THE UNIVERSITY OF CALGARY

Characterization of the 40 kDa Protease of *Burkholderia cepacia*

by

Vandana Sood

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA

JUNE, 1997

ABSTRACT

Burkholderia cepacia strain Pc715j produces two proteases, PSCP (*Pseudomonas cepacia* protease) and a 40 kDa protease. Both PSCP and the 40 kDa protease had a pH optimum of 6 for activity, a temperature optimum of 50°C and a pI of 5.2. The 40 kDa protease was categorized as a zinc metalloprotease due to inhibition by metal chelators and restoration of activity by zinc. The 40 kDa protease could degrade collagen, fibronectin, immunoglobulins and transferrin. Primers were designed to internal peptides from the digested 40 kDa protease and were used to screen *B. cepacia* Pc715j genomic digests by Southern hybridization. No specific binding was observed. The primers did not amplify products by PCR which corresponded to the 40 kDa protease amino acid sequence from the Pc715j genome. A cosmid (pCP19) gene bank of Pc715j was constructed and screened in *Escherichia coli* strain HB101, *Pseudomonas aeruginosa* strain PA103-11 and *B. cepacia* strain 22-12. The inserts were unstable in PA103-11 and did not yield a protease-positive cosmid in 22-12 or HB101.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Dr. P. A. Sokol, whose support, guidance and encouragement has been indispensable throughout my graduate studies. My sincere gratitude and appreciation are extended for having had the opportunity to experience independent scientific research under Dr. Sokol's tutelage. My appreciation is extended to Dr. D. E. Woods and Dr. R. R. Read for their support and encouragement. I would like to thank Dr. K. E. Sanderson for his support and advice.

The past and current residents of B724 and friends in the department have been an integral part of my graduate school achievement and will be missed dearly. Included on this list are Patricia Darling, Dr. Cora Kooi, Dr. Eric LaFontaine, Brenda Beckthold, Shawn Lewenza, Sallene Wong, Carla Lewis and Lisa Paterson. Dr. Cora Kooi has been a knowlegeable, helpful and indispensable collaborator on much of my graduate research.

My parents, Prem and Indu Sood, are the source of my ambition and my motivation and along with my brother Rohith and my sister Geeta are my greatest source of support, understanding and love.

My deepest gratitude extends to my best friend Robert Hackl for his love, patience, sincere interest, understanding and friendship.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	base pair
CBB	Coomassie Brilliant Blue
CF	Cystic Fibrosis
Da	Dalton
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
3,4-DCL	3,4-dichlorisocoumarin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
g	gram
H_2O	water
HCl	hydrochloric acid
IEF	isoelectric focusing
Ig	immunoglobulin
kb	kilobase

LIST OF ABBREVIATIONS CONTINUED

kDa	kilodalton
LB	Luria-Bertani
LPS	lipopolysaccharide
M	Molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
N	Normal
ng	nanogram
nM	nanomolar
nm	nanometer
N-terminus	amino terminus
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate

TCA	trichloroacetic acid
μg	microgram
μl	microliter
μM	micromolar
V	volts

1.0 INTRODUCTION

1.1 *Burkholderia cepacia* – General Introduction and Physiology

Burkholderia (formerly *Pseudomonas*) *cepacia* is a gram-negative bacillus that was initially recognized in the 1950's as a plant pathogen responsible for soft rot in onions (Burkholder, 1950). Since then it has gained prominence in medical microbiology as an opportunistic pathogen in cystic fibrosis patients, a cause of documented cases of septicemia, an organism possessing multiresistance to antibiotics (Isles *et al.* 1984, Rosenstein and Hall 1980), and a nutritionally versatile organism (Goldmann and Klinger 1986). As additional research sheds light on the pathology of *B. cepacia*, its importance in causing infection and the search for therapeutic mechanisms becomes paramount.

B. cepacia is a non spore-forming aerobic bacillus which is motile, catalase and oxidase positive and capable of producing some non-fluorescent pigments; it grows optimally at 30-35°C. Natural habitats of this organism include soil, vegetation and water (Holmes, 1986). *B. cepacia* is a highly metabolically versatile organism that can proliferate in distilled water, in disinfectants and antiseptics, and can use penicillin G, herbicides and industrial waste as carbon sources (Goldmann and Klinger 1986). In contrast, approximately 45% of *B. cepacia* isolates from cystic fibrosis (CF) and bronchiectasis patients exhibit auxotrophy for amino acids such as cysteine, methionine, histidine, phenylalanine and tyrosine in comparison to prototrophic environmental and non-CF clinical isolates (Barth *et al.* 1995).

B. cepacia's metabolic versatility and multiple antibiotic resistance may stem partly from its complex genetic makeup. The *B. cepacia* 6.5-7 Mb genome consists of three replicons and a cryptic plasmid (Cheng *et al.* 1994). *B. cepacia* strains also contain a large number of insertion sequences which can be involved in genomic rearrangement, insertional gene inactivation and recruitment of foreign genes (Tyler *et al.* 1996). In Tyler's study, a unique combination insertion sequence element (IS402-IS536) was associated exclusively with an epidemic clone of *B. cepacia* CF isolates exhibiting enhanced transmissibility and virulence. This apparent variation in the pathogenic potential of *B. cepacia* clinical CF isolates may indicate a link between the genomic complexity of *B. cepacia* and its virulence, adaptibility, versatility and recent clinical appearance.

Notable is the current investigation in agricultural microbiology of *B. cepacia* as a bioremediation agent in degrading herbicides, in industrial microbiology for degrading industrial waste, and for its ability to produce antifungal and antibacterial agents (Shields and Reagin 1992, Fridlander *et al.* 1993). The issue of environmental release of *B. cepacia* for agricultural and industrial purposes versus the possible social transmission of this organism demonstrates the current concern over the pathogenic potential of *B. cepacia* particularly in pulmonary infections in CF patients.

1.2 *B. cepacia* -- Opportunistic pathogen

Until the early 1980's, *B. cepacia* was primarily recognized as a plant pathogen (Burkholder, 1950). Then Isles *et al.* (1984) documented the increasing prevalence of

B. cepacia infection in CF patients and an increased morbidity and mortality over non-colonized CF patients. Cystic fibrosis results from a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene responsible for cAMP-dependent chloride ion transport resulting physiologically in an imbalance of fluid and ion movement across epithelia, thickened mucous and blocked exocrine ducts in the lung, pancreas and intestine (Davidson *et al.* 1995). Three primary clinical courses of infection were identified in Isles' study: a chronic asymptomatic colonization; a progressive deterioration similar to most major CF pathogens such as *P. aeruginosa* characterized by fever, weight loss, increasing humoral response, episodes of acute pulmonary exacerbation and increasing lung disease; and the unique 'cepacia syndrome' observed in ~20% of colonized patients (Isles *et al.* 1984). This syndrome was characterized by a necrotizing pneumonia, fever, bacteremia and leukocytosis resulting in a rapid and often fatal clinical deterioration; often in previously mildly affected patients. One of the first documented cases of *B. cepacia* infection in 1980 described the additional complication of septicemia, emphasizing the difference in the 'cepacia syndrome' and bacteremia in comparison to a more localized infection by *P. aeruginosa* (Rosenstein and Hall 1980). In a post-mortem study of *B. cepacia*-infected CF patients, lung pathology of the patient population experiencing the cepacia syndrome exhibited a more extensive necrotizing pneumonia, abscess formation and lymphocytic infiltration with a subset exhibiting septicemia (Tomashefski *et al.* 1988). These features were not observed in the patient group experiencing the more typical slower deterioration observed with most major CF pathogens. A study by the Center

for Disease Control in conjunction with the Rainbow Babies and Children's Hospital in Clevelend, Ohio noted that *B. cepacia* infection was associated with increasing age, colonized siblings, prior hospitalization and the severity of the underlying lung disease; four cases with an accompanying bacteremia proved to be fatal (Tablan *et al.* 1987).

The question of whether *B. cepacia* colonization in CF patients is simply a marker of severe lung disease or a contributing cause of the lung disease has begun to be addressed using animal models in a CF background. The development of a transgenic CF mouse containing a 'leaky' *cftr* insertional mutant has proven to be a better comparison to human CF disease than the null *cftr* mutant mice due to the low-level expression of wild-type *cftr* in human CF patients, allowing direct examination of the effects of different CF pathogens in this animal model (Davidson *et al.* 1995). Controlled exposure of these CF mice with asymptomatic uninfected lungs to *B. cepacia* resulted in severe bronchopneumonia and mucous retention, similar to the rapidly fulminating pneumonia experienced by human CF patients (Davidson *et al.* 1995).

Thus the role of *B. cepacia* as an opportunistic pathogen and as more than a marker of infection in CF patients seems certain. Of clinical concern then are the aforementioned unique 'cepacia syndrome', and also the multiresistance of *B. cepacia* to antibiotics and the possible person-to-person transmission of certain epidemic clones by social contact. *In vitro* susceptibility of clinical CF *B. cepacia* isolates to antibiotics is often contradicted by resistance during treatment and little to no reduction in bacterial numbers in sputum samples. Rosenstein's 1980 study described

the *in vivo* resistance of *B. cepacia* to most aminoglycosides despite demonstrated *in vitro* susceptibility; trimethoprim-sulfamethoxazole (TMP-SMZ) was the successful course of treatment for the patient. Typical documented resistance includes polymyxins and aminoglycosides, ticarcillin and first and second generation cephalosporins. Many strains are now resistant to TMP-SMZ; *in vitro* susceptibility to third generation cephalosporins (such as ceftazidime), imipenem, aztreonam and ciprofloxacin have been explored as new alternatives for treatment (Goldmann *et al.* 1986). Ceftazidime, however, although improving clinical conditions in some patients and greatly reducing concurrent *P. aeruginosa* infections, resulted in little decrease in the density of *B. cepacia* in sputum (Goldmann and Klinger 1986). Thus, clinical treatment of *B. cepacia* infection continues to pose a challenge today.

The possibility of transmission between CF patients by social contact has continued to be both a cause of concern and of controversy in attempting to identify sources of social, nosocomial and sibling acquisition to clarify the epidemiology of this opportunist. Lipuma *et al.* in 1990 documented the person-to-person transmission and subsequent spread of a clinical strain through one CF centre. A highly transmissible epidemic strain was identified in 1993 by Govan *et al.* as being spread through social contact within and between regional CF centres. However, a study by Steinbach *et al.* in 1994 of CF lung transplant patients detected non-related low transmissible strains, with infected CF patients harboring the same strain before and after lung transplants. This and other studies have in fact led to the rejection of *B. cepacia*-infected CF patients as transplant candidates (Sun *et al.* 1995), and has also questioned the theory

of highly transmissible epidemic strains. A study of fifteen isolates from a Toronto CF centre indicated clonal relatedness both within themselves and also to the related epidemic isolates described in Govan's 1993 study, indicating that these isolates arose from a single clonally related lineage, and intercontinental spread occurred between Edinburgh and Toronto. Common to all these isolates was the expression of the cable (*cbl*) adhesion pili; the identical sequence for the *cblA* gene between these isolates, and the significantly higher adherence of these isolates to mucin and to *cftr* mutant airway epithelial cells (Govan *et al.* 1993, Sun *et al.* 1995). The unrelated *B. cepacia* CF isolates were $cblA^-$ and less adherent, with the one unrelated $cblA^+$ strain exhibiting marked sequence polymorphism from the epidemic $cblA^+$ sequence (Sun *et al.* 1993). This clonal group termed ET12 may have first been documented in the Isles study (1984), and demonstrated the identification of a dominant ribotype corresponding to ET12 among sixteen British and Irish CF centres (Pitt *et al.*, 1996). It should be emphasized that the epidemic nature of this clone refers to its enhanced transmissibility, and not to an enhanced virulence by this strain over infections with other *B. cepacia* strains. The resistance profiles of isolates within the ET12 group are markedly variable, possibly due to insertion sequence migration within the strain.

1.3 *Burkholderia cepacia* -- Virulence factors

An increased interest in the virulence potential of *B. cepacia* has led to growth in research on this organism's potential virulence factors. Included among these are virulence factors involved in colonization, adherence and invasion, iron-chelating

siderophores, extracellular virulence factors, cell surface antigens, factors involved in immune system evasion and factors contributing to the multiresistance of *B. cepacia* to antibiotics.

Adherence and colonization of respiratory mucosal surfaces is essential in the establishment of infection for *B. cepacia*. A 1990 study by Saiman *et al.* demonstrated polar pili and flagella in electron micrographs of *B. cepacia* cells. Adherence to respiratory cells by *B. cepacia* was enhanced by the presence of *P. aeruginosa* exoproducts; the reverse was not true. This is interesting in light of the ~80% rate of co- or pre-colonization of *B. cepacia* infected CF patients with *P. aeruginosa*. In this study, outer membrane proteins were hypothesized as mediating adherence (Saiman *et al.* 1990). *B. cepacia* fimbriae (pili) were subsequently found to be similar to *P. aeruginosa* fimbriae, being composed of a 16 kDa fimbrial subunit; and fimbriated strains exhibiting 20 to 40 times more adherence to tissue culture cells over non-fimbriated *B. cepacia* strains (Kuehn *et al.* 1992). CF clinical isolates predominantly from patients with more advanced lung disease preferentially adhered to mucin; the mucin receptors were carbohydrate in nature, and exogenous mucin could inhibit binding of *B. cepacia* piliated isolates to buccal epithelial cells (Sajjan *et al.* 1992). Examination of piliated isolates demonstrated mucin bound sparsely along the length of the pili; a 22 kDa mucin binding component on the pili surface was subsequently identified (Sajjan and Forstner, 1992). Piliated isolates also bound to a 55 kDa receptor on buccal epithelial cells (BEC's) via this 22 kDa protein; nonpiliated isolates

bound to the BEC's via a separate adhesion system which was glycolipid in nature and did not involve mucin (Sajjan and Forstner, 1993).

Two companion papers describe the distinct classes of pili expressed by various *B. cepacia* isolates. A group of clonally related CF isolates originating from an epidemic in a Toronto CF center were examined, as well as other *B. cepacia* CF and non-CF clinical and environmental isolates (See section 1.2 for discussion of this epidemic clone called ET12) (Sajjan *et al.* 1995). The clonally related isolates expressed a dense, fine, mesh-like pili (Msh), in addition to a thick, large cable-type pili (Cbl) (Goldstein *et al.* 1995). The gene for a 17 kDa major subunit protein composing the *cbl* pili was cloned. This gene was only detected in the clonally related isolates, and the 22 kDa adhesin was found along the length of the Cbl pili. A group of unrelated CF isolates expressed the Msh pili and a filamentous-type (Fil) pili. Non-CF clinical isolates expressed spine-type (Spn) pili, and environmental isolates expressed the Msh pili and a fifth morphological group of spike-type (Spk) pili. Ribotyping analysis demonstrated that all isolates studied were distinct from one another with the exception of the clonally related group (Goldstein *et al.* 1995). The higher affinity binding of nonpiliated isolates to BEC's was to a galactose containing glycolipids, located in a major receptor on two different human respiratory epithelial cell lines (Sylvester *et al.* 1996).

A recent paper documented the ability of *B. cepacia* to invade a human epithelial carcinoma cell line (A549 cells) (Burns *et al.* 1996a). Electron microscopy demonstrated one or more organisms within vacuoles; and thickening of the epithelial

cell membrane at the initial point of contact followed by apparent endocytosis by microvilli. Killed organisms did not enter cells and intracellular replication was demonstrated. Cytochalasin D was found to inhibit invasion; thus implicating possible utilization of host cell microfilaments for invasion (Burns *et al.* 1996a). Invasion could explain the bacteremia and persistence associated with a *B. cepacia* infection due to evasion of host defense; protection from antibiotics and systemic spread (Burns *et al.* 1996a).

To establish and maintain infection, nutritional requirements must be met. The iron-limited environment of the respiratory tract is a host defence due to the presence of iron-sequestering molecules, thus infecting organisms must overcome this defence using their own virulence factors. Iron-chelating siderophores are essential in this function. Sokol (1986) showed that roughly half of the CF isolates studied produced the siderophore pyochelin, which correlated with severity and fatality of the infection, with the patients infected with non-pyochelin producing isolates exhibiting mainly a mild or moderate infection. Pyochelin was also found to stimulate the growth *of B. cepacia in vitro* in the presence of transferrin (Sokol, 1986). A second siderophore, cepabactin, was described by Meyer *et al.* (1989). Cepabactin added exogenously could stimulate *B. cepacia* growth and promote iron-uptake in an iron-poor medium (Meyer *et al.* 1989). Salicyclic acid (formerly azurechelin) was identified by Sokol *et al.* (1992). This siderophore increased *B. cepacia* growth in an iron-poor medium and could possibly compete with transferrin for iron (Sokol *et al.* 1992). The ornibactins were recently described by Meyer *et al.* (1995) as siderophores being produced in

greater amounts than pyochelin and cepabactin, and were shown to facilitate iron uptake.

Potential extracellular virulence factors produced by *B. cepacia* include protease (see section 1.6), hemolysin and lipase. A study of 48 *B. cepacia* CF strains showed that 42 produced protease, 33 produced lipase and one produced hemolysin; no extracellular cytotoxic products were detected (McKevitt *et al.* 1984). A similar survey of 120 clinical isolates of *B. cepacia* showed that most produced protease, lecithinase and lipase while only 5 produced hemolysin (Nakazawa *et al.* 1987). A hemolysin characterized by Nakazawa *et al.* (1987) ws heat-labile with a pH optimum of 5.5, and its activity could be inhibited by sterols. Vasil *et al.* (1988) cloned at least two genes from *B. cepacia* in *E. coli* required for a hemolytic and a phospholipase C activity.

Lipase activity was observed beginning in log phase and continuing well into stationary phase with the pH optimum being in the alkaline range (Lonon *et al.* 1988). Purified lipase was not cytotoxic and did not affect intravenously injected mice (Lonon *et al.* 1988). This lipase was found to decrease phagocytosis by alveolar macrophages visualized in electron micrographs by a rounding of phagocytes in contrast to the usual spreading shape with pseudopodia and other projections (Straus *et al.* 1992). The structural gene *lipA* had a typical N-terminal signal sequence, and a downstream gene *limA* was found necessary for efficient lipase expression (Jorgensen *et al.* 1991). Renaturation of the urea-denatured lipase was only possible in the presence of the *limA* gene product (Hobson *et al.* 1993). LimA did not effect the activity of

intracellular prelipase and did not preferentially associate with prelipase over mature lipase; thus suggesting a potential role as a molecular chaperone due to the absence of a preference for the signal peptide (Hobson *et al.* 1995). LimA seems to be a transmembrane protein located on the inner membrane and may thus be involved in the conformational activation and translocation of lipase into the periplasm, followed by export via the general secretory pathway (Hobson *et al.* 1995).

Characteristic of *B. cepacia* infection in CF patients is an intense, potentially damaging host immune response. Cell surface antigens such as lipopolysaccharide (LPS) present on the outer membrane of gram negative bacteria such as *B. cepacia* may contribute to such an endotoxic effect. Colonized CF patients produced a higher titre humoral IgG response to core (rough) LPS from *B. cepacia* than did non-colonized CF patients or healthy blood donors. These antibodies were not cross-reactive with core LPS from *P. aeruginosa* though serum to whole organisms is cross-reactive (Nelson *et al.* 1993). *B. cepacia* LPS from CF strains was four to five times more endotoxic and induced nine times as much TNF-α (tumor necrosis factor alpha) from human mononuclear leukocytes when compared to *P. aeruginosa* LPS from CF strains (Shaw *et al.* 1995). TNF-α is instrumental in responding to LPS and in initiating and amplifying the host immune response thus possibly accounting for the destructive pulmonary inflammatory response in *B. cepacia* infected CF patients. *B. cepacia* infection is characterized by a huge influx of neutrophils. Interleukin-8 (IL-8) is produced by these activated neutrophils to signal more neutrophils, release neutrophil elastase and aid adhesion. *B. cepacia* cells from a CF patient stimulated

high levels of IL-8 production from lung epithelial cells. Purified LPS from *B. cepacia* elicited high IL-8 production from peripheral blood monocytes though not from lung epithelial cells, perhaps significant in associated cases of bacteremia (Palfreyman *et al.* 1996).

This massive neutrophil influx fails to eradicate the infecting *B. cepacia* isolate; examination of patients with chronic granulamatous disease (CGD) is important in elucidating the evasion of *B. cepacia* of this host defence. CGD-derived neutrophils (PMNs or polymorphonuclear leukocytes) cannot generate oxygen radicals to kill catalase-positive bacteria such as *B. cepacia* or *P. aeruginosa*. However, only *B. cepacia* is a significant pathogen in patients with CGD (Speert *et al.* 1994). These CGD-derived neutrophils could kill *P. aeruginosa* but not *B. cepacia*. Oxidant scavengers from normal neutrophils also exhibited this effect. Thus, the often pre-existent *P. aeruginosa* infection in the lung and the scavenging of oxygen radicals by alginate produced by mucoid *P. aeruginosa* may predispose the patient to infection with *B. cepacia*, which is subsequently resistant to non-oxidative killing by neutrophils (Speert *et al.* 1994). *B. cepacia* CF isolates were also all found to produce catalase, statistically significant from the 86% of control isolates (non-CF or environmental) producing catalase (Gessner *et al.* 1990).

The multiresistance of *B. cepacia* to antibiotics is due to three major mechanisms: decreased membrane permeability, β-lactamase production and active efflux. *B. cepacia* is highly resistant to most β-lactams and aminoglycosides, and CF isolates are more resistant to ceftazidime than non-CF isolates. Selecting for a

ceftazidime-resistant mutant of *B. cepacia* resulted in a 40-fold increase in expression of the β-lactamase (Aronoff *et al.* 1988). The cloned β-lactamase gene along with a transcriptional activator was identified (Proenca *et al.* 1993). Membrane permeability was equal to the multiresistant *P. aeruginosa*. Isolated porins were also comparable in their small size (Parr *et al.* 1987). The 81 kDa porin complex (OpcPO) was composed of a 36 kDa (OpcP1) and a 27 kDa protein (OpcP2). A ciprofloxacin-resistant mutant expressed greatly decreased levels of the 27 kDa protein as compared to the parent, and this protein was completely absent in two resistant CF isolates correlating with decreased membrane permeability (Aronoff *et al.* 1988, Tsujimoto *et al.* 1997). Expression of β-lactamases in these isolates was comparable (Aronoff *et al.* 1988). *B. cepacia* infections in CF patients are often treated with chloramphenicol, trimethoprim and ciprofloxacin (Burns *et al.* 1996b). A DNA fragment isolated from a CF strain resistant to these three agents encoded a gene homologous to *oprM* from *P. aeruginosa*, an outer membrane protein component encoded on a multiple antibiotic efflux operon. An upstream sequence from the *oprM* homolog exhibited similarity to the *mexB* gene of *P. aeruginosa*, also part of this operon (Burns *et al.* 1996b). Thus *B. cepacia* may employ an antibiotic efflux pump as one of its mechanisms of antibiotic resistance.

1.4 Bacterial Metalloproteases

The four families of bacterial extracellular proteases are grouped according to the catalytic residue at the active site: the serine proteases, the aspartic proteases, the

cysteine proteases and the metalloproteases. In the CF lung, bacterial extracellular proteases may act on biologically important substrates such as basement membrane proteins and immune effector molecules, be involved in effecting extensive host inflammatory and immune response and aid in the establishment of infection. Bacterial metalloproteases have been studied in other non-CF pathogens for their virulence potential and have thus been targeted as possible therapeutic targets.

Bacterial metalloproteases are grouped within the superfamily of zinc metalloproteases; the bacterial zinc metalloendoproteinases contain one catalytic zinc atom and several structural calcium atoms (Vallee *et al.* 1990). Of the six enzyme classes established by the International Union of Biochemistry, zinc is essential in the function of at least one enzyme in four of the groups and hydrolases contain the largest group of zinc enzymes (Vallee *et al.* 1990). The zinc metalloendoproteinases (hereafter referred to as metalloprotease) exist within this hydrolase class. Membership in this group requires the HEXXH motif containing the first two zinc-binding ligands, the two histidine residues (Jiang *et al.* 1992). The tetrahedral zinc-binding site contains a water molecule as the fourth ligand, the third ligand subdivides the metalloproteases into two large families, the thermolysins, and the metzincins. The metzincins are comprised of the astacins, the adamalysins, the matrixins and the serralysins. The bacterial metalloproteases belong either to the thermolysin or the serralysin class or have not yet been classified.

The thermolysin class contains the additional NEXXSD consensus sequence; where the glutamate residue is the third zinc ligand around ~20 residues from the

HEXXH motif. The serralysin class has an extended zinc-binding consensus sequence; HEXBHXBGXHZ (Z=P in the serralysins) containing all three zinc ligands where the third zinc ligand is proposed to be the third histidine residue; with a conserved methionine turn sequence common to the metzincins comprising an SBMS-Y sequence in the serralysins (Bode *et al.* 1993). Thus the thermolysins and the serralysins are distinguished for the purposes of classification by the position and identity of the third zinc ligand; and the metzincins are further subdivided according to the identity of the 'Z' residue in the consensus sequence, and by the sequence of the conserved methionine turn (Bode *et al.* 1993). Functional and structural differences will be discussed below.

1.4.1 Thermolysins

The bacterial metalloproteases in the thermolysin family are zinc-containing, calcium-stabilized neutral metalloendopeptidases. The signature member of this group is thermolysin from *Bacillus thermoproteolyticus*. The thermolysins possess a typical N-terminal signal sequence for secretion by the *sec*-dependent general secretory pathway; and a propeptide that is removed from the mature protease (Hase and Finkelstein, 1993). Significant members of this family include PSCP from *Burkholderia cepacia* (see section 1.6), elastase from *Pseudomonas aeruginosa* (see section 1.5.1), the metalloprotease from *Legionella pneumophila*, the HA/protease from *Vibrio cholerae* and a mucinase from *Helicobacter pylori*.

The *Legionella pneumophila* metalloprotease exhibits hemolytic and cytotoxic activity. It is produced *in vivo* and can elicit pulmonary necrosis. It may interfere with

the host cellular immune response by cleaving T cell CD4 receptors and by inactivating IL-2 and TNF-α which are required for T cell proliferation (Mintz *et al.* 1993, James *et al.* 1997). This 38 kDa protease can also degrade collagen, gelatin and casein. Pure protease causes lesions similar to that caused by the whole organism in guinea pig lung, and protease negative mutants exhibited attenuated virulence such that macrophage activation was allowed to limit the extent of an acute, necrotizing inflammatory response (Moffat *et al.* 1994b). The virulence of this organism is significantly attenuated under iron-limited conditions along with a significant decrease in metalloprotease activity. Examination of organisms under these conditions could not detect any siderophore production. Addition of exogenous human holotransferrin to a steady-state, iron-limited chemostat culture stimulated growth by a doubling of culture density in 30 hours. The metalloprotease was shown to be digesting the transferrin and thus possibly providing an iron source for *L. pneumophila* under iron-limited conditions (James *et al.* 1997).

The *Vibrio cholerae* hemagglutinin/protease (HA/protease) can cleave mucin, fibronectin, lactoferrin, elastin, casein, and can nick and thus activate the A subunit of the cholera enterotoxin and CT-related enterotoxins (Hase and Finkelstein, 1990). HA/protease can also compete with whole *V. cholerae* organisms for adherence to intestinal cells (Hase and Finkelstein, 1990). The cloned gene for HA/protease contained a putative N-terminal signal sequence, was homologous to *P. aeruginosa* elastase, and was synthesized as a large precursor before processing to the mature form. A protease negative mutant did not exhibit attenuated virulence in an infant

rabbit model, however, the mutant strain could not detach from cultured human intestinal epithelial cells after initial adhesion (Finkelstein *et al.* 1992). Purified HA/protease could compete with the whole organism for attachment to human intestinal epithelial cells; thus the protease is a detachase which allows organism to free themselves from the intestinal epithelium (Finkelstein *et al.* 1992). Both the *L. pneumophila* metalloprotease and *V. cholerae* HA/protease contained the three zinc-binding ligands the N-terminal signal sequence and the propeptide indicating membership in this family.

Helicobacter pylori is a causative agent of gastritis, peptic ulcer and gastric carcinoma. *H. pylori* is able to digest the protective gastric mucin layer *in vitro*, also demonstrated by examination of biopsy specimens. A secreted zinc metalloprotease gene cloned from this organism exhibited high similarity to the *V. cholerae* HA/protease gene and may belong to the thermolysin family of bacterial metalloproteases (Smith *et al.* 1994).

1.4.2 Serralysins

The serralysin family of bacterial metalloproteases is characterized by a more alkaline pH optimum, the absence of a typical N-terminal signal sequence and a repeated consensus sequence at the C-terminus. This consensus sequence is recognized by unique secretory proteins belonging to the ABC transporter family (Hase and Finkelstein, 1993). The ABC protein translocators are comprised of an outer membrane protein and two inner membrane proteins, one of which contains a consensus sequence for binding ATP (Baumann *et al.* 1993). Significant members of

this family include alkaline protease from *P. aeruginosa* (see section 1.5.1), the metalloproteases from *Serratia marcescens* and *Erwinia chrysanthemi*, and metalloproteases from *Proteus mirabilis* and *Burkholderia pseudomallei* (Hase and Finkelstein, 1993).

Erwinia chrysanthemi, a plant pathogen, produces four highly homologous metalloproteases (Prt A-D). It also has a protease inhibitor gene and three secretory genes encoding the ABC transporter (Binet and Wandersman, 1995). The PrtSM metalloprotease of *Serratia marcescens*, an organism under investigation for biotechnological processes, is highly homologous to the *E. chrysanthemi* metalloproteases, and encodes its own ABC transporter at a site distant to the *prtSM* structural gene (Letoffe *et al.* 1993).

The ZapA metalloprotease of *Proteus mirabilis* contains the extended zinc-binding site consensus sequence, the conserved methionine turn and the consensus C-terminal sequences characteristic of serralysins. *P. mirabilis* is involved in complicated urinary tract infections, and ZapA as a virulence factor can cleave immunoglobulins *in vitro*, as well as cleaving casein (Wassif *et al.* 1995).

Burkholderia pseudomallei is the causative agent of melioidosis, a disease whose manifestations include pneumonia and septicemia. The metalloprotease it produces is probably a serralysin due to its more alkaline pH optimum, size and substrate variability in this class, and the high EDTA concentration (chelates metals such as zinc and calcium required for metalloprotease function) required for inhibition in contrast to the thermolysins. This zinc metalloprotease can cleave complement

component C3, all of the major immunoglobulins, transferrin, hemoglobin, collagen type VIII and elastin (Sexton *et al.* 1994).

