Phyllosphere-mediated bacterial degradation of airborne phenol

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Phyllosphere-mediated bacterial degradation of airborne phenol

by

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Program of Study Committee:
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Abstract

Bacterial degradation of organic pollutants in soil has been studied extensively but little is known about the availability and degradation of pollutants by bacterial resident on leaf surfaces. Here, we examined availability and degradation of phenol on leaves by introduced cells of a known phenol degrader and by natural microbial communities. Phenol-responsive gfp-expressing *Pseudomonas fluorescens* A506 bioreporter cells detected airborne phenol as well as phenol that had been adsorbed by leaf surfaces. The degradation of airborne $^{14}$C-phenol by leaf-associated bacteria showed that *Pseudomonas sp.* strain CF600 released eight times more $^{14}$CO$_2$ than a non-degrading mutant after introduction onto leaves. This study provided the first direct evidence of degradation of an organic air pollutant by leaf-associated bacteria. We evaluated phenol degradation by natural microbial communities on green ash leaves that were collected from a field site rich in airborne organic pollutants. We found that significantly more phenol was mineralized by these leaves when the communities were present than by these leaves following surface sterilization. Thus, phenol-degrading organisms were present in these natural communities and were metabolically capable of phenol degradation. We isolated and identified phenol-degrading bacterial isolates within these natural microbial communities. Ten phenol-degrading bacterial isolates were obtained that were most closely related to the genera *Alcaligenes*, *Acinetobacter* and *Rhodococcus*. The genetic characterization of these isolates revealed little diversity as phenol hydroxylase (PH) enzymes of all of the Gram-negative isolates were type II PHs, which have a moderate affinity for phenol, and these isolates harbored the *ortho* pathway of phenol degradation. Lastly, we attempted to understand the mechanism of phenol toxicity and tolerance in bacteria by comparing the transcriptional profiles of cells exposed to phenol and cells exposed to agents causing membrane and oxidative stress using the leaf-associated bacterial species, *Pseudomonas syringae* pv. tomato. Phenol resulted in the induction of heat shock genes that were not induced by the other stresses tested. The role of these genes in cellular and membrane protein damage in response to phenol is discussed.
General Introduction

Bacterial availability and degradation of organic pollutants has been studied extensively in the rhizosphere. Microbial population sizes and their pollutant degradation activity are generally much higher in the rhizosphere than in bulk soil due to the release of oxygen and nutrients by plant roots. Pollutants are brought into the rhizosphere from the surrounding soil by the plant roots via the transpiration stream. Pollutants can be translocated to shoots via evapotranspiration where they may undergo various fates, such as degradation by the plant enzymes to less toxic metabolites, compartmentalization or localization into specific plant tissues, and volatilization back into the air. Volatile organic compounds (VOCs), such as toluene and phenol, can also be taken up directly from air by leaves or adsorbed onto the leaf surfaces. Plant leaves make up a vast surface area of the terrestrial habitat and are known to be colonized by bacterial populations as high as $10^7$-$10^8$ cells per g of leaf. Therefore, leaf surfaces of terrestrial plants could provide an excellent venue for promoting microbial access to VOCs. In the present study, we evaluated the ability of bacteria to access and degrade airborne organic pollutants on plant leaves by using phenol as a model compound. This was done not only with phenol degraders that were introduced onto leaf surfaces but also with naturally occurring bacterial communities on leaves taken from a site rich in airborne organic compounds. In addition, we attempted to isolate, identify and genetically characterize phenol-degrading bacterial isolates within the natural leaf-associated microbial communities. Identification and molecular characterization of the catabolic genes of pollutant-degrading bacterial communities in contaminated environments is important to determine the major players in degradation as well as the functional and genetic diversity of the degradation pathways involved. This information can be helpful in developing effective bioremediation strategies as well as in better understanding the microbial ecology of pollutant-rich sites. Because effective bioremediation of pollutants by bacteria requires the survival and maintenance of large populations in pollutant-rich sites, it is important to understand the mechanisms of toxicity in bacteria as well as the mechanisms that bacteria employ to tolerate stresses imposed by these toxic compounds. Previous studies have shown that phenol affects bacterial cell membranes, disrupting their integrity and function. Phenol has also been
suggested to cause oxidative stress in some bacterial species due to the action of enzymes catalyzing its degradation. To obtain a comprehensive understanding of the effects of phenol on bacterial cells, and to determine the toxicity and tolerance mechanisms of bacteria to phenol, we performed a transcriptome analysis of bacteria exposed to phenol, membrane stress, and oxidative stress. A model for the action of phenol was developed based on the expression data obtained.

**Dissertation organization**

The dissertation is divided into five chapters. The first chapter provides a review of the literature on the availability and degradation of airborne phenol by leaf-associated bacteria, and the mechanism of phenol toxicity and tolerance in bacteria. The second chapter presents studies on the availability and degradation of airborne phenol by leaf-associated bacteria and phenol degradation by naturally occurring microbial communities from leaves. This chapter was published in *Environmental Microbiology* and was highlighted in the research highlights section of *Nature Reviews in Microbiology*. The third chapter describes the isolation, identification, and phylogenetic and genetic characterization of phenol-utilizing bacterial communities from leaves taken from a site rich in airborne volatile organic compounds. The fourth chapter presents the transcriptional profiling of the leaf-associated bacterial species *Pseudomonas syringae* in response to phenol to understand the mechanism of tolerance to, and toxicity of, phenol in these bacteria. This was done by comparing the transcriptome profiles of bacterial cells exposed to phenol and cells exposed to membrane stress (caused by sodium chloride and polyethylene glycol), and oxidative stress (caused by hydrogen peroxide). The fifth chapter contains my general conclusions and my possible future directions.
Chapter 1. Literature Review

Bacterial degradation of pollutants has been extensively studied in the rhizosphere. It is known that the rhizosphere harbors higher bacterial populations than the bulk soil due to the nutrients and oxygen released by plant roots. These microbial populations have been shown to play a major role in the degradation of pollutants brought into the rhizosphere from surrounding soil by the transpiration stream. The pollutants present in the soil can also be taken up by roots and translocated via the transpiration stream to the leaves, where these pollutants can undergo various fates, such as localization into specific plant parts, transformation into less toxic metabolites by plant enzymes, or volatilization back into the air. Leaves can also take up pollutants directly from the air. Because leaf surfaces are often colonized by bacteria, with populations as high as $10^7$-$10^8$ cells per gram, these leaf-associated bacteria may be able to access and degrade pollutants. The degradation of pollutants by leaf-associated bacteria remains unexplored. In the present study, we hypothesized that phyllosphere bacteria may have access to and degrade the pollutants taken up by leaves, and tested this using phenol, an organic pollutant, as a model compound. We also attempted to identify and genetically characterize the phyllosphere bacteria responsible for degradation of phenol on leaves and to understand the mechanism of toxicity of phenol in a bacterial cell.

Plant uptake of pollutants and pollutant availability to leaf-associated bacteria.

Bacterial degradation of pollutants has been extensively studied in the rhizosphere (Kuiper et al., 2004). Roots not only provide a nutrient-rich habitat for microbes, but also release secondary metabolites that can induce microbial genes involved in pollutant degradation or can act as co-metabolites to facilitate microbial degradation (Gilbert and Crowley, 1997; Rentz et al., 2005). Plants can also mobilize and accumulate considerable amounts of pollutants from the surrounding soil (Harvey et al., 2001); these pollutants can exert a positive selection pressure for pollutant-degrading bacteria in the rhizosphere (Miya, 2000) or in endophytic sites in the roots or throughout the plant (Siciliano et al., 2001).
Plants can mediate the removal of organic pollutants from the environment not only by extracting pollutants from soil into the roots but also by translocating them to the shoots via the transpiration stream. For example, polyaromatic hydrocarbons such as pentachlorophenol have been shown to be taken up by a few plant species, including rice (Weiss et al., 1982), spinach and soybean (Casterline et al., 1985), and to be distributed in both roots and shoots after root uptake. A radiolabeled pesticide, bromacil, and two industrial waste compounds, phenol and nitrobenzene, were also shown to be taken up by soybean roots and translocated to the shoots when provided in hydroponic solution (McFarlane et al., 1987). Plants can take up organic compounds not only from soil through roots but also from air through leaves. The compounds can penetrate leaves in two ways: through stomata or through the cuticle of the epidermis. Stomatal uptake of gaseous pollutants is a major uptake pathway for small inorganic compounds such as sulfur dioxide, nitrogen oxides, and ozone (Slovik et al., 1996). *Phaseolus vulgaris* leaves exposed to gaseous ammonia in leaf chambers were also shown to take up ammonia via stomata whereas transport via the cuticle was negligible (Van Hove et al., 1987). Another study done to measure the uptake of a gaseous organic compound, $^{14}$C-toluene, by soybean leaves demonstrated that majority of the toluene provided was taken up by the stomatal pathway (Jen et al., 1995). The second route by which organic compounds penetrate leaves is the cuticle. The cuticle, being a lipophilic polymer membrane covering leaf surfaces, has been shown to take up or adsorb lipophilic compounds such as polychlorinated biphenyls (Barber et al., 2002) and polyaromatic hydrocarbons such as anthracene (Wild et al., 2004; Wild et al., 2005). Using two-photon excitation microscopy, Wild et al. (2004; Wild et al., 2005) could track the movement of anthracene, provided as an aqueous solution to intact maize leaves, through the epicuticular wax and plant cuticle and finally into the cell cytoplasm of maize leaves. These studies demonstrated that anthracene initially formed a thin band in the epicuticular wax, suggesting that cuticular uptake can result in the accumulation of organic compounds before diffusion into the epidermal cells. The other studies suggesting the sorption of organic compounds to the cuticle were performed with isolated plant cuticles. For example, Shafer and Schonherr (1985b) showed that organic compounds, such as phenol, 2-nitrophenol and 4-nitrophenol, sorbed onto the isolated cuticles of various plant species and suggested that this sorption was
due to hydrogen-bonding between the phenol and the free hydroxyl groups in the cutin polymer.

Leaves are colonized by a variety of microorganisms, including bacteria, yeasts and filamentous fungi, with bacteria being the most numerous (Hirano and Upper, 2000; Lindow and Brandl, 2003). Leaves often support bacterial populations as high as $10^7$-$10^8$ cells per gram of leaf tissue (fresh weight). Like rhizosphere microbial communities, phyllosphere microbial communities are comprised of many distinct genera and species, with bacteria of the genus *Pseudomonas* widespread in both phyllosphere and rhizosphere communities (Legard et al., 1994; Benizri et al., 2005). Bacteria in this genus, along with those in related genera, exhibit a high level of metabolic diversity. Moreover, they are common among the bacteria known to degrade organic pollutants (Gulensoy and Alvarez, 1999; Whiteley et al., 2001), suggesting that pollutant degraders may be present on leaves as they are on roots. In addition, the majority of the leaf-associated bacteria are located on the leaf surface in depressions such as epidermal cell wall junctions and along the veins, although some, primarily pathogens, may establish large populations in the intercellular spaces. The bacteria present on leaf surfaces may, thus, have access to organic pollutants adsorbed by leaf surfaces. Some previous studies have suggested a role for leaf-associated bacteria in the removal of pollutants from air. For example, bacteria present on leaves of a common indoor plant have been shown to augment the removal of an airborne organic pollutant, toluene (De Kempeneer et al., 2004). Similarly, genetically modified endophytic *Burkholderia cepacia* reduced the amount of toluene volatilized from the aerial parts of plant by 50-70% after uptake from hydroponic solution containing toluene (Barac et al., 2004). These studies suggest that bacteria resident on or within the above ground regions of plants, i.e., the phyllosphere, may play an important role in removing organic pollutants from the environment.

**Phenol - a model organic pollutant.**

In the present study, we evaluated the availability and degradation of airborne organic pollutants by leaf-associated bacteria using phenol as a model compound. Phenol is a priority
pollutant of the Environmental Protection Agency and is found in both indoor and outdoor air. It is a component of the emissions from wood, coal, and municipal solid waste combustion, as well as automobile exhaust and cigarette smoke (ATSDR, 1998). Moreover, it is an abundant pollutant generated by microbial communities during the anaerobic decomposition of livestock waste and is a major contributor to the odor intensity of animal wastes (Zahn, 2001). Moreover, several distinct phenol degradation pathways have been identified in *Pseudomonas* species (Feist and Hegeman, 1969; Herrmann et al., 1995; Whiteley et al., 2001), as well as in other bacterial species (Hughes et al., 1984; Arai et al., 1999; Duffner et al., 2000; Kim et al., 2002), supporting the possibility that phenol-degrading bacteria are widespread. Diverse phenol-degrading bacteria, such as *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Acinetobacter*, *Variovorax*, *Comamonas*, and *Klebsiella*, have been isolated from the natural microbial communities of various ecosystems, including wastewater, rivers, soils and marine environments (van Schie and Young, 1998; Bastos et al., 2000; Abd-El-Haleem et al., 2002; Arutchelvan et al., 2005; Garcia et al., 2005; Geng et al., 2006).

**Phenol degradation and genetic characterization of phenol-degrading bacteria.**

Aerobic bacterial phenol degradation occurs via two main pathways, *ortho* and *meta*. The first step that involves the hydroxylation of phenol to catechol is common for both the pathways and is catalyzed by phenol hydroxylase. Catechol is a common catabolic intermediate formed during the degradation of a variety of aromatic compounds, such as benzene, toluene, and naphthalene. Catechol is then metabolized via the *ortho* or *meta* pathway via the enzymes catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O), respectively, resulting in intermediates used for energy generation via the tricarboxylic acid pathway, pyruvate metabolism and glycolysis. Bacteria can possess one or both of these pathways and in organisms with both, the expression of each pathway appears to be determined by the chemical nature of the aromatic substrate that the bacteria are grown with (Feist and Hegeman, 1969).
Genetic characterization of pollutant-degrading bacterial communities is important to identifying the structure and function of these communities in the environment. Phenol hydroxylase, the first enzyme in the phenol catabolic pathway, catalyzes the conversion of phenol into catechol, which is a rate-limiting step in phenol degradation. Phenol-degrading Gram-negative bacteria have been categorized into three groups based on the conserved amino acid residues of the large subunit of the phenol hydroxylase gene (LmPH). The three distinct kinetic groups of Gram-negative bacteria are groups I, II and III, which exhibit high, moderate and low affinity for phenol, respectively. Group-specific monitoring of phenol hydroxylase genes in contaminated soils has been shown to be important in predicting the catabolic potential of indigenous TCE-degrading bacterial populations (Futamata et al., 2001a; Watanabe et al., 2002) and thus could help in developing effective bioremediation strategies. No such grouping has been suggested for Gram-positive bacteria.

The type of phenol catabolic pathway present in bacteria can be determined by the presence of C12O, an enzyme for the ortho pathway, or C23O, an enzyme for the meta pathway. In previous studies, catechol dioxygenase genes have been used as targets to determine the composition and behavior of microbial communities present in a polluted environment (Sei et al., 2004). Specifically, Sei et al (2004) used these genes to monitor the behavior of aromatic compound-degrading bacterial populations and changes in microbial community structure in a sea water microcosm and found that bacterial populations possessing C12O genes were the primary degraders of phenol and benzoate and C23O-harboring bacteria dominated only under higher substrate concentrations. These studies demonstrate that genetically characterizing communities can identify the functionally relevant bacterial populations and indicate changes in microbial community structure in a contaminated environment.

**Bacterial responses to phenol.**

*Pseudomonas syringae* was used as a model bacterial species for transcriptome studies for examining bacterial responses to phenol. *P. syringae* is both an epiphyte and a
pathogen colonizing a wide range of plant species. Enumeration of the *P. syringae* populations on healthy plant leaves has shown that 75 to 90% of the bacteria were epiphytic, i.e., residing on the leaf surfaces (Hirano and Upper, 2000). Epiphytic bacteria are exposed to various harsh and fluctuating environmental conditions such as variable temperatures, humidity, and UV radiation as well as toxic airborne pollutants. These conditions can adversely affect bacterial survival and their population sizes on leaf surfaces. Because effective removal of airborne pollutants from the environment by leaf-associated bacteria requires survival and maintenance of good population sizes, it is important to understand the mechanisms of toxicity and tolerance of epiphytic bacteria to the toxic pollutant compounds. Therefore, in the present study we used *P. syringae* as a model epiphytic bacterial species to study the effects of phenol on cellular physiology using transcriptome analysis.

Organic solvents, such as phenol, benzene, and toluene have been suggested to affect bacterial cells by partitioning into the cell membrane resulting in the disruption of membrane integrity and function. Most studies have suggested that bacterial cell membranes are modified in response to organic solvents. For example, the exposure of bacteria to phenol results in changes in cell membrane fatty acid composition by cis-to-trans isomerization of the fatty acids (Heipieper et al., 1992). It has been suggested that cis-to-trans conversion increases membrane ordering and consequently decreases the membrane fluidity (Sikkema et al., 1995). Most studies have examined the effect of phenol on cell membranes without considering its effects on cytoplasmic proteins or targets after entry into the cell. A few proteomic and transcriptome studies have shown that phenol induces heat shock proteins (Benndorf et al., 2001; Tam le et al., 2006). Heat shock proteins (HSPs) are the general stress proteins induced by a variety of stresses that denature proteins, including high temperature and toxic substances such as heavy metals, alcohols, and organic solvents. Denatured proteins in the form of incomplete peptides, unfolded and misfolded proteins, and protein aggregates, which are produced in response to stress exposure, induce the transcription of HSPs, suggesting that the HSPs play a crucial role in removing damaged proteins in the cell, thereby helping the cell adapt or survive under stress. Apart from the induction of heat shock proteins in bacteria in response to phenol, phenol has also been suggested to cause oxidative
stress. Proteomic analysis of *Pseudomonas putida* upon exposure to phenol revealed the upregulation of antioxidant genes, including catalases that convert H$_2$O$_2$ to H$_2$O and minimize oxidative stress (Santos et al., 2004). Another study has suggested that the bacterial degradation of aromatic substrates, such as phenol and 4-chlorophenol by mono-and dioxygenase enzymes, also produces reactive oxygen species that cause oxidative stress (Tamburro et al., 2004). To generate a comprehensive picture of the effect of phenol on *P. syringae*, we compared the stress response of *P. syringae* cells to phenol, stressors that cause membrane damage (NaCl and PEG), and to an agent that causes oxidative stress (H$_2$O$_2$).

**Oxidative stress response.**

Oxygen radicals resulting from aerobic growth, exposure to oxidative agents, such as H$_2$O$_2$, and ionizing radiation cause oxidative stress in bacteria. Oxygen radicals, including superoxide, peroxide and hydroxyl radicals are known to damage cell macromolecules, such as DNA, lipids and amino acids by oxidation. This results in the induction of antioxidant genes, such as superoxide dismutases that convert superoxide into H$_2$O$_2$, and catalases that convert H$_2$O$_2$ to H$_2$O and oxygen; these activities help prevent cell damage. Some antioxidant genes, including *ahpC* (alkyl hydroperoxide reductase, subunit C), *sodB* (superoxide dismutase), *tpx* (thiol peroxidase), *dsbA* (thiol:disulfide interchange protein) and *tig* (trigger factor presumably required for protein folding or accelerated protein export), have been shown to be induced by phenol in *P. putida* suggesting that phenol induces some oxidative stress in bacteria (Santos et al., 2004). The extent of oxidative stress caused by phenol, therefore, may be determined by comparing the genes induced by H$_2$O$_2$ and phenol in *P. syringae*.

