CHAPTER 1

LITERATURE REVIEW

IRONIC THAT WE AS HUMANS, THE TOP OF THE EVOLUTIONARY CHAIN, OR SO WE THINK, NEED TO TURN TO THE SIMPLEST OF LIFE FORMS, MICROBES, FOR ALL THE ANSWERS

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1.1 GENERAL INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium and highly successful in colonizing a diversity of environments (Costerton *et al.*, 1995; Costerton *et al.*, 1987). Not only is it an opportunistic pathogen of humans, causing infections in immunocompromised patients such as those with cancer or AIDS as well as those suffering from cystic fibrosis and burns (Van Delden and Iglewski, 1998), but it has also been shown to infect plants and insects (Rhame *et al.*, 1995). The success of *P. aeruginosa* in colonizing these diverse environments is attributed to its ability to synthesize a large number of different factors such as alginate, pili and lipopolysaccharides, and secreted virulence factors, including toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA protease and alkaline protease), and haemolysins (phospholipase and rhamnolipid) (Van Delden and Iglewski, 1998; Lazdunski *et al.*, 1990; Liu, 1974).

In the vast majority of ecological niches, *P. aeruginosa* can grow in association with surfaces, which leads to the formation of biofilms (Costerton *et al.*, 1995). Biofilms have been defined as structured communities of bacterial cells that are enclosed in a self-produced polymeric matrix and adhere to biotic and abiotic surfaces (Costerton *et al.*, 1995). Biofilm formation occurs in response to a variety of environmental signals (Davey and O'Toole, 2000) that leads to a number of changes in gene regulation that cause the adhering cells to become phenotypically (Costerton *et al.*, 1995; Davies and Geesey, 1995) and metabolically (Davey and O'Toole, 2000) distinct from their planktonic counterparts. The complex biofilm architecture also provides an opportunity for metabolic cooperation, and niches are formed within the spatially well-organized systems. Consequently, the bacteria are exposed to an array of distinct physicochemical conditions within a biofilm that can result in differential gene expression (Steyn *et al.*, 2001; Whiteley *et al.*, 2001; Davey and O'Toole, 2000; O'Toole *et al.*, 2000). The phenomenon of genes being differentially expressed by *P. aeruginosa* cells growing as a biofilm is not unique to only *P. aeruginosa*. Prigent-Combaret *et al.* (1999) have reported that 22% of the *Escherichia coli* genes were up-regulated in the biofilm mode of growth, and 16% were down-regulated. Similarly, Oosthuizen *et al.* (2002) have shown that 6% of proteins were up-regulated in 18-h old biofilms of *Bacillus cereus* DL5 compared to planktonic cells.

Often the expression of a cloned gene in a selected host organism is one of the main reasons for cloning genes for biotechnological purposes. The phenomenon of up-regulated gene expression when bacteria grow in association with surfaces, in addition to the higher metabolic rates and lower nutrient requirements of such biofilm-associated bacterial cells (Chandy and Angels, 2001; Frias *et al.*, 2001), may prove to be useful for biotechnological purposes. The successful overexpression of the cloned gene in the selected host organism usually depends on several factors and no single strategy exists whereby all genes can be successfully expressed in all hosts. An important consideration is, however, the stability of the expressed protein. The stability of the protein often depends on its cellular location and proteins secreted into the extracellular environment not only tend to be stable, but they can also be readily purified from the culture supernatant (Kitai *et al.*, 1988). By contrast to Gram-positive bacteria, which lack an outer membrane, Gram-negative bacteria face special challenges, as secreted proteins have to cross the periplasm and the outer membrane in addition to the cytoplasmic or inner membrane (Pugsley and Schwartz, 1985). Consequently, Gram-negative bacteria have developed various secretion strategies and these may range from simple one-component systems to complex multi-component assemblies (Thanassi and Hultgren, 2000). An understanding of the different secretion pathways may allow for the exploitation of these existing pathways in the secretion of expressed proteins. In this chapter, literature relevant to the ability of *P. aeruginosa* to form biofilms, the pathways whereby extracellular proteins are secreted from Gram-negative bacterial cells as well as approaches and strategies that may be useful for the overexpression of proteins in Gram-negative host organisms, will be reviewed.

1.2   BIOFILM FORMATION BY *P. aeruginosa*

Over the past few years, *P. aeruginosa* has become a model for studying biofilms of Gram-negative bacteria as well as for studies relating to surface- and biofilm-induced gene expression (Steyn *et al.*, 2001; Whiteley *et al.*, 2001; O'Toole *et al.*, 2000; Costerton *et al.*, 1995). The formation of a mature biofilm is believed to occur in a sequential process of transport of microorganisms to a surface, initial microbial attachment, formation of microcolonies, and formation of mature biofilms (Van Loosdrecht *et al.*, 1990; Marshall, 1985) (Fig. 1.1). Extensive biophysical, structural and chemical analysis of bacterial biofilms has led to a basic model for biofilm structure (Tolker-Nielsen *et al.*, 2000; Costerton *et al.*,

1995). In this model, bacteria form microcolonies surrounded by copious amounts of exopolysaccharide and between the microcolonies are water-filled channels (Costerton *et al.*, 1995). It has been suggested that these channels serve to promote the influx of O_2, organic substrates and nutrients, and the efflux of CO_2 and metabolic by-products (Costerton *et al.*, 1995).
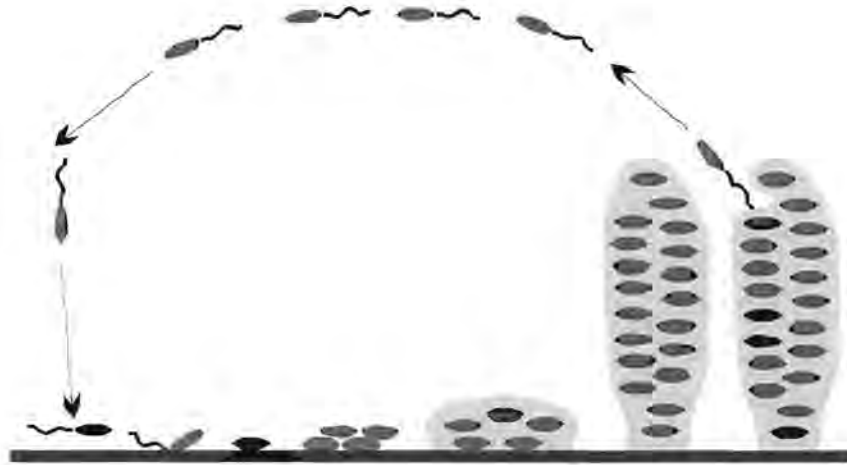

Fig. 1.1 Schematic presentation of the steps in biofilm formation (Modified from O'Toole *et al.* (2000).

Transposon insertion mutation analysis has greatly facilitated the characterization of bacterial structural components required for attachment of bacteria to surfaces. Specific structural components of *P. aeruginosa* shown to play a critical role in facilitating initial bacterial interaction with surfaces include flagella, pili and adhesins, while extracellular polysaccharides may contribute to the maintenance of the biofilm structure. Furthermore, changes in gene expression are also required for the bacterial cells to initiate the formation of a biofilm and to prepare the organism for its life on a solid surface (Davey and O'Toole, 2000; Costerton *et al.*, 1995). This is evidenced by the greater antibiotic resistance of biofilm cells compared to their planktonic counterparts (Cochran *et al.*, 2000; Stewart, 1996; Nickel *et al.*, 1985).

1.2.1 Bacterial structural components required for biofilm formation

O'Toole and Kolter (1998a; 1998b) have generated transposon insertion mutants of *P. aeruginosa* PA14 that were termed surface attachment deficient (*sad*) mutants, as they were unable to produce substantial biofilm. Three of the mutants had become non-motile, and the insertion sequence of one was located in a DNA sequence having identity to the *flgK* gene

sequence. The *flgK* gene codes for the flagellum-associated hook protein 1, and its loss leads to the production of nonfunctional flagella and loss of motility. A second class of *sad* mutants was identified in which the transposon was located in the genes coding for the synthesis of type IV pili (*pilB*, *pilC* and a *pilYI* homologue). These appendages are responsible for twitching motility. Twitching motility refers to surface translocation mediated by type IV pili and appears to be widespread among Gram-negative bacteria (Wall and Kaiser, 1999). Whereas the non-motile mutants failed to attach to the surface, the mutants deficient in type IV pili formed dispersed monolayers but failed to develop densely packed monolayers and microcolonies. Taken together, these findings indicate that flagella or flagella-mediated motility is important for transport and initial cell-to-surface interactions (O'Toole and Kolter, 1998a), while pili and pilus-associated adhesins are important for the initial adherence to and early colonization of surfaces (O'Toole and Kolter, 1998b; Pratt and Kolter, 1998). Recently, Vallet *et al.* (2001) reported that a *cupA* mutant was defective in biofilm formation in *P. aeruginosa*. The *cupA* gene cluster encodes the components of a chaperone/usher protein secretion pathway that is involved in assembly of fimbrial subunits such as P and type I pili, but not type IV pili (Soto and Hultgren, 1999). These results may thus imply that pili, other than type IV pili, may play a role in the attachment of *P. aeruginosa* to surfaces.

Bacterial extracellular polysaccharides, such as lipopolysaccharides and alginate, may also influence attachment and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between the bacteria and substratum (Van Loosdrecht *et al.*, 1990). The adhesiveness of different *Pseudomonas* species has been reported to be related to the presence and composition of lipopolysaccharides (Williams and Fletcher, 1996). In the case of O-polysaccharide-deficient *Pseudomonas* spp., substantially reduced attachment to both biotic and abiotic surfaces has been observed (DeFlaun *et al.*, 1994; DeFlaun *et al.*, 1999). The extracellular polysaccharide alginate has also been reported to be required for formation of thick, three-dimensional *P. aeruginosa* biofilms and was reported to be the intercellular material of *P. aeruginosa* microcolonies (Nivens *et al.*, 2001).

1.2.2 Importance of signalling molecules in biofilm formation

Several reports have addressed the potential role of extracellular factors in biofilm formation and recent studies have linked quorum sensing and biofilm formation (De Kievit *et al.*, 2001; Davies *et al.*, 1998). Acylated homoserine lactones (AHSLs), which are quorum-sensing

signal molecules, have been demonstrated to be present both in aquatic biofilms grown on submerged stones (McLean *et al.*, 1997) and in biofilms formed on urethral catheters (Stickler *et al.*, 1998). Acyl-HSL synthesized by *P. aeruginosa* has been shown to play an important role in the formation of the three-dimensional architecture within the biofilm (De Kievit *et al.*, 2001; Davies *et al.*, 1998). In these studies, *lasI* mutant cells unable to synthesize N-3-(oxo-octanoyl)-L-homoserine lactone (3OC12-HSL) were shown to form biofilms with abnormal structure. The mutant biofilms contained cells that were tightly packed, lacked structural organization and the biofilms were much thinner and more susceptible to treatment with SDS compared to the wild-type biofilms. These findings led to speculation that cell-to-cell signaling induced by the high density of the bacteria within biofilms may play a role in the establishment of a biofilm-specific physiological state (Davies *et al.*, 1998). Moreover, Whiteley *et al.* (1999) identified nearly 40 widely different genes whose expression was found to be activated by AHLs. However, the role of these genes in biofilm development awaits further analysis.

Recently, the *P. aeruginosa crc* gene has been shown to be required for biofilm formation (O'Toole *et al.*, 2000) and it represents the first molecular link between the environment and the decision between planktonic and biofilm modes of growth. The Crc protein plays a role in sensing carbon source availability and has been shown to affect expression of the type IV *pilA* structural gene. Since type IV pili-mediated twitching motility is required for *P. aeruginosa* biofilm formation (O'Toole and Kolter, 1998b), Crc serves to link carbon availability to the decision whether or not to enter a biofilm mode of growth.

1.2.3   Changes in gene expression during biofilm development

Attachment of bacteria to surfaces is thought to initiate a cascade of changes in gene expression of the bacterial cells. Such surface-induced gene activation occurs in both the *algC* (Davies and Geesey, 1995; Davies *et al.*, 1993) and *algD* (Hoyle *et al.*, 1993) genes of *P. aeruginosa*. Both these genes form part of the *algACD* gene cluster, which controls alginate synthesis, while the *algC* gene is also involved in lipopolysaccharide core biosynthesis (Gacesa, 1998). The expression of *algC* was shown to be activated as early as 15 min after the bacterial cell attaches to either a Teflon or glass substratum and cells that did not undergo *algC* up-regulation were less able to remain attached to the surface relative to

cells in which expression is activated (Davies and Geesey, 1995). Thus, *algC* may not only be required for initial cell-to-surface attachment, but it may also be important to maintain attachment.

Recently, Garrett *et al.* (1999) have extended upon the above studies by reporting the existence of a link between the regulation of flagellar biosynthesis and alginate production. Induction of *algU*, an alternative sigma factor that controls alginate biosynthesis, resulted in decreased expression of the *fliC* gene, which encodes flagellin, the structural subunit of the flagellar filament. Thus, induction of *algU* results in increased alginate synthesis and a coordinate decrease in flagellum synthesis. Garrett *et al.* (1999) proposed that *algU* activates a negative effector of flagellum synthesis, although the precise mechanism by which *algU* modulates *fliC* expression is not known.

In a study undertaken by Weyers (1999), reporter gene technology was used to screen a genomic library of *P. aeruginosa* in order to isolate the regulatory elements of genes required for attachment of the bacterium to a surface followed by the formation of well-developed biofilms. Of the 1000 clones that were screened, representing approximately 3% of the genome, 325 clones were identified that displayed significant up-regulation of the reporter gene expression following attachment to a substratum. Subsequent nucleic acid sequence analysis of various of these regulatory elements indicated that they mapped to regions upstream of genes encoding hypothetical proteins, probable transcription regulators, proteins involved in motility or genes encoding different enzymes and membrane proteins (Meiring, 2002).

Gene expression in mature biofilms of *P. aeruginosa* has also been investigated. In a global analysis of gene expression, Brözel *et al.* (1995) showed that bacteria attached to a surface underwent changes in expression for at least 11 proteins. Subsequent proteomic analysis of planktonic and biofilm biomass have led to the identification of numerous proteins that are differentially expressed, up-regulated and/or repressed in *P. aeruginosa* growing as a biofilm (Steyn *et al.*, 2001). By making use of DNA microarrays to study gene expression in *P. aeruginosa* biofilms, Whiteley *et al.* (2001) indicated that 73 genes in biofilm cells were differentially expressed when compared to planktonic cells. These genes included, amongst other, bacteriophage genes, genes involved in motility and genes encoding for membrane and secretory proteins.

Studies of mature *P. aeruginosa* biofilms have also demonstrated heterogeneity in the gene expression and physiological activity of the cell populations at different depths within the biofilm structure (De Kievit *et al.*, 2001; Huang *et al.*, 1998; Xu *et al.*, 1998). Under conditions of phosphate starvation, *P. aeruginosa* was shown to express alkaline phosphatase in a relatively thin layer at the liquid medium-biofilm interface (Huang *et al.*, 1998). Subsequent studies by Xu *et al.* (1998) showed that alkaline phosphatase expression was limited to regions of oxygen availability in biofilms of *P. aeruginosa*, suggesting that oxygen levels may serve a regulatory function in gene expression. Reporter gene studies by De Kievit *et al.* (2001) indicated that *rhlI* expression fluctuated very little during biofilm development, but only 5 to 15% of the cells actually expressed *rhlI* and these cells were concentrated around the base of the biofilm.

## 1.3   SECRETION OF PROTEINS IN GRAM-NEGATIVE BACTERIA

In Gram-negative bacteria, secretion of extracellular proteins involves passing through the periplasm in addition to two membranes, the inner or cytoplasmic membrane, which surrounds the cytoplasm, and the outer membrane, which encloses the periplasm and acts as a barrier to the environment. Gram-negative bacteria face special challenges in this regard, as both the inner and outer membranes are quite complex (Pugsley and Schwartz, 1985). Consequently, they have evolved several pathways for protein secretion to the extracellular environment and their classification is broadly based on sequence, structure and function (Thanassi and Hultgren, 2000; Lory, 1998). Until now, at least five different pathways for protein secretion have been described in Gram-negative bacteria. Two of these pathways (types I and III) do not require the Sec translocase system and are thus capable of exporting proteins directly from the cytoplasm to the external environment. The other three pathways (types II, IV and V) export proteins with cleavable N-terminal signal sequences that require the Sec translocase system for translocation of the proteins across the inner membrane. Since most of the export pathways are dependent on the general secretory (Sec) pathway to export proteins across the inner membrane prior to secretion across the outer membrane, this section will therefore be introduced by a brief description of the general secretory pathway prior to reviewing each of the different export pathways.

1.3.1 The general secretory pathway (GSP)

1.3.1.1 Secretion across the inner membrane

The Sec secretion system of *E. coli* (Fig. 1.2) has been extensively studied and consists of a cytoplasmic secretion-specific chaperone (SecB), a protein translocation ATPase (SecA) and an integral membrane protein complex formed by at least six different protein subunits (SecY, SecE, SecD, SecF, SecG and YajC) (Pugsley *et al.*, 1997; Pugsley, 1993). *In vivo* studies have shown that only the SecY and SecE subunits are essential, whereas the other subunits are required only at low temperatures (Danese and Silhavy, 1998).
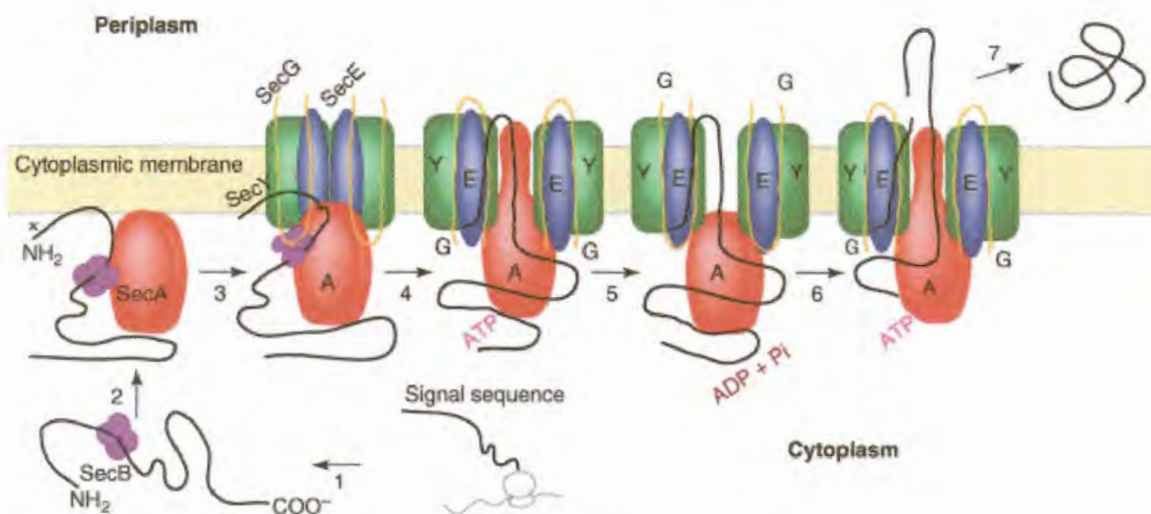
Fig. 1.2 Sec secretion process across the inner membrane. The precursor protein is represented by a black line and the signal sequence is represented by the gray region. Steps 1-3 are targeting of the precursor protein: The signal sequence and its immediate carboxy-terminal region comprise an initiation sequence that is recognized by the Sec machinery. SecB, the Sec-specific chaperone, channels the precursor protein to the Sec translocation pathway where it targets its precursor to the translocase by binding to SecA. The precursor protein-SecA complex then binds to the membrane at a high affinity SecA binding site. SecY, E, and G forms a hetero-trimeric complex, SecYEG, which provides the channel for the protein to cross the inner membrane. Steps 4 and 5 are translocaton initiation and requires ATP, but not hydrolysis. Step 6 is continuation of the translocation and requires ATP hydrolysis and/or proton-motive force. Translocation is thought to occur in a stepwise fashion involving 20 – 30 amino acid residues. Step 7 is completion of translocation where the protein is released into the periplasmic space. Little, however, is known about the precise kinetics involved in the release mechanism (Reprinted from TRENDS in Microbiology, Volume 9, Mori, H. and Ito, K., The Sec protein-translocation pathway, p 494, 2001, with permission from Elsevier).

