Effect of Carbon-Starvation on the Survival of and PNP Degradation by a *Moraxella* strain.

Author: Michael Moore
Student Number: 0161407
Supervisor: Dr. Kam Tin Leung

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ISBN: 0-494-00487-8
Our file Notre référence
ISBN: 0-494-00487-8

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Abstract of the Thesis

The effect of carbon-starvation on the stress resistance responses of a *p*-nitrophenol degrading *Moraxella* strain was examined in both, buffer and river water samples. The *Moraxella* strain showed optimal stress resistance responses in a minimal salt buffer when carbon-starved for 1-2 days. In the buffer system, the 1- and 2-day carbon-starved *Moraxella* cultures survived about 150-, 200- and 100-fold better than the non-starved cultures when exposed to 43.5°C, 2.7 M NaCl and 500 μM H_2O_2 for 4 hours, respectively. A green fluorescent protein gene- (*gfp*-) labelled derivative of the *Moraxella* strain was used to examine the stress resistance responses of the bacterium in natural river water microcosms. The carbon-starved *gfp*-labelled *Moraxella* strain also showed stress resistant responses against heat, osmotic and oxidative stresses in the river water samples. Despite the stress tolerant capability of the carbon-starved *gfp*-labelled *Moraxella* cells, they did not exhibit any survival advantage over their non-starved counterparts when inoculated into river water microcosms and incubated at 10° or 22°C for fourteen days.

The effect of carbon-starvation on the ability to degrade *p*-nitrophenol (PNP) was examined for both the *Moraxella* sp. strain and its *gfp*-labelled derivative. Over three days, the cell density of the *Moraxella* strain increased in a sterile carbon-depleted minimal salts medium (MSM) from 3.8×10^8 to 7.2×10^8 CFU/mL. Carbon starvation for 24 h decreased the induction time for PNP degradation by the bacterium in MSM from 6 to 1 h. However, it did not eliminate the induction time. *Moraxella* cells exposed to 2-day carbon starvation had an induction time of 3 h and the induction time of the 3-day-starved cells was 6 h, which was similar to that of the non-starved *Moraxella* cells. A 100% increase in density of the non-starved cells did not affect the induction time of PNP

degradation of the bacterium, indicating that the initial increase in cell density of the C-starved culture did not cause the faster onset of PNP degradation. However, the initial uptake of PNP of the 1-day C-starved *Moraxella* cells was three-fold higher than the non-starved cells. The *gfp*-labelled *Moraxella* (M6) cells were used to examine the survival of and PNP degradation by the bacterium in non-sterile river water samples. Similar PNP degradation behaviour was observed in the river water samples inoculated with the M6 cells. The time needed for complete degradation of PNP by the non-starved M6 was 19 to 27 and 33 h in samples spiked with 80, 200 and 360 μM PNP, respectively. However, the 1-day C-starved inocula required about 16 h to degrade the PNP completely regardless of PNP concentration in the water samples. Survival of the C-starved and non-starved M6 was similar in the river water regardless of the PNP concentration. In the absence of PNP, the cell density decreased continuously. At 200 and 360 μM PNP, the cell densities of M6 increased in the first 2-day of incubation and declined steadily afterward.

Effect of Carbon-Starvation on the Survival of and PNP Degradation by a *Moraxella* strain.

1. Literature Review.

1.1. Bacterial Starvation and Survival.

In aquatic and soil environments, carbon and other nutrients are scarce and when present may be unavailable to bacteria (Morita 1988, Roszack and Colwell 1987). These features of an oligotrophic environment lead to repeated experiences of starvation for the bacterial inhabitants. Bacteria in the environment are constantly switching between active growth due to a sudden influx of nutrients into the system and starvation due to the full consumption of these nutrients (Poindexter 1981). Some variables affecting survival of a starvation state are population density, nutritional status, growth rate, growth phase and the starvation environment (Dawes 1976). Bacterial starvation is important to study because starved bacteria have increased tolerance to various environmental stresses. They can display increased virulence in the case of some pathogens such as *Salmonella enterica Typhimurium* and *Staphylococcus aureus* (Hengge-Aronis 2000), and starved *E. coli* may better tolerate food preservation methods (Cheville *et al.* 1996).