The expression of many oxidative stress response systems is controlled by complex regulatory networks. One key regulator is OxyR, which senses the cytoplasmic redox state and is responsible for the activation of a number of genes involved in stress response networks in many bacteria. In *E. coli*, the OxyR transcription factor controls the expression of many H$_2$O$_2$-inducible genes, including hydroperoxidase I (catalase, *katG*), the 2 subunits of an alkyl hydroperoxide reductase (*ahpCF*), glutaredoxin 1(*grxA*), glutathione reductase
(gorA), the Fur repressor (fur), non-specific DNA binding protein (dps) and regulatory RNA (oxyS). In addition to protecting against H₂O₂-induced stress, OxyR-regulated genes also confer resistance to hypochlorous acid, organic solvents, and reactive nitrogen species.

To obtain a comprehensive picture of the effects of phenol in bacteria and the mechanism of bacterial tolerance to phenol, transcriptome analysis was performed by exposing *P. syringae* pv. tomato to phenol and agents that cause membrane stress, including NaCl and PEG, and oxidative stress, such as H₂O₂. There are numerous previous studies where transcriptome analysis has been used to evaluate bacterial responses to stress agents, including H₂O₂, phenol, catechol, and antibiotics (Zheng et al., 2001; Phadtare et al., 2002; Palma et al., 2004; Weber et al., 2005; Zeller et al., 2005; Tam le et al., 2006), and some genes that help bacteria survive under the stressful conditions have been identified. These studies have been effective at identifying the members of specific stress regulons, thus improving our understanding of both global and stress-specific regulatory networks that control the expression of these genes. Collectively these studies have provided important insights into bacterial stress physiology. In the present study, we have examined the mechanism of phenol toxicity in bacteria by evaluating the effects of phenol on genes influencing cell membranes and the cell cytosol, and the mechanism of phenol tolerance by evaluating the physiological role of the stress-responsive genes that were induced the most by phenol and were not induced by other stresses.

*Water stress response.*

In natural environments, such as soil, bacteria can experience stress due to low water availability. Water stress can be either due to limited water activity because of the high solute concentrations in the environment in which a bacterial cell is present or the physical unavailability of water because of the interactions of water with a matrix, such as soil (Papendick and Campbell, 1980). The former type of water stress is referred to as osmotic stress and the latter as matric stress. Because the cytoplasmic membrane of bacteria is permeable to water but not to most other metabolites, hyper- or hypoosmotic shock causes an instantaneous efflux or influx of water, respectively, which is accompanied by a concomitant
decrease or increase in the cytoplasmic volume (Csonka, 1989). Proteins and other biological macromolecules have evolved to function only within certain normal ranges of water activities, outside of which some essential cellular function(s) becomes impaired. Sudden exposure to a hyperosmotic environment causes plasmolysis, i.e., cell shrinkage due to loss of water from the cell cytosol, resulting in the inhibition of a variety of physiological processes ranging from nutrient uptake (Roth et al., 1985a; Roth et al., 1985b; Rudd and Menzel, 1987) to DNA replication (Meury, 1988). Importantly, water stress has been shown to cause changes in the membrane by altering the amounts of trans and cis isomers of monounsaturated fatty acids (Halverson and Firestone, 2000) or by differential synthesis of outer membrane proteins (Lugtenberg et al., 1976; Epstein, 1983). Therefore, a comparison of the genes induced by NaCl and PEG to those induced by phenol may help determine the extent and nature of the membrane stress caused by phenol in bacteria.

References


Chapter 2. Bacterial degradation of airborne phenol in the phyllosphere

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Abstract

Despite the vast surface area of terrestrial plant leaves and the large microbial communities they support, little is known of the ability of leaf-associated microorganisms to access and degrade airborne pollutants. Here, we examined bacterial acquisition and degradation of phenol on leaves by an introduced phenol degrader and by natural phyllosphere communities. Whole-cell gfp-based Pseudomonas fluorescens bioreporter cells detected phenol on leaves that had previously been transiently exposed to gaseous phenol, indicating that leaves accumulated phenol; moreover, they accumulated it in sites that were accessible to epiphytic bacteria and to concentrations that were at least 10-fold higher than those in the air. After inoculated leaves were exposed to gaseous $^{14}$C-phenol, leaves harboring the phenol-degrading Pseudomonas sp. strain CF600 released 8 times more $^{14}$CO$_2$ than did leaves harboring a non-degrading mutant, demonstrating that CF600 actively mineralized phenol on leaves. We evaluated phenol degradation by natural microbial communities on green ash leaves that were collected from a field site rich in airborne organic pollutants. We found that significantly more phenol was mineralized by these leaves when the communities were present than by these leaves following surface sterilization. Thus, phenol-degrading organisms were present in these natural communities and were metabolically capable of phenol degradation. Collectively, these results provide the first direct evidence that bacteria on leaves can degrade an organic pollutant from the air, and indicate that bacteria on leaves could potentially contribute to the natural attenuation of organic air pollutants.

Introduction

Plants can mediate the removal of organic pollutants from the environment. They can do this directly, such as by extracting pollutants from the soil, and indirectly by fostering populations of pollutant-degrading bacteria. The transpiration stream of plants promotes the movement of soil pollutants toward roots and their translocation to the shoots, thus allowing plants to act as wicks that remove pollutants from soil. Similarly, plants may remove pollutants from the air via stomatal uptake and sorption. Stomatal uptake of gaseous pollutants is a major uptake pathway for small inorganic compounds such as sulfur dioxide, nitrogen oxides, and ozone (Slovik et al., 1996), whereas sorption to the plant cuticle, a waxy layer on the leaf surface, can contribute to the uptake of airborne organic compounds (Schmitz et al., 2000). Following uptake, pollutants may be re-released into the environment, accumulated in the plant tissue, and transformed or degraded by plant metabolic processes or by photodegradation (Pilon-Smits, 2005; Wild et al., 2005). Soil contaminants may also be degraded by microbes in the rhizosphere. In this study, we explore the potential for phyllosphere, i.e., leaf-associated, microbial communities to access and degrade organic pollutants from the air.

The availability of organic air pollutants to phyllosphere microorganisms is likely influenced by their uptake pathway. Stomatal uptake should lead to movement of pollutants to the leaf intercellular spaces, a region typically devoid of significant microbial populations. In contrast, cuticular uptake may lead to accumulation on the leaf surface, a region often rich in microorganisms. Several studies support a cuticular uptake pathway for lipophilic gaseous organic compounds, including phenol (G.A. Beattie and J.S. Seibel, unpublished data; McCrady, 1994; Dienum et al., 1995; Keymeulen et al., 1995). Cuticular uptake can involve sorption to the outermost surface of the cuticle, diffusion into the underlying cuticle, and eventual movement into the epidermal cell wall and cytoplasm, as shown for anthracene and other compounds when applied as aqueous solutions to leaf surfaces (Schönherr and Riederer, 1989; Wild et al., 2004). We predict that a consequence of this sorption to the cuticle is enhanced availability to the microorganisms on the leaf surface.
Leaves often support diverse microbial communities with individual populations as large as $10^7 - 10^8$ cells per gram of leaf. The majority of these bacteria are present on the leaf surface, although some, primarily pathogens, may establish large populations in the intercellular spaces. Bacteria on leaf surfaces are not uniformly distributed, but rather may occur as individual cells or aggregates (Monier and Lindow, 2004). Roots also support diverse microbial communities, and these communities have been shown to promote pollutant degradation in the rhizosphere (Kuiper et al., 2004). Roots not only provide a nutrient-rich habitat for microbes, but also release secondary metabolites that can induce microbial genes involved in pollutant degradation (Gilbert and Crowley, 1997; Casavant et al., 2003) or can act as co-metabolites that facilitate degradation (Rentz et al., 2005). Roots can also mobilize and accumulate pollutants (Harvey et al., 2002), thus enriching pollutant-degrading bacteria near roots (Miya and Firestone, 2000) and in endophytic sites (Siciliano et al., 2001). Leaves may exhibit analogous properties, including releasing secondary metabolites that promote degradation and accumulating gaseous pollutants that enrich for pollutant-degrading bacteria. The large surface area of leaves in terrestrial ecosystems suggests that such processes could be significant to air quality and the ecology of leaf-associated microbes.

Here, we investigated the potential for pollutant degradation in the phyllosphere using phenol as a model compound and *Pseudomonas* species on bean (*Phaseolus vulgaris*) and maize (*Zea mays*) leaves as model phyllosphere systems. Phenol is a priority pollutant of the U.S. Environmental Protection Agency; it is a component of the emissions from wood, coal, and municipal solid waste combustion, as well as automobile exhaust and cigarette smoke (Agency for Toxic Substances and Disease Registry, 1998). Moreover, it is an abundant pollutant generated by microbial communities during the anaerobic decomposition of livestock waste (Zahn et al., 1997) and is a major contributor to the odor intensity of animal wastes (Zahn et al., 2001). In preliminary studies, we found that bean and maize leaves, which are known to support significant bacterial communities, removed detectable levels of gaseous phenol from the air in a closed system (A. Sandhu, J.S. Seibel and G.A. Beattie, unpublished data). The objectives of the present study were to evaluate 1) the availability of
phenol to leaf-associated bacteria following its uptake into bean and maize leaves, 2) the potential for phenol degradation by phenol-degrading bacteria on leaves, and 3) the phenol degradation ability of naturally-occurring epiphytic microbial communities from areas rich in airborne organic pollutants.

**Material and Methods**

**Bacterial strains, plasmids, and growth conditions**

*Pantoea agglomerans* strain BRT98 (Sabaratnam and Beattie, 2003), *Pseudomonas syringae* pv. syringae strain B728a (Loper and Lindow, 1987), *Ps. syringae* pv. tomato strain DC3000 (Whalen et al., 1991), *Ps. fluorescens* strain A506 (Wilson and Lindow, 1994), and *Escherichia coli* strain AW101 (Wise and Kuske, 2000), which contains a chromosomal fusion of the *dmp* promoter from *Pseudomonas* sp. Strain CF600 and *lacZ*, were grown in Luria-Bertani (LB) medium (Miller, 1972), or in yeast extract succinate (YES) medium (Stiner and Halverson, 2002) supplemented with Hutner’s mineral solution. *Pseudomonas* sp. strain CF600 (Shingler et al., 1989) harbors the phenol catabolic operon *dmp* on plasmid pVI150 and is able to degrade phenol. The CF600 derivative CF427 (Pavel et al., 1994) has a Km cassette in place of an internal *dmpR* fragment and does not degrade phenol. Strains CF600 and CF427 were grown in M9 minimal medium (Miller, 1972) containing 2.5 mM phenol or 2.5 mM glucose, respectively, and supplemented with Hutner’s mineral solution (Smibert and Krieg, 1994). Antibiotics were used at the following concentrations (µg/ml): rifampicin, 50; kanamycin, 50; and tetracycline, 20. Mercuric chloride was used at a concentration of 25 µg/ml.

**Bioreporter plasmid construction**

The 574-bp *dmp* promoter (*P_{dmp}*_) was amplified by PCR from *E. coli* strain AW101, ligated into pGEM-T easy vector (Promega, Madison, WI), and cloned as an EcoRI-BamHI fragment into pPROBE-KT’ (Miller et al., 2000) upstream of a promoterless *gfp* gene (Miller and Lindow, 1997). The plasmid pAW50-B23 (Wise and Kuske, 2000) was linearized with ClaI and inserted into a unique ClaI site in the *P_{dmp}-gfp*-fusion-containing pPROBE-KT’
plasmid. The resulting plasmid, pPhenol, was transferred from *E. coli* strain DH5α to the *P. agglomerans*, *Ps. fluorescens*, and *Ps. syringae* strains by conjugation in triparental matings with a helper strain containing the plasmid pRK2073 (Better and Helinski, 1983). Similarly, the plasmid pAW50-B24 (Wise and Kuske, 2000) was cloned into the ClaI site of pPROBE-KT* plasmid, forming pPhenB24, which was then transferred by conjugation into the *P. agglomerans*, *Ps. fluorescens*, and *Ps. syringae* strains.

**Phenol induction of P*dmp*-*gfp in culture medium**

YES-grown cells were washed twice and resuspended in YES (10⁶ cells/ml) in airtight glass tubes. Phenol was added to final concentrations of 0 to 100 µg/ml and after 4 h of incubation on a shaker, and GFP fluorescence by cells was measured using a Beckman-Coulter EPICS-XL-MCL flow cytometer, as described previously (Axtell and Beattie, 2002). Bacteria were identified based on their fluorescence. Samples were subjected to flow cytometry until either 25,000 fluorescent particles had been examined or the sample had been run for 30 sec. The geometric mean fluorescence was reported as the mean cellular fluorescence per sample. Phenol induction of strains containing pPhenB24 was characterized and was similar to that of strains with pPhenol.

**Availability of phenol to bacteria on leaves**

Bean (*Phaseolus vulgaris* L. cv. Bush Blue Lake 274) and maize (*Zea mays* L. inbred B73) seeds were grown in a sterile peat-perlite-soil (1-2-1) potting mixture at 45% relative humidity and 28°C with a 12-h photoperiod. Plants were used at the time of emergence of the first trifoliate (bean) or third leaf (maize), at which time the leaves that were used in the experiments were approximately 1 g (bean) or 0.3 g (maize). Inocula were prepared by suspending A506(pPhenol) cells grown on solid LB media in one-tenth strength YES (1/10 YES) (10⁸ cells/ml). Airtight exposure chambers were constructed using glass jars (3.75 L) with custom-machined polypropylene lids equipped with an injection port. This port was a 24-mm screw cap mininert valve that allowed liquid phenol to be introduced onto a glass
dish on a stainless steel holder attached to the lid; the phenol subsequently volatilized into the chamber.

To evaluate the availability of airborne phenol to bacteria on bean leaves, primary leaves of intact, potted plants were inoculated by submersion in the A506(pPhenol) inoculum or 1/10 YES. Plants were air dried until liquid was no longer visible on the leaves. Plants were severed approximately 3 cm below the point of attachment of the primary leaves, and the stem was placed in 2 ml of water in a microfuge tube, which was in a glass serum vial for support. Two plant leaves were introduced into each of 20 exposure chambers. Ten µl of a phenol solution (30 µg/ml in water) were injected into 12 of the chambers, 8 of which had plants inoculated with bacteria and 4 of which had plants inoculated with 1/10 YES. After incubation of the chambers at 25°C for 4 h, the leaves were removed from the chambers and the bacterial cells were recovered from each leaf by submerging in 20 ml of 1/10 YES medium and sonicating for 7 min (O'Brien and Lindow, 1989). Cells were harvested by centrifugation, resuspended in 1/10 YES, and examined using flow cytometry. The fluorescence of leaf-derived cells was compared to that of a suspension of cells (10^6 cells/ml) that were exposed to 1 µg/ml of phenol in 1/10 YES in airtight glass tubes for 4 h. Flow cytometry samples were run until either 25,000 fluorescent particles had been examined or the sample had been run for 50 sec.

To evaluate the availability of leafborne phenol to bacteria on bean and maize leaves, plants were exposed to airborne phenol prior to plant inoculation with A506(pPhenol) cells. Uninoculated plants were severed 3 cm below the primary leaves (beans) or 3 cm below the emergence point of the first true leaf (maize) and placed in water, as above, and in exposure chambers. Phenol was introduced into half of the chambers for each plant species. After a 4-h incubation at 25°C, the plants were removed from the chambers, inoculated with A506(pPhenol) cells by submersion, placed in new chambers that did not contain airborne phenol, and incubated for an additional 4 h before bacterial recovery and analysis.
Phenol degradation by bacteria on bean leaves

Gas-tight chambers (1L) were constructed by grinding the open ends of 85-mm diameter Pyrex glass beakers to conform to a flat surface and securing them to a glass plate with rubber bands that bound glass pegs fused to the beakers with metal pegs on the glass. Stopcock grease was used to make a gas-tight seal. Liquid phenol was introduced into a Pyrex dish via a mininert valve injection port that was fused to the chamber, and the phenol rapidly volatilized after its introduction.

Bacterial degradation of phenol on leaves was evaluated using Pseudomonas sp. strains CF600 and CF427. Bacterial inocula were prepared by growing cells in M9 minimal medium with 2.5 mM phenol or 2.5 mM glucose, respectively, at 28 °C for 24 h, washing cells twice, and resuspending cells in 10 mM phosphate buffer (PB) (10^8 cells/ml). Primary bean leaves of intact plants were submerged in bacterial inocula or in PB and were air dried for 1 h. For each treatment, four replicate leaves were used, and each was excised from the stem, placed in water in a microfuge tube, as described above, and then into a gastight chamber. Bacteria were recovered from the second primary leaf of each inoculated plant and were enumerated by plating. Ten \( \mu l \) of 0.054 \( \mu \)mol of \(^{14}\)C-phenol (Phenol-UL (uniformly labeled)-\(^{14}\)C, Sigma-Aldrich, St. Louis, MO) with a specific activity of 5.45 \( \mu \)Ci/\( \mu \)mol were injected into each chamber for an estimated final concentration of 17 \( \mu \)g/L of phenol. \(^{14}\)CO\(_2\) was trapped in 1 ml of 5M KOH in a glass vial (12 x 31 mm) in each chamber. After 24 h, bacteria were recovered from the leaves and enumerated, and the \(^{14}\)C in 1 ml of each the leaf wash, the KOH, and the water from the microfuge tubes supporting the leaves was measured after adding 15 ml of Scintisafe Plus 50% cocktail (Fisher Scientific, Fair Lawn, NJ).

Phenol degradation by naturally occurring microbial communities on leaves

Green ash (Fraxinus pennsylvanica) leaves were collected from trees growing within 5 to 6 m of a swine production facility in Hamilton County, Iowa, in August and October, 2005. A subset of the leaves were surface-sterilized by immersion in 0.02% Tween 20 for 20 s, 0.2% sodium hypochlorite for 3 min, and sterile water 3 times for at least 1 min; leaves were dried
in a laminar flow hood until surface water was no longer visible (approximately 30 min). Bacteria were recovered by sonication from unsterilized leaves, as well as from sterilized leaves to verify sterilization, and were enumerated on one-tenth strength tryptic soy agar (Microtech Scientific, Orange, CA) amended with the fungicide cycloheximide (100 µg/ml). At least three replicate leaves, each having 5 leaflets, were examined for each treatment. These leaves were placed in gastight chambers, with one leaf per chamber, and were exposed to radiolabeled phenol for 24 h. The radioactivity in the KOH traps, wash fluid from the leaves, and water from the microfuge tubes was measured, as described above.

**Statistical analysis**

Unless otherwise stated, \( P \)-values were determined based on Student’s \( t \)-test. Proportional data was subjected to arcsine squareroot transformation before analysis.

