CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Pseudomonas aeruginosa is a motile, rod-shaped Gram-negative bacterium that is found in a variety of environments. In addition to causing disease in insects, plants and animals, it is also an important opportunistic pathogen of humans, causing serious infections in immuno-compromised patients such as those with cancer or AIDS, as well as patients suffering from cystic fibrosis and severe burns (Van Delden and Iglewski, 1998). The success of *P. aeruginosa* to grow and cause infections in these diverse environments is attributed to its broad metabolic versatility and its many cell-associated and secreted virulence factors (Van Delden and Iglewski, 1998). In the vast majority of ecological niches, *P. aeruginosa* can grow in association with surfaces (Costerton *et al.*, 1995). Such surface-associated growth leads to the formation of biofilms that are defined as organized communities of cells embedded in an extracellular polysaccharide matrix attached to a biological or abiological surface (Costerton *et al.*, 1987, 1994; Palmer and White, 1997).

More often than not, biofilms are unwanted and may result in various problems. These may include dental plaque, medical implant-associated infections and corrosion of pipes in the oil industry (Palmer and White, 1997). In addition, biofilms may harbor pathogens in drinking water distribution systems, and food spoilage and pathogenic bacteria on food contact surfaces (Czhechowski, 1990; Matilla *et al.*, 1990). By contrast, some biofilms may also be beneficial to humans, e.g. in sewage treatment plants, biofilms are needed for the efficient degradation of xenobiotics (Wolfaardt *et al.*, 1994), while biofilms of lactic acid bacteria form part of the normal indigenous microbiota in humans and their maintenance is essential in the prevention of disease (Gottenbos *et al.*, 1999). Given the medical and economic consequences of biofilm formation, it is important to understand the formation of these complex communities in order to develop strategies whereby their formation can be prevented.

Previous studies have indicated that biofilm cells differ significantly from their planktonic counterparts in terms of their physiology (Anwar *et al.*, 1990). More recent studies regarding gene expression have indicated the differential expression of numerous genes in biofilm cells when compared to their planktonic counterparts (Prigent-Combaret *et al.*, 1999; Steyn *et al.*, 2001; Sauer and Camper, 2001; Whiteley *et al.*, 2001). One of the major limitations in investigating the regulation of gene expression during growth on solid surfaces is the lack of appropriate methods to properly monitor changes of gene expression *in situ*. However, these limitations can be overcome by making use of reporter gene technology. Consequently, relevant literature regarding the initial stages of biofilm formation, regulation of gene expression and reporter molecules whereby gene expression can be monitored in biofilm-growing bacteria, will be reviewed in this chapter.

1.2 BACTERIAL BIOFILMS

In most natural and artificial habitats, bacteria have a propensity to adhere to wet surfaces (Costerton *et al.*, 1987; 1994). Bacterial colonization of both biotic and abiotic surfaces is regarded as a fundamental aspect of their pathogenesis and ecology. Survival of environmental bacteria under starvation conditions, and of bacterial pathogens outside of their host has led to the development of two main bacterial survival strategies: entry into a dormant state, including spores and stationary phase cells (Grossman, 1995; Givskov *et al.*, 1994; Klotz and Hutcheson, 1992; Kolter *et al.*, 1993; Sarniguet *et al.*, 1995) or development of sessile communities in close association with surfaces, i.e. biofilms (Costerton *et al.*, 1994; O'Toole *et al.*, 2000b). Despite general agreement that biofilm bacteria are phenotypically different from their planktonic counterparts, the molecular mechanisms implicated in the biofilm formation process is still under intense investigation.

1.2.1 Formation of biofilms

The formation of a well-developed biofilm (Fig. 1.1) is believed to occur in a sequential process of transport of microorganisms; initial attachment; formation of microcolonies; and formation of well-developed biofilms (Marshall, 1985; Van Loosdrecht *et al.*, 1990). Prior to surface

colonization, a preconditioning film, composed of proteins, glycoproteins and organic nutrients, is believed to be formed on the attachment surface upon its immersion in liquid (Marshall *et al.*, 1971). Bacteria may be transported to the attachment surface through Brownian motion, sedimentation, liquid flow or active bacterial movement (Quirynen *et al.*, 2000). Initial attachment of bacteria to the preconditioned surfaces is then facilitated by Van der Waals forces, electrostatic interactions and specific interactions, or by a combination of these, depending on the proximity of the organisms to the attachment surface (Zottola and Sasahara, 1994; Razatos *et al.*, 1998). Initial attachment is followed by a phase during which production of bacterial exopolysaccharides (EPS) results in a more stable attachment by forming organic bridges between the cells and the substratum (Notermans *et al.*, 1991). Subsequent growth and multiplication of firmly attached primary colonizing organisms lead to the formation of microcolonies. Cells which are loosely attached may detach and these cells together with offspring of other biofilm cells may recolonize previously uncolonized surfaces (Korber *et al.*, 1989), extending the spatial boundaries of the biofilm. The well-developed biofilm is finally characterized by mushroom- or pillar-like structures surrounded by EPS and interspersed with fluid-filled channels (Kuchma and O'Toole, 2000). However, even established biofilms display dynamic behaviour, with periodic detachment and growth in new areas (Heydorn *et al.*, 2002). These dynamics may also be in response to environmental conditions (Nielsen *et al.*, 2000; Tolker-Nielsen *et al.*, 2000; Wolfaardt *et al.*, 1994).

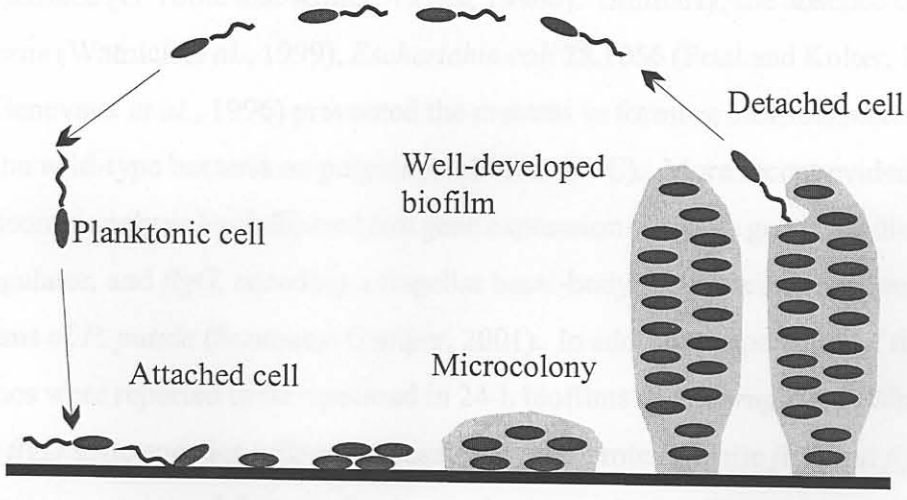

Fig. 1.1 Schematic presentation of the steps in biofilm formation (Modified from O'Toole *et al.*, 2000b).

1.2.2 Structural components required for initial attachment

Less is known about the cascade of events following adhesion than about the adhesion process itself. Of the processes leading to a well-developed biofilm, bacterial structural components required for initial attachment have been best characterized. These include flagella and pili, but membrane proteins and extracellular polysaccharides may also influence initial attachment of bacteria to surfaces.

