Effect of *rpoS* inactivation on biofilm formation of a *p*-nitrophenol degrading *Pseudomonas putida* strain

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Table of Contents

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List of Tables

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List of Figures

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Abstract of the Thesis

With the widespread existence of environmental pollutants in the modern world, effective, non-destructive means of remediation are becoming more and more desirable as an alternative to the traditional excavation and incineration. Bioremediation, the use of pollutant-degrading biological processes to cleanse an area, is a highly viable and realistic option. In a polluted area, organisms must be able to withstand a considerable amount of environmental stresses. Both biofilms and the stationary-phase sigma factor, RpoS, have been shown to aid in bacterial survival and thus their relationship to each other may provide clues to increasing the survival of remediating organisms.

The effect of *rpoS* deletion on the biofilm forming ability of a *p*-nitrophenol degrading *Pseudomonas putida* strain was examined. When examined in a simple Crystal Violet assay the *rpoS*-knockout mutant showed a greater amount of cells attached to the glass test tubes than did the wild-type. This observation was mirrored by confocal microscopy images which showed a greater amount of growth, quicker growth, and a greater degree of mature biofilm structure for the knockout cultures versus the wild-type. Converted to quantitative results, all parameters of biovolume, percent substrate coverage, thickness and roughness showed a significantly greater difference between the knockout and the wild-type *P. putida*. There were no significant differences in hydrophobicity values for the two cultures and it is unlikely that hydrophobicity played a role in the observed biofilm differences. Taken together, these results indicate a close relationship between RpoS and biofilm formation in *P. putida*, and are promising for future bioremediation studies.

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1. Literature Review

1.1. Biofilms:

Although traditional pure-culture techniques using planktonic cells have given a vast amount of information to the scientific community, it is becoming apparent that more information is required concerning bacterial growth in nature versus current artificial laboratory systems. In the environment, bacteria are mainly found attached to surfaces as pure or mixed biofilm communities. These biofilms are defined as matrix enclosed communities of microorganisms tightly interacting with each other and, in most cases, supported by an abiotic surface (Costerton *et al.* 1987).

In studying biofilms, a great deal of effort has gone into defining the steps involved for their formation. According to Dalton and March (1998), biofilm development in the environment follows a logical series of steps . Initially, a surface is totally bare but is soon covered with a random assortment of biological molecules. Following this, bacterial attachment occurs, leading to intercellular attachments, mature biofilm formation and the continual accumulation of biological material. The current knowledge base in biofilm research has been gained through a combination of physiological and genetic approaches, with the majority of the research relating to the initial attachment stages.

Through the use of attachment-deficient mutants many researchers have found that motility via flagella and/or fimbriae (pili) is an important biofilm forming characteristic for many bacterial species. In the case of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, it has

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been observed that mutations affecting the flagellum prevented effective colonization (O'Toole and Kolter 1998b; Sauer *et al.* 2002), and that both the flagellum and type-IV pili are required for the mature biofilm to develop (O'Toole and Kolter 1998a; 1998b). Following bacterial attachment via flaggelar actions, the type-IV pili provide a twitching-motility and a means for the biofilm to achieve confluency and the typical microcolony formations (O'Toole and Kolter 1998a).

Flagellae have also been shown to be important for the initial attachment phase of *Escherichia coli* biofilms (Genevaux *et al.* 1996; Pratt and Kolter 1998) by establishing initial cell-surface contacts. Pratt and Kolter (1999) also found that in addition to flagella, type-I pili are required for *E. coli* biofilm growth on numerous surfaces including PVC, polycarbonate, polystyrene, and borosilicate glass , although another study suggested that the pili of *E. coli* may not play a role in abiotic surface attachments (Reid *et al.* 1996).

In addition to the type-I pili, studies using *E. coli* have shown another fimbriae to be important for the initial attachment, curli fimbriae (Jackson *et al.* 2002). In looking closer, Vidal *et al.* (1998) were able to show that it is not motility which is important but instead a surface adhesin, curli. In their research, isolates lacking any motility were able to form biofilms via the presence of the curli fimbriae .

Additional studies on the effect of pili in bacterial biofilm formation have shown that the type-IV mannose-sensitive hemagglutinin pilus of *Vibrio cholerae* El Tor plays a role in bacterial attachment during pathogenic colonization of a host (Thelin and Taylor 1996) but is not needed on abiotic borosilicate glass (Watnick *et al.* 1999). Following this discovery, other abiotic surfaces were tested for colonization of *V. cholerae* and it was found that the same pilus was not needed for colonization of a nutritive chitin surface (Watnick *et al.* 1999). This indicates

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that another adhesion mechanism is active for *V. cholerae* colonization of chitin, and more importantly that bacteria have many avenues and methods for colonization of different materials and environments.

Studies have shown that motility is not the only factor required for bacterial adhesion. Research into pathogenic *Streptococcus* adhesion to hosts has provided a model for the two-step process of initial adhesion. The first step involves reversible hydrophobic interactions between the host cells and lipoteichoic acid of the bacterial surface (Hasty and Courtney 1996), and the second irreversible adhesion step involves the surface adhesin, M protein (Courtney *et al.* 1997). This model may be applied to environmental settings with alterations as to what the initial attractive forces are and which surface proteins are involved.

During initial adhesion, the bacterium *Staphylococcus epidermidis* uses protein and polysaccharide adhesions to bind surfaces (Rupp *et al.* 1999). In a study, Heilmann *et al.* (1996) provided additional support that a specific molecule can be responsible for biofilm development . Through transposon-mediated mutation of *S. epidermidis,* a mutant deficient in intercellular adhesion, biofilm development and production of a specific cell-surface polysaccharide was formed. When complemented with a plasmid-clone of the wild-type gene for the polysaccharide all phenotypes were reversed. The most striking aspect, however, was that this same plasmid-clone of the polysaccharide gene was able to create biofilm producing isolates of a non-biofilm forming *Staphylococcus carnosus*.

The closely related *Staphylococcus aureus*, like *S. epidermidis* has been shown to use a specific biofilm-associated protein in initial adhesion and intercellular adhesion of biofilms (Cucarella *et al.* 2001). Additional studies on *S. aureus* in a host situation have indicated the requirement for two proteins for binding fibronectin (Fn) (Greene *et al.* 1995). Fn is an

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important component of the matrix surrounding the biofilm. In addition, glass coverslips were coated with Fn by Greene *et al.* and bacterial attachment followed the same pattern as in the host. This demonstrates a potential for adhesion and sustainability of pathogenic bacteria in the environment, providing appropriate materials are available. Fn binding is also important in the binding of *Campylobacter jejuni* through a specific surface protein (Konkel *et al.* 1997). Of interest is the fact that this protein is 52% similar to the root adhesion protein of *P. fluorescens*. Such homology suggests that bacterial proteins such as adhesins may perform similar tasks in very diverse environments. As well, it is an indication that a single structure may have different roles in different bacteria under different conditions.

Following attachment and microcolony formation, the biofilm matures. Maturation is an ongoing, dynamic process leading to a sustained biofilm population maintained within a supporting polysaccharide matrix. Just as there are numerous bacterial species, there are also numerous forms of a mature biofilm. The biofilm structure of a *P. aeruginosa* pure-culture has often been used as the model in biofilm study (Toutain *et al.* 2004). In the model, biofilm growth is proposed as dispersed microcolonies surrounded by copious amounts of extracellular materials. Throughout the community is a system of interstitial voids or channels through which water and material flow has been observed (Stoodley *et al.* 1994). In a three dimensional view the microcolonies are raised from the substratum by the extracellular materials, looking much like mushrooms between which the bulk-fluid flows.

Many different factors have been found to play an important role in the maturation process including routine binary division increasing microcolony size (Heydorn *et al.* 2000b; Tolker-Neilsen *et al.* 2000) and relocation of bacterial cultures by movement of the bulk-fluid (Stoodley *et al.* 2000; 1999a). In addition, the role played by quorum-sensing (QS) has realized

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a great deal of attention. QS in bacterial populations is the means through which the regulation of many important changes in gene expression occur (Swift *et al.* 2001). Bacterial cells sense the relative density and number of their population through the use of freely-diffusing signal molecules such as acylated homoserine-lactone (AHL). The autoinduced positive feedback which results allows for appropriate phenotypic adjustments to be made in relation to environmental conditions or required population differentiation.

When looking at biofilms, it is reasonable to believe that such a concentrated population of cells would rely on the effects of QS molecules. The first study to show that AHL signaling occurs in a biofilm population was performed by McLean *et al.* (1997). Also in 1997 cell-density regulated phenotypes were demonstrated in relation to *P. aeruginosa* biofilms (Batchelor *et al.* 1997). In 1998, Davies *et al.* grew biofilms of a wild-type and a *lasI* mutant deficient in AHL production. The mutant biofilms were sparse in comparison to the wild-type, and returned to a wild-type phenotype with the addition of AHL to the media (Davies *et al.* 1998). In addition, the results from another study that used a halogenated furanone to successfully interfere with AHL signals provided support for the role of a Q system in *P. aeruginosa* biofilms (Hentzer *et al.* 2002). This compound from a red algae was shown to penetrate the biofilm, shutting down signaling through competition for binding to AHL receptors, with a result of destabilizing the biofilm and inducing sloughing from the surface.

The enclosing matrix of biofilms, EPS, referred to as "extracellular polymeric substances" or "extracellular polysaccharides" is also important for the function and structure of the mature biofilm. According to Rickard *et al.*, the EPS is responsible for enveloping the attached cells, strengthening cellular adhesion to the substrate, and providing receptors for coaggregates of single and multi-species biofilms (Rickard *et al.* 2003). This is, however, a

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simplified view of the complex roles performed by the EPS matrix. When examining EPS, one must be aware of the large diversity between the EPS components of different species, mixed-species biofilms, and how growth conditions will effect the final compositions of either (Sutherland 2001). It is for this reason that Davey and O'Toole (2000) warn against making such broad generalizations regarding any bacterial EPS .

EPS is composed of a complex mixture of polysaccharides, nucleic acids and/or proteins (Flemming *et al.* 2000; Sutherland 2001). Of these, the polysaccharide alginate from *P. aeruginosa* is one of the best studied and has been shown to play an important role in the mushroom-mound structure formation of mature mucoid biofilms (Hentzer *et al.* 2001; Nivens *et al.* 2001). However, it is not important for biofilm formation in non-mucoid environmental strains (Wozniak *et al.* 2003). Although less studied, other sugar molecules have also been shown to play important roles in biofilm structure, including glucose, galactose, and colonic acid (Danese *et al.* 2000; Jackson *et al.* 2002; Yildiz *et al.* 2001). Because of the difficult procedures required to separate the EPS from the cells of a biofilm, it has been speculated whether or not the nucleic acids and proteins are a true component of the EPS, or if they are cellular debris artifacts. A study by Whitchurch *et al.* (2002) has suggested that the nucleic acids may play a structural role in young (<60h) biofilms. In their study, DNase I was added to young biofilm cultures, and the cells were observed to dissipate in the absence of nucleic acids (Whitchurch *et al.* 2002).

In addition to the structural role played by the EPS of holding cells together and allowing the formation of the mushroom-mound structure by holding cells away from the surface and forming channels (Costerton *et al.* 1995; Watnick *et al.* 1999), a number of other benefits are realized by the biofilm. The EPS provides shelter from the environment and a means of maintaining homeostasis for the cells it contains (Davey and O'Toole 2000). Such shelter may

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be in the form of restricted diffusion of compounds such as antimicrobials via ion exchange (Gilbert *et al.* 1997), protection against desiccation (Ophir and Gutnick 1994), protection from UV radiation (Elasir and Miller 1999), or protection from any other number of environmental stresses including pH shifts and osmotic shock (Flemming 1993). To aid in survival and homeostasis, dissolved organic compounds are absorbed by the EPS, effectively concentrating essential nutrients and growth components for use by the cells (Wolfaardt *et al.* 1998). The EPS has also been shown to sequester heavy metals, cations and toxins from the environment (Decho 1990; Flemming 1993), a function which gives a much broader-based role for biofilms on a whole as environmental protectants.

Studies on mature biofilm communities have shown there to be a number of distinct differences between planktonic cells and sessile biofilm cells. Throughout 12 days of biofilm development, *P. aeruginosa* has been shown to pass through five distinct phases of genetic expression corresponding to the development stages of (i) reversible detachment (ii) irreversible detachment (iii) maturation-1 with microcolonies several cells thick embedded in EPS (iv) maturation-2 with cell clusters reaching maximum thickness and (v) dispersion (Sauer *et al.* 2002). Additional evidence collected on *P. putida* from both genetic microarray and proteomic studies indicate differences at similar stages in biofilm development. Through microarray analysis, it has been shown that genes related to flagellum, amino acid metabolism and the outer membrane lipoprotein NlpD expression were downregulated; genes related to type IV pili and polysaccharide synthesis were upregulated; and polysaccharide synthesis showed differential changes in expression (Sauer and Camper 2001). In the same study, proteomic analysis revealed the same downregulation of NlpD expression and amino acid metabolism. Microarray analysis

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was also applied by Whitely *et al.* in 2001, where similar results of downregulation of flagella, pilin and *rpoS* genes were observed in biofilm cells (Whitely *et al.* 2001).

The general physiology of the sessile cells themselves also shows variability within the community. In studies of both *Klebsiella pneumoniae* and *P. aeruginosa* differences in the metabolic activity at various depths of the biofilm has been observed through the response of alkaline phosphatase during phosphate starvation (Huang *et al.* 1998; Xu *et al.* 1998). Specifically, the active cells of mature *P. aeruginosa* biofilms were restricted to a thin liquid-interface layer. Looking closer, microelectrode probes showed that the dissolved oxygen concentration dropped rapidly below this layer, too low for the bulk-cells to be active. As such, it can be presumed that the cellular activity is controlled though both carbon source and electron acceptor availability (Xu *et al.* 1998). In general, it was observed that cells contained within the bulk of the biofilm were no longer physiologically active, a trait which may account for the heightened antibiotic and stress resistance of biofilm communities.