**Results**

**Phenol induction of \( P_{dmp-gfp} \) in *Pseudomonas fluorescens* A506 and other bacterial strains**

The plasmid pPhenol (Fig. 1) was constructed as a reporter of phenol exposure by bacteria. pPhenol carries a transcriptional fusion between the promoter of the \( dmp \) phenol catabolic operon and \( gfp \), as well as \( dmpR-B23 \), which encodes a derivative of the transcriptional activator DmpR that mediates an enhanced response to phenol (Wise and Kuske, 2000). \( P_{dmp-gfp} \) and \( dmpR-B23 \) are divergently transcribed in pPhenol. This plasmid was introduced into *Pantoea agglomerans* strain BRT98, *Pseudomonas syringae* pv. syringae strain B728a, *Ps. fluorescens* strain A506 and *Ps. syringae* pv. tomato strain DC3000. Nonpathogenic bacteria such as *P. agglomerans* BRT98 and *Ps. fluorescens* A506 have previously been shown to colonize primarily or exclusively external leaf surface sites (Wilson et al., 1999; Sabaratnam and Beattie, 2003), whereas phytopathogenic bacteria, including *Ps. syringae* pv. syringae B728a and *Ps. syringae* pv. tomato DC3000, can colonize internal leaf sites, at least on host plants (Boureau et al., 2002; Sabaratnam and Beattie, 2003). The \( P_{dmp-gfp} \) fusion on pPhenol exhibited relatively low levels of induction by phenol in BRT98(pPhenol) and
B728a(pPhenol) (Fig. 2A), in contrast to its responsiveness to phenol in A506(pPhenol) (Fig. 2B) and DC3000(pPhenol) (Fig. 2C). Thus, the responsiveness of $P_{dmp}$ to phenol in these strains was not correlated with their taxonomy, nor was it correlated with their sensitivity to phenol based on their growth in the presence of up to 100 µg/ml of phenol (data not shown). Regression analyses of the A506(pPhenol) and DC3000(pPhenol) data resulted in sigmoidal curves with equations $y = 34.85/(1+(x/1.453)^{-1.405})$ ($R^2 = 0.95, P < 0.0001$) and $y = 51.17/(1+(x/6.529)^{-0.966})$ ($R^2 = 0.95, P < 0.0001$), respectively. Moreover, the induction response of A506(pPhenol) and DC3000(pPhenol) by phenol was linear from 0.3 µg/ml to 25 µg/ml phenol (Figs. 2B and 2C insets), indicating that this range provided the most dynamic response and thus optimal efficacy for these strains as bioreporters of phenol. In leaf colonization studies over 24 h, A506(pPhenol) exhibited 30-fold better survival than DC3000(pPhenol) during the population decline that frequently occurs on leaves following bacterial inoculation (data not shown). Because of its superior leaf surface survival, A506(pPhenol) was selected as the bioreporter for the following studies.

**Fig. 1.** Map of pPhenol. The 21.5-kb plasmid has a fusion between the $dmp$ promoter and $gfp$ and carries the regulatory gene $dmpR-B23$ and the pVS1 origin of replication (Heeb et al., 2000). T1 represents the *E. coli* rrnB transcriptional terminator. The arrows indicate the direction of transcription.
Fig. 2. Induction of the $P_{dmp}$-gfp fusion in bioreporter cells in YES medium after phenol exposure for 4 h. A) *P. agglomerans* BRT98(pPhenol) (●) and *Ps. syringae* pv. syringae B728a(pPhenol) (○), B) *Ps. fluorescens* A506(pPhenol), and C) *Ps. syringae* pv. tomato DC3000(pPhenol). In B and C, distinct symbols indicate data generated in separate, independent experiments. Each data point is the mean of 3 independent measurements.

Relative fluorescence units (RFU) at 0 µg/ml of phenol were 3.0, 1.7, 1.5 and 0.5 for BRT98(pPhenol), B728a(pPhenol), A506(pPhenol) and DC3000(pPhenol), respectively. Linear regression of the RFU values at 0.3 to 25 µg/ml phenol (insets) yielded the equations B) $y = 18.62x + 13.42$ ($R^2 = 0.90$, $P < 0.0005$) and C) $y = 23.27x + 7.11$ ($R^2 = 0.83$, $P = 0.01$).

**Availability of phenol to A506(pPhenol) on bean leaves**

The availability of phenol to bacteria on leaves was evaluated based on the fluorescence of A506(pPhenol) cells recovered from leaves that were or were not exposed to phenol in closed chambers. When uninoculated leaves were exposed to phenol and the leaf washings were examined by flow cytometry (Fig. 3, trt 1), the number of fluorescent particles and their fluorescence was low, indicating a low background. Most or all of these particles were probably leaf debris. When leaves inoculated with A506(pPhenol) cells were incubated in chambers without phenol (Fig. 3, trt 2), the number of fluorescent particles and their fluorescence was also low, indicating that leaves did not produce phenolic molecules that induced fluorescence in A506(pPhenol). When leaves inoculated with A506(pPhenol) cells were incubated with phenol for 4 h (Fig. 3, trt 3), the A506(pPhenol) cells were significantly more fluorescent than the cells from leaves that were not exposed to phenol (Fig. 3, trt 2).
(P<0.0005), indicating that epiphytic A506(pPhenol) cells detected phenol that had been introduced into the air. The fluorescence of the A506(pPhenol) inoculum cells exposed to 1 µg/ml of phenol in broth culture (Fig. 3, trt 4) was similar to that predicted by the dose-response curve (Fig. 2B). Based on this dose-response curve, the A506(pPhenol) cells on leaves sensed 2 to 5 µg/ml, with an average of 3 µg/ml, of phenol. This is substantially greater than the amount of phenol introduced into the air (0.08 µg/ml), suggesting that the phenol was concentrated at the leaf surface, presumably because of sorption to the leaf.

Fig. 3. Availability of phenol provided in gaseous form to A506(pPhenol) on bean leaves. In trt 1, uninoculated leaves were incubated for 4 h following the introduction of 300 µg of phenol into the chambers. In trt 2, A506(pPhenol) cells were recovered from inoculated leaves after incubating for 4 h without phenol exposure. In trt 3, A506(pPhenol) cells were inoculated onto leaves and the leaves were incubated for 4 h following the introduction of 300 µg of phenol into the chambers. In trt 4, A506(pPhenol) cells were exposed to 1 µg/ml of phenol in broth culture for 4 h. The fluorescence of the bacterial cells was measured by flow cytometry. Symbols represent the mean fluorescence of the cells recovered from individual leaves, with the values on the right reflecting the mean fluorescence per treatment standard error of the mean (SEM) and mean number of fluorescent particles SEM (n = 4, 16 and 16 leaves for trts 1-3, respectively, and n = 3 cultures for trt 4).
**Availability of leafborne phenol to A506(pPhenol) on bean and maize leaves**

The availability of phenol to epiphytic bacteria following transient exposure of plants to phenol was also evaluated. Bean and maize leaves were exposed to phenol for 4 h and then were removed from the chambers, inoculated with A506(pPhenol) cells, and placed in chambers with no airborne phenol. A506(pPhenol) cells recovered from leaves that had not been exposed to phenol showed a relatively low level of fluorescence induction (Fig. 4, trts 1 and 3). The wide variability in the mean fluorescence of the samples from maize leaves (Fig. 4, trt 3) suggests that either maize leaves produce compounds that induce P<sub>dpn</sub> expression or leaf-derived fluorescent particles, such as leaf debris, are recovered at a higher rate from maize leaves than from bean leaves. A506(pPhenol) cells recovered from leaves that were exposed to phenol exhibited significantly more fluorescence than those recovered from leaves of the same species that had not been exposed to phenol (Fig. 4) (P<0.05). The amount of phenol accessible to bacteria on bean leaves under these conditions was estimated to be between 1.5 and 1.8 µg/ml, with an average of 1.7 µg/ml based on the dose-response curve shown in Fig. 2B. In contrast, A506(pPhenol) cells from maize leaves exhibited a much broader range in their mean fluorescence, from 11.2 to 36.7, with an average mean fluorescence of 27.0 ± 1.8, which was significantly higher than that of the cells from bean leaves (P<0.05). Based on the dose response curve, this reflects exposure to 1.0 to 3.5 µg/ml of phenol, with an average of 2 µg/ml. These estimates of phenol exposure only approximate the true exposure levels because they do not account for possible differences in cellular physiology between the cells on the leaves and the cells in the cultures used to generate the dose-response curves. The broader range of fluorescence among the cells recovered from maize than from bean leaves suggests that either the two plant species differed in the distribution of phenol adsorbed onto the leaf surfaces or that the bacterial cells were less uniformly distributed on maize leaves than on bean leaves. This greater fluorescence was observed for cells recovered from the first leaf of the maize seedlings as well as those separately recovered from the second leaf (data not shown). The fluorescence of bacteria that were present on leaves during phenol exposure (Fig. 3, trt 3) was significantly higher than the fluorescence of bacteria that were on leaves previously exposed to phenol (Fig. 4, trt 2).
(P<0.05). This result suggests that bacteria on leaves can access both airborne and leafborne phenol.

**Bioavailability to A506(pPhenol) of phenol that had been taken up and accumulated in bean (trts 1 and 2) and maize (trts 3 and 4) leaves.** Excised leaves were incubated in the chambers without phenol exposure (trts 1 and 3) or with 300 µg of phenol (trts 2 and 4) for 4 h after which the leaves were removed from the chambers, inoculated with A506(pPhenol) cells and incubated for another 4 h. In trt 5, A506(pPhenol) cells were exposed to 1 µg/ml phenol in 1/10 YES. The bacteria were recovered from each leaf and their fluorescence was measured by flow cytometry. Symbols and values are as described in Fig. 3, with n=15, 16, 15, 16, and 3 for trts 1-5, respectively.

**Phenol degradation by bacteria on bean leaves**

The ability of bacteria to degrade phenol while resident on leaves was evaluated based on detectable mineralization or cellular incorporation of radiolabeled phenol. Bacteria were suspended on a filter disk, which was placed on water agar and exposed to $^{14}$C-phenol for 24 h in the presence of a KOH trap for $^{14}$CO$_2$. The amount of radioactivity in KOH incubated with *Pseudomonas* sp. strain CF600 was 79% of the radioactivity introduced into the chamber as compared to only 12% for CF427, a mutant strain defective in the *dmpR* regulatory gene, and 6% for a filter disk without bacteria. Thus, mineralization ability was confirmed for *Pseudomonas* sp. strain CF600 and was confirmed to be greatly reduced for strain CF427. To evaluate if CF600 can degrade phenol when present on leaves, primary bean leaves were submerged in a CF600 cell suspension, a CF427 cell suspension, or phosphate buffer; these leaves were then exposed to uniformly labeled $^{14}$C-phenol (specific
activity 5.45 μCi/μmol) for 24 h in chambers that each contained a KOH trap. The CF600 population on bean leaves did not change under these conditions for at least 24 h, remaining at approximately $10^8$ CFU/g leaf. After incubation with $^{14}$C-phenol, the radioactivity in the KOH was significantly higher (approximately 8-fold) in the chambers with the CF600-inoculated leaves than in the chambers with the CF427-inoculated leaves or in those with the uninoculated leaves (single factor ANOVA, $P<0.005$) (Fig. 5), indicating that CF600 cells on leaves could access and degrade phenol introduced into the air. The low level of radioactivity in the KOH from chambers with the uninoculated leaves provides strong evidence against plant-mediated phenol mineralization, at least under the conditions examined. Although more radioactivity was assimilated into the CF600 cells than into the CF427 cells, this difference was not significant (Fig. 5). The negligible amount of radioactivity in the water in the microfuge tubes supporting the leaves (Fig. 5) indicates that the gaseous radiolabeled phenol did not partition appreciably into the water; this is attributable, in part, to the small surface area of the water. The CF600 and CF427 population sizes on leaves after 24 h of exposure to phenol were not different than they were at 0 h (data not shown), indicating that the degradation of phenol by CF600 was not due to a larger population size on leaves and that degradation activity did not detectably influence epiphytic population sizes.
Fig. 5. $^{14}$C-phenol degradation by Pseudomonas sp. strains CF600 and CF427 on bean leaves in closed chambers. The radioactivity was measured in the KOH from reservoirs placed in the chambers, in the bacterial suspension recovered from washing the leaves, and in the water from the microfuge tubes supporting the leaves. Values represent the mean percentage ± SEM of the total radioactivity introduced into the chambers at 0 h ($n = 4$).

Phenol degradation by naturally occurring microbial communities on leaves

Leaves were collected from a stand of green ash trees adjacent to a swine production facility, and thus from an environment that was likely rich in airborne volatile organic compounds, including phenol (Zahn et al., 1997). Leaves from both sampling times harbored $10^5$ to $10^7$ CFU/g bacteria based on culturable counts. Subsets of the leaves were surface-sterilized with 0.2% sodium hypochlorite; the effectiveness of this sterilization was confirmed based on the subsequent inability to recover culturable cells from the treated leaves. After exposure of the unsterilized and surface-sterilized leaves to radiolabeled phenol in closed chambers for 24 h, the amount of radioactivity that accumulated in the KOH in chambers with the unsterilized leaves was significantly greater than that in chambers with the sterilized leaves ($P < 0.005$); this was observed with leaves sampled in August, in which the difference was 3.1-fold (data not shown), and in October, in which the difference was 2.5 fold (Fig 6). These results indicate that organisms that were susceptible to the surface-sterilization treatment were
capable of mineralizing airborne phenol. The relatively low level of radioactivity in the KOH from chambers with the sterilized leaves provides good evidence against plant-mediated phenol mineralization by the green ash leaves, as was observed with the uninoculated bean leaves (Fig. 5). The amount of radioactivity in the microbial cells washed from the unsterilized leaves was significantly higher (approximately 10-fold) than in the wash fluid recovered from the sterilized leaves ($P<0.0005$), suggesting that phenol was assimilated into at least some of the microbial cells recovered from the green ash leaves.

![Fig. 6](image)

**Fig. 6.** $^{14}$C-phenol degradation by the naturally occurring microbial community on green ash leaves sampled in October from an area near a swine production facility. Data are as described in Fig. 5, with $n = 4$ and 3 for the sterilized and unsterilized treatments, respectively.

**Discussion**

Leaf surfaces of terrestrial plants provide an excellent venue for promoting microbial access to volatile organic compounds (VOCs). Here, we found that in addition to their known ability to foster significant microbial communities, plant leaves can accumulate the VOC phenol and make it available for subsequent degradation by phyllosphere bacteria.
Furthermore, we found that introduced bacteria can degrade phenol during leaf colonization and that such degradation may be a common feature of some terrestrial ecosystems based on the degradation activity detected in natural phyllosphere communities. The relatively rapid rate of degradation by these natural communities, as evidenced by detectable degradation within 24 h, suggests that phyllosphere communities near sites rich in airborne pollutants are naturally primed for such degradation. Collectively, these studies indicate that phylloremediation, so named by analogy to rhizoremediation in roots, may serve as a natural attenuation mechanism for air pollutants as well as could provide an alternative source of energy and nutrients to bacteria on leaves.

Phylloremediation of VOCs has been suggested by several previous studies. In particular, De Kempeneer et al. (2004) found that the rate of removal of airborne toluene by azalea leaves in a closed system was greater when leaves were inoculated with a toluene-degrading enrichment culture of *Pseudomonas putida* than when they were mock-inoculated with water. Although this study did not directly evaluate bacterial degradation activities, and thus did not rule out enhanced plant uptake in the presence of *Ps. putida*, it indicated that phyllosphere bacteria can contribute to reductions in airborne toluene. Another study suggests that phylloremediation can attenuate pollutants that become airborne via evapotranspiration. Specifically, the leaves of yellow lupine plants grown in a hydroponic system amended with toluene released less toluene by evapotranspiration when the plants were colonized with an endophytic toluene-degrading *Burkholderia cepacia* than when they were colonized with non-degrading *B. cepacia* (Barac et al., 2004). And lastly, a *Methylobacterium* sp. that was isolated from leaves and other hybrid poplar tissues was able to degrade xenobiotic compounds, including several volatile organic explosives (Van Aken et al., 2004), demonstrating that bacteria capable of VOC degradation are present in at least some phyllosphere communities.

We have provided direct evidence of active VOC degradation by natural phyllosphere communities. These communities were on leaves grown near a swine production facility, a site where VOCs are often released due to anaerobic degradation of organic compounds by
bacteria. Up to 168 airborne chemicals have been identified near swine production facilities (Curtis, 1983), with volatile fatty acids, phenols, alcohols, ammonia, and sulfur-containing compounds being the most abundant. Among 29 swine production facilities examined, phenol was present at an average air concentration of 0.025 µg/ml, which is similar to the levels used in this study (0.02 to 0.08 µg/ml). The natural bacterial communities on the sampled leaves contained culturable bacterial populations of $10^5$ to $10^7$ CFU/leaf and included a broad diversity of colony types. Based on the diversity of organisms that can degrade phenol and are common to phyllosphere communities, including Acinetobacter, Arthrobacter, Bacillus, Pseudomonas, Ralstonia, and Rhodococcus (Mishra et al., 2001; Kirchner et al., 2003; Shingler, 2004; Lambais et al., 2006), multiple candidate organisms could have been responsible for phenol degradation on these leaves.

We found that bean and maize leaves were highly effective bioaccumulators of phenol. Phenol was introduced into the closed chambers at a concentration of 0.08 µg/ml, assuming a uniform distribution, whereas the bioreporter cells detected concentrations of 1 to 3.5 µg/ml on leaves, demonstrating an increase in concentration of over 10-fold. The leaf surface properties of bean and maize are highly distinct, suggesting that phenol accumulation occurs in many plant species. VOCs may accumulate in the cuticle, as indicated by the visible localization of anthracene in the outermost layer of the cuticle of maize leaves, with subsequent diffusion into the epidermal cell cytoplasm over a 96-h period (Wild et al., 2004). We predict that phenol sorbed to the cuticle in the 4-h periods used in our studies based on its detection by the Ps. fluorescens bioreporter cells, which as nonpathogenic bacteria are likely restricted to leaf surface sites (Wilson et al., 1999; Sabaratnam and Beattie, 2003).

The dmp catabolic pathway in Pseudomonas sp. CF600 mediated complete mineralization of phenol on leaves. Such mineralization accounted for approximately 20% of the introduced radiolabel, whereas 8% was associated with the CF600 cells, presumably due to incorporation into macromolecules. These results indicate that phenol was used primarily as a source of energy and secondarily as a source of carbon under these conditions. This is consistent with the lack of significant population growth of CF600, based on
culturable counts, during the 24-h incubation period with $^{14}$C-phenol (data not shown). Although we did not detect such growth, detectable population increases due to the presence of a VOC as an additional nutrient source would likely require exposure periods longer than 24 h.