The SecB chaperone functions to maintain precursor proteins in a secretion-competent state and to target them to SecA. SecA then targets the precursor protein-SecB complex to the Sec complex in the membrane for export across the inner membrane. Translocation of the precursor protein is initiated by the binding of ATP to SecA, which promotes insertion of the

signal peptide and part of the mature protein sequence together with a SecA domain into a translocation channel consisting of SecYEG (Duong and Wickner, 1999). SecA translocates approximately 2.5 kDa of the exported protein (20-30 amino acids) upon ATP binding (Driessen *et al.*, 1998). Subsequent ATP hydrolysis results in the release of SecA from the partially translocated protein and the Sec complex in the inner membrane. This process is then repeated by SecA binding to another region of the precursor protein and thus promoting translocation of a further 2.5 kDa section. The translocation intermediates are driven forward by the proton-motive force (PMF), which also promotes the release of SecA from the Sec complex. ATP and PMF serve as energy sources at different stages of the translocation process, with ATP being required first. ATP is essential for Sec-mediated secretion across the inner membrane, whereas the PMF enhances the translocation rates (Economou, 1998). After several cycles, the precursor protein is translocated across the inner membrane but remains bound to the inner membrane by its amino-terminal signal peptide.

After translocation across the inner membrane, signal peptides are cleaved by the appropriate membrane-bound signal peptidase. A typical signal sequence contains a stretch of approximately 18 to 30 amino acids divided into three general domains: an N domain containing positively-charged amino acids that associates the presecretory protein with the inner membrane and correctly orientates the protein for translocation, an H domain containing a core of hydrophobic amino acids that inserts the signal sequence into the inner membrane, and a C domain containing a cleavage site recognized by the signal peptidase (Pugsley, 1993; Salmond and Reeves, 1993). There are three types of signal peptidases and each type possesses a different substrate specificity (Paetzel *et al.*, 2000; Pugsley, 1993). Whereas the type I signal peptidases are responsible for proteolytic processing of the N-terminal signal peptides of secreted proteins, the type II signal peptidases are responsible for proteolytic processing of secreted lipoproteins only (Paetzel *et al.*, 2000). The type III signal peptidases are responsible for proteolytic processing of type IV prepilins and posses two enzymatic activities: an endoproteolytic activity, which is required for the removal of the signal peptides of type IV pilin subunits, and N-methylation activity for posttranslational modification of type IV pilins (Paetzel *et al.*, 2000; Lory and Strom, 1997). After proteolytic processing by the appropriate signal peptidase, the mature polypeptide is usually released into the periplasm, whereas its signal peptide is further degraded by protease IV (Ichihara *et al.*, 1986).

1.3.1.2 Protein folding in the periplasmic space

Protein stability in the periplasm is an important issue in the secretion of proteins via the GSP. Secreted proteins exposed temporarily to the periplasmic environment may need to remain partially unfolded before they are transported across the outer membrane. A number of periplasmic proteins have therefore been identified that catalyse folding of cell envelope and secreted proteins. These include the DsbA and DsbC proteins, which are required for the formation of disulphide bonds in proteins that are present in the periplasm (Bardwell, 1994). Whereas DsbA is the predominant dithiol oxidant in the cytoplasm, DsbC is the main periplasmic disulfide isomerase. In addition, there are at least four periplasmic proteins (SurA, PpiA, PpiD and FkpA) catalyzing peptidyl-propyl isomerization in *E. coli* (Dartigalongue and Raina, 1998; Lazar and Kolter, 1996). Isomerization of peptidyl-proline bonds of periplasmic proteins containing proline residues may be required for proper folding of these proteins.

In addition to enzymes required for proper folding of proteins, the periplasm also contains proteins that can prevent secreted proteins from folding into their final conformation or can assist proteins in folding correctly. These latter processes are mediated by chaperones and are essential for the secretion of extracellular proteins that must be maintained in a properly folded state in the periplasm before transport across the outer membrane. Known periplasmic chaperones include the P pilus-specific chaperone PapD (Holmgren and Branden, 1989), the porin-specific chaperone Skp (De Cock *et al.*, 1999) and LolA, a chaperone that is required for proper localization of outer membrane lipoproteins (Tajima *et al.*, 1998). The periplasm also contains several proteases that play important roles in the physiology of the bacterial cell. One major function of the periplasmic proteases is to activate turnover of damaged or misfolded proteins. The protease, DegP, is considered to be among the primary proteases responsible for turnover of misfolded proteins in the periplasm (Pallen and Wren, 1997).

1.3.1.3 Secretion across the outer membrane

The final step of the general secretion pathway is translocation of proteins to be secreted across the outer membrane. Whereas all proteins secreted by the GSP traverse the inner membrane via the Sec translocation complex, the terminal step can take several different routes, referred to as terminal branches. The main terminal branch of the GSP is referred to as

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the type II secretion pathway, while the chaperone/usher, the autotransporter and type IV secretion pathways each represent alternate terminal branches of the GSP. These *sec*-dependent protein secretion pathways will subsequently be reviewed in more detail in the following sections.

1.4   *SEC*-DEPENDENT PROTEIN SECRETION PATHWAYS

1.4.1   The type II secretion pathway

Following export of proteins, which are to be secreted, into the periplasm via the Sec translocase system, the proteins may undergo further modifications before they are finally translocated across the outer membrane. This final translocation step requires several accessory proteins, collectively referred to as the type II secretion apparatus or secreton. This apparatus is highly specific and is capable of distinguishing proteins to be secreted from resident periplasmic proteins and, with a few exceptions, it can discriminate between its own secreted proteins and those induced from other species (Filloux *et al.*, 1998; Lindeberg *et al.*, 1996; Michel *et al.*, 1995; Filloux *et al.*, 1990).

The type II secretion systems are widely distributed and appear to be the primary pathway for the secretion of extracellular degradative enzymes by Gram-negative bacteria (Fig. 1.3) (Russel, 1998; Pugsley *et al.*, 1997; Hobbs and Mattick, 1993). Examples include, amongst other, the *out* pathway of *Erwinia* spp. for the secretion of pectic enzymes and cellulases, and the *xcp* pathway for the secretion of elastase, lipase, phospholipase C and alkaline phosphatase by *P. aeruginosa* (Filloux *et al.*, 1998; Tomassen *et al.*, 1992; Bally *et al.*, 1992; Filloux *et al.*, 1987). Recently, a second type II secretion pathway was discovered in *P. aeruginosa*, termed *hxcp* for homologous to *xcp*, and was reported to be dedicated to the secretion of alkaline phosphatase (Ball *et al.*, 2002). The *hxcp* pathway is thought to be important for improving alkaline phosphatase secretion and inorganic phosphate acquisition under conditions in which the Xcp system becomes limiting (Ball *et al.*, 2002).
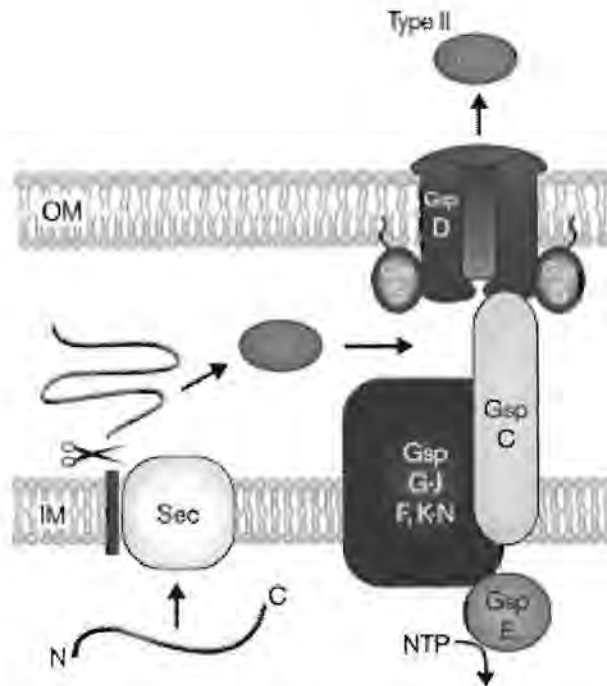
Fig. 1.3 Model for type II secretion. Components of the type II secretion system are indicated using the GSP nomenclature. Type II substrates cross the inner membrane via the Sec system as depicted in Figure 1.2. The GspD secretin and GspS lipoprotein, serves as a gated channel for secretion of substrates to the cell surface. GspC may transmit energy from the inner membrane, presumably generated by the cytoplasmic GspE nucleotide-binding protein, to the outer membrane. GspG – J exhibit homology to the pilin subunit PilA. GspO cleaves the leader sequence on the periplasmic side of the inner membrane prior to its secretion (Reprinted from Current Opinion in Cell Biology, Volume 12, Thanassi, D.G. and Hultgren, S.J., Multiple pathways allow protein secretion across the bacterial outer membrane, p 420, 2000, with permission from Elsevier).

Pullulanase export in *Klebsiella oxytoca* represents the prototypical type II secretion pathway (Pugsley *et al.*, 1997; Pugsley, 1993). Pullulanase (PulA) is an oligomeric extracellular lipoprotein that belongs to the α-amylase family. Secretion of PulA across the outer membrane is reported to involve the products of at least 14 specific genes located in the *pul* gene cluster (Pugsley *et al.*, 1997). At least four inner membrane proteins designated PulG, PulH, PulI and PulJ exhibit homology to the type IV pilus structural subunit, pilin. These type IV pilin-like proteins (pseudopilins) contain prepilin peptidase cleavage and methylation sites and undergo processing by the PulO inner membrane protein, a prepilin peptidase that is essential for PulA secretion (Pugsley *et al.*, 1997). It has been suggested that these pseudopilin proteins may assemble into a pilus-like structure across the periplasm that subsequently facilitates PulA transport to the outer membrane (Pugsley, 1996; Hobbs and Mattick, 1993).

The secretion components PulC, PulM, PulN, PulK and PulF are predicted to be anchored in the inner membrane by a single N-terminal transmembrane spanning segment followed by a

1.4.2   The chaperone/usher secretion pathway

The chaperone/usher pathway is dedicated to the assembly and secretion of various adhesive virulence structures on the Gram-negative bacterial surface (Thanassi *et al.*, 1998). The best characterized of these are the P and type 1 pili of uropathogenic *E. coli* (Roberts *et al.*, 1994). These pili are composite structures consisting of a thin, flexible tip fibrillum connected to a rigid, helical rod (Kuehn *et al.*, 1992). Secretion across the outer membrane by this pathway requires only two components: a periplasmic chaperone and an outer membrane protein termed an usher. A cluster of genes, termed *cupA*, has recently been identified in *P. aeruginosa* that specifies the components of a chaperone/usher pathway and was shown to function at distinct stages of biofilm formation (Vallet *et al.*, 2001).

Research on P pili has elucidated many molecular details of the chaperone/usher secretion pathway. Pilus subunits emerging from the Sec translocation complex into the periplasm must interact with the PapD periplasmic chaperone and also require DsbA for proper disulphide bond formation (Jacob-Dubuisson *et al.*, 1994a). The pilus subunits contain motifs in their N- and C-terminal regions that target them to the PapD chaperone (Soto *et al.*, 1998; Kuehn *et al.*, 1993). PapD is the prototypical periplasmic chaperone in a family that includes more than 30 members (Lindberg *et al.*, 1989). It is a boomerang-shaped two-domain molecule with each domain having an immunoglobulin-like (Ig) fold (Holmgren and Branden, 1989). PapD has three primary functions: it binds and caps interactive surfaces on pilus subunits to prevent them from precipitating in non-productive interactions (Soto *et al.*, 1998), it facilitates the release of pilus subunits into the periplasm (Jones *et al.*, 1997) and it may facilitate subunit folding (Soto *et al.*, 1998). In the absence of the chaperone, subunits form aggregates that are degraded by the DegP periplasmic protease (Danese and Silhavy, 1997).

The periplasmic PapD-subunit complexes target to the PapC usher in the outer membrane. The PapC usher assembles into 15 nm ring-shaped oligomeric complexes with central channels of 2-3 nm in diameter (Thanassi *et al.*, 1998). Interaction of the chaperone-subunit complex with the usher triggers dissociation of the chaperone from the subunit, exposing subunit assembly surfaces and thus allowing for incorporation of the subunit into the pilus fiber. In the absence of the usher, the chaperone-subunit complexes accumulate in the periplasm but no pili are assembled. Pilus assembly is proposed to occur at the periplasmic

face of the usher, concomitant with secretion through the usher pore (Valent *et al.*, 1995). Therefore, a newly synthesized pilus rod would be constrained to a linear fiber while traversing through the usher channel and upon reaching the cell surface, the rod could coil into its final helical conformation. The chaperone/usher pathway does not require input of external energy for secretion of pili across the outer membrane. The energy for secretion by this pathway is thought to be derived from the protein-protein interactions involved in assembling the pilus organelle and coiling of the rod on the cell surface may also facilitate the outward translocation of pili (Jacob-Dubuisson *et al.*, 1994b).

1.4.3   The type IV secretion pathway

The type IV secretion systems (TFSS) have only recently been discovered, but the list of organisms harbouring type IV secretion systems has grown rapidly. The type IV secretion systems are known to transport either proteins or protein-DNA complexes (Christie, 2001; Burns, 1999; Christie, 1997). The type IV secretion systems that transport protein-DNA complexes include the conjugative-transfer systems of IncN plasmid pKM101 (Pohlman *et al.*, 1994), IncP plasmid RP4 (Lanka and Wilkens, 1995) and the crown gall tumor-inducing T-DNA transfer system of *Agrobacterium tumefaciens* Ti plasmids (Fullner, 1998; Christie, 1997). Type IV systems that appear to be dedicated exclusively to protein transfer include the *cag* pathogenicity island of *Helicobacter pylori* (Censini *et al.*, 1996), which secretes a 145 kDa CagA protein into gastric epithelial cells, and the Ptl secretion system for pertussis toxin of *Bordotella pertussis* (Weiss *et al.*, 1993). Recently, *Legionella pneumophila* was found to encode a type IV system that appears capable of secreting protein virulence factors and transporting DNA by conjugative transfer (Segal *et al.*, 1999; Vogel and Isberg, 1999).

Most information about the type IV secretory apparatus has come from studying the VirB system of *Agrobacterium tumefaciens* that facilitates translocation of oncogenic T-DNA into plant cells (Fig. 1.4) (Burns, 1999; Beaupré *et al.*, 1997). The *virB* locus consists of 11 genes, ten of which (*virB2* through *virB11*) are critical for DNA transfer (Berger and Christie, 1994). Although *virB1* is not absolutely essential, deletion of this gene leads to a lower level of efficiency of DNA transfer (Berger and Christie, 1994). A number of VirB proteins are membrane-associated, interact with each other and themselves, and are present in multiple copies (Burns, 1999). The VirB1 protein is processed followed by export of its C-terminal portion to the exterior of the cell (Baron *et al.*, 1997). Both the VirB2 and VirB3 proteins are

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exported (Beijersbergen *et al.*, 1993), with VirB2 localizing to the surface of the cell (Lai and Kado, 1998). The VirB7 to VirB10 proteins fractionate with both the inner and outer membranes and are proposed to contain large periplasmic domains, suggesting the formation of a complex that spans the periplasm (Thorstenson *et al.*, 1993). VirB7 is an outer membrane-associated lipoprotein that forms a disulfide-bonded heterodimeric complex with VirB9 (Fernandez *et al.*, 1996; Spudich *et al.*, 1996). Both the VirB7 and VirB9 proteins have been reported to be important for the stability of other VirB proteins and has thus led to a model in which the VirB7 - VirB9 complex at the outer membrane nucleates and stabilizes assembly of the secretion apparatus (Beaupré *et al.*, 1997; Spudich *et al.*, 1996).
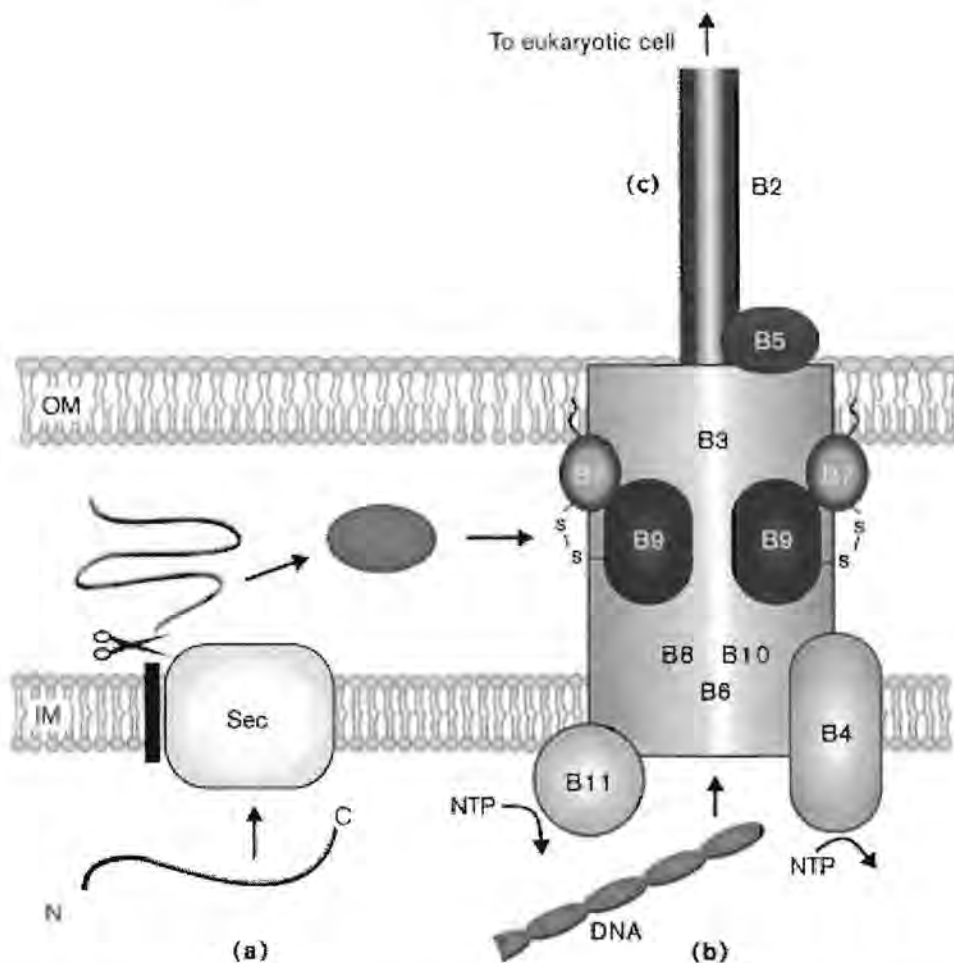
Fig. 1.4 Model for type IV secretion. Components of the type IV apparatus are labeled according to the VirB system. (a) Protein secretion by the type IV pathway may take place via a periplasmic intermediate, where substrates are first translocated across the inner membrane by the Sec machinery. (b) DNA secretion probably takes place from the cytoplasm without a periplasmic intermediate. VirB4 and VirB11 contain nucleotide-binding capability and most likely provide energy for the secretion process. (c) VirB2 is a major pilus component and VirB5 a minor component. The pilus may serve as a secretion tube for translocation of proteins or DNA into the target eukaryotic cell (Reprinted from Current Opinion in Cell Biology, Volume 12, Thanassi, D.G. and Hultgren, S.J., Multiple pathways allow protein secretion across the bacterial outer membrane, p 420, 2000, with permission from Elsevier).

Both the VirB4 and VirB11 proteins localize to the inner membrane. Whereas VirB4 is believed to be an integral cytoplasmic membrane protein with two periplasmic domains (Dang *et al.*, 1999), VirB11 is believed to be located on the inner side of the cytoplasmic membrane (Christie *et al.*, 1989). VirB4 has been shown to possess ATPase activity, while VirB11 has been reported to have weak ATPase and autokinase activity (Dang *et al.*, 1999; Stephens *et al.*, 1995; Christie *et al.*, 1989). They are therefore considered likely candidates for the motor behind the secretion process. Alternatively, these proteins might serve to signal the opening of a gate or channel via kinase activity, or act as molecular chaperones in the assembly of the transporter or during the transport process itself (Burns, 1999).