Not only do biofilm populations show differences in physiological activity at different depths, but subpopulations have been observed in pure-culture situations. Examination of *Pseudoalteromonas* S9 biofilms has shown there to be two distinctly active groups (Baty *et al.* 2000a; 2000b). The first degraded chitin while the second was completely chitinase inactive, but was actively replicating and releasing daughter cells to the bulk fluid. It was theorized that the nutritive products from the first subpopulation were exported via the EPS and channels to the second for use. In these studies, the authors suggested the presence of holistic integration between the biofilm populations and the planktonic daughter cells.

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1.2. Study of Biofilms:

As odd as it may seem, the study of biofilms was the first method of studying microbes. The original microbial studies performed by Leeuwenhoek were performed with microbes on surfaces: a biofilm. Microbiological studies quickly moved into pure-culture systems, which have provided a vast amount of knowledge. However, the tools required for studying pure culture suspensions provide little benefit in the examination of attached biofilms. Since the resurgence of interest in biofilms, the last 20 years have seen researchers struggling with limited scanning electron microscopy (SEM) and traditional techniques (Prakash *et al.* 2003). However, new methods have been developed and specialized equipment has been made within the last decade that have provided the non-invasive means required for biofilm research.

The most influential piece of equipment to modern biofilm research has been the scanning confocal laser microscope (SCLM) (Lawrence *et al.* 1991). The previous imaging tool, SEM, required biofilm samples be fixed, and all water removed. The images resulting from the dehydrated samples showed a very simplified concept of what the biofilm looked like, in addition to being unnatural and incomplete. Because the SCLM allows the biofilm to be viewed fully hydrated, the complex structure can be seen undisturbed, and can also be modeled into 3D views in real-time.

The means of cultivating the biofilm have also evolved from the traditional liquid suspensions and plated colonies. Current researchers have at their disposal an arsenal of techniques to choose from including chemostats, flow-cells, microstats and colonization tracks. In particular, the flow-cell has proven invaluable for allowing the growth and examination of a fully complete and undisturbed biofilm by microscopy.

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Although there were numerous methods for genetic analysis following the removal of nucleic acids from cultures, few useful *in situ* techniques existed for application to biofilms. The exploitation of rRNA differences for bacterial identification helped to introduce the use of fluorescent rRNA probes to identify cells in mixed cultures (Poulsen *et al.* 1997). Chromosomal tagging of different species has also shown valuable results in tracking movements in mixed-culture systems, as in the study by Tolker-Neilsen *et al.* (2000) where both *gfp* and *dsRed* were used. Gene expression studies have also been conducted, though both the use of fluorescent protein-promoter fusions (Moeller *et al.* 1998) and chromogenic substrates of β-galactosidase (Poulsen *et al.* 1997). The fusion reporter constructs allowed for both a temporal and spatial distribution of genes to be observed throughout biofilm development, although there is a limited number of genes that can be examined in a single experiment. Overall, a combination of molecular and *in situ* techniques provide the best data for modern biofilm studies.

When examining biofilms, there are a number of variables that can affect biofilm development. Areas to consider when designing simulations and experiments include system hydrodynamics, nutrient composition, the surface or intersurface, and if a mixed culture system is desired, the community composition (Stoodley *et al.* 1997). One of the accepted models for the mature biofilm morphology contains rounded microcolonies interspaced with voids. These biofilms are seen when growth is allowed to occur in laminar flow situations. When the flow is changed into a high-shear turbulent scenario, the growth pattern changes into a filamentous system with the microcolonies elongating into streamers (Stoodley *et al.* 1999b). These high-shear flow systems also influence the biofilm density and strength (Liu and Tay 2001). High shear forces also affect biofilms that are not in flow systems. For example, dental plaque

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exposed to the shear stress of chewing produces biofilms that are stratified and compact when compared against the rounded microcolony model (Bowden and Li 1997).

The media composition is another variable that has been shown to alter the biofilm phenotype. The roughness of *Pseudomonas aureofaciens* biofilms is inversely related to the concentration of citrate in the media (Heydorn *et al.* 2000b). Although not specifically attributed to one component of a growth media, differences in biofilm phenotype between thick wild-type growth (Davies *et al.* 1998) and flattened biofilms of the same strain (Hentzer *et al.* 2001) were attributed to differences in the media used.

The surface chosen to grow the biofilm on also has an impact on the results obtained. It has been shown that colonization increases as the surface roughness increases (Characklis *et al.* 1990). It may be that as the surface roughness increases surface area increases, but shear forces diminish. Aside from roughness, the material of the surface should be considered. A higher rate of attachment is seen for hydrophobic, non-polar materials like Teflon or plastics, versus hydrophilic glass or metal (Bendinger *et al.* 1993). Perhaps the hydrophobicity of the surface has a relationship and attraction to the lipid membranes of the bacterial cells.

Lastly, the cells themselves should be considered. Ghigo (2001) showed that the natural presence of conjugative plasmids induces biofilm formation. It could be hypothesized that the higher cell densities of biofilms favor horizontal plasmid DNA transfers, and as such biofilm development evolved a response to the presence of plasmid DNA. Or in the case of mixed-species biofilms, the researcher should be aware that each species may react differently to the parameters mentioned above, as well as differently to each other. The study of mixed-species biofilms is still in its infancy, and we are greatly lacking in information on what does or does not alter the biofilm formation potentials of such systems.

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1.3. The Stationary Phase Sigma Factor, RpoS:

Sigma factors are positive regulators of gene expression that bind directly to promoter sequences and by recruiting the core RNA polymerase, effectively direct the initiation of transcription. The stationary phase sigma factor gene, *rpoS,* encodes an alternate sigma factor which directs the transcription of a large subset of bacterial genes through altering the RNA polymerase core specificity (Ishihama 2000). In fact, Hengge-Aronis (2000; 2002a) stated that more than 70 genes in *E. coli* are regulated by RpoS. These genes may code for adaptation to nutrient-limiting conditions, environmental stresses and the induction of virulence factor production to name a few (Suh *et al.* 1999). The name "stationary phase sigma factor" came from the initial observation that RpoS activates the expression of genes to maintain cellular viability during the stationary phase of growth (Kolter *et al.* 1993; Loewen and Hengge-Aronis 1994).

Originally identified in *E. coli* and *Salmonella typhimurium*, RpoS protein has now been identified in *Pseudomonas* and many of the γ-proteobacteria (Venturi 2003), where the RpoS protein levels increase on entry to the stationary phase (Fujita *et al.* 1994). In comparison to *E. coli*, the role played by RpoS is less defined in the pseudomonads (Schuster *et al.* 2004). For example, *rpoS* mutants of *Pseudomonas* spp. have shown a less pronounced susceptibility to stresses than *E. coli rpoS* mutants (Jorgensen *et al.* 1999; Suh *et al.* 1999). In addition, RpoS activity is controlled at the transcriptional, post-transcriptional and post-translational levels (Hengge-Aronis 2002b). All three of these may differ between the two species, and may account for the observed variations in RpoS effects.

Taking a closer look at the variations between *E. coli* and *Pseudomonas* spp., it is seen that RpoS regulates more than just stress responses. In studies involving planktonic cultures of

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P. aeruginosa it is also reported to cross-regulate with the global QS regulon (Whitely *et al.* 2000), and the GacA-GacS two-component system which is involved in phenotypic control, pathogenicity, biofilm formation, and enzyme secretion (Bertani and Venturi 2004). It is, however, still unclear how the systems are connected, nor are the molecular mechanisms of the interactions clearly defined (Bertani and Venturi 2004; Schuster *et al.* 2004). In examining the relationship, it has been observed that QS boosts *rpoS* transcription by 2-fold in planktonic cultures (Schuster *et al.* 2003). From this it has been hypothesized that RpoS may control the timing of induction for many of the quorum-controlled genes as cell density increases and thus the timing of biofilm initiation (Schuster *et al.* 2004), although additional studies in biofilm systems are required to demonstrate this conclusively. Indeed, 40% of the genes controlled by QS are also controlled by RpoS (Schuster *et al.* 2004). Also from the observed link between RpoS and QS has come the theory that this link may aid in coordinating the regulation of virulence factors (QS) and survival in stationary phase (RpoS) for pathogenic species (Venturi 2003).

In 1999 Adams and McLean looked at the biofilm forming capability of a *rpoS* mutant of a Δ*lacZ E. coli* ZK126 (Adams and McLean 1999). They observed a 50% decrease in the biofilm cell density (CFU/cm^2) for the Δ*rpoS* and obvious differences in cell arrangement. In subsequent studies involving *E. coli*, RpoS deletion has been seen to negatively affect adhesion during the stationary phase via the *rpoS*-dependent transcription of the transcription regulator *cpxR* (Prigent-Combaret *et al.* 2001). In addition, *E. coli* MG1655 *rpoS* mutants were incapable of forming sessile communities within a laminar flow tube in 40 hours (Schembri *et al.* 2003). As adhesion is important to biofilm structure and development, such observations may help to explain the decrease noted by Adams and McLean.

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In contrast to these studies, Corona-Izquierdo and Membrillo-Hernández observed an increase in biofilm growth for their *E. coli* K12 Δ*rpoS* strain, especially during the exponential phase of growth (Corona-Izquierdo and Membrillo-Hernández 2002). They found indications that an extracellular factor was present in the spent media of the *rpoS* mutant, promoting the production of biofilm. When this spent media was added to wild-type cultures, abundant growth similar to the mutant biofilm structures was seen. This occurred even when the spent media was from a culture in exponential phase of growth, before the predicted onset of RpoS, indicating production of the extracellular factor was negatively influenced by the presence of the *rpoS* gene. Corona-Izquierdo and Membrillo-Hernández surmised that RpoS plays a role in the amount and timing of the initiation of biofilm formation (Corona-Izquierdo and Membrillo-Hernández 2002). Although the current information regarding the role of RpoS in the development of *E. coli* biofilms is not consistent, all researchers have agreed that *rpoS* does play an important role in their formation.

In comparison to *E. coli*, studies examining the role of RpoS in *Pseudomonas* show more consistency between results. When grown in flow chambers, Δ*rpoS* isolates produced a thicker biofilm than the wild-type counterparts, although the two biofilms were similar in roughness (Heydorn *et al.* 2000a; 2000b). Continuing these studies, Heydorn *et al.* (2002) observed an additional temporal difference in the biofilm development, with the wild-type lagging behind the mutant. Again, the mutant biofilms were more densely-packed and thicker than the wild-type.

In addition to the direct growth observations, genetic microarray studies on *rpoS* mutants show the repression of *rpoS* expression in 5 day old *P. aeruginosa* biofilms (Whitely *et al.* 2001). A protein assay using β-galactosidase, however, showed there to be a greater *rpoS* expression in the biofilm communities (Xu *et al.* 2001). This study, however, was normalized to

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the total protein of the isolate, EPS and cell membrane and cell contents combined. Due to the fact that the EPS may have contained high levels of protein this may have been a confounding factor in interpreting the final results. Returning to genetic support of there being a role for *rpoS* in biofilm communities, microarray observations in *E. coli* show 46% of the *rpoS*-dependent genes listed by Loewen *et al.* (1998) were altered in their expression during biofilm growth (Schembri *et al.* 2003). As it has been well documented that there are large differences in the RpoS activities between the two species *E. coli* and *P. aeruginosa* (Venturi 2003), the differences in its effects on biofilm formation might be considered reasonable and expected.

1.4. *p*-Nitrophenol:

p-Nitrophenol (PNP), also known as 4-nitrophenol/para-nitrophenol, is a man-made water soluble solid with no evidence of natural formation. It is an intermediate in the formation of some organophosphorus pesticides such as parathion, methyl-parathion and nitrofen, as well as some medical analgesics and fungicides (Boehncke *et al.* 2000). Additional commercial uses of PNP include dyes and the darkening of leathers.

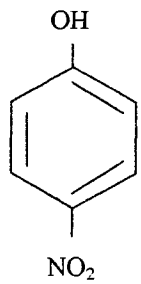

Figure 1.1: *p*-nitrophenol

Environmental release occurs to the air, water and soil through a number of sources and methods. Vehicles emissions can release PNP (formed during combustion) to the atmosphere at a rate of 1000ug/m^3 (Tremp *et al.* 1993), while hydrolytic and photolytic degradation of some organophosporus pesticides releases PNP to all three spheres at an estimate of several thousand tonnes annually. When quantifying the amount of PNP in the air, however, the levels in the gas phase are higher than expected from physiochemical data due to extensive particle binding (Boehncke *et al.* 2000). Looking at the toxicity of PNP, it should be noted that it is more toxic than its isomer 2-nitrophenol, and poses a risk to the more sensitive of aquatic organisms. In

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addition, *in vitro* evidence exists for PNP causing chromosomal aberrations, and sensitization of the skin has been observed in factory workers. The measured bioconcentration factors (BCF) of 11-57, however, indicate a low potential for bioaccumulation (Freitag *et al.* 1982; Geyer *et al.* 1981). BCF is an indication of a chemical concentration in an animal as compared to surrounding water. Thus, a BCF of 2 is twice the concentration in the water. Some substances such as the insecticide permethrin have BCFs as high as 1900 (Schimmel *et al.* 1983).