Collectively, terrestrial leaves provide a vast surface area, as much as 400,000,000 km$^2$ globally (Myneni et al., 1997; Morris and Kinkel, 2002), for the sorption and uptake of air pollutants and as a habitat for microorganisms. Exposure of phyllosphere communities to airborne organic pollutants could affect their ecology by augmenting phyllosphere nutrient pools, enriching for pollutant-degraders, and inhibiting pollutant-sensitive organisms. This exposure appears to occur on the leaf surface, based on the probable surface location of our *Ps. fluorescens* bioreporter cells (Wilson et al., 1999; Sabaratnam and Beattie, 2003), but could also occur in endophytic sites, where degradation would affect pollutant phytotoxicity as well as airborne pollutant levels. We found that a bioreporter derivative of *Ps. syringae* pv. tomato (*Pst*) was similar to the *Ps. fluorescens* A506 bioreporter in its responsiveness to phenol, but as a pathogen, *Pst* can establish large endophytic populations in host plants with a delay of at least 48 h before disease symptoms occur in some plants (Boureau et al., 2002). This strain would thus be appropriate for future investigations into the bioavailability of phenol in endophytic sites. Similarly, future studies aimed at quantifying the impact of phylloremediation on airborne VOC concentrations are needed to understand the role of phylloremediation in natural pollutant attenuation and to design strategies for minimizing indoor and outdoor airborne organic pollutants.

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References


Chapter 3. Identification and genetic characterization of phenol-degrading bacteria from leaf microbial communities

Introduction
A wide diversity of phenol-degrading bacteria, including *Pseudomonas*, *Ralstonia*, *Acinetobacter*, *Burkholderia*, *Comamonas* and *Variovorax*, have been isolated from natural microbial populations in various ecosystems including wastewaters, rivers, seas, and soils (van Schie and Young, 1998; Bastos et al., 2000; Abd-El-Haleem et al., 2002; Arutchelvan et al., 2005; Garcia et al., 2005; Geng et al., 2006). The presence of pollutants in these sites can select for pollutant-degrading bacteria (Ghiorse et al., 1995; Van der Meer, 2006). Whereas the structure and function of microbial communities in contaminated wastewaters and polluted soils have been well characterized (Shafer and Schonherr, 1985a; Watanabe et al., 2002; Sei et al., 2003; Brakstad and Lodeng, 2005; DeRito et al., 2005) the nature of those on plant leaves, especially near sites abundant in airborne organic compounds, has not. In our previous study (Sandhu et al., 2007), we provided the first direct evidence of phenol degradation by bacteria on plant leaves; we observed this with introduced bacteria and natural microbial communities present on green ash (*Fraxinus pennsylvanica*) leaves that were taken from a site rich in airborne phenol. We found that significantly more phenol was mineralized when microbial communities were present on the leaves than when leaves were surface-sterilized. A few previous studies have shown that bacteria capable of pollutant degradation are present in at least some phyllosphere communities. For example, phyllosphere bacteria related to the genera *Acinetobacter*, *Alcaligenes*, *Bacillus*, and *Micrococcus* were isolated from the leaves of various tropical plants and shown to degrade the hydrocarbons diesel, kerosene and lubricating oil in culture (Ilori et al., 2006). In another study, a *Methylobacterium* sp. that was isolated from leaves and other hybrid poplar tissues was able to degrade xenobiotic compounds, including several volatile organic explosives (Van Aken et al., 2004). Although these studies clearly show that pollutant-degrading
bacteria can be present in the phyllosphere, little has been done to identify and characterize these pollutant-degrading phyllosphere bacterial communities. In the present study, we identified members of the microbial communities that are capable of degrading airborne pollutants on green ash leaves, thus improving our understanding of phyllosphere microbial ecology, at a site rich in airborne organic pollutants.

Characterization of the genetic and functional diversity of pollutant-degrading bacterial communities can provide insight into the catabolic potential of those communities (Watanabe et al., 2002). Identification of the bacterial species that are capable of degrading most of the pollutants in contaminated sites should help in the development of effective bioremediation strategies (Watanabe et al., 1998; Watanabe et al., 2002). Molecular characterization of pollutant-degrading isolates is important for developing genetic markers for monitoring the presence and expression of catabolic genes in situ. For example, genes encoding the catabolic enzymes phenol hydroxylase (Watanabe et al., 1998; Futamata et al., 2001a) and catechol dioxygenase (Okuta et al., 1998) have been commonly used to assess the genetic diversity of phenol-degrading bacterial populations in soil and marine environments (Sei et al., 2004). The sequence diversity of catabolic genes in environmental samples may reflect the diversity in functional potential within the microbial communities in those samples.

Phenol is a priority pollutant of the US Environmental Protection Agency (ATSDR, 1998); it is a component of the emissions from wood, coal and municipal solid waste combustion, as well as automobile exhaust and cigarette smoke. It is also one of the compounds responsible for malodors associated with livestock production facilities resulting from the anaerobic decomposition of animal wastes (Zahn et al., 1997; Zahn, 2001). The objectives of the present study were to (i) isolate and identify phenol-degrading bacteria on green ash leaves, and (ii) characterize the genetic diversity of the phenol catabolic genes present in the phenol-degrading isolates and total leaf communities. This information will help in identifying bacterial contributors to phenol degradation and in assessing the diversity of the phenol degradation genes or pathways employed by these bacteria in the phyllosphere.
Material and Methods

**Confirmation of phenol degradation by naturally occurring microbial communities on leaves.**

Green ash (*Fraxinus pennsylvanica*) leaves were collected from trees growing within 5-6 m of a swine production facility in Hamilton County, Iowa, in September, 2006 and were transported in plastic bags in a cooler and stored at 4°C until used. A subset of leaves were surface-sterilized by immersion in 0.02% Tween 20 for 20 s, 0.2% sodium hypochlorite for 3 min, and sterile water three times for at least 1 min; leaves were dried in a laminar flow hood until surface water was no longer visible (approximately 30 min). Two replicate leaves, each having five leaflets, were examined for each treatment. These leaves were placed in gastight chambers, with one leaf per chamber, and were exposed to 10 µl of 0.054 µmol of $^{14}$C-phenol (Phenol-UL (uniformly labeled)-$^{14}$C, Sigma-Aldrich, St. Louis, MO) with a specific activity of 5.45 µCi/µmol for 24 h. For each chamber, the radioactivity was measured after adding 15 ml of Scintisafe Plus 50% cocktail (Fisher Scientific, Fair Lawn, NJ) to each 1 ml of the KOH used for trapping $^{14}$CO$_2$ released due to microbial phenol degradation, 1 ml of the wash fluid from the leaves, and 1 ml of water from the microfuge tube supporting the leaves. This experiment was performed to evaluate the phenol degradation activity of the phyllosphere community; such activity was observed with green ash leaves collected in August and October, 2005.

**Recovery of microbial communities from green ash leaves.**

Microbial communities were recovered from each leaf by suspending the leaf, with its 5-6 leaflets, in 25 ml of 10 mM phosphate buffer (PB), sonicating for 7 min and vortexing. The leaf was removed from the tube and the suspension was centrifuged at 5000x g for 5 min to remove soil particles. The supernatant was centrifuged at 8000x g for 10 min and the final pellet was resuspended in 1 ml of M9 minimal medium with no carbon source.
Isolation of phenol-utilizing bacteria

Enrichments. Three leaves were used to enrich for phenol-utilizing isolates. Serial enrichments were performed on the communities recovered from each of the three leaves by increasing the phenol concentration during each subculturing. A 1 ml bacterial suspension was transferred into 4 ml of M9 minimal medium with 1 mM phenol as a carbon source and incubated for 4 days at 28°C. For the second enrichment, 500 µl of cells from the first enrichment were transferred to 4.5 ml of M9 medium with 2 mM phenol and grown for 2 days; the remainder was stored at -20°C for microbial community DNA isolation. The third enrichment was performed in the same manner as the second, also with 2 mM phenol and grown for 2 days. Dilutions were plated on M9 plates with 2 mM phenol and streaked for purity. A total of 17 pure isolates were obtained by this method.

Colony autoradiography. In addition to subjecting the communities recovered from each of the three leaves to enrichments, the communities recovered from a different set of three leaves were directly plated onto 1/10 strength Tryptic Soy Agar (1/10 TSA) plates for autoradiography. From these plates, which supported densely packed colonies, two hundred colonies, each likely impure to the high density of the source plate, were transferred as short streaks onto 1/10 TSA plates and the plates were incubated overnight at 28°C. The plates were exposed to 0.2 µCi of $^{14}$C-phenol in an airtight glass container for 2 days to allow cells that degraded phenol to accumulate $^{14}$C. The colonies were replica printed on filter paper (Whatman no. 3), the filter was exposed to a phosphorscreen (Amersham Biosciences) for 2 days, and the image was analyzed using a Typhoon 9410 phosphorimager (Amersham Biosciences, Piscataway, NJ). The colonies that accumulated phenol appeared dark in the image and were streaked for purity. A total of 16 pure isolates were obtained by this method.

Evaluation of the phenol-mineralization activity of isolates.

To confirm that the purified isolates were able to degrade phenol, we examined each one for its ability to release $^{14}$CO$_2$ via $^{14}$C-phenol mineralization. Bacteria were grown in M9 medium supplemented with 5 mM sodium succinate at 28°C, washed thrice in M9 medium without carbon and resuspended in M9 medium without carbon to an OD$_{590}$ of 0.2-0.3. One
hundred µl of this bacterial suspension was inoculated into screw-capped glass tubes containing 5 ml of M9 medium with 2 mM of non-labeled phenol. Caps with mininert valves were used to make the tubes airtight. Ten µl of \(^{14}\)C-phenol (0.01 µCi) was introduced into the tubes using a gas-tight syringe and the tubes were incubated at 28°C on a shaker. M9 medium without bacteria was included as a control to determine the amount of \(^{14}\)C-phenol being trapped along with \(^{14}\)CO\(_2\). One ml of the tube headspace was sampled using a gas-tight syringe immediately after adding \(^{14}\)C-phenol, i.e., at 0 h, and again at 48 h. The \(^{14}\)CO\(_2\) was trapped in 1 ml of 5 M KOH, which was then mixed with 15 ml of Scintisafe Plus 50% cocktail (Fisher Scientific, Fair Lawn, NJ) to measure the radioactivity.

**Genomic DNA extraction and amplification of 16S rDNA for identification of bacterial isolates.**

Bacteria were grown in ½ strength TSA broth at 28°C and genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI). The 16S rDNA was amplified using the primers 8F (5′-AGAGTTTGATCCTGGCTC-3′) and 1404R (5′-GGGCCGGWGTGTACAAGGC-3′; where W=A or T). PCR was performed by using a 50-µl reaction mixture containing 1X PCR buffer (New England Biolabs, Ipswich, MA), each dNTP at a concentration of 200 µM, each primer at a concentration of 0.4 µM, 100 ng genomic DNA, and 2 U Ex Taq (TaKaRa, Madison, WI). The PCR conditions were 1 min at 94°C and then 35 cycles of 30 sec at 95°C, 30 sec at 45°C, and 1 min at 72°C, and a final 5 min extension at 72°C. The 1396-bp PCR product was visualized by electrophoresis using a 1% agarose gel and was sequenced using the 8F and 1404R primers.

**Characterization of the phenol catabolic genes.**

To test for the presence of the gene encoding the first enzyme in the phenol catabolic pathway, phenol hydroxylase (PH), PCR was performed using the universal primers to PH, PheUf/PheUr (Futamata et al., 2001a) for Gram-negative isolates, and primers that we designed, Pheh-3F (5′-CGKATGACSTACGGCTGGATGGGC-3′; where K=G or T and S=G or C) and Pheh-3R (5′-ACGTCTTGTTAGATCTCCTTGGATCCGC-3′) for Gram-positive isolates. The latter primers were based on pheA\(_1\) of *Rhodococcus* RHA1 (Accession
# NC008268), pheA1 of Rhodococcus erythropolis CCM2595 (Accession # AJ973228), and a gene encoding a putative phenol hydroxylase large subunit of Nocardia farcinia IFM 10152 (locus tag nfa43650, Accession # AP006618). The PCR program used to amplify a 620-bp band with the primer set PheUf/PheUr was a touchdown program as follows: 1) 94°C for 10 min, 2) five cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, 3) five cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, 4) 25 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, and 5) a 10-min extension at 72°C. The PCR program used for amplification of a 872-bp band with Pheh-3F/Pheh-3R primers was 94°C for 10 min, 30 cycles of 94°C for 1 min, 60.5°C for 1 min, and 72°C for 1 min followed by a final extension step at 72°C for 5 min.

To determine the type of phenol catabolic pathway employed by the Gram-negative isolates, PCR was performed using the degenerate C23O-specific primers C23OF (5’ – GGTCTGATYGAAATGGAYCGCGA-3’) and C23OR (5’- CGTTCGTTSAACCCGCTGTGG3’), and the catechol 1,2-dioxygenase (C12O)-specific primers C12OF (5’-CCTGARCBGTHGGYTTTTCGCATGATGA-3’) and C12OR (5’- TCACGRGTGCRWARGCATGCAAAAGTC-3’). The C23OF/C23OR primers were designed based on the C23O gene sequence of two Acinetobacter sp. strains, PC19 (Accession # AY887962) and YAA (atdB gene, Accession # AB008831), and Alcaligenes sp. KF711 (Accession # S77084). The C12OF/C12OR primers were designed based on the C12O gene sequences of Acinetobacter sp. ADP1 (catA, Accession # CR543861), Acinetobacter lwoffii K24 (catA3, Accession # DQ874401), Acinetobacter calcoaceticus (Accession # Z36909) and Acinetobacter radioresistens (isoA, Accession # AF380158). The 816-bp and 480-bp products for C23O and C12O, respectively, were visualized by electrophoresis using a 1% agarose gel and were sequenced using the C23OF/C23OR and C12OF/C12OR primers, respectively.

**Qualitative assay for catechol 2,3-dioxygenase activity.**

The catechol 2,3-dioxygenase activity of each isolate was examined using a modified version of an assay described by Pankhurst et al. (1965). The phenol-degrading bacterial isolates
were streaked onto M9 minimal medium plates with 2.5 mM phenol as the carbon source and grown for 4-5 days at 28°C. A bacterial strain, *Pseudomonas* sp. strain CF600 harboring a C23O gene on a plasmid, was included as a positive control. The colonies from the plates were lifted onto filter paper (Whatman no. 3) and sprayed with 0.1 M of catechol. The appearance of yellow color around colonies exhibiting C23O activity occurred within 30 min.

**Total leaf community DNA extraction and amplification of phenol hydroxylase and catechol dioxygenase genes.**

To determine the proportion of the total genetic diversity in the leaf community represented by the phenol-degrading isolates obtained in this study, total microbial community DNA was isolated from 3 communities recovered from leaves by using the PowerSoil DNA isolation kit (MoBio Labs, Carlsbad, CA); however, the DNA yield from only one set of leaves was sufficiently high to use in subsequent studies. An aliquot of the DNA from this community was amplified using the GenomiPhi kit (GE Healthcare, Amersham Place, UK). PCR was performed with the unamplified and amplified total community DNA samples using primers PheUf/PheUr and Pheh-3F/Pheh-3R to amplify the phenol hydroxylase genes in the Gram-negative and Gram-positive components of the communities, respectively. PCR products of the appropriate sizes were gel purified and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Twenty clones resulting from the unamplified community DNA, and ten from the GenomiPhi-amplified community DNA, were selected, the plasmids were isolated, and the cloned fragments were sequenced using the primers R1 (Reverse M13 primer 5’-CAGGAAACAGCTATGACC-3’) and T7-1 (5’-AATACGACTCACCTAG-3’).

**Sequence analysis.**

The taxonomic identification of the isolates was determined by comparing the 16S rDNA sequences obtained to the Ribosomal Database (Maidak et al., 2001) to identify the closest matches. The phenol hydroxylase sequences were translated and the amino acid sequences were aligned using ClustalW (Thompson et al., 1994). The identity of the specific residues
was used to place each phenol hydroxylase gene into one of the three groups described by Futamata (2001).

**Results**

*Confirmation of phenol degradation activity of naturally occurring microbial communities on green ash leaves.*

The degradation activity of the microbial community on green ash leaves was demonstrated by exposing surface-sterilized and unsterilized leaves to radiolabeled phenol in gastight chambers for 24 h. The amount of radioactivity in KOH in chambers with the unsterilized leaves was significantly greater than that in chambers with the sterilized leaves ($P < 0.005$) (Fig. 1), indicating that organisms that were susceptible to the surface-sterilization treatment were capable of mineralizing airborne phenol. Similar results were observed in a previous study (Sandhu et al., 2007). Collectively, these studies performed with leaves sampled in Aug and Oct 2005 and Sept 2006 suggest that the degradation activity of these leaf-associated microbial communities is a common characteristic of these communities under these conditions.

![Fig. 1. Phenol mineralization activity of naturally occurring microbial communities on green ash leaves sampled in September 2006 from an area rich in airborne organic compounds.](image-url)
Values represent the mean percentage ± SEM of the total radioactivity introduced into the chambers at 0 h (n = 2).

**Isolation of phenol-utilizing bacteria from green ash leaves.**

The phenol-utilizing bacteria from green ash leaf microbial communities were isolated using two methods: enrichment and colony autoradiography. Direct selection of phenol utilizers on M9 minimal media plates with 2.5 mM phenol as the sole carbon source was attempted but was not successful because bacterial colonies developed on plates without any carbon source, indicating that bacteria were able to use residual nutrients in the agar to support growth. Also, the phenol-utilizing bacteria may need to adapt to lower phenol concentrations than those used in plates to induce enzymes involved in phenol degradation. Leaves harbored total communities with at least $10^5$-$10^7$ cfu g$^{-1}$ bacteria based on culturable counts on 1/2TSA with cycloheximide (200 µg/ml). Enrichments using minimal media with phenol provided as the sole carbon source exhibited visible turbidity within a week, whereas tubes without phenol had no detectable turbidity. After enrichment, a total of 17 colonies with distinct morphologies were selected from three independent leaf replicates.

In addition to enrichments, colony autoradiography was employed to screen for phenol-utilizing bacteria within the microbial communities on green ash leaves. Colony autoradiography is a technique used to identify cells that can utilize or incorporate $^{14}$C substrates. Of two hundred bacterial samples that were transferred from plates supporting densely-packed colonies from green ash leaves to 1/10 TSA plates (Fig. 2A), seven samples exhibited evidence of $^{14}$C accumulation (Fig. 2B). These samples were streaked for purity, and all were found to be mixtures; 16 isolates with distinct colony morphologies were identified. These isolates were subsequently screened for $^{14}$C-phenol mineralization (described below).
Fig. 2. Identification of phenol-utilizing bacteria by colony autoradiography. Bacterial samples were patched onto two 1/10 TSA plates (A) that were exposed to $^{14}$C-phenol for 48 h and were lifted onto filter disks (B), which were subsequently exposed to a phosphorscreen to indicate those samples that accumulated $^{14}$C.

