J. E., Saraste, M., Ruswick, M. J., and Gay, N, J. (1982). Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide

binding fold. EMBO J. 1, 945-951.

Wandersman, C. (1992). Secretion across the bacterial outer membrane. Trends Genet. 8, 317-321.

Wandersman, C., and Delepelaire, P. (1990). TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA 87, 4776-4780.

Wandersman, C., Delepelaire, P., and Letoffe, S. (1990). Secretion processing and activation of *Erwinia chrysanthemi* proteases. Biochimie. 72, 143-146.

Wandersman C., and Letoffe, S. (1993). Involvement of lipopolysaccaharide in the secretion of *Escherichia coli* α-hemolysin and *Erwinia chrysanthemi* proteases. Mol. Microbiol. 7, 141-150.

Wang, R. C., Seror, S. J., Blight, M., Pratt, J. M., Broome-Smith, J. K., and Holland, I. B. (1991). Analysis of the membrane organization of an *Escherichia coli* protein translocator, HlyB, a member of a large family of procaryote and eucaryote surface transport proteins. J. Mol. Biol. 217, 441-454.

Ward, J. E., Akiyoshi, D. E., Regier, D., Datta, A., Gordon, M. P., and Nester, E. W. (1988). Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. 263, 5804-5814.

Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). Distantly related sequences in the α- and β- subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 945-951.

Whitchurch, C. B., Hobbs, M., Livingstone, S. P., Krishnapillai, V., and Mattick, J. S. (1991). Characterization of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialized protein export system widespread in bacteria. Gene 101, 33-44.

Woestyn, S., Allaoui, A., Wattiau, P., and Cornelis, W. R. (1994). YscN, the putative energizer of the *Yersinia* Yop secretion machinery. J. Bacteriol. 176, 1561-1569.

Wohlgemuth, K., Pierce, R. L., and Kerkbude, C. A. (1972). Bovine abortion associated with *Aeromonas hydrophila*. J. Am. Vet. Med. Assoc. 160, 1001-1002.

Wong, K. R., and Buckley, J. T. (1989). Proton motive force involved in protein

transport across the outer membrane of *Aeromonas salmonicida*. Science. 246, 654-656.

Wong, K. R., Green, M. J., and Buckley, J. T. (1989). Extracellular secretion of cloned aerolysin and phospholipase by *Aeromonas salmonicida*. J. Bacteriol. 171, 2523-2527.

Wong, K. R., Mclean, D. M., and Buckley, J. T. (1990). Cloned aerolysin of *Aeromonas hydrophila* is exported by a wild type marine *Vibrio* strain but remains periplasmic in pleiotropic export mutants. J. Bacteriol. 172, 372-376.

Wretlind, B., and Pavlovskis, O. R. (1984). Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. J. Bacteriol. 158, 801-808.

Yanagida, N., Uozumi, T., and Beppu, T. (1986). Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli*. J. Bacteriol. 166, 937-944.

Zubay, G. (1980). The isolation and properties of CAP, the catabolite gene activator. Methods Enzymol. 65, 856-877.

Zuker, M., and Stiegler, P. (1981). Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. 9, 133-148.