1.2.2.1 Flagella, pili and adhesins

Although earlier studies have suggested that simple chemical models could account for the bacterial behaviour during the initial stages of attachment (Marshall *et al.*, 1971; McEldowney and Fletcher, 1986), subsequent studies, mainly through transposon mutagenesis, have shown that structural components such as flagella, pili and adhesins, play a critical role in bacterial interaction with the surface. The primary function of flagella in biofilm formation is assumed to be in transport and in initial cell-to-surface interactions. These assumptions have been based on observations that the absence of flagella impaired *Pseudomonas fluorescens* and *Pseudomonas putida* in colonization of potato and wheat roots (DeFlaun *et al.*, 1994; De Weger *et al.*, 1987) and reduced cellular adhesion of *P. aeruginosa* and *P. fluorescens* WCS365 to a polystyrene surface (O'Toole and Kolter, 1998a; 1998b). Similarly, the absence of flagella in *Vibrio cholerae* (Watnick *et al.*, 1999), *Escherichia coli* 2K1056 (Pratt and Kolter, 1998) and *E. coli* K-12 (Genevaux *et al.*, 1996) prevented the mutants in forming biofilms resembling those formed by the wild-type bacteria on polyvinylchloride (PVC). More recent evidence obtained through proteomic analysis has indicated that gene expression of *fleN,* a gene encoding a flagellar synthesis regulator, and *flgG,* encoding a flagellar basal-body rod protein were down-regulated in 6-h biofilms of *P. putida* (Sauer and Camper, 2001). In addition, expression of the *flgD, fliD* and *flgE* genes were reported to be repressed in 24-h biofilms of *P. aeruginosa* (Whiteley *et al.*, 2001). The *flgD* gene encodes a flagellar basal-body rod protein, while *fliD* and *flgE* encode a flagellar capping protein and flagellar hook protein, respectively. These observations seem to indicate that after initial cell-to-surface contact, the flagella become dispensable for further biofilm development.

Pili and pilus-associated adhesins have also been shown to be important for the adherence to and colonization of surfaces. Expression of *sfaA*, the gene encoding S-fimbrial adhesins, of a pathogenic strain of *E. coli* has been reported to be up-regulated upon attachment (Schmoll *et al.*, 1990). In *P. aeruginosa* PA14, type IV pili mutants were reduced in their ability to form microcolonies (O'Toole and Kolter, 1998a). In *E. coli*, attachment is reduced by mutations in the *csgA* gene, a biosynthetic curlin gene (Dorel *et al.*, 1999; Vidal *et al.*, 1998), and in the type I pili biosynthetic gene *fimH*, which encodes the mannose-specific adhesin (Pratt and Kolter, 1998). 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 pathway that is involved in assembly of fimbrial subunits such as P pili and type I pili, but not type IV pili (Soto and Hultgren, 1999).

Mutations in the mannose-sensitive hemaglutinin pilus of *V. cholerae* El Tor (Watnick *et al.*, 1999), in *ica*, the gene for the polysaccharide intercellular adhesin of *Staphylococcus epidermidis*, and in *atlE*, the gene for autolysin of *Staphylococcus aureus* (Heilmann *et al.*, 1997; Mack *et al.*, 1994; Rupp *et al.*, 1999) all have been reported to reduce adhesion to surfaces. The importance of pili in the initial stages of biofilm formation has been supported by proteomic analysis indicating the up-regulation of gene expression of *pilR, pilC* and *pilK* in 6-h old biofilms of *P. putida* (Sauer and Camper, 2001). Whereas *pilR* encodes a two-component response regulator, both *pilC* and *pilK* encode for proteins involved in the biogenesis of type IV pili. In well-developed biofilms of *P. aeruginosa*, expression of the *pilA* gene, which encodes the type IV pilin subunit, was repressed. This may indicate that although type IV pili are involved in the initial steps of biofilm formation, they may not be required for maintenance of the well-developed biofilm (Whiteley *et al.*, 2001).

1.2.2.2 Membrane proteins and extracellular polysaccharides

Membrane proteins have been reported to have a substantial influence on attachment and may also play a role in early biofilm development. Mutations in surface and membrane proteins, including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential glutathione-regulated K^+ efflux pump caused defects in attachment of *P. putida* to corn seeds

(Espinosa-Urgel *et al.*, 2000). The expression of genes encoding several membrane proteins has also been shown to be up-regulated in *P. putida* biofilm cells grown on silicone tubing. These included *nlpD,* the gene encoding an outer membrane lipoprotein, *potB*, the gene encoding a component of the polyamine ABC transporter, *mexA,* the gene for a resistance/nodulation/cell division/multidrug efflux pump, *ybaL*, the gene encoding a probable K^+ efflux transporter and *xcpS*, the gene encoding the general secretion pathway protein F. In *P. aeruginosa*, the expression of *tatA* and *tatB*, genes encoding translocation proteins, *tolA*, the gene encoding a product which affects lipopolysaccharide (LPS) structure, and the *omlA* gene, which encodes an outer membrane protein, were up-regulated in well-developed biofilm cells compared to their planktonic counterparts (Whiteley *et al.*, 2001).

Bacterial extracellular polysaccharides may also influence attachment and initial biofilm development, since these factors contribute to cell surface charge, which affects electrostatic interactions between bacteria and the substratum (Van Loosdrecht *et al.*, 1989). Adhesiveness of *Pseudomonas* species has been reported to be related to the presence and composition of polysaccharides (Williams and Fletcher, 1996). Substantially reduced attachment to biotic and abiotic surfaces was observed in O-polysaccharide-deficient *Pseudomonas* spp. (DeFlaun *et al.*, 1999; Dekkers *et al.*, 1998), while changes in *P. aeruginosa* lipopolysaccharide (LPS) resulted in an altered attachment behaviour (Makin and Beveridge, 1996). For example, a *P. aeruginosa* strain containing a mutant B-band LPS showed reduced attachment to hydrophilic surfaces and increased attachment to hydrophobic surfaces. *E. coli* W3110 strains with mutations in the LPS core biosynthetic genes *rfaG, rfaP* and *galU* also displayed reduced attachment to surfaces (Genevaux *et al.*, 1999a). The extracellular polysaccharide alginate appears to be required for formation of thick three-dimensional *P. aeruginosa* biofilms and has been shown to be the intercellular material of *P. aeruginosa* microcolonies (Nivens *et al.*, 2001).

1.2.3 Gene expression following attachment

Following attachment of bacteria to a surface, numerous changes in gene expression are initiated which enable the bacteria to adapt to the changing environment. In *P. aeruginosa*, expression of the *algC* and *algD* genes has been reported to be up-regulated following bacterial adhesion

(Davies *et al.*, 1993; Davies and Geesey, 1995; Hoyle *et al.*, 1993). Both the *algC* and *algD* genes are involved in the biosynthesis of the exopolysaccharide alginate. In addition, *algC* is also involved in LPS core biosynthesis. By contrast, expression of *mucC*, a negative regulator of alginate synthesis, was found to be up-regulated in biofilm cells of *P. putida* (Boucher *et al.*, 2000; Nunez *et al.*, 2000; Sauer and Camper, 2001), indicating that alginate expression is down-regulated in biofilm cells of *P. putida* following attachment. Following adhesion of *P. putida* to a surface, the expression of genes involved in carbon and energy metabolism and co-factor biosynthesis (e.g., *gltR*, *rbsK*, *leuS* and *thiE*) as well as *recB* and *ksgA* are repressed (Sauer and Camper, 2001). The *gltR* gene encodes a two-component response regulator, the *rbsK* gene encodes a ribokinase, *leuS* encodes a leucyl-tRNA synthase and *thiE* encodes a phosphate pyrophosphorylase. The *recB* and *ksgA* genes encode an exoribonuclease and a rRNA dimethyltransferase, respectively. Several Pf1 bacteriophage genes as well as genes involved in translation and metabolism were shown to be differentially expressed in *P. aeruginosa* biofilm cells when compared to planktonic cells (Whiteley *et al.*, 2001). Changes in gene expression that correlate with attachment to surfaces have also been described for antibiotic resistance in *P. aeruginosa* (Giwercman *et al.*, 1991), and for antibiotic production such as phenazine synthesis in *P. aureofaciens* (Wood *et al.*, 1997). After attachment of *E. coli* to a surface, up-regulation was observed for colanic acid exopolysaccharide production, a nickel high-affinity transport system (*nikA*), which is known to be up-regulated by anaerobiosis, an aminotripeptidase (*pepT*), induced by autoinducer PAI-1, and three genes induced by high osmolarity (*ompC, proU and wcaB*) (Prigent-Combaret *et al.*, 1999). The *ompC* gene encodes a porin protein, while the *proU* operon encodes a high-affinity glycine betaine transport system and *wcaB* encodes for a protein involved in the synthesis of colanic acid.