**Evaluation of the phenol-mineralizing activity of the isolates.**

A total of 33 bacterial isolates were screened for phenol-mineralizing activity in a minimal medium containing $^{14}$C-phenol. Bacterial isolates were first grown in a minimal medium with unlabeled phenol as the sole carbon source. After introduction of $^{14}$C-phenol into the tubes, air in the headspace was sampled at 0 and 48 h and was injected into KOH to trap $^{14}$CO$_2$, and the KOH was evaluated for radioactivity. A total of 10 isolates, 7 from the enrichments and 3 identified by colony autoradiography, exhibited significantly higher radioactivity in the air sampled at 48 h than at 0 h (Fig. 3A and B), demonstrating that these bacterial isolates were in fact phenol degraders based on their ability to mineralize $^{14}$C-phenol to $^{14}$CO$_2$. A negative control with no bacterial cells in the medium was included to determine the amount of airborne $^{14}$C-phenol sampled along with the $^{14}$CO$_2$, and it showed that the amount of radioactivity in air either did not change or decreased 48 h following $^{14}$C-phenol introduction (Fig. 3). Tubes with the bacterial strain *Pseudomonas syringae* DC3000, which does not utilize phenol, were also included and did not show an increase in radioactivity at 48 h, as expected. These tubes also showed that a negligible amount of radiolabel was incorporated into the non-phenol degrading bacterial cells as compared to in the phenol degraders.
Fig. 3. \(^{14}\)C-phenol mineralization ability of selected isolates from the enrichments (A) and as identified by colony autoradiography (B). Isolates were grown in M9 minimal medium with 2 mM of non-labeled phenol and 0.1 µCi of \(^{14}\)C-phenol as the carbon source. The amount of \(^{14}\)CO\(_2\) released due to bacterial \(^{14}\)C-phenol mineralization was measured by sampling 1 ml of the tube headspace and trapping in KOH, and the counts per minute (DPM) in the one-ml headspace samples were determined. Values represent the mean DPM ± SEM (n = 2). The isolates that did not show phenol degradation are not shown.

**Taxonomic identification of the phenol-degrading isolates.**

The 16S rDNA sequences were determined for the ten bacterial isolates that degraded \(^{14}\)C-phenol and were compared to those of known organisms in the Ribosomal Database (Maidak et al., 2001). While molecular phylogeny based on the ribosomal genes is standard for bacterial classification, there are no universally accepted criteria for the level of similarity among 16S rDNA sequences that indicates that two isolates are from the same genus or species. While 8 out of 10 of the 16S rDNA sequences showed greater than 97% homology to the closest related sequence (Table 1) (Drancourt et al., 2000; Kim et al., 2006), two sequences (E-9 and 10) shared ≤ 95% with known organisms (Table 1). Interestingly, all enrichment isolates were Gram-negative, out of which five were most closely related to the
genus *Acinetobacter* and two to the genus *Alcaligenes*, whereas all isolates identified by colony autoradiography were Gram-positive and belonged to the genus *Rhodococcus* (based on a sequence similarity of >99.5%). Previous studies have shown that *Acinetobacter*, *Alcaligenes* and *Rhodococcus* strains can be metabolically versatile (Yang et al., 2001; Barbe et al., 2004; Larkin et al., 2005; Rivas et al., 2007). These species have been previously shown to be members of phyllosphere communities (Jurkevitch and Shapira, 2000; Cohen et al., 2004; Ilori et al., 2006).

### Table 1. Identity of phenol-degrading bacterial isolates obtained from leaves

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Closely related organism based on 16S rDNA gene sequence</th>
<th>Identity</th>
<th>Phenol hydroxylase identity</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-1</td>
<td>E</td>
<td><em>Acinetobacter lwoffii</em></td>
<td>99.4%</td>
<td><em>Acinetobacter calcoaceticus, mphN</em></td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>E-9</td>
<td>E</td>
<td><em>Acinetobacter sp.</em></td>
<td>95.4%</td>
<td><em>A. calcoaceticus</em></td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>E-10</td>
<td>E</td>
<td><em>Acinetobacter sp.</em></td>
<td>93.1%</td>
<td><em>Acinetobacter radioresistens</em></td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>E-12</td>
<td>E</td>
<td><em>Acinetobacter seohaensis</em></td>
<td>97%</td>
<td><em>Acinetobacter sp. PD12</em></td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>E-4</td>
<td>E</td>
<td><em>A. lwoffii</em></td>
<td>99.4%</td>
<td><em>A. calcoaceticus, mphN</em></td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td>E-3</td>
<td>E</td>
<td><em>Alcaligenes sp. CC-ESB2</em></td>
<td>99.7%</td>
<td><em>Alcaligenes faecalis BC2001</em></td>
<td>β-Proteobacteria</td>
</tr>
<tr>
<td>E-6</td>
<td>E</td>
<td><em>Alcaligenes sp. A72 or CC-ESB2</em></td>
<td>100%</td>
<td><em>A. faecalis BC2001</em></td>
<td>β-Proteobacteria</td>
</tr>
<tr>
<td>T-2</td>
<td>CA</td>
<td><em>Rhodococcus sp. CHNTR32</em></td>
<td>100%</td>
<td><em>Rhodococcus sp. RHA1</em></td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>T-5</td>
<td>CA</td>
<td><em>Rhodococcus sp. CHNTR32</em></td>
<td>100%</td>
<td><em>Rhodococcus sp. RHA1</em></td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>T-10</td>
<td>CA</td>
<td><em>Rhodococcus sp. CHNTR32</em></td>
<td>99.5%</td>
<td><em>Rhodococcus sp. RHA1</em></td>
<td>Actinobacteria</td>
</tr>
</tbody>
</table>

*a Method used for selection of phenol-utilizing bacterial isolates. E, Enrichment; CA, Colony autoradiography.

**Genetic characterization of the phenol catabolic genes in the phenol-degrading isolates.**

To characterize the 7 Gram-negative isolates for the presence of phenol hydroxylase, the first enzyme involved in the degradation of phenol, we used universal primers to amplify the gene encoding the largest subunit of the multi-component phenol hydroxylase. This gene has been
used in other studies as a molecular marker to assess the diversity of phenol-degrading bacteria in the environment (Zhang et al., 2004). A 620-bp band was amplified from each isolate and sequenced. Futamata et al. (2001a) aligned the amino acid sequences of the large subunit of phenol hydroxylases from several Gram-negative bacteria and grouped them into three classes, designated I, II and III, which correspond to the previously determined kinetic groups, low-$K_s$, moderate-$K_s$, and high-$K_s$, exhibited by phenol-degrading bacteria during TCE degradation (Futamata et al., 2001b). Based on the sequences of the PH genes in the Gram-negative isolates, all of these isolates had class II PHs. Phenol hydroxylase sequences belonging to isolates E-1 and E-4 were 86% similar to the phenol hydroxylase gene $mphN$ of Acinetobacter calcoaceticus; the genes in E-3 and E-6 were 96% similar to an Alcaligenes faecalis strain BC2001 PH gene; the E-9 PH gene was 88% similar to the A. calcoaceticus PH gene; E-10 PH was 92% similar to the gene for the oxygenase component of the PH of Acinetobacter radioresistens, and the E-12 PH gene was 98% similar to the gene for the oxygenase component of the PH of Acinetobacter sp. PD12. The phenol hydroxylase gene sequences of all three Gram-positive isolates were 84 to 86% similar to the $Rhodococcus$ sp. RHA1 phenol hydroxylase gene.

The type of phenol degradation pathway, ortho or meta, in our isolates was determined using PCR and an enzymatic assay for C23O. PCR indicated the presence of the C12O gene, but not the C23O gene, in all of the Gram-negative isolates, suggesting that either all of the Gram-negative isolates harbored only the C12O gene or that the C23O primers were not degenerate enough to amplify the C23O gene sequences from the isolates obtained in this study. The C12O gene sequences of the isolates were similar to those of the Alcaligenes and Acinetobacter sp., suggesting the presence of at least an ortho pathway for phenol degradation in the Gram-negative isolates. The type of pathway present in the $Rhodococcus$ sp. isolates could not be determined by PCR because the available sequences of the $Rhodococcus$ sp. C12O and C23O genes were too dissimilar to design primers effective for multiple strains. None of the Gram-negative or Gram-positive isolates tested positive for C23O activity in an enzymatic assay specific for C23O activity.
Efforts to characterize the phenol hydroxylase gene diversity present in a leaf microbial community.

To compare the phenol hydroxylase gene diversity in the total bacterial community to that in the cultured isolates obtained in this study, we amplified and cloned the phenol hydroxylase genes from the DNA isolated from a microbial community recovered from a green ash leaf, with and without subsequent GenomiPhi genomic DNA amplification. Twenty clones obtained with unamplified total community DNA and 10 obtained with amplified community DNA were sequenced. All of the sequences were identical and exhibited 86% identity to an *Acinetobacter calcoaceticus* phenol hydroxylase gene. Reasons underlying the recovery of only a single PH sequence from the community include the possibility that a single phenol-degrading species, perhaps *A. calcoaceticus*, dominated this phyllosphere bacterial community, that the phenol hydroxylase genes of other phenol-degrading species were present at a sufficiently low concentration that they were not amplified, cloned, and/or selected for sequencing, and that the primers used for amplifying the phenol hydroxylase genes were less effective for other bacterial species. No amplification was obtained with community DNA using the Pheh-3F/Pheh-3R primers specific for the *Rhodococcus* isolates. This could be due to a low gene copy number in the community DNA, as well as poor lysis of the Gram-positive cells, and degradation of DNA during leaf or DNA storage, thus losing the sensitivity needed to detect minority community members. Furthermore, the community DNA was isolated from a distinct set of leaves from the set used for culturing phenol-utilizing bacteria; thus, the lower phenol hydroxylase gene diversity identified in the total community DNA than in the cultured isolates may reflect differences in the microbial communities on these distinct leaf sets.

Discussion

Leaf surfaces of terrestrial plants near sites rich in airborne pollutants provide an excellent habitat for pollutant-degrading bacterial communities. Although phenol-degrading bacteria have been isolated from a number of ecosystems, such as polluted river waters and
contaminated soils, leaf surfaces remain an unexplored habitat for these organisms. Here, we isolated and characterized phenol-degrading bacteria from the natural microbial communities residing on leaves that were taken from a site rich in airborne volatile organic compounds, including phenol (Zahn et al., 1997). Using a culture-based approach, we found ten phenol-degrading bacterial isolates related to three different genera, the Gram-negative genera *Acinetobacter* and *Alcaligenes* and the Gram-positive genus *Rhodococcus*. Although bacteria belonging to these genera have been isolated from phenol-contaminated waters (Xu et al., 2000) and soils (Saadoun, 2002; Mandri and Lin, 2007), they have also been shown to be a part of phyllosphere communities. For example, *Acinetobacter sp.* has been shown to be present in the phyllosphere of variety of plant species such as *Citrus sinesis* (Yang et al., 2001), chestnut trees (Valverde et al., 2005), and field-grown maize (Kadivar and Stapleton, 2003), and *Alcaligenes* and *Acinetobacter* have been found to be a part of the phyllosphere community of tropical plants (Ilori et al., 2006) and tomato plants (Jurkevitch and Shapira, 2000), with some isolates exhibiting the ability to degrade hydrocarbons, such as diesel, kerosene and lubricating oil (Ilori et al., 2006). Ilori et al (2006) found that these hydrocarbon-utilizing bacteria isolated from leaves belonged to the genera *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Micrococcus*, *Bacillus*, *Pseudomonas* and *Corynebacterium*. The *Rhodococcus* genus has also been found on the leaves the aquatic fern *Azolla pinnata* (Cohen et al., 2004). Collectively, these studies indicate that pollutant-degrading bacteria are a part of naturally occurring microbial communities on leaves.

We identified ten phenol-degrading bacterial isolates from natural phyllosphere communities of green ash leaves. Five of these bacterial isolates were most closely related to the genus *Acinetobacter*, two to *Alcaligenes*, and three to *Rhodococcus*. Bacteria of these genera have been isolated from numerous contaminated natural or engineered environments (Watanabe et al., 1998; Abd-El-Haleem et al., 2002; Arutchelvan et al., 2005) and have been shown to be capable of degrading a variety of aromatic compounds, including phenol and its derivatives (Lenke et al., 1992; Paller et al., 1995; Hill et al., 1996; Abd-El-Haleem et al., 2002). In fact, bacteria belonging to the genus *Rhodococcus* are among the most metabolically versatile of the Gram-positive bacteria (Karlson et al., 1993; van der Geize and
Dijkhuizen, 2004; Larkin et al., 2005). Phenol-degrading isolates may be common in natural communities based on several reports documenting the presence of these genera on leaves, as described above (Jurkevitch and Shapira, 2000; Yang et al., 2001; Ilori et al., 2006), although how common pollutant degradation activities are among phyllosphere isolates of these genera are not known.

Characterization of the phenol catabolic genes of the isolates obtained from leaves did not reveal much genetic diversity. All of the Gram-negative isolates possessed an ortho-pathway for phenol degradation and phenol hydroxylases belonging to group II, the moderate affinity PHs. A few studies (Eichner et al., 1999; Boon et al., 2000; Iwamoto et al., 2000; Ogino et al., 2001) looking at the quantitative changes in overall microbial community structure during pollutant degradation processes have shown a decrease in the microbial diversity and domination by a few functionally important bacterial populations. Different types of PHs, class I, II and III, are present in different bacterial genera or strains and a previous study showed that one strain can even harbor two different multi-component PH genes (Zhang et al., 2004), and this was suggested to help it to adapt to a highly fluctuating environment. Also, in our study none of the isolates tested positive for C23O, an enzyme required for the meta pathway of phenol degradation. This supports a finding in previous studies that bacterial populations possessing C12O are the primary phenol degraders that are dominant under low phenol-conditions (Muller and Babel, 1996; Sei et al., 2004), assuming that leaves have a low phenol content. For example, Sei et al. (2003) demonstrated that in low-phenol seawater microcosms, bacterial populations possessing C12O were the primary degraders, whereas bacterial populations possessing C23O increased under high phenol concentrations. We were not able to test phenol-utilizing Rhodococcus isolates for the presence of C23O and C12O genes by PCR because the sequence similarity of C23O and C12O genes among Rhodococcus strains was too low to design general PCR primers; however, like the Gram-negative isolates, these Rhodococcus strains appeared to lack the C23O gene based on the absence of C23O activity.
Phylloremediation of volatile organic compounds has been suggested by several previous studies and one study has demonstrated pollutant-degrading ability of leaf-associated bacteria (Van Aken et al., 2004). In this study by Van Anken et al., a *Methylobacterium* sp. strain that was isolated from leaves and other hybrid poplar tissues was able to degrade xenobiotic compounds, demonstrating that bacteria capable of pollutant degradation are present in some phyllosphere communities. In our previous study (Sandhu et al., 2007), we provided direct evidence of active phenol degradation by the natural phyllosphere communities on green ash leaves that were taken from trees near a swine production facility. This site was rich in airborne VOCs, based on the level of odor, and likely included phenol (Zahn et al., 1997). So far few studies have explored leaf surfaces as a habitat for the isolation and identification of pollutant-degrading bacteria.

Characterization of the genes involved in the phenol catabolic pathway revealed that all of the *Acinetobacter*- and *Alcaligenes*-like isolates have group II phenol hydroxylase genes and that the three *Rhodococcus* isolates have phenol hydroxylase genes similar to that of *Rhodococcus* sp. RHA1. The phenol-degrading Gram-negative bacteria are diverse and their PHs have been categorized based on specific amino acid residues into three groups with distinct kinetics, classes I, II and III, which exhibit high (low $K_s$), moderate and low (high $K_s$) affinity for phenol, respectively (Futamata et al., 2001a). Examples of organisms with PHs in these classes are *Comamonas testosteroni* R5, *Pseudomonas* sp. strain CF600, and *Pseudomonas putida* P-2, for class I, II, and III, respectively (Futamata et al., 2001a). Phylogenetic classification of PHs corresponds to the kinetic classification of phenol-degrading bacteria (Futamata et al., 2001a), thus facilitating the group-specific monitoring of the different types of phenol-degrading bacteria in microbial communities. It has been suggested in previous studies that bacteria with a high affinity for phenol are present at sites with low phenol concentrations (Massol-Deya et al., 1997) and those with low affinity are present at sites with high phenol concentrations (Watanabe et al., 1996; Jiang et al., 2004; Jiang et al., 2006), as would be predicted based on the kinetics. Thus, the phenol available to bacteria on leaves at a site rich in airborne organic compounds was likely high enough to select bacteria with moderate affinity for phenol. Group-specific monitoring of phenol
hydroxylase genes that can cometabolize trichloroethylene (TCE) in contaminated soils has been shown to be important in predicting the catabolic potential of indigenous TCE-degrading bacterial populations (Futamata et al., 2001a; Watanabe et al., 2002) and thus could help in developing effective bioremediation strategies.

The proportion of the phenol hydroxylase gene diversity represented by the phenol-degrading isolates in the total leaf community was determined by amplification of the PH genes within the total community DNA. No amplification was obtained with the primers specific for *Rhodococcus* PH genes, suggesting that *Rhodococcus* species were a minor population on leaves and thus their PH genes were below the limit of detection by PCR. In contrast, the PheUf/PheUr primers successfully amplified PH genes, the sequences of which were identical for all of the twenty cloned genes and were 86% similar to a known *Acinetobacter* PH gene. This indicates either that the organism with this gene was a dominant population in the phyllosphere of green ash leaves, possibly because its growth was favored by the airborne phenol, or that the PCR amplification was biased toward this *Acinetobacter*-like PH gene. The PheUf/PheUr primers were designed based on the phenol hydroxylase gene sequences of pseudomonads, *Comamonas* species and *Burkholderia* species (Futamata et al., 2001a) and therefore may not have been efficient in amplifying genes of other bacterial species. In such cases, where degenerate primers cannot be used to capture the total bacterial diversity in environmental samples, other approaches, such as using several PCR primers (Llyod-Jones et al., 1999), need to be employed.

Bacterial phenol degradation occurs mainly via two pathways, initiated by either the *ortho* or *meta* cleavage (Muller and Babel, 1996). The *ortho* pathway is catalyzed by the enzyme C12O, which cleaves the catechol ring at the *ortho* position, while the *meta* pathway is catalyzed by C23O that cleaves the ring at the *meta* position. Bacteria can have one or both of these enzymes (Song et al., 2000; Jiang et al., 2006), which may be expressed under different conditions. Catechol dioxygenase genes have been used as targets in previous studies to monitor types of and changes in bacterial populations in environmental samples contaminated with aromatic compounds. In the present study, we used catechol dioxygenase
gene-specific primers to determine gene diversity in phenol-degrading isolates obtained from
the phyllosphere of green ash leaves. We found that all phenol-degrading Gram-negative
isolates possessed C12O genes based on PCR while no amplification was obtained with the
Gram-positive *Rhodococcus* species. None of the isolates tested positive for C23O in an
assay for C23O activity, suggesting little diversity in the type of phenol degradation
pathways that are possessed by phyllosphere bacteria. It is also possible that the low phenol
concentrations (approximately 0.25 µM) around the swine facility selected for bacteria
possessing C12O genes, similar to the finding in a previous study (Tay et al., 2005) that high
phenol concentrations (3mM) were associated with the dominance of C23O-possessing
bacteria in a bioreactor, consistent with the lower affinity of C23O than C12O for phenol
(Muller and Babel, 1996; Filonov et al., 1997). In another study (Sei et al., 2004), behavior of
aromatic compound-degrading bacterial populations and changes in the microbial community
structure in sea water microcosms revealed that the bacterial populations possessing C12O
genes were the primary degraders of phenol and benzoate and that the C23O-harboring
bacteria only dominated under higher substrate concentrations.