1.4.3 Other Bacterial Metalloproteases

Bacterial metalloproteases that do not appear to belong to either the thermolysin or serralysin group by homology include the anthrax toxin lethal factor, the botulinim and tetanus toxins, the immunoglobulin A1 protease from *Streptococcus pneumoniae* and the metalloprotease toxin from *Bacteroides fragilis.*

The importance of inactivation of IgA in the colonization of mucosal surfaces of the respiratory tract is exemplified by the numerous pathogenic bacteria such as *Strep. pneumoniae*, producing IgA1 (a subclass of IgA) proteases. This IgA1 protease is a zinc metalloprotease possessing the zinc-binding active site consensus sequence and is predominantly cell-associated rather than extracellular (Wani *et al.* 1996).

The anthrax toxin lethal factor from *Bacillus anthracis* also contains the zinc-binding site sequence thus identifying it as a zinc metalloprotease. The lethal factor (LF) was not inhibited by phosphoramidon, an inhibitor of certain bacterial metalloproteases, but was inhibited by aminopeptidases. An actual substrate for LF has not yet been identified (Klimpel *et al.* 1994).

Bacteroides fragilis is an anaerobe known for causing abdominal abscess, bloodstream infection and diarrhea. A toxin produced was found to be a zinc metalloprotease and was possibly synthesized as a precursor possessing a propeptide. This toxin can hydrolyze monomeric or G actin (Franco *et al.* 1997).

The tetanus and botulinim neurotoxins are zinc metalloproteases due to the presence of the zinc-binding active site consensus sequence. The substrate specificity is however exceedingly narrow comprising components of the neuroexocytosis apparatus such as synaptobrevin, syntaxin or a synaptosome-associated protein (Yamasaki *et al.* 1994). This activity results in the inhibition of acetylcholine release by the botulinal neurotoxins and the inhibition of neurotransmitter release by tetanus toxin. Phosphoramidon, EDTA and 1,10-phenanthroline (preferentially chelates zinc cations) effectively inhibited the activity of these neurotoxins in preventing neurotransmitter release (Schiavo *et al.* 1994).

1.5 *Pseudomonas aeruginosa* – Pathogenesis and Virulence factors

P. aeruginosa is a major CF pathogen with over 80% of CF patients eventually becoming infected (reviewed in Koch and Hoiby 1993). Extensive colonization of the respiratory tract, virulence factors and the resulting inflammatory response contribute to pathogenesis (Wang *et al.* 1996). By the late teenage years, most CF patients are chronically colonized with mucoid *P. aeruginosa*, with an extremely small chance of eradication (Fitzsimmons 1993).

Major virulence factors of *P. aeruginosa* include exotoxin A, exoenzyme S, hemolysin/phospholipase C, elastase, alkaline protease and alginate (Vasil *et al.* 1986a). Adhesion of *P. aeruginosa* to the apical membrane of CF epithelial cells is mediated by pili and thought to occur preferentially to epithelial cells expressing the CFTR mutation via modification of receptors compared to normal epithelial cells

(Saiman and Prince 1993). Overproduction of alginate results in the mucoid phenotype in *P. aeruginosa* which is observed primarily in CF isolates (>80%) compared to less than 2.5% occurrence in other clinical strains (Vasil *et al.* 1986a). The transition from an initial infection to a chronic presence usually coincides with conversion to the mucoid phenotype (rev. in Koch and Hoiby 1993). Effects on virulence ascribed to alginate include a physical barrier to phagocytosis and opsonization, a possible immunomodulatory function and a possible role in biofilm phenomena such as adhesion and antibiotic resistance (Pederson 1992). The mucoid phenotype may be related to a stress-response system in *P. aeruginosa* in that a deregulated sigma factor similar to heat-shock sigma factors in other gram-negative bacteria results in the overproduction of alginate (Martin *et al.* 1994).

P. aeruginosa produces two toxins, exotoxin A and exoenzyme S. Exotoxin A is an inhibitor of protein synthesis, exhibits necrotizing activity in exposed tissues resulting in lesions similar to those in clinical infections, and is involved in parenchymal invasion and dense intra-alveolar mononuclear cell infiltrations in chronic lung infections (Pollock 1984). Exoenzyme S expression is correlated with the ability of *P. aeruginosa* to spread from epithelial colonization sites to the bloodstream of infected patients (Hovey and Frank 1995). Loss of either toxin results ina loss of the ability to cause local lung damage (Nicas and Iglewski 1985).

1.5.1 *P. aeruginosa* Proteases -- Clinical significance and Molecular Background

P. aeruginosa produces at least three proteases: elastase, alkaline protease and LasA. Alkaline protease, a zinc-dependent metalloprotease, can inhibit the activity of human gamma-interferon, and can degrade the C1q and C3 proteins of serum complement (Guzzo *et al.* 1990, Baumann *et al.* 1993). Expression of alkaline protease was correlated with declining clinical status in CF patients infected with *P. aeruginosa*, and the protease could degrade purified and tissue-associated basement membrane laminin, found in all vascular tissues (Heck *et al.* 1986a, Jaffar-Bandee *et al.* 1995). The structural gene (*apr*) was cloned on an 8.8 kb fragment and expressed and secreted protease in *E. coli*. Mutations in the general secretory pathway of *P. aeruginosa* did not affect secretion of alkaline protease (Guzzo *et al.* 1991). Homology of the structural gene to the *E. chrysanthemi* metalloprotease was demonstrated, and supplying the *E. chrysanthemi* ABC transporter secretion genes *in trans* allowed secretion of mature, active alkaline protease in *E. coli* from a 2.7 kb subclone containing the *apr* structural gene only. Subsequently, the genes encoding the ABC transporter for alkaline protease in *P. aeruginosa* were identified adjacent to the structural gene and were shown to have homology to the *E. chrysanthemi* secretion genes (Duong *et al.* 1992). Alkaline protease has a pH optimum of 7.9 and an isoelectric point of 4 (Morihara, 1963). Characterization of the alkaline protease gene revealed the absence of the conventional N-terminal signal sequence, the consensus repeats at the C-terminus, secretion via its own independent ABC transporter and the extended active site consensus sequence present in members of the serralysin family of zinc metalloproteases (see section 1.4 and 1.4.2).

The elastolytic phenotype of *P. aeruginosa* was initially thought to be due to the activity of elastase (*lasB* gene product) alone. A second protease, LasA, that could degrade elastin was identified by complementation of a mutant exhibiting diminshed elastolytic activity and less lung damage in the rat chronic lung infection model than the parent strain (Toder *et al.* 1991). Elastin degradation is clinically significant as it is a major component of connective tissue, blood vessels and lung tissue (Gustin *et al.* 1996). Activity against elastin was demonstrated in an elastase-negative mutant supplied with copies of the *lasA* gene *in trans* thus demonstrating its elastase activity independent of the LasB protease. The two proteases do, however, act synergistically against elastin (Gustin *et al.* 1996). The *lasA* sequence contains a typical N-terminal signal sequence and a propeptide and predicts a 41 kDa protein though the mature form is 22 kDa in size (Gustin *et al.* 1996). The LasA protease was later shown to possess staphylolytic activity in that it rapidly lysed *Staphylococcus aureus* cells by cleaving pentaglycine cross-links in the cell wall peptidoglycan thus placing it in a new family of bacterial proteases called beta-lytic endopeptidases (Gustin *et al.* 1996). Activity of LasA by a staphylolytic assay was subsequently found to be inhibited by 1,10-phenanthroline, high concentrations of EDTA, $ZnCl_2$, but not by serine protease inhibitors (Kessler *et al.* 1997). The DNA sequence was found to be 69% identical to a metalloprotease from *Aeromonas hydrophila*; thus, LasA is a putative zinc-dependent metalloprotease (Kessler *et al.* 1997).

A protease with elastase activity was purified and characterized by Wretlind and Wadstrom in 1977. The pI of this protease, elastase (*lasB* gene product; Bever

and Iglewski, 1988) was 6.6 and the pH optimum was 8.0 in phosphate buffer and 6.5 in Tris/maleate buffer. Elastase has since been examined for its potential as a virulence factor in *P. aeruginosa* infections in CF patients. Elastase has been found to degrade a number of potentially relevant substrates *in vitro*: human collagen type I, III and IV; complement components and complement-derived peptides, alpha-1-proteinase inhibitor, IgA, IgG, secretory IgA, airway lysozyme and purified or tissue-associated basement membrane laminin (Heck *et al.* 1986a, Heck *et al.* 1986b, Heck *et al.* 1990). Proteolytic destruction in the lung tissue of CF patients infected with *P. aeruginosa* was also demonstrated. Histologic examination of lung tissue from CF patients post-mortem demonstrated fragmented elastic fibers in bronchial-based inflammatory lesions, ulcers and abscesses (Bruce *et al.* 1985). The degradation of type III collagen, an interstitial collagen found in the lung; and type IV collagen, a basement membrane collagen found in all vascular tissues as well as tissue-associated basement membrane laminin signifies the role of elastase in destruction of the extracellular matrix in respiratory infection. Elastase was also able to increase the permeability of alveolar epithelial cells by altering the epithelial tight junctions between cells (Azghani *et al.* 1990).

Meeting the nutritional requirements of the pathogen in question as mentioned before in section 1.3 is essential in the establishment and maintenance of infection. Mechanisms of iron acquisition by *P. aeruginosa* help to circumvent the iron-limited environment of the respiratory tract. Transferrin and lactoferrin are iron-sequestering molecules present in airway secretions; and these molecules have been shown to be

degraded by elastase *in vitro*. *P. aeruginosa* siderophores can more easily scavenge iron from the products of this degradation, and these new iron chelates can catalyze the formation of the highly cytotoxic hydroxyl free radical from the oxidants produced by the high levels of neutrophils present in the CF lung during infection (Britigan *et al.* 1993). *In vivo* cleavage of transferrin and lactoferrin in the CF lung is attributable to both *P. aeruginosa* elastase and neutrophil-elastase; and transferrin may additionally function as an antioxidant in sequestering the potentially catalytic iron chelates that can mediate extensive oxidative damage to the lung tissue (Britigan *et al.* 1993, Miller *et al.* 1996). Augmentation of this cell injury can also occur by the induction of production of superoxide and hydrogen peroxide by the *P. aeruginosa* product pyocyanin thus acting with iron liberated from elastase-mediated cleavage of transferrin to produce this hydroxyl free radical (Miller *et al.* 1996). Interaction of bacterial elastase and neutrophil elastase in causing this effect is demonstrated by *P. aeruginosa* elastase's ability to activate three types of human matrix metalloproteinases thus directly and indirectly mediating damage to the extracellular matrix (Okamoto *et al.* 1997).

The *P. aeruginosa* elastase structural gene, *lasB* was cloned by Schad *et al.* in 1987. The DNA sequence contained a putative N-terminal signal sequence followed by a putative propeptide and the mature protease; expression of this fragment led to production of a 50 kDa and 54 kDa protein (Bever and Iglewski 1988). The zinc-binding active site consensus sequence was identified in the sequence, as well as a calcium-binding site for stability of the enzyme, thus placing elastase in the thermolysin

family of zinc metalloproteases (Fukushima *et al.* 1989). The native structure of elastase was solved by Thayer *et al.* in 1991 and found to be similar to the structures of other zinc metalloproteases in the thermolysin family. Processing of elastase involves removal of the signal peptide from the 54 kDa preproelastase and transport of the 50 kDa proelastase in to the periplasm, cleavage of the 18 kDa propeptide in the periplasm followed by a non-covalent interaction between the propeptide and the 33 kDa mature protease, followed by export of the 33 kDa mature protease into the extracellular milieu via the general secretory pathway (Galloway 1991, Kessler *et al.* 1992). Site-directed mutagenesis of two of the zinc-binding ligands abolished proteolytic and elastolytic activity. Mutation of the third zinc-binding ligand interfered with proelastase processing and subsequent secretion of the mature protease (Kawamoto *et al.* 1993, McIver *et al.* 1993). The propeptide was found to bind to and inhibit the activity of periplasmic elastase (activity was comparable to mature elastase in its free form) and mature elastase (Kessler and Safrin 1994). Deletion of the propeptide resulted in an inactive elastase in *E. coli* and prevention of secretion in *P. aeruginosa*. Supplying the propeptide on a separate plasmid restored activity in *E. coli* and activity and extracellular secretion in *P. aeruginosa* (McIver *et al.* 1995). Thus, the propeptide is a putative chaperone in facilitating extracellular secretion of the mature elastase (McIver *et al.* 1995).

1.6 *B. cepacia* – Proteases

An extracellular protease was purified and characterized from *B. cepacia* strain Pc715j (a clinical CF strain) by McKevitt *et al.* in 1989. This protease, PSCP, was 34 kDa in size by gel filtration and activity could be inhibited by EDTA and 1,10-phenanthroline. Activity was regained by the addition of Zn^{2+}, Ca^{2+} and to a lesser extent Mg^{2+}, thus putatively categorizing it as a zinc metalloprotease. Its pH optimum was 6 and temperature optimum was 45°C. PSCP was shown to degrade human collagen types I, IV and V *in vitro* but CF strains failed to exhibit elastolytic activity by plate assays (McKevitt *et al.* 1984, McKevitt *et al.* 1989). Polyclonal serum to PSCP cross-reacted with *P. aeruginosa* elastase and anti-elastase polyclonal serum cross-reacted with PSCP (McKevitt *et al.* 1989). Intratracheal injection of purified PSCP into rat lungs resulted in bronchopneumonia (McKevitt *et al.* 1989).

In 1994, Kooi *et al.* identified a second less active 40 kDa protease in *B. cepacia* strain Pc715j supernatants. Monoclonal antibodies (MAb's) were raised against PSCP (36-6-6 and 36-6-8) and to the 40 kDa protease (G-11). The PSCP and 40 kDa MAbs cross-reacted on immunoblots with PSCP, the 40 kDa protease, elastase, alkaline protease, *V. cholerae* HA/protease and more weakly with *B. pseudomallei* metalloprotease (Kooi *et al.* 1994). The PSCP MAb 36-6-8 could neutralize the activity of PSCP, elastase, *V. cholerae* HA/protease and the *B. pseudomallei* protease, but not alkaline protease. The 40 kDa protease MAb G-11 only neutralized PSCP activity, neutralization of 40 kDa protease activity was not performed due to its low specific activity (Kooi *et al.* 1994). Further characterization of the PSCP monoclonal antibodies 36-6-6 and 36-6-8 demonstrated recognition on

immunoblots (in addition to those previously described) of thermolysin, the *S. marcescens* metalloprotease, LasA from *P. aeruginosa* and the *Aeromonas hydrophila* metalloprotease. Anthrax lethal factor produced a weak reaction (Kooi and Sokol 1996). Neutralization by 36-6-6 or 36-6-8 occurred with elastase and *V. cholerae* HA/protease but not with alkaline protease or the *S. marcescens* metalloprotease. Thus MAb 36-6-6 and 36-6-8 appear capable of distinguishing between the serralysin and thermolysin family of metalloprotease by their neutralization abilities (Kooi and Sokol 1996). To identify the specific epitopes on elastase being recognized by these monoclonal antibodies elastase was digested with N-chlorosuccinimide, which cleaves after tryptophan residues, into nine peptide fragments. A 13.9 kDa peptide recognized by both PSCP MAbs was subjected to epitope mapping studies in which a series of sixty 9-mer peptides were synthesized spanning this peptide. The MAbs 36-6-6 and 36-6-8 bound most strongly to two of the 9-mer peptides, 15 and 42. The first peptide (15) overlapped the active site of elastase: $_{341}$HGFTEQNSG$_{349}$; where the second histidine in the HEXXH motif is His-341. The second peptide (42), $_{395}$RYNDQPSRD$_{403}$ is between the third zinc-binding ligand and a proton donor at the active site (Kooi *et al.* 1997). Polyclonal antisera to peptides 15 and 42 recognized (on immunoblots) and neutralized thermolysin, *V. cholerae* HA/protease, elastase and PSCP; but not alkaline protease or the *S. marcescens* metalloprotease thus distinguishing between the thermolysin and serralysin family of bacterial zinc metalloproteases (Kooi *et al.* 1997).

Abe and Nakazawa in 1996 used a transposon mutagenesis protocol followed by complementation with a gene bank to generate *B. cepacia* protease-negative mutants and to subsequently complement the mutated gene (Abe *et al.* 1996). Fourteen such mutants were generated, 13 were both protease and lipase-negative. Of these, one retained 5% proteolytic activity, and one retained 5% lipase activity (Nakazawa and Abe 1996). One mutant was protease negative and lipase positive. Complementation of this mutant yielded a homolog of *dsbB* of *E. coli* and *P. aeruginosa* (Abe and Nakazawa 1996). DsbB is a disulfide bond-forming protein involved in processing and folding of protein precursors via disulfide bond formation in the periplasm before export across the outer membrane by the general secretory pathway. The *B. cepacia dsbB* mutant only produced the 40 kDa protease but not PSCP in culture supernatants and was immotile. Mutants of *dsbB* in *E. coli* were immotile due to the inability to form disulfide bonds in the P-ring protein of flagellar basal bodies (Abe and Nakazawa 1996). Motility and PSCP production in the *B. cepacia* mutant was restored by the addition of L-cystine or by complementation with the *B. cepacia dsbB* DNA fragment (Abe and Nakazawa 1996).

Complementation of the lipase negative and 5% protease positive mutant yielded a DNA fragment homologous to an inner membrane protein of the general secretory pathway present in *P. aeruginosa* (*xcpS*); upstream of this fragment was a sequence homologous to a cytoplasmic ATP-binding protein of the general secretory pathway from *P. aeruginosa*, *xcpR*. Thus, PSCP and lipase appear to be secreted by

the general secretory pathway and the *dsbB* gene product is required for processing and secretion of PSCP in *B. cepacia* (Nakazawa and Abe 1996).

1.7 Hypothesis and Objectives

The hypothesis of this project is that the 40 kDa protease is a precursor of PSCP. Alternatively the 40 kDa protease may share common epitopes with PSCP but be coded for by a separate protease gene. The constitutive high-level expression of the 40 kDa protease may signify it as an important virulence factor in *B. cepacia* infection of the respiratory tract in CF patients. The purposes of this project were to determine the relationship of the 40 kDa protease to PSCP, and to determine the properties of the 40 kDa protease. In clinical strain Pc715j, both PSCP and the 40 kDa protease are produced with the 40 kDa protease being the predominant extracellular protein throughout the growth cycle.

Three objectives were defined to fulfill the purposes of this project. The objectives of this study were to purify the 40 kDa protease, chemically digest it and perform N-terminal sequencing on internal peptides to obtain amino acid sequences to design degenerate probes. These probes were to be used to attempt to clone the gene for the 40 kDa protease. Additionally, the structure and function of the 40 kDa protease was to be determined via physical characterization, substrate specificity, homology studies and expression studies.

2.0 MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

Bacterial strains used in this study are described in Table 1 and were stored in 10% (w/v) skim milk (Difco Laboratories, Detroit, MI) at -70°C. Plasmids used in this study are described in Table 2; plasmid DNA was stored at -20°C.

2.2 Growth Media and Culture Conditions

Growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* for genetic manipulations was performed in Luria (L-B) broth, or on Luria agar (L-B) plates (Gibco BRL, Life Technologies, Gaithersburg, MD).

Strains to be cultured were maintained on solid media: *E. coli* on L-B; *P. aeruginosa* and *B. cepacia* on L-B or M-9 (1 g/L NH_4Cl, 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 5 g/L NaCl, 0.5% glucose (w/v), 0.1 g/mL $MgSO_4$, 15 g/L agar) (Pugsley and Reeves, 1976).

For protease purification (2.4) and for TCA precipitation of extracellular proteins (2.8), *B. cepacia* strains were grown in peptone-tryptone soy broth (PTSB) liquid media (Difco Laboratories, Detroit, MI) (5% Bacto-Peptone(w/v), 0.25% tryptic soy broth (w/v)).

For detection and analysis of protease expression, strains were plated on skim milk/brain heart infusion (SM/BHI) agar (Sokol *et al.* 1979).

For transduction (2.20), *E. coli* strain HB101 was grown in TM broth (1% Bacto-Tryptone (Difco Laboratories, Detroit, MI) (w/v), 0.5% NaCl (w/v), 0.2% maltose (w/v)).

When appropriate, antibiotics were added at the following concentrations to solid and liquid media: *E. coli* -- ampicillin 50-100 μg/mL, tetracycline 15-30 μg/mL, kanamycin 30-50 μg/mL; *P. aeruginosa* strain PA103-11 -- tetracycline 100 μg/mL; *B. cepacia* strain Pc22-12 -- tetracycline, 100-200 μg/mL.

2.3 Enzymes and Chemicals

Restriction endonucleases, DNA and protein molecular weight markers, Taq polymerase, agarose, Tris (Hydroxymethyl)Aminomethane, acrylamide, glycine and urea were purchased from Gibco-BRL, Life Technologies, Gaithersburg, MD. Lysozyme, RNaseA, EDTA (ethylenediaamine tetraacetic acid, disodium salt), X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside), salmon sperm DNA, glutathione, maleic acid, Tricine, bovine serum albumin (BSA), hide powder azure, elastin congo red, ethidium bromide, Coomassie Brilliant Blue R-250, holotransferrin, apotransferrin, lactoferrin (iron-saturated and iron-depleted forms), IgG, IgM, IgA, secretory IgA, fibronectin from bovine plasma, PMSF (phenylmethylsulfonylfluoride) and DTT (dithiothreitol) were purchased from Sigma Chemical Co., St. Louis, MO. DEAE-ion exchange resin, Sephadex G-75, 2'-deoxynucleoside-5' triphosphates, PhastGel IEF 3-9, Broad pI Calibration Kit and the Oligolabelling Kit were purchased from Pharmacia Biotech, Uppsala, Sweden. The DuPont NEP 5'-End Labelling

System and GeneScreen Plus nylon membranes were purchased from NEN Research Products, Dupont, Boston, MA. The GENECLEAN II kit was purchased from Bio 101 Inc., La Jolla, CA. T4 DNA ligase was purchased from Promega. Tris-hydrochloride was purchased from Terochem Laboratories, Edmonton, AB. ATP[γ-^{32}-P] and 3,4-DCL (3,4-dichloroisocoumarin) were obtained from ICN Pharmaceuticals Inc., Costa Mesa, CA. SDS (sodium dodecyl sulfate) and the Bio-Rad Protein Assay were purchased from Bio-Rad Laboratories, Hercules, CA. The Pierce BCA Protein Assay was purchased from Pierce, Rockford, IL. Polyethylene glycol (PEG 8000), cesium chloride, sodium thioglycollate and maltose were purchased from Fisher Scientific Company, Fair Lawn, NJ. NCS (N-chlorosuccinimide) was purchased from Aldrich Chemical Company Inc., Milwaukee, WI. The Gigapack II packaging extract was purchased from Stratagene Cloning Systems, La Jolla, CA. The TA Cloning Kit was purchased from the Invitrogen Corporation, San Diego, CA. The Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit was obtained from Applied Biosystems, Perkin Elmer, Norwalk, CT.

2.4 Protease Purification from *Burkholderia cepacia* strain Pc715j

PSCP and the 40 kDa protease were purified from strain Pc715j using the protease purification protocol from McKevitt *et al.* (1989). A 10 mL starter culture of strain Pc715j in PTSB media was used to inoculate 4 L of PTSB which was then grown overnight at 32°C for a minimum of 20 hours. Preparations were kept at 0°C-

4°C for all subsequent steps. The cultures were centrifuged for 20 min at 10 000 x g; supernatants were then precipitated by slowly adding ammonium sulfate to 60% saturation, and stirred overnight. The precipitate was recovered by centrifuging for 20 min at 10 000 x g; pellets were then resuspended in 15 mL of 50 mM Tris pH 8.0 4°C and dialysed overnight (12000-14000 MW cutoff) against 4 L of 10 mM Tris pH 8.0 4°C. The dialysate was assayed for proteolytic activity by the hide powder azure assay (2.9.1) and was then stored at -80°C.

The 40 kDa protease was purified by a 0-1 M NaCl gradient on a DEAE-Sephacel ion-exchange column (Pharmacia). The resin was equilibrated in 50 mM Tris buffer (pH 8.0) at 4°C in a 2.6 x 40 cm column at 4°C. The dialysate (15 mL) was applied to the column. Elution with the equilibrating buffer yielded PSCP in the first 150 mL; 100 mL was then washed through and discarded. A 0-1 M NaCl gradient was applied to the column, and fractions (90 x 5 mL) were collected. The fractions were read for absorbance at 280 nm and then electrophoresed on an SDS-PAGE gel (2.6). Fractions containing the 40 kDa protease were pooled, dialyzed against 4 L of 10 mM Tris pH 8.0 4°C, lyophilized, resuspended in deionized water and stored at -70°C.

The 40 kDa protease was further purified from PSCP and other contaminating proteins using a Sephadex G-75 gel filtration column (Pharmacia). The resin was equilibrated in 10 mM Tris pH 8.0 4°C buffer in a 1.6 x 40 cm column. The 40 kDa protease sample was loaded onto the column; 25 mL was then washed through with the equilibrating buffer. Fractions (90 x 0.5 mL) were collected, read for absorbance

at 280 nm and electrophoresed by SDS-PAGE gel electrophoresis (2.6). Fractions containing the 40 kDa protease were pooled and stored at -70°C. The amount of 40 kDa protease in these samples was quantified by the Pierce BCA Protein Assay.

2.5 NCS(N-chlorosuccinimide) digestion of the strain Pc715j proteases (Lischwe and Ogs, 1982)

Chemical digestion of the Pc715j 40 kDa protease was performed using NCS (Aldrich). The digestion reaction (35 µg 40 kDa protease, 7.5 M urea, 45% acetic acid (v/v), 100 µL deionized water, final volume of 1 mL) had 75 µl removed as a non-digested control; then 125 µL of fresh 0.11 M NCS was added for a final concentration of 0.014 M NCS. This reaction was incubated at 37°C for 60 min and then stopped by adding 500 µL of cold 20% trichloroacetic acid (v/v) (TCA) plus 10 µg BSA and precipitated at -20°C for one hour. After centrifuging at 15 000 x g for 30 min at 4°C, pellets were washed twice with 1:1 ethanol:ether. Pellets were then air-dried and resuspended in 40 µL10 mM Tris pH 8.0 4°C. Sample buffer (recipe: 2.7) was added to the digested protease and samples were electrophoresed on a Tricine SDS-PAGE gel (2.6.1). Digestion of the 40 kDa protease yielded a 21, 24 and 28 kDa fragment.

2.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The discontinuous system of Laemmli (1970) with a 5% stacking gel and a 7.5%, 12.5% or 15% resolving gel was used to electrophorese protein samples.

Samples were boiled for 5 min in a 1X sample buffer (0.25 M Tris-HCl, pH 6.8, 10.0% (w/v) SDS, 50.0% (v/v) glycerol, and 5.0% (w/v) 2-mercaptoethanol, pH with 10.0 N NaOH to 6.8). Electrophoresis was carried out at 25 mA constant current until samples reached the resolving gel, when the current was increased to 35 mA. Gels were first fixed (20% methanol (w/v), 10% trichloroacetic acid (w/v)); stained with Coomassie Blue stain (0.25% Coomassie Brilliant Blue R-250 (Sigma) (w/v), 25.0% methanol (v/v), 10.0% acetic acid (v/v)), and then destained (10.0% methanol (v/v), 10.0% acetic acid (v/v)).

2.6.1 Tricine SDS-PAGE

The Tricine SDS-PAGE system (Schagger and Von Jagow, 1987) was used for better resolution of the peptides obtained from NCS digestion of the 40 kDa protease. The anode buffer (0.2 M Tris, pH to 8.9 with HCl), the cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS (w/v), pH 8.25) and 30% acrylamide (w/v) (30:0.6 acrylamide:bis-acrylamide (w/v)) were filtered to remove impurities when electroblotting gels onto PVDF membranes (2.7) for amino-terminal amino acid sequencing. Gels were poured using one gel buffer (3.0 M Tris base, 0.3% SDS (w/v), pH to 8.45 with HCl) for all three components(15.0% bottom resolve gel, 10.0% middle resolve gel, 5.0% stacking gel). For electroblotting onto a PVDF membrane for Edman degradation (2.7), the Tricine SDS-PAGE gel was electrophoresed prior to loading for two hours at 3 mA constant current with 25 μM glutathione in the cathode buffer in the upper reservoir. Sample buffer was as for the SDS-PAGE protocol (2.6) but excluded 2-mercaptoethanol; samples were not boiled

but instead heated to 37°C for 5 min and then electrophoresed on the Tricine SDS-PAGE gel (2.6.1) at 15 mA for 20 hours with 0.1 mM thioglycollate fresh cathode buffer in the upper reservoir.

2.6.2 Isoelectric focusing (IEF) using Pharmacia PhastSystem

Isoelectric focusing was performed with the Pharmacia PhastSystem using PhastGel IEF 3-9 preformed gels and the Pharmacia Broad pI Calibration Kit. The preformed gels were prefocused for 75 volthours (Vh). Samples were focused on the preformed gels according to the manufacturer's instructions. Samples were loaded in duplicate at the cathode and the anode end of the gel. The 40 kDa protease (~150 ng) and the pI markers (~8 ng of each pI marker) were focused on the gel for 500 Vh; PSCP (~300 ng) required 600 Vh to come to its pI. Gels were then silver stained in the PhastSystem development chamber according to the manufacturer's instructions

2.7 Blotting SDS-PAGE onto PVDF (polyvinylidene difluoride, Bio-Rad) membranes for Edman degradation

Tricine SDS-PAGE gels (2.6.1) were electroblotted onto Bio-Rad TransBlot Transfer PVDF membranes (Bio-Rad Laboratories, Hercules, CA) for amino-terminal amino acid sequencing (Edman degradation). The PVDF membrane was equilibrated in methanol and then in 0.5X Towbin buffer (250 mM Tris-Base, 1.92 M glycine, dilute 20X and add 10.0% methanol (v/v)) (Towbin, 1979) and electroblotted at 1 mA constant current for 2 hours (Hoefer Scientific Instruments, TE Series Transphor Electrophoresis Unit, San Francisco, CA). The PVDF blot was stained with PVDF

CBB R-250 membrane stain (0.025% Coomassie Brilliant Blue R-250 (w/v), 40.0% methanol (v/v)) for 5 min, then destained for 15 min with 50.0% methanol (v/v) and rinsed with dH_2O. The blot was dried and bands corresponding to the 40 kDa protease peptide fragments were cut out and sent to the protein sequencing facility.

2.7.1 Primer design from N-terminal amino acid sequences

The amino-terminal amino acid sequences obtained by Edman degradation of the 21 and 28 kDa peptide fragments from the 40 kDa protease were utilized as a basis for designing a DNA primer in the reverse complementarity from each fragment. These primers were used to screen the Pc715j genome for the structural gene for the 40 kDa protease (2.14, 2.15). The PC/GENE (PROBE) program was utilized to search for the least redundant region in the amino acid sequences for designing a DNA primer. This was combined with selecting for the most probable codon for redundant amino acids by utilizing the *Pseudomonas* codon usage table (Wada *et al.* 1992). The *Pseudomonas* codon usage table is based on all of the *Pseudomonas spp.* genes (259) in the GenBank Genetic Sequence Data Bank at the time of publication before the transfer of the group II *Pseudomonas* species to the *Burkholderia* genus (Wada *et al.* 1992, Yabuuchi *et al.* 1992). This allowed the primer with the probable closest similarity to the actual DNA sequence of the 40 kDa protease structural gene to be designed.