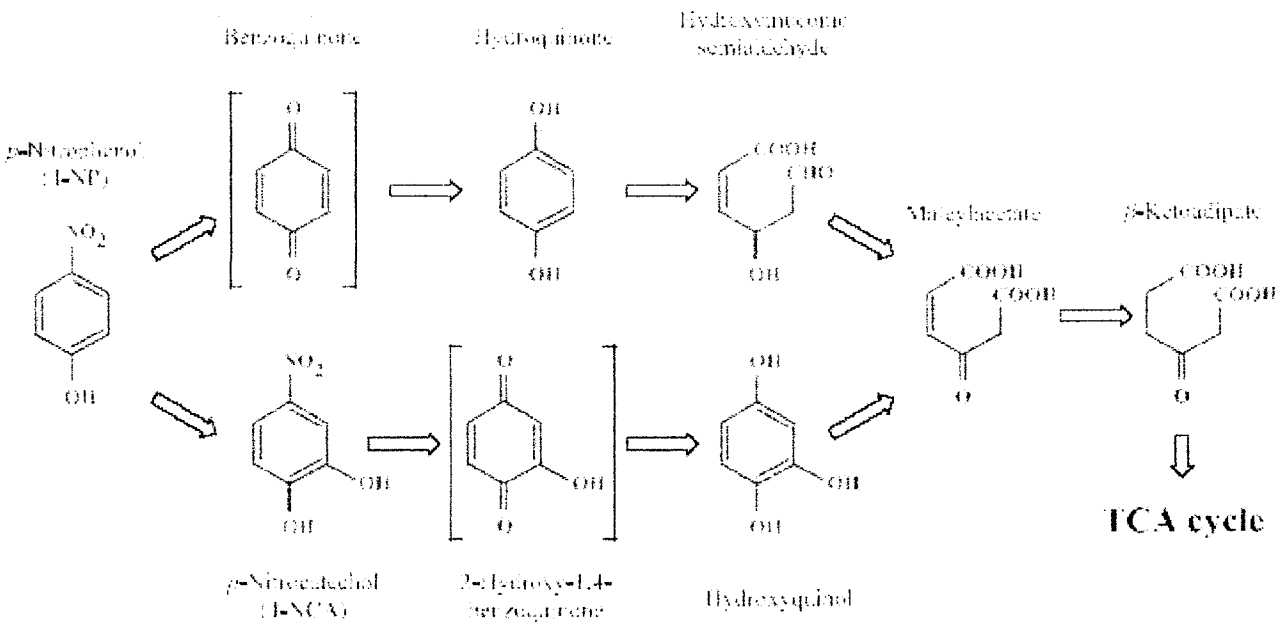

Figure 1.2: Metabolic pathways for the biodegradation of *p*-nitrophenol via hydroquinone (top) or 4-nitrocatechol and hydroxyquinone (bottom) (Kitigawa *et al.* 2004)

Although PNP is readily degraded within 13.7 days in water under aerobic conditions, mineralization under anaerobic conditions requires extended adaptations of the microbial community (Boehncke *et al.* 2000). Aerobic degradation of PNP has been observed in a number of bacterial species, including *Moraxella* spp. (Spain and Gibson 1991), *Bacillus* spp. (Kadiyala and Spain 1998), *Pseudomonas* spp. (Prakash *et al.* 1996), *Arthrobacter* spp. (Jain *et al.* 1994), *Burkholderia* spp. (Bhushan *et al.* 2000), *Sphingomonas* spp. (Leung *et al.* 1997), and

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Flavobacterium spp. (Raymond and Alexander 1971). Aerobic degradation follows two major pathways; the hydroquinone pathway and the hydroxyquinol pathway (Kitigawa *et al.* 2004). The hydroquinone pathway is seen most often in gram-negative species such as *Moraxella* spp. and *Burkholderia* spp. (Prakash *et al.* 1996; Spain and Gibson 1991), using hydroquinone as an intermediate between PNP and maleylacetate. The alternate hydroxyquinone pathway is found in the gram-positive species such as *Bacillus* spp. and *Arthrobacter* spp. (Jain *et al.* 1994; Kadiyala and Spain 1998) and converts PNP through hydroxyquinol /4-nitrocatechol (Fig. 1.2).

1.5. Bioremediation:

Bioremediation refers to the use of biological processes to solve an environmental pollution or contamination problem. This is an increasingly important scientific challenge as well as an increasing economic business as it offers an inexpensive alternative to physical methods (Sreenivasulu and Aparna 2001). The traditional moving of contaminated soils or other environmental materials to be incinerated requires complicated equipment and a great deal of expense, whereas bioremediation potentially requires little in the way of supplies or equipment and can be performed directly on-site.

Effective bioremediation releases non-toxic end products such as CO_2 and H_2O into the environment that will not be detrimental and that can be easily accommodated by the biome (Atlas and Bartha 1997). A common practice for bioremediation is to add an organism or organisms capable of degrading a specific pollutant or group of pollutants to the desired area, although bioremediation may also be accomplished through manipulation of the environmental parameters to conditions favoring biodegradation (Bartha 1986). Both of these two available methods, however, rely on a specific set of parameters to be fully effective; effectiveness depends on the nature and amount of pollutant, the ambient and seasonal conditions, and the

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composition of the microbial community (Atlas and Bartha 1997). As such, it is much more effective in soils and controllable situations, than oil coated ocean surfaces which are a much more open system. Survivability of the degrading organisms is of concern as well, as they are exposed to numerous competition influences from the multiple organisms resistant to the pollution (although not degrading it) and environmental stresses of the contaminated sites once introduced. For this reason, the examination of biofilms for degrading organisms may provide a an understanding of how to increase their survival, and thus their effectiveness, in cleansing a desired area. In addition to biofilms, RpoS is also known to increase bacterial survival in response to environmental stresses and so the examination of the relationship between RpoS and biofilms may aid in the construction of a more effective bioremediation agent.

1.6. Objectives of the Thesis:

The first objective of this thesis is the construction of a *rpoS*-knockout mutant strain from the PNP-degrading *P. putida* isolated from activated sludge by Spain *et al.* in 1979. This will be achieved though:

(i) Detection and sequencing of the *rpoS* gene belonging to the PNP-degrading *P. putida*, and confirmation of taxonomic identity.

(ii) Construction of a *rpoS*-deficient mutant through engineering of a suicide vector containing an interrupted fragment of this gene followed by subsequent conjugation and homologous crossing-over of the interrupted *rpoS* gene to the wild-type genome.

(iii) Confirmation of both the knockout status of the engineered mutant as well as maintenance of the PNP-degrading ability within the mutant.

The second objective of this thesis is evaluation of the biofilm forming ability of the *rpoS*-knockout mutant in comparison to the original wild-type strain. This will be achieved through:

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(i) Growth and comparison of both mutant and wild-type biofilms through two separate methods of a simple Crystal Violet assay and examination under a confocal microscope combined with statistical analysis of the images through the PHLIP software.

(ii) Comparison of the relative hydrophobicity between the mutant and the wild-type cultures.

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2. Construction of a PNP-degrading *P. putida rpoS*-knockout mutant

2.1. Introduction:

Bioremediation refers to the use of biological processes to solve an environmental pollution or contamination problem. Effectiveness of these processes depends on the nature and amount of pollutant, the ambient and seasonal conditions, and the composition of the microbial community (Atlas and Bartha 1997). Survivability of the pollutant-degrading organisms is of concern, as they are exposed to numerous competition influences from the multiple organisms resistant to the pollution (although not degrading it) and environmental stresses of the contaminated sites. The formation of biofilms provides natural populations with protection against such adversities, and so the examination of biofilms of degrading organisms may provide a means to increasing their survival, and thus their effectiveness, in remediating a desired area.

The stationary phase sigma factor gene, *rpoS,* encodes an alternate sigma factor that directs the transcription of a large subset of bacterial stress-response genes by altering the specificity of RNA polymerase core subunits (Ishihama 2000) and is thus responsible for increased survivability of bacterial cells. Originally identified in *E. coli* and *S. typhimurium*, RpoS has now been identified in *Pseudomonas* and many of the γ-proteobacteria (Venturi 2003). The name "stationary phase sigma factor" came from the initial observation that RpoS activates the expression of genes to maintain cellular viability during the stationary phase of growth (Kolter *et al.* 1993; Loewen and Hengge-Aronis 1994). Recently, RpoS has been shown to play a role in the formation of bacterial biofilms, with *P. aeruginosa rpoS* mutants having increased biofilm production, while both positive and negative effects have been observed for *E. coli rpoS*-mutants depending on experimental conditions (Adams and McLean 1999; Corona-Izquierdo and

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Membrillo-Hernández 2002; Heydorn *et al.* 2002; Prigent-Combaret *et al.* 2001; Schembri *et al.* 2003) and as such may provide insight into aiding bacterial survival at contaminated sites.

Many bacteria have been isolated with the capability to degrade certain environmental pollutants and are considered good candidates for bioremediation. One such pollutant is PNP, which is readily degraded by a number of bacterial species including *Moraxella* spp. (Spain and Gibson 1991), *Bacillus* spp. (Kadiyala and Spain 1998), *Pseudomonas* spp. (Prakash *et al.* 1996), *Arthrobacter* spp. (Jain *et al.* 1994), *Burkholderia* spp. (Bhushan *et al.* 2000), *Sphingomonas* spp. (Leung *et al.* 1997), and *Flavobacterium* spp. (Raymond and Alexander 1971). PNP is an intermediate in the formation of organophosphorus pesticides such as parathion, methyl-parathion and nitrofen, as well as some medical analgesics and fungicides (Boehncke *et al.* 2000). Aerobic degradation follows two major pathways; the hydroquinone pathway and the 4-nitrocatechol/hydroxyquinol pathway (Kitigawa *et al.* 2004).

The formation of genetic knockout mutants has aided bacterial research immensely since the advent of genetic studies. By comparing the effects of a mutant defective in a specific gene to wild-type cultures, theories can be tested in relation to the gene's role under the defined experimental conditions. The aim of this study was to engineer a genetic knockout of the *rpoS* gene in a PNP-degrading *P. putida* (formerly identified as a putative *Moraxella* strain) isolated from activated sludge (Spain *et al.* 1979). This mutant will be used to examine the role of the RpoS gene in the formation of biofilms of *P. putida*.

2.2. Materials and Methods:

2.2.1. Bacterial strains, plasmids, and media: Bacterial strains used were the wild-type and Δ*rpoS* of a PNP-degrading *P. putida*, as well as *E. coli* JM109 and S17-1λ vector hosts. The *P.*

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putida was originally identified to be a putative *Moraxella* strain isolated from an activated sludge (Spain *et al.* 1979), identified through 16S rRNA sequencing. Plasmids used in this study were pCR®2.1-TOPO® cloning vector (Invitrogen, Burlington ON, www.invitrogen.com) in *E. coli* JM109 hosts, pJQ200sk suicide plasmid (Quandt and Hynes 1993) in *E. coli* S17-1λ, and pGEM®-T Easy cloning vector (Promega, Nepean ON, www.promega.com) in *E. coli* JM109 hosts. *E. coli* cells were grown at 37°C in tryptic soy broth (TSB (BBL, Sparks MD)) or on tryptic soy agar (TSA) plates (30g/L TSB, 15g/L agar (FisherBiotech, Fair Lawn NJ)). *P. putida* strains were maintained on TSA at 30°C with 10μg/ml chloramphenicol for the wild-type and TSA with 50μg/ml kanamycin and 10μg/ml chloramphenicol for the *rpoS* mutant. Both *P. putida* strains were grown 24 h in TSB with appropriate antibiotics followed by 24 h in TSB without antibiotics prior to experimentation. Minimal salt medium (MSM) was used when comparing PNP-degrading capabilities (1.25mM KH_2PO_4, 3.73mM K_2HPO_4, 0.4mM $MgSO_4$-$7H_2O$, 0.02mM $FeSO_4$-$7H_2O$, 1.4mM NH_4Cl, pH 7.0).

2.2.2. 16S rRNA sequencing and identification: To determine the taxonomic identity of the putative PNP-degrading *Moraxella* strain isolated by Spain *et al.* (1979), 16S rRNA primers [forward-27bp 5`GTGCTGCAGAGAGTTTGATCCTGGCTCAG; reverse-1492bp 5`CACGGATCCTACGGGTACCTTGTTACGACTT] were used in a PCR reaction to amplify a large (approximately 1.5kb) portion of the 16S rRNA gene. PCR was performed in a 50μl reaction using reagents from Fermentas (Burlington ON, www.fermentas.com): 5μl 10X PCR buffer, 5μl 25mM $MgCl_2$, 5μl 2mM dNTPs, 1U *Taq* DNA polymerase, 1μl each 25μM primer (forward and reverse), 1μl template DNA, and 31μl sterile ddH_2O. Template DNA was prepared as per the Promega Wizard® Genomic DNA Purification kit (Madison WI, www.promega.com).

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The PCR protocol was performed with one initial denaturing cycle of 3 min at 94°C, 30 cycles of denaturing at 94°C for 30 s, annealing at 61°C for 30 s, extending at 72°C for 2 min, and one cycle of extending at 72°C for 10 min with a hold at 4°C. The resulting amplicon of about 1.5kb was sequenced (Mobix, McMaster Univ., Hamilton, ON) and a BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) search performed to determine the taxonomic identity of the PNP-degrading bacterium.

2.2.3. Detection and sequencing of the rpoS gene: To locate the *rpoS* gene of the PNP-degrading *P. putida* strain, a set of universal *rpoS* PCR primers was designed. Known *rpoS* sequences (Table 2.1 and Appendix A) were selected from the GenBank archives (http://www.ncbi.nlm.nih.gov/), and aligned through the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/#). Areas of high homology near the beginning and end of the sequences were chosen as PCR primers [forward 5`GAAGAAGAAGTKYATTTTGCSC; reverse 5`ACCTGRATCTGSCGWACACGY]. Degenerate nucleotides are defined as follows: K=G+T, Y=C+T, S=G+C, R=A+G, W=A+T. PCR was performed on genomic DNA of the *P. putida* strain, extracted as per the protocol of the Promega Wizard® Genomic DNA Purification kit (Madison WI, www.promega.com). PCR was performed in a 25µl reaction mixture containing 2.5µl 10X PCR buffer, 2.5µl 25mM $MgCl_2$, 2.5µl 2mM dNTPs, 0.5µl formamide, 1U *Taq* DNA polymerase, 1µl each 25µM primer (forward and reverse), 1µl 409µg/ml template DNA, and 14.5µl sterile ddH_2O. The PCR protocol was performed with one cycle of 3 min at 94°C, 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 2 min, and one cycle of 72°C for 10 min with a hold at 4°C. Two amplicons of approximately 900bp and 700bp were obtained and gel purified for sequencing (Mobix, McMaster Univ., Hamilton, ON) using the MoBio UltraClean™

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15 DNA Purification Kit From Gels and Solutions (MoBio, Salana Beach, CA, www.mobio.com). BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed to identify the 700bp segment as *rpoS* and the 900bp segment as *rpoD*. Using this fragment, two sets of internal, outward-facing inverse PCR (iPCR) primers were selected [forward1 5`CTCACCGACAAGCAGCGCGA; reverse1 5`ATCGAATTTCTCCACGGCC; forward2 5`GGATAAAACCCTGCTGGACA; reverse2 5`GGTCTGATTCATGATCGCGC]. iPCR was performed on self-ligated products of the genomic DNA digested by either *BamH*I and *Xho*I restriction enzymes. Fifty μg of genomic DNA was digested at 37°C for 2 h with each enzyme, and heat inactivated at 65°C for 10 min. Self-ligations were performed as per a modified Fermentas method (Burlington ON, www.fermentas.com: 200ng digested DNA, 4U T4 ligase, 5μl ligation buffer, dH_2O to 50μl; 22°C 1 h, deactivate at 65°C 10 min). For the initial iPCR reaction, 66ng of the self-ligation was added to the same 50μl PCR reaction mixture as was used in the 16S rRNA protocol above with the forward1 and reverse1 primers. The iPCR protocol was performed with one cycle of 5 min at 94°C, 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 5 min, and one cycle of 72°C for 10 min with a hold at 4°C. Following the initial iPCR, the reaction products were diluted by half with sterile ddH_2O and run through a 50μl nested PCR reaction with forward2 and reverse2 primers, containing the same mixture as the 16S rRNA protocol above. The PCR protocol was one cycle of 3 min at 94°C, 27 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2.5 min, and one cycle of 72°C for 10 min with a hold at 4°C. Of the two different digests and self-ligations performed, the resulting *BamH*I product was cloned into the pGEM®-T Easy cloning vector as per the instructions included with the system and sequenced with SP6 and T7 primers. The *Xho*I product was sequenced directly from the nested PCR

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(Mobix, McMaster Univ., Hamilton, ON). Sequence results from both enzymes were combined to construct the full *rpoS* gene sequence.