In conclusion, we examined an unexplored habitat, the phyllosphere, to identify and
characterize the phenol-degrading bacterial species. Although ecosystems such as polluted
seawaters, rivers and soils have been examined for phenol-degrading bacteria and monitored
for changes in bacterial populations, the diversity of phenol-degrading taxa in the
phyllosphere was unexplored prior to this study. We isolated ten phenol-degrading bacterial
species most closely related to the genera *Acinetobacter*, *Alcaligenes* and *Rhodococcus* from
leaves at a site rich in airborne organic compounds. We expected a wide diversity of the
phenol-degrading bacteria on leaves, in part because the diversity of bacteria associated with
leaves is known to be high (Wilson and Lindow, 1992; Yang et al., 2001). Approaches such
as RNA-SIP (stable isotope probing) may be employed in future studies to determine the
total diversity of phenol-degrading bacterial populations and to characterize the catabolic
genes present in these populations. This technique would thus help in linking microbial
phylogeny with function, which is important in developing strategies for bioremediation of
polluted environments.
References


Chapter 4. Effect of phenol on *Pseudomonas syringae* as determined by a transcriptome analysis of the response of *P. syringae* to phenol and other abiotic stresses

**Introduction**

The bacterial genes or proteins induced in response to a chemical agent may reveal the mechanism of toxicity of that compound. For example, the induction of the oxidative stress response involving anti-oxidative enzymes of the *OxyR* and *SoxRS* regulons in response to hydrogen peroxide (H$_2$O$_2$) suggests that H$_2$O$_2$ causes oxidation of cellular components, such as the DNA, lipids and proteins (Zheng et al., 2001). Similarly, the exposure of *E. coli* to potassium tellurite resulted in the induction of anti-oxidative enzymes, namely superoxide dismutases, suggesting that tellurite causes oxidative stress, possibly via the production of superoxide radicals in the cell cytoplasm. Yet another study showed that *E. coli* exposure to selenium oxides induced manganese and iron superoxide dismutases (*sodA* and *sodB*) and went further to show increased sensitivity of superoxide dismutase mutants to selenium oxide, demonstrating that superoxide production is a major mechanism of selenium toxicity in these cells (Bebien et al., 2002). These studies illustrate how identifying the types of proteins induced in response to toxic compounds can be helpful in understanding the modes of action of the compounds and may provide insight into the mechanisms that bacteria employ to tolerate the stresses imposed by these toxic compounds.

Phenol is an organic solvent that is considered hydrophobic enough to partition into the bacterial cell membrane (Heipieper et al., 1994). Previous studies have suggested that organic solvents affect the cell membrane by disrupting its structural integrity and function, resulting in increased membrane fluidity and permeabilization (Heipieper and de Bont, 1994; Weber and de Bont, 1996; Segura et al., 1999). For example, in *E. coli*, phenol was found to result in the leakage of potassium ions and ATP (Heipieper et al., 1991) and in *Pseudomonas putida*, the two major mechanisms of phenol tolerance included changes in cell membrane
composition and the level of saturation of membrane fatty acids (Diefenbach et al., 1992; Heipieper et al., 1992).

Surprisingly, little is known about the toxic effects of phenol on constituents in the bacterial cell cytoplasm once phenol has passed through the cell membrane. This is particularly unexpected given that phenol has been suggested to cause oxidative stress in bacteria. For example, degradation of aromatic substrates, including phenol and 4-chlorophenol by mono-and dioxygenases, produces reactive oxygen species in Ochrobactrum anthropi (Tamburro et al., 2001). Similarly, exposure of P. putida to phenol also resulted in up-regulation of some oxidative stress proteins (Santos et al., 2004). The exposure of Bacillus subtilis to phenol and catechol caused an induction of the Spx (suppressor of clpP and clpX) disulfide stress regulon and a thiol-specific oxidative stress response, respectively. These results suggest that phenol generates non-native disulfide bonds and catechol generates oxygen species, both of which reflect the imposition of oxidative stress by these organic solvents (Tam le et al., 2006).

In the present study, we aimed to understand the mechanism of phenol tolerance and toxicity in bacteria during its entry through cell membranes and after it enters the bacterial cell. Cells were exposed not only to phenol but also to H$_2$O$_2$, with the extent of overlap between these stress regulons used to evaluate the extent of oxidative stress caused by phenol in bacteria. Bacterial cells were also exposed to water stress, to evaluate the effects of a stress known to impact the bacterial cell membrane. The cells were exposed to these stresses for 15 min to understand the short-term adaptation response in bacteria upon exposure to phenol. Lack of availability of water to a bacterial cell imposes two kinds of stress (Halverson and Firestone, 2000), osmotic stress (Csonka, 1989) and matric stress (van de Mortel and Halverson, 2004). Osmotic stress occurs when bacteria are present in an environment high in solutes, such as sodium chloride, whereas matric stress occurs when a cell is present in an environment where water is physically absent or is bound to particles such as soil or high molecular weight polyethylene glycol (PEG) (MW $\geq$ 8000), a compound that cannot cross the outer membrane. Osmotic stress is known to cause changes in the bacterial cell
membrane, including a shift in the cis/trans ratio of unsaturated fatty acids in the membrane to maintain membrane fluidity (Heipieper et al., 1996; Halverson and Firestone, 2000). The extent of overlap between genes affected by water stress and phenol will provide insight into the extent to which phenol damages the cell membrane.

The majority of the previous studies examining the effect of toxic compounds in bacteria have been done with bacterial strains that can degrade these compounds. For example, the effects of the aromatic compounds phenol, catechol, and benzoate have been examined in Acinetobacter species (Benndorf et al., 2001; Pessione et al., 2003), a soil bacterium that can metabolize various xenobiotic compounds. A proteomic study with Pseudomonas putida strain KT2440, a model bacterial strain with diverse metabolic capabilities, was performed to gain insight into global mechanisms underlying solvent toxicity and tolerance (Santos et al., 2004). In terms of the mechanisms of tolerance, degradation of toxic organic solvents may reduce solvent toxicity, thus increasing bacterial tolerance (Ferrante et al., 1995). The modes of toxicity of a compound can be different in bacteria capable of degrading a toxic compound versus a non-degrading strain, as suggested by the finding that polychlorinated biphenyl (PCB) toxicity in Burkholderia xenovorans resulted from the production of toxic intermediates produced during metabolism under PCB-degrading conditions (Parnell et al., 2006). To better understand the mode of toxicity of phenol, we examined its effects on Pseudomonas syringae pv. tomato strain DC3000, a non-phenol degrading bacterial strain. P. syringae cells commonly reside on leaf surfaces where they may be exposed to a variety of stresses, such as airborne pollutants and rapidly fluctuating temperatures, humidity, and solar radiation. These stressful conditions on leaf surfaces can affect bacterial survival and thus the effective removal of airborne pollutants by leaf-associated bacteria.

Global gene expression analysis is a powerful tool for exploring bacterial responses to chemical or environmental stresses, providing insights into the mechanisms that bacteria use for stress tolerance. A number of such studies have been performed with the model bacterial species E. coli and B. subtilis to identify the mechanisms that bacteria employ for adaptation
or tolerance to antibiotics, H$_2$O$_2$, and organic solvents (Zheng et al., 2001; Phadtare et al., 2002; Hayashi et al., 2003; Tam le et al., 2006). In this study, we investigated the mechanism of phenol tolerance by comparing the genes induced in response to phenol to those induced by oxidative stress and low water availability, including both osmotic and matric stress. To accomplish this, we performed a transcriptome analysis of exponentially-growing *P. syringae* pv. tomato DC3000 cells exposed to phenol, H$_2$O$_2$, NaCl, and PEG, and proposed a model for phenol toxicity in bacteria based on the induction of heat shock genes.

### Material and Methods

**Bacterial strains and growth conditions.**
The strains used in this study were *Pseudomonas syringae* pv. tomato strain DC3000 (Whalen et al., 1991) for microarray experiments and *E. coli* strain DH5α for standard cloning procedures (Sambrook et al., 1989). DC3000 was grown in either $\frac{1}{2}$ 21C medium (Halverson and Firestone, 2000; Axtell and Beattie, 2002) supplemented with 0.3% glucose and 5 mM of sodium succinate (designated $\frac{1}{2}$ 21C) or MinA minimal medium (Miller, 1972) supplemented with 5 mM of sodium succinate (designated MinA) and plated on King’s medium B (KB) (King et al., 1954) agar medium for cell counts when determining EC$_{50}$ values. *E. coli* strains were grown in LB (Luria-Bertani) medium (Miller, 1972). The antibiotics used were rifampicin (50 µg/ml) and kanamycin (50 µg/ml).

**Determination of EC$_{50}$ of phenol and hydrogen peroxide.**
The effective concentration (EC$_{50}$) of phenol and hydrogen peroxide, defined as the concentration of a compound required to cause a 50% decrease in the growth rate, was determined in test tubes containing 5 ml of $\frac{1}{2}$ 21C, various concentrations of phenol or hydrogen peroxide, and DC3000 cells at an initial density of $10^8$ cells/ml. The cultures were incubated for 9-12 h at 28°C and the optical density (OD$_{600}$) was measured over time. The colony forming units (CFU) per ml were determined from the standard curve and the growth rates were determined from the slope of the curve obtained by plotting mean log CFU/ml versus time for each inhibitor concentration. These growth rates were used to calculate the
percent rate of growth of treated cells relative to that of the untreated cells. The EC\textsubscript{50} values were determined for phenol and hydrogen peroxide by plotting the % rate of growth versus inhibitor concentration.

**DNA microarray experiments and data analysis.**

On each of four days, two replicate cultures were prepared. For each, a colony of DC3000 from KB agar was inoculated into ½ 21C medium and grown until dense for 24 h. The cells were sub-cultured twice in fresh medium, grown to mid-log phase (10\textsuperscript{8} cells/ml), centrifuged at 5000 x g for 10 min, and resuspended in pre-warmed ½ 21C medium. For each culture, two replicate tubes were prepared in which 2.5x10\textsuperscript{8} cells were exposed to either 5 mM of phenol, 1 mM of H\textsubscript{2}O\textsubscript{2}, 220 mM of NaCl, 16% PEG or no stress in a 1.1-ml final volume. The cells for each of the five treatments were incubated on a shaker for 15 min at 28°C, immediately after which they were amended with RNAprotect bacterial reagent (Qiagen, Valencia, CA) to stabilize the RNA. The cells from replicate tubes for each treatment were combined and the RNA was isolated using the manufacturer’s instructions. After repeating the treatments and the RNA isolation each day for four days, the RNA isolated for a given treatment on days 1 and 3 were pooled, and that collected on days 2 and 4 were pooled, resulting in two RNA samples that represented each treatment. The quality of the RNA samples was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

RNA samples were sent to NimbleGen Systems Inc. (Madison, WI) for cDNA synthesis, hybridization and scanning. The DC3000-specific DNA microarray was designed and manufactured by NimbleGen Systems Inc., and included 5,608 ORFs, each represented by at least 17 unique 24-mer probe pairs, which were synthesized \textit{in situ}; each ORF was represented in duplicate, designated as blocks, on the glass slide. Each probe pair consisted of a sequence perfectly matched to the ORF and a sequence that included two mismatched bases to determine background and cross-hybridization. After labeled cDNA synthesis and hybridization, the microarrays were scanned to obtain the signal intensity for each probe. The average value across the probe sets for a single gene was determined for each block, and the
values for the two blocks were averaged. The values were normalized with the quantile normalization method and analyzed with the RMA algorithm (Bolstad et al., 2003; Irizarry et al., 2003) by NimbleGen Systems, Inc. For each gene, a multiple comparisons test using a blocked design with 5 treatments and 2 replicates was performed using SAS (SAS Statistical Institute, Cary, NC). This yielded a $P$-value and $Q$-value (False Discovery Rate, FDR) for each gene. The extent of induction of a gene by a stress was calculated relative to its expression level in non-stressed cells. Genes were identified as responding to a treatment based on the following criteria: FDR of $\leq 2\%$, $P$-value of $\leq 0.05$, and at least a two-fold change in response to phenol, NaCl or PEG, and at least a $\geq 1.3$-fold change in response to hydrogen peroxide. A two-fold change cut-off value has been most commonly used in microarray studies to identify genes with altered expression levels; however, the two-fold cut-off value was too stringent for $H_2O_2$ treatment based on the number of genes obtained that were significantly up-or down-regulated and the level of change in genes commonly associated with oxidative stress.

**Construction of Pst DC3000 heat shock gene mutants.**

An internal fragment of each of the four induced heat shock genes $ftsH$, $htpX$, $clpA$ and $lon2$ was PCR amplified from DC3000 using the following forward and reverse primers: FtsH-F (5'-GAACAGCAGAGCATCT GGACC-3'), FtsH-R (5'-AATCTGCTCGCGACACGAAT-3'), HtpX-F (5'-AATGAG CACCGGTACTCAGATC-3'), HtpX-R (5'-ATACGGGCGAAGAACATCAC-3'), ClpA-F (5'-ATCTTTCCTCAGGCAATCCTCTG-3'), ClpA-R (5'-ATAGGCACCCGCT TCG TCA AT-3'), Lon2-F (5'-AAGGAAGTCAAGTGCGACAGTT-3') and Lon2-R (5'-GGCGCAGGTCCAGGTAATGAT-3'). Each of these fragments was ligated into XcmI-digested T-vector pTOK2 (Kitten and Willis, 1996) into which a $lacZ$ gene containing an XcmI site had been introduced (Chiliang Chen, unpublished data). The plasmids containing these gene fragments were electroporated into $E. coli$ strain DH5α and were then transferred to DC3000 by conjugation in a triparental mating using a helper strain containing the plasmid pRK2073 (Better and Helinski, 1983). The plasmids were inserted into the DC3000 heat shock genes by homologous recombination, causing insertional inactivation of the heat shock genes and resulting in $htpX$, $clpA$ and $lon2$ mutants of DC3000. We were not
able to obtain a DC3000 ftsH mutant, possibly because ftsH is an essential gene in P. syringae as it is in E. coli (Tomoyasu et al., 1993; Akiyama et al., 1994). The mutations were confirmed by PCR using the forward primer carol-1 (5'-CCCAGTCACGACGTTGTAAAACG-3'), which targets the pTOK2 plasmid, and the reverse primer for the appropriate heat shock gene.

**Phenol tolerance and toxicity assays.**

Phenol tolerance was evaluated by identifying the maximum concentration at which DC3000 cells could initiate growth based on an increase in turbidity over time. The cells were grown in MinA medium until log phase (OD$_{600}$=0.3-0.4), washed and used as inoculum for fresh MinA containing various phenol concentrations. The growth was measured spectrophotometrically over time. Phenol toxicity was assessed by identifying the concentration at which DC3000 cells exhibited significant death based on culturable counts after phenol exposure for 2-3 h. Phenol toxicity in DC3000 was determined in both exponential (OD$_{600}$ of 0.2-0.3 ) and stationary phase (OD$_{600}$ of 1.0-1.2) cells grown in MinA medium. The cells were exposed to various phenol concentrations for 2 to 3 h, appropriate dilutions were plated on KB plates, and cfu/ml were calculated.

**Results and Discussion**

**Selection of stressful conditions used in the microarray studies.**

To study the gene expression profile of DC3000 in response to phenol and hydrogen peroxide at sub-lethal concentrations, we determined the EC$_{50}$ value, i.e., the concentration that caused a 50% reduction in the growth rate relative to the rate of growth in the absence of each compound. The EC$_{50}$ values for phenol and H$_2$O$_2$ were 5 mM and 1 mM, respectively (Fig. 1), and thus these concentrations were used to evaluate the stress response of DC3000 to phenol and H$_2$O$_2$. DC3000 cells were also exposed to 220 mM NaCl and 16% PEG as these concentrations result in a water potential of approximately -1 MPa in $\frac{1}{2}$ 21C, which was estimated as the amount of water stress that was sensed by DC3000 cells during a hypersensitive response in Arabidopsis thaliana leaves (Wright and Beattie, 2004).
Fig. 1. Dose-response curve of *P. syringae* pv. tomato DC3000 growth rate in the presence of phenol (A) and hydrogen peroxide (B) in ½ 21 C medium. The growth rate was determined from the slope of the curve obtained by plotting the mean log CFU/ml versus time for each inhibitor concentration. These growth rates were used to calculate the percent rate of growth of treated cells relative to that of the untreated cells.

**Overview of gene expression changes.**

To determine the extent of membrane and oxidative stress caused by phenol, DC3000 cells were exposed to phenol, NaCl, PEG and H$_2$O$_2$. A total of 288 genes were significantly induced (Fig. 2A) and 372 were significantly repressed (Fig. 2B) by at least one treatment based on a *P*-value ≤ 0.05, a *Q*-value ≤ 0.021, and a change in expression that was at least 2-fold for all treatments and at least 1.3-fold for H$_2$O$_2$. Out of the 288 induced genes, 171 genes (35%) exhibited induction by phenol and at least one other stressor, indicating that these genes might be involved directly or indirectly in tolerance to a variety of stresses and not just phenol. Of the 171 genes, 70 genes (24% of the total genes induced) were induced only by phenol. Of the 103 genes that were repressed by phenol and at least one other stressor, 23 genes (10% of the total genes repressed) were repressed only by phenol, suggesting that these genes are involved in responding specifically to phenol. To better understand the effects of phenol in bacteria and the mechanisms underlying phenol tolerance or protection, the genes that were induced or repressed by phenol are discussed here.
Fig. 2. Venn diagrams showing the number of genes induced (A) and repressed (B) by phenol, H$_2$O$_2$, NaCl and PEG and the extent of overlap in the number of genes whose expression changed in response to multiple stresses. The numbers next to the stress in parentheses indicates total number of genes induced (A) or repressed (B) by that stress irrespective of the induction or repression by other stresses.

**Genes that were induced by phenol.**

The genes induced by phenol were categorized based on the cellular processes they are involved in according to the *Pseudomonas* website (www.pseudomonas.com).

**Heat shock response.**

Heat shock proteins (hsps) are general stress proteins induced by a variety of stresses in which proteins are denatured, including high temperature and exposure to toxic substances such as heavy metals, alcohols, and organic solvents. Denatured proteins in the form of incomplete peptides, unfolded and misfolded proteins, and protein aggregates can result from exposure to these stresses and induce the transcription of hsps. Their induction suggests that the hsps play a role in removing abnormal proteins, which may help the cell adapt to or tolerate the stresses. Our study showed that the majority of the genes known to be involved in
the heat shock response in *E. coli* were induced by phenol and not by other stresses in DC3000 (Fig. 3).

**Fig. 3.** Expression of heat shock genes after exposure of DC3000 to phenol, H\(_2\)O\(_2\), NaCl and PEG. The genes *htpX, clpA, ftsH* and *lon-2* were induced more than 2-fold by phenol.
Positive values indicate the fold induction and negative values indicate the fold repression relative to unstressed cells.

Among the heat shock genes induced by phenol in DC3000 were genes that were induced by phenol in other species. For example, **grpE** was induced by phenol in *P. putida* KT2440, and moreover was induced to similar levels in DC3000 (1.8-fold) and *P. putida* (1.5- to 1.7-fold) (Santos et al., 2004). The heat shock genes **dnaK**, **groEL**, and **groES** were induced by phenol in *B. subtilis* (Tam le et al., 2006), as they were in DC3000. In fact, the study by Tam le et al. (2006) showed that phenol induced a classical heat shock response in a 10-20 min exposure in *B. subtilis* based on transcriptome and proteome analyses. Similarly, phenol induced a heat shock response in *Acinetobacter calcoaceticus* whereas catechol, which differs from phenol only in the number of hydroxyl groups, did not, although it induced an oxidative stress response (Benndorf et al., 2001).