2.8 Trichloroacetic acid (TCA) precipitation of extracellular proteins from *B. cepacia* strains

TCA precipitation of the extracellular supernatants of putative protease-negative *B. cepacia* strains was performed to concentrate the extracellular protein for the purpose of determining whether the strains were producing mature, extracellular PSCP or 40 kDa protease. Possible protease-negative strains could then be utilized as hosts in screening the Pc715j cosmid gene bank for protease expression (2.20). Strains to be tested were inoculated from M-9 plates to 10 mL PTSB starter cultures, incubated overnight at 37°C, subcultured the next day to 30 mL PTSB cultures and grown to an optical density of 1.4 at 600 nm to look for production of the *B. cepacia* PSCP and 40 kDa extracellular proteases. Cultures were centrifuged to remove cells and an aliquot of the extracellular supernatant was tested for protease activity by the hide powder azure assay (2.9.1). The proteins in the remainder of the supernatant were precipitated by adding 1 mL of 100% TCA to 14 mLs of supernatant and incubated for a minimum of one hour at 4°C. The precipitated supernatant was centrifuged and the pellet (containing the precipitated extracellular proteins) was resuspended in 0.4 mL of 10 mM Tris buffer pH 8.0 37°C. The protein content in the sample was quantitated by the Bio-Rad Protein Assay, then analyzed by SDS-PAGE gel electrophoresis (2.6).

2.9 Assays for protease activity

2.9.1 Hide powder azure assay

The hide powder azure assay (Sigma) was used to assay for proteolytic activity of the 36 and 40 kDa proteases. Protease was added to 20 mg of hide powder azure

in a final volume of 1.5 mL. The assays were then incubated with shaking for 3 hrs at 37°C. After centrifuging at 15 000 x g for 5 minutes, 1 mL of the supernatant was removed and the absorbance at 595 nm determined. All assays were performed in triplicate. A negative control was included containing buffer and hide powder azure only. For the 40 kDa protease, one unit of enzyme activity was defined as that amount which yielded an increase in absorbance of 0.5 A_{595} units for 3 hours at 37°C at pH 6; for PSCP, one unit of enzyme activity was that which yielded an increase in absorbance of 1.0 A_{595} units for 2 hours under the conditions described for the 40 kDa protease.

To determine the optimum pH for activity of one unit of the 40 kDa protease and PSCP, assays were performed for 3 hours in Tris-maleate buffer at 37°C at pH 5.2, 6, 7, 8 and 8.6; and in glycine-NaOH buffer at pH 8.6, 9, 10 and 10.6. To determine the optimum temperature for activity of one unit of the 40 kDa protease and PSCP, assays were performed at pH 6 for 3 hours in Tris-maleate buffer at 32, 37, 45, 50 and 55°C. Subsequently, hide powder azure assays described below for the 40 kDa protease were performed in Tris-maleate buffer at pH 6 at 47°C for 3 hours.

In some experiments, 1 unit of the 40 kDa protease was preincubated for 30 min at 4°C in Tris-maleate buffer pH 6 with 0.1, 1 or 10 mM EDTA (chelates divalent cations; inhibits metalloproteases), 1,10-phenanthroline (preferentially chelates zinc divalent cations; inhibits zinc metalloproteases), DTT (inhibits cysteine proteases), PMSF (inhibits serine proteases) and 3,4-DCL (inhibits serine proteases) prior to the hide powder azure assays.

In addition, one unit of the 40 kDa protease was preincubated for 30 min at 4°C with 0.1 mM EDTA and various divalent cations: 10, 100 and 1000 μM $ZnCl_2$; 0.1, 1 and 10 mM $CaCl_2$, 0.1, 1 and 10 mM $MnCl_2$, 0.1 and 1 mM $MgCl_2$ and 0.1 and 1 mM $FeCl_3$ prior to performing the hide powder azure assay.

2.9.2 Neutralization assays

One unit of the 40 kDa protease was preincubated for 30 min at 4°C with the monoclonal antibody 36-6-8 (Kooi *et al.*, 1994) at 1/250 dilution; controls were included without antibody containing control ascitic fluid. Similar experiments were performed with polyclonal sera to peptide 15 and peptide 42 from elastase (Kooi *et al.* 1996) also at 1/250 dilution; controls were included without antisera using rabbit serum. The hide powder azure assay was subsequently performed on these preparations.

2.9.3 Elastin Congo Red assay

The 40 kDa protease was tested for elastase activity using the elastin congo red (ECR) assay (Bjorn *et al.* 1979). Five units of the protease were incubated with 10 mg of ECR in 1 mL of 0.1 M Tris-maleate buffer pH 7.0 containing 1 mM $CaCl_2$ for 3 hours at 47°C; the blank contained buffer and substrate only, and elastase (10 μg) was included as a positive control. The reaction was terminated by adding 0.5 mL of sodium phosphate buffer pH 6.0, centrifuged and the absorbance at 495 nm determined on 1 mL of the supernatant.

2.9.4 Substrate studies

The 40 kDa protease (3 μg) was incubated with 10 μg of various substrates for 48 hours (24 hours for fibronectin and collagen VIII) at 47°C in Tris-maleate buffer pH 6.0 supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Substrates tested included human holo- and apo-forms of transferrin and lactoferrin, IgG, IgM, IgA, secretory IgA, fibronectin (from bovine plasma) and human collagen type VIII. Controls included the 40 kDa protease and each substrate incubated alone. Reactions were terminated by adding 1X sample buffer with β-mercaptoethanol, and subjected to SDS-PAGE gel electrophoresis (2.6).

2.10 Isolation of Total Genomic DNA from *Burkolderia cepacia* strain Pc715j (Goldberg and Ohman, 1984)

Cultures were grown overnight in 50 mL of Luria broth. Cells were recovered by centrifuging for 10 min at 4°C at 6000 x g, and washed with 25 mL sterile PBS (phosphate buffered saline: 0.06 M Na_2HPO_4, 0.05 M NaH_2PO_4, 0.15 M NaCl). The pellet was resuspended in 10 mL of cold ET buffer (10 mM Tris pH 8.0, 10 mM EDTA). One mg of lysozyme dissolved in one mL ET buffer was added to the cell suspension and incubated for 15 min at 37°C. Cells were lysed with 1.2 mL Sarkosyl-pronase solution (10% Sarkosyl (w/v), 5 mg pronase) in ET buffer; this mixture was incubated with gentle shaking for 1 hour at 37°C. The lysed cells were then extracted twice with 10 mL TES-saturated phenol (TES: 10 mM Tris pH 8.0 4°C, 1 mM EDTA, 0.05 M NaCl) and twice with 10 mL chloroform:isoamyl alcohol (24:1);

saving the top layer each time. The DNA was precipitated by adding 0.5 mL of 7.5 M ammonium acetate and by slowly layering 20 mL of isopropanol on top of the mixture. The DNA was spooled onto a glass rod by mixing the two phases. The DNA was blotted to remove excess isopropanol and was then dissolved in 8 mL of TE buffer (10 mM Tris pH 8.0 4°C, 1 mM EDTA) and stored at 4°C.

2.11 Agarose gel electrophoresis

DNA samples were electrophoresed on agarose gels ranging from 0.6% to 1.4% in Tris-borate buffer (90 mM Tris base, 2 mM EDTA, 90 mM boric acid). When isolating DNA fragments from an agarose gel (2.12), Tris-acetate buffer (40 mM Tris-acetate, 2 mM sodium EDTA, pH 7.9) was used. Sample dye (0.1% bromophenol blue (w/v), 33% glycerol (v/v)) was added to the DNA samples before loading on the gel. Electrophoresis was performed at 100 V constant voltage for one to three hours or overnight at 10 V. Agarose gels were then stained with ethidium bromide (0.002 mg/mL), and the stained DNA samples were viewed with ultraviolet light.

2.12 Isolating DNA fragments from agarose gels

DNA samples were first digested with restriction endonucleases and then electrophoresed on agarose gels in Tris-acetate buffer (2.11). The desired DNA fragments were excised from the agarose gel and purified using the GENECLEAN II Kit (Bio 101 Inc). Fragments were dissolved at 65°C in two volumes of 6 M sodium

iodide. Glassmilk (10 μL) was added to the suspension and incubated at room temperature for 10 minutes. The suspension was centrifuged at 15 000 x g at room temperature for 30 seconds, and the supernatant was discarded. The Glassmilk pellet was resuspended and then pelleted three times by centrifugation at 15 000 x g for 30 seconds with 600 μL of NEW WASH buffer. To elute the DNA from the Glassmilk, the Glassmilk-DNA pellet was resuspended in 13 μL of sterile deionized water and incubated at 50°C for 2 minutes. After centrifuging at 15 000 x g for 3 minutes, 10 μL of supernatant was carefully removed to a new microfuge tube and stored at -20°C.

2.13 End-labelling oligonucleotides with P^{32}-γdATP

Oligonucleotides were end-labelled with P^{32}-γdATP using the Du Pont NEP-101 5'-End Labelling System for DNA hybridization (2.14). The oligoprobe (2 μg/10 μL), 5 μL of 10X kinase phosphorylation buffer, 24 μL of deionized water, 10 μL of P^{32}-γdATP (10 μCi/μL) and 1 μL of T4 nucleotide kinase were incubated at 37°C for one hour. The reaction was stopped by adding 400 μL of cold Reagent A (0.1 M Tris-HCl, 1.0 mM EDTA, 10 mM triethylamine, pH 7.7). A Nucleic Acid Purification Cartridge (Biotechnology Systems NEN Research Products, Dupont, Boston, MA) was used for purification of the end-labelled oligonucleotides. The column was activated by washing with methanol followed by 2 mL of reagent A. The reaction sample was loaded on the column and washed with 2 mL of reagent A. The end-

labeled oligonucleotides were eluted with 1 mL of 20% ethanol (v/v); they were contained in the first 400 μL.

2.14 DNA Hybridization

Agarose gels (2.11) were blotted using a Pharmacia vacuum blotter onto Gene Screen Plus Hybridization Transfer Membranes (Dupont). DNA in agarose gels was depurinated for 10 min at room temperature in 0.25 N HCl, denatured for 5 min at room temperature in 1.5 M NaCl, 0.5 M NaOH and neutralized for 5 min at room temperature in 1 M Tris, 2 M NaCl, pH 5.0. The transfer membrane was equilibrated in 5XSSC (0.7 M NaCl, 0.07 M trisodium citrate ($Na_3C_6H_5O_7.2H_2O$), pH to 7.0 with citric acid). Gels were transferred to the membrane in transfer solution (20X SSC: 3 M NaCl, 0.3 M trisodium citrate, pH to 7.0 with citric acid),. After transfer, membranes were dried under a red heat lamp.

Blots were equilibrated in 15 mL of hybridization buffer (10% dextran sulfate (w/v), 1% SDS (w/v)) in a hybridization bag. The radiolabelled DNA containing 10 x 10^6 counts per minute (cpm) was denatured by boiling for 5 minutes; 100 μL of salmon sperm DNA solution (10 mg/mL) was then added to the radiolabelled DNA. This mixture was then added to the hybridization bag and incubated overnight with shaking at the desired hybridization temperature. Following this incubation, the blot was washed twice each with 100 mL of 2XSSC (0.3 M NaCl, 0.03 M trisodium citrate) for 5 min at room temperature, 200 mL of 2XSSC plus 1% SDS (w/v) for 30 min at 5°C above the hybridisation temperature, and 200 mL of 0.1X SSC (0.015 M

NaCl, 0.0015 M trisodium citrate) for 30 min at room temperature. The blot was then dried under a red heat lamp and subjected to autoradiography at -70°C using Kodak XAR 5 Scientific Imaging Film (Eastman Kodak Co.).

2.15 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify portions of the gene encoding the 40 kDa protease of *Burkholderia cepacia* strain Pc715j (Perkin-Elmer Cetus DNA Thermal Cycler, Norwalk, CT). The primers designed from the amino acid sequences obtained by Edman degradation of the 40 kDa protease and its NCS-digested peptides (2.7) were used for amplification.

A typical reaction consisted of 50 pmol each of the 40 kDa amino-terminal forward primer, and the 40-21 or 40-28 reverse primer, 200 ng-1 μg of Pc715j chromosomal DNA (boiled for 5 min) (2.10), 5% dimethyl sulfoxide (DMSO) (v/v), 3 mM $MgCl_2$, 0.2 mM dNTP's, 2.5 U (units) Taq polymerase, 1X PCR reaction buffer (Gibco-BRL), and deionized water for a final reaction volume of 100 μL. Mineral oil (75 μL) was then layered on top of the reaction mixture. Reactions were initally denatured at 95°C for 3 min. The DNA was then denatured at 95°C for 1 min, primers were annealed at 55°C or 60°C for 1 min, and products were extended at 72°C for 1 min; for 25-35 cycles. Products were then analyzed by agarose gel electrophoresis (2.11).

2.16 Cloning and sequencing of PCR fragments

2.16.1 Cloning of PCR fragments

PCR fragments were cloned using the TA Cloning Kit (Invitrogen). Ligation reactions containing 5-10 ng of the PCR fragment to be cloned and 50 ng of the pCRII vector were carried out at 12°C for 12 to 18 hours in final volumes of 10 or 20 μL with 1 μL of ligation buffer per 10 μL volume and 1 μL of T4 DNA ligase per reaction. This mixture was transformed by heat shock at 42°C for 30 seconds into *Escherichia coli* strain INVαF' cells, plated on L-B plates containing 50 μg/mL ampicillin and overlayed with 10 μL of 10% (w/v) X-gal, and incubated overnight at 37°C. The following day small-scale plasmid preparations (2.19.1) were performed on white colonies. Restriction digests (2.17) of the plasmids with *Eco*RI separated the insert (PCR fragment) from the vector; allowing the PCR fragment to be identified by its size. Clones were then stored at -70°C.

2.16.2 PCR Cycle Sequencing

Plasmids were isolated from clones of interest using the modified alkaline plasmid preparation protocol (2.19) recommended by UCDNA Sequencing Services (University of Calgary). The plasmids were then either sent directly to the UCDNA Sequencing Services for reaction preparation and sequence analysis, or sequencing reactions (fluorescence-based dideoxy sequencing reactions) were first prepared using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Perkin Elmer) and then sent for sequence analysis. Briefly, the PCR sequencing reaction consisted of 9.5 μL of the terminator premix, 1 μg of the ds DNA plasmid template and 5 pmol of one primer in a final reaction volume of 20 μL.

Reactions were overlayed with mineral oil and then subjected to PCR for 25 cycles (Perkin Elmer Thermal Cycler 480) with the following parameters: denaturation (96°C for 30 seconds), annealing (50°C for 15 seconds) and extension (60°C for 4 minutes). The extension products were then column-purified using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ); the eluant was ethanol-precipitated and the vacuum-dried pellet was then sent to UCDNA Sequencing Services.

2.17 Restriction endonuclease digestion

All restriction endonuclease digestions were performed according to the manufacturers recommendations to ensure optimal enzyme activity and to avoid non-specific DNA cleavage. DNA was routinely digested for 3 to 18 hours.

2.18 Sucrose gradients for DNA

Sucrose gradients (10% to 40%) were prepared using a gradient maker filled with 5.2 mL of 10% sucrose (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM NaCl, 70 mM sucrose) in the far reservoir and 40% sucrose (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM NaCl, 0.3 M sucrose) in the near reservoir. Gradients were prepared in 14 mm by 89 mm polyclear centrifuge tubes (Seton Scientific, Sunnyvale, CA). The DNA sample (75 μg) was layered on top of the gradient and centrifuged overnight at 67 000 x g (based on r_{av}) for 18 hours at 25°C. The following day, 0.5 mL fractions were collected and analyzed by agarose gel electrophoresis (2.11).

2.19 Isolation of plasmid DNA

2.19.1 Alkaline lysis plasmid preparation

Cultures plus the appropriate antibiotic marker were incubated overnight at 37°C and harvested by centrifugation the next day. Pellets were resuspended in 100 µL of GTE buffer (0.5 mM glucose, 0.1 mM EDTA, 2.5 mM Tris HCl pH 8.0) containing 4 mg/mL lysozyme. Cells were lysed by adding 200 µL of lysing solution (1 M NaOH, 1% SDS (w/v)) for 5 minutes at room temperature, and the solution was neutralized by adding 150 µL of 3 M potassium acetate solution, pH 4.8. Insolubles were removed by centrifugation, and RNA was removed by incubating for 30 minutes at 37°C with 1 µL of 10 mg/mL RNaseA. The supernatant was then extracted once with phenol:chloroform:isoamyl alcohol (25:24:1). Plasmid DNA was precipitated with 95% ethanol (v/v) for 30 minutes at -20°C, vacuum-dried and resuspended in 50 µL of sterile deionized water. The plasmid preparations were stored at -20°C.

Alkaline lysis plasmid DNA preparation protocol was modified as recommended by UCDNA Sequencing Services (University of Calgary) when preparing plasmids for automated sequencing (2.16.2). The overnight culture was centrifuged in 1.5 mL aliquots, and pellets were resuspended in 200 µL of the GTE buffer (no lysozyme added). Cells were lysed for 5 min on ice with 300 µL of lysing solution. The solutions were neutralized by adding 300 µL of 3 M potassium acetate pH 4.8. The supernatant was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform. Plasmid DNA was precipitated with 100% isopropanol. Pellets were washed once with 70% ethanol (v/v), vacuum-dried for 3

min and resuspended in 32 µL of deionized water. This preparation was precipitated by adding 8 µL of 4 M NaCl and 40 µL of sterile 13% PEG 8000 (w/v) and incubated on ice for 20 min. Pellets were rinsed with 70% ethanol (v/v) and vacuum-dried for 3 min. Pellets were then resuspended in 20 µL of sterile deionized water and quantified by absorbance at 260 nm. The sample was diluted to 0.5 µg/µL, and 10 µL was provided for sequencing to the UCDNA Sequencing Services.

2.19.2 Cesium chloride gradient large-scale plasmid preparation

Cultures (250 mL) plus the appropriate antibiotic marker were incubated overnight at 37°C. Cultures were centrifuged and were resuspended in 6 mL of glucose buffer (50 mM glucose, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0, filter-sterilized), 1 mL of glucose buffer plus 20 mg lysozyme was added, and the cells were then lysed with 14 mL of lysing solution (0.2 N NaOH, 1% (w/v) SDS). The solution was neutralized with 3 M sodium acetate pH 4.8, centrifuged to remove insolubles and extracted once with phenol:chloroform:isoamyl alcohol (25:24:1). Plasmid DNA was precipitated with isopropyl alcohol; pellets were air-dried and resuspended in 700 uL of TE buffer. Cesium chloride (1.1 g) was dissolved in the preparation, and 80 uL of 10 mg/mL ethidium bromide was added. Gradients were prepared in a 3.0 mL polyallomer Quick-Seal tube (Beckman Instrument, Palo Alto, CA). The tube was half-filled with 65% CsCl (w/v), the DNA solution was layered under the CsCl solution, and the remainder of the tube was filled up with the 65% CsCl solution. The gradient was centrifuged at 344 000 x g for 18 hours at 18°C. Plasmid DNA was removed from the gradient under ultraviolet light, extracted with TES-saturated

butanol to remove the ethidium bromide, and dialyzed overnight in 4 L of 10 mM TE buffer. Plasmid DNA was stored at -20°C.

2.20 Construction of Pc715j Cosmid Gene Bank and Screening by Conjugation in Target Strains

Transduction was used to introduce cosmids (pCP19) ligated (2.17) to large fragments (9-20 kb) of *B. cepacia* Pc715j chromosomal DNA obtained from sucrose gradients (2.18) into *E. coli* strain HB101 to create a cosmid library. The Gigapack II packaging extract (Stratagene) was used for packaging the ligated cosmids into recombinant lambda phage. The host strain (HB101) was incubated for 5 hours at 37°C in 50 mL of TM broth. The cells were harvested by centrifugation and resuspended in 10 mL of L-B broth supplemented with 10 mM $MgSO_4$. Packaging extracts were thawed on ice, 1 µg of ligated cosmid DNA was added to the extract and the packaging mix was incubated for 2 hours at room temperature. The packaging reaction was terminated by adding 500 µL of SM buffer (0.1 M NaCl, 1 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin (w/v), 50 mM Tris-HCl pH 7.5) followed by 25 µL of chloroform. The *in vitro* packaged extract was added to 1 mL of the prepared HB101 cells and incubated at room temperature for 10 min. The transduced cells were then incubated with 3 mL of L-B broth at 37°C for one hour. The cells were plated (100 µL/plate) on selective media (L-B media plus 15 µg/mL tetracycline) and incubated overnight at 37°C. The following day, colonies were pooled by adding 1 mL L-B broth plus 15 µg/mL tetracycline to each plate and scraping off all the colonies.

Glycerol was added to the pooled colonies at a concentration of 16% (v/v), and the cosmid library was stored at -70°C in 2 mL aliquots.

The Pc715j cosmid gene bank in HB101 (100 μL) was inoculated to 50 mL of L-B broth plus 15 μg/mL tetracycline and incubated overnight at 37°C. The absorbance at 600 nm was determined for the culture and 50 μL of a 10^{-6} dilution of the culture for obtaining single colonies was plated on 40 SM/BHI plates to assess possible protease expression in the *E. coli* HB101 host.

Triparental matings were used to conjugate the *B. cepacia* Pc715j cosmid library into *P. aeruginosa* strain PA103-11 and *B. cepacia* strain Pc22-12. The donor strain (Pc715j cosmid library in HB101) and the helper strain which provides the conjugation functions *in trans* (*E. coli* HB101 [pRK2013]) were grown overnight at 37°C and the recipient strain (PA103-11 or Pc22-12) at 42°C to prevent activation of restriction systems. The following day, 0.3 mL of each strain was mixed together and centrifuged; the pellet containing the cells was then resuspended in 0.1 mL PBS (phosphate-buffered saline: 60 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.15 M NaCl). The suspension was then placed on sterile 2.5 cm diameter filters (Sartorious Canada Inc.) on L-B plates and incubated overnight at 37°C. The following day, the filter was vortexed in 3 mL PBS, and the cell suspension was plated on M-9 plates supplemented with 100 μg/mL (PA103-11) or 200 μg/mL tetracycline (Pc22-12) to select for the recipient strain containing the donor plasmid. These plates were incubated at 37°C; colonies were then picked to SM/BHI plates to select for clones exhibiting protease expression.

Table 1 -- Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/Reference
I. *Escherichia coli*		
HB101	Sm^r, *pro*, *leu*, *thi*, *lac*Y, *hsd*-20, *end*A, *rec*A, *ara*-14, *gal*K, *xyl*-5, *mtl*-1, *sup*E44	Boyer *et al.* (1969)
INVαF'	F'*end*A1, *rec*A1, *hsd*R17 (r_k^-,m_k^+), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169, λ-	Invitrogen
II. *Pseudomonas aeruginosa*		
PAO	prototypical strain	Holloway *et al.* (1979)
PA103-11	elastase negative, EMS mutant of PA103 deficient in alkaline protease production	Guzzo *et al.* (1991)
III. *Burkholderia cepacia*		
Pc715j	CF	McKevitt & Woods (1984)
K30-6	CF	McKevitt & Woods (1984)
K56-2	CF	McKevitt & Woods (1984)
Pc22-12	plant strain, protease negative	Cleveland, OH
15278	isolated from skull bone infection	Dr. R. Read (1993)
K36-2	CF	McKevitt & Woods (1984)
K37-3	CF	McKevitt & Woods (1984)
K43-3	CF	McKevitt & Woods (1984)

Table 2 -- List of Plasmids

Plasmid	Characteristics	Source/Reference
pUC19	Amp^r, MCS of M13mp19, α-peptide of lacZ, 2.69 kb, high copy number	Yanisch-Perron (1985), BRL
pRK2013	RK2 tra, Km^r, ColE1	Figurski and Helinski (1979)
pCRII	Amp^r, Km^r, α-peptide of lacZ, lac promoter, ColE1, T7 and Sp6 promoters and primers, M13 (-20 and -40) forward and M13 reverse primers, f1 origin	Invitrogen
pCP19	Tc^r, IncP λcos^+, broad-host range cloning cosmid vector derived from pLAFR1, multiple cloning site (six sites)	Friedman et al. (1982)
pVS21-180	pCRII with *Eco*RI-*Eco*RI 180 bp PCR product amplified from Pc715j by primers 40N and R40-21, Amp^r, Kan^r	This study
pVS21-340	pCRII with *Eco*RI-*Eco*RI 340 bp PCR product amplified from Pc715j by primers 40N and R40-21, Amp^r, Kan^r	This study
pVS21-425	pCRII with *Eco*RI-*Eco*RI 425 bp PCR product amplified from Pc715j by primers 40N and R40-21, Amp^r, Kan^r	This study
pVS28-1000	pCRII with *Eco*RI-*Eco*RI 1000 bp PCR product amplified from Pc715j by primers 40N and R40-28, Amp^r, Kan^r	This study
pVS21-1050	pCRII with *Eco*RI-*Eco*RI 1050 bp PCR product amplified from Pc715j by primers 40N and R40-21, Amp^r, Kan^r	This study

3.0 RESULTS

3.1 Protease purification from *Burkholderia cepacia* strain Pc715j

B. cepacia strain Pc715j (a clinical CF strain) produces at least two extracellular proteases, PSCP (36 kDa by SDS-PAGE, 34 kDa by gel filtration) and the 40 kDa protease. PSCP was previously purified and characterized by McKevitt *et al.* (1989) as the major extracellular proteolytic enzyme produced by *B. cepacia* strain Pc715j. The 40 kDa protease was purified by Kooi *et al.* (1994) and described as a second extracellular protease produced by strain Pc715j.

In this study, purification of the 40 kDa protease was performed for the purposes of characterization of its physical parameters for optimal enzymatic activity, its classification within the bacterial protease superfamily, and its substrate specificity in reference to its role as a virulence factor in opportunistic respiratory infections in cystic fibrosis patients. Purified 40 kDa protease was also chemically digested by NCS (N-chlorosuccinimide) for the purposes of N-terminal sequencing on internal peptides to obtain primers from the amino-terminal amino acid sequences for cloning the protease's structural gene.

B. cepacia Pc715j PTSB cultures were grown for 18-20 hours, and ammonium sulfate-precipitated culture supernatants (Fig. 1, lane 2) were chromatographed on a DEAE-Sephacel ion exchange column. PSCP was pooled from the first 250 mL of the Tris-HCl wash. It had previously been found in the first 150 mL both by McKevitt *et al.* (1989) and by Kooi *et al.* (1994). A 0-1 M NaCl gradient applied to the column

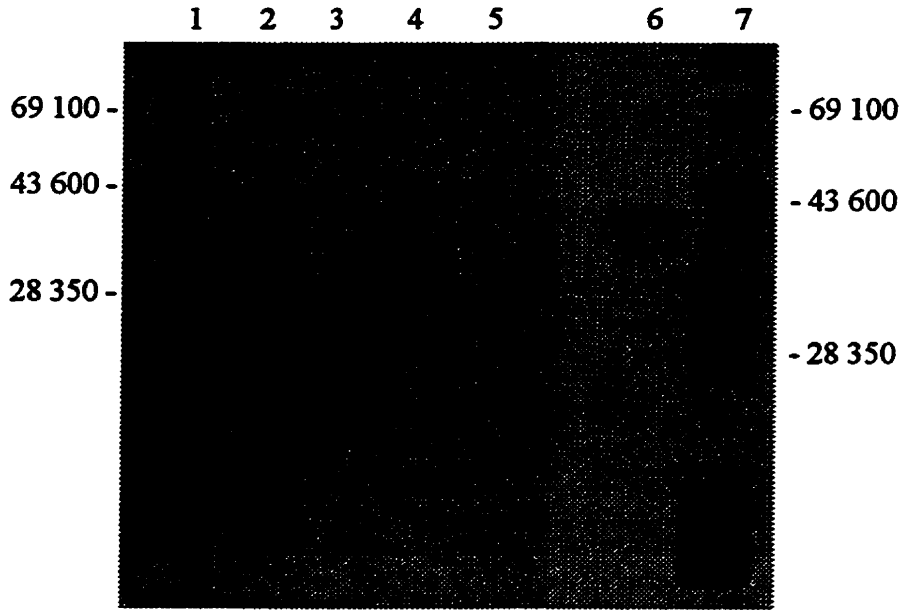


Figure 1. SDS-PAGE (12.5%) (Coomassie blue stain) analysis of the purification steps of the *B. cepacia* Pc715j 40 kDa protease and of a PSCP preparation. Lanes 1 and 7, Gibco BRL prestained molecular markers (indicated in kilodaltons); lane 2, ammonium sulfate-precipitated culture supernatants (25 uL); lane 3, 40 kDa protease eluted from DEAE-Sephacel (12 uL); lane 4, 40 kDa protease eluted from G-75 gel filtration column (5 ug in 64 uL) ; lane 5, second 40 kDa protease preparation eluted from G-75 gel filtration column (3 ug in 64 uL); lane 6, PSCP eluted from DEAE-Sephacel (2 ug in 5 uL).

(Fig. 2) eluted the 40 kDa protease in the first 43 fractions (215 mL), with the first 31 fractions (155 mL) containing the 40 kDa protease with few contaminants as observed by SDS-PAGE (Fig. 1, lane 3). Previous protease purifications in this laboratory identified the presence of the 40 kDa protease by SDS-PAGE as being associated with proteolytic activity when measured by the hide powder azure assay. Lyophilization and dialysis of the pooled fractions containing the 40 kDa protease yielded samples sufficiently pure for chemical digestion (Fig. 9, lane 3) as described in section 3.3 for the purposes of N-terminal sequencing. The 40 kDa protease was further purified by Sephadex G-75 gel filtration chromatography (Fig. 1, lanes 4 and 5) for characterization studies described in section 3.2. The PSCP preparation used for characterization of the pH and temperature optimum (ref. 3.2.1) is shown in figure 1, lane 6.

3.2 Characterization of the 40 kDa protease

The 40 kDa protease was characterized to determine its possible relationship to PSCP as a precursor or related protease, to classify it within the realm of bacterial extracellular proteases, and to ascertain its potential as a virulence factor in opportunistic respiratory *B. cepacia* infections in cystic fibrosis patients.