Although the molecular architecture of type IV secretion systems have been unravelled, very little is known about the series of events that occur during the transport process. It is unclear whether transport occurs as a one-step process by crossing both inner and outer membranes simultaneously or as a two-step process by crossing the inner and outer membranes individually. It would appear that different members of the type IV secretion system family may differ in this regard. The single-stranded T-DNA, which is transported by the VirB system, must be transported by a mechanism whereby it remains intact as the periplasm contains many nucleases that may degrade the T-DNA. Thus, it has been postulated that the VirB system probably utilizes a one-step transport process (Christie, 1997; Thorstenson *et al.*, 1993), possibly through a channel formed by VirB proteins (Burns, 1999). By contrast, the pertussis toxin of *B. pertussis* is thought to be secreted by a two-step process, despite the existence of Ptl homologues for each of the VirB proteins, except VirB1 and VirB5. Since the individual subunits of the toxin each possess their own signal sequence (Winans *et al.*, 1996; Pizza *et al.*, 1990), they may cross the inner membrane by a *sec*-like system (Nicosia *et al.*, 1986) whereafter the subunits are assembled in the periplasmic space and the holotoxin is then transported via the Ptl apparatus across the outer membrane (Burns, 1999).

1.4.4 The type V autotransporter secretion pathway

Bacterial proteins that are targeted to the microbial surface or released into the environment often depend on periplasmic proteins and almost always require outer membrane proteins to promote their secretion. The autotransporter family of Gram-negative bacterial proteins is a unique subset of secreted proteins that do not rely on other proteins for transit from the

periplasm to the bacterial surface (Jacob-Dubuisson *et al.*, 2001; Henderson *et al.*, 2000; Henderson *et al.*, 1998). These proteins possess a C-terminal domain that mediates targeting to and translocation across the outer membrane. This type of secretion pathway is responsible for the export of proteins with diverse functionalities and include, amongst other, proteases, toxins, adhesins and invasins (Henderson *et al.*, 2000; Henderson *et al.*, 1998). Members of this group include the *Neisseria gonorrhoeae* IgA1 protease (Pohlner *et al.*, 1987), the *Haemophilis influenza* Hap adhesin (Hendrixson *et al.*, 1997), the *E. coli* Tsh haemaglutinin (Stathopoulos *et al.*, 1999) and the *Shigella flexneri* IcsA protein (Egile *et al.*, 1997).

A typical autotransporter is synthesized as a precursor protein that can be divided into three characteristic domains: an N-terminal signal sequence, an internal passenger domain (also termed α, functional or mature domain), and a C-terminal β-domain (Henderson *et al.*, 1998) (Fig. 1.5). Once translated, the protein is exported across the inner membrane, presumably using the N-terminal signal sequence and Sec translocase. After cleavage of the signal sequence, the C-terminus of the protein inserts into the outer membrane. As the periplasm contains many proteases that could degrade proteins, it has been postulated that periplasmic chaperones might be involved in protecting the presecretory protein or that the inner and outer membrane translocation process is transiently coupled so as to minimize exposure to the periplasm (Henderson *et al.*, 1998). Secondary structure analysis performed on the C-terminus of several autotransporter proteins predicts the formation of a β-barrel. The β-barrel is believed to function as a porin, with a central hydrophobic channel (Shannon and Fernandez, 1999). The β-barrel pore structure consists of between 10 and 18 amphipathic antiparallel β-sheets and the first and last β-sheets spontaneously associate with each other by hydrogen bonding resulting in a pore that is 2 nm in diameter (Veiga *et al.*, 2002; Shannon and Fernandez, 1999). Whereas the alternating hydrophobic side chains are embedded within the hydrophobic lipid bilayer, the hydrophilic side chains project towards the centre of the channel. A linker region connecting the passenger and β-domain is essential for export and may guide the passenger region through the β-domain channel (Maurer *et al.*, 1999). As with the chaperone/usher pathway, secretion across the outer membrane does not appear to require input of external energy.

Once secreted, the passenger domain may either be retained on the bacterial surface by noncovalent interactions or it may be released into the extracellular environment by a
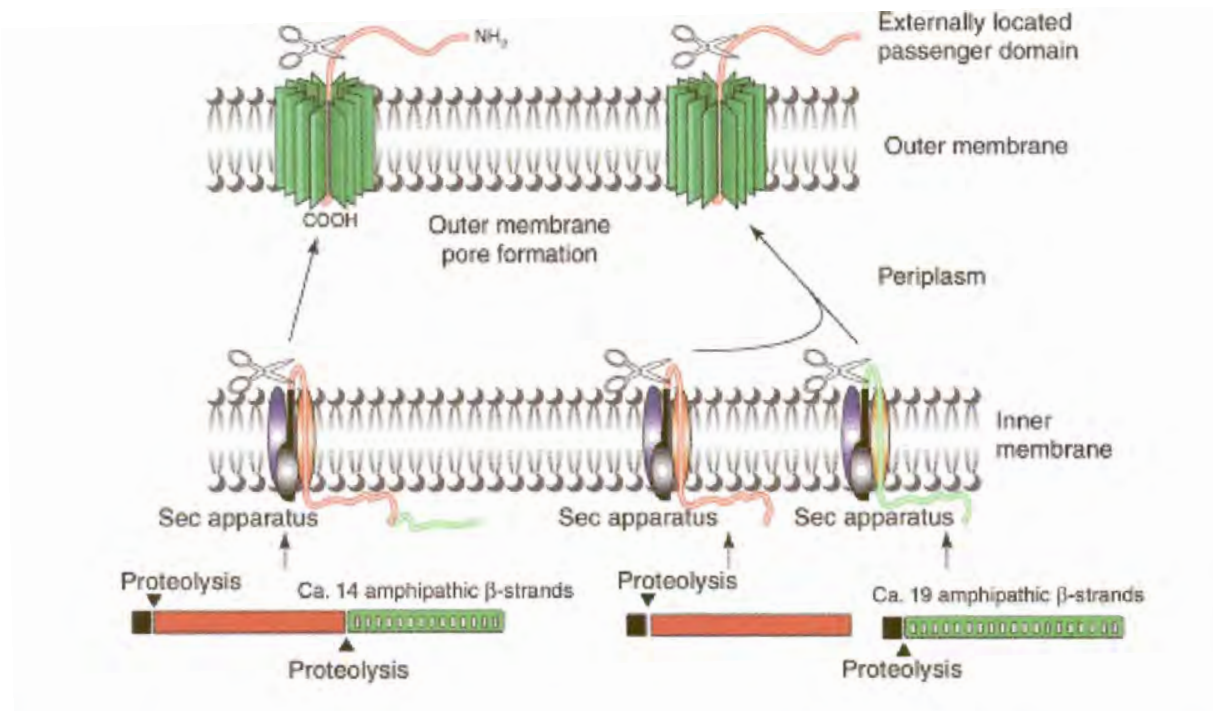
Fig. 1.5 The secretion mechanism of the true autotransporters are depicted in the bottom left of the diagram and the 'unlinked' autotransporters in the bottom right. The three protein domains are displayed: the leader sequence (black), the passenger domain (red), and the β-domain (green). Both the true and 'unlinked' autotransporters cross the inner membrane into the periplasmic space by means of the Sec machinery (coloured ovals). Once through the inner membrane, the signal sequence is cleaved by means of specific peptidases and the β-domain inserts itself in into the outer membrane in such a manner as to form a pore structure through which secretion is to occur. The passenger domain is inserted into the pore, crosses the outer membrane and is secreted where subsequent modifications might occur (Reprinted from TRENDS in Microbiology, Volume 9, Mori, H. and Ito, K., The Sec protein-translocation pathway, p 494, 2001, with permission from Elsevier).

proteolytic event. Cleavage of the passenger domain from the β-domain occurs through autoproteolysis or is mediated by another outer membrane protease, depending on the particular autotransporter. For many autotransporters, the passenger domain contains a serine protease motif that is responsible for autoproteolysis (Hendrixson *et al.*, 1997; Pohlner *et al.*, 1987). By contrast, the *S. flexneri* IcsA autotransporter is cleaved by an outer membrane protease called SopA (IcsP), which displays a high level of sequence homology to the AmpT and OmpP outer membrane serine proteases of *E. coli* (Egile *et al.*, 1997; D'Hauteville *et al.*, 1996).

An alternative autotransporter pathway has recently been identified in both *B. pertussis* and *Serratia marcescens* that requires a single accessory factor for secretion of the *B. pertussis* filamentous haemagglutinin (Jacob-Dubuisson *et al.*, 2001) and the *S. marcescens* haemolysin (Könninger *et al.*, 1999) across the outer membrane. This pathway has also been referred to as the single accessory pathway or two-partner secretion (TPS) pathway. In this

pathway, the accessory protein is thought to form a β-barrel pore in the outer membrane for secretion of the proteins to the cell surface. Although this single accessory pathway appears to be functionally similar to autotransporters, there is no sequence homology between the two systems and consequently the two systems may have evolved independently (Reumann *et al.*, 1999).

1.5 *SEC*-INDEPENDENT PROTEIN SECRETION PATHWAYS

1.5.1 The type I secretion pathway

Type I secretion pathways, also referred to as ATP-binding cassette (ABC) protein export systems, are employed by a wide range of different Gram-negative bacteria and are responsible for the secretion of toxins, proteases and lipases (Binet *et al.*, 1997; Fath and Kolter, 1993). Type I secretion is exemplified by the *E. coli* α-haemolysin secretion system (Mackman *et al.*, 1986) and other members of this group include the protease secretion system from *Er. chrysanthemi* (Létoffé *et al.*, 1990), the haemoprotein secretion system from *S. marcescens* (Létoffé *et al.*, 1994) as well as the AprX (Duong *et al.*, 2001) and alkaline protease (Guzzo *et al.*, 1991) secretion system from *P. aeruginosa* (also refer to Section 1.6). The proteins secreted by the type I pathway are not subject to proteolytic cleavage and therefore lack cleavable N-terminal leader peptide sequences (Binet *et al.*, 1997). Instead, the secretion signal is located within the C-terminal 60 amino acids of the secreted protein (Binet *et al.*, 1997; Duong *et al.*, 1996).

The type I secretion apparatus (Fig. 1.6) consists of three proteins: an inner membrane transport ATPase, which provides the energy for protein secretion, a membrane fusion protein (MFP), which is anchored in the inner membrane and spans the periplasmic space, and an outer membrane protein, which is exported via the *sec* pathway (Binet *et al.*, 1997). The genes encoding the secretion apparatus and the gene encoding the secreted protein are usually clustered in a single operon (Binet *et al.*, 1997; Létoffé *et al.*, 1994; Duong *et al.*, 1992; Létoffé *et al.*, 1990). It is thought that the outer membrane protein presumably functions as the outer membrane secretion channel. The periplasmic membrane fusion protein interacts with both the outer membrane protein and ABC transporter (Létoffé *et al.*, 1996).

These proteins typically contain a hydrophobic amino terminus, which is believed to span the inner membrane or is anchored in the inner membrane by lipid modification of the N terminus (Johnson and Church, 1999). The bulk of the membrane fusion protein is thought to extend across the periplasm to contact the outer membrane protein and/or the outer membrane itself.
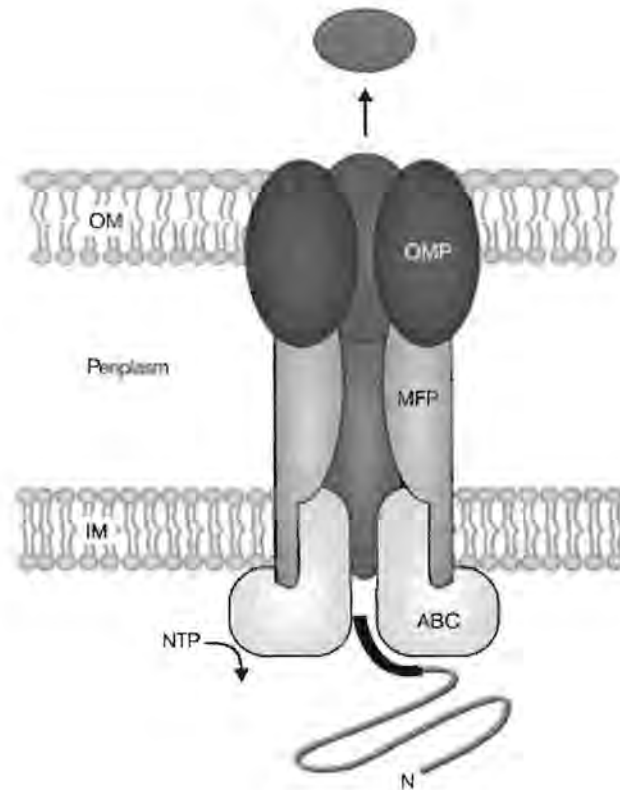
Fig. 1.6 Type I or ABC secretion pathway. The type I pathway does not provide for a cleavable N-terminal signal sequence to be recognized by the Sec machinery, instead it has a non-cleavable C-terminal recognition sequence. The C-terminal sequence targets the protein to the inner membrane protein. The inner membrane ABC export protein is linked to the outer membrane protein (OMP) via the membrane fusion protein (MFP). The outer membrane protein and the MFP are thought to assemble as trimers. ATPase activity by the ABC protein may energize substrate release into the medium. (Reprinted from Current Opinion in Cell Biology, Volume 12, Thanassi, D.G. and Hultgren, S.J., Multiple pathways allow protein secretion across the bacterial outer membrane, p 420, 2000, with permission from Elsevier).

The membrane fusion protein is thought to facilitate protein secretion without a periplasmic intermediate and current experimental evidence has led to two related models for secretion by the type I pathway. In the case of *E. coli* α-haemolysin secretion, the ABC transporter and membrane fusion protein associate prior to binding of the protein to be secreted. Binding of the protein to be secreted to this complex then triggers contact of the membrane fusion protein with the outer membrane protein. This bridging is transient and collapses after export of the protein. ATP hydrolysis by the ABC transporter drives release of the protein outside

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the cell and is not required for substrate binding or assembly of the complex (Thanabalu *et al.*, 1998). By contrast, secretion of proteases from *Er. chrysanthemi* involves an ordered set of associations in which the protein first binds to the ABC transporter, which then triggers binding of the membrane fusion protein. This complex in turn interacts with the outer membrane protein, allowing secretion of the proteases (Létoffé *et al.*, 1996).
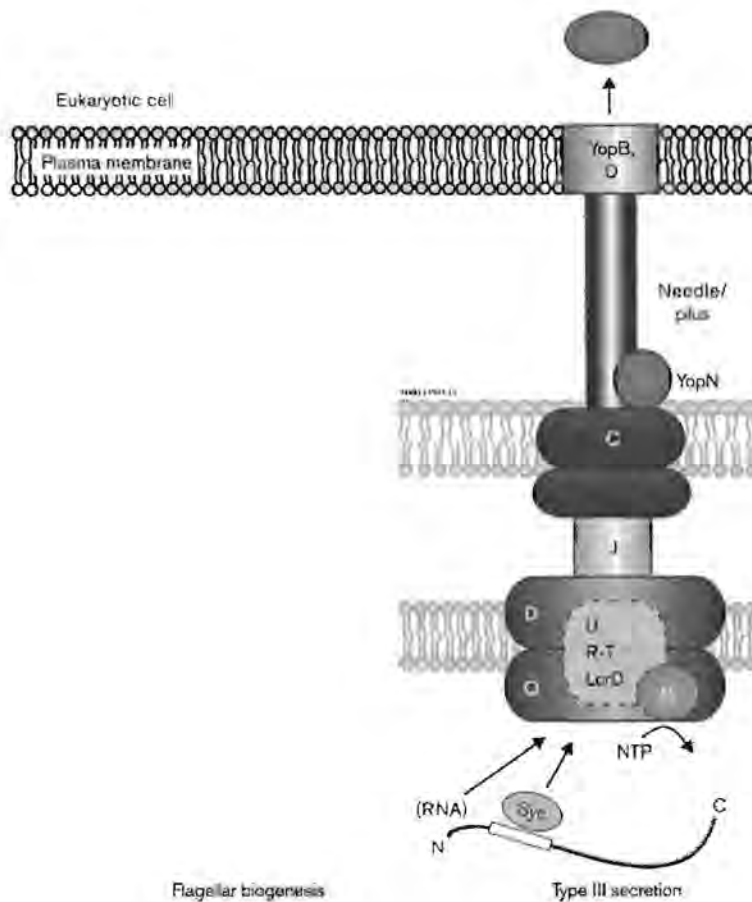
1.5.2   The type III secretion pathway

Similar to the type I secretion pathway, type III secretion is independent of the Sec translocase system and thus does not involve N-terminal processing of the secreted proteins. However, assembly of the type III secretion apparatus probably requires the *sec* pathway, since several components of the type III secretory apparatus carry *sec*-characteristic N-terminal signal sequences (Hueck, 1998). By contrast to the secretion process in type I and type II systems, type III secretion is triggered when a pathogen comes in close contact with host cells and has thus been called contact-dependent secretion (Plano *et al.*, 2001). Consequently, type III protein secretion systems are present in various human and animal pathogens, amongst other, *Yersinia* spp., *Shigella flexneri*, *Salmonella typhimurium*, *P. aeruginosa* and enteropathogenic *E. coli* (EPEC) as well as in several plant pathogens, *e.g. P. syringae*, *Erwinia* spp., *Ralstonia solanacearum* and *Rhizobium* spp. (Hueck, 1998).

The type III secretion apparatus is composed of approximately 20 proteins, most of which are located in the inner membrane (Woestyn *et al.*, 1994). Most of the inner membrane proteins are homologous to components of the flagellar biosynthesis apparatus of both Gram-negative and Gram-positive bacteria. Although the type III secretion does not involve distinct periplasmic intermediates of the secreted proteins, transport through the inner membrane is genetically separable from secretion through the outer membrane (Plano *et al.*, 2001). As in type I and type II secretion pathways, most proteins in the type III secretion systems, including effector proteins, regulatory proteins, structural proteins and chaperones, are encoded by genes that belong to several large operons, which are clustered together on plasmids in some species, and on the chromosome in others (Bergman *et al.*, 1994; Groisman and Ochman, 1993).

The proteins secreted via the type III pathway are not subjected to N-terminal processing during secretion. Although the signal for secretion was initially thought to reside within the

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N-terminal 15-20 amino acids of the secreted proteins (Sory *et al.*, 1995; Michiels and Cornelis, 1991), subsequent studies have shown that the secretion signal resides in the 5' region of the mRNA which encodes the secreted proteins (Anderson and Schneewind, 1997). Furthermore, the secreted proteins require small cytoplasmic proteins with chaperone functions to protect the secreted factors from premature interaction with other components of the secretion system (Anderson and Schneewind, 1997).

The type III secretion apparatus in *Yersinia* spp., which is used for the secretion of *Yersinia* outer proteins (Yops), has been the most intensively investigated (Plano *et al.*, 2001; Thanassi and Hultgren, 2000) (Fig. 1.7). Although it consists of a secretion apparatus (the Ysc apparatus) composed of 25 proteins, only one protein in the system, YscN, has been shown to hydrolyse ATP and is a likely candidate for generating energy to drive secretion (Woestyn *et al.*, 1994). YscN is predicted to be a cytoplasmic protein, closely associated with the inner membrane. Several proteins essential for secretion, including LcrD, YscD, YscR, YscS, YscT and YscU, are known or predicted to reside in the inner membrane (Plano and Stratley, 1995; Plano *et al.*, 1991). At the outer membrane, only one protein, YscC, and two lipoproteins, YscJ and VirG (Allaoui *et al.*, 1995a; 1995b), appear essential for proper secretion. The roles and subcellular locations are not known for several more essential proteins YscE, YscF, YscG, YscI, YscK and YscL, and how all of these proteins interact with one another to form the secretion apparatus is not yet understood (Plano *et al.*, 2001). The correct assembly of the apparatus is not only required for secretion, but also for normal synthesis of effector molecules (Plano and Stratley, 1995; Woestyn *et al.*, 1994). Two proteins, YopB and YopD, are loosely associated with the outer membrane and are crucial for the efficient delivery of effector molecules into target cells (Håkansson *et al.*, 1993). These two proteins use the type III secretion system to reach the bacterial cell surface and presumably form a pore in the target cell through which the effector molecules pass. Several chaperone proteins play important roles in secretion by binding to effector molecules in the bacterial cytoplasm. These chaperones have several proposed functions. For example, chaperone binding may stabilize and prevent proteins from folding into conformations that are impossible to secrete or, alternatively, they may prevent effector molecules from improperly associating with one another before secretion. Furthermore, the chaperones may also aid in delivering effector molecules to the secretion apparatus (Frithz-Lindsten *et al.*, 1995; Wattiau *et al.*, 1996).