Recent studies have also linked quorum sensing and biofilm formation (Davies *et al.*, 1998). *P. aeruginosa* possesses two complete, semi-independent quorum sensing systems, designated *las* and *rhl*. These two quorum sensing systems are inter-related in that LasR activates the expression of the *rhlR* and *rhlI* genes (Latifi *et al.*, 1996; Pesci *et al.*, 1997; Whiteley *et al.*, 1999). Each quorum sensing system consists of a transcriptional activator, LasR (Gambello *et al.*, 1993) or RhlR (Brint and Ohman, 1995), and an autoinducer synthetase, LasI or RhlI. LasI directs the synthesis of the autoinducer PAI-1, N-(3-oxododecanoyl)-$_L$-homoserine lactone

(Pearson *et al.*, 1994), while RhlI directs the synthesis of the autoinducer PAI-2, N-butyryl-$_L$-homoserine lactone (Pearson *et al.*, 1995). Whereas a *P. aeruginosa lasI* mutant lacked the three-dimensional architecture of the wild-type biofilm and was susceptible to treatment with SDS, a *rhlI* mutant biofilm closely resembled the wild-type biofilm, suggesting that the *las* quorum sensing system, but not the *rhl* quorum sensing system, is important for *P. aeruginosa* biofilm development into three-dimensional structures (Davies *et al.*, 1998). In addition, reporter gene studies by De Kievit *et al.* (2001) indicated that up-regulation of the *lasI* gene corresponded with the metamorphosis of microcolonies to the three-dimensional architecture characteristic of well-developed biofilms. By contrast, *rhlI* expression fluctuated very little during biofilm development. However, only about 5 to 15% of the cells expressed *rhlI*, and these cells were concentrated around the base of the biofilm.

1.2.4 Regulation of gene expression in biofilms

From the above, it is evident that numerous genes and proteins have been identified that are involved in the initial attachment of bacteria to surfaces and the subsequent development of well-developed biofilms. However, in only a few cases has regulation of their expression been investigated. Proteins that have associated regulatory functions have been identified by transposon mutagenesis. These include the two-component sensor systems CpxA/CpxR and EnvZ/OmpR, which are involved in the regulation of curli synthesis in *E. coli* (Dorel *et al.*, 1999; Vidal *et al.*, 1998), and two proteins acting at a posttranslational level, DsbA in *E. coli* (Genevaux *et al.*, 1999b) and ClpP in *P. fluorescens* (O'Toole and Kolter, 1998b). DsbA is a disulfide isomerase that facilitates subunit folding and is required for pilus biogenesis (Jacob-Dubuisson *et al.*, 1994), while ClpP is a protease, suggesting that there is a protease-sensitive regulator involved in biofilm development, although the target of this protease is not known. The observation adds biofilm formation to the list of bacterial systems that are regulated by proteases (Damerau and St.John, 1993; De Crecy-Lagard *et al.*, 1999; Jenal and Fuchs, 1998; Lazazzera and Grossman, 1997; Msadek *et al.*, 1998). Expression of the *P. aeruginosa pilA* gene, which encodes the pilin subunit, has been reported to be regulated by the global regulator of carbon metabolism, Crc (O'Toole *et al.*, 2000a). The mechanism of regulation is still unclear, but is though to occur in response to nutritional signals (O'Toole *et al.*, 2000a). Analysis of the

regulation of *algD* gene expression, a gene up-regulated by attachment, indicates a possible role for two different sigma factors, although this regulation may not necessarily be ascribed to attachment *per se.* Boucher *et al.* (2000) indicated that two different pathways of conversion to mucoidy exist in *P. aeruginosa*; AlgU (AlgT, σ^E)-dependent *algD* expression and σ^{54} (RpoN)-dependent *algD* expression followed by alginate production. Earlier, Totten *et al.* (1990) and Mohr *et al.* (1990) reported that *algD* transcription was not regulated by RpoN in mucoid strains, whereas Kimbara and Chakrabarty (1989) reported that *algD* transcription was regulated by RpoN in non-mucoid strains. In general, σ^{54}-RNA polymerase transcribes genes with diverse roles in various organisms as well as diverse roles within a single organism (see Section 1.5.1).

The *fleS, fleR* and *fleQ* genes are also involved in the regulation of mucin adhesion and flagellar expression in *P. aeruginosa*. Products of *fleS* and *fleR* are homologous to members of the two-component systems involved in transcription regulation of a number of genes with σ^{54}-dependent promoters. Promoter fusion experiments have indicated that the *fleSR* operon is regulated by σ^{54} and FleQ, a transcriptional regulator (Arora *et al.*, 1997). The involvement of σ^{54} in the synthesis of flagella, pili and adhesins, supports the hypothesis that σ^{54} may be involved in the up-expression of biofilm-specific regulatory elements.

Earlier, Martin *et al.* (1994) have indicated that the *algR*, *algD* and *algU* genes are under control of *algU*. The *algR* gene encodes a regulatory protein, *algD* encodes a protein involved in the biosynthesis of alginate while *algU* encodes a sigma factor. Garret *et al.* (1999) showed by mutational analysis that the alternative sigma factor σ^{22} (AlgT / AlgU) modulates gene expression which leads to expression of the alginate operon, and it appears to inhibit flagellum synthesis and motility. This σ^{22}-mediated inhibition occurs through transcriptional control of *fliC,* a flagellar filament protein, most probably by controlling the expression of a negative effector of flagellum synthesis (Garret *et al.*, 1999). It is still unclear which quorum sensing-controlled genes are important for biofilm maturation. In addition, both quorum sensing systems in *P. aeruginosa* are involved in the regulation of twitching motility, a flagellum-independent mode of surface translocation which requires functional type IV pili (Glessner *et al.*, 1999). While the *rhl* system was shown to affect pilin export and assembly, it was proposed that the *las* quorum sensing system is required for maintaining optimal spacing between cells which migrate together in rafts.

1.3 GENE EXPRESSION

1.3.1 Importance of sigma factors

The term gene expression refers to the entire process whereby the information encoded in a particular gene is decoded into a particular protein (Lodish *et al.*, 1995). Although it being a complex process involving many different steps, transcription initiation through promoter clearance and release from the RNA polymerase is the most important control point in determining whether or not most genes are expressed (Ishihama, 1988; Lodish *et al.*, 1995; Reznikoff *et al.*, 1985). However, before genes can be transcribed from specific DNA promoter sequences, the bacterial core RNA polymerase (with a subunit composition of $\alpha_2\beta\beta'$) must combine with a dissociable sigma subunit (σ) to form a RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma$). The σ factors are thus central to the function of the RNA polymerase holoenzyme (Burgess *et al.*, 1969; Helmann and Chamberlin, 1988; McClure, 1985) (Table 1.1). The reversible binding of various different alternative σ factors, which are expressed under specific conditions or may be sequestered by complementary anti-sigmas until needed (Arthur and Burgess, 1998), allows formation of different holoenzymes able to distinguish groups of promoters required for different cellular functions (Stragier and Losick, 1990; Gross *et al.*, 1992; Blattner *et al.*, 1997). In addition to double-stranded DNA promoter recognition and binding, σ proteins also play a role in promoter melting (DeHaseth and Helmann, 1995; Fenton *et al.*, 2000; Guo and Gralla, 1998; Marr and Roberts, 1997; Wösten, 1998), inhibition of non-specific transcription initiation and they are often targets for activators (Gribskov and Burgess, 1986; Gross *et al.*, 1998; Helmann and Chamberlin, 1988).