3.2.1 The effect of pH and temperature on proteolytic activity

Physical classification was the first step in studying the similarities between the 40 kDa protease and PSCP, and in determining the optimal physical parameters for enzymatic activity of the 40 kDa protease. The 40 kDa protease (one unit) and PSCP (one unit) were assayed for optimum activity in the pH range 5.2-8.6 in Tris-maleate

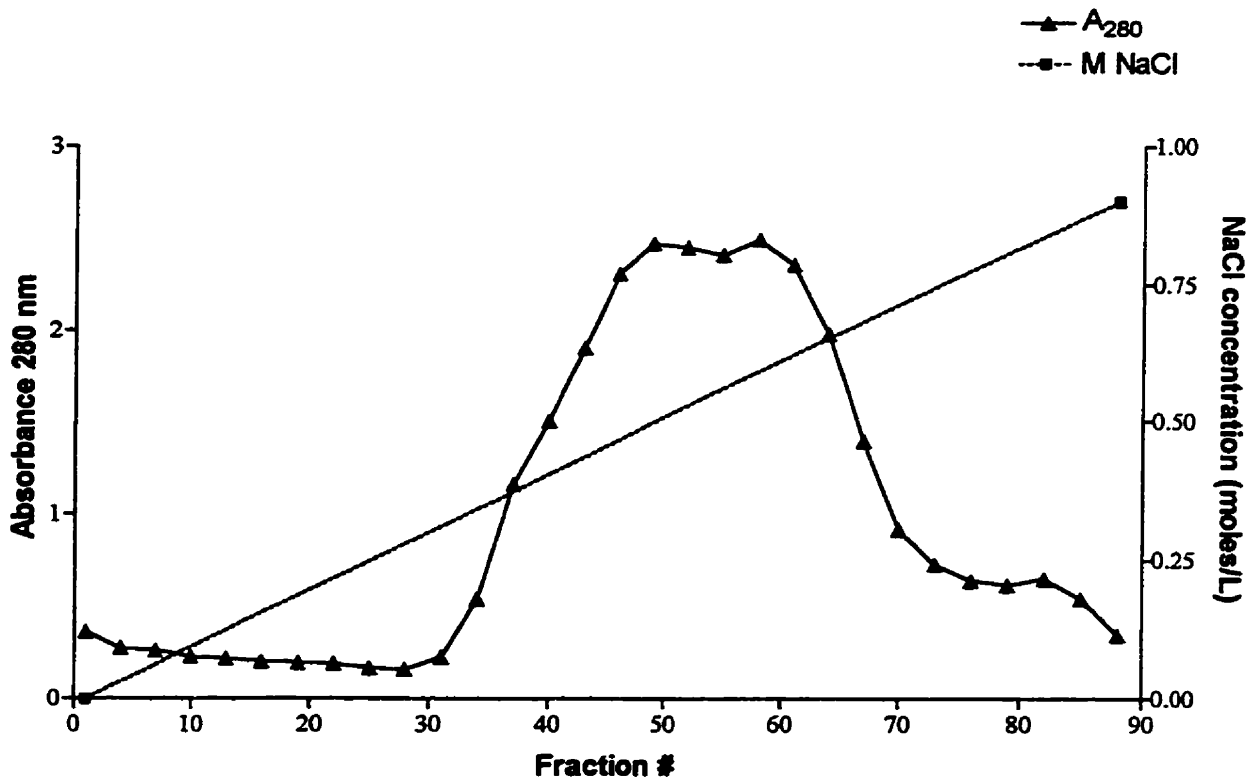


Figure 2. Elution profile of 0 to 1 M NaCl gradient applied to DEAE-Sephacel ion-exchange chromatography. Elution was monitored by A_{280} to measure protein. Five mL fractions 1 to 12, 13 to 31 and 32 to 43 were pooled and concentrated according to the presence of the 40 kDa protease as assessed by SDS-PAGE.

buffer and from pH 8.6-10.6 in glycine/NaOH buffer by the hide powder azure assay. The 40 kDa protease was found to be optimally active at pH 6.0 in Tris-maleate buffer; PSCP was optimally active at pH 6-7, as was previously reported by McKevitt *et al.* (1989) (Fig. 3). The temperature optimum was also determined by the hide powder azure assay for the 40 kDa protease and for PSCP. The two proteases were assayed at 32, 37, 45, 50 and 55°C; both were found to be optimally active at 50°C (Fig. 4). Thus, the two proteases had identical pH and temperature for optimal proteolytic activity.

3.2.2 Determination of the isoelectric points (pI) of the *B. cepacia* proteases

The isoelectric point is a defining property of the protein in question reflecting the amino acid composition of the protein and the ratio of its charged groups. Thus, determining the isoelectric point of PSCP and the 40 kDa protease was important in further ascertaining the relationship between these two proteases. The pI of the two proteases was determined on a broad-range pH 3-9 gradient preformed gel using the PhastGel system from Pharmacia. The Pharmacia broad-range pI markers from pH 3 to 9 were used to determine the pI of PSCP and the 40 kDa protease. PSCP required more volthours than did the 40 kDa protease to come to its final position in the gel; at which it lined up with the 40 kDa protease at a pI of ~5.2 (Fig. 5). Thus, both proteases appeared to have the same isoelectric points, perhaps reflecting a highly similar amino acid composition.

3.2.3 Determination of the class of protease for the 40 kDa enzyme

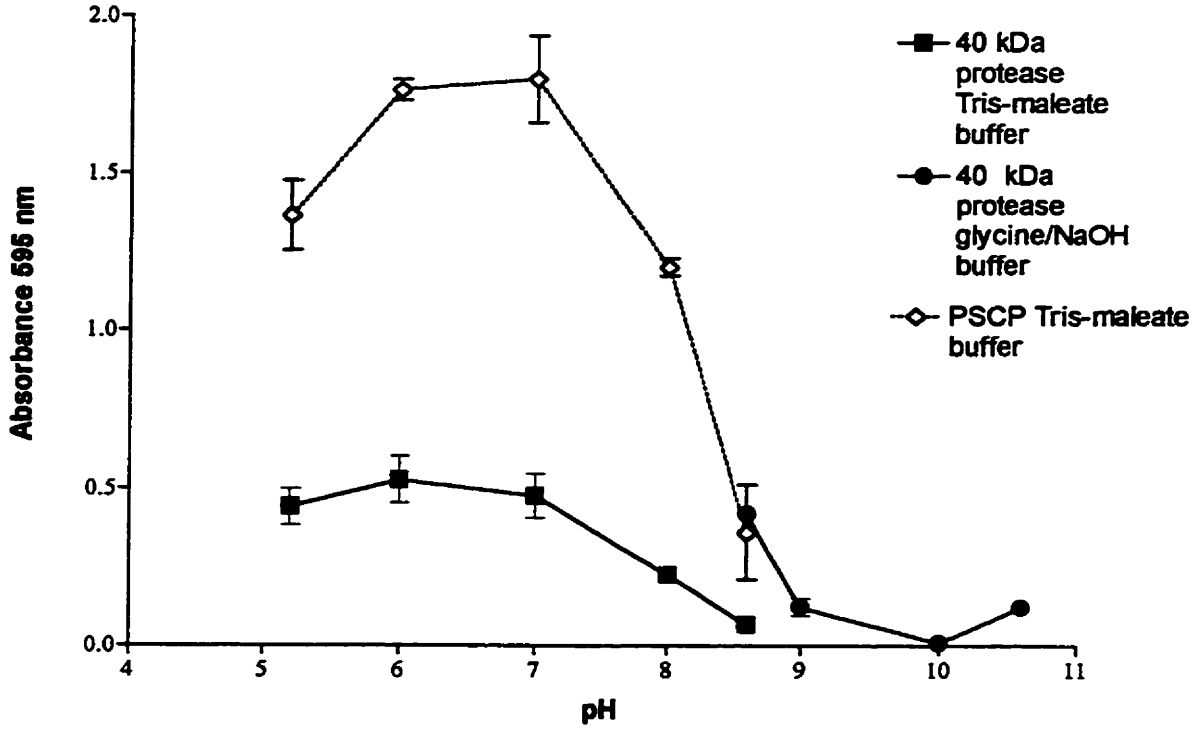


Figure 3. Effect of pH on the activity of *B. cepacia* 40 kDa protease. Activity was determined using hide powder azure as a substrate. Values represent the mean (+/- standard deviation) A_{595} of triplicate assays.

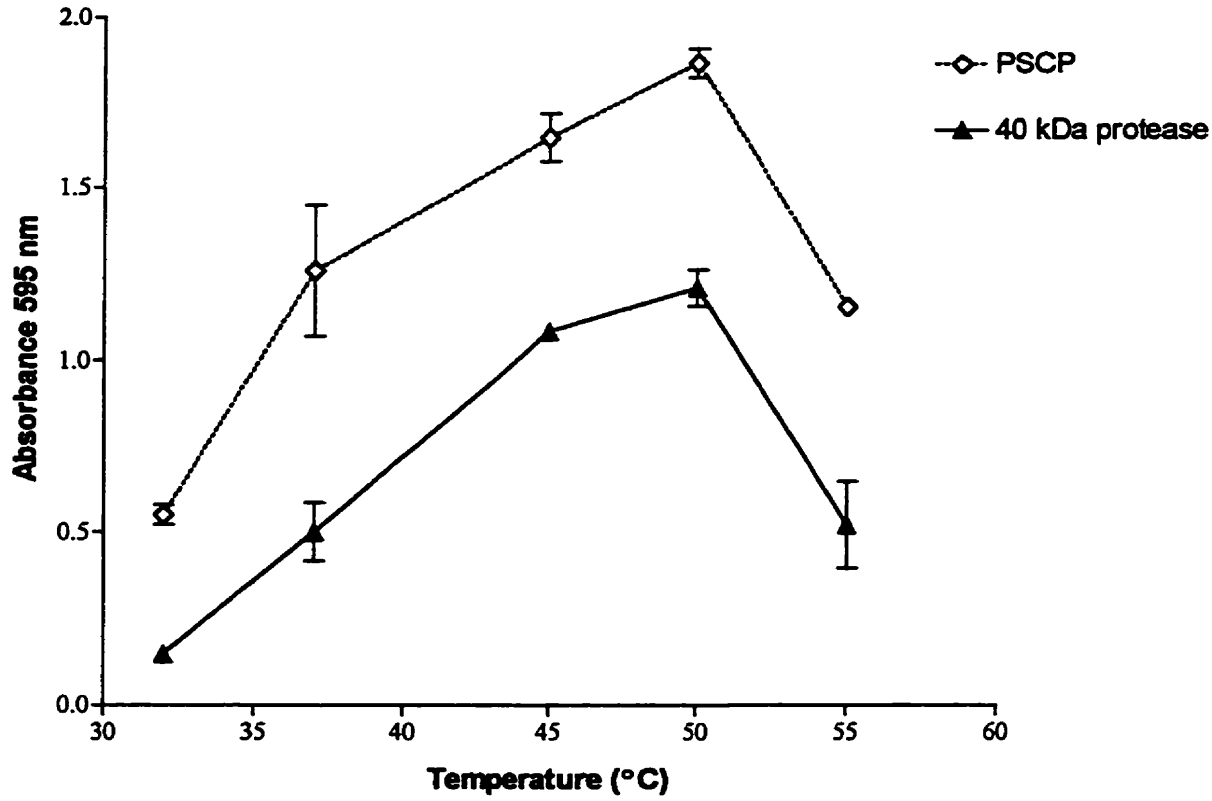


Figure 4. Effect of temperature on the activity of *B. cepacia* 40 kDa protease. Activity was determined using hide powder azure as a substrate. Values represent the mean (+/- standard deviation) A_{595} of triplicate assays.

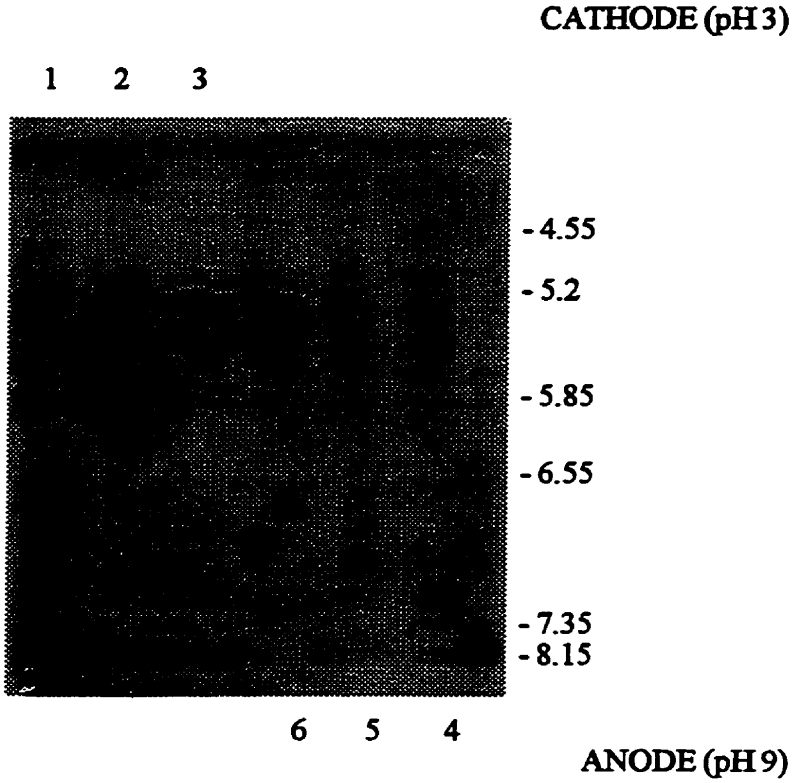


Figure 5. Pharmacia PhastGel IEF 3-9 (silver stain) of the 40 kDa protease and PSCP. Lanes 1 and 4, Pharmacia broad-range pI calibration markers; lanes 2 and 5, PSCP (200 ng); lanes 3 and 6, 40 kDa protease (150 ng).

The inhibition of activity by the 40 kDa protease by various class-specific protease inhibitors was instrumental in classifying the 40 kDa protease. Inhibitors chosen encompassed three classes of the bacterial extracellular proteases: the cysteine proteases (DTT), the serine proteases (PMSF and 3,4-DCL) and the metalloproteases (EDTA and 1,10-phenanthroline) (Beynon and Bond, 1989).

Activity of the 40 kDa protease was assayed by the hide powder azure assay in the presence of specific protease inhibitors (Table 3). The level of inhibition was calculated as a percentage of the proteolytic activity with no inhibitors present. Dithiothreitol (DTT) partially inhibited 40 kDa activity (31% remaining activity) at high (10 mM) concentrations. Serine protease inhibitors had mixed effects: phenylmethylsulfonylfluoride (PMSF) (can also inhibit cysteine proteases) could totally inhibit 40 kDa protease activity at low (0.1 mM) concentrations; 3,4-dichlorisocoumarin (3,4-DCL) only partially inhibited 40 kDa protease activity (39.5% remaining activity) at high (100 μM) concentrations. The lack of inhibition by the more specific serine protease inhibitor, 3,4-DCL, helped to eliminate the serine protease category in classifying the 40 kDa protease (Beynon and Bond, 1989). EDTA (ethylenediaaminetetracetic acid), which chelates divalent cations, totally inhibited 40 kDa protease activity at low (0.1 mM) concentrations as did 1,10-phenanthroline. Inhibition by EDTA indicated that the 40 kDa protease required divalent metal cations for activity. Inhibition by 1,10-phenanthroline, which preferentially chelates zinc cations, helped to place the 40 kDa protease in the zinc

TABLE 3. Effect of protease class-specific inhibitors on *B. cepacia* 40 kDa protease activity

Addition to reaction		Proteolytic activity[a]	% control
Buffer	**Inhibitor**		
control	none (control)	0.53 ± 0.03[b]	100
	10 mM EDTA	-0.30 ± 0.00 **	0
	1 mM EDTA	-0.30 ± 0.00 **	0
	0.1 mM EDTA	-0.26 ± 0.03 **	0
	10 mM DTT	0.16 ± 0.21 **	30.6
	1 mM DTT	0.62 ± 0.06	>100
	0.1 mM DTT	0.70 ± 0.17	>100
+ 300 µL methanol	none (control)	0.50 ± 0.09	100
+ 300 µL methanol	10 mM 1,10-Phe	-0.20 ± 0.07 **	0
+ 30 µL methanol	none (control)	0.75 ± 0.15	100
+ 30 µL methanol	1 mM 1,10-Phe	0.03 ± 0.13 **	3.7
+ 3 µL methanol	none (control)	0.46 ± 0.08	100
+ 3 µL methanol	0.1 mM 1,10-Phe	-0.10 ± 0.18 **	0
+ 75 µL methanol	none (control)	0.67 ± 0.10	100
+ 75 µL methanol	10 mM PMSF	-0.04 ± 0.03 **	0
+ 7.5 µL methanol	none (control)	0.72 ± 0.16	100
+ 7.5 µL methanol	1 mM PMSF	0.16 ± 0.16 *	22.5
+ 0.75 µL methanol	none (control)	0.55 ± 0.05	100
+ 0.75 µL methanol	0.1 mM PMSF	-0.00 ± 0.03 **	0
+ 30 µL DMSO	none (control)	0.68 ± 0.09	100
+ 30 µL DMSO	100 µM 3,4-DCL	0.27 ± 0.11**	39.5
+ 3 µL DMSO	none (control)	0.78 ± 0.17	100
+ 3 µL DMSO	10 µM 3,4-DCL	0.63 ± 0.06	80.6
+ 0.3 µL DMSO	none (control)	0.67 ± 0.10	100
+ 0.3 µL DMSO	1 µM 3,4-DCL	0.62 ± 0.18	93.5

[a] A_{595} of hide powder azure assays; the background with no protease but containing buffer (and organic solvent, see (b)) was subtracted in each case.

[b] Each value represents the mean ± standard deviation of three determinations. The footnote symbols indicate that the values are significantly different from the values for the control by the two-tailed t test as follows: *, $P<0.05$, **, $P<0.01$.

metalloprotease class (Beynon and Bond, 1989). Thus, preliminary results indicated that the 40 kDa protease belongs to the zinc metalloprotease class as does PSCP.

To confirm the classification of the 40 kDa protease in the zinc metalloprotease category, various concentrations of divalent cations were added back to preparations of the 40 kDa protease containing 0.1 mM EDTA to determine if they were able to restore activity. Hide powder azure assays were then performed on these preparations to measure restoration of activity (Table 4). Full restoration of activity occurred with the addition of 100 μM Zn^{2+} and 1 mM Mn^{2+}. Some activity recovery was observed with 0.1 mM Fe^{3+} (64%). Only partial recovery of activity occurred with 1 mM Ca^{2+} (8%). Mg^{2+} had no effect on activity.

The full recovery of activity with low concentrations of Zn^{2+} (100 μM) established the 40 kDa protease as a member of the zinc metalloprotease family. Partial activity recovery with the higher concentrations of other metal cations indicated the substitution of Zn^{2+} with these other cations at the active site (Beynon and Bond, 1989). These experiments, therefore, placed the 40 kDa protease in the same class as PSCP.

3.2.4 Neutralization assays

Kooi et al. (1994) described the generation of monoclonal antibodies to PSCP. MAb 36-6-6 and 36-6-8 are monoclonal antibodies capable of neutralizing PSCP, *P. aeruginosa* elastase and *V. cholerae* HA/protease (the latter two belong to the thermolysin class of bacterial extracellular zinc metalloproteases). To identify the specific epitopes on PSCP being recognized by 36-6-6 and 36-6-8, elastase (a protease hypothesized to be similar to PSCP) was partially digested with N-chlorosuccinimide.

TABLE 4. Effect of metal ions on activity of *B. cepacia* 40 kDa protease

Metal ion added	Proteolytic activity of EDTA-inhibited 40 kDa protease with added metal ion[a]	% activity restored
none	0.72 ± 0.05 (no EDTA)	100
none (control)	0.00 ± 0.00[b]	0
1 mM Zn^{2+}	0.32 ± 0.13 **	44
100 μM Zn^{2+}	0.90 ± 0.13 **	125
10 μM Zn^{2+}	0.01 ± 0.07	1
10 mM Ca^{2+}	0.24 ± 0.13 **	33
1 mM Ca^{2+}	0.31 ± 0.05 **	43
0.1 mM Ca^{2+}	0.23 ± 0.04	32
1 mM Mg^{2+}	0.01 ± 0.02	1
0.1 mM Mg^{2+}	-0.02 ± 0.02	0
10 mM Mn^{2+}	0.39 ± 0.03 **	54
1 mM Mn^{2+}	0.76 ± 0.15 **	105
0.1 mM Mn^{2+}	0.33 ± 0.09 **	46
1 mM Fe^{3+}	-0.03 ± 0.00	0
0.1 mM Fe^{3+}	0.56 ± 0.01 **	78

[a]A_{595} of hide powder azure assays; the background with no protease but containing buffer was subtracted in each case.

[b]Each value represents the mean ± standard deviation of three determinations. The footnote symbols indicate that the values are significantly different from the values for the control (40 kDa protease plus 0.1 mM EDTA) by the two-tailed t test as follows: **, $P<0.01$.

A 13.9 kDa peptide recognized by both PSCP MAbs was subjected to epitope mapping to identify the epitopes being recognized by 36-6-6 and 36-6-8, and to determine if antisera raised against peptides containing the identified epitopes could neutralize various bacterial metalloproteases. The peptide was mapped by sixty overlapping 9-mer peptides spanning its sequence. Two of these peptides, 15 and 42, reacted strongly with both 36-6-6 and 36-6-8. Peptide 15 overlapped the HEXXH zinc-binding active site of elastase, and peptide 42 was close to the third zinc-binding ligand of elastase (Kooi *et al.* 1997). Polyclonal antisera to peptides 15 and 42 recognized (on immunoblots) and neutralized thermolysin, *V. cholerae* HA/protease, elastase and PSCP but not alkaline protease or the *S. marcescens* metalloprotease thus distinguishing between the thermolysin and serralysin family of bacterial zinc metalloproteases (Kooi *et al.* 1997). In this study, neutralization assays were performed on the 40 kDa protease using monoclonal antibody 36-6-8 and polyclonal sera to peptides 15 and 42. Proteolytic activity was 71% neutralized by 36-6-8. No neutralization was observed with the polyclonal anti-peptide sera (Table 5).

Neutralization by 36-6-8 suggests that PSCP and the 40 kDa protease are closely related and that the 40 kDa protease may belong to the thermolysin class of bacterial extracellular zinc metalloproteases (Kooi *et al.* 1996). The lack of neutralization by the antisera to peptides 15 and 42 from *P. aeruginosa* elastase may suggest that the 40 kDa protease is less related to elastase than is PSCP.

3.2.5 Substrate specificity of 40 kDa protease

In the study by McKevitt *et al.* (1989) describing the characterization of PSCP as a possible virulence factor in the pathogenesis of respiratory infection in CF patients

TABLE 5. Neutralization of *B. cepacia* 715j 40 kDa protease by MAbs to PSCP and by polyclonal sera to *P. aeruginosa* elastase peptides 15 and 42

MAb or polyclonal sera (1/250)	Proteolytic activity[a]	Proteolytic activity with MAb or polyclonal sera	% control
MAb 36-6-8	1.16 ± 0.04[b]	0.28 ± 0.10 **	24
Anti-peptide 15	0.95 ± 0.00	0.98 ± 0.07	>100
Anti-peptide 42	0.95 ± 0.00	0.95 ± 0.01	100

[a]A_{595} of hide powder azure assays containing control ascitis fluid for MAb; and control rabbit serum for polyclonal sera; the background with ascitic fluid or rabbit serum but with no protease was subtracted in each case.

[b]Each value represents the mean ± standard deviation of three determinations; the footnote symbols indicate that the values are significantly different from the values for the control by the two-tailed t-test as follows: **, P<0.01.

TABLE 6. Elastase activity of the 40 kDa protease using the Elastin Congo Red Assay

Sample	Elastase activity[a]
P. aeruginosa elastase, 10 μg	0.41
B. cepacia 40 kDa protease	0.01 ± 0.01[b]

[a]A_{495} of elastin congo red assays;; the background with no protease but containing buffer was subtracted in each case.

[b]Each value represents the mean ± standard deviation of three determinations.

the digestion of substrates by PSCP was investigated focusing on substrates that would be significant in this clinical scenario. These substrates included immunoglobulins such as secretory IgA which is an important mucosal surface defense in mucosal secretions in the respiratory tract, and components of the basement membrane such as collagen commonly found in the respiratory tract. In addition to the immunoglobulins and collagen substrates tested in McKevitt *et al.* (1989), the present study included other major components of the basement membrane such as fibronectin and elastin, and the iron-binding molecules transferrin (found in the blood and saliva) (Mietzner *et al.* 1994) and lactoferrin (found in mucosal secretions) (Weinberg 1984).

Degradation of IgG, IgA, IgM, secretory IgA, holotransferrin, apotransferrin, hololactoferrin, apolactoferrin, collagen VIII and fibronectin was investigated by incubating the 40 kDa protease with the potential substrate and then analyzing the results by SDS-PAGE gel electrophoresis. The 40 kDa protease was able to cleave holotransferrin (iron-saturated form) and apotransferrin (iron-deficient form) in the 48 hour incubation period into defined digestion products in the 55 kDa size range. Little to no degradation was observed for hololactoferrin and apolactoferrin (Fig. 6). Significant degradation was noted for IgG, IgM, IgA and secretory IgA (Fig. 7). No identifiable products were observed, although the amount of undigested immunoglobulins in the reaction with the 40 kDa protease was significantly less than that in the reaction without any protease. The 40 kDa protease was able to cleave all of the fibronectin yielding specific digestion products in the 35, 50, 60 and 140 kDa range, and to degrade all of the collagen VIII in a 24 hour incubation period (Fig. 8).

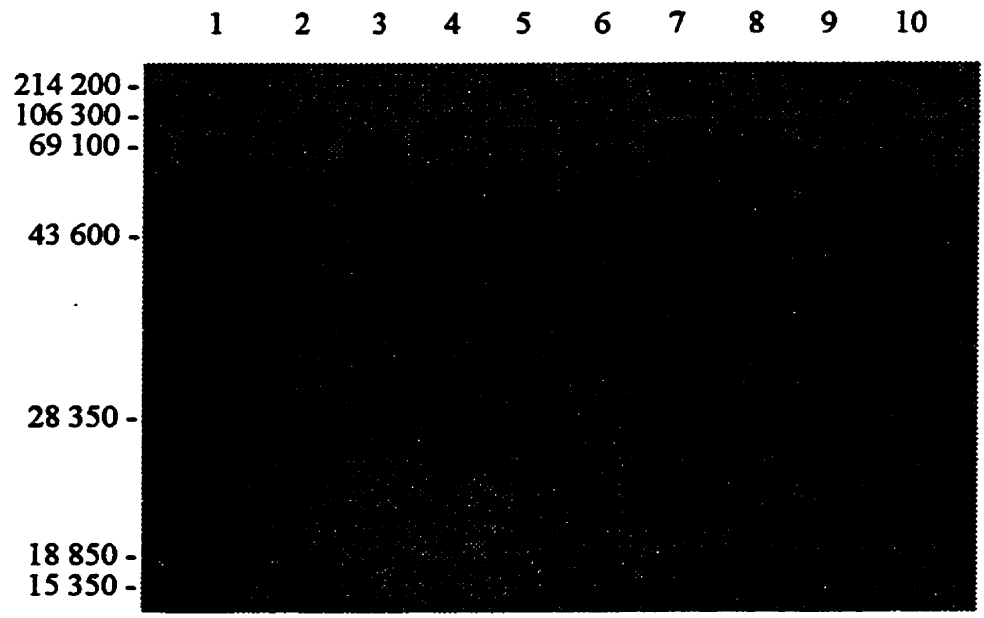


Figure 6. SDS-PAGE (12.5%) (Coomassie blue stain) analysis of 40 kDa protease digestions of transferrin and lactoferrin, separated under reducing conditions. Lane 2, 40 kDa protease (3 ug). In lanes 3, 5, 7 and 9 are10 ug each of holotransferrin, apotransferrin, hololactoferrin and apolactoferrin, respectively, incubated without enzyme. In lanes 4, 6, 8 and 10 are 10 ug each of holotransferrin, apotransferrin, hololactoferrin and apolactoferrin, respectively, incubated with 3 ug of 40 kDa protease; lane 1, Gibco BRL prestained molecular mass markers (indicated in kilodaltons from the top): myosin (heavy chain), phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, B-lactoglobulin, lysozyme.

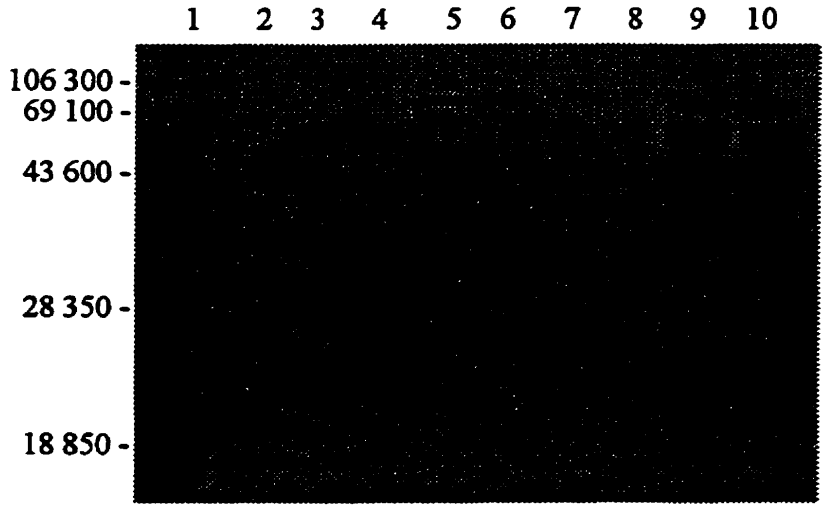


Figure 7. SDS-PAGE (12.5%) (Coomassie blue stain) analysis of 40 kDa digestions of human immunoglobulins separated under reducing conditions. Lane 2, 40 kDa protease, 3 ug. In lanes 3, 5, 7 and 9 are 10 ug of IgG, IgM, IgA and secretory IgA, respectively, incubated without protease. In lanes 4, 6, 8 and 10 are 10 ug each of IgG, IgM, IgA and secretory IgA, respectively, incubated with 3 ug of 40 kDa protease; lane 1, molecular mass markers (indicated in kilodaltons, see Fig. 5 legend);

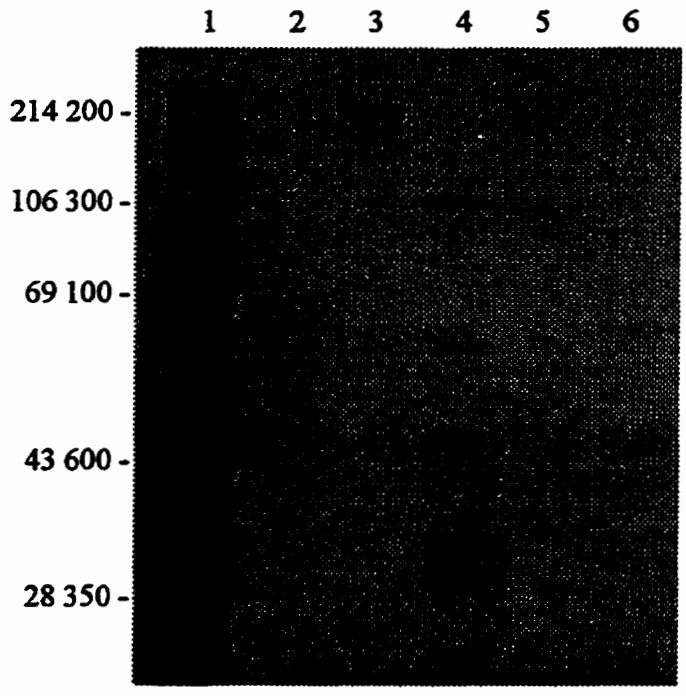


Figure 8. SDS-PAGE (12.5%) (Coomassie blue stain) analysis of 40 kDa protease digestions of fibronectin and collagen VIII, separated under reducing conditions. Lane 2, 40 kDa protease (3 ug). In lanes 3 and 5 are 10 ug each of fibronectin (from bovine plasma) and human collagen VIII, respectively, incubated without enzyme. In lanes 4 and 6 are 10 ug each of fibronectin and collagen VIII, respectively, incubated with 3 ug of 40 kDa protease; lane 1, Gibco BRL prestained molecular mass markers (indicated in kilodaltons).