Among the heat shock genes induced by phenol in DC3000 were genes encoding a chaperone, ClpA, and two proteases, Lon-2 and FtsH, all of which were induced approximately 2-fold (Table 1 and Fig. 3). Although the protease HtpX was induced by both phenol and H$_2$O$_2$ (Fig. 3), the induction was much higher by phenol (5-fold) than by H$_2$O$_2$ (1.4-fold). FtsH and HtpX are membrane proteases while Lon-2 is a cytoplasmic protease. FtsH is a membrane-bound ATP-dependent zinc metalloprotease known to degrade unstable membrane proteins (Akiyama et al., 2004; Ito and Akiyama, 2005), including SecY (Kihara et al., 1995), a subunit of protein translocase, and the F$_0$ subunit of the proton ATPase (Akiyama et al., 1996) when they fail to assemble. FtsH also degrades some unstable cytoplasmic proteins such as the heat shock regulator σ$_{32}$ (Herman et al., 1995; Tomoyasu et al., 1995). HtpX is another membrane-bound zinc metalloprotease induced by phenol. It is an energy-independent endopeptidase that can also act on both membrane and cytoplasmic proteins. The induction of genes for membrane proteases by phenol suggests that while crossing the cell membrane phenol may denature some membrane proteins, resulting in the induction of FtsH and HtpX to remove or repair damaged membrane proteins. Two other genes induced by phenol encode ClpA, a chaperone, and Lon-2, a protease that acts primarily
on damaged cytoplasmic proteins. The ClpA chaperone forms a multisubunit complex with the ClpP protease to recognize, unfold, and degrade damaged cytoplasmic proteins, with the unfolding requiring ATP hydrolysis. Lon is an ATP-dependent cytosolic protease involved in the degradation of various regulatory and abnormal proteins (Gottesman, 1989; Goldberg, 1992; Goldberg et al., 1994). Lon was shown to be a major protease degrading abnormally folded proteins in *E. coli* based on that *E. coli* mutants lacking Lon, although viable, contained three times more aggregated proteins than wild type (Rosen et al., 2002). The induction of cytoplasmic proteases by phenol suggests that upon entry into the cell cytosol, phenol causes the production of abnormal proteins by unfolding or misfolding them. These abnormal proteins may aggregate in the cytoplasm causing the induction of chaperones and proteases to either unfold the misfolded proteins and refold them into their proper conformation or remove them by degradation.

Our transcriptome data suggests that the induction of heat shock genes is important to phenol tolerance in DC3000 cells. This has also been suggested in another study where transcriptional analysis of *Clostridium acetylbutylicum* exposed to butanol resulted in Lon induction (Tomas et al., 2004) and thus may be involved in solvent tolerance. Based on the finding that all of the heat shock genes that were induced by phenol were induced specifically by phenol and not by other environmental stresses, we propose a model for the effect of phenol in which protein damage (protein misfolding, unfolding, aggregation, etc.) occurs as phenol enters the cell through the outer membrane, into the periplasmic space and through the inner membrane into the cytosol (Fig. 4). The induction of both membrane and cytoplasmic proteases by phenol suggests that periplasmic or membrane proteins as well as cytoplasmic proteins are damaged by phenol. The damaged proteins cause the induction of heat shock proteins including chaperones, such as ClpA and proteases FtsH, HtpX, and Lon-2 to remove the damaged proteins or refold them into proper conformation.
Fig. 4. Schematic diagram. Model for the effect of phenol on bacterial cell protein quality. The entry of phenol (represented by hexagons) through the cell outer membrane (OM), periplasmic space (PP), cell membrane (CM) into the cytosol results in protein denaturation by misfolding, unfolding, and aggregation of proteins. The denatured proteins sensed by extracytoplasmic and cytosolic sigma factors result in increased transcription of chaperones, such as ClpA and/or proteases, FtsH, HtpX, and Lon-2, to either degrade or refold the proteins into their native conformation.
Table 1. Induction of the putative heat shock genes in *P. syringae* pv. tomato DC3000 to 15 min of exposure to phenol, H$_2$O$_2$, NaCl and PEG based on a microarray analysis. Genes with $P \leq 0.05$, $Q \leq 2.1\%$ and at least a 2-fold induction in response to at least one treatment, or at least a 1.3-fold induction in response to H$_2$O$_2$, were considered significantly induced and are shown in bold.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene #</th>
<th>Fold induction relative to unstressed cells</th>
<th>Annotated Function</th>
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<td>heat shock protein, Hsp20 family</td>
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Response to low water availability.

Phenol and water stress strongly induced the extracytoplasmic sigma factor AlgU, an *E. coli* RpoE homolog, and its regulators MucA and MucB (Table 2). AlgU/RpoE is crucial in the adaptation of *Pseudomonas fluorescens* to dry conditions and hyperosmolarity (Schnider-Keel et al., 2001), as well as is induced in *E. coli* in response to stresses that result in the accumulation of misfolded proteins in the periplasmic space (Mecsas et al., 1993; Raina et al., 1995; Missiakas et al., 1996; Rouviere and Gross, 1996). AlgU is also known to upregulate the expression of extracytoplasmic proteases and chaperones as well as genes involved in the synthesis of the extracellular polysaccharide alginate. The induction of extracytoplasmic proteases by AlgU in response to the accumulation of abnormal proteins in the periplasmic space suggests that phenol may cause damage to outer membrane and or periplasmic proteins, and that this damage results in the induction of AlgU to repair or degrade the damaged proteins. There were 91 genes that were induced by phenol and water stress (Fig. 2) whereas only 26 genes were induced by both phenol and hydrogen peroxide. Based on the relative number of genes induced, the effect of phenol is more similar to that of water stress than H$_2$O$_2$, suggesting greater membrane damage than oxidative damage within the cytosol.

The genes involved in the synthesis of the compatible solutes N-acetylglutaminylglutamine amide (NAGGN) and trehalose were also highly induced by phenol, NaCl and PEG (Table 3). The two genes PSPTO1632 and PSPTO1633 are 68% and 75% identical to acetyltransferase PA3460 and glutamine amidotransferase PA3459, respectively, which have been shown to be involved in NAGGN synthesis in *P. aeruginosa* (Aspedon et al., 2006). The genes of three putative trehalose biosynthetic operons and those involved in periplasmic glucan biosynthesis were also induced by water and phenol stress (Table 3). These results further support a similarity in the responses of DC3000 to phenol and water stress.
Table 2. Induction of DC3000 genes putatively involved in the synthesis or regulation of alginate by phenol, H$_2$O$_2$, and osmotic and matric stress.

<table>
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<th>Gene symbol</th>
<th>Gene #</th>
<th>Fold induction relative to unstressed cells</th>
<th>Annotated Function</th>
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Table 3. Induction of DC3000 genes putatively involved in the biosynthesis of osmoprotectants and periplasmic glucan by phenol, H$_2$O$_2$, and osmotic and matric stress.

<table>
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<th>Gene #</th>
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**Oxidative stress response.**

Phenol has been suggested to cause an oxidative stress in *P. putida* KT2440 due to the increased expression of some genes that encode antioxidants, including AhpC (Alkyl hydroperoxide reductase, subunit C), SodB (Superoxide dismutase), Tpx (Thiol peroxidase), DsbA (Thiol:disulfide interchange protein) and Tig (Trigger factor, presumably required for protein folding or accelerated protein export) (Santos et al., 2004). None of these genes were induced by phenol in DC3000 and there was little overlap between the genes induced by H$_2$O$_2$ and those induced by phenol (Table 4), suggesting that, unlike in *P. putida*, phenol probably does not result in an oxidative stress response in DC3000. Also, the genes that were most strongly induced by H$_2$O$_2$, the catalase-encoding *katB* and *katG* genes and *ahpC* and *ahpF* genes, were not induced by phenol (Fig. 5). AhpC and AhpF are the antioxidant enzymes involved in the catalysis of organic peroxide reduction and KatG is a catalase-peroxidase involved in the detoxification of H$_2$O$_2$. KatG, AhpF and AhpC are regulated by the transcription factor OxyR (VanBogelen et al., 1987; Tartaglia et al., 1989) and have been shown to be strongly induced by H$_2$O$_2$ (Farr and Kogoma, 1991; Zheng et al., 2001; Zeller and Klug, 2004). Our results are consistent with a previous proteomic study that showed induction of AhpF and AhpC upon exposure to H$_2$O$_2$ but not to phenol (Benndorf et al., 2001). Hydrogen peroxide causes oxidative stress by producing reactive oxygen species that can damage cytoplasmic and membrane lipids and proteins, and DNA by peroxidation, thereby, resulting in the upregulation of catalases and hydroperoxidases involved in the removal of reactive oxygen radicals (Demple, 1991). Our results indicate that the toxic effects of phenol in DC3000 are different than those of H$_2$O$_2$. 
Table 4. Induction of the putative oxidative stress genes in DC3000 by phenol, $H_2O_2$, NaCl, and PEG.

<table>
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**Fig. 5.** Expression of genes involved in oxidative stress response 15 min after exposure to phenol, H$_2$O$_2$, NaCl and PEG in DC3000. The genes encoding the antioxidant enzymes, KatB, KatG, AhpC and AhpF were induced > 1.3-fold only by H$_2$O$_2$. Positive values indicate the fold induction and negative values indicate the fold repression relative to unstressed cells.
Functions of cell membrane proteins.

Despite the apparent induction of membrane stress suggested by the overlap between the phenol- and water stress-responsive regulons, as described above, the expression of many membrane proteins was not affected by phenol (Table 5). For example, the *cti* gene encoding the cis/trans isomerase, an enzyme involved in fatty acid composition changes during bacterial adaptation to organic solvents including phenol, was not induced; however, activation of this enzyme likely occurs post-transcriptionally (Kiran et al., 2005) and *cti* expression in *P. putida* is known to be constitutive (Heipieper et al., 1992). Consistent with the finding that efflux transporters are often induced by organic solvents, several of the efflux transporter proteins in DC3000 were induced by phenol (Table 4). Interestingly, the *tolC* gene (PSPTO4977), which encodes a homolog of an outer membrane efflux protein that was induced by phenol in *P. putida* (Santos et al., 2004), was not induced.
Table 5. Induction of DC3000 genes putatively encoding cell membrane proteins by phenol, H$_2$O$_2$, NaCl, and PEG.

<table>
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<td>1.0</td>
<td>1.2</td>
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<tr>
<td>PSPTO4305</td>
<td>1.3</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>PSPTO4442</td>
<td>0.9</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>PSPTO4443</td>
<td>1.3</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>PSPTO4444</td>
<td>1.9</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>PSPTO4445</td>
<td>2.1</td>
<td>0.9</td>
<td>1.5</td>
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<tr>
<td>PSPTO4446</td>
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<td>1.8</td>
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<td>PSPTO4447</td>
<td>2.0</td>
<td>1.0</td>
<td>1.7</td>
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<td>mxeE</td>
<td>PSPTO3099</td>
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<td>mxeF</td>
<td>PSPTO3100</td>
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<td>oprN</td>
<td>PSPTO3101</td>
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<td>0.9</td>
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<td>0.9</td>
</tr>
<tr>
<td>tolC</td>
<td>PSPTO4977</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>
General metabolic functions.

Although there was a general decrease in metabolism in DC3000 as expected based on the slowed growth of DC3000 when exposed to 5 mM phenol (Fig. 1) and based on the repression of various metabolic genes described below, some genes involved in general metabolism were induced in response to phenol (Table 6). These included several genes involved in carbohydrate catabolism, such as \( \text{gpm}A \), \( \text{gnl} \) and \( \text{gcd} \), which encode a putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, gluconolactonase, and glucose dehydrogenase, respectively. The gene \( \text{gpm}A \) is homologous to \( \text{pgm} \) of \( P. \text{putida} \), which was also induced in response to phenol (Santos et al., 2004). The two genes \( \text{gnl} \) and \( \text{gcd} \) encode enzymes that are involved in the pentose phosphate pathway, a pathway that contributes to the production of energy and ribose sugars for biosynthesis. This may suggest that in DC3000 some genes involved in energy production are upregulated to produce energy that could be used to provide phenol tolerance to the cells. Other genes that were induced by phenol and are involved in energy production encode a putative formate dehydrogenase, which allows the use of formate during anaerobic respiration, and azurin, which allows electron transfer during anaerobic growth in bacteria. The induction of these genes adapted for anaerobic growth suggests that the phenol-exposed cells may have encountered oxygen limitation.

Consistent with the induction of some genes involved in amino acid metabolism in \( P. \text{putida} \) in response to phenol (Santos et al., 2004), a few DC3000 genes related to amino acid metabolism were induced, although most were not. For example, the \( \text{arg}A \) and \( \text{leu}D \) genes involved in arginine degradation and leucine biosynthesis, respectively, were induced in \( P. \text{putida} \) in response to toluene, whereas \( \text{arg}B \) involved in arginine biosynthesis was induced in DC3000 and \( \text{arg}A \) and \( \text{leu}D \) were not (Table 5).
Table 6. Induction of DC3000 genes putatively involved in general metabolic functions following exposure to phenol, H$_2$O$_2$, NaCl, and PEG.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene #</th>
<th>Fold induction relative to unstressed cells</th>
<th>Annotated Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phenol</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>Carbohydrate organic acid metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSPTO5407</td>
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<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>fdhD</td>
<td>PSPTO 5406</td>
<td>3.6</td>
<td>1.0</td>
</tr>
<tr>
<td>gloA</td>
<td>PSPTO3106</td>
<td>2.7</td>
<td>1.0</td>
</tr>
<tr>
<td>fumC-1</td>
<td>PSPTO1731</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>gnl</td>
<td>PSPTO1345</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>gpmA</td>
<td>PSPTO5327</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>azu</td>
<td>PSPTO4923</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>gcd</td>
<td>PSPTO4196</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>gabD</td>
<td>PSPTO0300</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSPTO4171</td>
<td>2.6</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>PSPTO1343</td>
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<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>argB</td>
<td>PSPTO0082</td>
<td>2.1</td>
<td>1.2</td>
</tr>
<tr>
<td>argA</td>
<td>PSPTO0324</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>leuD</td>
<td>PSPTO2173</td>
<td>0.9</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Transcriptional regulators.

At least five genes encoding proteins involved in transcriptional regulation were induced specifically in response to phenol exposure (Table 7). These were psrA (a TetR family Pseudomonas sigma regulator), tvrR (for TetR-like virulence regulator), SigW, and two others belonging to the TetR and LysR family of regulators. The tetracycline repressor (TetR) family of bacterial regulatory proteins allow bacteria to sense and respond quickly to their environment, thus modulating the expression of genes in response to environmental signals, such as hyperosmolarity (Lamark et al., 1991) and antibiotics. In this study, the induction of DC3000 TetR-type regulators by phenol suggests that they may regulate genes important in adaptation and tolerance to phenol. In P. aeruginosa, PsrA positively regulates the rpoS gene at the onset of stationary phase (Kojic and Venturi, 2001; Kojic et al., 2002; Kojic et al., 2005), as well as other novel targets such as an acyl-CoA dehydrogenase, an electron transfer flavoprotein, and an electron transfer flavoprotein-ubiquinone oxidoreductase (Kojic et al., 2005). The acyl-CoA dehydrogenases, in particular, may promote the degradation of fatty acids for energy production to help bacteria cope with stresses such as starvation during the stationary phase. In DC3000, PsrA may also be involved in the induction of acyl-CoA dehydrogenase (PSPTO0500) based on that it was induced 5.5-fold by phenol. TvrR, another TetR family regulator, has been identified as a novel transcriptional regulator that is required for virulence of DC3000 in Arabidopsis thaliana plants and has been speculated to be involved in sensing environmental signals (Preiter et al., 2005). Lastly, the RNA polymerase sigma factor SigW is an extracytoplasmic sigma factor (ECF) of B. subtilis, and thus is known to respond to signals from the extracytoplasmic environment, such as the presence of misfolded proteins in the periplasm. In a previous study (Steil et al., 2003), the SigW regulon was shown to be induced in B. subtilis within 10 min of exposure to high salinity. Of the 75 B. subtilis genes induced immediately after salt shock, 31 were assigned to the SigW regulon, which included transporters for osmoprotectants and enzymes catalyzing the synthesis of compatible solutes. The induction of SigW in DC3000 suggests that phenol may be causing damage to the periplasmic proteins and/or cell membrane, sending signals to this SigW ECF sigma factor to promote the induction of other genes.
Table 7. Induction of putative transcriptional regulator genes of DC3000 following exposure to phenol, H₂O₂, NaCl, and PEG.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene #</th>
<th>Fold induction relative to unstressed cells</th>
<th>Annotated Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phenol</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Transcriptional regulators</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>psrA</td>
<td>PSPTO3508</td>
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</tr>
<tr>
<td></td>
<td>PSPTO5462</td>
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<td>1.2</td>
</tr>
<tr>
<td>tvrR</td>
<td>PSPTO3576</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>PSPTO0378</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>sigW</td>
<td>PSPTO2298</td>
<td>2.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Other cellular functions.

Five of the six genes of an operon involved in tellurium resistance, terA, B, D, Z, and a membrane protein belonging to terC family were induced more than 2-fold by phenol, with terD and terZ induced some by H₂O₂ (Table 8). Tellurite causes toxicity in bacteria by oxidizing and replacing sulfur in cellular proteins, rendering them nonfunctional (Summers and Jacoby, 1977; Garberg et al., 1999). A fivefold induction in tellurium resistance genes as well as significant induction of oxidative stress-responsive genes upon exposure to the antibiotic 4,5-dihydroxy-2-cyclopenten-1-one has also been shown in E. coli (Phadtare et al., 2002), suggesting that some kind of oxidative stress might be responsible for up-regulating tellurium resistance genes. Another study (Turner et al., 1995) has suggested that after tellurium enters a cell through a phosphate transport uptake system it is acted upon by nitrate reductase, which causes reduction of tellurite in the cytoplasm as a basal level of defense against tellurium toxicity. The induction of these genes by phenol suggests that phenol may cause some oxidation of cellular components.
Table 8. Induction of DC3000 genes involved in tellurite resistance following exposure to phenol, H$_2$O$_2$, NaCl, and PEG.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene #</th>
<th>Fold induction relative to unstressed cells</th>
<th>Annotated Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phenol</td>
<td>H$_2$O$_2$</td>
</tr>
<tr>
<td>terZ</td>
<td>PSPTO0940</td>
<td>3.9</td>
<td>1.3</td>
</tr>
<tr>
<td>terB</td>
<td>PSPTO0942</td>
<td>3.6</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>PSPTO2691</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>terA</td>
<td>PSPTO0941</td>
<td>3.0</td>
<td>1.2</td>
</tr>
<tr>
<td>terD</td>
<td>PSPTO0944</td>
<td>2.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Proteins of unknown function.