Based on structural and functional criteria, the different σ factors identified in bacterial cells can be grouped into two classes. One class contains σ^{54} (also referred to as σ^{N}) as the only member, while the remainder of σ factors belong to the σ^{70} class (Merrick *et al.*, 1987; Sasse-Dwight and Gralla, 1990; Kustu *et al.*, 1989; Thöny and Hennecke, 1989; Lonetto *et al.*, 1992). The σ^{70} class of sigma factors can be further classified into primary, e.g. σ^{70}, and alternative sigma factors, e.g. σ^{32}, σ^{28}, etc. (Lonetto *et al.*, 1992). Despite the lack of any significant sequence similarity and vast differences in their transcription mechanism, both classes of σ factors bind the same core

RNA polymerase, but result in holoenzymes with very different properties. In *E. coli*, both σ^{70} and σ^{54} are always present, but the synthesis of other minor σ^{70} class members is induced under certain stress conditions such as high osmolarity or low temperature (Jishage and Ishihama, 1997). Although σ^{54} and σ^{70} are unrelated by primary amino acid sequence, they both share a significant overall structural similarity as indicated by X-ray scattering data (Svergun *et al.*, 2000), as well as by protein footprinting studies (Traviglia *et al.*, 1999).

Table 1.1 Functions of *E. coli* core RNA polymerase subunits

Subunit	Gene	Function
α	*rpoA*	Association of β and β' subunits Promoter recognition, by binding to upstream (UP) elements in some promoters Indirectly involved in some promoters by providing a contact point for the activator protein
β	*rpoB*	Catalysis of RNA synthesis (initiation and elongation) Recognition of terminators Binding of substrate ribonucleotide 5'-triphosphates Binding of product RNA Stringent control Autogenous regulation of ββ' synthesis Binding of rifampicin and streptolidogen
β'	*rpoC*	Binding of template DNA Binding of sigma subunits

(Compiled from Ishihama, 1988; Record *et al.*, 1996)

1.3.2 Sigma 54-dependent gene transcription

1.3.2.1 Occurrence and function of σ^{54}

By contrast to σ^{70} factors which are involved in expression of most genes during exponential growth (Lonetto *et al.*, 1992), σ^{54} appears to play a role in many different apparently unrelated biological activities across the eubacteria (Merrick, 1993; Barrios *et al.*, 1999). Genes encoding σ^{54} have been identified in many proteobacteria (Merrick, 1993) as well as in *Bacillus subtilis*, where σ^{54} is involved in utilization of arginine and ornithine (Gardan *et al.*, 1997) and transport of fructose (Debarbouille *et al.*, 1991). Among the proteobacteria, the functions carried out by the products of σ^{54}-dependent transcription include utilization of various nitrogen and carbon sources, energy metabolism (Lenz *et al.*, 1997), RNA modification (Genschik *et al.*, 1998), chemotaxis, development, flagellation, electron transport, response to heat and phage shock (Weiner *et al.*, 1991), and expression of alternative σ factors (Barrios *et al.*, 1999; Kustu *et al.*, 1989; Studholme and Buck, 2000). In *P. aeruginosa*, σ^{54} is important for transport of dicarboxylic acid, catabolism of toluene and xylene, nitrogen fixation, flagella and pilus synthesis, glutamine prototrophy, non-pilus adhesin synthesis and osmoprotection (Ishimoto and Lory, 1989; Totten *et al.*, 1990; Ramphal *et al.*, 1991; Merrick, 1993; Sage *et al.*, 1997).

The lack of any obvious theme in the diverse functions carried out by products of σ^{54}-dependent transcription, points to σ^{54} being biologically important and advantageous. The ability of a single σ^{54} polypeptide to mediate transcriptional responses to such diverse physiological needs is due to the independent regulation of σ^{54}-RNA polymerase at a wide variety of genes by means of sequence-dependent enhancer proteins with promoter-specific binding sites (Collado-Vides *et al.*, 1991). Since initiation of transcription at a σ^{54}-dependent promoter requires the activity of the cognate activator protein, transcription can therefore be tightly regulated, with low levels of leaky expression (Wang and Gralla, 1998). It furthermore allows the capability to vary transcriptional efficiency at a given promoter over a wide range without the use of a separate repressor. Thus, genes can be either silent or highly expressed, depending on the physiological or environmental conditions (Buck *et al.*, 2000).

1.3.2.2 Domain structure of the σ^{54} protein and σ^{54}-dependent promoter sequences

Based on sequence alignments, mutation analysis and protein fragmentation studies, σ^{54} has been divided into three regions (Region I to III) by sequence conservation (Cannon *et al.*, 1995; Gallegos and Buck, 1999; Merrick *et al.*, 1987; Sasse-Dwight and Gralla, 1990; Wong *et al.*, 1994; Merrick, 1993) (Fig. 1.2). Region I performs two distinct functions in that it inhibits polymerase isomerization and transcription initiation in the absence of activator (Cannon *et al.*, 1999a; Syed and Gralla, 1998; Wang *et al.*, 1995), and it stimulates transcription initiation in response to activation (Sasse-Dwight and Gralla, 1990; Syed and Gralla, 1998; Casaz *et al.*, 1999; Gallegos and Buck, 2000). Region II has been implicated in DNA melting, in the transition from the closed to open complex (Wong and Gralla, 1992) and in assisting σ^{54} binding to homoduplex and heteroduplex DNA (Cannon *et al.*, 1999b). Region III is associated with the primary DNA-binding activity for recognition of double-stranded promoter DNA sequences (Merrick and Chambers, 1992; Gallegos and Buck, 1999). The primary DNA-binding sequences include a DNA cross-linking region (Cannon *et al.*, 1994), a helix-turn-helix motif (Merrick and Chambers, 1992) and an RpoN box (Taylor *et al.*, 1996) near the C-terminus. Region III also appears to play a role in maintaining the closed promoter complex in a transcriptionally silent state and in generating polymerase isomerization upon activation (Chaney and Buck, 1999). By contrast to Regions I and III, Region II is variable and it appears that none of its activities are essential (Buck *et al.*, 2000).

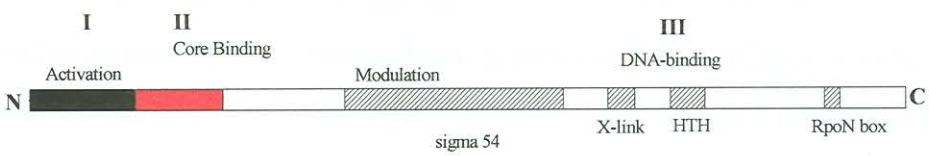

Fig. 1.2 Domain organization of the σ^{54} protein (Modified from Buck *et al.*, 2000). Whereas Region I is required for activator responsiveness, Region II has been implicated in core binding functions. DNA-binding functions and associated motifs (DNA cross-linking [X-link] region, helix-turn-helix [HTH] motif and RpoN box) reside in the Region III.