The initial collagen stock was partially degraded. Degradation yielded ~45 kDa and ~65 kDa fragments.

The elastin congo red assay was performed on the 40 kDa protease to assess possible elastase activity. However, no significant elastase activity was observed for 5 units of the 40 kDa protease (Table 6).

Degradation of the basement membrane components, collagen VIII and fibronectin which are found in abundance in the respiratory tract, and of the immunoglobulins, especially secretory IgA, certainly suggests a potential role for the 40 kDa protease in virulence. Degradation of transferrin may be relevant in documented cases of *B. cepacia* septicemia as well as in respiratory infections. The basement membrane component, elastin, has so far only been shown to be digested by a few bacterial proteases such as *P. aeruginosa* elastase and not by proteases such as PSCP or the *B. cepacia* 40 kDa protease.

3.3 Determination of partial amino acid sequences of 40 kDa protease

Cloning of the 40 kDa protease structural gene was attempted in order to elucidate the relationship between the 40 kDa protease as a possible precursor of PSCP or as a related protease. The cloning of the 40 kDa protease structural gene was initiated by chemical digestion of the purified protease using NCS, (N-chlorosuccinimide; cleaves after tryptophan residues) followed by amino-terminal sequencing of the internal peptides by N-terminal sequencing. The amino-terminal end of the intact 40 kDa protease had previously been sequenced by the University of

Calgary Protein Sequencing Laboratory. The amino-terminal amino acid sequence was as follows: AGTG**INFTGEI**VAGAxGIDSNSVNQT. A primer to this sequence was previously designed to the highlighted sequence in the forward direction by Dr. Andrew Cox and Dr. P. Sokol: 5'-ATCAACTTCACCGGCGAGATC-3'. In Southern hybridization experiments the recognition of a genomic restriction fragment with one primer from the 40 kDa protease amino acid sequence could be confirmed by recognition of the same genomic fragment with another primer from the 40 kDa protease amino acid sequence. In the case of multiple bands on a hybridization membrane, recognition of a band by more than one primer could help locate the fragment corresponding to the 40 kDa protease structural gene. In addition, amino acid sequences exhibiting less degeneracy were sought. A primer designed to an internal fragment could be used in conjunction with the above N-terminal primer in PCR (polymerase chain reaction) reactions as a tool for amplifying a portion of the 40 kDa protease structural gene.

NCS (N-chlorosuccinimide) digestion of the 40 kDa protease yielded three peptides sized 21, 24 and 28 kDa as analyzed by Tricine SDS-PAGE (Fig. 9, lane 4). Complete digestion of a protein by NCS is difficult to achieve, thus partial digestion may be occurring. Tricine SDS-PAGE gel electrophoresis demonstrated improved resolution of the 40 kDa protease fragments. Previous SDS-PAGE electrophoresis of the 40 kDa protease peptide fragments using the standard protocol with glycine as the counter-ion did not exhibit satisfactory resolution for the purposes of purifying peptide fragments for N-terminal sequencing. Electroblotting of Tricine gels onto PVDF

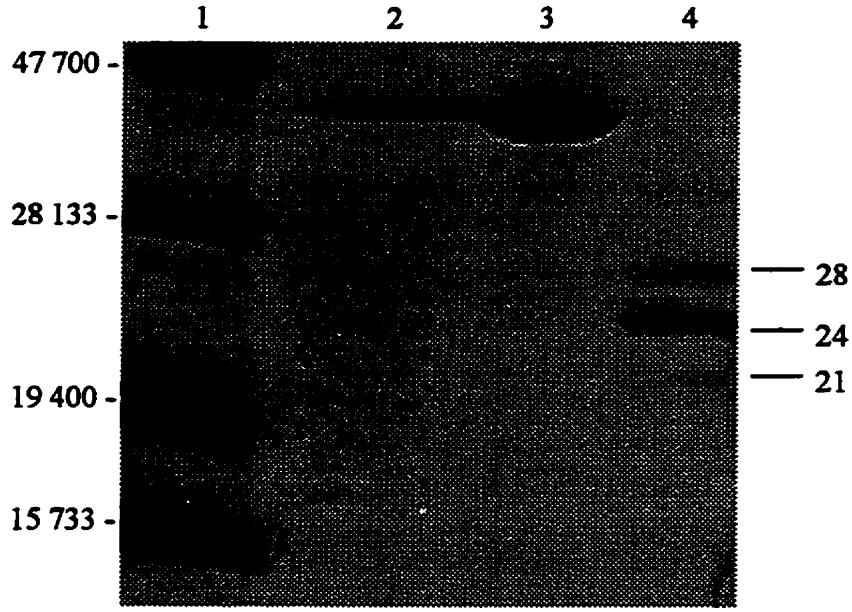


Figure 9. SDS-PAGE (12.5%) (Coomassie blue stain) analysis of NCS-digested *B. cepacia* 40 kDa protease. Lane 2, 40 kDa protease (5 ug); lane 3, 40 kDa protease (35 ug); lane 4, 40 kDa protease (35 ug) with NCS (peptide fragments indicated by dashes); lane 1, Gibco BRL prestained molecular mass markers (indicated in kilodaltons from the top): ovalbumin, carbonic anhydrase, B-lactoglobulin, lysozyme.

membranes yielded efficient transfer of the 40 kDa protease fragments; higher molecular weight markers were partially transferred. The peptide fragment bands were cut out of the PVDF membrane for N-terminal sequencing.

The 21, 28 kDa fragments and 40 kDa protease (previously) were sent to the University of Victoria for Edman degradation (amino-terminal sequencing). High signals for at least the first twenty amino acids were obtained for both peptides. Sequences were as follows: 21 kDa fragment: GGV**QPIGNGA**SGGLLTALSTSIA; 28 kDa fragment: NDVVSPYQDATLYPTQLV**NQIGVHQ**. The 24 kDa fragment was not initally sent for N-terminal sequencing at the same time as the 21 and 28 kDa fragment as it was sufficient to begin screening initially with primers to the amino acid sequences of the 21 and 28 kDa fragment. The 24 kDa fragment was later sent for N-terminal sequencing; however, there were contaminants present in the sample; thus a high signal was not obtained from the 24 kDa fragment sequence.

The program PCGENE using the *Pseudomonas* codon usage table (Wada *et al.*, 1992), was utilized to design the least redundant primers exhibiting reverse complementarity to the above sequences (indicated above by bold regions). The 28 kDa primer (R40-28) was designed to amino acid 19-25: 5'-TGGTGIACGCCGATCTGGTT-3' (T_m=75.3°C); the 21 kDa primer (R40-21) was to amino acids 4-10: 5'-GCGCCGTTGCCGATIGGCTG-3' (T_m=81.5°C). Primers were synthesized by the DNA Synthesis Laboratory at the University of Calgary.

3.4 DNA Hybridization using R40-21 and R40-28

The primers R40-21 and R40-28 were expected to have a reasonable degree of similarity to the actual DNA sequence of the 40 kDa protease due to the low degeneracy of the amino acids in the selected sequence. The high-GC content of *Burkholderia spp.* genomic DNA was used to preferentially select the third base of degenerate codons as G or C and highly degenerate codons were designed with a third inosine base which can recognize all four dideoxy nucleotides thus reducing the degeneracy of the primer design. Southern hybridization was performed to determine if the designed primer hybridized to *B. cepacia* genomic DNA. Genomic DNA from *P. aeruginosa* strain PAO was included in the hybridizations as a negative control.

The radiolabelled oligonucleotides R40-21 and R40-28 were hybridized to restriction digests of *B. cepacia* Pc715j genomic DNA using various conditions. Genomic DNA was digested with enzymes from the multiple cloning site of pUC19 to allow for future cloning in this vector: *Acc*I, *Eco*RI, *Hinc*II, *Hind*III, *Kpn*I, *Pst*I, *Sal*I and *Sma*I yielded the most complete digestion (Fig. 10). DNA was transferred to GeneScreen nylon membranes using vacuum blotting and first hybridized at conditions judged to be optimal when using degenerate primers: the T_m minus 25°C for each primer, minus 5°C for each possible base pair mismatch. R40-21 and R40-28 were first separately hybridized to Pc715j genomic DNA digests at 50°C; no binding was observed for either primer. At a lower stringency (27°C) no bands were obtained with either primer; some faint nonspecific binding in the *Pst*I genomic digest (three bands) and faint smears in other lanes was observed with R40-21 (data not shown). To attempt to avoid this nonspecific binding, 40°C hybridizations with R40-21 were

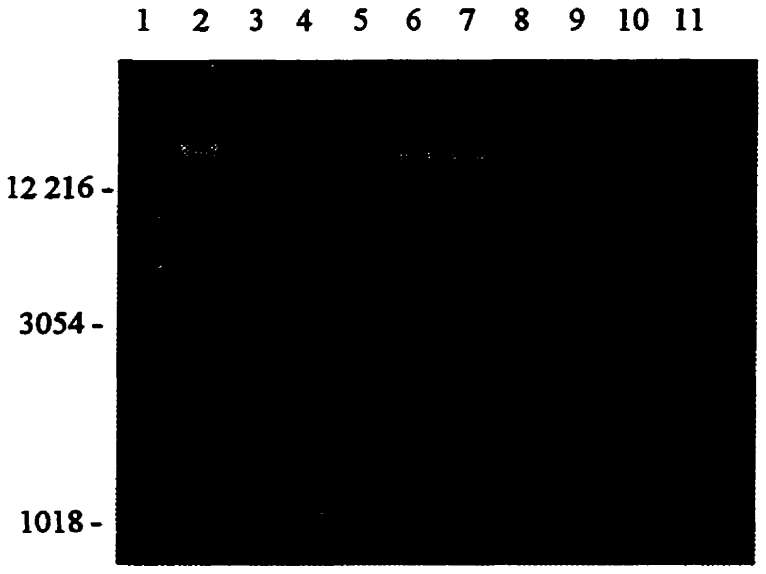


Figure 10. Restriction endonuclease digestions of *B. cepacia* Pc715j genomic DNA. Lane 1, 1 kb DNA standard (1-12 kb range). Genomic DNA of *P. aeruginosa* strain PAO (lane 2, undigested) and *B. cepacia* strain Pc715j (lanes 3-11) digested with: Lane 3, *Acc*I; lane 4, *Eco*RI; lane 5, *Hinc*II; lane 6, *Hind*III; lane 7, *Kpn*I; lane 8, *Pst*I; lane 9, *Sal*I; lane 10, *Sma*I; lane 11, *Sph*I.

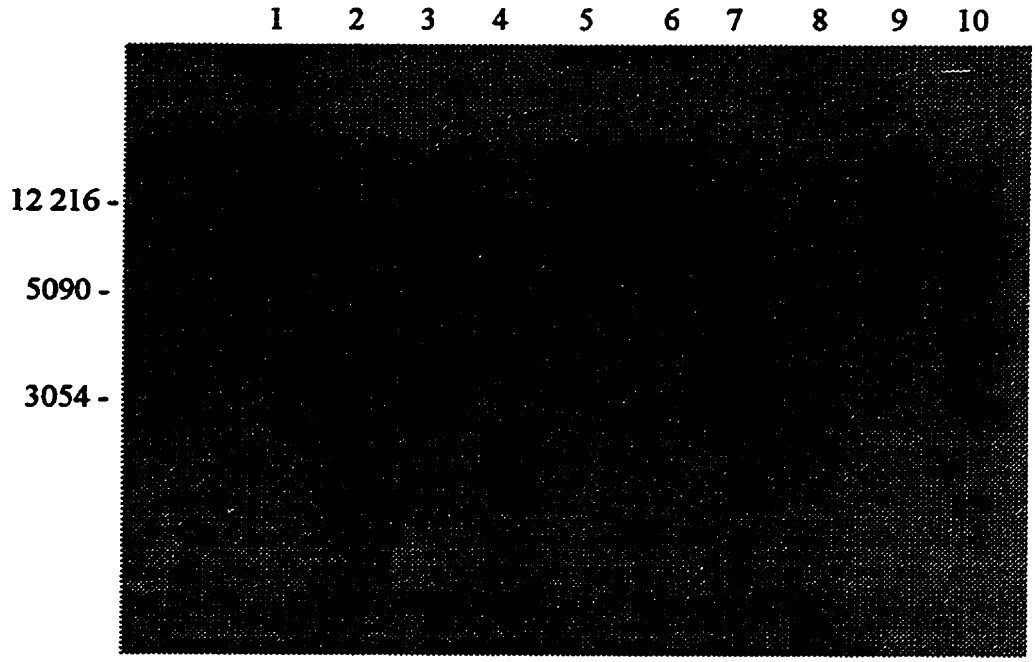


Figure 11. Southern hybridization analysis of *B. cepacia* Pc715j genomic DNA restriction digests hybridized with R40-21. Genomic DNA of *P. aeruginosa* strain PAO (lane 1-undigested); and *B. cepacia* strain Pc715j (lanes 2-10) digested with: lane 2, *Acc*I; lane 3, *Eco*RI; lane 4, *Hinc*II; lane 5, *Hind*III; lane 6, *Kpn*I; lane 7, *Pst*I; lane 8, *Sal*I; lane 9, *Sma*I; lane 10, *Sph*I; 1 kb DNA standard (1-12 kb range). Autoradiograms were scanned using Hewlett Packard Scan Jet 4c and Hewlett Packard Deskscan II Software (Hewlett Packard Co.)

performed, however, no binding occurred (data not shown). R40-21 demonstrated nonspecific binding in two separate experiments at 35°C. The first experiment demonstrated faint multiple bands in the *Eco*RI and *Acc*I genomic digests, and faint smears were observed in other lanes. The second experiment is demonstrated in Figure 11 where multiple bands were observed with all genomic digests. Hybridizations with R40-21 at 40°C demonstrated faint multiple bands with all genomic digests. At 35 and 40°C, R40-28 demonstrated no binding to Pc715j genomic DNA digests. Repeated hybridizations at 37°C for R40-28 and at 32, 37, 40 and 42°C for R40-21 did not demonstrate any binding at all to the genomic digests. Standard hybridization protocol consisted of prehybridization and hybridization in a solution of 50% dextran sulfate, deionized water, 10% SDS and salmon sperm DNA with the hybridization solution also containing the radiolabelled oligonucleotide. A different hybridization protocol utilizing a prehybridization solution consisting of 45% formamide (v/v), 0.2X SSC (NaCl and trisodium citrate), 5X Denhardt's solution, 20 mM sodium phosphate(v/v) and salmon sperm DNA was performed for 30 minutes at 42°C; hybridization was performed at 42°C overnight in a solution containing 60% formamide (v/v), 7X SSC, 1.5X Denhardt's solution and salmon sperm DNA with R40-21. This hybridized blot exhibited no binding at all. Thus, R40-28 did not exhibit any binding to Pc715j genomic DNA, and R40-21 demonstrated either no binding or nonspecific binding via multiple bands or smears without any banding observed.

The primers designed from the amino acid sequences of internal peptides from the 40 kDa protease may not be close enough to the actual DNA sequence of the 40

kDa protease structural gene to hybridize to restriction digests of Pc715j genomic DNA.

3.5 Polymerase chain reaction with 40 kDa protease primers

Another approach to cloning the 40 kDa protease structural gene was by PCR amplification. PCR was performed with the 40N forward primer and either the reverse complementary R40-21 or R40-28 primer. PCR using 0.5 μg of template, 4 mM $MgCl_2$ and R40-21 in a 50 μL final reaction volume with the following cycle conditions: initial 3 minute denaturation at 94°C; thirty cycles of one minute denaturation at 92°C, 1 minute annealing at 35°C, 1 minute extension at 72°C; final 7 minute extension at 72°C; yielded a 340 basepair (bp) product (Fig. 12, lane 3). The initial screening of the Pc715j genome by PCR using 40N and either R40-21 or R40-28 was performed under various conditions; no products were observed for any of the reactions using R40-28, while R40-21 yielded the above 340 bp product from Pc715j genomic DNA. Reamplification of these previous reaction products also yielded this 340 bp product (Fig 12, lanes 6-7) as did reamplification of the product in lane 3 of Figure 12 (data not shown). Reamplification of previous reaction products was performed to increase the amount of product being synthesized so as to have enough product for radiolabelling and subsequent colony hybridizations to clone the whole gene. As indicated in section 3.5.1 below, sequencing of this 340 bp reamplification product indicated it was not from the 40 kDa protease structural gene as it did not

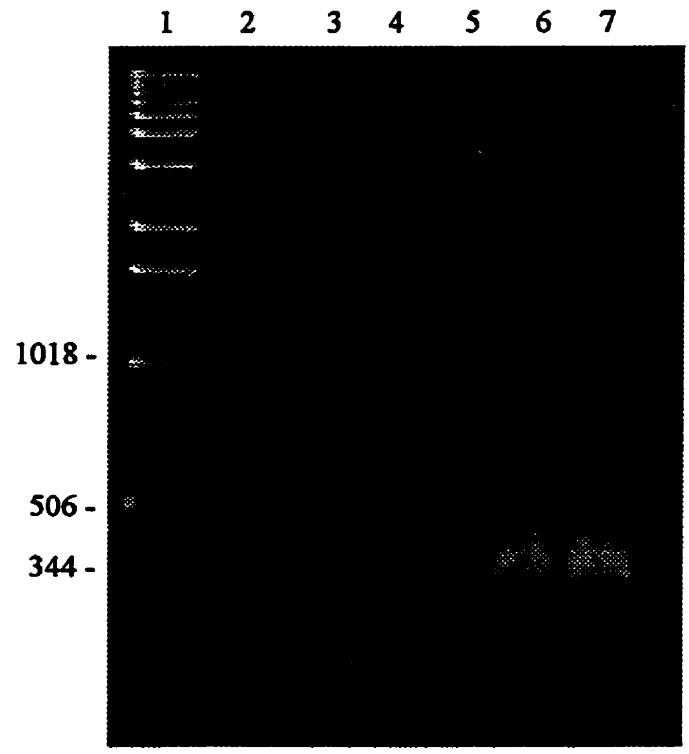


Figure 12. PCR amplification of a 340 bp product from the *B. cepacia* Pc715j genome. Primers R40-21 and 40N amplification from the Pc715j genome (unless otherwise indicated). Lane 1, 1kb DNA standard (0.1-12 kb range), Lane 2, PAO, primers 40N and R40-21; lane 3, 500 ng Pc715j genomic DNA, primers 40N and R40-21; lane 4, 250 ng Pc715j genomic DNA, primers 40N and R40-21; lane 5, 500 ng Pc715j genomic DNA, primers R40-28 and 40N; lanes 6 and 7, previous PCR product reamplification, primers 40N and R40-21.

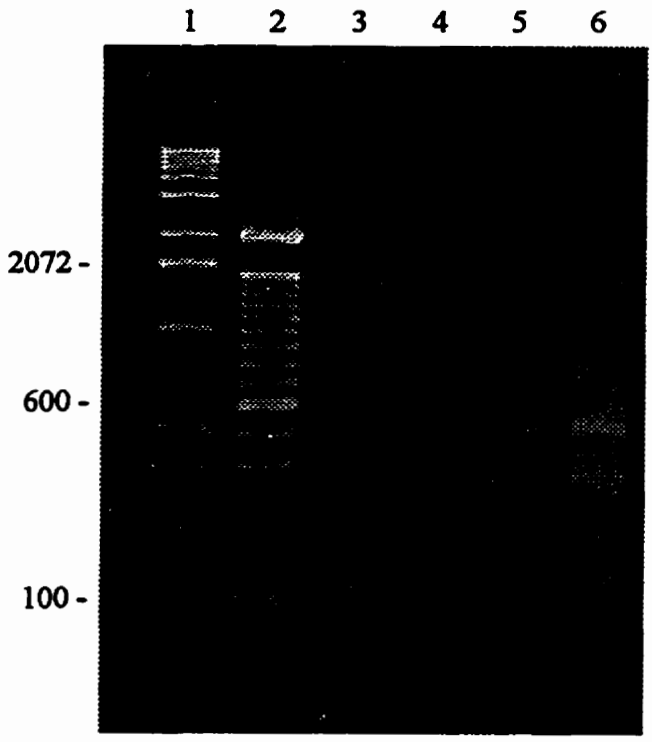


Figure 13. PCR amplification of 180, 340 and 425 bp products from the *B. cepacia* Pc715j genome. Primers 40N and R40-21 amplification from the Pc715j genome under various $MgCl_2$ concentrations. Lane 1, 1kb DNA standard (0.1-12 kb range), Lane 2, 100 bp DNA standard (100-2000 bp range), Lane 3, water control ; lane 4, 2 mM $MgCl_2$; lane 5, 3 mM $MgCl_2$; lane 6, 4 mM $MgCl_2$.

originate from the amino acid sequence obtained from N-terminal sequencing (see section 3.3).

Reaction conditions were modified to attain a higher level of specificity. Figure 13 lane 5 indicates three products: 180 bp, 340 bp and 425 bp obtained using primers 40N and R40-21 and ethanol-precipitated and washed template (1 μg), 3 mM $MgCl_2$ and modified cycle conditions: initial denaturation for 3 minutes at 95°C, denaturation at 1 minute at 95°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C for 35 cycles. Reactions under these conditions with 40N and R40-28 did not yield specific products. Denaturation of the GC-rich template couples 5% DMSO with the higher denaturation temperature of 95°C. Sequencing of the 180, 340 and 425 bp products (see section 3.5.1 for sequence analysis) indicated these products were not from the 40 kDa protease structural gene when compared to the N-terminal amino acid sequences. Subsequent PCR reactions using 40N and R40-21 with the elimination of possible contaminating instruments or reagents, higher annealing temperatures and decreasing the amount of template to 0.5 μg to increase reaction specificity continued to yield the 180, 340 and 425 bp products; no products were observed with the R40-28 primer.

Decreasing the amount of template to 250 ng started to yield products other than the 180, 340 and 425 bp products; so to confirm the specificity of these products, single primer reactions were used to identify specific versus nonspecific products from the primers since it was possible that products were arising from nonspecific priming at both ends of the product. In addition, the annealing temperature was increased for

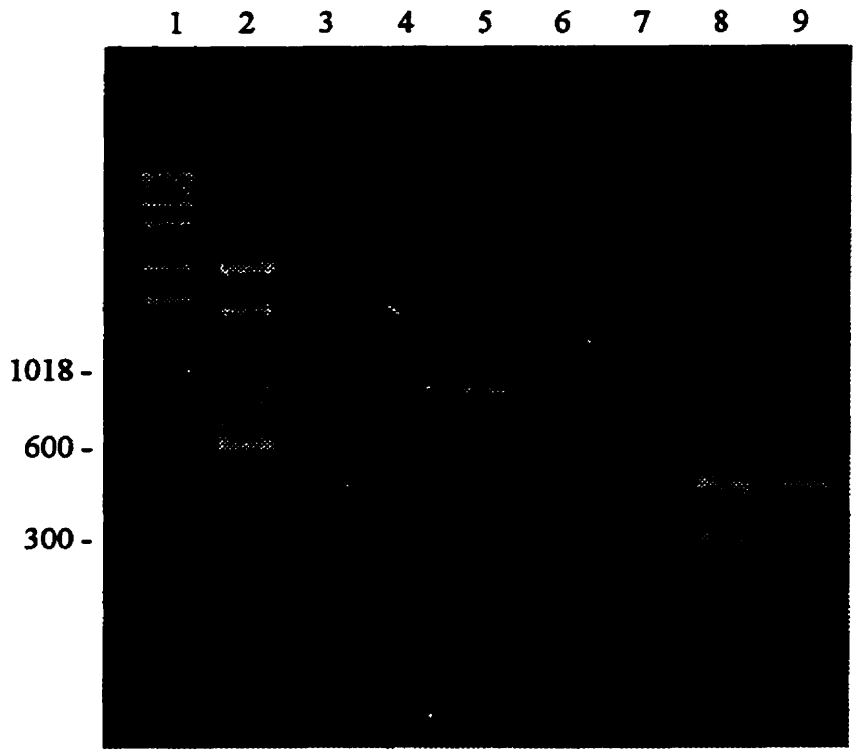


Figure 14. PCR amplification of 1000 and 1050 bp products from the *B. cepacia* Pc715j genome. Primers 40N and R40-21 or R40-28 amplification from the Pc715j genome with 4 mM $MgCl_2$ (unless otherwise indicated). Lane 1, 1kb DNA standard (0.2-12 kb range), Lane 2, 100 bp DNA standard (200-2000 bp range), Lane 3, water control; lane 4, 40N; lane 5, R40-21; lane 6, R40-28; lane 7, 40N and R40-28; lane 8, R40-21 and 40N; lane 9, 40N and R40-21 with 2.5 mM $MgCl_2$.

the first 5 cycles to yield specific products and then lowered to continue amplifying from these specific products: 65°C for 5 cycles then 60°C for 30 cycles. When 40N and R40-28 were each used separately to amplify products primed on both ends by the same primer; no nonspecific products were observed; however R40-21 amplified a 900 and 1200 bp product. A 1000 bp fragment (Fig. 14, lane 7) was amplified by 40N and R40-28. Amplification by 40N and R40-21 continued to yield the 180, 340 and 425 bp products as well as the 900 bp product primed on both ends by R40-21; however, when the $MgCl_2$ concentration was lowered to 3 mM, an additional 1050 bp product was observed (Fig 14, lane 9). The 1000 bp product from 40N and R40-28 and the 1050 bp product from 40N and R40-21 were analyzed by sequencing in section 3.5.1.

3.5.1 Analysis of Pc715j PCR products

Automated DNA sequencing was performed on the PCR products obtained in section 3.5 to confirm the product as originating from the 40 kDa structural gene by comparisons to the deduced amino acid sequences described in section 3.3. NCBI GENBANK BLAST searches were also performed to look at relevant homologies to other sequences and to look at the possibility of the primers amplifying a related gene such as that belonging to another *B. cepacia* Pc715j protease.

The PCR products obtained in section 3.5 were analyzed by cloning into the pCRII vector and transformation into *E. coli* strain INVαF' and PCR cycle sequencing of the insert (the cloned PCR product). These included the initial 340 bp product amplified by 40N and R40-21 (Fig. 12, lane 3), the 180, 340 and 425 bp products amplified by 40N and R40-21 (Fig. 13, lane 5), the 1000 bp product amplified by 40N

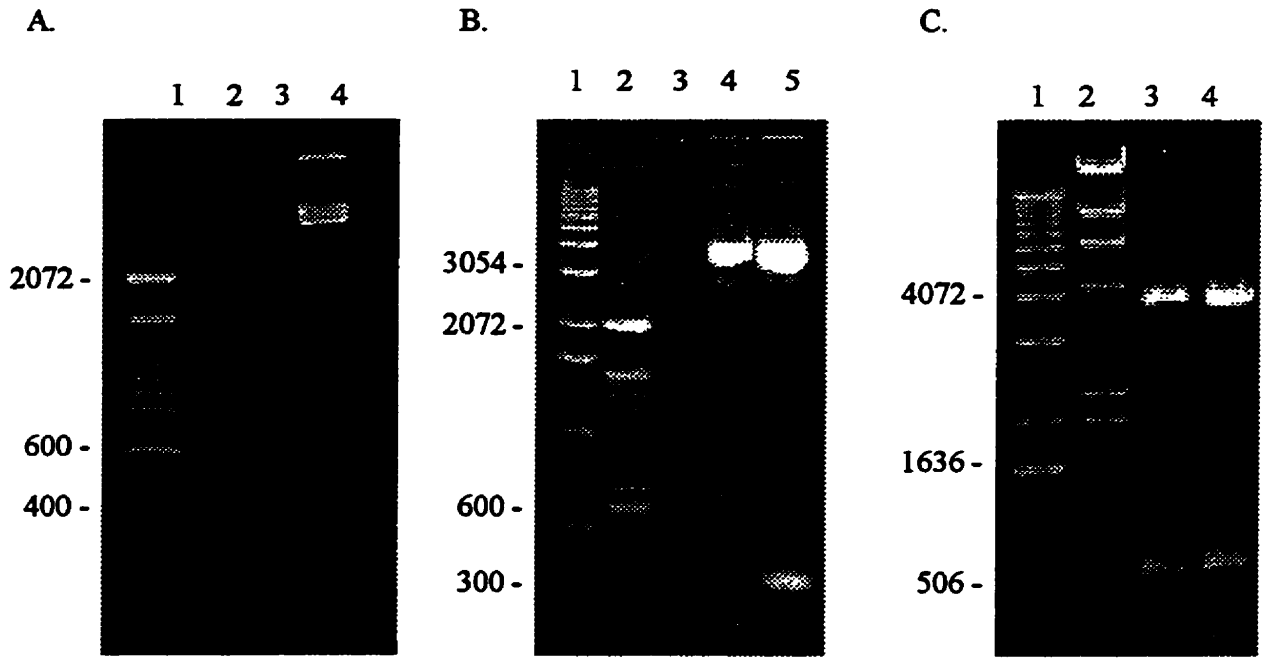


Figure 15. Agarose gel (1.5%) analysis of *Eco*RI digested pCRII:PCR product plasmid constructs. A. Lane 1, 100 bp DNA standard (200-2000 bp range), lane 4, pVS21-425, B. Lane 1, 1 kb DNA standard (0.3-12 kb range), lane 2, 100 bp DNA standard (200-2000 bp range), lane 4, pVS21-180; lane 5, pVS21-340. C. Lane 1, 1 kb DNA standard (1-12 kb range), lane 2, lambda *Hind*III DNA standard (2-23 kb range), lane 3, pVS28-1000; lane 4, pVS21-1050. All agarose gels were scanned using Hewlett Packard Scan Jet 4c and Hewlett Packard Deskscan II Software (Hewlett Packard Co.)