The ten genes that were most induced by phenol (22- to 106-fold) encoded hypothetical proteins with no known function (Table 9). Of the 33 genes that were induced ≥ 6-fold, 24 genes encoded hypothetical proteins. Future studies should be aimed at identifying these genes and their role in DC3000 stress tolerance.

Table 9. Induction of DC3000 genes with unknown functions following exposure to phenol, H$_2$O$_2$, NaCl, and PEG.

| Gene symbol | Gene # | Fold induction relative to unstressed cells |  |
|-------------|--------|------------------------------------------|  |
|             |        | Phenol  | H$_2$O$_2$  | NaCl  | PEG  |  |
| Hypothetical proteins |        | 106    | 1.5       | 61.1   | 77.9  |  |
| PSPTO2049    |        | 69.0   | 1.3       | 46.1   | 57.5  |  |
| PSPTO0332    |        | 47.3   | 1.5       | 27.2   | 29.0  |  |
| PSPTO0507    |        | 43.7   | 1.5       | 17.0   | 25.3  |  |
| PSPTO4546    |        | 38.1   | 1.4       | 10.9   | 17.2  |  |
| PSPTO4484    |        | 33.6   | 1.7       | 24.9   | 30.9  |  |
| PSPTO1848    |        | 33.3   | 1.1       | 22.2   | 21.9  |  |
| PSPTO1304    |        | 33.2   | 1.1       | 32.7   | 39.1  |  |
| PSPTO4516    |        | 29.8   | 1.4       | 12.4   | 17.4  |  |
| PSPTO1507    |        | 22.3   | 1.8       | 12.8   | 22.3  |  |
| PSPTO1596    |        | 15.4   | 1.3       | 4.5    | 5.4   |  |
| PSPTO0318    |        | 13.5   | 1.3       | 3.7    | 5.7   |  |
| PSPTO1485    |        | 13.2   | 1.3       | 4.3    | 4.6   |  |
| PSPTO0156    |        | 12.7   | 1.9       | 11.2   | 17.5  |  |
| PSPTO2377    |        | 12.5   | 1.3       | 8.6    | 11.3  |  |
| PSPTO0155    |        | 9.8    | 1.2       | 7.1    | 10.8  |  |
| PSPTO1417    |        | 9.0    | 1.8       | 2.9    | 3.2   |  |
| PSPTO2895    |        | 8.7    | 1.1       | 2.8    | 2.5   |  |
**Genes that were repressed by phenol**

The majority of the genes that were repressed by phenol were predicted to be involved in general metabolism, flagellar biosynthesis and chemotaxis, or protein synthesis. Phenol seemed to result in some membrane stress as suggested by the repression of the genes involved in flagellar biosynthesis (Table 10). This is consistent with the repression in *P. putida* of the genes involved in chemotaxis and motility as well as the *fliC* gene encoding the flagellin FliC, which is essential to the formation of flagella, in response to phenol (Santos et al., 2004). The flagellar biosynthesis genes were also repressed by water stress, consistent with water stress causing membrane changes.

A previous proteomic study has shown that exposure of *P. putida* to toluene results in the upregulation of proteins involved in glucose metabolism to provide energy to extrude toluene out of the cell through efflux pumps (Segura et al., 2005). These proteins were succinyl coenzyme A synthetase (SucC), fructose-1,6-bisphosphate aldolase (Fda), glucokinase (Gck), and oxoglutarate dehydrogenase, lipoamide dehydrogenase component (LpdG). In contrast, DC3000 showed significant repression of the genes in the operons involving SucC and LpdG (Table 10), suggesting a decrease in metabolic activity in response to phenol. In addition, the majority of the genes encoding ribosomal proteins were repressed by phenol but not by other stresses, suggesting a slowed cell growth as a result of phenol exposure. This was expected because the level of phenol used in this study was chosen based on the fact that it resulted in slowed growth without killing of the cells.
Table 10. Repression of various DC3000 genes following exposure to phenol, H$_2$O$_2$, NaCl, and PEG. Only genes specifically repressed by phenol are shown.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene #</th>
<th>Fold repression relative to unstressed cells</th>
<th>Annotated Function</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Phenol H$_2$O$_2$ NaCl PEG</td>
<td></td>
</tr>
<tr>
<td><strong>Flagellar biosynthesis and chemotaxis proteins</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>flgC</td>
<td>PSPTO1934</td>
<td>3.0 0.6 4.3 4.9</td>
<td>flagellar basal-body rod protein</td>
</tr>
<tr>
<td>flgB</td>
<td>PSPTO1933</td>
<td>2.7 0.6 3.7 3.8</td>
<td>flagellar basal-body rod protein</td>
</tr>
<tr>
<td>flgD</td>
<td>PSPTO1935</td>
<td>2.6 0.7 3.2 3.0</td>
<td>basal-body rod modification protein</td>
</tr>
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<td>flaG</td>
<td>PSPTO1950</td>
<td>3.2 0.9 3.8 6.1</td>
<td>flagellin, putative</td>
</tr>
<tr>
<td>cheZ</td>
<td>PSPTO1981</td>
<td>2.1 0.7 2.4 4.8</td>
<td>chemotaxis protein</td>
</tr>
<tr>
<td><strong>Proteins involved in general metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSPTO2199</td>
<td>4.3 1.4 1.2 1.8</td>
<td>2-oxoglutarate dehydrogenase, E1 component</td>
</tr>
<tr>
<td>sucD</td>
<td>PSPTO2203</td>
<td>3.4 1.1 1.8 2.4</td>
<td>succinyl-CoA synthase, alpha subunit</td>
</tr>
<tr>
<td>sucC</td>
<td>PSPTO2202</td>
<td>3.2 1.0 1.6 1.7</td>
<td>succinyl-CoA synthase, beta subunit</td>
</tr>
<tr>
<td>lpdG</td>
<td>PSPTO2201</td>
<td>2.9 1.1 1.2 1.1</td>
<td>2-oxoglutarate dehydrogenase, E3 component, lipoamide dehydrogenase</td>
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<tr>
<td></td>
<td>PSPTO2200</td>
<td>2.8 1.1 1.2 1.4</td>
<td>2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase</td>
</tr>
<tr>
<td>gck</td>
<td>PSPTO1289</td>
<td>1.7 1.1 1.4 1.7</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>fda</td>
<td>PSPTO0390</td>
<td>0.6 1.0 1.1 1.2</td>
<td>fructose-bisphosphate aldolase, class II</td>
</tr>
<tr>
<td><strong>Translation, ribosomal proteins</strong></td>
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<td></td>
</tr>
<tr>
<td>rpmF</td>
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<td>3.2 1.6 1.1 1.5</td>
<td>50S ribosomal protein L32</td>
</tr>
<tr>
<td>rpsU</td>
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<td>2.9 1.2 1.1 0.9</td>
<td>30S ribosomal protein S21</td>
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<tr>
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<td>Translation elongation factor Tu</td>
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<tr>
<td>rpsT</td>
<td>PSPTO0802</td>
<td>2.5 1.2 1.4 1.6</td>
<td>30S ribosomal protein S20</td>
</tr>
<tr>
<td>rpmE-2</td>
<td>PSPTO5136</td>
<td>2.4 1.3 1.4 1.6</td>
<td>50S ribosomal protein L31</td>
</tr>
<tr>
<td>rpsA</td>
<td>PSPTO1750</td>
<td>2.3 1.4 1.0 1.1</td>
<td>30S ribosomal protein S1</td>
</tr>
<tr>
<td>rplI</td>
<td>PSPTO4930</td>
<td>2.3 1.9 1.4 2.1</td>
<td>ribosomal protein L9</td>
</tr>
<tr>
<td>rplL</td>
<td>PSPTO0618</td>
<td>2.2 1.2 1.5 1.4</td>
<td>ribosomal protein L7/L12</td>
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<td><strong>Hypothetical proteins</strong></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PSPTO2729</td>
<td>3.2 0.6 0.7 1.0</td>
<td></td>
</tr>
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<td></td>
<td>PSPTO5423</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PSPTO0307</td>
<td>2.2 0.5 1.0 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSPTO1429</td>
<td>2.1 1.0 0.9 1.4</td>
<td></td>
</tr>
</tbody>
</table>
**Phenol tolerance and toxicity in DC3000 heat shock mutants.**

The role of phenol-induced heat shock proteins, HtpX, ClpA and Lon-2, in providing tolerance to phenol in DC3000 was determined by exposing heat shock mutants to various phenol concentrations in minimal media. We were not able to obtain a DC3000 *ftsH* mutant, possibly because *ftsH* is essential and the mutation in this gene is lethal, as has been shown for *E. coli* (Ogura et al., 1999). In phenol tolerance assays, the cells were grown in phenol and their OD$_{600}$ was measured over time whereas in toxicity assays, the mutants were exposed to a sudden shock of a high phenol concentration for 2 to 3 h and their survival was determined from viable plate counts. Under the experimental conditions used in this study, we did not see a difference between wild-type DC3000 and any of the mutants in their ability to tolerate phenol (Fig. 6). However, this does not rule out the role that heat shock proteins may have in protecting the cells from phenol toxicity. Because heat shock proteins have redundant functions it is possible that in the absence of one protease the other takes over the function of removing abnormal proteins from the cell and helping bacterial cells survive under stress.

**Fig. 6.** Phenol tolerance of DC3000 and the heat shock mutants *htpX*, *clpA*, and *lon-2* in MinA minimal medium amended with phenol at the concentrations indicated.
Conclusion

Based on our gene expression data, we proposed a model for the mode of action of phenol in a bacterial cell. Upon entry into the cell through cell membrane, phenol causes denaturation of outer and inner membrane proteins, and periplasmic proteins by misfolding and/or unfolding, resulting in the activation of envelope stress response. The misfolding of proteins in the outer membrane is sensed by AlgU (5.9-fold induced), an *E. coli* sigma E homologue and an extracytoplasmic sigma factor, which is induced by various agents that cause envelope stress, and the transcription of sigma H, an AlgU regulon member and a positive transcriptional regulator of heat shock genes, is upregulated. Sigma H, in turn induces the transcription of heat shock genes, such as *clpA*, *lon*, and *ftsH* producing proteases, that degrade denatured cytosolic and membrane proteins. AlgU also upregulates the expression of extracytoplasmic proteases and chaperones directly that help degrade or refold the damaged periplasmic and membrane proteins. This constitutes the AlgU signal transduction pathway, which is regulated by two negative regulatory proteins, MucA (induced 5.5-fold) and MucB (induced 5.4-fold), homologues of RseA and RseB of *E. coli*. MucA is an inner membrane anti-sigma factor that binds to AlgU in the absence of envelope stress. MucB is a periplasmic protein that binds to MucA and stabilizes it. Upon sensing the presence of misfolded periplasmic proteins MucB leaves MucA and binds to the abnormal proteins, thereby destabilizing MucA, which releases AlgU to turn on the transcription of stress response genes.

In conclusion, the transcriptome analysis of *P. syringae* pv. tomato DC3000 in response to phenol revealed a mechanism of toxicity in bacteria, which was different from the modes of action of other stress agents, including H$_2$O$_2$, PEG, and NaCl. It was interesting to observe that although heat shock response is considered a general stress response that is induced in bacteria by various stresses, including ethanol, antibiotics, and heavy metals, in our study only phenol exposure to DC3000 resulted in the up-regulation of heat shock genes. This suggested that phenol caused denaturation of cytoplasmic, membrane, and/or periplasmic proteins resulting in the activation of sigma 32 regulated-heat shock response. The phenol tolerance and toxicity assays suggested that the heat shock proteins induced due
to the appearance of abnormal proteins in the cell have overlapping functions, where one protease takes over for, or complements, the function of the other in its absence under stress.

References


Chapter 5. Conclusion and future directions

Extensive work has been done on bacterial degradation of organic pollutants in soil but the availability and degradation of airborne pollutants by bacteria present on leaf surfaces has not been explored. Pollutants can be translocated from soil to leaves via the transpiration stream and can also be taken up or adsorbed directly from air by the leaves. Leaf surfaces have also been shown to be colonized by bacterial populations as high as $10^7$-$10^8$ cfu/g of leaf. Therefore, it is possible that bacteria present on the leaf surfaces have access to and degrade the pollutants adsorbed onto the leaf surfaces or translocated to leaves from the soil. In the present study, we demonstrated the availability and degradation of airborne organic pollutants on leaves by leaf-associated bacteria using phenol as a model pollutant.

With the help of phenol-responsive A506 biosensor cells constructed in this study, we showed that bacteria present on the leaf surfaces can access airborne phenol as well as the phenol that had been adsorbed by the leaf surfaces; moreover, the concentrations accessible to bacteria were at least 10-fold higher than those in the air, suggesting that leaves accumulate phenol in sites accessible to bacteria (chap 2). The phenol accessible to leaf-associated bacteria was also available for degradation as evident by the degradation of $^{14}$C-phenol by phenol-degrading bacteria, *Pseudomonas sp.* strain CF600, after inoculation on leaf surfaces. A previous study indirectly suggested a role for bacteria inoculated on leaf surfaces in the removal of airborne toluene by measuring air concentrations of toluene (De Kempeneer et al., 2004); however, our study provided the first direct evidence of pollutant degradation by bacteria present on leaf surfaces by measuring the amount of radiolabeled CO$_2$ released due to bacterial degradation of $^{14}$C-phenol. In this chapter, we also showed that naturally occurring bacterial communities on green ash leaves taken from trees around a swine production facility have the ability to degrade airborne phenol.

We also identified and genetically characterized some members of the phenol-degrading naturally occurring bacterial communities from green ash leaves (chap 3). This
characterization is important to assess the genetic diversity, functional diversity, and catabolic potential of these organisms (Watanabe et al., 2002) and to identify the main bacterial species responsible for the majority of the pollutant degradation. This information is also important to developing bioremediation strategies (Watanabe et al., 1998; Watanabe et al., 2002). Molecular characterization of pollutant-degrading isolates may also help in developing genetic markers for monitoring the presence of catabolic genes and their specific catabolic activities in situ. In this study, 10 bacterial isolates were obtained, out of which 7 were Gram-negative and 3 were Gram-positive. The Gram-negative isolates were most closely related to the genera Acinetobacter and Alcaligenes, whereas the three Gram-positive isolates belonged to the genus Rhodococcus. Bacterial species belonging to Acinetobacter, Alcaligenes, and Rhodococcus are metabolically versatile and have been shown to degrade a range of aromatic compounds. It is possible that bacteria belonging to these three genera are capable of efficient phenol degradation at a concentration present in the air around the swine production facility.

The phenol-degrading bacterial isolates were characterized genetically to determine the presence and type of phenol catabolic genes. This was done based on the conserved amino acid residues of the large subunit of multi-component phenol hydroxylases (PH), and the presence of catechol dioxygenase genes. Phenol hydroxylase is the first enzyme of the phenol catabolic pathway in which phenol is converted into catechol, a common intermediate in the aerobic degradation of a variety of aromatic compounds. The catechol is then metabolized into tricarboxylic acid cycle intermediates via either the ortho pathway by catechol-1,2 dioxygenase or the meta pathway by catechol-2,3 dioxygenase. PHs from Gram-negative bacteria have been previously classified into class I, II and III PHs, which have low, moderate and high affinity for phenol, respectively. In our study, we found that the PHs from all of the Gram-negative isolates belonged to class II PHs and all of the Gram-negative isolates possessed the ortho pathway for phenol degradation, suggesting that either the phyllosphere or the bacterial isolation techniques used did not support much genetic diversity.
In the present study, we also attempted to understand the mechanism of toxicity of phenol in bacteria by transcriptome analysis of a plant pathogen, *Pseudomonas syringae* pv. tomato strain DC3000, upon exposure to phenol, membrane stress caused by sodium chloride (NaCl) and polyethylene glycol (PEG), and oxidative stress caused by H$_2$O$_2$. Our analysis indicated that phenol induces a heat shock response and membrane stress in DC3000, but not much oxidative stress. We proposed a model of cellular responses to phenol in which phenol causes denaturation of membrane, periplasmic and cytoplasmic proteins that result in the activation of the sigma factor AlgU. AlgU then activates the transcription of sigma H, a heat shock gene regulator. This regulator then turns on the transcription of heat shock genes that encode proteases and chaperones to repair or remove the damaged cellular proteins. The gene expression analysis also revealed a number of genes with unknown functions that were up-regulated more than 20-fold by phenol. Identification of these genes and their functions would be important to further enhance our understanding of the effects of phenol in a bacterial cell.

**Future directions**

In the present study we were able to identify some bacterial species with the ability to degrade phenol among the naturally occurring phyllosphere communities. The techniques we used did not reveal much phylogenetic and genetic diversity; therefore, it seems more work is required in this area to determine the total diversity of phenol-degrading bacteria, including non-culturable bacterial species, in the phyllosphere. One way to determine the phyllosphere community diversity would be to use culture-independent approaches such as Stable Isotope Probing (SIP) (Radajewski et al., 2000) and Reverse transcriptase-PCR (RT-PCR). SIP involves the use of $^{13}$C-labeled substrate to label cellular RNA/DNA, which can then be used to identify bacteria that have the ability to use $^{13}$C labeled substrate. This will not only help in determining the non-culturable phenol-degrading bacteria among the phyllosphere community, but also in identifying the ecologically relevant bacterial species in the phyllosphere community. The combination of microautoradiography and fluorescent *in situ* hybridization (FISH) can also be used to link phylogeny with function of bacterial population *in situ* (Lee et al., 1999). It is also important to characterize the genetic diversity of bacterial
populations involved in pollutant degradation as it can help in identifying the functionally dominant bacterial populations in the environment. This information would help in developing effective bioremediation strategies. A number of previous studies have used catabolic genes to identify bacterial genetic diversity. The differences in catabolic gene sequence is important as it may reflect differences in substrate specificity or affinity (Beil et al., 1998). For example, the phylogenetic diversity of functional catabolic genes such as catechol 2,3-dioxygenase has been determined by amplified functional DNA restriction analysis (AFDRA) (Junca and Pieper, 2003), a technique adapted from amplified ribosomal DNA restriction analysis (ARDRA). AFDRA can be applied to define the predominant polymorphism of a functional gene present in the environmental DNA extracts. Determination of the catabolic gene sequence diversity in environmental samples will significantly increase our knowledge of the functional potential of microbial communities.

Future studies aimed at confirming the action of phenol on cellular proteins are required to understand the mechanism of phenol toxicity in bacteria. We initiated this in the present study by constructing three heat shock DC3000 mutants in genes, htpX, clpA, and lon-2, but we did not see any difference between wild type and mutants in phenol toxicity or tolerance based on the measurement of growth. Construction of double mutants or even examining the presence and amount of protein aggregates in the mutants vs. wild type by labeling the proteins would help in understanding the role of heat shock proteins in phenol tolerance. Additionally, several hypotheses were suggested by the microarray data regarding other effects of phenol, such as changes in the membrane and cross-protection provided by exposure to one stress over the other. The cross-protection studies would yield important information because bacterial cells are not exposed to one stress but various stresses simultaneously in the environment. Identification of the function of hypothetical genes that were highly up-regulated by phenol could also provide insights into the mechanism of action of phenol in a bacterial cell, and therefore, need to be examined in order to generate a complete picture of the effect of phenol on bacteria. This information can then be applied to other bacterial species that have important biotechnological and bioremediation applications.
References