In a laboratory, there are two main ways to initiate starvation in bacteria: either suspension in a nutrient-deficient medium, or culturing the bacteria in a nutrient-limited medium and allowing them to deplete the limited nutrient (Siegele *et al.* 1993). Although the latter is less stressful for the bacteria, it could be difficult to determine the precise point at which the bacteria entered a starvation-state. Conversely, the former can be stressful or fatal to the bacteria due to repeated centrifugation and suspension steps, but

the point at which the cells entered a starvation-state would be more consistent. Both methods are used throughout the literature.

Several reviews exist for bacterial starvation and the stationary phase (Gutierrez 1999, Groat *et al.* 1986, Hengge-Aronis 1993, Siegele and Kolter 1992, Kolter *et al.* 1993, Kjelleberg *et al.* 1993, Lazazzera 2000, Nyström 1999, Matin *et al.* 1989, Spector 1998, Takayama and Kjelleberg 2000, Wai *et al.* 1999). From these reviews, a general profile of cellular changes and characteristics has been compiled for non-differentiating (i.e. non spore-forming) bacteria. The focus in this thesis will be on non-differentiating bacteria, as opposed to spore-forming bacteria, because a non-differentiating Gram-negative bacterium, i.e. *Moraxella* sp. strain, is used in this project.

A number of processes are associated with non-differentiating cells entering a starvation state: altered protein synthesis and changes in the cellular protein profile as well as increased protein turnover rate, changes in cell wall and cell membrane composition, altered nucleic acid composition and reductive division (Kolter *et al.* 1993). During the initial few hours of starvation, the protein profile of the bacteria changes significantly. During this period there are 30-50 new proteins expressed (Groat *et al.* 1986, Lange and Hengge-Aronis 1991), including proteases that degrade old proteins associated with exponential growth. The new proteins expressed are usually different for each deficient macronutrient (i.e. carbon, nitrogen, phosphorus and sulphur). However, for *Escherichia coli*, there is a set of starvation proteins synthesized regardless of the type of limiting nutrient (Matin *et al.* 1989).

The functions of most of these general stress proteins are unknown. However, those proteins with known functions typically are involved in stress-resistance or nutrient

scavenging and others for exiting starvation when nutrients become available (Matin *et al.* 1991). Cell wall alterations caused by starvation such as increased trehalose production and thickening of the peptidoglycan layer make the cells more resistant to autolysis and thermolysis (Cashel and Rudd 1987, Hengge-Aronis *et al.* 1991, Nyström & Kjelleberg, 1989). Structures are added to the cell membrane, the lipid profile is altered, and exopolymeric substances are secreted to facilitate adhesion to different surfaces including other bacteria, and to form biofilms, clumps or aggregates (Kjelleberg *et al.* 1987, Siegele and Kolter 1992). Cellular division without an associated increase in cell mass, known as reductive division, is also a common phenomenon and produces smaller (dwarf) cells (Lange and Hennge-Aronis 1991, Kjelleberg *et al.* 1987).

After the initial changes to the bacteria, the metabolic rate and the cellular ATP level decreases significantly, but then remains at a constant low level (Siegel and Kolter 1992). Cyclic AMP (cAMP) levels in *E. coli* have also been shown to increase with starvation (Botsford and Drexler 1978), resulting in de-repression of some nutrient transport systems known to be affected by carbon-starvation and which rely on high levels of cAMP in the cell to maintain function (Death and Ferenci 1994, Death *et al.* 1993). This de-repression leads to increased activity of high-affinity transport systems as a way to increase nutrient scavenging (Albertson *et al.* 1990). Examples of the low-affinity transports affected by this de-repression include those involving carbohydrates in *E. coli* (Death *et al.* 1993, Death and Ferenci 1994) and amino acid transport (Kay and Grondlund 1969, Marden *et al.* 1987). However, not all starvation proteins and transporters are dependent on cAMP levels, specifically those associated with starvation of individual nutrients such as nitrogen or phosphorus (Schultz *et al.* 1988).

The metabolic adjustments may not be experienced by all bacterial species upon entering a starvation state and the response to starvation by different bacterial species can vary greatly. For Gram-negative microorganisms, most of the processes are initiated and controlled by RpoS, the stationary phase sigma-factor. RpoS is a RNA polymerase transcription factor (originally designated KatF). Concentrations of RpoS increase at the onset of starvation and RpoS acts as a regulator, allowing expression of genes related to survival in carbon-limited conditions, while inhibiting those associated with exponential growth (Loewen and Hengge-Aronis 1994).