TABLE 7. Comparison of PCR product sequences to *B. cepacia* Pc715j amino-terminal amino acid sequences

Name[b]	Sequence
R40-21	[GC GCC GTT GCC GAT CGG CTG][a] NAC NCC NCC
pVS21-180	GC GCC GTT GNC GAT GGG CTG CTC GCG GTG
pVS21-340	GC GCC GTT GCC GAT GGG CTG GGA GCA GCA
pVS21-425	GC GCC GTT GCC GAT GGG CTG CAC ATG TCG
pVS21-1050	GC GCC GTT GCC GAT GGG CTG CGG AAC ACG
pVS21-1050	GC GCC GTT GCC GAT GGG CTG TCA GCG TCG
R40-28	[TG GTG IAC GCC GAT CTG GTT] NAC NAG/A/T T/CTG
pVS28-1000	TG GTG GAC GCC GAT CTG GTT TAT CCC GAT
40N	[ATC AAC TTC ACC GGC GAG ATC] ATN GTN GCN GGN
pVS21-180	ATC AAC TTC ACG GGG GCA GAT CGT TTG TGG ACA
pVS21-340	ATC AAC TTC ACG GGC GAG ATG TTG ACC GCG AGC
pVS21-425	ATC AAC TTC ACG GGG GAG ATG CGG AAA ACG GGC

[a]Brackets indicate primer DNA sequence.
[b]Primer name.

and R40-28 (Fig. 14, lane 7) and the 1050 bp product amplified by 40N and R40-21 (Fig. 14, lane 9).

The 180, 340, 425, 1000 and 1050 bp pCRII:PCR product plasmid constructs were confirmed as containing the appropriate PCR product insert by *Eco*RI digestions (*Eco*RI restriction sites flank the insertion site) indicated in figure 15. The PCR product inserts were sequenced and analyzed. None of these products possessed any significant homologies to genes related to or coding for a bacterial protease via BLAST searches. Table 7 indicates the comparisons of the PCR product sequences to the 40 kDa protease amino-terminal amino acid sequences. None of the PCR products originated from the 40 kDa protease structural gene as indicated by comparison to the amino acid sequence obtained from N-terminal sequencing. The 1000 bp PCR product (pVS28-1000) amplified from 40N and R40-28 had a different sequence from a second clone of this product; this clone showed significant homology to UDP-N-acetylglucosamine 1-carboxyvinyltransferase from *Enterobacter cloacae*, *Haemophilus influenzae*, *Escherichia coli* and *Acinetobacter calcoaceticus* (data not shown) indicating the unpredictable nature of the products being amplified. The 1050 bp product (pVS21-1050) amplified by 40N and R40-21 was primed on both ends by R40-21 and was thus a nonspecific product. The initial 340 bp PCR reamplification product was cloned and sequenced, but it did not originate from the 40 kDa protease amino acid sequence, nor were any homologies identified after a BLAST search (data not shown). Reamplification of PCR products increases the probability of turning up a contaminant rather than the desired specific product. The initial 340 bp reamplification

product and the second 340 bp product (pVS21-340) (obtained in conjunction with the 180 bp and 425 bp product (pVS21-180 and pVS21-425)) amplified by 40N and R40-21 had different sequences further indicating the nonspecificity of the products. This common occurrence of clones originating from the same PCR product having different sequences indicated the nonspecificity of the products being amplified. Three different clones of the 425 bp product amplified from 40N and R40-21 did, however, have the same sequence.

Thus, the DNA sequence of the primers designed from the amino acid sequence of the 40 kDa protease structural gene was probably not similar enough to the actual DNA sequence of the 40 kDa protease gene to be used for cloning the structural gene for the 40 kDa protease. The degree of purity of the initial 40 kDa protease preparation from section 3.1 used in the amino-terminal sequencing (ref. 3.3) was a possible problem in that contaminant proteins may have been sequenced thus accounting for the PCR products amplified by the primers not originating from the 40 kDa protease gene. In addition, the N-terminal sequencing procedure itself could have been detecting major contaminant proteins in the sample rather than the digested 40 kDa protease fragments.

3.6 Construction and screening of *B. cepacia* Pc715j cosmid gene bank

Since the cloning using oligonucleotide primers was unsuccessful, cloning of the 40 kDa protease gene was attempted via the construction and screening of a cosmid gene bank in *E. coli* for protease expression. In this approach, the entire

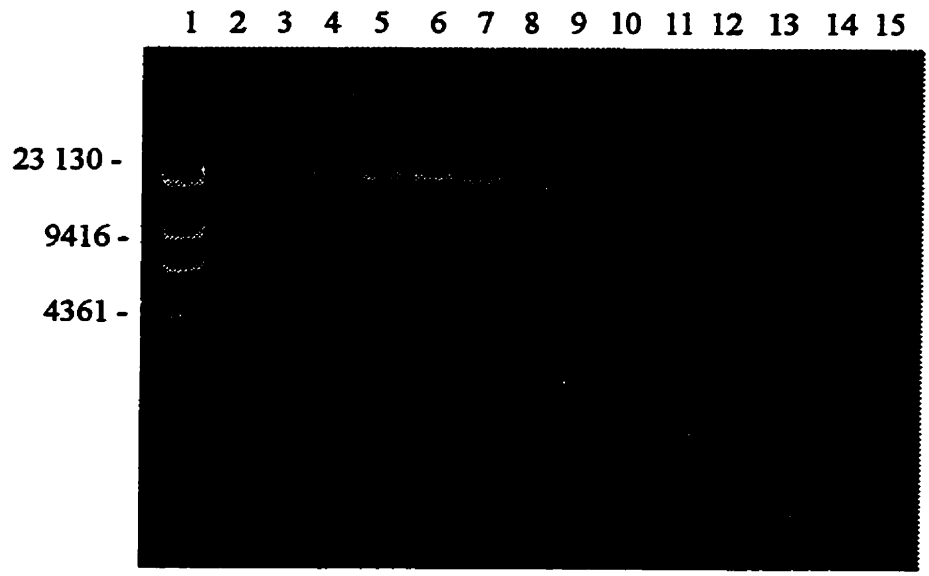


Figure 16. Agarose gel (0.75%) analysis of sucrose gradient of *Hind*III digested *B. cepacia* Pc715j genomic DNA. Lane 1, lambda *Hind*III DNA standard (2-23 kb range), lanes 2-15, fractions 1-14 (0.5 mL) of the DNA sucrose gradient.

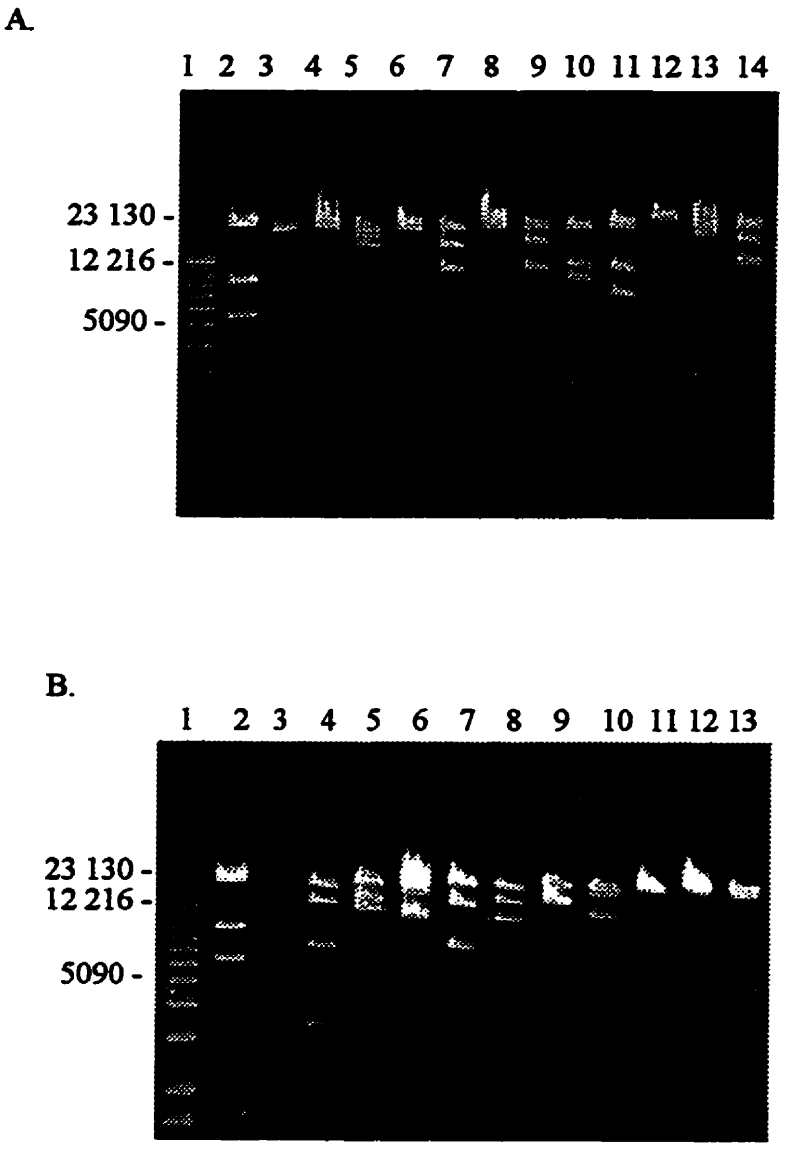


Figure 17. Agarose gel (0.6%) analysis of *Hind*III digestions of cosmids from first *B. cepacia* Pc715j gene bank. A. Lane 1, 1kb DNA standard (1-12 kb range), lane 2, lambda *Hind*III DNA standard (2-23 kb range), lane 3, pCP19 (uncut); lanes 4-14, pCP19 with *Hind*III Pc715j genomic DNA inserts from HB101. B. *Hind*III digestions of cosmids from second *B. cepacia* Pc715j gene bank. Lane 1, 1kb DNA standard (1-12 kb range), lane 2, lambda *Hind*III DNA standard (2-23 kb range), lanes 4-13, pCP19 with *Hind*III Pc715j genomic fragment inserts from HB101.

genome of *B. cepacia* strain Pc715j would be contained in large fragments in a cosmid vector, stored in an appropriate host, and screened by conjugation (triparental mating) in a host capable of allowing expression of Pc715j genes. The pCP19 cosmid vector (23 kb) contains a λ *cos* site and a multiple cloning site. The λ recombinant phage package DNA fragments in the 34-52 kb range thus inserts range in size from 15-31 kb with an average insert size of 23 kb (Friedman *et al.* 1992). A fragment encoding a protease structural gene would be detected by a phenotypic skim milk/brain heart infusion (SM/BHI) agar plate assay designed to detect protease expression by a clearing of the agar around the colony due to proteolysis of the casein in the skim milk. This approach might make it possible to detect a structural gene for any protease expressed in *E. coli* that could degrade casein.

B. cepacia Pc715 genomic DNA was partially digested with *Hind*III, and sucrose gradients were used to isolate 9-20 kb fragments (Fig. 16). After ligation of the 9-20 kb fraction (Fig. 16, lane 8 and 9) into the cosmid vector pCP19, this mixture was transduced into *E. coli* strain HB101. The first gene bank when packaged, amplified in *E. coli* HB101 and harvested had a low yield of only ~700 colonies. However, it must be considered that many of the clones contain more than one genomic fragment from Pc715j. Figure 17A indicates the presence of inserts in approximately seven of ten randomly selected plasmids from the gene bank. Of these plasmids, three appeared the same with two fragments of 11 kb and 16 kb. The remaining four had inserts of 10.5 and 12 kb; 9 and 12 kb, 4 and 18 kb, and 25 kb.

Thus, the average size of insert in this gene bank was 23.5 kb as expected for packaging of cosmid vectors by recombinant λ phage, and the unique insert frequency for the first gene bank was 50%. The remainder of the ligation was packaged into *E. coli* HB101 with a yield of ~2300 clones and a unique insert frequency of approximately 70% demonstrated in Figure 17B. These seven clones appeared to be different: 3.3, 7.5 and 16 kb; 13 and 17 kb; 12 kb; 8 and 17 kb; 12 and 18 kb; 20 kb and 13 kb. The average size of the Pc715j genomic inserts in this gene bank was 22.4 kb. When combining both gene banks the total coverage is 45 080 kb of unique inserts. Since 9-20 kb genomic fragments were ligated into the cosmid vector pCP19, the first gene bank contains 8 distinct genomic fragments from ten cosmid clones, and the average insert size was 13 kb. The second gene bank contains nine distinct genomic fragments in ten cosmid clones with an average insert size of 13 kb. When combining the gene banks together, a total of 34 190 kb is included in the gene bank. Thus, with the *B. cepacia* genome being ~7 Mb, the gene banks cover 4-5 times the size of the *B. cepacia* genome.

The Pc715j cosmid gene bank in *E. coli* HB101 was screened on SM/BHI plates to determine if protease expression could occur in *E. coli* with the occurrence of a protease-positive phenotype. No protease-positive clones were identified. Protease structural genes and/or genes related to protease production such as regulatory or secretory genes may not be properly expressed or processed to allow secretion of mature protease(s) into the extracellular medium.

3.6.1 Screening of the two Pc715j gene banks in PA103-11

The first host selected was the protease negative *P. aeruginosa* strain PA103-11. This strain is phenotypically elastase negative, and an EMS (ethylmethanesulfonate) mutant of alkaline protease (Guzzo *et al.* 1990). *B. cepacia* (and all *Burkholderia spp.*) formerly belonged to the *Pseudomonas* genus, the *Pseudomonas* genus was divided into five groups according to rRNA-DNA hybridization. Homology group II was reclassified as *Burkholderia spp.* according to 16S rRNA sequences and certain phenotypic characteristics (motility, carbon sources, cellular lipid and fatty acid analysis) (Yabuuchi *et al.* 1992). The two genera are related in predominant characteristics such as being gram-negative, aerobic, nutritionally versatile, possessing a high G-C content in their genomes, etc. thus expression of *B. cepacia* Pc715j genes could possibly occur in PA103-11. The two gene banks were first screened by conjugation in PA103-11 to look for clones exhibiting a protease positive phenotype on skim milk agar plates. The recipient (PA103-11) was incubated at 42°C to attempt to inactivate its restriction system. Transconjugants were screened on skim milk agar (1300 from the first gene bank and 2600 from the second gene bank), however, the protease positive phenotype was not observed. The PA103-11 transconjugants carrying the plasmids were examined to determine if the Pc715j inserts were stable in this host. Figure 18 indicates that the vectors in the PA103-11 transconjugants did not contain inserts. Thus, it appeared that PA103-11 was not similar enough *to B. cepacia* Pc715j to allow the presence of Pc715j genomic fragments, since the fragments appeared to be degraded by the

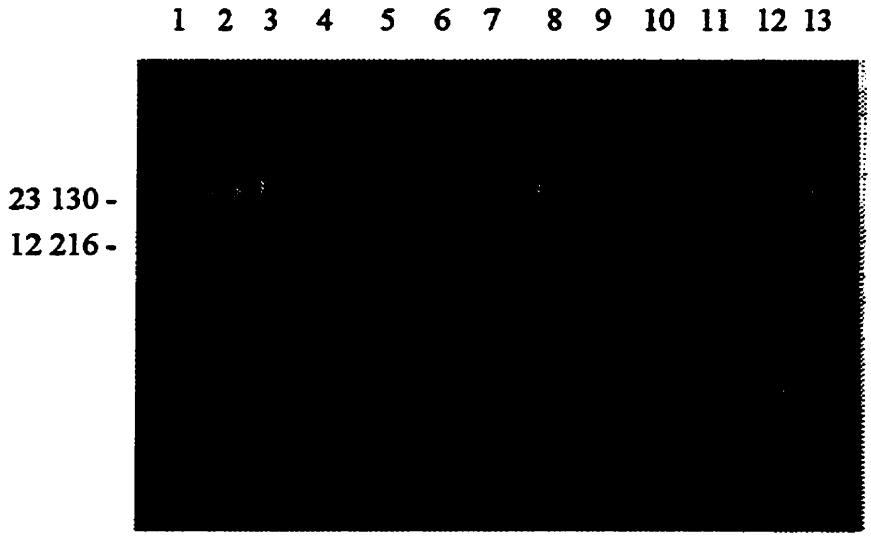


Figure 18. Agarose gel (0.7%) analysis *Hind*III digestions of pCP19 *B. cepacia* Pc715j gene bank transconjugants in PA103-11. Lane 1, 1 kb DNA standard (1.6-12 kb range), lane 2, lambda *Hind*III DNA standard (2-23 kb range), lane 3, pCP19, lanes 4-13, PA103-11 transconjugants.

restriction system of strain PA103-11. Expression of Pc715j DNA could not then be studied in this host.

3.6.2 Determination of protease expression in *B. cepacia* strains

Since *Pseudomonas spp.* seemed to recognize *Burkholderia spp.* DNA as foreign and degraded these fragments via its restriction system, a phenotypically protease-negative *Burkholderia cepacia* strain was sought. Previous screening studies in Dr. P. Sokol's lab by Dr. Andrew Cox and Carla Lewis of *B. cepacia* strains on SM/BHI plates identified a preliminary set five of strains exhibiting protease-negative phenotypes.

The *B. cepacia* strains suspected of exhibiting a protease-negative phenotype were subjected to analysis of proteolytic expression by incubation at 37°C for up to 96 hrs on SM/BHI plates, and by TCA precipitation of extracellular supernatants. The strains studied included 22-12 (plant strain), 15278 (clinical strain from skull infection), K36-2, K37-3 and K43-3 (latter three all CF clinical strains); with Pc715j as a positive control. All strains were initially protease negative on SM/BHI plates; however, later studies demonstrated some proteolytic zone formation by K36-2, K37-3 and K43-3.

For analysis of culture supernatants for secreted protease, 15 mL cultures were grown in PTSB media at 37°C to an A_{600} of 1.4, which ranged from 6 hrs for 15278 to 11 hrs for K36-2, K37-3 and K43-3. At this point, 14 mL of the culture was precipitated with TCA overnight at 4°C. The protein concentration of the resuspended protein precipitate was determined by the Bio-Rad protein assay and adjusted to ~0.9 μg/μL. SDS-PAGE analysis of the samples (Fig. 19) indicated that

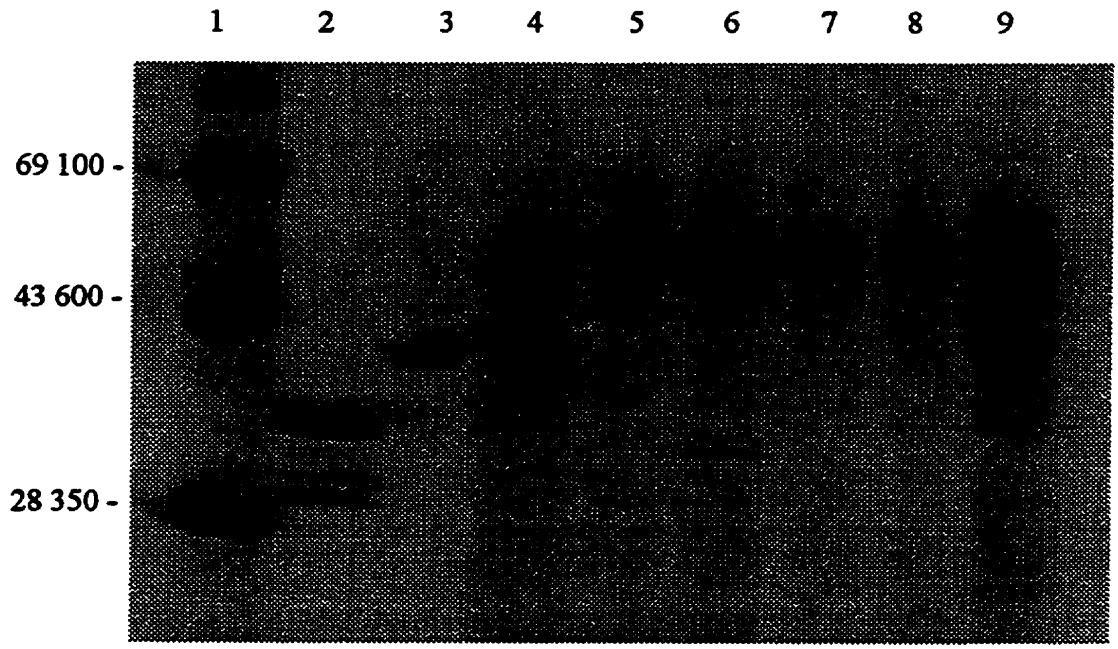


Figure 19. SDS-PAGE (12.5%) (Coomassie Blue (CBB) Stain) analysis of TCA precipitated supernatants of *B. cepacia* strains; each lane contains ~60 ug of protein. Lane 2, PSCP; lane 3, 40 kDa protease; lane 4, Pc715; lane 5, 22-12; lane 6, 15278; lane 7, K36-2; lane 8, K37-3; lane 9, K43-3; PSCP and 40 kDa protease indicated by dashes (-); lane 1, prestained molecular mass markers (indicated in kilodaltons, see Fig. 5 legend). All SDS-PAGE gels were scanned using Hewlett Packard Scan Jet 4c and Hewlett Packard Deskscan II Software (Hewlett Packard Co.)

strains 22-12 and 15278 did not appear to express either the 40 kDa protease or PSCP (Fig. 19, lanes 5 and 6), whereas K36-2, K37-3, K43-3 and Pc715j expressed both proteases (Fig. 19, lanes 4, and 7-9). These results correlated with the preliminary SM/BHI screening studies. Strain 22-12 was chosen over 15278 as the host for the Pc715j cosmid gene bank due to the unknown nature and origin of strain 15278 and the established protease negative phenotype of most *B. cepacia* plant (rhizosphere) strains (Bevivino *et al.* 1994).

3.6.3 Screening of the Pc715j cosmid gene banks in *B. cepacia* strain 22-12

B. cepacia strain 22-12 was the isolate chosen in which to screen the Pc715j cosmid gene banks for protease expression. Any evidence of proteolytic expression by a cosmid clone could be due to a protease structural gene or required secretory or regulatory genes which are not present in 22-12 since the protease negative phenotype of strain 22-12 could be due to a number of reasons: the absence or mutation of the protease structural gene in the genome, or the absence or mutation of a specific regulatory or secretory gene involved in expression, maturation and export of the protease.

The two gene banks (2500 transconjugants screened from the triparental mating with the first gene bank and 5700 transconjugants screened from the triparental mating with the second gene bank) were screened by conjugation in *B. cepacia* strain 22-12. Figure 20 shows that inserts were present in the 22-12 transconjugants (four out of five for the first gene bank and five out of five for the second), however, no protease positive clones were observed. For both gene banks combined, the unique

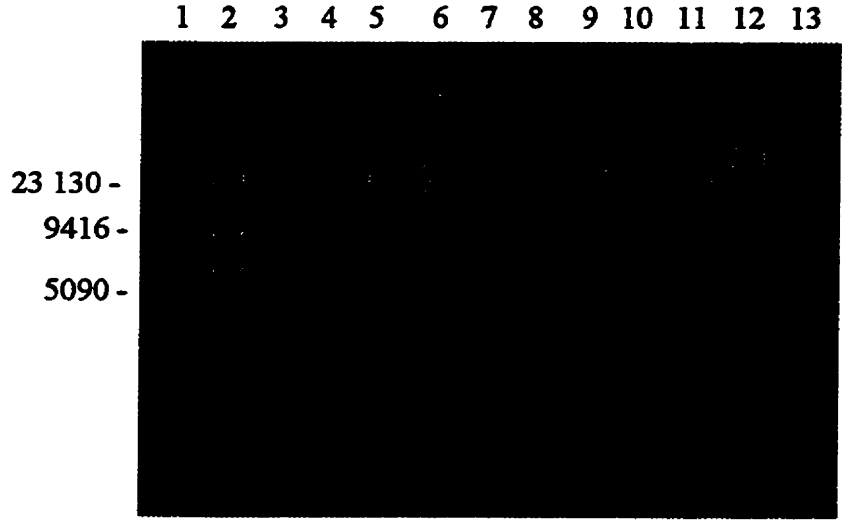


Figure 20. Agarose gel (0.8%) analysis of *Hind*III digestions of pCP19 *B. cepacia* Pc715j gene bank transconjugants in 22-12. Lane 1, 1 kb DNA standard (1.6-12 kb range), lane 2, lambda *Hind*III DNA standard (2-23 kb range), lane 3, pCP19; lanes 4-8, 22-12 transconjugants from first gene bank; lanes 9-13, 22-12 transconjugants from second gene bank.

insert frequency was 90%; with an average insert size of 21 kb, comprising a total of 107 415 kb being screened in 22-12. The unique genomic fragment frequency is 100%, with an average unique genomic fragment size of 10 kb, covering a total of 69 300 kb. Thus, the *B. cepacia* genome was screened nine-fold above the size of the 7 Mb genome in strain 22-12. However, the absence of any proteolytic expression could be due to random fragments of the genome that were not included in the gene bank either by the insufficient number of initial cosmid clones obtained when constructing the gene bank or by the specificity of the *Hind*III restriction site selecting against genomic fragments larger than 20 kb that do not contain the *Hind*III site. Restriction enzymes often preferentially cleave certain sites in a genome; leading to overrepresentation of the fragments containing these sites. Amplification of the cosmid gene bank may also lead to overrepresentation of certain genomic fragments due to some advantage conferred to the host from the insert. Alternatively, all of the cosmid clones may not have been transferred over to the host. The possibility also exists that the fragment containing one of the protease structural genes was cleaved by *Hind*III in the middle of the gene, or that other genes necessary for expression and maturation of the protease were not present on the same fragment.

4.0 DISCUSSION

Extracellular zinc metalloproteases have been implicated in the pathogenesis of a number of diseases such as *Pseudomonas aeruginosa* respiratory infection in CF patients. *Burkholderia cepacia*, also a CF pathogen, produces at least two proteases which may be involved in the pathogenesis of this organism in the CF lung. PSCP, a 34 kDa protease, has been demonstrated to cause bronchopneumonia when instilled intratracheally into rat lungs. *In vitro* it can cleave collagen types I, IV and V, where collagen type III is an interstitial collagen found in the lung and collagen type IV is a basement membrane collagen found in all vascular tissues (McKevitt *et al.* 1989). PSCP activity can be inhibited by metal chelators such as EDTA and 1,10-phenanthroline and activity of PSCP in the presence of EDTA can be restored by the addition of zinc, calcium or magnesium ions. Thus, putative classification placed PSCP in the zinc metalloprotease family of bacterial proteases (McKevitt *et al.* 1989). The purification of a second 40 kDa protease with less specific activity by the hide powder azure assay than PSCP by Kooi *et al.* (1994) raised the possibility of its being a precursor of PSCP or a related protease to PSCP. Further characterization of PSCP by Nakazawa and Abe (1996) has demonstrated that the expression of homologs to *P. aeruginosa* XcpR(cytoplasmic ATPase) and XcpS(inner membrane protein) from the general secretory pathway, and of the disulfide-bond forming protein, DsbB, are required in *B. cepacia* for the expression of extracellular, mature PSCP. The hypothesis of this study is that the 40 kDa protease is either a precursor of PSCP, or it is a protease sharing common epitopes with PSCP. As the main,

constitutively produced extracellular product of *B. cepacia* strain Pc715j (a clinical CF strain), it is possibly an important virulence factor in the pathogenesis of *B. cepacia* respiratory infections in CF patients. This study endeavoured to understand the properties of the 40 kDa protease in relation to its role as a virulence factor, and to its relation to PSCP and to the physiology of *B. cepacia*. Towards this goal, the characterization of the 40 kDa protease was performed, and the cloning of the structural gene for the 40 kDa protease, PSCP or other proteases produced by *B. cepacia* strain Pc715j was attempted.

Purification of the 40 kDa protease was performed as described in McKevitt *et al.* in 1989 and in Kooi *et al.* in 1994. After ammonium sulfate precipitation of the culture supernatants and dialysis, the proteases were purified by DEAE Sephacel ion-exchange chromatography. PSCP predominantly eluted off the column with a buffer wash in the unbound fraction. The 40 kDa protease was eluted off with a 0-1 M NaCl gradient. Often the gradient elution also eluted off small remaining amounts of PSCP with the 40 kDa protease. Thus, the two proteases appear to have some structural differences in their interaction with the DEAE ion-exchange resin where more negatively charged proteins will bind more tightly to the positively-charged DEAE moiety on the resin. However, the 40 kDa protease eluted off the column in the first half of the NaCl gradient with some PSCP. No 40 kDa protease eluted off in the buffer wash. Thus, the two proteases appear to have minor structural differences in their overall interaction with the ion-exchange resin. Subsequent gel filtration of the 40 kDa protease NaCl fractions allowed salt removal from the sample and further separation from other contaminating proteins in the sample. Small

amounts of PSCP did, however, remain in the sample of 40 kDa protease used in the subsequent characterization studies.

Characterization of the 40 kDa protease began with determination of the pH and temperature optima for activity by the hide powder azure assay. The pH optimum was found to be 6, which is the same pH optimum determined by McKevitt *et al.* (1989) for PSCP. The pH optimum determined for PSCP in this study was pH 7, though the activity at pH 6 was very close; thus setting the pH optimum in a range of 6-7. The pH optimum of the 40 kDa protease at 6 was very close to the activity observed at pH 5 and pH 7, thus suggesting more of a range of optimal activity for this protease. The pH of the thermolysins (neutral metalloproteases) versus the serralysins such as *P. aeruginosa* alkaline protease (typically have a more alkaline pH optimum) suggests that the 40 kDa protease is in the thermolysin family of metalloproteases. Further experiments described below classify the 40 kDa protease as a putative zinc metalloprotease. The temperature optimum for the 40 kDa protease was determined to be 50°C which was 5°C above the temperature optimum previously reported for PSCP by McKevitt *et al.* (1989). In a comparison of rhizosphere versus clinical isolates of *B. cepacia* by Bevivino *et al.* (1994), the optimal temperature for growth for the clinical isolates was at 42°C compared to the wider temperature range preferred by the rhizosphere isolates. The higher temperature optimum of the 40 kDa protease is then appropriate as it originates from a clinical strain of *B. cepacia* (strain Pc715j). Thus, preliminary comparison of the 40 kDa protease with PSCP suggested a similarity in the physical properties necessary for optimal activity.

Determination of the isoelectric point of a protein yields very specific information about the structural properties of that protein. The isoelectric point (pI) depends on the ratio of surface-exposed charged amino acids in a protein, where the ratio of negatively charged amino acids in the protease sequence to positively charged amino acids exposed to the surface in the protease's native conformation determines the protease's pI. Thus, this property reflects the intrinsic structure of the protein both in its primary amino acid sequence and in its native tertiary structure. The isoelectric points of the 40 kDa protease and of PSCP were determined on a broad-range isoelectric focusing (IEF) gel with a preformed pH gradient in the 3-9 range. The 40 kDa protease and PSCP were found to be extremely close in their pI determinations as both proteins focused at pH 5.2. PSCP did require more volthours to come to its final focused position than did the 40 kDa protease reflecting the differences in certain properties of the two proteases. However, the near identity of the isoelectric points of the two proteases suggests a strong similarity between the primary and tertiary structures of the 40 kDa protease and PSCP especially in the ratios of their surface-exposed charged amino acids.