Table 2.1: Known *rpoS* sequences by species used in the creation of universal *rpoS* primers for locating and amplifying the unknown *rpoS* sequence of the PNP-degrading *P. putida*.

Species	Accession number	Length	Primer pos. (forward, reverse)
Escherichia coli	AF275947	1101 bp	254, 937
Erwinia carotovora	AJ238884	1004 bp	229, 912
Vibrio cholerae	AF000945	1008 bp	221, 904
Pseudomonas syringae	AF208000	1008 bp	233, 916
Yersinia enterocolitica	U16152	996 bp	221, 904
Salmonella typhi	Y17610	1155 bp	218, 901
Pseudomonas putida	Y19122	1008 bp	236, 919
Pseudomonas fluorescens	I73366	1005 bp	236, 919

2.2.4. Construction of the rpoS-knockout: The *rpoS*-knockout mutant was constructed through use of a suicide vector, pJQ200sk (Quandt and Hynes 1993). The initial 700bp partial *rpoS* fragment was cloned into the pCR®2.1-TOPO® vector as per the product instructions (Invitrogen, Burlington ON, www.invitrogen.com) to form TOPO::*rpoS*. A kanamycin-resistance cassette from the plasmid p34S-Km (Dennis and Zylstra 1998) and the TOPO::*rpoS* were then digested using *Sal*I, and the kanamycin resitance cassette inserted to a *Sal*I site in the middle of the *rpoS* fragment to produce TOPO::*rpoS*::Km. Using the M13 reverse and T7 sequences as primer sites, the 2kb *rpoS*::Km fragment was PCR amplified and digested with *Eag*I, while pJQ200sk was digested with *Not*I. This was done as *Eag*I and *Not*I both share the same sticky-end sequences. Electroporation was used to transform the resulting pJQ200sk::*rpoS*::Km suicide plasmid into the *E. coli* S17-1λ host. Conjugation was performed between the PNP-degrading *P. putida* strain and the pJQ200sk::*rpoS*::Km as follows: 5ml of each overnight culture was spun 10min, 3200x*g* and washed twice with sterile chilled 10mM $MgSO_4$. These were resuspended in 1.0ml of

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10mM $MgSO_4$ and mixed in a 1:1 ratio. Fifty µl of the mixed cultures was dropped on a sterile 0.22µm filter placed on a TSA plate and incubated at 30°C for 24 h. Each filter was vortexed in 5ml sterile 10mM $MgSO_4$, the cells spun down and resuspended in 1ml 10mM $MgSO_4$, and plated to TSA plates with 10µg/ml chloramphenicol and 50µg/ml kanamycin. Resulting colonies were PCR screened as homolgous double cross-overs by both the presence of the 2kb *rpoS*::Km fragment (forward 5`AATCGTGGCTTGTCGCTG; reverse 5`CGGCGTACAACCACCTCG) and the Km resistance cassette (forward 5`TCTCTGATGTTACATTGCACAAGA; reverse 5`GAGGGAGCCACGGTTGATG), as well as the absence of genes *sacB* (forward 5`CGCACTGCTGGCAGGAGGC; reverse 5`GGCTAATGCAAAGACGATGTGG) and *aacC1* (forward 5`GCCCTGCCTCCGGTGCTCGC; reverse 5`GCCTCGGGCATCCAAGCAGC) of the pJQ200sk plasmid. The four different primer sets were run in PCR reactions performed using the same 50µl mixture as the 16S rRNA protocol above. The PCR protocol was one cycle of 3 min at 94°C, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2.5 min, and one cycle of 72°C for 10 min with a hold at 4°C.

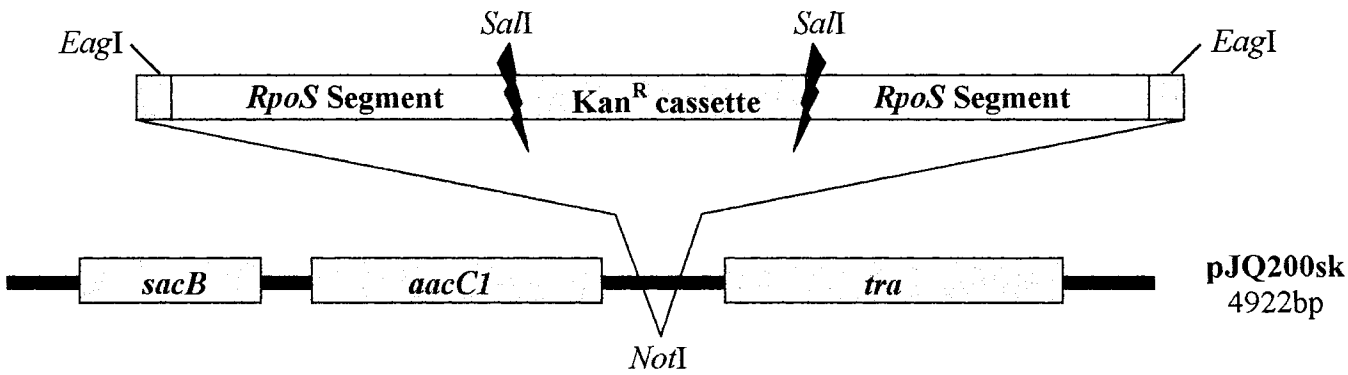

Figure 2.1: Insertion of the constructed 2kb *rpoS*::Km into the pJQ200sk plasmid through ligation of sticky ends from an *Eag*I and a *Not*I digestion. The *rpoS*::Km insert formed in pCR®2.1-TOPO® vector by first cloning in the 700bp partial *rpoS* fragment, then interrupting the fragment with a kanamycin resistance cassette.

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2.2.5. Growth curve determination: Knockout and wild-type cultures were grown for 24 h at 30°C, 100 rpm in 50ml TSB with 10μg/ml chloramphenicol (wild-type) or 10μg/ml chloramphenicol + 50μg/ml kanamycin (Δ*rpoS*). One ml of this was added to 50ml fresh TSB in a 200ml flask and grown an additional 24 h at 30°C and shaken at 100 rpm to ensure prolific growth of both cultures before analysis. To begin the curve, 1.0 ml of the non-antibiotic culture was transferred to 50ml fresh TSB in a 200ml flask and set for growth at 30°C and shaken at 100 rpm. One ml samples were taken at timed intervals, beginning at time=0. Cell density (CFU/ml and OD_{600nm}) was determined for each sample and graphed.

2.2.6. PNP degradation assay: Knockout and wild-type cultures were grown to mid log-phase growth (approximately 6 h, 50ml TSB in a 200ml flask at 30°C and shaken at 100rpm) and spun for 10min at 3200x*g*. Cells were washed once in sterile MSM and resuspended to an OD_{600nm} of 0.2. One ml of the OD_{600nm} 0.2 suspension was added to 50ml MSM supplemented with 150μM PNP and incubated at 30°C and shaken at 200 rpm. One ml samples were taken at timed intervals starting at time=0 and the cells pelleted. Five hundred μl of the supernatant was mixed with 500μl 0.5M NaOH and the OD_{420nm} recorded (MSM+NaOH blank). Readings were compared to a prepared standard curve and the degradation graphed.

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2.3. Results:

2.3.1. 16S rRNA sequencing and identification: The PNP-degrading putative *Moraxella* strain isolated by Spain *et al.* (1979) was identified through 16S rRNA genetic sequencing to be a strain of *P. putida*. The resulting partial 16S rRNA sequence returned a BLASTn search (http://www.ncbi.nlm.nih.gov/BLAST/) having a 99% homology to known *P. putida* 16S rRNA sequences (Figure 2.2).

```
1       TGAACGCTGG CGGCAGGCCT AACACATGCA AGTCGAGCGG ATGAAGAGAG CTTGCTCTCT
61      GATTCAGCGG CGGACGGGTG AGTAATGCCT AGGAATCTGC CTGGTAGTGG GGGACAACGT
121     TTCGAAAGGA ACGCTAATAC CGCATACGTC CTACGGGAGA AAGCAGGGGA CCTTCGGGCC
181     TTGCGCTATC AGATGAGCCT AGGTCGGATT AGCTAGTTGG TGAGGTAATG GCTCACCAAG
241     GCGACGATCC GTAACTGGTC TGAGAGGATG ATCAGTCACA CTGAAACTGA GACACGGTCC
301     AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT GGGCGAAAGC CTGATCCAGC
361     CATGCCGCGT GTGTGAAGAA GGTCTTCGGA TTGTAAAGCA CTTTAAGTTG GGAGGAAGGG
421     CAGTAAGCGA ATACCTTGCT GTTTTGACGT TACCGACAGA ATAAGCACCG GCTAACTCTG
481     TGCCAGCAGC CGCGGTAATA CAGAGGGTGC AAGCGTTAAT CGGAATTACT GGGCGTAAAG
541     CGCGCGTAGG TGGTTTGTTA AGTTGGATGT GAAAGCCCCG GGCTCAACCT GGGAACTGCA
601     TTCAAAACTG ACAAGCTAGA GTATGGTAGA GGGTGGTGGA ATTTCCTGTG TAGCGGTGAA
661     ATGCGTANAT ATAGGAAGGA ACACCANTGG CGAAGGCNAC CACCTGGACT GATACTGACA
721     CTGANGTGCG AAAGCGTGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
781     CGATGTCAAC TAGCCGTTGG GAGCCTTGAG CTCTTAGTGG CGCAGCTAAC GCATTAAGTT
841     GACCGCCTGG GGAGTACGGC CGCAAGGTTA AAACTCAAAT GAATTGACGG GGGCCCGCAC
901     AAGCGGTGGA GCATGTGGTT TAATTCGAAG CAACGCGAAG AACCTTACCA GGCCTTGACA
961     TCCAATGAAC TTTCCAGAGA TGGATNGGTG CCTTCGGGAA CATTGAGACA GGTGCTGCAT
1021    GGCTGTCGTC AGCTCGTGTC GTGAGATGTT GGGTTAAGTC CCGTAACGAG CGCAACCCTT
1081    GTCCTTAGTT ACCAGCACGT TATGGTGGGC ACTCTAAGGA GACTGCCGGT GACAAACCGG
1141    AGGAAGGTGG GGATGACGTC AAGTCATCAT GGCCCTTACG GCCTGGGCTA CACACGTGCT
1201    ACAATGGTCG GTACAGAGGG TTGCCAAGCC GCGAGGTGGA GCTAATCCCA TAAAACCGAT
1261    CGTAGTCCGG ATCGCAGTCT GCAACTCGAC TGCGTGAAGT CGGAATCGCT AGTAATCGCG
1321    AATCAGAATG TCGCGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG
1381    GGAGTGGGTT GCACCAGAAG TAGCTAGTCT AACCTTCGGG AGGACGGTTA CCACGGTGTG
1441    ATTCATGACT GGGGG
```

Figure 2.2: Sequence of 16S rRNA 1455bp PCR amplicon identified as having a 99% homology to *P. putida*. N=ambiguous base-pair identification.