Fig. 1.7 Model for type III secretion. The components depicted in the type III secretion model are labeled according to the *Yersinia* nomenclature (Ysc proteins or as indicated). Certain type III secretion substrates contain two amino-terminal signal sequences for targeting to the secretion machinery. One is encoded by the mRNA and the second serves as a binding site for cytoplasmic Syc chaperones. In a similar fashion, type III substrates (Yop proteins) may travel through a central channel in the type III needle or pilus. Translocation of Yops into the target eukaryotic cell may take place via a channel formed in the plasma membrane by YopB and YopD (Reprinted from Current Opinion in Cell Biology, Volume 12, Thanassi, D.G. and Hultgren, S.J., Multiple pathways allow protein secretion across the bacterial outer membrane, p 420, 2000, with permission from Elsevier).

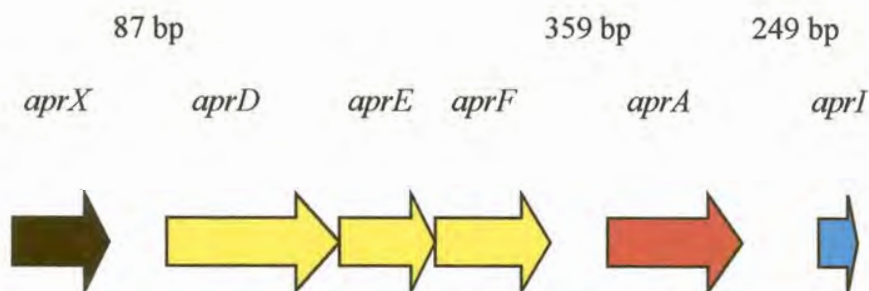## 1.6   ALKALINE PROTEASE SECRETION IN *P. aeruginosa*

P. aeruginosa is capable of secreting a wide variety of toxins and enzymes of which the extracellular release is achieved by dedicated secretion systems. A type I secretion system is used for secretion of alkaline protease (Duong *et al.*, 1992), AprX (Duong *et al.*, 2001) and HasAp (Létoffé *et al.*, 1998). A type II secretion system is used to secrete proteins as diverse as elastase, lipase, phospholipase and exotoxin A (Filloux *et al.*, 1998; Lazdunkski *et al.*, 1990), while exo-enzymes S and other coordinately expressed proteins are secreted by a

type III secretion pathway (Yahr *et al.*, 1997). Since secretion of the *P. aeruginosa* alkaline protease is closely linked to the aims of this investigation, the following section will attempt to provide a more detailed overview of the protein components required for alkaline protease secretion as well as the mechanism whereby the protease is secreted from *P. aeruginosa* cells.

1.6.1 The secretory apparatus

Evidence for the secretion of alkaline protease by a pathway other than the type II (*xcp*) secretion pathway was obtained through studying *xcp* mutants in which the secretion of alkaline protease appeared to be unaffected (Filloux *et al.*, 1987). Subsequent investigations by Guzzo *et al.* (1990; 1991) and Duong *et al.* (1992) resulted in the identification of an 8.8-kb DNA fragment, which contained the structural gene for alkaline protease, and led to the secretion of the enzyme into the extracellular medium when expressed in *E. coli* (Guzzo *et al.*, 1990). Further characterization of the DNA fragment by insertion mutagenesis (Guzzo *et al.*, 1991) and nucleic acid sequencing (Duong *et al.*, 1992) indicated the existence of a gene cluster consisting of five genes, *i.e. aprA* which represents the structural gene encoding the alkaline protease, *aprI* which encodes a protein that may serve either as an intracellular inhibitor or chaperone of alkaline protease, and three genes, designated *aprD*, *aprE* and *aprF* which are involved in alkaline protease secretion. Recently, an additional gene, termed *aprX*, was identified upstream from the *aprD* gene in the *apr* operon and appears to be recognized by the AprDEF secretory apparatus, suggesting that it is an ABC-transported exoprotein (Duong *et al.*, 2001) (Fig. 1.8). The function of this exoprotein is, however, not known at present.

87 bp		359 bp	249 bp

aprX *aprD* *aprE* *aprF* *aprA* *aprI*

Fig. 1.8 The alkaline protease operon indicating all the genes needed for efficient alkaline protease secretion. The structural genes are indicated in yellow and constitute genes *aprD*, *aprE* and *aprF*. The red arrow denotes the alkaline protease structural gene *aprA* and the protease inhibitor, *aprI*, is represented by the blue arrow. The black arrow is representative of , *aprX*, an upstream protease recently reported by Duong *et al.* (2001).

1.6.2 Characteristics of the Apr proteins

The AprD protein consists of an N-terminal hydrophobic domain with four putative membrane-spanning regions and a hydrophilic C-terminal domain, which displays an ATP-binding motif (Duong *et al.*, 1992). The C-terminal domain may serve to couple ATP hydrolysis to the secretion process, whereas the N-terminal domain may be part of or act as the translocator itself. The AprE protein has one hydrophobic membrane-spanning region close to the N-terminus. This N-terminal domain may be part of the translocator, whereas the large C-terminal domain may bridge the periplasm to contact the outer membrane component of the system (Duong *et al.*, 1992). The outer membrane protein AprF is synthesized in a precursor form with a typical N-terminal signal peptide (Duong *et al.*, 1992).

The alkaline protease (AprA) displays a significant degree of sequence identity to the *Er. chrysanthemi* proteases B and C, and to a metalloprotease of *S. marcescens* (Duong *et al.*, 1992). All of these proteases are synthesized as inactive precursors with short N-terminal extensions, which do not resemble a signal peptide. The propeptides are not involved in the secretion process, but may serve to protect the proteins against intracellular proteolytic activity (Tommassen *et al.*, 1992). Consistent with the lack of a signal sequence is the observation that secretion of alkaline protease occurs via a *sec*-independent pathway (Guzzo *et al.*, 1991). The alkaline protease possesses a conserved tandem series of glycine-rich hexapeptides located just upstream from the C-terminal targeting sequence (Duong *et al.*, 1994). These peptides seem to favor secretion by possibly acting as a flexible hinge to separate the catalytic N-terminal domain from the C-terminal secretion signal, thus resulting in a better presentation of the signal to the translocator (Binet *et al.*, 1997). By contrast, both the AprX (Duong *et al.*, 2001) and HasAp (Létoffé *et al.*, 1998) proteins require an extreme C-terminal motif consisting of a negatively charged amino acid followed by several hydrophobic amino acids for efficient secretion by the secretory apparatus. The *aprA* gene is followed by a gene that encodes the AprI protein. The actual role and involvement of AprI in the regulation of protease secretion is, however, not clear (Duong *et al.*, 1992).

1.6.3 Model for the secretion of the *P. aeruginosa* alkaline protease

A model for the secretion of alkaline protease of *P. aeruginosa* has been proposed by Tommassen *et al.* (1992). According to this model, the AprE protein may contact both AprD in the inner membrane and AprF in the outer membrane, thereby facilitating the direct interaction of these cytoplasmic and outer membrane proteins during secretion. The alkaline protease contains a secretion signal at the C-terminus, which is recognized by AprD, and thus initiates its secretion from the cytoplasm directly into the extracellular medium. The AprD protein is the translocator in the inner membrane and provides energy to the secretion process by ATP hydrolysis. The AprF protein forms a pore in the outer membrane through which the protease is secreted and the pore may only be opened during the secretion process, thus serving as a gated channel.

1.7 RECOMBINANT PROTEIN EXPRESSION

The objective of gene cloning for biotechnological purposes is usually the expression of the cloned gene in a selected host organism. Among the many systems that are available for heterologous protein production, the Gram-negative bacterium *E. coli* has remained one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of a large number of cloning vectors and mutant host strains (Old and Primrose, 1994; Balbas and Bolivar, 1990). Although there is no guarantee that a recombinant gene product will accumulate in *E. coli* at high levels in a full-length and biologically active form, a considerable amount of effort has been directed at improving its performance and versatility (Old and Primrose, 1994). Many of the approaches and strategies that have been developed for *E. coli* may also be applicable to a wide variety of other microorganisms. In this part of the literature review, some of these strategies and approaches that have been found to be useful for the overproduction of proteins will be highlighted.

1.7.1 Transcriptional regulation

1.7.1.1 Promoters

The minimum requirement for an effective gene expression system is the presence of a promoter sequence upstream from the cloned gene. Such a promoter should, however, have several properties to render it suitable for high-level protein synthesis (Goldstein and Doi, 1995). The promoter must be strong, resulting in the accumulation of protein making up 10 to 30% or more of the total cellular protein. The promoter should exhibit a minimal level of basal transcriptional activity as large-scale gene expression usually employs cell growth to high density and minimal promoter activity, followed by induction or derepression of the promoter. The tight regulation of a promoter is also essential for the synthesis of proteins that may be detrimental to the host cell (Suter-Crazzolara and Unsicker, 1995; Wülfling and Plückthun, 1993). Furthermore, incompletely repressed expression systems may cause plasmid instability, a decrease in cell growth rate and consequently may prevent the efficient production of recombinant protein (Chen *et al.*, 1991; Mertens *et al.*, 1995).

Another important characteristic of a promoter is its inducibility in a simple and cost-effective manner. The most widely used promoters for large-scale protein production use thermal induction (λP_L) or chemical inducers (*lac*, *tac* and *trp*) (Friesen and An, 1983). Not only do each of these promoters interact with repressors, which provide a controllable switch for transcription of adjacent cloned genes, but they are also recognized by the sigma 70-RNA polymerase holoenzyme, which is present in much larger quantities than other minor sigma factors in bacterial cells (Record *et al.*, 1996). Consequently, transcription is not limited because of a shortage of available sigma factor. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoters *lac* (Donovan *et al.*, 1996) or *tac* (de Boer *et al.*, 1983) are powerful and widely used for basic research, but the use of IPTG for the large-scale production of human therapeutic proteins is undesirable because of its toxicity and high cost (Figge *et al.*, 1988).

Other promoters have been characterized that may provide alternative options for high-level gene expression systems. For example, cold-responsive promoters have been shown to facilitate efficient gene expression at reduced temperatures (Giladi *et al.*, 1995). The

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promoter of the *E. coli* major cold shock gene *cspA* was demonstrated to be active at about 15 to 20°C (Vasina and Baneyx, 1997; Goldstein *et al.*, 1990). The rationale behind the use of cold-responsive promoters for gene expression is based on the proposition that the rate of protein folding will be only slightly affected at lower temperatures, whereas the rates of transcription and translation, being biochemical reactions, will be substantially decreased. This, in turn, will provide sufficient time for protein refolding, yielding active proteins and avoiding the formation of inactive protein aggregates, *i.e.* inclusion bodies, without reducing the final yield of the target protein (Giladi *et al.*, 1995).

Several reports have also demonstrated that sequences upstream of the core promoter play an important role in determining transcription efficiency by increasing the rate of transcription initiation *in vivo* (Ross *et al.*, 1998; Rao *et al.*, 1994; Ross *et al.*, 1993; Gourse *et al.*, 1986). Gourse and colleagues have shown that a DNA sequence, the UP element, located upstream of the −35 region of the *E. coli* rRNA promoter *rrnB* P1, stimulates transcription by a factor of 30 *in vitro* and *in vivo*. The UP element functions as an independent promoter module because when it is fused to other promoters such as *lacUV5*, it stimulates transcription (Rao *et al.*, 1994; Ross *et al.*, 1993).

1.7.1.2 Transcriptional terminators

In prokaryotes, transcription termination is effected by either a *rho*-dependent or rho-independent mechanism (Richardson, 1993). The *rho*-dependent transcription termination depends on the hexameric protein Rho, which causes the release of the nascent RNA transcript from the template. By contrast, the *rho*-independent termination depends on signals encoded in the template, specifically, a region of dyad symmetry that encodes a hairpin or stem-loop structure in the nascent RNA and a second region that is rich in dA and dT and is located 4 to 9 bp distal to the dyadic sequence. Efficient transcription terminators are indispensable elements of expression vectors, because they serve several important functions. Transcription through a promoter may inhibit its function, a phenomenon known as promoter occlusion (Adhya and Gottesman, 1982). This interference can be prevented by the proper placement of a transcription terminator downstream of the coding sequence to prevent continued transcription through another promoter. Similarly, a transcription terminator placed upstream of the promoter that drives expression of the gene of interest minimizes background transcription (Nishihara *et al.*, 1994). In addition, transcription terminators enhance mRNA

stability and can thus substantially increase the level of protein production (Vasquez *et al.*, 1989; Wong and Chang, 1986; Hayashi and Hayashi, 1985).

1.7.2 Translational regulation

1.7.2.1 Translational issues

Putting a cloned gene under control of a regulatable, strong promoter, although essential, may not be sufficient to maximize the yield of the cloned gene product. Other factors, such as the efficiency of translation and the stability of newly transcribed mRNA may also affect the amount of product. Because of the close coupling between transcription and translation in prokaryotes, engineering of the translation initiation region is considered a powerful tool for modulating gene expression in a promoter-independent fashion (Ringquist *et al.*, 1992). Initiation of translation of prokaryotic mRNAs requires a Shine-Dalgarno (SD) sequence complementary to the 3' end of the 16S rRNA, followed by an initiation codon, which is most commonly AUG (Gualerzi and Pon, 1990; Shine and Dalgarno, 1974). Stable mRNA secondary structures encompassing the SD sequence and/or the initiation codon have been reported to dramatically reduce gene expression by interfering with ribosome binding (de Smit and van Duin, 1990; Hall *et al.*, 1982). It is believed that the occlusion of the SD region and/or the AUG codon by a stem-loop structure precludes accessibility to the 30S ribosomal subunits and inhibits translation (Ramesh and De Nagaraja, 1994; Gheysen *et al.*, 1982). This problem can be circumvented by increasing the homology of SD regions to the consensus (Coleman *et al.*, 1985; Stanssens *et al.*, 1985) and by raising the number of A residues in the initiation region through site-directed mutagenesis (Chen *et al.*, 1994; Olsen *et al.*, 1989).

An additional mRNA feature affecting translation initiation is the downstream box (DB), which is located after the initiation codon and complementary to bases 1469-1483 of the 16S rRNA. Evidence has been presented that suggests that DBs play a major role as translational enhancers (Etchegaray and Inouye, 1999; Sprengart *et al.*, 1990). Deletion of the DB was shown to abolish translational activity, while shifting of the DB to upstream of the initiation codon to the position of the SD sequence rendered it nonfunctional (Sprengart *et al.*, 1996). Although introduction of a consensus DB at the 5' end of genes encoding recombinant proteins would change their amino acid sequence, increasing the homology of this region to

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that of the DB by using synonymous codons may improve translational initiation of certain transcripts (Etchegaray and Inouye, 1999).

1.7.2.2   mRNA stability

The process of mRNA degradation provides a major control point of gene expression in virtually all organisms (Ross, 1995). Prokaryotic mRNAs are rather unstable, with half-lives ranging between 30 s and 20 min. The major enzymes involved in mRNA degradation are two $3' \rightarrow 5'$ exonucleases (RNase II and polynucleotide phosphorylase (PNPase) and the endonuclease RNase E (Carpousis et al., 1999; Coburn and Mackie, 1999). The catalytic activity of RNase E is located at the protein N-terminus, whereas the C-terminus serves as a scaffold for the assembly of a highly efficient RNA 'degradasome' involving PNPase, the RNA helicase RhlB and enolase. Two classes of protective elements are known to stabilize mRNAs in E. coli and may subsequently be used to prolong the half-life of heterologous mRNAs (Emory et al., 1992; Chen et al., 1991; Duvoisin et al., 1986). One class consists of sequences in the 5' untranslated regions (UTRs) of mRNAs, and the other class consists of 3' UTR sequences that can form stem-loop structures, thereby blocking exonucleolytic degradation of the transcripts from the 3' terminus. In either case, stable secondary structures present in the 5' UTR of certain transcripts as well as 3' rho-independent terminators can both increase mRNA stability and consequently increase the level of protein production (Carpousis et al., 1999).

1.7.3   Protein targeting

1.7.3.1   Cytoplasmic expression

Overproduction of heterologous proteins in the cytoplasm of E. coli is often accompanied by their misfolding and segregation into insoluble aggregates known as inclusion bodies (Rudolph and Lilie, 1996). The formation of inclusion bodies remains a significant barrier to gene expression in the cytosol. In addition, protein degradation is more likely to occur in the cytoplasm than in other compartments, because of the greater number of proteases located there (Gottesman, 1996; Talmadge and Gilbert, 1982; Swamy and Goldberg, 1982). These proteases are important for the degradation of abnormal and defective proteins, which is a housekeeping function that is necessary for the continued viability of the cells. Another

difficulty that affects cytosolic gene expression is the need to purify the soluble target protein from the pool of the intracellular proteins. Although inclusion body formation can greatly facilitate protein purification, there is no guarantee that the *in vitro* refolding will yield large amounts of biologically active product. The precise physicochemical parameters that contribute to the formation of inclusion bodies remain unclear (Rudolph and Lilie, 1996; Krueger *et al.*, 1989; Mitraki and King, 1989), but properties of the expressed protein such as charge average, turn-forming residue fraction, cysteine and proline fractions, hydrophilicity, and total number of residues may all aid in inclusion body formation (Wilkinson and Harrison, 1991).

Some traditional approaches to reduce protein aggregation include growth of bacterial cultures at lower temperatures (Schein, 1993; Cabilly, 1989), addition of non-metabolizable sugars to the growth medium (Bowden and Georgiou, 1988) and alteration of the pH of the culture medium (Sugimoto *et al.*, 1991). The realization that *in vivo* protein-folding is assisted by molecular chaperones, which promote the proper izomerization and cellular targeting of other polypeptides by transiently interacting with folding intermediates, and by foldases, which accelerate rate-limiting steps along the folding pathway (Thomas *et al.*, 1997), has provided useful alternative approaches to combat the problem of inclusion body formation. Consequently, several experimental approaches have been used to minimize the formation of inclusion bodies and improve protein folding. These include the substitution of selected amino acid residues (Dale *et al.*, 1994; Rinas *et al.*, 1992), the co-production of chaperones (Battistoni *et al.*, 1993) and the use of *E. coli* thioredoxin as a fusion partner (Yasukawa *et al.*, 1995; La Vallie *et al.*, 1993).

1.7.3.2 Periplasmic expression

The periplasm offers several advantages for protein targeting. By contrast to the cytosolic compartment, the periplasm contains only 4% of the total cell protein or approximately 100 proteins (Pugsley and Schwartz, 1985). The target protein can thus be effectively concentrated, and its purification is considerably less tedious. The oxidizing environment of the periplasm facilitates the proper folding of proteins, and the cleaving *in vivo* of the signal peptide during translocation to the periplasm is more likely to yield the authentic N-terminus of the target protein. Protein degradation in the periplasm is also less extensive (Missiakas and Raina, 1997; Talmadge and Gilbert, 1982).

Protein transport to the bacterial periplasm is a particularly complex process and normally requires a signal sequence (Thanassi and Hultgren, 2000; Pugsley, 1993). However, the presence of a signal peptide alone does not always ensure efficient protein translocation through the inner membrane (Cheah *et al.*, 1994). Most signal sequences derived from naturally occurring secretory proteins support the efficient translocation of heterologous polypeptides across the inner membrane when fused to their N-termini. In some cases, preproteins are not readily exported and either become lodged in the inner membrane, accumulate in precursor inclusion bodies or are readily degraded within the cytoplasm (Gottesman, 1996; Wilcox and Studnicka, 1988; Talmadge and Gilbert, 1982). In such instances, translocation of proteins to the periplasm may be improved by overproduction of components involved in protein transport and processing, particularly the signal peptidase I (van Dijl *et al.*, 1991).

1.7.2.3 Extracellular secretion

The targeting of synthesized proteins for secretion to the culture medium presents significant advantages, such as a low level of proteolysis, simpler purification strategies and improved protein folding (Kitai *et al.*, 1988). However, *E. coli* normally secretes very few proteins and the manipulation of the various transport pathways to facilitate secretion of foreign proteins remains a formidable task (Pugsley *et al.*, 1997; Pugsley, 1993).