The σ^{54}-RNA polymerase recognizes promoter sequences with conserved GG and GC elements located -24 and -12 nucleotides upstream from the transcriptional start site (Thöny and Hennecke, 1989; Cases and De Lorenzo, 2001; Barrios *et al.*, 1999). However, several exceptions do exist, e.g. the σ^{54} promoters of the *nifH* gene of *Rhizobium leguminosarum* biovar *viciae* and the *glnB* gene of *Rhodospirillum rubrum* both contain GG-N_{10}-GA, while the σ^{54} promoters of the *E. coli glnH* gene and the *P. aeruginosa oprE* gene contain sequences of GG-N_{10}-TC and GG-N_{10}-CC, respectively (Yamano *et al.*, 1998; Nohno and Saito, 1987; Roelvink *et al.*, 1990; Johansson and Nordlund, 1996). Optimal spacer length in σ^{54}-dependent promoters are 10 nucleotides, while deletions of one or more nucleotides in the region between the -24/-12 elements abolish promoter function (Keseler and Kaiser, 1995; Buck, 1986; Mullin and Newton, 1989; Mullin and Newton, 1993).

1.3.2.3 Regulation of transcription from σ^{54}-dependent promoters

On its own, the σ^{54}-RNA polymerase forms stable, closed complexes with promoters and does not result in melting of the DNA (Sasse-Dwight and Gralla, 1988; Popham *et al.*, 1989). Basal, unactivated transcription from the closed complex is intrinsically very low, consistent with the lack of repressors associated with σ^{54}-dependent promoters (Collado-Vides *et al.*, 1991). Transcription initiation from these complexes is dependent on one or several activator proteins (Morett and Segovia, 1993) that bind to upstream enhancer sequences (UAS), because hydrolysis of ATP, a process catalyzed by the activator proteins (Wedel and Kustu, 1995), is required for the formation of an open complex. These activators, which include NR1/NtrC, FhlA and NifA, are also referred to as prokaryotic enhancer binding proteins (EBPs) (Cases and De Lorenzo, 2001). Whereas the binding sites of the NR1/NtrC activators occur in a number of positions between -90 and -160, binding sites for FhlA and NifA can, in addition to these positions, also occur at much greater distances upstream from the promoter region. For example, a functional FhlA site was recently found more than 700 bp upstream of the transcription start site and a NifA site has been mapped to a position 250 bp upstream of the transcription start site (Gralla and Collado-Vides, 1996). Once an activator that has bound to the UAS receives a specific signal, an upstream nucleoprotein complex is formed which loops round and contacts the σ^{54} factor of the holoenzyme prebound to the -12/-24 sequences of the promoter. ATP hydrolysis is then

channelled through an as yet unknown mechanism into DNA strand separation and subsequent transcription initiation (Vicente *et al.*, 1999). At some promoters, the looping-out of the intervening DNA is facilitated by integration host factor (IHF), a DNA bending protein (Hoover *et al.*, 1990).

Integration host factor (IHF) is a heterodimeric protein that bends DNA by approximately 160° and binds to the promoter region of many σ^{54}-dependent promoters (Wassem *et al.*, 2000) as well as some σ^{70}-dependent promoters (Goosen and Van de Putte, 1995; Bertoni *et al.*, 1998). Earlier reports have indicated that IHF may directly stimulate transcription from the Pe promoter of bacteriophage Mu (Goosen en Van de Putte, 1984; Krause and Higgins, 1986), the pL1 promoter of bacteriophage λ (Giladi *et al.*, 1990) and the P_G2 promoter of the *ilvGMEDA* operon of *E. coli* (Pagel and Hatfield, 1991), without the involvement of any other transcription factor. Although the mechanism whereby IHF may directly stimulate transcription is still unclear, it is thought that IHF binds to a site located just upstream from the promoter, and appears to function in enhancing closed-complex formation (Krause and Higgins, 1986; Giladi *et al.*, 1992). In most cases, however, IHF functions by facilitating the loop formation that is required to bring together the activator protein and the σ^{54}-holoenzyme (Carmona and Magasanik, 1996; Carmona *et al.*, 1997) (Fig. 1.3). The outcome of looping is an activator-dependent isomerization of the closed complex into an open one, which subsequently leads to initiation of transcription (Bertoni *et al.*, 1998). This mechanism is not observed for the σ^{70}-holoenzyme, where both highly stable closed complexes and looping are rarely used for activation (Gralla, 1996). However, IHF can also function as a repressor by occluding the binding of an activator by directly occupying its specific binding site (Bewley *et al.*, 1998). EBPs can also form stable complexes on non-specific DNA (Popham *et al.*, 1989; Wedel *et al.*, 1990) and non-sequence-specific DNA such as superhelical regions of a supercoiled plasmid that may then function as a generalized enhancer sequence (Brahms *et al.*, 1995; Revet *et al.*, 1995). In these cases, IHF generally acts as an inhibitor of activation from σ^{54} promoters from non-specific sites by generating a bend which prevents EBPs from making productive contact with the σ^{54}-RNA polymerase (Claverie-Martin and Magasanik, 1991; Austin *et al.*, 1994). Therefore, IHF may be bi-functional; enhancing transcription when the EBP binds to specific, appropriate sites and repressing transcription when the activator binds to non-specific, inappropriate sites.

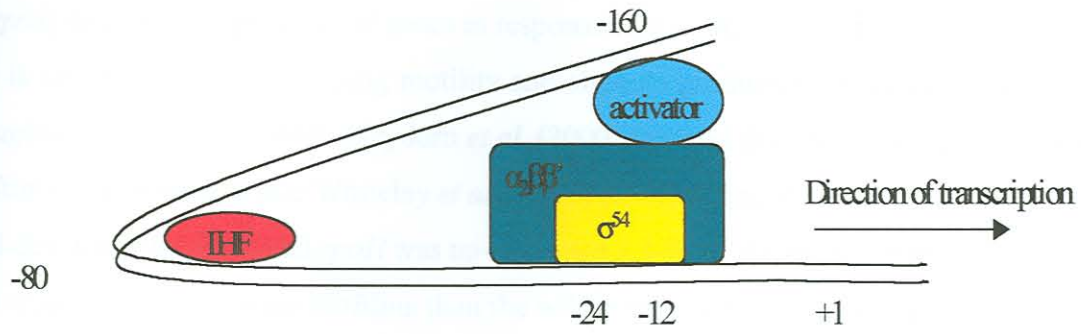

Fig. 1.3 Regulation of the σ^{54}-holoenzyme by activator proteins. Activators of the σ^{54}-holoenzyme bind to sites pre-upstream of the promoter, and are brought into close contact with the σ^{54}-holoenzyme, pre-bound to the -24 -12 promoter elements, by a sharp bend in the DNA that is induced by the integration host factor (IHF). The activator catalyzes the isomerization of the closed complex between the σ^{54}-holoenzyme and the promoter to an open complex, through a process that requires ATP hydrolysis (Modified from Hoover *et al.*, 1990).