Classification of the 40 kDa protease was necessary to ascertain the mechanism of action of this protease and to determine its structural similarity to PSCP. Bacterial proteases belong to four major groups according to the catalytic group present at the active site: the serine proteases, the cysteine proteases, the aspartic proteases and the metalloproteases (Hase and Finkelstein, 1993). Preliminary classification of a protease begins with the study of the effect of class-specific inhibitors on the activity of the protease. Inhibitors chosen included the cysteine protease inhibitor dithiothreitol, a

reducing agent inhibiting cysteine proteases or other proteases containing disulfide bridges. Serine protease inhibitors were PMSF, a serine protease inhibitor that can also inhibit some cysteine proteases and 3,4-DCL, a specific serine protease inhibitor. Metalloprotease inhibitors can be chelators of divalent metal cations, or can specifically inhibit bacterial metalloproteases. The chelator EDTA was chosen as the initial inhibitor of metalloproteases or other proteases requiring divalent cations for their activity; and 1,10-phenanthroline was utilized for its preference for chelating Zn^{2+} ions over other divalent cations such as Ca^{2+} (Beynon *et al.* 1989). Inhibition of the 40 kDa protease at effective concentrations of EDTA as indicated in Beynon *et al.* (1989) could preliminarily identify the 40 kDa protease as a metalloprotease and inhibition with effective concentrations of 1,10-phenanthroline (Beynon *et al.* 1989) could preliminarily classify the 40 kDa protease as a zinc-containing metalloprotease. Total inhibition of activity of the 40 kDa protease occurred with low (0.1 mM) concentrations of the metal chelators EDTA and 1,10-phenanthroline (Table 5). These results preliminarily classified the 40 kDa protease as a zinc metalloprotease. Significant activity inhibition occurred with 0.1 mM PMSF, however, no significant inhibition of 40 kDa protease activity was observed with the specific serine protease inhibitor 3,4-DCL thus confirming the preliminary classification of the 40 kDa protease as a zinc metalloprotease.

Confirmation of the 40 kDa protease as a zinc-requiring metalloprotease was achieved with the measurement of activity recovery of the 40 kDa protease in the presence of the metal chelator EDTA by the addition of various concentrations of divalent metal cations. Beynon *et al.* (1989) states that μM amounts of Zn^{2+} should restore activity to a

zinc metalloprotease in the presence of a metal chelator and that mM amount of Zn^{2+} would be inhibitory to the activity of the protease. The activity of the 40 kDa protease when in the presence of 0.1 mM EDTA was restored to control levels by 100 μM Zn^{2+}, and mM amounts of Zn^{2+} were inhibitory to the protease's activity (Table 4). Thus, the classification of the 40 kDa protease as a zinc metalloprotease was confirmed placing it in the same category of bacterial proteases as PSCP. Other metal cations restoring activity of the 40 kDa protease to significant levels were manganese and iron. One mM Mn^{2+} restored protease activity to control levels and 0.1 mM Fe^{3+} significantly restored activity to 64% of control levels. Metalloproteases such as elastase also have calcium-binding sites for stability of the protease (Fukushima *et al.* 1989). However, calcium ions demonstrated little to no ability to restore activity to the 40 kDa protease in the described experiments (ref. 3.2.3). One mM Ca^{2+} could only restore 8.2% of the activity of the 40 kDa protease. Other metal ions indicated as having effects on activity recovery in zinc metalloproteases include magnesium ions, but, no activity recovery with Mg^{2+} was noted for the 40 kDa protease. As zinc metalloproteases often exhibit more similarity in their tertiary structure than in their primary sequence, it has been noted that various divalent metal cations can substitute at the active site of the zinc metalloproteases with varying degrees of effective substitution when observing activity recovery. Thus, the 40 kDa protease may also differ from the bacterial zinc metalloproteases such as elastase in that manganese ions may more effectively substitute at the active site of the 40 kDa protease than calcium or magnesium. Previous metal replacement experiments did indicate recovery of 40 kDa protease activity to 66% of control levels with 1 mM Ca^{2+} (data not

shown); these results were not observed in latter experiments. McKevitt *et al.* (1989) reported that activity of PSCP in the presence of 10 mM EDTA was 50% restored by 10 mM Ca^{2+}, 35% restored by 10 mM Zn^{2+} and 45% restored by 100 mM Mg^{2+}. The present study focused on utilizing lower concentrations of metal cations in replacement studies; 10 mM Ca^{2+}, 10 mM Zn^{2+}, 10 mM and 100 mM Mg^{2+} were not included in the metal replacement experiment. Thus, the efficiency of metal cations substituting at the active site of zinc metalloproteases may be dependent on the concentrations of the protease, the metal chelator and the substituted metal ion as well as the efficiency with which the metal cation fits into the active site and catalyzes activity of the protease.

Kooi *et al.* (1994) produced six MAbs, five to PSCP (including 36-6-6 and 36-6-8), and one to the 40 kDa protease (G-11). The PSCP MAbs 36-6-6 and 36-6-8 were capable of distinguishing between bacterial metalloproteases belonging to the thermolysin class (eg. *B. cepacia* PSCP, *P. aeruginosa* elastase, *V. cholerae* HA/protease, thermolysin) and those belonging to the serralysin class (eg. *P. aeruginosa* alkaline protease, *S. marcescens* metalloprotease) by neutralization of only the thermolysin-type metalloproteases (Kooi and Sokol 1996). Activity of the 40 kDa protease was neutralized to 24% of control levels with 36-6-8 suggesting classification in the thermolysin class of bacterial metalloproteases (Table 5). Kooi *et al.* (1997) further defined the epitopes being neutralized by these monoclonal antibodies by epitope mapping of a 13.9 kDa peptide from *P. aeruginosa* elastase that was strongly recognized by 36-6-6 and 36-6-8. Since the PSCP sequence was not available, elastase was chosen for the epitope mapping studies as it is a protease hypothesized to be similar to PSCP. The 13.9 kDa peptide was used to

generate a series of overlapping 9-mer peptides; peptides 15 and 42 produced the strongest reactions on ELISAS with 36-6-6 and 36-6-8. Peptide 15 overlapped the zinc-binding active site of elastase, and peptide 42 was in between the third zinc-binding ligand from elastase and a proton donor at the active site (Kooi *et al.* 1997). The anti-peptide polyclonal sera was also reported to distinguish between the thermolysin and serralysin class of metalloproteases by neutralization of bacterial metalloproteases belonging to the thermolysin class such as elastase, PSCP, thermolysin and *V. cholerae* HA/protease. The 40 kDa protease was not neutralized by either anti-peptide 15 or anti-peptide 42 polyclonal sera (Table 5). Previous repeated experiments in this study failed to show neutralization of PSCP or the 40 kDa protease by the anti-peptide polyclonal sera; neutralization of elastase and thermolysin with the antisera occurred only with fresh preparations of these proteases. It is possible that the polyclonal sera is unstable in its storage conditions since different preparations of monoclonal or polyclonal antibodies can exhibit varying stability according both to the specific MAbs or polyclonal sera in question, and to the storage conditions. It is also possible that the epitopes being recognized by the anti-peptide 15 and anti-peptide 42 polyclonal sera in the 40 kDa protease are not close enough to elastase in the primary sequence and tertiary zinc-binding site and active site structure to be effectively neutralized by these polyclonal antibodies.

To assess the substrate specificity of the 40 kDa protease, incubation of the protease with substrates relevant to the possible role of the 40 kDa protease as a virulence factor in *B. cepacia* respiratory infections in CF patients was performed. The purpose of these experiments was to assess the ability of the 40 kDa protease to degrade the

substrates under laboratory conditions as a starting point for determining the substrate specificity of this protease. Substrates tested included IgG, with functions including activation of complement and facilitation of opsonization and constituting 80% of total serum immunoglobulin; IgM, the first immunoglobulin class produced in response to an antigen and accounting for 5-10% of the total serum immunoglobulin; IgA, comprising 10-15% of the total immunoglobulin in the serum; and secretory IgA; the predominant immunoglobulin class in external secretions such as mucous of the respiratory tract. Secretory IgA can bind to the surface structures of bacterial pathogens thus preventing their attachment to mucosal cells (Kuby 1992). The 40 kDa protease was able to degrade all of the above immunoglobulin classes resulting in a nonspecific degradation and presumably rendering the immunoglobulins ineffective in their function (Fig. 7). This inactivation of the immunoglobulins in an infection would significantly reduce the effectiveness of the host humoral response in eradication of the pathogen especially in *B. cepacia* infections where eradication is rarely achieved (Isles *et al.* 1984). Fibronectin (from bovine plasma) and collagen VIII, both components of the basement membrane, were totally degraded by the 40 kDa protease in half the incubation time utilized in the other substrate experiments yielding fibronectin products in the 140 kDa range, 60 kDa range, 50 kDa range and the 35 kDa range. Collagen VIII degradation resulted in ~65 kDa and ~45 kDa products (Fig. 8). Fibronectin is an adhesive glycoprotein in the extracellular matrix facilitating adhesion by cells to the extracellular matrix. Collagen type VIII is a significant component of all tissues such as those in the respiratory tract (Alberts *et al.* 1989). Thus, degradation of these two components would significantly add to the

tissue damage exhibited in more severe *B. cepacia* infections in CF patients. Another component of the extracellular matrix of the lung, elastin, was not degraded by the 40 kDa protease in the chromogenic elastin Congo Red assay (Table 6). Few bacterial proteases exhibit elastase activity (Galloway 1991). The iron-sequestering molecules transferrin, found in abundance in the saliva, plasma, and also in the respiratory tract, and lactoferrin, present in abundance in mucosal secretions are an important host defense for bacterial pathogens encountering the iron-limited environment of the respiratory tract (Meitzner *et al.* 1994, Weinberg 1984). The 40 kDa protease was able to degrade holo-transferrin (iron-saturated) and apo-transferrin (iron-limited) forms of transferrin to products in the 55 kDa size range (Fig. 6). Little to no degradation was observed for holo-lactoferrin and apo-lactoferrin. The occurrence of *B.cepacia*-associated bacteremia in CF patients demonstrates the potential for *B. cepacia* to acquire iron in the blood via degradation of circulating transferrin. In addition, degradation of transferrin by elastase in *P. aeruginosa* infections in the CF lung produced iron chelates capable of reacting with the oxidants produced by neutrophils in the lung to produce highly cytotoxic hydroxyl free radicals capable of causing extensive damage to the lung tissue (Britigan *et al.* 1993, Miller *et al.* 1996). Severe *B. cepacia* infections in the CF lung have demonstrated extensive damage and necrosis in the lung tissue. Thus, transferrin degradation by the 40 kDa protease provides a possible route to this clinical condition (Isles *et al.* 1984, Tomashefski *et al.* 1988).

The question of whether the 40 kDa protease is a precursor of PSCP is uncertain. Both proteases share common physical characteristics such as pH and temperature optima

and, significantly, have the same isoelectric point. They are both zinc metalloproteases, though EDTA inhibition of the proteases followed by the addition of magnesium ions only shows activity recovery with PSCP. Substrate specificity is also comparable (immunoglobulin and transferrin degradation by PSCP has been demonstrated in our lab; unpublished results). However, digestion of the 40 kDa protease with trypsin did not produce PSCP. Digestion of PSCP with N-chlorosuccinimide which cleaves after tryptophan residues (this lab, unpublished results) does yield a highly similar digestion pattern to that of the 40 kDa protease. Digestion of PSCP yields five fragments: 17, 18, 21, 24, 29.5 and 33.5 kDa. Digestion of the 40 kDa protease yields four fragments: 21, 24, 28 and 34 kDa, with a possible 17 kDa fragment. This suggests similarities in the primary sequence of both proteases agreeing with the similarity in the pI's of the two proteases. Exoenzyme S (ExoS) and exoenzyme T (ExoT) from *P. aeruginosa* are examples of two protein sharing common structural and physical characteristics yet are encoded by separate genes. The ExoS high molecular weight aggregate was found to migrate on SDS-PAGE gels as two proteins with molecular weights of 49 and 53 kDa (Olson *et al.* 1997). These two proteins are immunologically cross-reactive, share common amino-terminal sequences and yield common fragments when digested with trypsin, chymotrypsin or cyanogen bromide. Exoenzyme S (49 kDa) is enzymatically more active *in vitro* than Exoenzyme T (53 kDa) However, these two proteins are encoded by separate genes and share 75% sequence identity (Olson *et al.* 1997). In addition, the three metalloproteases (50, 53 and 55 kDa) produced by *Erwinia chrysanthemi* are closely related yet are encoded by separate genes that share a high level of homology with each

other (Wandersman *et al.* 1987). Thus, although two proteins may seem highly similar in their physical and structural characteristics, they may be distinct proteins encoded by separate genes. It is certain that the *B. cepacia* proteases are strongly related according to their amino acid sequences, physical characteristics, putative structural characteristics and substrate specificity. However, to properly assess the relationship between these two proteases the structural genes need to be cloned and further characterization experiments need to be performed.

Cloning of the structural gene for PSCP had previously been unsuccessfully attempted in our lab by Dr. A.D. Cox. The amino-terminal end of PSCP was sequenced by Edman degradation, and a primer was designed to a portion of this amino acid sequence. Southern hybridization experiments and PCR with this primer failed to locate the PSCP structural gene. Since it was hypothesized that the 40 kDa protease could be a precursor of PSCP and also could be a potentially significant virulence factor due to its high-level constitutive expression, the amino-terminal end of this protease was also sequenced by Edman degradation. A primer was designed to this amino acid sequence, and Pc715j genomic digests were screened by Southern hybridizations using this primer. Again, these experiments did not locate the structural gene for the 40 kDa protease (this lab, unpublished results). The present study examined the possibility of obtaining internal amino acid sequences of the 40 kDa protease to apply to cloning strategies such as Southern hybridization and PCR. A primer to an internal sequence of the protease in combination with the primer to the amino-terminal end of the protease could be used in PCR reactions to amplify the fragment of the structural gene in between these two

primers. In addition, the bacterial zinc metalloproteases tend to be more highly conserved in central regions than at the amino-terminal end. Thus, an internal fragment could contain a more conserved sequence allowing the primer to preferentially recognize the sequence for the 40 kDa protease structural gene and to avoid nonspecific binding. The internal peptide sequence could contain a region allowing primer design with less degeneracy than that of the amino-terminal primer. Digestion of the 40 kDa protease with N-chlorosuccinimide, (NCS) which cleaves after tryptophan residues, consistently yielded three peptides: 21 kDa, 24 kDa and 28 kDa. Comparison of the amino acid sequences for the amino-terminal end of the 40 kDa protease and of the three internal peptides did not exhibit any overlap. Thus longer contiguous amino acid sequences were not available for primer design or homology searches. Amino-terminal sequencing of the 21 kDa and 28 kDa fragments produced high-signal quality sequences; the 24 kDa fragment was later subjected to N-terminal sequencing, but the signal was not as high (data not shown). Primers were designed to the 21 kDa sequence (R40-21) and the 28 kDa sequence (R40-28) in the reverse complementarity.

The two primers were first screened in Southern hybridization experiments to genomic digests of *B. cepacia* strain Pc715j DNA. Hybridizations with R40-28 to genomic digests failed to exhibit any binding at all at any stringency; no binding occurred at 27, 35, 37 or 40°C. R40-21 exhibited variable binding to Pc715j genomic digests though no specific binding was observed. Initial screening of R40-21 hybridizations began with the suggested optimal temperature for binding of this primer: the melting temperature of the primer (T_m) minus 25°C, minus 5°C for each possible base pair mismatch. The

optimal temperature was 45-50°C for hybridization of R40-21 or R40-28 to their DNA sequences in the Pc715j genome. No binding was observed at 50°C for R40-21 indicating this stringency was too high for the specificity of this primer for its DNA sequence. The lower stringency, 27°C, was found to be too low in that nonspecific binding of R40-21 was observed by three bands in the *Pst*I genomic digest and faint non-banding smears in the other genomic digests. The temperature was raised to 40°C; no binding was observed for R40-21. An intermediate stringency of 35°C, attempted twice with one of the experiments demonstrated in figure 11, produced high levels of nonspecific binding by R40-21 via intense, multiple bands in all genomic digests. Due to the occurrence of nonspecific binding via multiple bands by R40-21 and the absence of any visible binding by R40-28 at multiple stringencies, R40-21 was pursued as the primer more likely to recognize the sequence of the 40 kDa protease structural gene. When hybridization was repeated at 40°C, faint nonspecific binding occurred though no binding had occurred the first time. To attempt to attain the optimal stringency for binding of R40-21 to genomic digests specifically and to avoid binding to nonspecific sequences in the Pc715j genome, a stringency of 37°C was attempted with no binding occurring by R40-21. Repeated experiments at 32, 40 and 42°C (twice) did not demonstrate any binding at all by R40-21. Formamide is often added to prehybridization and hybridization solutions allowing a lower stringency to be used while at the same time obtaining specific results characteristic of a higher stringency (Vasil *et al.* 1986b). Hybridization at 42°C with solutions containing formamide with R40-21 produced binding to the entire blot; repeated exposure simply yielded a gray blot with no evident banding, specific or nonspecific. The absence of a

positive control for hybridization with the radiolabelled primers in these experiments did not allow detection of whether the primers were binding to the chromosomal DNA digests. Thus, there is a possibility that the absence of binding by the primers was caused by poor hybridization conditions. Overall, it seems that the question was not of finding the optimal stringency for binding of these primers, especially R40-21, to the Pc715j genomic digests; but rather that the similarity of the nucleotide sequence of these primers to the actual DNA sequence of the 40 kDa protease structural gene was too distant to allow specific detection by Southern hybridization using R40-21 or R40-28.

A different approach to cloning the structural gene for the 40 kDa protease structural gene was to use the complementary primer from the amino-terminal end of the undigested 40 kDa protease with either the reverse complementary R40-21 or R40-28 primer to amplify the intervening region. Initial screening of the genome with the two primer combinations using various annealing temperatures failed to amplify any detectable specific products. Effective denaturation of the *B. cepacia* GC-rich genome via the addition of 5% DMSO and higher denaturation temperatures yielded a 340 bp product in small quantities amplified from 40N and R40-21 (Fig. 12, lane 3); 40N and R40-28 failed to amplify any products under these conditions. Reamplification of previous reaction products again yielded this 340 bp product; sequencing revealed that it was not a fragment of the 40 kDa protease structural gene. Reamplification of PCR products is a useful procedure but highly subject to contamination; thus, this result was not unexpected. Increasing the specificity of the reaction by lower template and $MgCl_2$ concentrations allowed amplification of three products directly from the *B. cepacia* Pc715j genome by

40N and R40-21: 180 bp, 340 bp and 425 bp (Fig. 13, lane 5). Again, 40N and R40-28 failed to amplify any products under these conditions. The three products amplified by 40N and R40-21 (pVS21-180, pVS21-340, pVS21-425,) when analyzed by sequencing, were not fragments from the structural gene for the 40 kDa protease (Table 7). The initial reamplification 340 bp product and the pVS21-340 had different sequences indicating the nonspecific nature of the primer's affinity for *B. cepacia* Pc715j genomic DNA and possibly implicating the reamplification product as a contaminant amplified from one of the reagents in the PCR reaction. Three clones of the 425 bp product did have the same sequence removing the cloning process as a possible factor in the PCR product's failure to correspond with the amino acid sequence from the 40 kDa protease structural gene. By further increasing the specificity of the reaction via lower template amounts and higher annealing temperatureas, the 180, 340 and 425 bp products and other products were amplified. To identify which products were amplified by single primers at both ends control reactions with single primers were performed. When these products were eliminated, 40N and R40-21 amplified a 1050 bp product (Fig. 14, lane 9), and 40N and R40-28 amplified a 1000 bp product (Fig. 14, lane 7); however, these products (pVS21-1050 and pVS28-1000) were not fragments originating from the structural gene for the 40 kDa protease (Table 7). Two clones of the 1000 bp product demonstrated different sequences; one had significant homology to a UDP-N-acetylglucosamine 1-carboxyvinyltansferase in various gram-negative bacteria. The 1050 bp product was primed on both ends by R40-21. The primer's tendency to anneal to sequences in the *B. cepacia* Pc715j genomic DNA that do not originate from the 40 kDa protease structural

gene is striking considering the 40N, R40-21 and R40-28 primers design from the amino acid sequence of the 40 kDa protease and the specificity of the PCR reactions by the high denaturation and annealing temperatures, low template amounts, and lower $MgCl_2$ concentrations. In addition, these conditions produced more amplification products than the more nonspecific conditions previously utilized. However, higher template amounts can inhibit efficient amplification of products; thus, perhaps the lower template amounts served to allow the primers more access to the fully denatured genome, allowing amplification of additional nonspecific products. As stated before, the sequence of the primers is probably not close enough to the actual DNA sequence of the 40 kDa protease gene to allow specific recognition of the sequence over other nonspecific sequences. Another possibility that must be addressed is the degree of purity of the 40 kDa protease preparation used in the NCS digestions. Cleavage yielded three fragments that probably originated from the 40 kDa protease but could not be confirmed. The Edman degradation procedure can detect minute amounts of protein and signals obtained from this procedure are usually intermingled with lower signals from contaminating proteins; the observer's task is to deduce the primary protein in the mixture being sequenced. The purity of the peptide fragments is thus also a possible factor in that a contaminant protein was sequenced, and the amino acid sequences do not originate from the 40 kDa protease amino acid sequence. However, the most probable factor in the failure to amplify a portion of the 40 kDa protease gene is the aforementioned dissimilarity of the primers to the actual 40 kDa protease nucleotide sequence.

The inability of the primers to locate the structural gene of the 40 kDa protease led to the use of a different approach to clone the protease gene. A cosmid gene bank of *B. cepacia* strain Pc715j was constructed and packaged in *E. coli* strain HB101 with a sufficient number of transductants containing unique genomic fragments to allow for coverage of the entire *B. cepacia* 7 Mb genome (Cheng *et al.* 1994). Screening for a protease-positive phenotype on skim milk agar plates could locate the structural gene for any protease expressed by *B. cepacia* if in combination with the appropriate secretory and regulatory genes either located on the genomic fragment or provided by the host. When combining the unique insert frequencies and the average insert size for both gene banks, the kilobases covered are 45.1 Mb. When considering the 9-20 kb genomic fragments contained in the gene bank, the combined unique genomic fragment frequency and the average genomic fragment size yields a total of 34.2 Mb. Thus, with either method of calculation, it appears that *B. cepacia* Pc715j genomic fragments less than 20 kb in size containing at least one *Hind*III site are represented in this gene bank. Screening of the gene bank in the *E. coli* HB101 host failed to exhibit the protease-positive phenotype. This result could be due to the absence of processing and/or secretion apparatus for maturation of the *B. cepacia* protease(s). The genus *Burkholderia* used to be part of the *Pseudomonas* genus; thus the two genera are more related to each other than to other genera of gram-negative bacteria (Goldmann *et al.* 1986). In addition, both *P. aeruginosa* and *B. cepacia* are CF pathogens present in the later teenage to adult years of CF patients with an ~80% occurrence of concurrent infection (rev. in Govan *et al.* 1996). Antibodies in serum from CF patients to elastase cross-react with PSCP from *B. cepacia* and vice-

versa (McKevitt *et al.* 1989). Thus, a *P. aeruginosa* protease-negative strain, PA103-11 (elastase negative, chemical mutant of alkaline protease) was chosen as the host for the Pc715j gene bank. Transconjugants however did not contain inserts indicating the host was recognizing the Pc715j genomic fragments as foreign and degrading them even though the host was grown at 42°C to inactivate the restriction system. It seems that the creation of a separate genus for *Burkholderia spp.* was prudent as the two genera are actually quite different from each other in many aspect (Yabuuchi *et al.* 1992).

A number of *B. cepacia* strains were previously screened in this laboratory for the protease phenotype by the skim milk agar assay. Putative protease negative strains were analyzed for protease production by examination of their extracellular supernatants for PSCP or the 40 kDa protease. Although negative on skim milk agar; three clinical CF strains produced both PSCP and the 40 kDa protease as well as exhibiting some proteolysis of casein in the skim milk after prolonged incubation (Fig. 19, lanes 7-9). Other *B. cepacia* strains have exhibited differences in proteolysis of substrates such as the casein in skim milk agar versus hide powder azure where strains often appear positive even though they are negative by the skim milk assay. Thus, the expression of both proteases by these strains was not surprising. In addition, Bevivino *et al.* (1994) demonstrated that only clinical strains exhibited a proteolytic activity when compared to rhizosphere isolates of *B. cepacia*. The strain isolated from a head wound did not produce protease (Fig. 19, lane 6); nor did a plant isolate, 22-12 (Fig. 9, lane 5). Due to the atypical characteristics of the head isolate and the lack of information on its properties;

strain 22-12 was chosen as the host of the gene bank. The degradation of Pc715j genomic fragments in the same species, but different strain, was highly improbable.

Transconjugants of the triparental mating of the Pc715j gene bank in *E. coli* HB101 into *B. cepacia* 22-12 did contain inserts. Calculation of the amount of kilobases being screened in 22-12 took into account the insert frequency in the original gene bank as well as that in the transconjugants. According to the unique insert frequency, 107.4 Mb of Pc715j inserts was screened in 22-12; according to the unique genomic fragment frequency, 69.3 Mb of Pc715j *Hind*III genomic fragments was screened in 22-12. Thus, it can be assumed that the entire gene bank was screened in strain 22-12. However, no clones exhibiting a protease-positive phenotype on skim milk agar were observed. The *Hind*III restriction site is six base pairs long and is thus a specific restriction site when compared to restriction enzymes recognizing a four base pair site. There is a good possibility that there exist fragments in the *B. cepacia* genome that do not contain a *Hind*III site in 20 kb of sequence thus eliminating a portion of the genome from being screened. The restriction site could cleave in the middle of the protease structural gene though this is less probable due to the partial restriction digest of the *B. cepacia* Pc715j genome for construction of the gene bank. The reason for the protease-negative phenotype of strain 22-12 is unknown; necessary secretory and/or regulatory functions could be absent, thus preventing extracellular secretion of a protease expressed from the Pc715j genomic fragment. Mutations in general secretory pathway proteins or in the disulfide bond-forming protein DsbB in *B. cepacia* abolished expression and extracellular secretion of PSCP (Nakazawa and Abe, 1996). If Pc715j protease structural genes were

not contained in the gene bank or were interrupted by a *Hind*III restriction site, the proteases may not have been expressed. The classification of the 40 kDa protease as a thermolysin-type protease is putative at this point; it could be a serralysin-type protease or a non-classified bacterial metalloprotease. If it is a serralysin, then it needs the unique secretory protein required by the serralysins for extracellular secretion of a mature, functioning protease (Hase and Finkelstein, 1993), and these genes may not be on the same genomic fragment as the 40 kDa protease structural gene or they could be interrupted. Through equilibrium dynamics, all of the cosmid clones may not have been transferred to the host strain. Additionally, both in the construction of the gene bank and in the growth of the gene bank for triparental matings, certain transductants and/or transconjugants may have been selected for due to some advantage conferred by the genes expressed from the Pc715j genomic fragment or by the cosmid vector preferentially ligating certain size fragments or the packaging strain of bacteriophage λ preferentially packaging specific cosmid constructs. It is highly probable that one of these mechanisms caused a portion of the *B. cepacia* genome to not be contained in the gene bank and/or in the 22-12 transconjugants; or the protease gene could not express a mature protease for the reasons mentioned above.

Future studies should begin by modifying the cosmid gene bank approach for cloning protease structural genes as it is an approach that could locate a protease structural gene. As previously demonstrated, PSCP is probably a thermolysin-type metalloprotease with secretion by the general secretory pathway; thus, a protease structural gene should be expressed and exported in its mature form in a *B. cepacia*

protease negative host such as 22-12. However, a new gene bank should be constructed with a restriction enzyme exhibiting a shorter recognition site to allow for a more random selection of genomic fragments, such as *Sau*3A or *Hae*III (recognition site GG/CC). This latter enzyme would in fact be a good choice due to the high G-C content of its recognition site which should be present in abundance in the *B. cepacia* genome. The gene bank should also be constructed such that it contains a higher number of cosmid clones containing unique genomic fragments by transducing the packaged cosmid constructs into an aliquot of concentrated, viable, log-phase *E. coli* HB101 cells. This would help to ensure that the whole *B. cepacia* genome was being represented in the gene bank. Repeated triparental matings with this gene bank into *B. cepacia* strain 22-12 should allow for the entire gene bank being screened in 22-12.

The primers to the 40 kDa protease, 40N, R40-21 and R40-28 should be utilized together in hybridizations to *B. cepacia* Pc715j genomic digests to increase the signal observed by these radiolabelled oligonucleotides. In the case where multiple banding occurs to the Pc715j genomic digests by R40-21, additional primers would serve to intensify the signal corresponding to the specific recognition of the 40 kDa protease structural gene by R40-21.

The other approach that should be explored is to clone the gene for any protease produced by *B. cepacia* via mutagenesis. Mutagenesis protocols such as transposon mutagenesis have the advantage of creating only one transposon insertion per bacterium; thus, a mutant exhibiting a protease-negative phenotype on skim milk agar would definitely contain a transposon inserted inside a gene relevant to the production of

protease. This approach has been attempted in this laboratory. A protease-negative mutant was found to contain the transposon insertion in the *xcpR* homolog from *P. aeruginosa*, also reported by Nakazawa and Abe (1996). In addition, Nakazawa and Abe have 12 uncharacterized mutants negative for protease and lipase production, though the mutation in both protease and lipase make it unlikely that a structural gene for a *B. cepacia* protease has been interrupted. It is more likely that additional secretory and regulatory genes have transposon insertions.

The other mutagenesis approach that should be considered is chemical mutagenesis followed by complementation with the above gene bank. Chemical mutagenesis is a non-selective random procedure for inactivating a gene or genes in the selected host. The structural gene for *P. aeruginosa* alkaline protease was cloned in this manner (Guzzo *et al.* 1990). Ethyl methanesulfonate mutagenesis of PA103, an elastase-deficient prototroph, was followed by screening of mutants on skim milk agar. Mutants exhibiting a protease-negative phenotype by the skim milk assay were complemented with a gene bank of *P. aeruginosa* strain PAO1 resulting in cloning of the alkaline protease structural gene (Guzzo *et al.* 1990). This approach would avoid the selectivity exhibited by transposon insertion where though insertion is basically random, transposons do insert at sequence 'hot spots' in a bacterial genome. Although chemical mutagenesis could inactivate more than one gene, the protocol could be modified such that in most cases, only one gene is being inactivated per bacterium. This approach could locate protease structural genes or regulatory or secretory genes with equal success.

When characterizing the 40 kDa protease, the cloning of the structural gene is highly desirable for expressing sufficient amounts of the pure protease for characterization studies where the purity of the protease is certain. However, in the absence of this structural gene, the preparations of PSCP and the 40 kDa protease could be further resolved via a higher capacity (greater height) gel filtration column, or by HPLC (high-performance liquid chromatography). The resultant characterization studies would be more accurate with a higher certainty of the purity of the protease preparation being utilized.