The methodological approaches to protein secretion can be divided into two categories, *i.e.* the exploitation of existing pathways for "truly" secreted proteins (Stader and Silhavy, 1990), and the use of signal sequences, fusion partners, permeabilizing proteins, nutrients or other agents that may effect protein secretion as a result of "leakage" or selective and limited permeability of the outer membrane (Ko *et al.*, 1995; Suominen *et al.*, 1987). The first approach offers the advantage of specific secretion of the protein of interest and thus minimum contamination by nontarget proteins (Kern and Ceglowski, 1995; Stader and Silhavy, 1990; Holland *et al.*, 1990). The second approach relies on the induction of limited leakage of the outer membrane to cause protein secretion (Ko *et al.*, 1995; Suominen *et al.*, 1987) and is facilitated by either the co-expression of bacteriocin release protein (van der Wal *et al.*, 1998) or the co-expression of the *kil* gene for membrane permeabilization (Kato *et al.*,

1987; Kitai *et al.*, 1988). These latter studies have reported that there was no appreciable *E. coli* cell lysis and that the protein yields were modest.

1.7.4   Other factors of importance

1.7.4.1 Metabolic Load

The introduction and expression of foreign DNA in a host organism often change the metabolism of the organism in ways that may impair normal cellular functioning. The phenomenon, which is a multifaceted biological response, is due to a metabolic load that is imposed upon the host by the foreign DNA. A metabolic load can occur as the result of a variety of conditions (Glick, 1995; Glick and Whitney, 1987). For example, increasing plasmid copy number and/or size requires increasing amounts of cellular energy for plasmid replication and maintenance, and the limited amount of dissolved oxygen in the growth medium is often insufficient for both host cell metabolism and plasmid maintenance and expression. In such cases, plasmid-bearing cells grow more slowly than untransformed cells, often resulting in the loss of the recombinant plasmid or a portion of the plasmid DNA. Since cells growing in the presence of a metabolic load generally have a decreased level of energy available for a variety of cellular functions, the cell's energy-intensive metabolic processes such as protein synthesis are invariably adversely affected by a metabolic load (Glick and Whitney, 1987). Furthermore, overproduction of foreign proteins may deplete the pools of certain aminoacyl-tRNAs and/or drain the host cell of its energy in the form of ATP or GTP. Such a high level of expression of a foreign protein can initiate a cellular stress response, including increased synthesis of cellular proteases, so that the recombinant protein is rapidly degraded. The depletion of amino acid pools may also occur as the consequence of the synthesis of highly expressed antibiotic resistance genes (Glick, 1995).

1.7.4.2 Plasmid copy number and maintenance

To achieve high gene dosage, the genes to be expressed are typically cloned into plasmids that replicate in a relaxed fashion and are present at high copy numbers, ranging from 15 to a few hundred copies per cell. Under laboratory conditions, such multicopy plasmids are randomly distributed during cell division and, in the absence of selective pressure, are lost at low frequency, primarily as a result of multimerization (Summers, 1998). However, plasmid

loss can increase in the case of very high copy number plasmids, when plasmid-borne genes are toxic to the host or otherwise significantly reduce its growth rate, or when cells are cultivated at high density or in continuous processes (Summers, 1998).

The simplest way to address this problem is to take advantage of plasmid-encoded antibiotic-resistance markers and supplement the growth medium with antibiotics to kill plasmid-free cells. The drawbacks of this approach are loss of selective pressure as a result of antibiotic degradation, or inactivation and the contamination of the product or biomass by antibiotics, which may be unacceptable in the production of human therapeutic proteins (Old and Primrose, 1994; Glick and Whitney, 1987). A number of alternative strategies have therefore been developed to ensure that plasmid-free cells will not overtake a culture. In most cases, cloning vectors are engineered to carry gene(s) or repressors that cause cell death upon plasmid loss (Williams *et al.*, 1998). Alternatively, the heterologous genes may be directly inserted within the chromosome of the host organism (Hinds *et al.*, 1999; Olson *et al.*, 1998; Chopin *et al.*, 1989). In this case, problems associated with plasmid instability may be overcome and the transformed host cell will also not waste its resources synthesizing unwanted and unneeded antibiotic resistance marker gene products.

1.8   AIMS OF THIS STUDY

Most microorganisms in natural habitats possess the ability to adhere to surfaces. Cellular growth and reproduction of the surface-associated microorganisms ultimately leads to the formation of biofilms. The biofilm-associated cells can be differentiated from their planktonic counterparts by the generation of an extracellular polymeric matrix, reduced growth rates, and the up- and downregulation of several genes (Davey and O'Toole, 2000; O'Toole *et al.*, 2000; Costerton *et al.*, 1995). The biofilm cells are consequently responsible for much of the microbial activity found in natural habitats and the potential for altered gene expression at surfaces and in biofilms could be advantages in biotechnological applications. One such an application may be the development of a biofilm-specific expression system whereby products or enzymes of commercial importance can be produced in membrane bioreactors.

Expression systems are quite diverse and there does not exist a universal strategy for achieving maximal expression of every gene in all bacteria, or even in Gram-negative

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bacteria. Although *E. coli* has been extensively used as a host for the expression of foreign genes (Old and Primrose, 1994; Balbas and Bolivar, 1990), it may not necessarily be the microorganism of choice for the expression of all foreign proteins. However, many of the approaches and strategies that have been developed for the overexpression of proteins in *E. coli*, could also be useful with a variety of other microorganisms. In this investigation, *P. aeruginosa* was used as model organism for the development of a biofilm-specific expression system. Although *P. aeruginosa* does not have GRAS status, it was nevertheless used as a model organism as it readily forms biofilms (Tolker-Nielsen *et al.*, 2000; Costerton *et al.*, 1995), its molecular biology has been characterized to a reasonable degree (Davey and O'Toole, 2000; O'Toole *et al.*, 2000) and several genes and promoters that are actively up-regulated in biofilm cells have been identified (Whiteley *et al.*, 2001; Weyers, 1999; Davies and Geesey, 1995; Hoyle *et al.*, 1993). In addition, an alkaline protease, AprA, is naturally secreted by *P. aeruginosa* into the extracellular culture medium via a type I secretion pathway (Guzzo *et al.*, 1991; Tommassen *et al.*, 1992). As alkaline proteases are commonly used in detergent and washing powder formulations, overexpression of the extracellular alkaline protease of *P. aeruginosa* was considered to be an attractive model in this investigation.

Towards the development of a biofilm-specific expression system for *P. aeruginosa*, the aims of this investigation were the following:

(i)   To generate an alkaline protease-deficient isogenic mutant strain of *P. aeruginosa* DSM1707 by directed insertional mutagenesis using an allelic exchange vector.

(ii)   To construct several expression cassettes, comprising various different promoters and the structural gene of the alkaline protease of *P. aeruginosa*, in a high-copy-number plasmid vector as well as pseudomonad-specific chromosomal integration vector.

(iii) To examine different recombinant *P. aeruginosa* strains by means of quantitative azocasein hydrolysis assays to determine whether the different types of promoters, increased gene dosage and different growth phases may lead to increased extracellular alkaline protease expression levels.

CHAPTER 2

GENERATION OF AN ALKALINE PROTEASE-DEFICIENT MUTANT STRAIN OF *Pseudomonas aeruginosa* DSM1707

2.1   INTRODUCTION

In Gram-negative bacteria, proteins that are secreted into the extracellular medium have to traverse a cell envelope consisting of two membranes, separated by the periplasmic component. Two main pathways for the secretion of proteins can be distinguished (Tommassen *et al.*, 1992; Pugsley and Schwartz, 1985). In the first pathway, the exoproteins gain direct access to the external environment, thereby bypassing the periplasmic space. This is achieved by the formation of a channel-like structure, which spans both cell membranes and selectively secretes the target protein. This process does not require the help of a classic signal peptide. Exoproteins following the second pathway cross the inner membrane in a signal peptide-dependent pathway and several *sec* genes regulate the process. Following inner membrane translocation, the proteins are translocated across the outer membrane in a separate step requiring the proteins encoded by a different set of genes. In the case of *P. aeruginosa*, a large number of extracellular proteins are produced (Filloux *et al.*, 1998; Kadurugamuwa and Beveridge, 1995; Lazdunski *et al.*, 1990; Liu, 1974). Amongst them, alkaline protease follows the signal peptide-independent type I pathway (Guzzo *et al.*, 1991), while the great majority of exoproteins, including elastase, phospholipase C, exotoxin A, alkaline phosphatase and lipase, follow the signal peptide-dependent type II pathway (Ball *et al.*, 2002; Filloux *et al.*, 1998; Filloux *et al.*, 1990; Lazdunski *et al.*, 1990).

The fact that the *P. aeruginosa* alkaline protease has it's own specific and independent secretion pathway is of particular interest to this study. Genetic studies have shown that a DNA segment flanking the alkaline protease-encoding gene, *aprA*, encodes the functions required for alkaline protease secretion (Duong *et al.*, 1992; Guzzo *et al.*, 1990; Filloux *et al.*, 1987). Nucleic acid sequence analysis and insertion mutagenesis have revealed the existence of four accessory genes. These are *aprI*, which encodes a protein that may serve as an intracellular inhibitor or chaperone of alkaline protease (Duong *et al.*, 1992; Létoffé *et al.*, 1989), and three genes, designated *aprD*, *aprE* and *aprF*, which encode membrane proteins involved in protease secretion (Duong *et al.*, 1992; Lazdunski *et al.*, 1990). All of these genes are organized in a single operon and the *aprA* and *aprI* genes are located downstream from the *aprF* gene.

As the alkaline protease secretion machinery is organised in a single operon, it represented an attractive system for investigating whether the level of extracellular alkaline protease may be

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increased through, amongst other, the use of different promoters and culturing conditions. Should this be possible, the alkaline protease secretion machinery may be exploited for the export of heterologous proteins expressed in *P. aeruginosa*. However, as a first step towards achieving this goal, it would be necessary to construct an alkaline protease-deficient *P. aeruginosa* strain, which could subsequently be used to assay the efficacy of different strategies in altering the level of extracellular alkaline protease.

Although various strategies for generating mutant bacterial strains have been described (Guzzo *et al.*, 1990), the use of plasmids that are conditional for their replication, also referred to as "suicide plasmids", are frequently used to create defined mutations within a target genome (de Vries and Wackernagel, 2002; Hinds *et al.*, 1999; Parish *et al.*, 1999; Tamakoshi *et al.*, 1997). In such instances, a plasmid containing a cloned copy of a chromosomal gene, which has been disrupted through the insertion of an antibiotic gene, is introduced into a recipient strain where the plasmid cannot replicate. By selecting for some property of the plasmid, such as the newly introduced antibiotic resistance marker, isolates that have integrated the cloned DNA fragment into the host genome via homology between the cloned DNA fragment and the corresponding region of the recipient chromosome can then be selected. Not only is it a powerful approach for identifying gene function (Tran *et al.*, 1998), but it may also facilitate the understanding of pathogenicity at a molecular level (Hinds *et al.*, 1999), the definition of structure-function relationships (Horrocks *et al.*, 2002) and the production of vaccine candidates (Parish *et al.*, 1999).

The aims of this part of the study were therefore (i) to generate an alkaline protease-deficient mutant *P. aeruginosa* DSM1707 strain by directed insertional mutagenesis using an allelic exchange vector and (ii) to characterize the newly generated mutant *P. aeruginosa* strain with regards to its extracellular proteolytic activity.

2.2   MATERIALS AND METHODS

2.2.1   Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used in this part of the study are listed in Table 2.1. The *E. coli* and *P. aeruginosa* strains were routinely cultured overnight in Luria-Bertani broth (LB:

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0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl, pH 7.4) at 37°C with shaking at 250 rpm. The *E. coli* cultures were maintained on LB-agar plates, while cultures of *P. aeruginosa* were maintained either on *Pseudomonas* Agar Base (PAB; Sigma-Aldrich) or on *Pseudomonas* Isolation Agar (PIA; Difco). The plates were incubated at 37°C for 12 to 20 h. The following antibiotics were used in order to maintain the plasmid DNA in *E. coli*: ampicillin at 100 μg.ml^{-1} and gentamicin at 7 μg.ml^{-1}. To maintain the chromosomal insertions in *P. aeruginosa* DSM1707, gentamicin was used at a concentration of 50 μg.ml^{-1}. All antibiotics were purchased from Sigma-Aldrich.

Table 2.1: Bacterial strains, plasmids and primers used in this study

Strains, plasmids or primers	Relevant properties	Source or reference
Strains:		
E. coli DH5α	*recA endA1 hsd*R17 *sup*E4 *gyr*A96 *rel*A1 Δ(*lac*ZYA-*arg*F)U169 (φ80d*lac*ZΔM15)	Promega
P. aeruginosa DSM1707	Prototroph (PAO1)	[#]DSM
DSMap0	Alkaline protease null mutant of *P. aeruginosa* DSM1707	This study
Plasmids:		
pFASTBAC™	Gm^R, Bac-to-Bac™ expression plasmid	Gibco-BRL
pGEM® – T Easy	Amp^R, cloning vector for PCR products	Promega
pUC18	ColE1, φ80d*lac*Z, *lacI*, Amp^R, cloning vector	Stratagene
pGEMGent	pGEM® – T Easy containing PCR-amplified gentamicin resistance cassette	This study
pAPR	pUC18 containing cloned *aprA* and *aprI* genes	This study
pIG	pAPR containing a gentamicin resistance cassette cloned into the *aprA* and *aprI* genes	This study
*Primers:		
APR1	5' – GCGAGCTCGAGCTGTGTCAGTTTGGAC – 3'	This study
APR2	5' – CGCTCTAGAGAGGTCGATCACAACCTG – 3'	This study
GENT1	5' – CGGATATCCCTTCCAGAAAACCGAGG – 3'	This study
GENT2	5' – GCGCGCTCAGTCCTCCAGTTATGCTGTG – 3'	This study
CONF	5' – CGATGAATGAGCTGTCCAAGGC – 3'	This study
CONR	5' – CATGAGATCGAAGGCATGATCG – 3'	This study
pUC/M13 Forward	5' – GTTTCCCAGTCACGAC – 3'	Roche
pUC/M13 Reverse	5' – GTAAAACGACGGCCAGT – 3'	Roche

[#] DSM - Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

* Underlined are the restriction endonuclease sites for *Sac*I in APR1, *Xba*I in APR2, *Eco*RV in GENT1 and *Bss*HII in GENT2

2.2.2 Polymerase chain reaction (PCR)

2.2.2.1 Genomic DNA extraction

P. aeruginosa DSM1707 genomic DNA, to be used as template DNA in PCR reactions, was isolated using hexadecyltrimethylammonium bromide (CTAB) according to the procedure described by Jansen (1995). Briefly, the cells from 1.5 ml of an overnight culture were collected by centrifugation at 12 000 × g for 2 min and suspended in 567 µl of 1 × TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.4). The cells were lysed by the addition of SDS to a final concentration of 0.5% (v/v) and the proteins were digested by the addition of Proteinase K to a final concentration of 100 µg.ml^{-1} in a total volume of 600 µl. After incubation at 37°C for 1 h, 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution was added and incubation was continued at 65°C for 10 min. The CTAB-protein/polysaccharide complexes were extracted by the addition of an equal volume of a chloroform/isoamyl alcohol mixture (24:1). Following centrifugation at 12 000 × g for 5 min, the supernatant, containing the chromosomal DNA, was recovered and further purified by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Following centrifugation (12 000 × g, 5 min), the supernatant was recovered and the chromosomal DNA precipitated by the addition of 0.6 volume isopropanol. The precipitated chromosomal DNA was pelleted by means of centrifugation, rinsed with 70% ethanol and then vacuum-dried before being suspended in 20 µl UHQ water.

2.2.2.2 PCR amplification of the *P. aeruginosa aprA* and *aprI* genes

For amplification of the *aprA* gene together with the downstream *aprI* gene, primers APR1 (containing a *Sac*I site) and APR2 (containing a *Xba*I site) were used (Table 2.1). The PCR reaction mixtures (50 µl) contained 100 ng of template chromosomal DNA, 1.5 mM MgCl₂, 1 × polymerase buffer (50 mM KCl, 10 mM Tris·HCl (pH 9.0), 0.1% [v/v] Triton X-100), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 25 pmol of each primer, 5% (v/v) dimethyl sulfoxide (DMSO) and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). The PCR reaction mixtures were placed in a Perkin-Elmer GeneAmp 2400 thermocycler using the following temperature profile: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min and

elongation at 72°C for 3 min. Following the last cycle, a final elongation step was performed at 72°C for 5 min. For control purposes, a reaction mixture containing all reagents except template DNA was included. Aliquots of the various reaction mixtures were subsequently analyzed by agarose gel electrophoresis as described in Section 2.2.3.

2.2.2.3 PCR amplification of a gentamicin resistance cassette

The gentamicin resistance cassette was obtained by PCR amplification using pFASTBAC™ plasmid DNA (Gibco-BRL) as template and oligonucleotide primers GENT1 (containing an *Eco*RI site) and GENT2 (containing a *Bss*HII site) (Table 2.1). The PCR reaction mixtures (50 μl) contained 10 ng of template DNA, 1.5 mM MgCl₂, 1 × polymerase buffer (50 mM KCl, 10 mM Tris·HCl (pH 9.0), 0.1% [v/v] Triton X-100), 0.2 mM of each dNTP, 25 pmol of each primer and 1 U of *Taq* DNA polymerase (Promega). The PCR reaction mixtures were placed in a Perkin-Elmer GeneAmp 2400 thermocycler using the following temperature profile: denaturation at 95°C for 3 min, followed by 10 cycles of denaturation at 95°C for 30 s, annealing at 40°C for 40 s and elongation at 72°C for 1 min. The cycle profile was then adjusted to consist of the same denaturation and elongation conditions as those described above, but annealing was allowed to occur at 61°C for 1 min and the number of cycles was 15. Following the last cycle, a final elongation step was performed at 72°C for 5 min. For control purposes, a reaction mixture containing all reagents except template DNA was included. Aliquots of the PCR reaction mixtures were subsequently analyzed by agarose gel electrophoresis as described in Section 2.2.3.

2.2.3 Agarose gel electrophoresis

Analysis of DNA was performed by means of agarose gel electrophoresis (Sambrook *et al.*, 1989). For this purpose, horizontal 1% (w/v) agarose slab gels were cast and electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris·HCl, 2 mM EDTA, 20 mM NaOAc acid, pH 8.5). To allow visualization of the DNA on a UV transilluminator, the gels were supplemented with 0.5 μg.ml⁻¹ ethidium bromide. The DNA fragments were sized according to their migration in the gel as compared to that of standard DNA molecular markers, namely phage lambda DNA digested with *Bam*HI and/or *Hind*III (Roche) or a 100 bp marker (Promega).

2.2.4 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using a silica suspension as described by Boyle and Lew (1995). Briefly, the DNA band of interest was excised from the agarose gel and mixed with 400 µl of a 6 M NaI solution. The agarose was dissolved by incubation at 55°C after which 10 µl of the silica suspension was added to the sample. The DNA was allowed to bind to the silica by incubation on ice for 30 min. The DNA-silica complex was pelleted by centrifugation and washed four times with NEW wash buffer (50 mM NaCl, 10 mM Tris·HCl, 2.5 mM EDTA, 50% [v/v] ethanol). The DNA was eluted from the silica at 55°C for 10 min in a volume of 10 µl with UHQ water. An aliquot of the eluate was electrophoresed on a 1% (w/v) agarose gel in order to estimate both the efficiency of the purification procedure as well as the DNA concentration.

2.2.5 Restriction endonuclease digestion

All restriction enzyme digestions were performed in sterile Eppendorf tubes in small reaction volumes (10-20 µl). The reactions contained the appropriate concentration of salt by using the 10 × buffer supplied by the manufacturer of the specific enzyme, and 5-10 U of enzyme per µg of plasmid DNA. The reactions were typically incubated for 1-1.5 h in a temperature-regulated water bath at 37°C. When digestions entailed the use of two enzymes requiring different salt concentrations for optimal activity, the enzyme requiring a lower salt concentration was used first after which the salt concentration was adjusted and the second enzyme added. All restriction enzymes were supplied by Roche or Promega. The digestion products were analyzed by gel electrophoresis in 1% or 2% (w/v) agarose gels as described in Section 2.2.3.