2.3.2. rpoS gene sequence: The PNP-degrading *P. putida rpoS* gene was located through use of universal primers designed by a multiple-sequence alignment of known *rpoS* genes. Following the amplification of a 700bp partial *rpoS* fragment, iPCR was utilized to determine the complete *rpoS* gene sequence. The resulting genetic sequence showed an 88% homology to known

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Pseudomonas chloraphis rpoS sequences (AY586457.1, AY336077.1) and 87% homology to known *P. fluorescens rpoS* sequences (CP000076.1, U34203.1) through a BLASTn search (http://www.ncbi.nlm.nih.gov/BLAST/) (Figure 2.3). The homology values for the PNP-degrading *P. putida rpoS* sequence to known *P. putida rpoS* sequences (AF178851.1, X91654.1) ranged between 82-85% homology. Translated to a protein sequence (Fig. 2.4), homology was 97% to *P. clororaphis* RpoS (AAP97086.1), 95% to *P. fluorescens* RpoS (AAB02846.1), and 91% to *P. putida* RpoS (CAB46191.1, AAN67244.1, NP 743780.1, AAF05319.1).

```
1       TCGGGTGGAC TTATGAGCTT GAGGTCGAAC TCACCAAAGG GACTATAACA ATGGCTCTCA
61      GTAAAGAAGT GCCGGAGTTT GACATCGACG ATGAGGTTCT CCTGATGGAG ACCGGCATCG
121     ATACGGATAC GATGTCGAAT GATGAAGGGG CGGCTCCACC TTCCGTTCGT GCCAAATCCA
181     AACACTCCGC TTCGCTAAAG CAACACAAAT ACATTGACTA CACGCGGGCA CTCGATGCCA
241     CGCAGCTGTA TCTCAATGAG ATCGGTTTTT CCCCACTATT GTCCCCAGAA GAAGAAGTTC
301     ATTTTGCGCG ACCGTCGCAA AGTGGCGATC CGGCCGGGCG CAAACGCATG ATTGAAAGTA
361     ACCTGCGGCT GGTGGTGAAA ATCGCCCGGC GTTACGTCAA TCGTGGCTTG TCGCTGCTGG
421     ATCTGATCGA AGAGAGCAAC CTTGGCTTGG TCAG                   CCCGAAC
481     GTGGCTTCCG CTTCTCGACC TACGCAACCT GGTGGATTCG TCAGACCATC GAGCGCGCGA
541     TCATGAATCA GACCCGGACC ATCCGGCTGC CGATCCATGT GGTCAAAGAG CTCAACGTGT
601     ACCTGCGGGC CGCACGGGAG CTGACGCAGA AGCTTGATCA TGAACCCTCA CCCGAAGAAA
661     TCGCCAACCT GCTGGAAAAA CCGGTGGGAG AGGTCAAGCG CATGCTGGGC TTGAACGAGC
721     GGGTGTCTTC GGTCGACGTC TCGCTGGGTC CGGATTCGGA TAAAACCCTG CTGGACACCC
781     TGACTGACGA TCGTCCAACC GATCCATGTG AACTGCTGCA GGATGACGAC CTGTCCCAGA
841     GCATCGATCA GTGGCTGTCG GAA                   GGTGGTT GTACGCCGCT
901     TCGGCCTGCG CGGCCATGAG AGCAGCACCC TCGAAGACGT AGGCCTGGAA ATCGGCCTGA
961     CCCGCGAACG GGTCAGACAG ATTCGGGTTG AAGGCCTGAA GCGTCTTCGT GAGATCCTGG
1021    AGAAAAACGG CCTGTCGAGC GAGTCGCTGT TTCAATAACA GACTCGCGTA GATGGCAACA
1081    AAAC
```

Figure 2.3: Sequence of the *rpoS* gene from the PNP-degrading *P. putida* strain isolated by Spain *et al.* (1979). ATG=start codon, TAA=termination codon, blue=universal primer, =iPCR primer set 1, red=iPCR primer set 2.

```
1       MALSKEVPEF DIDDEVLLME TGIDTDTMSN DEGAAPPSVR AKSKHSASLK QHKYIDYTRA
61      LDATQLYLNE IGFSPLLSPE EEVHFARPSQ SGDPAGRKRM IESNLRLVVK IARRYVNRGL
121     SLLDLIEESN LGLVRAVEKF DPERGFRFST YATWWIRQTI ERAIMNQTRT IRLPIHVVKE
181     LNVYLRAARE LTQKLDHEPS PEEIANLLEK PVGEVKRMLG LNERVSSVDV SLGPDSDKTL
241     LDTLTDDRPT DPCELLQDDD LSQSIDQWLS ELTDKQREVV VRRFGLRGHE SSTLEDVGLE
301     IGLTRERVRQ IRVEGLKRLR EILEKNGLSS ESLFQ*
```

Figure 2.4: Protein sequence of the RpoS from the PNP-degrading *P. putida* strain isolated by Spain *et al.* (1979).

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2.3.3. rpoS-knockout formation: The *rpoS*-knockout mutant was formed by interrupting the 700bp partial *rpoS* fragment with a kanamycin resistance cassette, placing this construct into the suicide vector pJQ200sk (Quandt and Hynes 1993) and causing a homologous cross-over into the PNP-degrading *P. putida* genome. Colonies showing kanamycin resistance and gentamicin susceptibility were further examined through PCR-amplification of four genetic sequences. The Δ*rpoS* isolate showed the presence of the desired 2kb *rpoS*::Km construct and the 1.3kb kanamycin resistance cassette, as well as the absence of the 282bp *sacB* suicide gene fragment

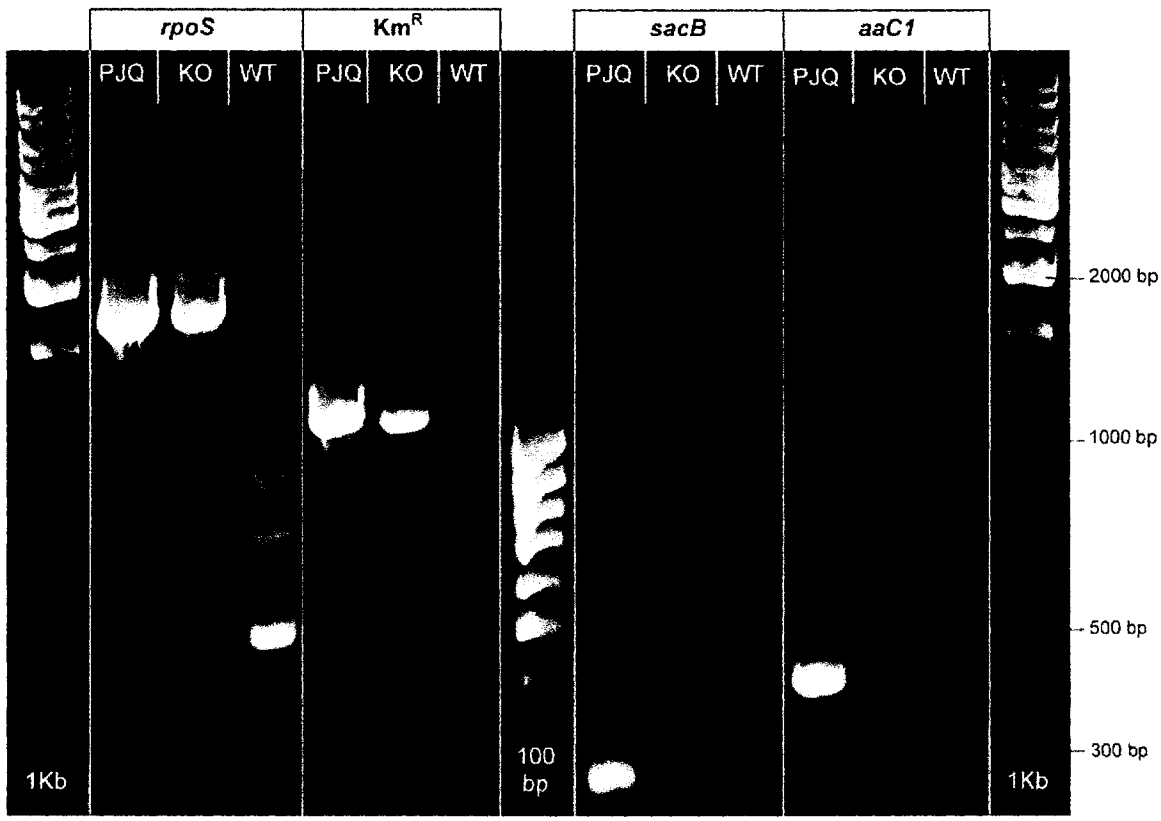

Figure 2.5: PCR confirmation of double homologous crossover of the *rpoS*::Km fragment into the wild-type PNP-degrading *P. putida* genome. Reaction sets are grouped as *rpoS* = 2kb *rpoS*::Km fragment, Km^R = 1.3kb kanamycin resistance cassette, *sacB* = 282bp suicide gene fragment, *aacC1* = 431bp gentamicin resistance gene fragment. 1Kb = 1Kb ladder, 100bp = 100bp ladder, PJQ = pJQ200sk::rpoS::Km, KO = Δ*rpoS* isolate, WT = wild-type *P. putida*.

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and the 431bp *aacC1* gentamicin resistance gene fragment of the pJQ200sk plasmid (Figure 2.5 - KO). None of the four target sequences were amplified from the wild-type control reactions (WT). All sequences were amplified from the pJQ200sk::*rpoS*::Km constructed suicide vector control reactions (PJQ).

2.3.4. Growth curve comparison: Growth curves were generated for both the Δ*rpoS* and wild-type isolates (Figure 2.6). The curves followed closely through the log and early stationary phases, beginning to diverge in late stationary/early death phase at 120 h. The Δ*rpoS* showed a quicker decline in death phase with complete death by 156 h; a 5 log difference from the wild-type culture. From a paired t-test of the times from 120-156 h, the results showed a significant difference at the 95% confidence level (P=0.054, T=3.08).

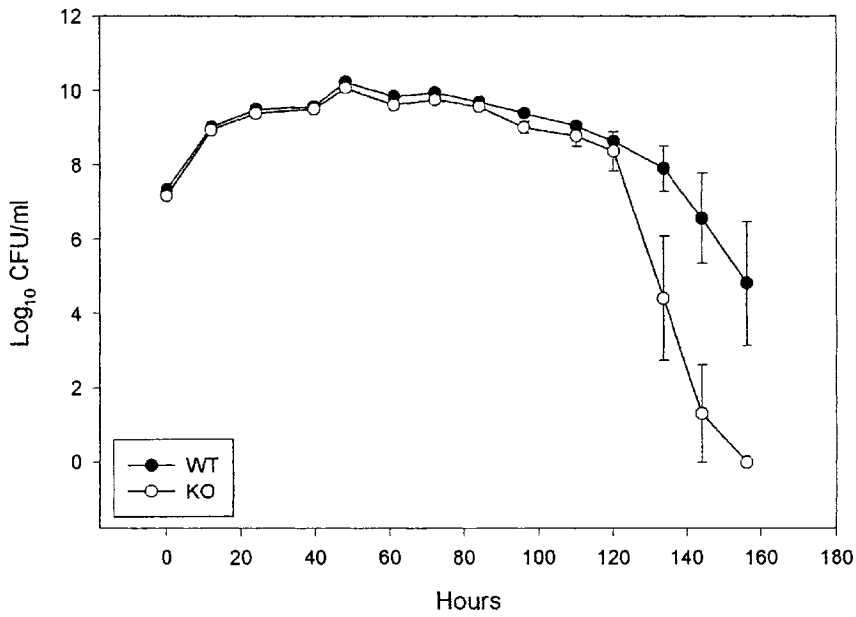

Figure 2.6: Growth curves for the wild-type and *rpoS*-knockout mutant cultures. Values shown are the means of determinations from 4 separate growth experiments. Standard error shown.

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*2.3.5. **PNP-degradation comparison***: PNP-degradation curves were generated for both the Δ*rpoS* and wild-type cultures (Figure 2.7). The curves both showed induction of the degradation at 6 h, with the wild-type completing the degradation within 9.5 h, approximately 1 h before the Δ*rpoS* culture. A paired t-test of the times from 6 h to 11 h showed the curves to have a significant difference at the 95% confidence level (P=0.016, T=2.88).

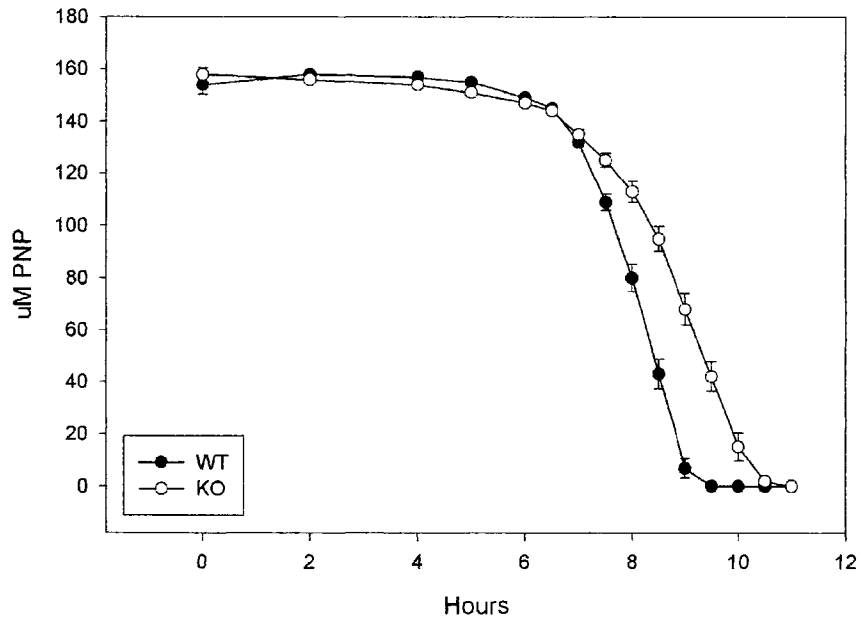

Figure 2.7: PNP-degradation curves for the wild-type and *rpoS*-knockout mutant cultures. Values are the means of 10 determinations taken between two experiments having 5 replicates in each experiment. Standard error shown.

2.4. Discussion:

RpoS is a master regulator for the stress responses of a number of γ-proteobacteria including *E. coli* and *Pseudomonas* spp. (Venturi 2003) and has also been implicated as having a role in biofilm formation (Adams and McLean 1999; Corona-Izquierdo and Membrillo-Hernández 2002; Heydorn *et al.* 2002; Prigent-Combaret *et al.* 2001; Schembri *et al.* 2003). Based on the 87-88% homology of the determined *rpoS* sequence to the known *rpoS* sequences

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of *Pseudomonas* spp., it is appropriate to claim the gene located though universal primers is indeed *rpoS*. Work was not continued on the 900bp fragment that co-amplified with the 700bp fragment, and BLASTn results showed this to have an 86% homology to the *rpoD* vegetative sigma factor of *P. fluorescens*. From this it can also be claimed that the gene targeted and interrupted is indeed the desired stationary-phase sigma factor gene.

Following conjugation between the *P. putida* and pJQ200sk::*rpoS*::Km, PCR screening confirmed the replacement of the *P. putida rpoS* gene by the interrupted *rpoS*::Km construct through amplification of both the desired 2kb full insert and the kanamycin resistance cassette itself. The inability to amplify either the *sacB* or the *aacC1* genes from the suicide plasmid indicated the absence of the plasmid in the suspect knockout isolate. This suggested that there had been a homologous double crossover effectively interrupting the *rpoS* gene, and not a single cross-over event that had inserted the suicide plasmid in its entirety into the *P. putida* genome.

When the growth curves of the Δ*rpoS* and the wild-type cultures were compared, it was shown that the survivability of the knockout mutant was compromised following stationary phase (Fig. 2.5). As RpoS is responsible for the induction of a large range of stress-response and stationary phase genes related to increased survival, this result was expected. The same reduction of longevity has been observed in many other studies involving cultures deficient in the *rpoS* gene (Lange and Hengge-Aronis 1991; Suh *et al.* 1999).