1.3.3. Sigma 70-dependent gene transcription

1.3.3.1 Occurrence and function of σ^{70}

Sequence conservation among the σ^{70} family of proteins suggests that this group of proteins may be divided into three groups: the primary sigmas which are responsible for most RNA synthesis (group 1), closely related but non-essential sigmas (group 2), and alternative sigmas which are responsible for transcription of specific regulons (group 3). While the primary sigma factors are phylogenetically related, the alternative sigma factors are functionally related (Lonetto *et al.*, 1992). The major sigma factor, σ^{70}, encoded by *rpoD*, is required for transcription of genes involved in the housekeeping functions of the cell, e.g. metabolism and biosynthesis during exponential growth (Record *et al.*, 1996). Sigma factors are able to regulate regulons in response to particular environmental stimuli by regulating the availability or activity of sigma factors alternative to the housekeeping σ^{70}. For example, the heat-shock response is mediated by the expression of σ^{H} (σ^{32} encoded by *rpoH*) and directs the expression of a large number of genes

involved in the heat-shock response, while the starvation response sigma factor, σ^S (σ^{38} encoded by *rpoS*) directs the expression of genes in response to starvation (Cases and DeLorenzo, 2001) and is also required for twitching motility and alginate production in a mucoid variant of *P. aeruginosa* (Suh *et al.*, 1999). Heydorn *et al.* (2000) reported that *rpoS* may serve some role in biofilm development, while Whiteley *et al.* (2001) reported that *rpoS* was repressed 2.3-fold in well-developed biofilms and *rpoH* was up-regulated 2.3-fold. An *rpoS* mutant of *P. aeruginosa* was reported to form larger biofilms than the wild-type strain (Heydorn *et al.*, 2000; Whiteley *et al.*, 2001). The expression of flagellum genes is mediated by σ^{28}, encoded by *rpoF/fliA* (Starnbach and Lory, 1992). Both AlgU and PvdS have been grouped with the extracytoplasmic function (ECF) sigma factors, for most of their activities deal with extracytoplasmic functions (Missiakas and Raina, 1998). In *P. aeruginosa*, the alternative sigma factor PvdS is not only required for the production of pyoverdine, which enables iron uptake under conditions where little free iron is available (Wilson *et al.*, 2001), but is also required for the synthesis of exotoxin A, a virulence factor, and PrpL, an extracellular proteinase (Wilson *et al.*, 2001).

1.3.3.2 Domain structure of the σ^{70} protein and σ^{70}-dependent promoter sequences

Based on sequence alignments, σ^{70} has been divided into four regions (Region 1 to 4) by sequence conservation (Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988; Stragier *et al.*, 1989; Lonetto *et al.*, 1992) (Fig. 1.4). Region 1 is conserved only among primary sigma factors, while the remainder of the protein is conserved among all σ^{70}-type sigma factors (Lonetto *et al.*, 1992). Region 2 is involved in core binding (Lesley and Burgess, 1989), in recognition of the -10 region of the promoter (Daniels *et al.*, 1990; Kahn and Ditta, 1991; Siegele *et al.*, 1989; Tatti *et al.*, 1991; Waldburger *et al.*, 1990; Zuber *et al.*, 1989), and possibly in DNA strand melting (Helmann and Chamberlin, 1988). There is some evidence that part of Region 3 may also be involved in core binding in some sigma factors (Zhou *et al.*, 1992). The helix-turn-helix motif (Brennan and Matthews, 1989; Gribskov and Burgess, 1986; Helmann and Chamberlin, 1988; Stragier *et al.*, 1989) in Region 4 is involved in the recognition of the -35 region of the promoter (Kenny and Moran, 1991; Kenney *et al.*, 1989; Siegele *et al.*, 1989; Waldburger *et al.*, 1990). By contrast to Regions 2 and 4, which are highly conserved and tend to be very basic, Regions 1 and 3 exhibit lower conservation and are acidic.

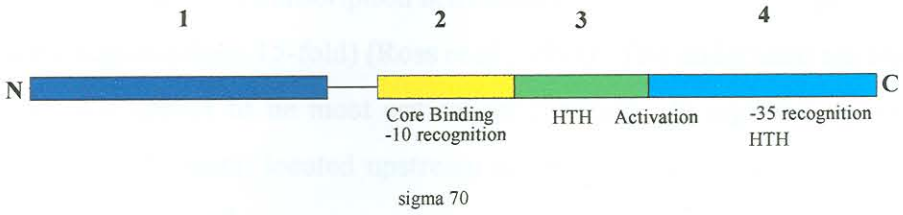

Fig. 1.4 Domain organization of the sigma 70 protein (Modified from Lonetto *et al.*, 1992; Record *et al.*, 1996). Region 1 is conserved among primary sigmas, Region 2 is involved in core binding and recognition of the -10 region of the promoter, Region 3 contains a helix-turn-helix (HTH) motif and may be involved in core binding, while Region 4 is involved in recognition of the -35 region of the promoter.

The σ^{70} family of RNA polymerases recognize diverse promoter sequences (Table 1.2) with conserved hexamers centred at -35 and -10 nucleotides upstream from the transcriptional start site (Hawley and McClure, 1983). In general, the *in vivo* strength of a promoter correlates with its qualitative homology with the consensus sequence and the favoured spacing of 17 bp between the -35 and -10 sequences (Record *et al.*, 1996; Reznikoff *et al.*, 1985). Although the optimal spacer length in σ^{70} promoters is 17 +/-1 nucleotide, functional promoters with a spacing between 15 and 20 non-conserved nucleotides have been reported (Hawley and McClure, 1983; DeHaseth and Helmann, 1995). The role of spacer length is to orientate the positions of the -35 and -10 hexamers (Record *et al.*, 1996) for recognition by the RNA polymerase enzyme. The level of transcription of genes under control of σ^{70}-dependent promoters may be affected by signals located upstream, downstream or between the core promoter elements. Some promoters appear to lack the -35 sequence and mutational studies have indicated that in these cases the sequence immediately upstream of the -10 region affects promoter activity. The optimal sequence for extended -10 sequences appears to be TnTGn followed by the -10 region (Record *et al.*, 1996). In the case of the *rrnB* P1 promoter, an A + T-rich region located at -40 to -60 relative to the transcription start site, the upstream (UP) element, has been shown to increase promoter activity, possibly by stabilizing the initial closed complex (Record *et al.*, 1996). The UP element contains two conserved regions, a 11 bp distal region [-57 to -47: AAA(a/t)(a/t)T(a/t)TTTT] and a 4 bp proximal region [-44 to -41: AAAA]. Each region can function independently, but the proximal

region confers a higher degree of transcription activation on the *rrn*B P1 core promoter (>100-fold) than the distal region (about 15-fold) (Ross *et al.*, 1998). The underlined nucleotides (-51 to -53 and -41 to -43) appear to be most critical for function. In addition, curved or bent sequences (e.g., rich in A tracts) located upstream of the -35 hexamers generally lead to an increase in the promoter strength.

Table 1.2 Conserved promoter sequences for different holoenzymes belonging to the σ^{70} family of *E. coli*

Holoenzyme & synonyms	Biological role	Promoter sequence	
		-35	-10
$E\sigma^{70}$, σ^{D}	Housekeeping functions	TTGACA	TATAAT
$E\sigma^{24}$, σ^{E} AlgU[#]	Stress response Alginate production	GAACTT	TCTGA
$E\sigma^{32}$, σ^{H}	Heat shock regulation	TCTCNCCCTTGAA	CCCCATNTA
$E\sigma^{28}$, σ^{F}	Motility and chemotaxis	CTAAA	CCGATAT
$E\sigma^{38}$, σ^{S}	Stationary phase regulation	?[$]	TATACT
$E\sigma^{19}$, FecI PvdS*	Iron uptake Pyoverdine biosynthesis	(G/C)(G/C)TAAAT (T/A)(G/C)	

[#]AlgU in *P. aeruginosa*

[$]No defined -35 sequence

*PvdS in *P. aeruginosa*

(Compiled from Lodish *et al.*, 1995; Reznikoff *et al.*, 1985; Martin *et al.*, 1994; Lonetto and Gross, 1996; Gross, 1996; Wilson *et al.*, 2001; Park *et al.*, 2001)