2.2.6 Construction of plasmid pGEMGent

The pGEM® – T Easy vector system (Promega) was used to clone the PCR-amplified and gel-purified gentamicin resistance cassette. Ligation of the purified amplicon and the linear pGEM® – T Easy vector was performed overnight (16 h) at 16°C in a total volume of 10 µl. The reaction mixture contained 5 µl of a 2 × DNA ligase buffer (60 mM Tris·HCl, 20 mM MgCl$_2$, 20 mM DTT, 1 mM ATP, 10% [w/v] PEG 6000, pH 7.8), 50 ng of pGEM® – T Easy

vector, approximately 150 ng insert DNA, 1 μl of T4 DNA ligase (3 U.μl⁻¹; Promega) and
UHQ water. The vector to insert ratio was typically 1:3.

2.2.7 Construction of plasmid pAPR

The amplicon comprising both the alkaline protease (*aprA*) and protease inhibitor (*aprI*)
genes was cloned into pUC18 vector DNA (Stratagene). The amplicon and pUC18 plasmid
DNA were enzymatically cleaved with both *Sac*I and *Xba*I after which the appropriate DNA
fragments were purified from the agarose gel and then ligated overnight (16 h) at 16°C. The
reaction mixture contained 1 μl of 10 × ligase buffer (300 mM Tris-HCl, 100 mM MgCl$_2$, 100
mM DTT, 10 mM ATP, pH 7.8), 1 U of T4 DNA ligase (1-3 U.μl⁻¹; Promega) and UHQ
water to a final volume of 10 μl. The ratio of vector to insert was typically in excess of 1:3.

2.2.8 Construction of the allelic exchange vector pIG

The cloning strategy used in the construction of the allelic exchange vector is shown in Fig.
2.1. The pAPR plasmid DNA was digested with *Eco*RV and *Bss*HII after which the digested
pAPR vector DNA fragment was excised from the agarose gel, purified using a silica
suspension and used in the subsequent cloning steps. The gentamicin resistance cassette was
recovered from pGEMGent by digestion with *Bss*HII, followed by partial restriction with
*Eco*RV at 37°C for 20 min. The DNA fragment was gel-purified and ligated into the digested
pAPR vector DNA, yielding the allelic exchange vector pIG. Insertion of the gentamicin
cassette into the *aprA* and *aprI* genes of pAPR resulted in 316 bp of upstream and 366 bp of
downstream *P. aeruginosa* DNA flanking the insertion.

2.2.9 Preparation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were prepared and transformed according to the procedure
described Chung and Miller (1988). A single colony of a freshly streaked culture of *E. coli*
DH5α was inoculated into 20 ml double-strength LB-broth (1% [w/v] yeast extract, 2% [w/v]
tryptone, 2% [w/v] NaCl, pH 7.4) and cultured overnight at 37°C with agitation at 250 rpm.
Following incubation, 1 ml of the overnight culture was inoculated into 100 ml sterile pre-
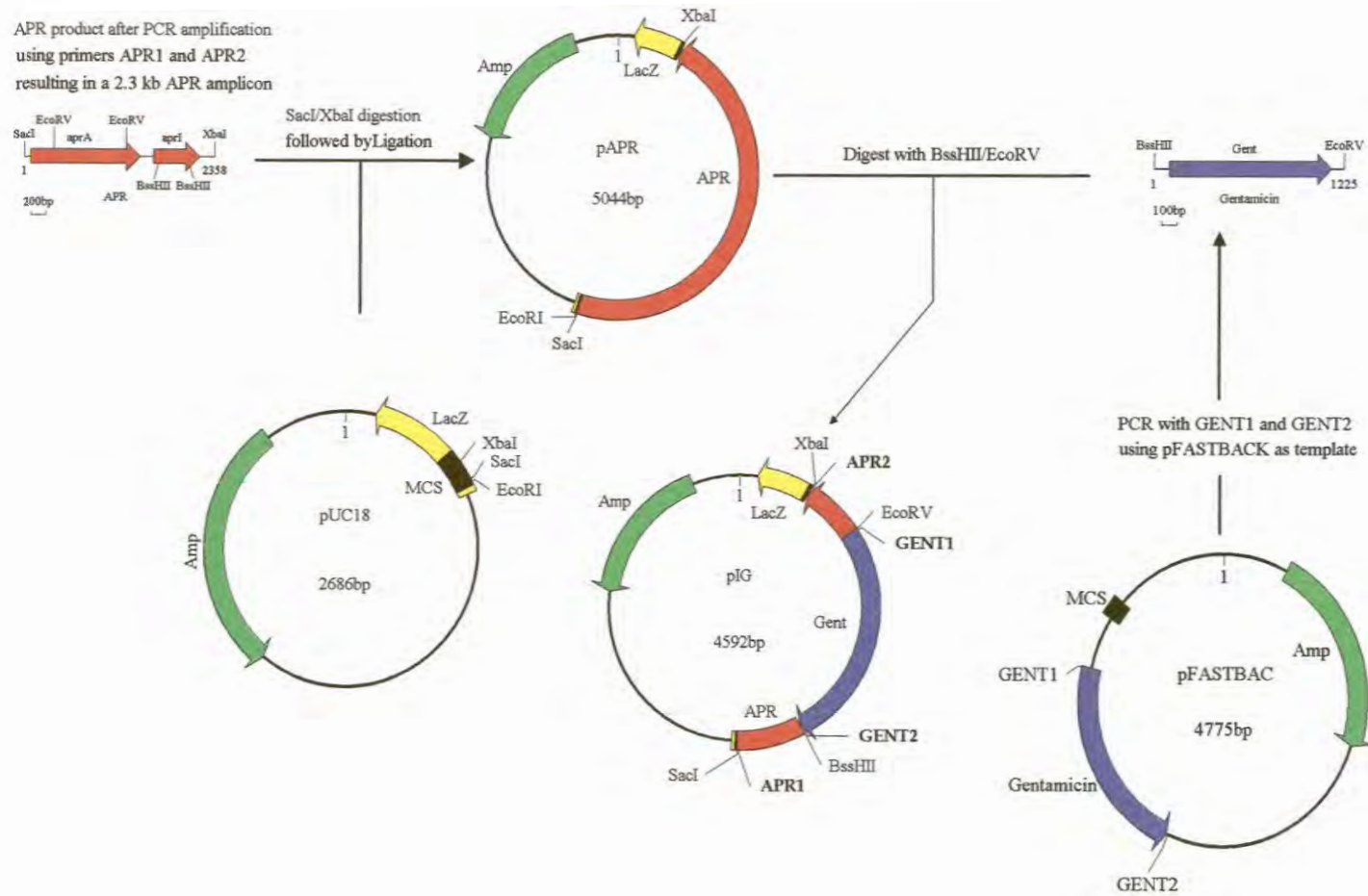
Fig. 2.1 Diagrammatic representation of the construction of the allelic exchange vector pIG as described in Materials and Methods Section 2.2.8.

warmed (37°C) double-strength LB-broth and incubated at 37°C with shaking to mid-exponential phase (OD_{600} of 0.3 to 0.4). The cells from 60 ml of the culture were pelleted in polypropolene tubes by centrifugation at $4000 \times g$ for 10 min at 4°C in a Sorval SS-34 rotor. The supernatant was decanted and the pellet suspended in 3 ml of TSS (93 ml LB-broth, 0.1 M $MgCl_2$, 10% [w/v] PEG 6000, 5% [v/v] DMSO, pH 6.5 – 6.8). Following incubation on ice for 15 min, the cells were aliquoted and stored at -70°C until needed.

2.2.10   Transformation of competent *E. coli* DH5α cells

The prepared competent *E. coli* DH5α cells were transformed by mixing the competent cells (100 μl) and the ligation mixture (5 μl) in a sterile glass transformation tube. The cells were maintained on ice for 1 h after which 500 μl pre-warmed (37°C) LB-broth was added and the transformation mixtures were incubated at 37°C for 2 h with agitation. The cells were plated in aliquots of 100 to 200 μl onto LB-agar plates supplemented with the appropriate antibiotic. Where applicable, recombinant transformants were selected by blue/white colour selection based on insertional inactivation of the *lacZ'* gene. For this purpose, the cells were spread with 40 μl of X-gal (2% [v/v] stock solution) and 10 μl IPTG (100 mM stock solution) over the surface of LB-agar plates supplemented with the appropriate antibiotic. The plates were incubated overnight at 37°C and investigated for the presence of recombinant transformants with a Gal⁻ phenotype.

2.2.11   Plasmid DNA extraction

Colonies were picked from the agar plates and inoculated into 20 ml of LB-broth supplemented with the appropriate antibiotic, and then incubated overnight at 37°C with agitation. Plasmid DNA was isolated from the cultures by the alkaline lysis method (Sambrook *et al.*, 1989), with the following modifications. After incubation, cells from 3 ml of each culture were collected in Eppendorf tubes by centrifugation at $8000 \times g$ for 2 min. The supernatant was discarded and the bacterial pellet suspended in 400 μl of Solution 1 (50 mM glucose, 25 mM Tris·HCl, 10 mM EDTA, pH 8.0). After incubation at room temperature for 10 min, 400 μl of Solution 2 (1% [w/v] SDS, 0.2 N NaOH) was added and the tubes were incubated on ice for 10 min. Following the addition of 300 μl of 7.5 M ammonium acetate (pH 7.6), the tubes were incubated on ice for 10 min and then centrifuged at $12\,000 \times g$ for 10

min. The plasmid DNA was precipitated from the supernatant by the addition of 650 μl isopropanol for 10 min at room temperature. The precipitated plasmid DNA was collected by centrifugation at 12 000 × g for 10 min and the supernatant discarded before the addition of 100 μl of 2 M ammonium acetate (pH 7.4). The tubes were incubated on ice for 10 min. Following centrifugation at 12 000 × g for 10 min, 110 μl of isopropanol was added to the recovered supernatant and the tubes were incubated at room temperature for 10 min. The precipitated DNA was collected, as above, and the pellets washed with 70% ethanol to remove residual salts. The DNA was vacuum-dried for 5 min and resuspended in UHQ water prior to analysis on a 1% (w/v) agarose gel.

2.2.12   Nucleic acid sequencing

Sequencing of the cloned insert DNA was performed using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin-Elmer), together with the universal pUC/M13 forward or reverse sequencing primers (Table 2.1). Each reaction mixture contained 80-100 ng purified template DNA, 12.5 pmol of sequencing primer, 2 μl Terminator Ready Reaction Mix and UHQ water in a final reaction volume of 5 μl. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp 2400 thermocycler using 25 of the following cycles: denaturation at 96°C for 30 s, primer annealing at 50°C for 15 s and elongation at 60°C for 4 min. The extension products were precipitated by the addition of 20 μl 60% ethanol and incubated at room temperature for 20 min in the dark. Following centrifugation at 12 000 × g for 30 min, the supernatant was carefully aspirated and the pellets washed with 25 μl 70% ethanol, vacuum-dried for 10 min and then stored at 4°C. Prior to electrophoresis, the purified extension products were suspended in 3.5 μl Blue dextran/EDTA loading buffer, denatured for 2 min at 90°C and loaded onto a Hitachi 3100 capillary array automated DNA sequencer.

2.2.13   Preparation of competent *P. aeruginosa* DSM1707 cells

Competent *P. aeruginosa* cells were prepared and transformed essentially by the procedures described by Olsen *et al.* (1982). A single colony of *P. aeruginosa* DSM1707, cultured overnight at 37°C on a TN-agar plate (0.5% [w/v] tryptone, 0.1% [w/v] dextrose, 0.25% [w/v] yeast extract, 0.4% [w/v] sodium nitrate, 1.2% [w/v] agar) (Olsen and Shipley, 1973),

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was inoculated into 30 ml TN-broth and grown at 37°C to an OD_{540} of between 0.3 and 0.5. The cells from 4 ml of the culture were collected in 2-ml Eppendorf tubes by centrifugation at $5000 \times g$ for 3 min in a Hettich microfuge. The pellet was suspended in 2 ml ice-cold filter-sterilized 0.15 M $MgCl_2$, incubated on ice for 5 min, pelleted as before and gently resuspended in 1 ml of the ice-cold $MgCl_2$ solution. After incubation on ice for 20 min, the cells were collected by centrifugation and the pellet resuspended in 100 µl of ice-cold $MgCl_2$.

2.2.14 Transformation of competent *P. aeruginosa* DSM1707 cells

The freshly prepared competent *P. aeruginosa* DSM1707 cells were transformed by the addition of 500 ng pIG plasmid DNA to 100 µl of the competent cells in a sterile glass transformation tube. Following incubation on ice for 1 h, the cells were incubated at 37°C for 3 min and chilled on ice for 5 min. After the addition of 500 µl of LB-broth, the transformation mixtures were incubated overnight at 37°C with shaking. An aliquot (50 µl) of the overnight culture was used to inoculate 20 ml LB-broth supplemented with 20 $µg.ml^{-1}$ gentamicin and the culture was then incubated for a further 24 h at 37°C. The procedure was repeated once more, except that the LB-broth was supplemented with gentamicin to a final concentration of 50 $µg.ml^{-1}$. Following incubation, the culture was serially diluted in sterile dH_2O and 150 µl of the serial dilutions (10^{-6} to 10^{-8}) were plated onto LB-agar plates supplemented with 50 $µg.ml^{-1}$ gentamicin. The agar plates were incubated overnight at 37°C and observed for the presence of gentamicin-resistant transformants.

2.2.15 Characterization of alkaline protease-deficient mutants of *P. aeruginosa* DSM1707

2.2.15.1 Proteolytic activity assays

The extracellular proteolytic activity of the parental *P. aeruginosa* DSM1707 and isogenic mutant strains was investigated by streaking of the respective cultures onto milk agar plates as well as by cup plate assays. Single colonies of the parental and putative mutant strains were selected and streaked onto milk agar plates (0.5% [w/v] yeast extract, 0.1% [w/v] KH_2PO_4, 0.05% [w/v] KCl, 0.02% [w/v] $MgSO_4 \cdot 7H_2O$, 0.01% [w/v] $CaCl_2 \cdot 2H_2O$, 15% [w/v] milk powder, 1% [w/v] glucose, 1.2% [w/v] agar, pH 7.4) (Smibert and Krieg, 1994). For cup plate assays, 100 µl of the cell-free culture supernatant was added to wells punched

into casein agar plates (1% [w/v] casein, 1.2% [w/v] agar). Following incubation of the agar plates at 37°C for 16-20 h, the cultures were investigated for a reduction or lack of hydrolysis zones by comparison to the parental control.

2.2.15.2 PCR assays

Mutant strains were analyzed for the presence of the gentamicin resistance cassette within the *aprA* and *aprI* genes by different PCR analyses. Oligonucleotide primers CONF and GENT1 were used to amplify a 2.071-kb hybrid amplicon consisting of the 5' end of the interrupted *aprA* gene and the 3' end of the gentamicin resistance cassette. Alternatively, oligonucleotide primers CONR and GENT2 were used to amplify a 1.7-kb hybrid amplicon consisting of the 5' end of the gentamicin resistance cassette and the 3' end of the interrupted *aprI* gene. The PCR reactions were performed as described in Section 2.2.2.2, except that primer annealing was performed at 55°C for 2 min in both instances. Oligonucleotide primers CONF and CONR were used to amplify the interrupted *aprAI* genes plus the gentamicin resistance cassette in mutant chromosomal DNA, resulting in a product of 2.581 kb. To confirm the presence of the gentamicin resistance cassette, oligonucleotide primers GENT1 and GENT2 were used to amplify the gentamicin resistance cassette of 1.2 kb present in the interrupted *aprAI* genes. The PCR reactions were performed as described previously in Sections 2.2.2.3. For all of the above analyses, UHQ water served as negative control, while chromosomal DNA extracted from the parental and isogenic mutant strains provided sample template DNA. The products were analyzed by gel electrophoresis on a 1% (w/v) agarose gel.

2.2.16   Proteolytic assays using azocasein

2.2.16.1 Extracellular protease assay

The extracellular proteolytic activity of the parental *P. aeruginosa* DSM1707 and isogenic mutant strains toward azocasein was determined according to the method of Smibert and Krieg (1994). The strains were cultured overnight at 30°C with shaking at 150 rpm in 200 ml TSB-broth (17% [w/v] pancreatic-digested casein, 3% [w/v] papaic digest of soyabean, 5% [w/v] NaCl, 2.5% [w/v] di-basic potassium phosphate, 2.5% [w/v] glucose, pH 7.3) (Oxoid). Following incubation, the cells from 40 ml of each culture were harvested by centrifugation at $4500 \times g$ for 8 min and the recovered supernatants were transferred to dialysis tubing

(Pierce, Snakeskin™, 10 000 MWCO). Dialysis was performed against 30 mM Tris buffer (pH 7.5) for 30 h (Jensen *et al.*, 1979). The dialysates were then concentrated by lyophilization and suspended in the same buffer at $1/10^{th}$ of the original volume. The activity assays were performed by incubating 80 µl of the dialysate with 80 µl of azocasein substrate (Sigma-Aldrich) at a final concentration of 2 mg.ml^{-1}, in 30 mM Tris buffer (pH 7.5). Following incubation at 30°C for 30 min, the reaction was terminated by the addition of 800 µl 8% (w/v) trichloroacetic acid (TCA). The tubes were briefly centrifuged and 400 µl 1 M NaOH was added to the recovered supernatants. After incubation at room temperature for 10 min, readings were taken at A_{420}. The reaction buffer without dialysate was used to zero the absorbency readings. All assays were performed in triplicate. Where required, the above activity assays were performed in the presence of the protease inhibitor, N-α-ρ-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma-Aldrich), at a final concentration of 1 mM.

2.2.16.2 Intracellular protease assay

Following overnight culturing of the parental DSM1707 and isogenic mutant strains in 200 ml TSB-broth, cells from 300 µl of each culture were harvested by centrifugation at $5\ 000 \times g$ for 3 min. The supernatants were collected and transferred to sterile Eppendorf tubes and assayed separately. The pelleted cells were washed in dH$_2$O and suspended in 300 µl 30 mM Tris buffer (pH 7.5). The cells were subsequently subjected to a freeze-thaw cell lysis procedure. Briefly, bacterial suspensions were snap-frozen in liquid nitrogen followed by incubation at 37°C until completely thawed. These cycles were completed five times after which the cells were sonicated by three 20-s pulses using a Cole-Palmer Series 4700 Ultrasonic Homogenizer. Both the supernatant and lysed cellular fractions were assayed for proteolytic activity using azocasein as substrate, as described above (Section 2.2.16.1).

2.2.17 Determination of bacterial growth

The parental *P. aeruginosa* DSM1707 and isogenic mutant strains were cultured overnight at 37°C in LB-broth supplemented with the appropriate antibiotics. The overnight cultures (10 ml) were diluted to an optical density at 540 nm of 0.05, and 2 ml of the diluted cultures were then used to inoculate LB-broth in 28-ml McCartney bottles. The bacterial growth was followed by measuring the optical density at 540 nm every 2 h for 16 h.

2.3 RESULTS

The primary aim of this part of the study was to generate an alkaline protease-deficient *P. aeruginosa* DSM1707 strain. Towards achieving this goal, the *aprA* and *aprI* genes on the *P. aeruginosa* genome were targeted for insertion mutagenesis. Whereas the *aprA* gene encodes the alkaline protease, the *aprI* gene encodes a protein that may play a role as an intracellular inhibitor or chaperone of alkaline protease (Duong *et al.*, 1992). An allelic exchange vector, pIG, was thus constructed that contained the wild-type *P. aeruginosa aprA* and *aprI* genes disrupted by the insertion of a cassette encoding gentamicin resistance. The constructed allelic exchange vector containing the null *aprAI*::Gent^R allele was subsequently used to generate mutant *P. aeruginosa* strains by homologous recombination via a double crossover event. The resultant mutant strains were then characterized with regards to their extracellular proteolytic activity using various different assays.

2.3.1 Construction of the allelic exchange plasmid pIG

2.3.1.1 Cloning of the *aprA* and *aprI* genes into pUC18

To obtain the *aprA* gene together with the downstream *aprI* gene, oligonucleotide primers were designed based on the published sequence of the *P. aeruginosa* PAO1 *apr* operon (Duong *et al.*, 1992). To allow for a sufficient amount of sequence similarity with the chromosomal DNA that would permit homologous recombination between the amplicon and chromosomal genes, the respective primers were designed to anneal 105 nt upstream of the *aprA* ORF and 154 nt downstream of the *aprI* ORF. Each of the primers were extended at their 5' ends by an additional eight nucleotides which incorporated unique restriction endonuclease recognition sites to facilitate subsequent cloning procedures.