In summary, the 40 kDa protease was characterized and compared in its properties to PSCP. The 40 kDa protease was found to have the same pH optimum (6) and temperature optimum (50°C) as PSCP. The isoelectric point of PSCP and the 40 kDa protease was determined and found to be the same at 5.2 for both proteases. The activity of the 40 kDa protease was inhibited by traditional metal chelators such as EDTA and zinc chelators such as 1,10-phenanthroline. Activity of the protease was regained with 100 μM Zn^{2+} confirming the identity of the 40 kDa protease as a zinc metalloprotease and classifying it in the same category as PSCP (Beynon *et al.* 1989). The 40 kDa protease was putatively categorized in the thermolysin class of zinc metalloproteases due to its more neutral pH optimum and its neutralization by the monoclonal antibody to PSCP, 36-6-8 (Kooi *et al.* 1996). The 40 kDa protease was able to degrade fibronectin, collagen VIII, holotransferrin and apotransferrin, IgG, IgM, serum IgA and secretory IgA. Primers designed to internal peptides of the 40 kDa protease were found to lack the specificity necessary to locate the 40 kDa protease structural gene either by Southern hybridization

or by PCR. A *Hind*III cosmid gene bank of *B. cepacia* strain Pc715j was constructed and screened in a protease negative strain (22-12) of *B. cepacia*, a protease-positive clone was not obtained. Thus, although the structural gene for the 40 kDa protease was not located, the characterization of the 40 kDa protease served to further clarify the relationship between this protease and PSCP in that the two protease are highly related and the 40 kDa protease is a possible precursor of PSCP. Further elucidation of this relationship and determination of the role of these two proteases as virulence factors in *B. cepacia* infections in CF patients will provide a clearer picture of the pathogenesis of this organism; and will allow research and design of therapeutic and preventive strategies for controlling infections by this organism.

5.0 REFERENCES

Abe, M., and T. Nakazawa. 1996a. The *dsbB* gene product is required for protease production by *Burkholderia cepacia*. Infect. Immun. **64**:4378-4380.

Abe, M., Tsuda, M., Kimoto, M., Inouye, S., Atsushi, N., and T. Nakazawa. 1996b. A genetic analysis system of *Burkholderia cepacia*: construction of mobilizable transposons and a cloning vector. Gene **174**: 191-194.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and J.D. Watson. 1989. Molecular biology of the cell. Garland Publishing Co., New York, USA.

Aronoff, S.C. 1988. Outer membrane permeability in *Pseudomonas cepacia*: diminished porin content in a β-lactam-resistant mutant and in resistant cystic fibrosis isolates. Antimicrob. Agents Chemother. **32**: 1636-1639.

Azghani, A.O., Connelly, J.C., Peterson, B.T., Gray, L.D., Collins, M.L. and A.R. Johnson. 1990. Effects of *Pseudomonas aeruginosa* elastase on alveolar epithelial permeability in guinea pigs. Infect. Immun. **58:**433-438.

Barth, A.L. and T.L. Pitt. 1995. Auxotrophy of *Burkholderia (Pseudomonas) cepacia* from cystic fibrosis patients. J. Clin. Microbiol. **33**: 2192-2194.

Bever, R.A. and B.H. Iglewski. 1988. Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. J. Bacteriol. **170:** 4309-4314.

Bevivino, A., Tabacchioni, S., Chiarini, L., Carusi, M.V., Del Gallo, M. and P. Visca. 1994. Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. Microbiol. **140:**1069-1077.

Beynon, R.J. and J.S. Bond. (ed.) 1989. Proteolytic enzymes a practical approach. IRL Press at Oxford University Press, Oxford, England.

Binet, R. and C. Wandersman. 1995. Protein secretion by hybrid bacterial ABC-transporters: specific functions of the membrane ATPase and the membrane fusion protein. EMBO J. **14:**2298-2306.

Bjorn, M.J., Sokol, P.A. and B.H. Iglewski. 1979. Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. J. Bacteriol. **138:** 193-200.

Bode, W., Gomis-Ruth, F-X. and W. Stockler. 1993. Astacins, serralysin, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments

(HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett. **331:** 134-140.

Boyer, H.W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. **41:** 459-472.

Britigan, B.E., Hayek, M.B., Doebbling, B.N. and R.B. Jr. Fick. 1993. Transferrin and lactoferrin undergo proteolytic cleavage in the *Pseudomonas aeruginosa*-infected lungs of patients with cystic fibrosis. Infect. Immun. **61:** 5049-5055.

Bruce, M.C., Poncz, L., Klinger, J.D., Stern, R.C., Tomashefski, J.F. Jr. and D.G. Dearborn. 1985. Biochemical and pathologic evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. Am. Rev. Respir. Dis. **132:** 529-535.

Burkholder, W.H. 1950. Sour skin, a bacterial rot of onion bulbs. Phytopath. **40:** 115-117.

Burns, J.L., Jonas, M., Chi, E.Y., Clark, D.K., Berger, A. and A. Griffith. 1996a. Invasion of respiratory epithelial cells by *Burkholderia (Pseudomonas) cepacia*. Infect. Immun. **64:** 4054-4059.

Burns, J.L., Wadsworth, C.D., Barry, J.J. and C.P. Goodall. 1996b. Nucleotide Sequence analysis of a gene from *Burkholderia (Pseudomonas) cepacia* encoding an outer membrane lipoprotein involved in multiple antibiotic resistance. Antimicrob. Agents Chemother. **40:** 307-313.

Cheng, H-P. and T.G. Lessie. 1994. Multiple replicons constituting the genome of *Pseudomonas cepacia* 17616. J. Bacteriol. **176:** 4034-4042.

Davidson, D.J., Dorin, J.R., McLachlan, G., Ranaldi, V., Lamb, D., Doherty, C., Govan, J. and D.J. Porteous. 1995. Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. Nature Gen. **9:** 351-357.

Deretic, V., Schurr, M.J. and H. Yu. 1995. *Pseudomonas aeruginosa*, mucoidy and the chronic infection phenotype in cystic fibrosis. Trends Microbiol. **3:** 351-356.

Duong, F., Lazdunski, A., Cami, B. and M. Murgier. 1992. Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. Gene **121:** 47-54.

Figurski, D.H. and D.R. Helsinki. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. **76:** 1648-1652.

Govan, J.R.W., Brown, P.H., Maddison, J., Doherty, C.J., Nelson, J.W., Dodd, M., Greening, A.P. and A.K. Webb. 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. Lancet **342:** 15-19.

Govan, J.R.W. and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:** 539-574.

Govan, J.R.W., Hughes, J.E. and P. Vandamme. 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. J. Med. Microbiol. **45:** 395-407.

Gustin, J.K., Kessler, E. and D.E. Ohman. 1996. A substitution at His-120 in the LasA protease of *Pseudomonas aeruginosa* blocks enzymatic activity without affecting propeptide processing or extracellular secretion. J. Bacteriol. **178:** 6608-6617.

Guzzo, J., Duong, F., Wandersman, C., Murgier, M. and A. Lazdunski. 1990. The secretion genes of *Pseudmonas aeruginosa* alkaline protease are functionally related to those of *Erwinia chrysanthemi* proteases and *Escherichia coli* α-haemolysin. Mol. Microbiol. **5:** 447-453.

Finkelstein, R.A., Boesman-Finkelstein, M., Chang, Y. and C.C. Hase. 1992. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect. Immun. **60:** 472-478.

Fitzsimmons, S.C. 1993. The changing epidemiology of cystic fibrosis. J. Pediatr. **122:** 1-9.

Franco, A.A., Mundy, L.M., Trucksis, M., Wu, S., Kaper, J.B. and C.L. Sears. 1997. Cloning and characterization of the *Bacteroides Fragilis* metalloprotease toxin gene. Infect. Immun. **65:** 1007-1113.

Fridlander, M., Inbar, J. and I. Chet. 1993. Biological control of a soil-borne plant pathogens by a beta-1,3 glucanase-producing *Pseudomonas cepacia*. Soil Biol. Biochem. **25:** 1211-1221.

Friedman, A.M., Long, S.R., Brown, S.E., Buikema, W.J. and F.M. Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:** 289-296.

Fukushima, J., Yamamoto, S., Morihara, K., Atsumi, Y., Takeuchi, H., Kawamoto, S. and K. Okuda. 1989. Structural gene and complete amino acid

sequence of *Pseudomonas aeruginosa* IFO 3455 elastase. J. Bacteriol. **171:** 1698-1704.

Galloway, D.R. 1991. *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. Mol. Microbiol. **5:** 2315-2321.

Gessner, A.R. and J.E. Mortensen. 1990. Pathogenic factors of *Pseudomonas cepacia* isolates from patients with cystic fibrosis. J. Med. Microbiol. **33:** 115-120.

Goldberg, J.B. and D.E. Ohman. 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158:** 1115-1121.

Goldmann, D.A. and J.D. Klinger. 1986. *Pseudomonas cepacia*: biology, mechanisms of virulence, epidemiology. J. Pediatr. **108:** 806-812.

Goldstein, R., Sun, L., Jiang, R-Z., Sajjan, U., Forstner, J.F. and C. Campanelli. 1995. Structurally variant classes of pilus appendage fibers coexpressed from *Burkholderia (Pseudomonas) cepacia*. J. Bacteriol. **177:** 1039-1052.

Hase, C.C. and R.A. Finkelstein. 1990. Comparison of the *Vibrio cholerae* hemagglutinin/protease and the *Pseudomonas aeruginosa* elastase. Infect. Immun. **58:** 4011-4015.

Hase, C.C. and R.A. Finkelstein. 1993. Bacterial extracellular zinc-containing metalloproteases. Microbiol. Rev. **57:** 823-837.

Heck, L.W., Alarcon, P.G., Kulhavy, R.M., Morihara, K., Russell, M.W. and J.F. Mestecky. 1990. Degradation of IgA Proteins by *Pseudomonas aeruginosa* elastase. J. Immun. **144:** 2253-2257.

Heck, L.W., Morihara, K. and D.R. Abrahamson. 1986a. Degradation of soluble laminin and depletion of tissue-associated basement membrane laminin by *Pseudomonas aeruginosa* elastase and alkaline protease. Infect. Immun. **54:** 149-153.

Heck, L.W., Morihara, K., McRae, W.B. and E.J. Miller. 1986b. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. Infect. Immun. **51:** 115-118.

Hobson, A.H., Buckley, C.M., Aaman, J.L., Jorgensen, S.T., Diderichsen, B. and D.J. McConnell. 1993. Activation of a bacterial lipase by its chaperone. Proc. Natl. Acad. Sci. **90:** 5682-5686.

Hobson, A.H., Buckley, C.M., Jorgensen, S.T., Diderichsen, B. and D.J. McConnell. 1995. Interaction of the *Pseudomonas cepacia* DSM3959 lipase with its chaperone, LimA. J. Biochem. **118:** 575-581.

Holloway, B.W., Krishnapillai, V. and A.F. Morgan. 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:** 73-102.

Holmes, B. 1986. The identification of *Pseudomonas cepacia* and its occurrence in clinical material. J. Appl. Bacteriol. **61:** 299-314.

Hovey, A.K. and D.W. Frank. 1995. Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon. J. Bacteriol. **177:** 4427-4436.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P. and H. Levison. 1984. *Pseudomonas cepacia* infection in cystic fibrosis: An emerging problem. J. Pediatr. **104:** 206-210.

Jaffar-Bandjee, M.C., Lazdunski, A., Bally, M., Carrere, J., Chazalette, J.P. and C. Galabert. 1995. Production of elastase, exotoxin A, and alkaline protease in sputa

during pulmonary exacerbations of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. J. Clin. Microbiol. **33:** 924-929.

James, B.W., Mauchline, W.S., Dennis, P.J. and C.W. Keevil. 1997. A study of iron acquisition mechanisms of *Legionella pneumophila* grown in chemostat culture. Curr. Microbiol. **34:** 238-243.

Jiang, W. and J.S. Bond. 1992. Families of metalloendopeptidases and their relationships. FEBS **312:** 110-114.

Jorgensen, S., Skov, K.W. and B. Diderichsen. 1991. Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes. J. Bacteriol. **173:** 559-567.

Kawamoto, S., Shibano, Y., Fukushima, J., Ishii, N., Morihara, K. and K. Okuda. 1993. Site-directed mutagenesis of Glu-141 and His-223 in *Pseudomonas aeruginosa* elastase: catalytic activity, processing, and protective activity of the elastase against *Pseudomonas* infection. Infect. Immun. **61:** 1400-1405.

Kessler, E. and M. Safrin. 1994. The propeptide of *Pseudomonas aeruginosa* elastase acts as an elastase inhibitor. J. Biol. Chem. **269:** 22726-22731.

Kessler, E., Safrin, M., Abrams, W.R., Rosenbloom, J. and D.E. Ohman. 1997. Inhibitors and specificity of *Pseudomonas aeruginosa* LasA. J. Biol. Chem. **272:** 9884-9889.

Kessler, E., Safrin, M., Peretz, M. and Y. Burstein. 1992. Identification of cleavage sites involved in proteolytic processing of *Pseudomonas aeruginosa* preproelastase. FEBS **299:** 291-293.

Klimpel, K.R., Arora, N. and S.H. Leppla. 1994. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol. Microbiol. **13:** 1093-1100.

Koch, C. and N. Hoiby. 1993. Pathogenesis of cystic fibrosis. Lancet **341:** 1065-1069.

Kooi, C., Cox, A., Darling, P. and P.A. Sokol. 1994. Neutralizing monoclonal antibodies to an extracellular *Pseudomonas cepacia* protease. Infect. Immun. **62:** 2811-2817.

Kooi, C. and P.A. Sokol. 1996. Differentiation of thermolysins and serralysins by monoclonal antibodies. J. Med. Microbiol. **45:** 219-225.

Kooi, C., Hodges, R.S. and P.A. Sokol. 1997. Identification of neutralizing epitopes on *Pseudomonas aeruginosa* elastase and effects of cross-reactions on other thermolysin-like proteases. Infect. Immun. **65:** 472-477.

Kuby, J. 1992. Immunology. W.H. Freeman and Co., New York, USA.

Kuehn, M., Lent, K., Haas, J., Hagenzieker, J., Cervin, M. and A.L. Smith. 1992. Fimbriation of *Pseudomonas cepacia*. Infect. Immun. **60:** 2002-2007.

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

Letoffe, S., Ghigo, J-M. and C. Wandersman. 1993. Identification of two components of the *Serratia marcescens* metalloprotease: protease SM secretion in *Escherichia coli* is TolC dependent. J. Bacteriol. **175:** 7321-7328.

Lipuma, J.L., Dasen, S.E., Nielson, D.W., Stern, R.C. and T.L. Stull. 1990. Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis. Lancet **336:** 1094-1096.

Lischwe, M.A. and D. Ochs. 1982. A new method for partial peptide mapping using N-chlorosuccinimide/urea and peptide silver staining in sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. **127:** 453-457.

Lonon, M.K., Woods, D.E. and D.C. Straus. 1988. Production of lipase by clinical isolates of *Pseudomonas cepacia*. J. Clin. Microbiol. **26:** 979-984.

Martin, D.W., Schurr, M.J., Yu, H. and V. Deretic. 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in Pseudomonas aeruginosa: relationships to σ^E and stress response. J. Bacteriol. **176:** 6688-6696.

McIver, K.S., Kessler, E., Olson, J.C. and D.E. Ohman. 1995. The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa*. Mol. Microbiol. **18:** 877-889.

McIver, K.S., Olson, J.C. and D.E. Ohman. 1993. *Pseudomonas aeruginosa lasB1* mutants produce an elastase, substituted at active-site His-223, that Is defective in activity, processing, and secretion. J. Bacteriol. **175:** 4008-4015.

McKevitt, A.I. and D.E. Woods. 1984. Characterization of *Pseudomonas cepacia* isolates from patients with cystic fibrosis. J. Clin. Microbiol. **19:** 291-293.

McKevitt, A.I., Bajaksouzian, S., Klinger, J.D. and D.E. Woods. 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia*. Infect. Immun. **57:** 771-778.

Meyer, J-M., Hohnadel, D. and F. Halle. 1989. Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. J. Gen. Microbiol. **135:** 1479-1487.

Meyer, J-M., Tran Van, V., Stintzi, A., Berge, O. and G. Winkelmann. 1995. Ornibactin production and transport properties in strains of *Burkholderia vietnamiensis* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*). Biometals **8:** 309-317.

Mietzner, T.A. and S.A. Morse. 1993. The role of iron-binding proteins in the survival of pathogenic bacteria. Annu. Rev. Nutr. **14:** 471-93.

Miller, R.A., Rasmussen, G.T., Cox, C.D. and B.E. Britigan. 1996. Protease Cleavage of iron-transferrin augments pyocyanin-mediated endothelial cell injury via promotion of hydroxyl radical formation. Infect. Immun. **64:** 182-188.

Mintz, C.S., Miller, R.D., Gutgsell, N.S. and T. Malek. 1993. *Legionella pneumophila* protease inactivates interleukin-2 and cleaves CD4 on human T cells. Infect. Immun. **61:** 3416-3421.

Moffat, J.F., Black, W.J. and L.S. Tompkins. 1994a. Further molecular characterization of the cloned *Legionella pneumophila* zinc metalloprotease. Infect. Immun. **62:** 751-753.

Moffat, J.F., Edelstein, P.H., Regula Jr., D.P., Cirillo, J.D. and L.S. Tompkins. 1994b. Effects of an isogenic Zn-metalloprotease-deficient mutant of *Legionella pneumophila* in a guinea-pig model. Mol. Microbiol. **12:** 693-705.

Morihara, K. 1962. *Pseudomonas aeruginosa* proteinase I. Purification and general properties. Biochim. Biophys. Acta. **73:** 113-124.

Nakazawa, T. and M. Abe. 1996. Pathogenesis of *Burkholderia cepacia* and export of protease by the general secretory pathway involving disulfide bond formation in the periplasm. In: Nakazawa, T. et al, ed. Molecular Biology of Pseudomonads. ASM Press, Washington, D.C., p. 462-471.

Nakazawa, T., Yamada, Y. and M. Ishibashi. 1987. Characterization of hemolysin in extracellular products of *Pseudomonas cepacia*. J. Clin. Microbiol. **25:** 195-198.

Nelson, J.W., Butler, S.L., Brown, P.H., Greening, A.P. and J.R.W. Govan. 1993. Serum IgG and sputum IgA antibody to core lipopolysaccharide antigen from *Pseudomonas cepacia* in patients with cystic fibrosis. J. Med. Microbiol. **39:** 39-47.

Nelson, J.W., Butler, S.L., Krieg, D. and J.R.W. Govan. 1994. Virulence factors of *Burkholderia cepacia*. FEMS Immunol. Med. Microbiol. **8:** 89-98.

Nicas, T.I. and B.H. Iglewski. 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. Can. J. Microbiol. **31:** 387-392.

Okamoto, T., Akaike, T., Suga, M., Tanase, S., Horie, H., Miyajima, S., Ando, M., Ichinose, Y. and H. Maeda. 1997. Activation of human matrix metalloproteinases by various bacterial proteinases. J. Biol. Chem. **272:** 6059-6066.

Olson, J.C., McGuffie, E.M. and D.W. Frank. 1997. Effects of differential expression of the 49-kilodalton exoenzyme S by *Pseudomonas aeruginosa* on cultured eukaryotic cells. Infect. Immun. **65:** 248-256.

Palfreyman, R.W., Watson, M.L., Eden, C. and A.W. Smith. 1997. Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia (Pseudomonas) cepacia* products. Infect. Immun. **65:** 617-622.

Parr, T.R., Moore, R.A., Moore, L.V. and R.E.W. Hancock. 1987. Role of porins in intrinsic antibiotic resistance of *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **31:** 121-123.

Pederson, S.S. 1992. Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. APMIS **100 (Suppl 28):** 1-79.

Pitt, T.L., Kaufmann, M.E., Patel, P.S., Benge, L.C.A., Gaskin, S. and D.M. Livermore. 1996. Type characterisation and antibiotic susceptibility of *Burkholderia (Pseudomonas) cepacia* isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland. J. Med. Microbiol. **44:** 203-210.

Pollock, M. 1984. The virulence of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **6:** S617-S626.

Proenca, R., Wei Niu, W., Cacalano, G. and A. Prince. 1993. The *Pseudomonas cepacia* 249 chromosomal pencillinase is a member of the AmpC family of chromosomal β-lactamases. Antimicrob. Agents Chemother. **37:** 667-674.

Pugsley, A.B. and P. Reeves. 1976. Iron uptake in colicin B-resistant mutants of *Escherichia coli* K-12. J. Bacteriol. **126:** 1052-1062.

Rosenstein, B.J. and D.E. Hall. 1980. Pneumonia and septicemia due to *Pseudomonas cepacia* in a patient with cystic fibrosis. Johns Hopkins Med. J. **147:** 188-189.

Saiman, L., Cacalano, G. and A. Prince. 1990. *Pseudomonas cepacia* adherence to respiratory epithelial cells is enhanced by *Pseudomonas aeruginosa*. Infect. Immun. **58:** 2578-2584.

Saiman, L. and A. Prince. 1993. *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. J. Clin. Invest. **92:** 1875-1880.

Sajjan, U.S., Corey, M., Karmali, M.A. and J.F. Forstner. 1992. Binding of *Pseudomonas cepacia* to normal human intestinal mucin and respiratory mucin from patients with cystic fibrosis. J. Clin. Invest. **89:** 648-656.

Sajjan, S.U. and J.F. Forstner. 1992. Identification of the mucin-binding adhesin of *Pseudomonas cepacia* isolated from patients with cystic fibrosis. Infect. Immun. **60:** 1434-1440.

Sajjan, U.S. and J.F. Forstner. 1993. Role of a 22-kilodalton pilin protein in binding of *Pseudomonas cepacia* to buccal epithelial cells. Infect. Immun. **61:** 3157-3163.

Sajjan, U.S., Sun, L., Goldstein, R. and J.F. Forstner. 1995. Cable (Cbl) type II pili of cystic fibrosis-associated *Burkholderia (Pseudomonas) cepacia*: nucleotide sequence of the *cblA* major subunit pilin gene and novel morphology of the assembled appendage fibers. J. Bacteriol. **177:** 1030-1038.

Schad, P.A., Bever, R.A., Nicas, T.I., Leduc, F., Hanne, L.F. and B.H. Iglewski. 1987. Cloning and characterization of elastase genes from *Pseudomonas aeruginosa*. J. Bacteriol. **169:** 2691-2696.

Schagger, H. and G. Von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:** 368-379.

Schiavo, G., Rossetto, O., Benfenati, F., Poulain, B. and C. Montecucco. 1994. Tetanus and botulinim neurotoxins are zinc proteases specific for components of the neuroexocytosis apparatus. Annals N. Y. Acad. Sci. p.65-75.

Sexton, M.M., Jones, A.L., Chaowagul, W. and D.E. Woods. 1994. Purification and characterization of a protease from *Pseudomonas pseudomallei*. Can. J. Microbol. **40:** 903-910.

Shaw, D., Poxton, I.R. and J.R.W. Govan. 1995. Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide. FEMS Immunol. Med. Microbiol. **11:** 99-106.

Shields, M.S. and M.J. Reagin. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. **58:** 3977-3983.

Smith, A.W., Chahal, B. and G.L. French. 1994. The human gastric pathogen *Helicobacter pylori* has a gene encoding an enzyme first classified as a mucinase in *Vibrio cholerae*. Mol. Microbiol. **13:** 153-160.

Sokol, P.A., Ohman, D.E. and B.H. Iglewski. 1979. A more sensitive plate assay for detection of protease production by Pseudomonas aeruginosa. J. Clin. Microbiol. **9:** 538-540.

Sokol, P.A. 1986. Production and utilization of pyochelin by clinical isolates of *Pseudomonas cepacia*. J. Clin. Microbiol. **23:** 560-562.

Sokol, P.A., Lewis, C.J. and J.J. Dennis. 1992. Isolation of a novel siderophore from *Pseudomonas cepacia*. J. Med. Microbiol. **36:** 184-189.

Speert, D.P., Bond, M., Woodman, R.C. and J.T. Curnutte. 1994. Infection with *Pseudomonas cepacia* in chronic granulamatous disease: role of nonoxidative killing by neutrophils in host defense. J. Infect. Dis. **170:** 1524-1531.

Steinbach, S., Sun, L., Jiang, R-Z., Flume, P., Gilligan, P., Egan, T.M. and R. Goldstein. 1994. Transmissibility of *Pseudomonas cepacia* infection in clinic patients and lung-transplant recipients with cystic fibrosis. N. Engl. J. Med. **331:** 981-987.

Straus, D.C., Lonon, M.K. and J.C. Hutson. 1992. Inhibition of rat alveolar macrophage phagocytic function by a *Pseudomonas cepacia* lipase. J. Med. Microbiol. **37:** 335-340.

Sun, L., Jiang, R-Z., Steinbach, S., Holmes, A., Campanelli, C., Forstner, J., Sajjan, U., Tan,Y., Riley, M. and R. Goldstein. 1995. The emergence of a highly transmissible lineage of *cbl*$^{+}$ *Pseudomonas (Burkholderia) cepacia* causing CF centre epidemics in North America and Britain. Nature Med. **1:** 661-666.

Sylvester, F.A., Sajjan, U.S. and J.F. Forstner. 1996. *Burkholderia* (basonym *Pseudomonas*) *cepacia* binding to lipid receptors. Infect. Immun. **64:** 1420-1425.

Tablan, O.C., Martone, W.J., Doershuk, C.F., Stern, R.C., Thomassen, M.J., Klinger, J.D., White, J.W., Carson, L.A. and W.R. Jarvis. 1987. Colonization of the respiratory tract with *Pseudomonas cepacia* in cystic fibrosis. Chest **91:** 527-532.

Thayer, M.M., Flaherty, K.M. and D.B. McKay. 1991. Three-dimensional structure of the elastase of *Pseudomonas aeruginosa* at 1.5-Å resolution. J. Biol. Chem. **266:** 2864-2871.

Toder, D.S., Gambello, M.J. and B.H. Iglewski. 1991. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. Mol. Microbiol. **5:** 2003-2010.

Tomashefski Jr., J.F., Thomassen, M.J., Bruce, M.C., Goldberg, H.I., Konstan, M.W. and R.C. Stern. 1988. *Pseudomonas cepacia*-associated pneumonia in cystic fibrosis. Arch. Pathol. Lab. Med. **112:** 166-172.

Towbin, H., Staehlin, T. and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. **76:** 4350-4354.

Tsujimoto, H., Gotoh, N., Yamagishi, J-i., Oyamada, Y. and T. Nishino. 1997. Cloning and expression of the major porin protein gene *opcP* of *Burkholderia* (formerly *Pseudomonas*) *cepacia* in *Escherichia coli*. Gene **186:** 113-118.

Tyler, S.D., Rozee, K.R. and W.M. Johnson. 1996. Identification of IS1356, a new insertion sequence, and its association with IS402 in epidemic strains of *Burkholderia cepacia* infecting cystic fibrosis patients. J. Clin. Microbiol. **34:** 1610-1616.

Vallee, B.L. and D.S. Auld. 1990. Active-site ligands and activated H_2O of zinc enzymes. Proc. Natl. Acad. Sci. **87:** 220-224.

Vasil, M.L. 1986a. *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. J. Pediatr. **108:** 800-805.

Vasil, M.L., Chamberlain, C. and C.C.R. Grant. 1986b. Molecular studies of Pseudomonas exotoxin A gene. Infect. Immun. **52:** 538-548.

Vasil, M.L., Krieg, D.P., Kuhns, J.S., Ogle, J.W., Shortridge, V.D., Ostroff, R.M. and A.I. Vasil. 1990. Molecular analysis of hemolytic and phospholipase C activities of *Pseudomonas cepacia*. Infect. Immun. **58:** 4020-4029.

Wada, K., Wada, Y., Ishibashi, F., Gojobori, T. and T. Ikemura. 1992. Codon usage tabulated from the GenBank genetic sequence data. Nucl. Acids. Res. **20, Supplement:** 2111-2118.

Wandersman, C., Delepelaire, P., Letoffe, S. and M. Schwartz. 1987. Characterization of *Erwinia chrysanthemi* proteases: cloning and expression of the protease genes in *Escherichia coli*. J. Bacteriol. **169:** 5046-5053.

Wang, J., Mushegian, A., Lory, S. and S. Jin. 1996. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. Proc. Natl. Acad. Sci. **93:** 10434-10439.

Wani, J.W., Gilbert, J.V., Plaut, A.G. and J.N. Weiser. 1996. Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*. Infect. Immun. **64:** 3967-3974.

Wassif, C., Cheek, D. and R. Belas. 1995. Molecular analysis of a metalloprotease from *Proteus mirabilis*. J. Bacteriol. **177:** 5790-5798.

Weinberg, E.D. 1984. Iron withholding: A defense against infection and neoplasia. Physiol. Rev. **64:** 65-102.

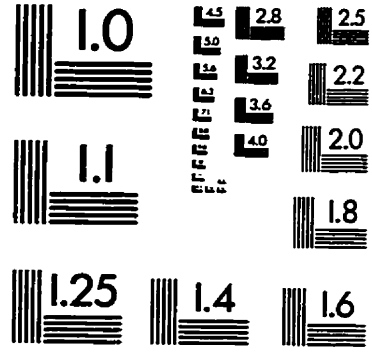
Wretlind, B. and T. Wadstrom. 1977. Purification and properties of a protease with elastase activity from *Pseudomonas aeruginosa*. J. Gen. Microbiol. **103:** 319-327.

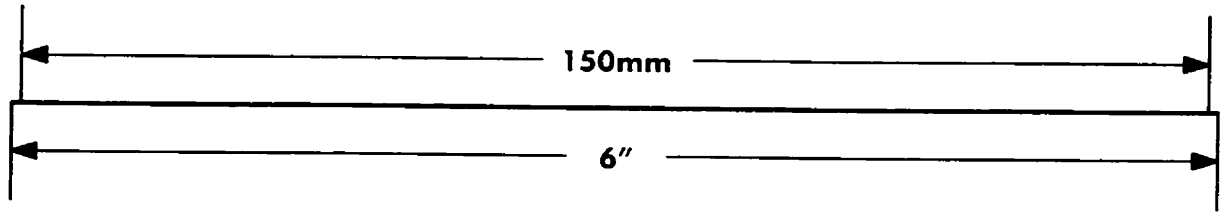
Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and M. Arakawa. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36:** 1251-1275.

Yamasaki, S., Hu, Y., Binz, T., Kalkuhl, A., Kurazono, H., Tamura, T., Jahn, R., Kandel, E. and H. Niemann. 1994. Synaptobrevin/vesicle-associated membrane protein (VAMP) of *Aplysia californica*: structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F. Proc. Natl. Acad. Sci. **91:** 4688-4692.

Yanisch-Perron, C., Vieira, J. and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **133:** 103-119.

IMAGE EVALUATION
TEST TARGET (QA-3)





APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989