The effect of *rpoS* deletion was also examined in relation to the PNP-degrading capabilities of the *P. putida* strain. Although comparison of the degradation curves between the knockout mutant and the wild-type cultures did show a significant statistical difference, the relevance of this difference is likely not substantial in application. Were the knockout mutant to be used in a bioremediation situation PNP degradation would still occur, and on a grand scale the

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difference of approximately 1 h before complete degradation is likely to be negligible although such situations need to be examined under physiochemical and environmental conditions to see the full effects. As such, the confirmed double cross-over *rpoS* knockout mutant does maintain the PNP-degrading capabilities and will be appropriate for use in further studies related to the *rpoS* role in biofilm formation to the end goal of possible bioremediation.

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3. Examination of *rpoS* inactivation effects on biofilm formation of a PNP-degrading *P. putida*

3.1. Introduction:

The stationary phase sigma factor gene, *rpoS*, encodes an alternate sigma factor which directs the transcription of a large subset of bacterial stress-response genes through altering the RNA polymerase core specificity (Ishihama 2000) and is thus responsible for increased survivability of bacterial cells. Originally identified in *E. coli* and *Salmonella typhimurium*, RpoS has now been identified in *Pseudomonas* and many of the γ-proteobacteria (Venturi 2003). The name "stationary phase sigma factor" came from the initial observation that RpoS activates the expression of genes to maintain cellular viability during the stationary phase of growth (Kolter *et al.* 1993; Loewen and Hengge-Aronis 1994).

A number of studies have shown the *rpoS* gene to play an important role in the biofilm formation of both *E. coli* and *P. aeruginosa* and as such this gene may provide insight into aiding bacterial survival at contaminated sites. Although results from studies involving *E. coli* are conflicting concerning whether *rpoS* enhances or reduces biofilm production (Adams and McLean 1999; Corona-Izquierdo and Membrillo-Hernández 2002; Schembri *et al.* 2003), results from studies on *Pseudomonas* spp. are more consistent and show that biofilms of *rpoS* deficient mutants produce a thicker biofilm more quickly than their wild-type counterparts (Heydorn *et al.* 2002; 2000a; 2000b). The purpose of this study is to examine the effects of *rpoS* deletion on biofilm formation of a PNP-degrading *P. putida* isolated from activated sludge (Spain *et al.* 1979).

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3.2. Materials and Methods:

*3.2.1. **Bacterial strains and media:*** Bacterial strains used were the wild-type and Δ*rpoS* of a PNP-degrading *P. putida*. The *P. putida* was originally believed to be a putative *Moraxella* strain isolated from an activated sludge (Spain *et al.* 1979), identified to species through 16S rRNA sequencing (data from chapter 2). *P. putida* strains were maintained on TSA (30g/L TSB, 15g/L agar (FischerBiotech, Fair Lawn NJ)) at 30°C with 10μg/ml chloramphenicol for the wild-type and TSA with 50μg/ml kanamycin and 10μg/ml chloramphenicol for the mutated strain. Both *P. putida* strains were grown 24 h in TSB (BBL, Sparks MD) with appropriate antibiotics followed by 24 h in TSB without antibiotics prior to experimentations.

3.2.2. *Crystal Violet biofilm assay*: Initial comparison of the biofilm forming ability of the two cultures was examined in glass test tubes through a modified method of O'Toole *et al.* (1999). Four and a half ml of TSB was mixed with 0.5ml of a 24 h culture. Tubes were incubated as triplicate sets for 24, 48, 72 and 96 h at 30°C without shaking, along with blanks of 5.0ml uninoculated TSB. Following incubation, 250μl of 0.1% (w/v) Crystal Violet was added and incubated at room temperature for 10min, rinsed thoroughly with water to remove residual dye and detached cells and 5ml of 95% ethanol added for 10min to solubilize the dye. Tubes were vortexed to fully mix the dye and ethanol prior to reading. Absorbance readings were taken at A_{590nm} in triplicate for each tube. The results were graphed and statistics calculated.

*3.2.3. **Biofilm formation***: Examination of biofilm formation was performed on sterile glass coverslips. A single coverslip was placed in a standard disposable Petri-dish containing 10.0ml sterile TSB with 200μl of a 24 h culture of either Δ*rpoS* or the wild-type and incubated at 30°C

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and 50rpm. Three coverslips were prepared for each incubation length of 24, 48, 72, 96, and 120 h. Spent media was removed every 24 h and replaced with 10.0ml fresh TSB. Following incubation, coverslips were rinsed with sterile deionized water to remove residual media and unattached cells and the upwards facing surface stained with 0.1% (w/v) acridine orange for 3 min. The stained coverslips were inverted onto concave slides filled with sterile deionized water to allow a hydrated viewing of the biofilms. Confocal microscopy images were taken, 5 Z-stacks per slide, with 3 slides per incubation length. Image analysis was performed using the computer program PHLIP (www.phlip.org) run through Matlab 7.0 (The MathWorks, Natick, MA, www.mathworks.com) for biovolume, percent surface coverage, thickness and roughness parameters. These were graphed and statistics calculated.

3.2.4. Hydrophobicity assay: Examination of the hydrophobicity for the wild-type and the *rpoS* deficient mutant cultures was performed using a modified bacterial adhesion to hydrocarbon (BATH) assay (Sweet *et al.* 1987). Cultures were grown in 50ml TSB at 30°C and shaken at 100rpm and samples taken at 6, 24 and 48 h growth. Two tubes of 3 ml were removed from each of four replicate growth flasks. One tube from each flask was vortexed with 0.1ml *n*-hexadecane for 20 s (A_b), the second tube was vortexed with no hydrocarbon (A_c). The phases were allowed to separate for 20 min, and the A_{600nm} read using a light spectrophotometer. Adhesion to hydrocarbon (i.e. hydrophobicity) was calculated as (%) = $(A_c - A_b)/A_c$ X 100.

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3.3. Results:

3.3.1. Crystal Violet biofilm assay: A bar graph was generated for each of the four incubation times of 24, 48, 72 and 96 h (Figure 3.1). Greater absorbance values were observed with the wild-type after 24 h of growth, however this reversed by 48 h and greater absorbance values were observed for the Δ*rpoS* mutant from 48 h through to 96 h (T=8.65, P=0.0113).

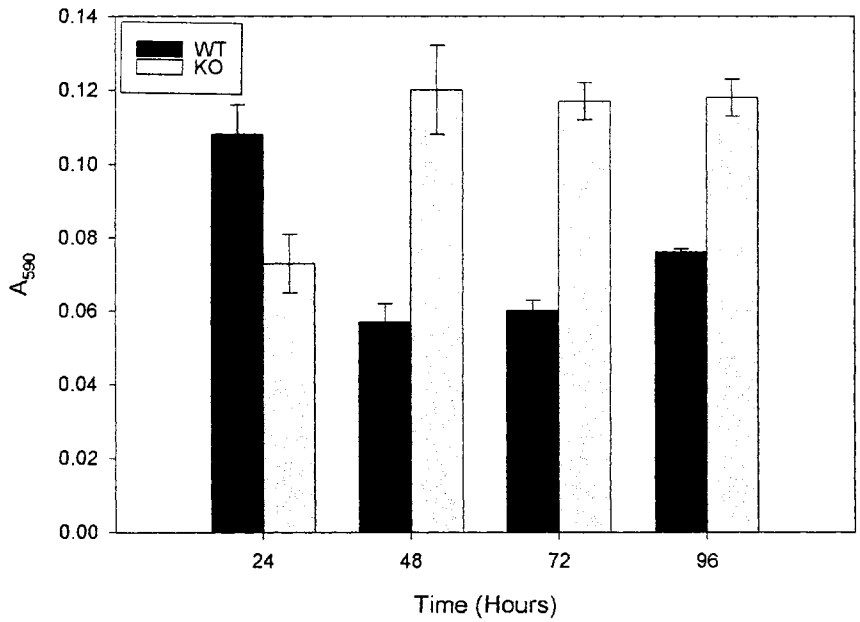

Figure 3.1: Absorbance at 590nm for the wild-type and *rpoS*-knockout mutant in relation to Crystal Violet staining of biofilm formation in glass test tubes at 30°C without shaking. Values are the means of repeated trials done in triplicate; standard error shown.

3.3.2. Biofilm formation: Confocal microscopy images were taken for each time period of 24, 48, 72, 96, and 120 h of incubation, three slides per culture at each time, with the experiment repeated in triplicate. As seen in Figure 3.2, the biofilms of the knockout-mutant were consistently more luxuriant than those of the wild-type cultures throughout the experiment. In addition, a higher level of microcolony formation, EPS production, and mature, interlinked mushroom-mound structures were observed in the knockout-mutant biofilm images. The initial

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formation of these structures were only observed late in the experiment for the wild-type biofilms, shown in the upper-centre of the 120 h wild-type image (Figure 3.2).

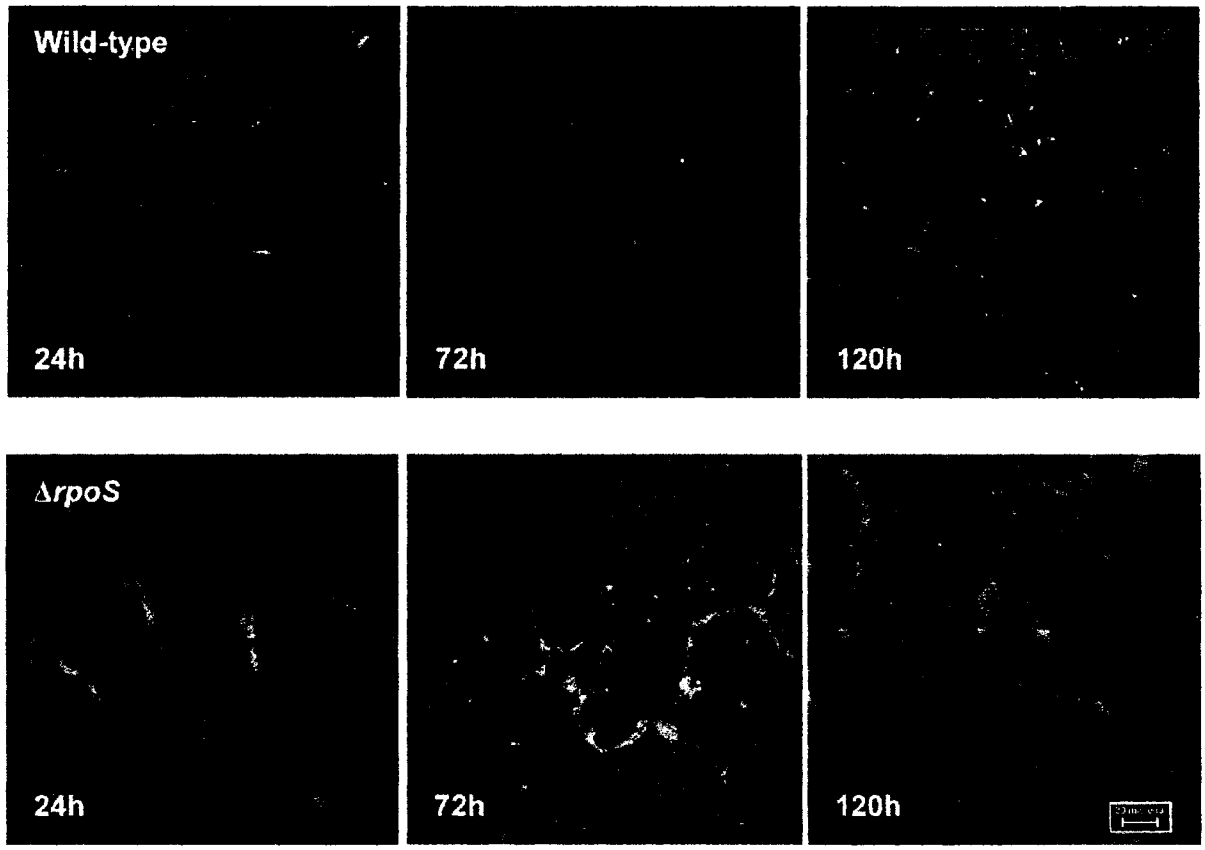

Figure 3.2: Confocal 2D images of the hydrated wild-type and Δ*rpoS* biofilms stained with 0.1% (w/v) acridine orange at 24, 72, and 120 h incubation. Scale indicates 30 microns length.

Through PHLIP analysis of the multiple z-slice images obtained from all three experiment repeats, additional striking differences were seen between the knockout-mutant and the wild-type biofilms. For all parameters of biovolume, substratum coverage, biofilm thickness and biofilm roughness, results for the knockout-mutant were considerably higher throughout the experiment (Figure 3.3), beginning with the first sampling after 24 h incubation.