1.3.3.3 Regulation of transcription from σ^{70}-dependent promoters

Transcription initiation by σ^{70}-RNA polymerases can be regulated by the action of regulatory proteins that inhibit (repress) or stimulate (activate) transcription initiation, as well as by regulation of the DNA topology and the modification of DNA sequences within the promoter sequence (Reznikoff *et al.*, 1985). Of these mechanisms, repression of transcription initiation is the most common means of regulation of *E. coli* σ^{70} promoters (Gralla and Colado-Vides,

Repressors bind to operator sequences which overlap the region contacted by the RNA polymerase holoenzyme bound to the promoter, and either sterically block polymerase binding, inhibit open complex formation or prevent transcription occurring from the promoter region (Lodish *et al.*, 1995). By contrast, activators of σ^{70}-RNA polymerase generally bind to DNA sequences on opposite sides of the double helix from the RNA polymerase in the region from -20 to -50, or just upstream of the RNA polymerase near -60 (Fig. 1.5). There are no indications as yet that other members of the σ^{70} family use fundamentally different mechanisms to control promoter function. This is to be expected as activators appear to work primarily through the α and σ subunits of the RNA polymerase. RNA polymerases within the σ^{70} family contain identical α and similar σ subunits and thus are in principle subject to similar mechanisms of activation (Gralla and Collado-Vides, 1996).

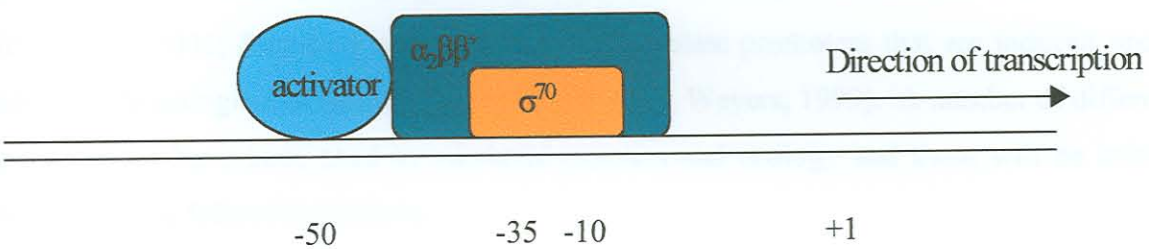

Fig. 1.5 Regulation of the σ^{70}-holoenzyme by activator proteins (Modified from Busby and Ebright, 1994). An activator protein binds to a site adjacent to the σ^{70} RNA polymerase-holoenzyme that is bound to the -35 -10 promoter elements and enhances transcription.

The superhelical character of the template DNA can affect the promoter activity, with many promoters being stimulated by negative superhelicity while others are inhibited (Balke and Gralla, 1987). Whereas DNA supercoiling in bacteria can be influenced by environmental factors (Higgins *et al.*, 1988), other factors, such as growth phase and nutritional limitation, have also been shown to affect DNA topology (Dorman *et al.*, 1988; Kusano *et al.*, 1996; Conter *et al.*, 1997; Schneider *et al.*, 1999). Methylation of *dam* sites has been reported to decrease transcription, by apparently inhibiting the recognition of σ^{70} promoters by the RNA polymerase holoenzyme (Reznikoff *et al.*, 1985).

1.4 ANALYSIS OF GENE EXPRESSION IN BIOFILM BACTERIA USING REPORTER GENES

Direct biochemical measurements of the activity or abundance of gene products are often sufficient to monitor gene expression in biofilm populations (Romani and Sabater, 2000). However, not all gene products are readily assayable and frequently such measurements are not adequately sensitive or readily adaptable to kinetic studies in adherent populations. Quantitative data about gene expression has been obtained by *in situ* reverse transcription followed by hybridization with fluorescently-labeled oligonucleotides (Møller *et al.*, 1998), but an appropriately equipped microscope is required and some method to permeabilize the biofilm cells is needed. A number of investigators have circumvented these limitations by making use of reporter gene technology. The use of reporter genes has proved to be extremely valuable in the study of environmental control of gene expression (Belas *et al.*, 1986; Davies *et al.*, 1993; De Kievit *et al.*, 2001; Sternberg *et al.*, 1999) and to isolate promoters that are induced under particular physiological conditions (Burne *et al.*, 1997; Weyers, 1999). A number of different reporter genes have been used in microbial genetics and ecology and these will be briefly discussed in the following sections.

1.4.1 Genes encoding chromogenic substrate cleavage enzymes

A number of genes encoding metabolic enzymes that are capable of cleaving chromogenic substrates have been described and used as reporter genes, e.g., *xylE* (Curcic *et al.*, 1994), *phoA* (Reuber *et al.*, 1991) and *celB* (Sessitsch *et al.*, 1996). The β-galactosidase (*lacZ*) gene of *E. coli* was one of the first genes of this class to be used as a reporter gene (Drahos *et al.*, 1986). Its popularity has been due to the ease by which the *lacZ* gene activity can be quantitatively assayed using a variety of relatively inexpensive chromogenic (o-nitrophenyl-β-D-galactoside [ONPG]) and fluorescent substrates (fluorescein-di-β-D-galactopyranoside [FDG] and methylumbelliferyl-β-D-galactoside [MUG]). The primary advantages of using the *lacZ* metabolic marker are that rapid visual screening is possible and the enzyme activity assay can be performed in cuvettes or in microtiter plates, and the samples can be read on an enzyme-linked immunosorbent (ELISA) plate reader, spectrophotometer, luminometer or fluorimeter, depending on the product of the

reaction (Burne and Chen, 2000; Schmoll *et al.*, 1990). Alternatively, epifluorescence or confocal laser scanning microscopy (CLSM) can be used, in combination with fluorescent substrates, to study expression of the reporter gene at a single cell level (Davies *et al.*, 1993; Davies and Geesey, 1995). Although *lacZ* is a versatile reporter, its utility may be hindered by the presence of endogenous microbial β-galactosidases (Bronstein *et al.*, 1994) and thus necessitates deletion of the native *lacZ* gene prior to its use. Further disadvantages of using *lacZ* are the stability of the enzyme, preventing the study of temporal gene expression, and the denaturation of the enzyme at pH values below neutrality (Burne and Chen, 2000).

1.4.2 Antibiotic resistance genes

A number of antibiotic resistance genes have been used as reporter genes in a variety of Gram-negative and Gram-positive bacteria. The genes conferring chloramphenicol (Cm^r or *cat*) resistance upon their host have been particularly useful, owing to the fact that the expression of these reporter genes can not only be selected for in growth medium, but also quantified via enzyme assays for chloramphenicol acetyltransferase (CAT) activity. The *cat* gene has been employed to monitor qualitative and quantitative gene expression in a wide variety of bacteria (Osbourne *et al.*, 1987). Although assays for CAT activity are not as easy or as inexpensive as those for LacZ activity, and the fluorescent substrates used in CAT assays are not able to penetrate cells, it may offer distinct advantages for studying biofilm bacteria. CAT seems fairly stable in cells growing at pH values between 4.0 and 7.5. It has also been reported that CAT, at least in a number of streptococci, has a short half-life, thereby enabling the assay of temporal gene expression (Burne and Chen, 2000). CAT activity may be measured by various methods, including kinetic assays and ELISAs with anti-CAT antibodies and anti-CAT substrates. The use of fluorescent CAT substrates may be adaptable to biofilms which have been gently fixed, but thus far no publication has reported such a method (Burne and Chen, 2000).