By making use of isolated *P. aeruginosa* DSM1707 genomic DNA as template and oligonucleotide primers APR1 and APR2, PCR amplification was carried out using the conditions described under Materials and Methods (Section 2.2.2.2). A single amplicon of 2.35 kb was observed following agarose gel electrophoresis of the reaction mixture. This corresponded to the combined size of the targeted *aprA* (1.439 kb) and *aprI* (0.393 kb) genes together with an intergenic region of 0.249 kb, as well as 0.105 kb of *P. aeruginosa* DNA

upstream of the *aprA* gene and 0.154 kb of *P. aeruginosa* DNA downstream of the *aprI* gene. No amplification products were observed in the negative control in which template DNA was omitted (Fig. 2.2). The gel-purified amplicon was subsequently digested with both *Xba*I and *Sac*I, and cloned into similarly digested pUC18 plasmid DNA. Following transformation of competent *E. coli* DH5α cells, resultant recombinant transformants with a Gal⁻ phenotype were identified by colorimetric screening on X-gal-containing indicator plates. The isolated plasmid DNA was characterized by agarose gel electrophoresis and by restriction enzyme analysis. Digestion of recombinant plasmid DNA with both *Xba*I and *Sac*I resulted in two fragments of approximately 2.7 kb and 2.35 kb (Fig. 2.3). These corresponded with the expected size of the parental linearized plasmid (pUC18) and DNA insert (*aprAI*), respectively. A recombinant plasmid DNA was designated pAPR and used in further DNA manipulations.

2.3.1.2 Cloning of the gentamicin resistance cassette into pGEM® – T Easy

To obtain the gentamicin resistance cassette, PCR was performed by making use of primers GENT1 and GENT2 and plasmid pFastBac™ as template DNA. An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single band of approximately 1.2 kb was observed (Fig. 2.4). The amplicon was gel-purified and ligated into pGEM® – T Easy vector DNA. Following transformation of competent *E. coli* DH5α cells, colonies with a Gal⁻ phenotype were selected from LB-agar plates supplemented with ampicillin. These putative recombinant transformants were subsequently restreaked onto LB-agar plates supplemented with gentamicin and the plasmid DNA from gentamicin-resistant transformants was isolated. The plasmid DNA was analyzed for the presence of a gentamicin gene-specific insert by digestion with *Eco*RV and *Bss*HII (Fig. 2.5). Since the gentamicin cassette contains an *Eco*RV recognition site 220 nt from the 5' end, complete digestion of the recombinant plasmid resulted in excision of a 1.0 kb DNA fragment. A recombinant plasmid DNA was designated pGEMGent and used in further DNA manipulations.
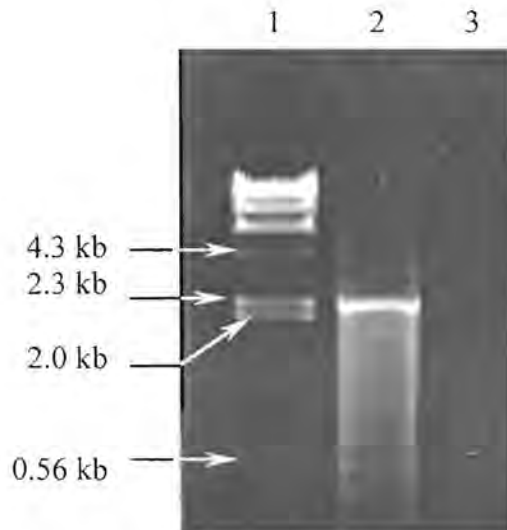
Fig. 2.2   Agarose gel electrophoretic analyses of the amplicon obtained by PCR amplification of the *aprA* and *aprI* genes using *P. aeruginosa* DSM1707 genomic DNA as template. Lane 1, DNA molecular weight marker; Lane 2, sample of the reaction mixture following the PCR; Lane 3, negative control reaction lacking template DNA. The sizes of the DNA molecular weight marker, phage lambda DNA restricted with *Hind*III, are indicated to the left of the figure.
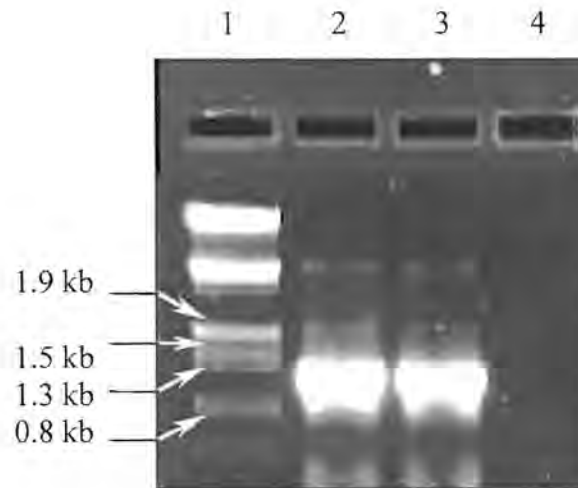

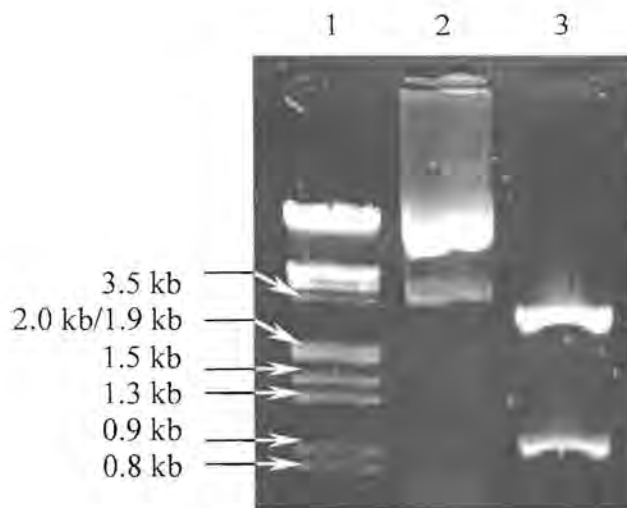Fig. 2.3   Agarose gel electrophoretic analysis of recombinant plasmid pAPR, constructed by cloning the *aprAI* genes into pUC18. Lane 1, DNA molecular weight marker; Lane 2, uncut plasmid pAPR; Lane 3, plasmid pAPR restricted with *Sac*I and *Xba*I. The sizes of the DNA molecular weight marker, phage lambda DNA restricted with *Hind*III, are indicated to the left of the figure.

Fig. 2.4   Agarose gel electrophoretic analysis of the amplicon obtained by PCR amplification of the gentamicin resistance cassette using plasmid pFASTBAC™ as template DNA. Lane 1, DNA molecular weight marker; Lanes 2 and 3, samples of the reaction mixtures following the PCR; Lane 4, negative control reaction lacking template DNA. The sizes of the DNA molecular weight marker, phage lambda DNA restricted with *Eco*RI and *Hind*III, are indicated to the left of the figure.

Fig. 2.5   Agarose gel electrophoretic analysis of recombinant plasmid pGEMGent, constructed by cloning of the gentamicin gene into the pGEM® – T Easy vector. Lane 1, DNA molecular weight marker; Lane 2, uncut plasmid pGEMGent; Lane 3, plasmid pGEMGent restricted with *Bss*HII and *Eco*RV. The sizes of the molecular weight marker, phage lambda DNA restricted with *Eco*RI and *Hind*III, are indicated to the left of the figure.

2.3.1.3 Nucleic acid sequencing of the cloned amplicons

To verify the integrity of the cloned *aprAI* genes as well as gentamicin resistance cassette, both the 5' and 3' terminal ends of each insert were characterized by automated sequencing using the universal pUC/M13 forward and reverse sequencing primers, as described in Section 2.2.12. Analysis of the obtained nucleotide sequences indicated no difference in the nucleotide sequences between the PCR-amplified and published sequences of the respective genes and furthermore confirmed that the full-length gentamicin gene was successfully cloned.

2.3.1.4 Construction of the allelic exchange vector pIG

The previously constructed pGEMGent and pAPR recombinant plasmids served as sources for the construction of the allelic exchange vector, pIG. Recombinant plasmid pAPR was digested with *Eco*RV, which cuts twice in the *aprA* ORF at nucleotide positions 261 and 1167, and *Bss*HII, which cuts once in the intergenic region at nucleotide position 1553 and once in the *aprI* ORF at nucleotide position 1937. Thus, digestion of pAPR with these enzymes resulted in four restriction DNA fragments corresponding in length to 3368, 906, 386 and 384 bp. The 3.3-kb recombinant vector DNA fragment was excised from the agarose gel, purified using a silica suspension and then used in subsequent ligation reactions. The full-length 1.2-kb gentamicin resistance cassette was recovered from pGEMGent by first digesting with *Bss*HII until completion, followed by a partial digest using *Eco*RV. The DNA fragment was gel-purified and ligated into the restricted pAPR vector. Following transformation of competent *E. coli* DH5α cells, plasmid DNA from gentamicin-resistant transformants were isolated and characterized by agarose gel electrophoresis and by restriction enzyme analysis. By digesting the recombinant plasmid DNA with both *Sac*I and *Xba*I, a 1.9 kb DNA fragment was excised (Fig. 2.6, lane 3). This corresponded with the size of the gentamicin resistance cassette together with 316 bp of upstream and 366 bp of downstream *P. aeruginosa* DNA flanking the insertion. Restriction of the recombinant plasmid DNA with both *Eco*RV and *Bss*HII resulted in restriction fragments of 1.2 and 1.0 kb, which corresponded in size to the full-length and truncated version of the gentamicin resistance cassette, respectively (Fig. 2.6, lane 4).
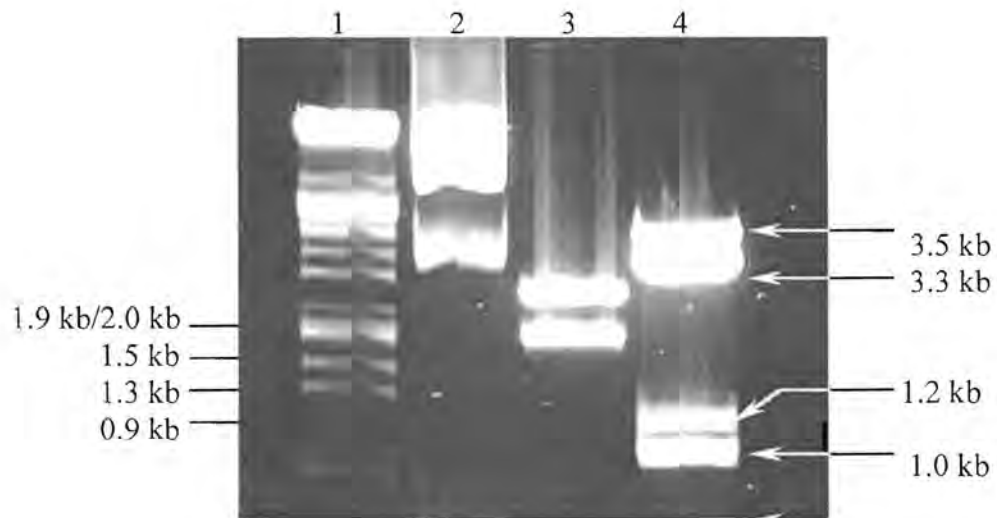
Fig. 2.6   Agarose gel electrophoretic analysis of recombinant plasmid pIG. Lane 1, DNA molecular weight marker; Lane 2, uncut plasmid pIG; Lane 3, plasmid pIG restricted with *Sac*I and *Xba*I; Lane 4, plasmid pIG restricted with *Eco*RV and *Bss*HII. The sizes of the molecular weight marker, phage lambda DNA restricted with *Eco*RI and *Hind*III, are displayed to the left of the figure.

2.3.2 Generation of an isogenic mutant of *P. aeruginosa* DSM1707 deficient in alkaline protease

Isogenic mutants of *P. aeruginosa* DSM1707 were constructed by introducing the allelic exchange plasmid pIG into the parental strain by transformation of competent *P. aeruginosa* cells, and selecting for subsequent double homologous recombination events between the *aprA* and *aprI* DNA flanking the gentamicin resistance cassette in the vector and the wild-type *aprA* and *aprI* loci on the genome. *P. aeruginosa* strains harbouring an integrated copy of the mutant allele were selected by culturing the transformed cells in LB-broth supplemented with increasing concentrations of gentamicin prior to plating onto selective medium. A number of gentamicin-resistant colonies were subsequently selected and screened for extracellular proteolytic activity.

2.3.3 Characterization of alkaline protease-deficient *P. aeruginosa* mutant strains

2.3.3.1 Identification of presumptive alkaline protease-deficient mutant strains

Since the alkaline protease of *P. aeruginosa* is secreted into the extracellular environment, it was to be expected that successful disruption of the *aprA* and *aprI* genes in the gentamicin-resistant strains would result in an absence of alkaline protease activity. The parental *P. aeruginosa* DSM1707 and isogenic mutant strains were therefore examined for extracellular proteolytic activity by first streaking the respective strains onto milk agar plates. After overnight incubation at 37°C, the agar plates were examined for zones of hydrolysis. By contrast to the parental DSM1707 strain, the mutant strains did not exhibit a detectable zone of proteolytic activity (Fig. 2.7). Similar results were obtained by cup plate assays in which 100 µl of the cell-free culture supernatants were pipetted into wells punched in casein agar plates (Fig. 2.8). One of the mutant strains displaying an apparent lack of extracellular proteolytic activity in both these assays was designated DSMap0 and characterized further on a genetic level.
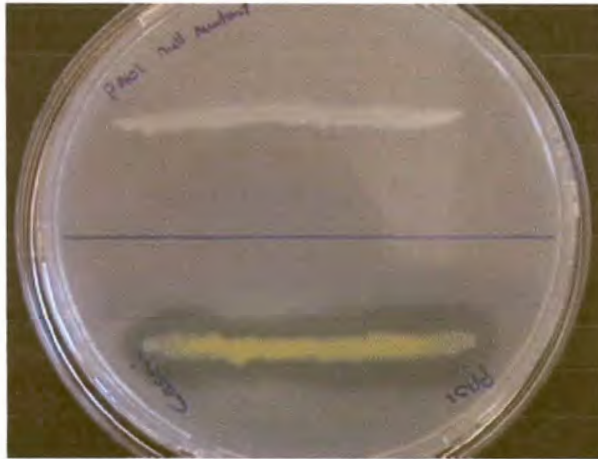
Fig. 2.7   Extracellular proteolytic activity of the parental *P. aeruginosa* DSM1707 and isogenic mutant DSMap0 strains. Cultures of the respective strains were streaked onto milk agar plates and incubated at 37°C. By contrast to the parental DSM1707 strain (bottom half of the petri dish), the isogenic DSMap0 strain (top half of the petri dish) did not display a detectable zone of hydrolysis.
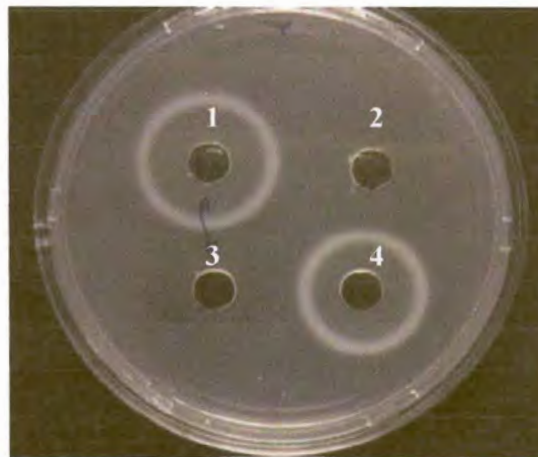

Fig. 2.8   Casein-based cup plate assay of the cell-free culture supernatants of parental *P. aeruginosa* DSM1707 and isogenic mutant DSMap0 strains. Whereas clear zones of extracellular proteolytic activity could be observed for the parental DSM1707 strain (wells 1 and 4), no similar activity could be observed in the case of the DSMap0 mutant strain (wells 2 and 3).

2.3.3.2 Presence of the gentamicin resistance cassette within alkaline protease-deficient mutant strains

Detection of the gentamicin resistance cassette within the disrupted *aprA* and *aprI* genes of the mutant DSMap0 strain was confirmed by different PCR analyses. In the first analysis, primers GENT1 and GENT2 were used to confirm the presence of the 1.2-kb gentamicin resistance cassette within the chromosome of the mutant DSMap0 strain. An expected 1.2-kb product was produced when DSMap0 chromosomal DNA was used as template, but no detectable product was generated when parental DSM1707 chromosomal DNA was used in the PCR reaction (Fig. 2.9, lanes 2 and 3, respectively).

Primers CONF and GENT1 as well as primers CONR and GENT2 were subsequently used to amplify a 2.071-kb and 1.784-kb hybrid product, respectively, only if the gentamicin resistance cassette was located within the disrupted *aprA* and *aprI* genes. The respective products were produced when DSMap0 chromosomal DNA was used as template, but these products were absent when parental DSM1707 chromosomal DNA was used as template in the respective PCR reactions (Fig. 2.9, lanes 4 through 7).

In the final analysis, primers CONF and CONR were used to amplify either a 3.032-kb *aprAI* product in the absence of the gentamicin resistance cassette, or a 2.581-kb product in the presence of the 1.2-kb cassette. As expected, a 2.581-kb product was produced when DSMap0 chromosomal DNA were used as template (Fig. 2.9, lane 8). Template DNA from parental DSM1707 generated the 3.032 kb product indicative of the absence of the gentamicin cassette within the *aprA* and *aprI* genes (Fig. 2.9, lane 9).

Cumulatively, the results of the above analyses indicated that the lack of extracellular proteolytic activity observed for the mutant DSMap0 strain might have been due to the successful disruption of the chromosomal *aprA* and *aprI* genes through insertion of a cassette encoding gentamicin resistance.
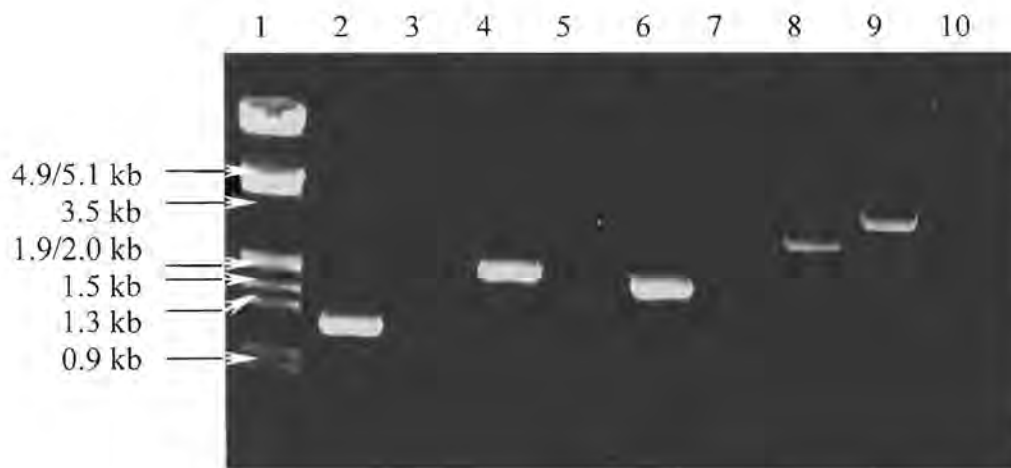
Fig. 2.9   Agarose gel electrophoretic analysis of the amplification products obtained following PCR analysis of *P. aeruginosa* DSM1707 and DSMap0 genomic DNA using primers GENT1 and GENT2 (lanes 2 and 3), CONF and GENT1 (lanes 4 and 5), CONR and GENT2 (lanes 6 and 7), and CONF and CONR (lanes 8 and 9). Lanes 2, 4, 6 and 8 represent genomic DNA from mutant strain DSMap0, where lanes 3, 5, 7 and 9 represent genomic DNA from parental strain DSM1707. A control PCR reaction containing no template DNA was included (lane 10). The sizes of the DNA molecular weight marker, phage lambda DNA restricted with *Eco*RI and *Hind*III (lane 1), are indicated to the left of the figure.