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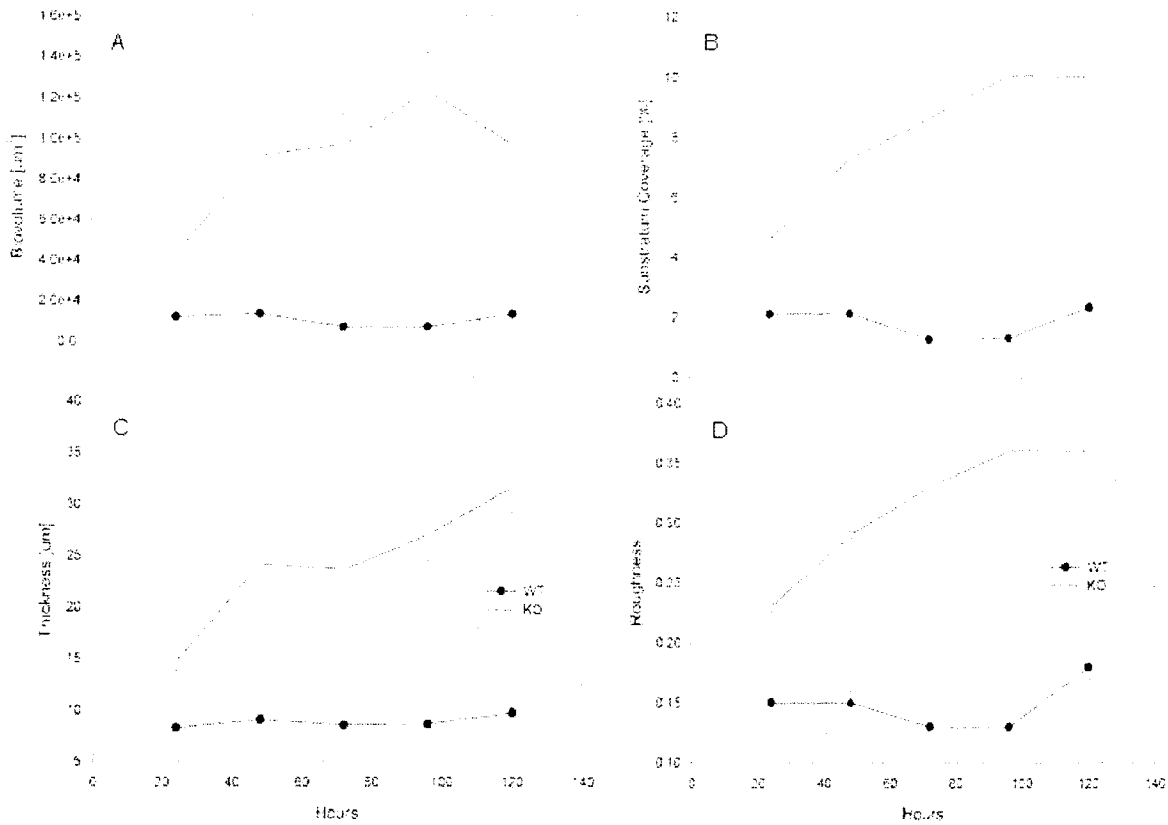

Figure 3.3: Summarized data from PHLIP analysis of wild-type and Δ*rpoS* biofilm characteristics: (A) biovolume, (B) percentage substratum coverage, (C) biofilm mean thickness, and (D) biofilm roughness. Values are the means of 40 z-slice confocal images from three repeat trials for each time period. Standard error shown.

3.3.3. Hydrophobicity assay: Hydrophobicity analysis comparing the wild-type and the knockout-mutant cultures was performed using a modified BATH method (Sweet *et al.* 1987). Neither the knockout or the wild-type culture adhered to the *n*-hexadecane hydrocarbon at 6 h incubation, while at 24 h incubation the percent adhesion of both was approximately 1.0%, showing no significant difference at the 95% confidence level with a two-sample t-test (P=0.648, T=0.47) (Fig. 3.4). Adhesion to hydrocarbon decreased for the knockout-mutant (less hydrophobic) at 48 h incubation, a statistically significant difference from the wild-type culture at the 95% confidence level (Two sample t-test, P=0.011, T=3.00). However, considering the

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percent adhesion of the two cultures is only 1% and the graphed standard deviations overlap, they are not practically different.

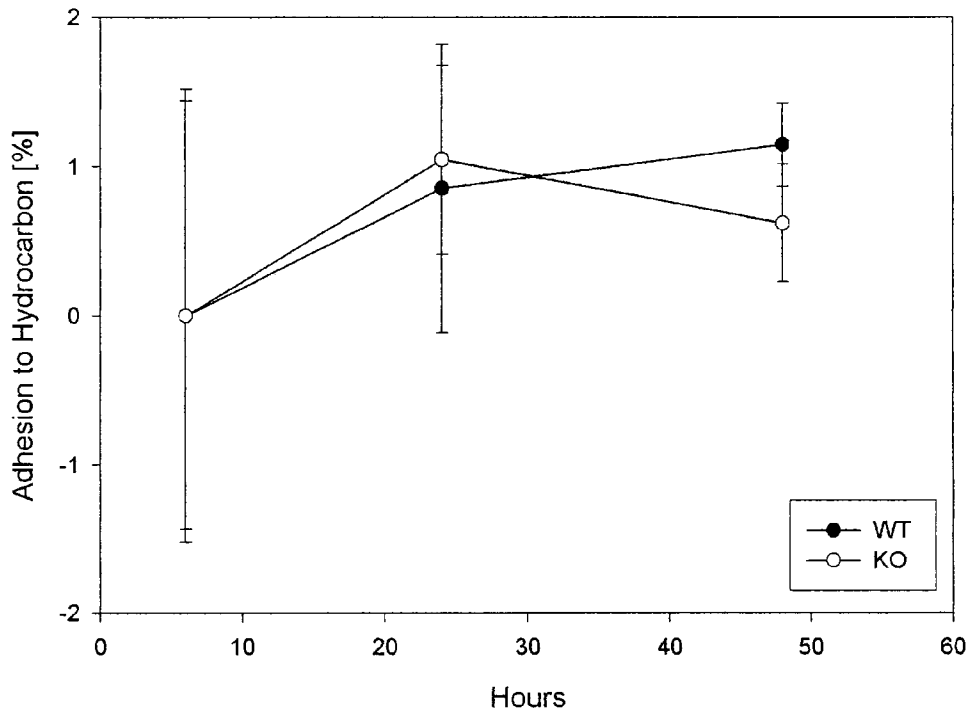

Figure 3.4: Hydrophobicity results for wild-type and *rpoS*-knockout mutant cultures at 6, 24, and 48 h, displayed as percent bacterial adhesion to hydrocarbon. Values are the means of 2 experiments, each with four replicates. Standard deviation shown.

3.4. Discussion:

RpoS is a master regulator for the stress responses of a number of γ-proteobacteria including *E. coli* and *Pseudomonas* spp. (Venturi 2003) and has also been implicated as having a role in biofilm formation (Adams and McLean 1999; Corona-Izquierdo and Membrillo-Hernández 2002; Heydorn *et al.* 2002; Prigent-Combaret *et al.* 2001; Schembri *et al.* 2003). With the knowledge that both RpoS and biofilms play important roles for the survival of bacteria in the environment, an understanding of how the master regulator RpoS affects biofilm formation may provide important insight into aiding bioremediation efforts.

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In the initial examination of the biofilm forming capabilities of the wild-type and the *rpoS*-knockout mutant using a simple Crystal Violet assay, it was observed that the mutant produced a much higher level of biofilm after 24 h. This result was similar to the findings of Corona-Izquierdo and Membrillo-Hernández (2002), who found greater biofilm development by a *rpoS* deficient *E. coli* mutant. These preliminary observations suggest RpoS plays a significant role in overall biofilm development of the *P. putida* strain, although by the nature of the assay, observations are limited to inference of attached cell numbers alone and no other biofilm characteristics.

Looking closer, confocal microscopy images reveal a similar trend in the biofilms formed by the wild-type and the knockout mutant when compared to previous findings. In accordance to those observations made by Heydorn *et al.* regarding *P. aeruginosa rpoS* mutant biofilm growth in flow-chambers (2002; 2000a; 2000b), the biofilms of the *rpoS* mutant developed much faster, and were significantly thicker than those of the wild-type. Throughout the 120 h experiment, the wild-type biofilm remained sparse with very little micro-colony formation, EPS, or mature mushroom-mound structures. The mutant, however, showed a great deal of microcolony formation as early as 24 h incubation. These biofilms communities contained a greater biovolume, and more highly developed mushroom-mound structures linked with multiple cell and EPS bridges.

Combined with PHLIP analysis, the confocal images allow for a more detailed picture of the differences between the wild-type and the knockout mutant biofilms. Again, in all aspects examined, the knockout mutant showed a higher level of biofilm development than the wild-type strain. Since more mushroom-mound structures were observed, the mutant biofilm roughness and thickness were expected to be much greater than the sparse wild-type biofilms, which was

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the case. The roughness observations contrast those of Heydorn *et al.* (2002) whose flow-chamber knockout biofilms were thicker but of equal roughness to the wild-type. However, it is very possible that differences in the experimental procedures may be responsible. A flow-chamber would have provided a steady laminar flow across the biofilm surface, whereas the rotational movement used in this study may have provided appropriate conditions that might favor roughness.

Concerning the observed higher biovolume of the knockout biofilm compared to the wild-type biofilm the wild-type; in chapter 2 it was shown that the planktonic exponential and stationary phase growth rates of both were equal and so had an equal biovolume of cells. Thus, in biofilm physiology, RpoS does have an effect which may be an altering of doubling time. This effect of RpoS is not present in planktonic growth as observed in chapter 2. It has been suggested that RpoS may limit macrocolony size and biofilm thickness in an attempt to maintain cell viability within the nutrient-limited central regions of a mature biofilm, where diffusion is reduced (Toutain *et al.* 2004). Such a theory holds well with both the role of RpoS as a stress-response regulator and with the observed biovolume differences between the *rpoS*-knockout mutant and the wild-type PNP-degrading *P. putida* strain of this study.

An alternative theory to the observed biovolume difference being caused through differential growth rates in the biofilm state is a possible difference in bacterial attachment between the wild-type and the knockout mutant. When comparing the biovolume and the Crystal Violet results, it was thought perhaps the unexpected observation at the 24 h incubation point may be due to initial attachment differences, as the biovolume experiment was performed with shaking and the Crystal Violet without. Added to this was the observed lower percentage

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substratum coverage for the wild-type, suggesting a reduced ability for bacterial attachment which may have delayed biofilm growth under shaking conditions.

In examining biofilm development, it is understood that hydrophobic interactions between the cell surface and the substratum do have an effect on the initial reversible-attachment period (Hasty and Courtney 1996; Teixeira and Oliveira 1999). No significant differences were observed between the two cultures at 6 and 24 h incubation, after which time attachment for both the knockout and wild-type was observed under the confocal microscope. By 48 h incubation both the wild-type and mutant hydrophobicites were still within the 1% range, and at this point all biofilm and Crystal Violet assay results were similar.

Recent hydrophobicity studies regarding bacterial/surface relationships have further defined the interactions and suggest that although the substrate hydrophobicity plays an important role, there is no relationship between the bacterial surface hydrophobicity and the extent of initial binding. Indeed, biofilm development was not dependent on the extent of the initial bacterial adhesion (Araujo *et al.* 2004; Cerca *et al.* 2005) and was more likely to depend on cell-to-cell attachments. Thus, the discrepancy at 24 h is more likely a result of factors not related to the initial attachment, as are the overall differences in biovolume and substrate coverage.

In conclusion, deletion of *rpoS* does have a noticeable effect on biofilm development of the PNP-degrading *P. putida* strain, and does not interfere with the PNP-degrading capabilities. Biovolume, percent substratum coverage, thickness and roughness were significantly greater in the *rpoS*-knockout mutant, and do not appear to be related to possible hydrophobicity differences between the two cultures. The survival implications of this increase in biofilm forming

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capabilities through *rpoS* deletion require further examination, and are promising for further bioremediation efforts.

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4. Appendix

4.1. Acknowledgements

This research was supported by a Natural Science and Engineering Research Council of Canada (NSERC) Discovery Grant granted to K.T. Leung. We thank Dr. J.C. Spain for providing the PNP-degrading *P. putida* strain.