1.4.3 Bioluminescence genes

Luciferase activity (bioluminescence) is frequently used as a reporter system in both eukaryotic and prokaryotic cells (Gould and Subramani, 1988; De Wet *et al.*, 1985; Palomares *et al.*, 1989;

Greener *et al.*, 1992; Stewart and Williams, 1992) as well as in biofilm studies (King *et al.*, 1990; Denyer *et al.*, 1991; Mittelman *et al.*, 1992; Rice *et al.*, 1995). Luciferase is an oxidase that catalyzes the oxidation of reduced flavin ($FMNH_2$) to form an intermediate peroxide, which then reacts with a long-chain aldehyde to give blue-green luminesence emitting at 490 nm and oxidized flavin plus the corresponding long-chain fatty acids as products (Hastings, 1996). The genes for this light-producing reaction have been obtained from the firefly (*Photorhabdus luminescens*; *luc*) or bacteria (*Vibrio fisheri*; *lux*). Although bioluminescence represents a powerful tool for genetic analysis, it may be problematic to use, since the reaction requires the addition of an exogenous substrate for the light reaction to occur. In the case of using the *luxAB* genes as reporter, an aldehyde substrate, usually *n*-decanal, must be supplied to the cells during the assay procedure. Although the substrate penetrates the cells readily, it can be toxic at relatively low doses. To circumvent the addition of the aldehyde substrate, investigators have cloned all five genes of the *lux* operon (*luxCDABE*), which allows a completely independent light-generating system that requires no additional substrate (Applegate *et al.*, 1998; Hay *et al.*, 2000). However, cells containing the entire *lux* operon exhibit reduced viability compared to cells containing only *luxAB* (De Weger *et al.*, 1991; Amin-Hanjani *et al.*, 1993). Often the assays also use extracts from samples, rather than whole cells, and therefore destructive sampling is required (Møller *et al.*, 1995). Bioluminescent reporter genes can be directly monitored by visual or microscopic observations (Masson *et al.*, 1993; Flemming *et al.*, 1994), by measuring the light input in a luminometer (Rattray *et al.*, 1990) or a scintillation counter in chemiluminescence mode (Belas *et al.*, 1986). The light output is indicative of a metabolically active population of cells, since the luciferase enzymes are dependent on cellular activity reserves, or reducing equivalents for bioluminescence. However, after long-term incubation, microbial cells often become starved or stressed and the light production from luciferase enzymes declines as a response to the change in cellular energy status (Duncan *et al.*, 1994). Therefore, *in situ* bioluminescence may not be a reliable indicator and limits its utility.

1.4.4 Green fluorescent protein genes

The *gfp* gene, encoding green fluorescent protein (GFP), has been isolated and cloned from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). Through an autocatalytic reaction, GFP forms

a cyclic peptide that is highly fluorescent and stable (Cody *et al.*, 1993). An advantage of GFP, over other reporters is the fact that no other energy source or substrate addition is required, other than oxygen during initial formation of the chromophore (Chalfie *et al.*, 1994; De Weger *et al.*, 1994; Inouye and Tsuji,1994; Stewart and Williams, 1992). In addition, GFP is stable in the presence of many denaturants and proteases, and persists at high temperatures (65°C) and pH values (6-12) (Ward *et al.*, 1980). It is, however, not yet clear how reliable GFP would be in the oxygen-restricted conditions that may develop in deep biofilms. A potential disadvantage of using *gfp* as a reporter gene is the extreme stability of the GFP protein (Tombolini *et al.*, 1997). Whereas stability of the protein is advantageous for the environmental monitoring of GFP-tagged cells, it can be problematic in studies regarding temporal changes in gene expression, since, once the reporter protein is synthesized, it may persist. To overcome this problem, various unstable variants of GFP have been constructed that are more susceptible to degradation by ClpXP-type protease complexes and therefore have shorter half-lives (Andersen *et al.*, 1998; Cormack *et al.*, 1996; Heim *et al.*, 1994; Keiler *et al.*, 1996). Additionally, various groups have obtained GFP mutants exhibiting diverse spectral properties that may allow simultaneous analysis of gene expression from a number of different promoters (Delgrave *et al.*, 1995; Ehrig *et al.*, 1995). Depending on the nature of the study, GFP fluorescence can be monitored by exposing bacterial colonies to UV light (Tresse *et al.*, 1998), by fluorometric detection (Burlage *et al.*, 1996), epifluorescence microscopy (Chalfie *et al.*, 1994; De Kievit *et al.*, 2001), confocal laser scanning microscopy (Eberl *et al.*, 1997) or flow cytometry (Tombolini *et al.*, 1997). Numerous *gfp*-based reporter systems have been developed for use in especially environmental applications to study GFP-tagged bacteria in soils, water systems, rhizospheres, activated sludges, root nodules and biofilms (Eberl *et al.*, 1997; Chalfie *et al.*, 1994). Recently, *gfp*-based reporter systems have also been used successfully to study gene expression in bacteria (De Kievit *et al.*, 2001; Heydorn *et al.*, 2002; Sternberg *et al.*, 1999).

1.5 AIMS OF THIS STUDY

As early as 1933, Henrici recognized the phenomenon that marine bacteria grow for the most part on submerged surfaces, rather than being free-floating. With the rediscovery that bacteria are found predominantly attached to surfaces in aquatic systems (Geesey *et al.*, 1977), much

attention has been paid to unravelling the molecular mechanisms underlying the formation and regulation of biofilms. To investigate, a number of regulatory elements from P. aeruginosa PAO (DSM1707) have been isolated in our laboratory. These are thought to control the expression of genes which may play roles in the attachment of *P. aeruginosa* to a surface, subsequent biofilm formation and/or detachment of cells from the biofilms.

In an attempt to isolate genetic elements up-regulated by attachment, Weyers (1999) constructed a chromosomal library of *P. aeruginosa* DSM1707 in the broad-host-range reporter vector, pALacZsd, by cloning genomic fragments upstream of the promoterless *lacZ* reporter gene. One thousand clones, representing *ca.* 3% of the genome, were screened for up-expression of β-galactosidase activity upon attachment of the transformed *P. aeruginosa* cells to a glass wool substratum. The results were expressed as a ratio between the β-galactosidase activity assayed for the cells grown in the presence of glass wool (attached; A) and for the cells grown in the absence of glass wool (planktonic; P). The clones were divided into four main groups according to their A/P ratio and basal level of activity of planktonic growing cells; Group I consisted of 337 clones and had an A/P ratio $\geq$ 3, Group II consisted of 338 clones and had an A/P ratio of between 2 and 3, Groups III and IV consisted of 325 clones, had an A/P ratio $<$ 2 and had low ($<$0.5) and high ($>$0.5) basal levels of activity of planktonic cells, respectively. The clones in Group I was further differentiated and a total of 131 clones (Group IAi) were regarded as being significantly affected by attachment, since they displayed a high level of β-galactosidase activity in the attached state (A$>$ 0.5) and a low level of β-galactosidase activity in the planktonic state (P$<$0.5). A total of 24 clones, consisting of 20 clones from Group IAi and 4 selected clones from Group II, were chosen for further characterization in this investigation.

Therefore, the aims of this investigation were the following:

a) To characterize selected attachment-induced genetic elements obtained from a *P. aeruginosa* genomic library.

b) To investigate the transcriptional activity of one such element under different growth conditions using reporter gene technology.

The research strategies for obtaining the primary objectives involved the following:

- Nucleotide sequencing of the genetic elements and analysis of the obtained sequence data in order to identify putative promoter sequences and to determine which genes may be expressed by these regulatory elements (Chapter 2).
- Preparation of *lacZ* and *gfp* transcriptional fusions, and analysis of the transcriptional activity of a selected attachment-induced regulatory element under different growth conditions as well as in developing and well-developed biofilms, respectively (Chapter 3).