2.3.4 Azocasein hydrolysis assays

2.3.4.1 Extracellular proteolytic activity

Recently, Caballero *et al.* (2001) have reported that the protease inhibitor TLCK could inhibit *P. aeruginosa* elastase and protease IV, but not alkaline protease activity. Thus, to obtain quantitative data regarding the extracellular proteolytic activity of the parental DSM1707 and mutant DSMap0 strains, the extracellular proteolytic activity of dialysed cell-free culture supernatants of the respective cultures were assayed spectrophotometrically, in the presence or absence of TLCK, using azocasein as substrate. The results of the analyses are shown in Fig. 2.10.

The results indicated that the DSMap0 strain was severely impaired in its extracellular proteolytic activity. The DSMap0 strain displayed a 6-fold reduction in extracellular proteolytic activity compared to the parental DSM1707 strain. However, TLCK did neither inhibit nor substantially reduce the extracellular proteolytic activity as similar levels of proteolytic activity were observed in both the presence and absence of the inhibitor. As similar results were also obtained for the parental DSM1707 strain, the residual proteolytic activity observed for the DSMap0 strain can be attributed to the presence of trace amounts of other extracellular enzymes(s). An increase in the concentration of TLCK inhibitor to 3 mM, did not result in any further reduction of the observed residual extracellular proteolytic activity (data not shown). Consequently, TLCK was omitted from all subsequent assays.

2.3.4.2 Intracellular proteolytic activity

To investigate whether the lack of extracellular proteolytic activity observed for the DSMap0 strain may have been due to intracellular accumulation of the alkaline protease, the bacterial cells from overnight cultures of the parental DSM1707 and isogenic mutant DSMap0 strains were collected by centrifugation and suspended in a Tris buffer prior to lysis of the bacterial cells by means of successive freeze-thaw cycles and sonication. The proteolytic activity of both the cell-free culture supernatants and prepared cell extracts were subsequently determined using azocasein as substrate. The results that were obtained (Fig. 2.11) suggested that the alkaline protease did not accumulate inside the cells to any great extent. Enzymatic assays of the cell extract and culture supernatant of the parental DSM1707 strain indicated
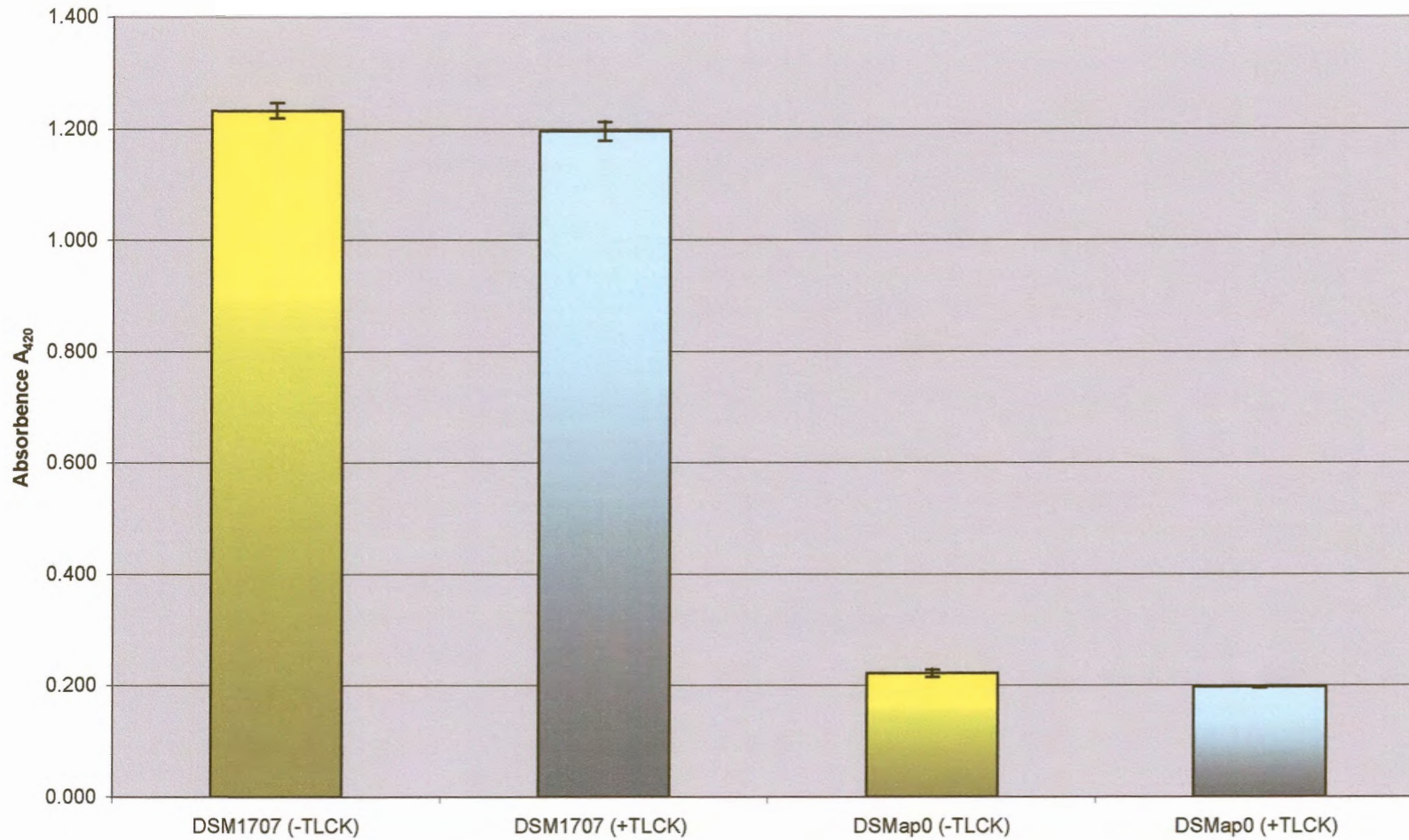
62

Fig. 2.10   Azocasein hydrolysis of the parental DSM1707 strain and mutant DSMap0 strain in the absence (-TLCK) or presence (+TLCK) of the protease inhinitor TLCK. Error bars denote one standard deviation of the mean.
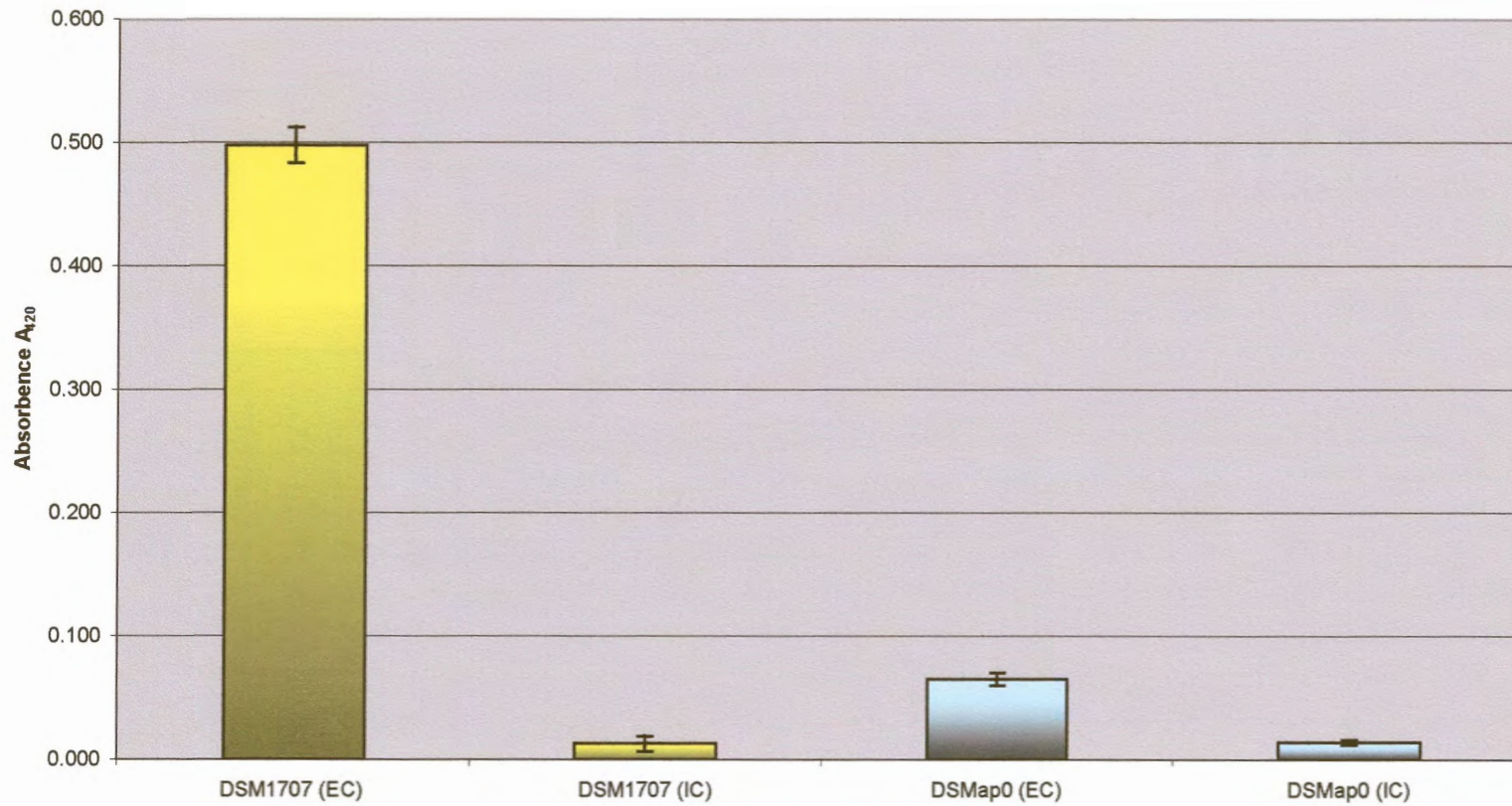
Fig. 2.11   Graph displaying the intracellular (IC) and extracellular (EC) proteolytic activity of the parental DSM1707 strain and the mutant DSMap0 strain. Error bars denote one standard deviation of the mean.

that the proteolytic activity was mainly detected in the culture supernatant, and almost no cell-associated activity was oserved. By contrast, almost no proteolytic activity was observed in either the culture supernatant or cell extract obtained from the mutant DSMap0 strain.

2.3.5 Growth curves of the parental DSM1707 and isogenic mutant DSMap0 strains

Various reports have noted that insertion mutagenesis may influence the growth properties of a particular mutant strain. Thus, the observed effect following mutagenesis may be due to growth impairment of the strain rather that inactivation of a specific gene. To exclude the possibility that the observed decrease in extracellular proteolytic activity of the mutant DSMap0 strain may have been the result of an impairment in its growth, the growth of the parental DSM1707 and isogenic DSMap0 mutant strains in LB-broth was followed by taking optical density readings every 2 h over a period of 16 h. The results (Fig. 2.12) indicated that the introduced mutation in the *aprA* and *aprI* genes of DSMap0 does not affect its growth, as the growth curves obtained for both the parental and isogenic mutant strains were near identical.

2.4 DISCUSSION

P. aeruginosa is capable of secreting several extracellular proteins (Filloux *et al.*, 1998; Kadurugamuwa and Beveridge, 1995; Lazdunski *et al.*, 1990; Liu, 1974) and consequently several distinct secretion pathways co-exist in this bacterium, each dedicated to the export of a different protein or a group of proteins. The extracellular alkaline protease enzyme, AprA, of *P. aeruginosa* is secreted by a dedicated type I secretion system of which the AprDEF proteins constitute the ABC transporter of the alkaline protease. Such ABC pathways are well documented (Binet *et al.*, 1997; Fath and Kolter, 1993) and they are usually specific for one polypeptide or a family of highly related proteins. In addition, the genes encoding the secretion proteins are in most cases clustered with the structural genes of the secreted proteins. This genetic organization of the *apr* operon therefore prompted an investigation into the possibility of exploiting this secretion system for the overexpression of extracellular alkaline protease in *P. aeruginosa*.
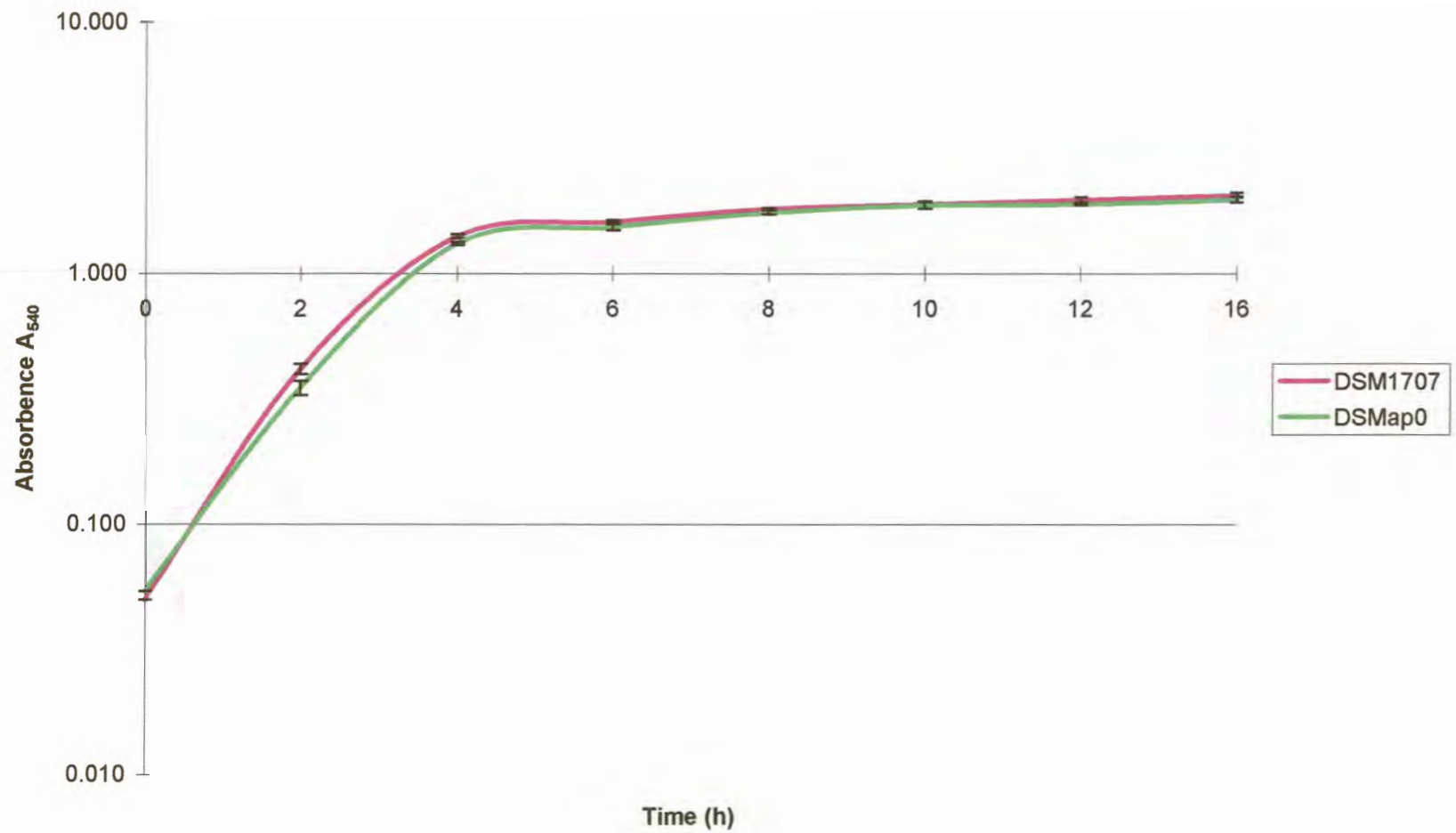
Fig. 2.12   Graph representing the comparitive growth rates of the parental DSM1707 and isogenic mutant DSMap0 strains. Error bars denote one standard deviation of the mean.

Since alkaline protease is naturally secreted by *P. aeruginosa*, the first step was to generate a mutant *P. aeruginosa* strain deficient in alkaline protease, but having no other genetic defects. Thus, the genes encoding the secretion proteins had to remain intact and only the structural gene for the alkaline protease had to be inactivated. The process of allelic exchange makes it possible to obtain a mutant in which the only genetic difference from the parent strain is the insertion of a selectable marker into the ORF of the particular gene of interest. Compared to alternative mutagenesis procedure such as random transposon insertion mutagenesis, mutations made by allelic exchange are targeted, making it therefore a more attractive method of mutagenesis (Horrocks *et al.*, 2002; Hinds *et al.*, 1999; Tran *et al.*, 1998). However, as with transposon mutations, it is possible to introduce polar mutations downstream of the insertion site. The gentamicin resistance cassette used to disrupt the *aprA* and *aprI* genes consists of the gene *aacC1*, which encodes the enzyme N-aminoglycoside acetyltransferase flanked by transcriptional and translational terminator signals (Luckow *et al.*, 1993). As a consequence of the cloning strategy used, the gentamicin resistance cassette would be integrated into the chromosomal DNA in the opposite transcriptional direction relative to that of the genes in the *apr* operon. The ORFs immediately downstream of the integration site are *aprDEF* whose products are required for secretion of the alkaline protease. Consequently, the inclusion of the 5' and 3' termination signals on the gentamicin resistance cassette was assumed to be sufficient to prevent promoter occlusion and polar mutations from occurring.

To enable the generation of an alkaline protease-deficient mutant strain, an allelic exchange vector was constructed by disrupting the *aprA* and *aprI* genes through insertion of a gentamicin resistance cassette. The allelic exchange vector was subsequently transformed into *P. aeruginosa* and presumptive mutant strains were selected following culturing in selective medium. Although the presumptive mutants did not produce zones of clearing on milk and casein agar plates, *P. aeruginosa* secretes various extracellular proteases, amongst other, elastase A, elastase B, and protease IV, in addition to alkaline protease (Kamath *et al.*, 1998; Kadurugamuwa and Beveridge, 1995). As alkaline protease does not possess unique substrate specificity, it is therefore difficult to conclude that the mutant is specifically affected in this protease. However, several lines of evidence seem to suggest that the isogenic mutant DSMap0 strain is deficient only in alkaline protease. In the first instance, four different but complimentary PCR analyses demonstrated that the chromosomal DNA between mutant and parental strains differed only by the *aprAI* loci disrupted by a cassette encoding

gentamicin resistance (Fig. 2.9). Analysis of the dialysed cell-free culture supernatants of the mutant and parental strains grown in TSB-broth indicated a 6-fold reduction in azocasein substrate hydrolysis by the mutant DSMap0 strain compared to the parental DSM1707 strain (Fig. 2.10) and no intracellular accumulation of the protease was detected for DSMap0 (Fig. 2.11). Also, the DSMap0 mutant exhibited no growth deficiency when compared to the parental DSM1707 strain in broth culture (Fig. 2.12).

Some residual extracellular proteolytic activity by strain DSMap0 was detected when azocasein was used as the substrate. A protease inhibitor, TLCK, was reported to specifically inhibit the extracellular *P. aeruginosa* elastase and protease IV enzymes, but not alkaline protease (Caballero *et al.*, 2001). However, inclusion of this inhibitor in the reaction buffers did not result in complete inhibition of the extracellular proteolytic activity. In the assay described by Caballero *et al.* (2001), poly-L-lysine was used as a substrate, but elastase A and B do not have an affinity for this substrate. In this study, azocasein, which is commonly used as a substrate for proteases (Smibert and Krieg, 1994), was used for assaying of the extracellular proteolytic activity. Moreover, *P. aeruginosa* secretes multiple proteases. In addition to the proteases mentioned above, two additional enzymes have also been reported, namely PS-1, which is a lysine-specific serine protease (Elliot and Cohen, 1986), and LasD, which has been suggested to function as a staphylolytic protease (Park and Galloway, 1995). Thus, the residual proteolytic activity observed for DSMap0 could have been due to trace amounts of these or other proteolytic enzyme(s). Nevertheless, the residual activity was thought not to represent a significant problem as the difference in extracellular proteolytic activity displayed by the *P. aeruginosa* parental DSM1707 and mutant DSMap0 strains could easily be distinguished.

The alkaline protease-deficient *P. aeruginosa* DSMap0 strain, of which the construction has been detailed in this Chapter, was subsequently used in studies aimed at increasing the level of extracellular expression of alkaline protease. The details of these investigations are given in the following Chapter (Chapter 3).