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4.2. CLUSTAL W (1.82) multiple sequence alignment

```
E. COLI          ATGTTCGTTAAGGGATTAGGGTAGGAGCCACCTTATGAGTCAGAATATGTTGAAAGTT  60
S. TYPHI         ------------------------------------ATGAGTCAGAATACGTTGAAAGTT  24
E. CAROTOVAORA   ------------------------AGGAGCCAC-TTATGAGCCAAAGTATGTTGAAAGTT  35
Y. ENTERO        ------------------------------------ATGAGCCAAAATACGCTGAAAGTT  24
V. CHOLERAE      ----------------------------ATGAGTGTCAGCAATACCGTAACCAAAGTA  30
ALT. P. FLUOR    ----------------------------ATGGCTCTCAATAAAGAAGCGCCGGAGTTT  30
P. PUTIDA        ----------------------------ATGGCTCTTGGCAACGAAGCGCCGGAGTTT  30
P. SYRING        ----------------------------ATGGCTCTCAGTAAAGAAGTGCCGGAGTTT  30
                                              *     *           *  *

E. COLI          CATGATTTAAATGAAGA--TGCGGAATTTGATGAGAACGG-------AGTTGAGGTTTTT  111
S. TYPHI         CATGATTTAAATGAAGA--CGCGGAATTTGATGAGAACGG-------AGTAGAGGCTTTT  75
E. CAROTOVORA    AACGAGTTACATGAAGA--GACCGATTTCGACGAAAATGG-------ACTTGACGTTTTT  86
Y. ENTERO        AACGAGTTGCATGAAGA--TGCTGATTTTGATGAAAACAGTACGG-AAACTGAAATTTTC  81
V. CHOLERAE      GAAGAGTTCGATTTTGA--AGATGAAG-CACTGGAAGTGC---------TAGAAACTGAT  78
ALT. P. FLUOR    GACATCGACGATGACGTCCTCCTAATGGAGACGGGCATCGTTTTGGAAACGGATGTGGTG  90
P. PUTIDA        GACATCGATGATGAAGTGCTCCTTATGGAAGCCGGCATTGTCCTG-AAGGAAGCGTCAAA  89
P. SYRING        GACATCGACGATGAAGTTCTCCTTATGGAGACGGATACCGCCGCT-GATTCGATGTCGAA  89
                  *        **   *        *

E. COLI          GACGA----AAAGGCCTTAGTAGAAGAGGAACCCAGTGATAACGATTTGGCCGAAGAGGA  167
S. TYPHI         GACGA----AAAAGCCTTGAGTGAAGAGGAACCCAGTGATAACGACCTGGCTGAAGAAGA  131
E. CAROTOVORA    GACGA----TAAAGCGCTGGCAGAGGAAGATACCAATGATAGTGACTCGGCGGAAGACGA  142
Y. ENTERO        GATGA----AAAAGCATTAGTAGAAGATGAACCTACTGAAAGCGAGTTAGCAGAAGATGA  137
V. CHOLERAE      GCCGA----GCTCACCAGTGATGAAGAATTAGTTGCTGTTGAAGGGGCAAGTGAAGACGT  134
ALT. P. FLUOR    TCAGACG-AACCTGCTGTACCTTCGGTTCGGACCAGGGCCAAGTCCGGCTCTTCGCTCAA  149
P. PUTIDA        CGAGAAGCAGCCAGCCGCTGTCTCGGGACGTACCAAGGTCAAGCATTCAACATCGCTCAA  149
P. SYRING        TGAGG---GATCTGCTGTACCTTCAGTTCGTGCCAAATCCAAACACTCCGCTTCATTGAA  146
                    *          *          *

E. COLI          ACTGTTATCGCAGGGAGCCACACAGCGTGTGCTGGACGCGACTCAGCTTTACCTTGGTGA  227
S. TYPHI         GCTGTTATCGCAAGGGGCCACACAGCGTGTGTTGGACGCGACTCAGCTTTACCTTGGTGA  191
E. CAROTOVORA    GCTGTTATCGCAAGGGGTCCCACAGCGTGTTTTAGACGCAACACAGCTCTATTTGGGAGA  202
Y. ENTERO        GCTGTTGGCGCAAGGTGTTAC-CAGCG-GTGCT-GATGCGACACAGCTCTATCTTGGTGA  194
V. CHOLERAE      TCGTGAAGAGTTTGATGCTTCTGCGAAAAGTCTTGATGCGACCCAGATGTATCTCAGCGA  194
ALT. P. FLUOR    GCAGCACAAGTACATCGATTACACTCGGGCACTTGATGCTACCCAGTTGTACCTCAACGA  209
P. PUTIDA        ACAACACAAGTACATTGACTACACGCGGGCGCTCGATGCTACCCAGCTGTACCTCAATGA  209
P. SYRING        ACAACATAAATACATTGATTACACGCGGGCGCTCGACGCGACGCAGTTGTATCTCAATGA  206
                  *              *                * ** ** ** *** * **  *    **

E. COLI          GATTGGTTATTCACCACTGTTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTCGCGCACT  287
S. TYPHI         GATTGGGTATTCACCACTGTTAACAGCCGAAGAAGAAGTCTATTTTGCGCGTCGCGCACT  251
E. CAROTOVORA    GATCGGCTATTCGCCGCTTTTAACCGCAGAAGAAGAAGTTTATTTTGCCCGACGCGCGCT  262
Y. ENTERO        GATTGGTTATTCGCCGTTGTTGACCGCAGAAGAAGAGGTTTATTTTGCCCGGCGTGCATT  254
V. CHOLERAE      AATTGGTTTTTCACCGCTCCTTACTGCCGAAGAAGAAGTGCTTTATGCTCGTCGTGCCTT  254
ALT. P. FLUOR    AATCGGCTTCTCCGCCTCTGCTGTCGCCGGAGGAGGAAGTGCATTTTGCGCGCCTGTCGCA  269
P. PUTIDA        GATCGGTTTTTCTCCTCTATTGTCCCCGGAAGAAGAAGTTCATTTTGCGCGTCTGTCGCA  269
P. SYRING        AATCGGCTTTTCCCCATTGCTCTCTCCGGAAGAAGAAGTTCATTTTGCGCGTCTTTCACA  266
                  ** ** *  ** **  *  *  *  CGGAAGAAGAAGTTCATTTTGC (Forward Primer)
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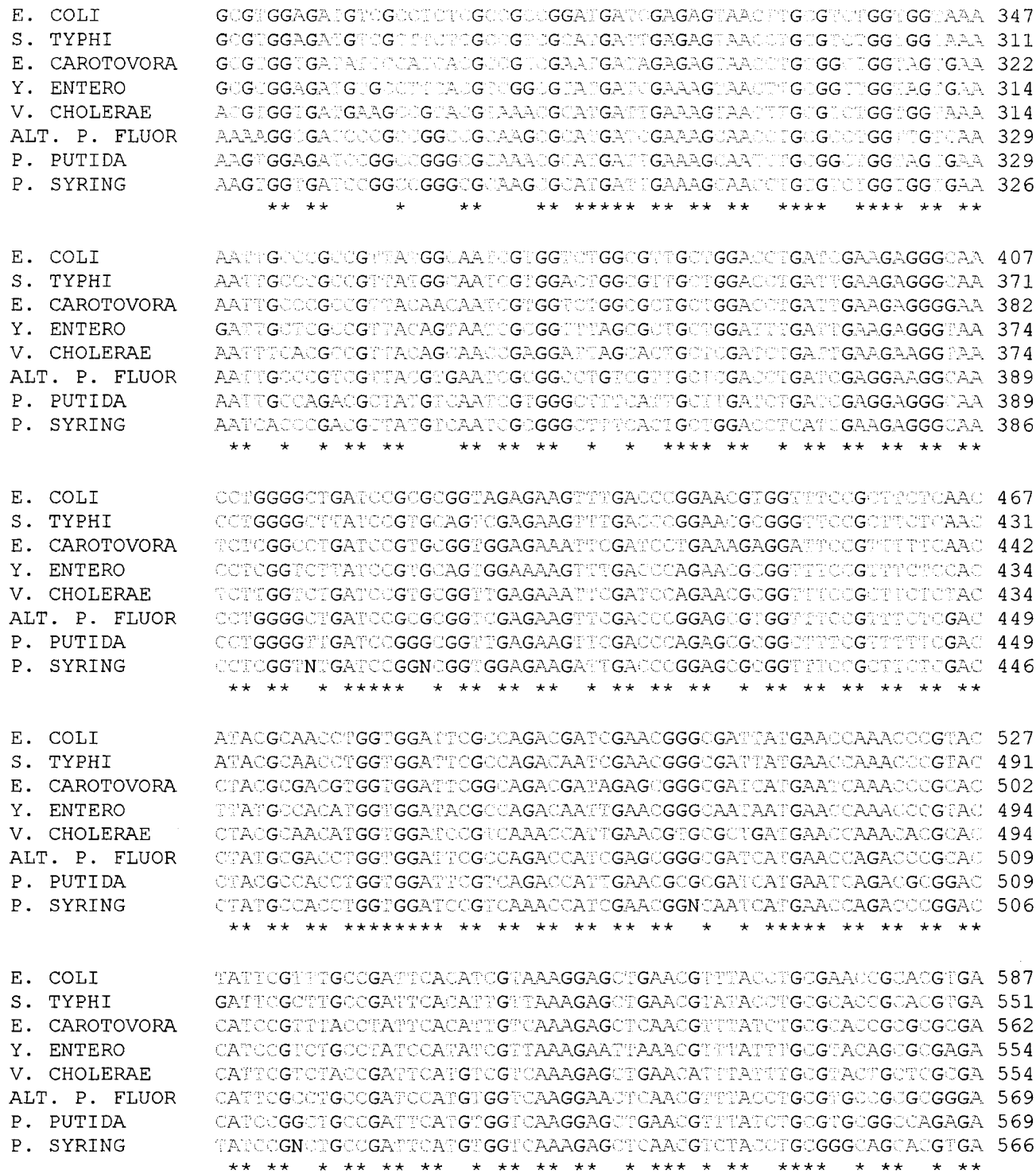

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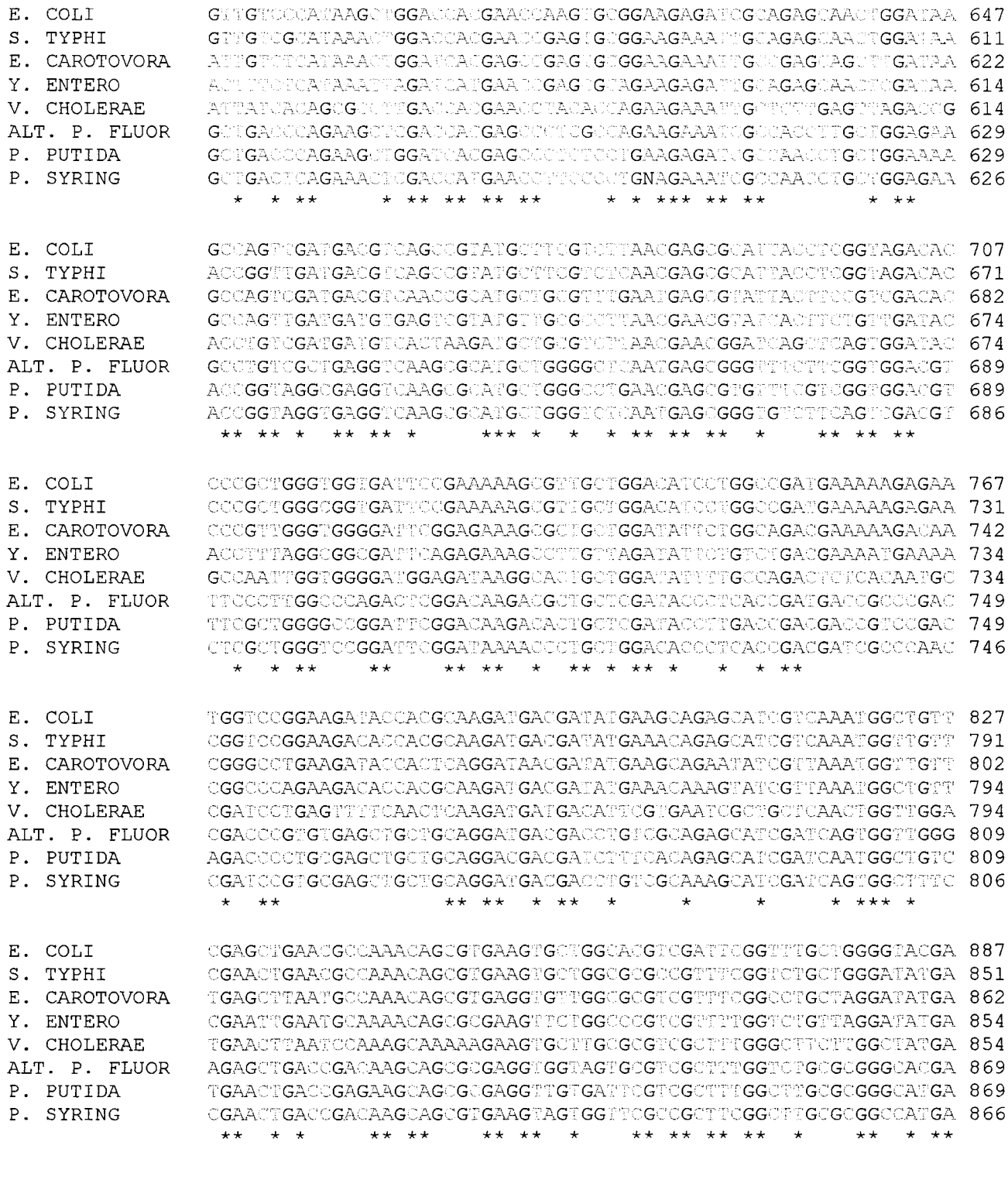

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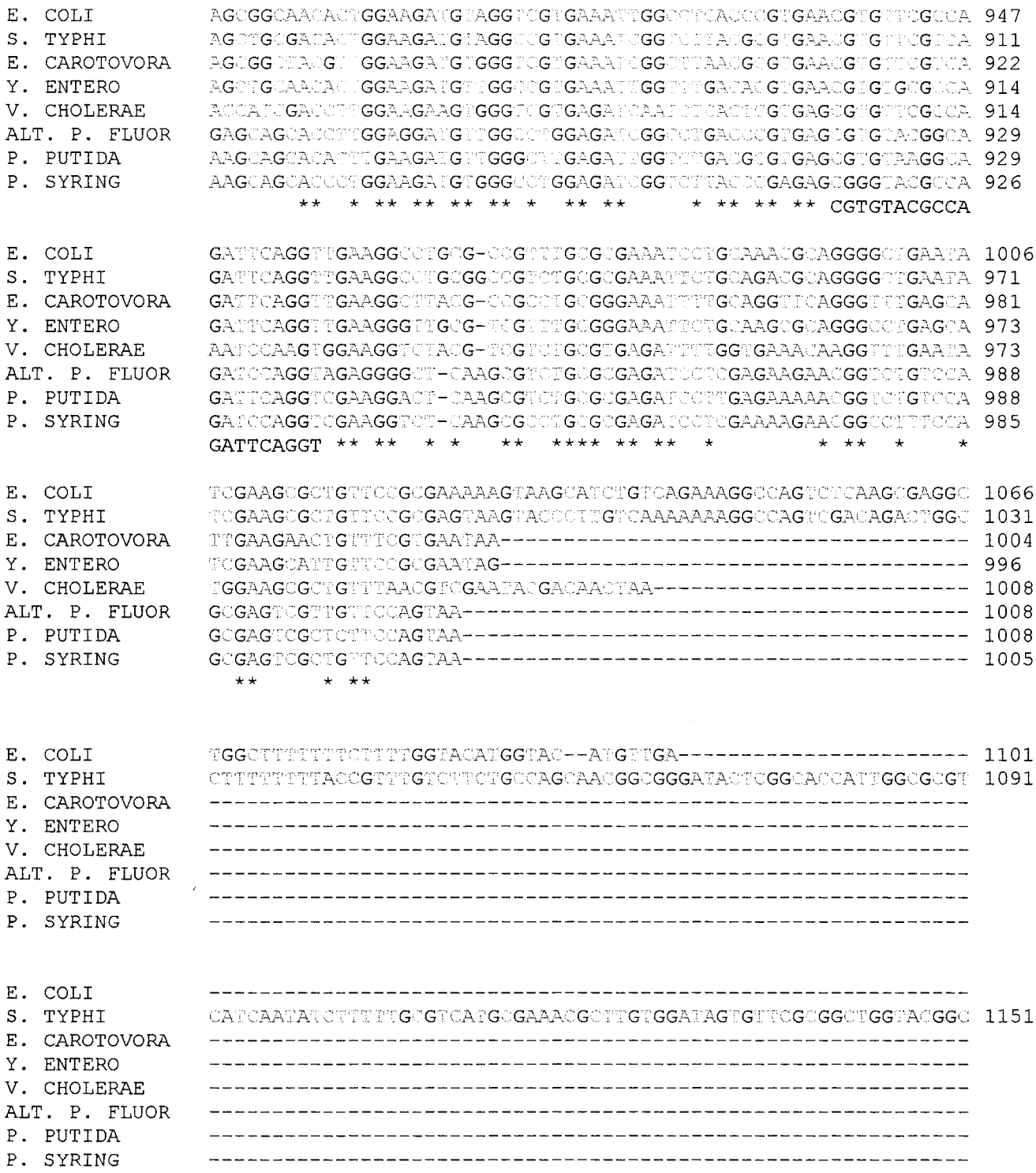

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