1.2. Carbon-Starvation and Stress-Resistance.

There have been several review articles published on the effect of carbon-starvation on stress-resistance (Hengge-Aronis 1993, Kolter *et al.* 1993, Lazazzera 2000, Matin 1991, McDougald *et al.* 2002, Nyström 1999, Pichereau *et al.* 2000). For some bacteria experiencing carbon-starvation, there is an associated increased stress-resistance or tolerance. Some bacteria known to exhibit this type of response include: *Escherichia coli* (Jenkins *et al.* 1990, Jenkins *et al.* 1988, Jouper-Jaan *et al.* 1992), *Pseudomonas fluorescens* (van Overbeek *et al.* 1995), *Vibrio* sp. (Jouper-Jaan *et al.* 1992, Nyström *et al.* 1992), and *Staphylococcus aureus* (Watson *et al.* 1998). Stress-resistance responses in the described bacteria include increased heat-tolerance and increased resistance to osmotic pressure and hydrogen peroxide, ethanol, extreme pH, bleach and UV irradiation, though this list is not exhaustive (Pichereau *et al.* 2000) The listed bacterial species did not all exhibit the same degree of resistance or show resistance to the same stresses. The stress-resistance response of the bacteria seems to be variable, depending on the bacterial species and the starvation conditions. Carbon-starvation elicits a cellular response as previously described including the synthesis of

30-50 starvation-related proteins. Some of these new proteins are responsible in part for the increased stress-resistance. For example, during carbon-starvation, a DNA binding protein, DPS, is expressed at higher levels. DPS acts to condense the structure of the bacterial genome and can protect the DNA from oxidative stresses (Almirón *et al.* 1992). Some heat shock proteins are produced during carbon-starvation and help protect cells during times of thermal stress (Jenkins *et al.* 1991, Jenkins *et al.* 1988). Another starvation protein expressed to increase the stress-resistance of some bacterial species is the regulatory protein RpoE. RpoE, similar to RpoS, is a RNA polymerase transcription factor. However, RpoE has a narrower area of influence and is only thought to be involved in osmotic and oxidative stress-resistance (McDougald *et al.* 2002)

1.3. Environmental contamination.

Environmental pollution is a major problem around the world and represents a significant drain on the economic resources in North America and elsewhere. There is great concern over many different chemicals that have made their way into the environment, especially those that are acutely toxic or recalcitrant to degradation. These chemicals enter the environment either through a point source, such as industrial outflow or spills, or through a non-point source such as agricultural chemical application/run-off and automobile engine exhaust. While pollutants like polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pentachlorophenol (PCP) and trichloroethane (TCE) are the focus of many studies and much effort for removal from the environment, there are many less prominent but important chemicals that need to be addressed: The chemical *p*-nitrophenol (PNP) is the pollutant of interest in this thesis.

1.4. *p*-Nitrophenol

p-Nitrophenol (PNP) is an anthropogenic compound that is mainly used in the production of pesticides such as methyl-parathion, parathion and nitrofen. The pharmaceutical industry also uses PNP to manufacture some analgesics such as acetaminophen and tuberculostatic compounds such as 4-aminosalicylic acid (Boehncke *et al.* 2000). Alternatively, PNP is used as a fungicide and leather preservative. It is listed in the potentially toxic list of ingredients in pesticides (U.S. E.P.A.) and the Priority List of Hazardous Chemicals (U.S. E.P.A. 2003).

Environmental contamination by PNP occurs from various sources: industrial waste disposal (Shackelford and Keith 1976), direct spillage of PNP and agricultural applications of parathion (Munnecke and Hsieh.1976). Airborne pollution is also an issue since PNP is present in gasoline and diesel exhaust and can be released at levels of 1 µg/L (Tremp *et al.* 1993). PNP accumulates in fog and cloud water up to 20 and 5 µg/L respectively and is estimated to enter the environment from rain, along with its isomer 2-nitrophenol, at a rate of several thousand tonnes per year worldwide (Boehncke *et al.* 2000). Hydrolysis of parathion or methyl-parathion in the environment leads to release of PNP. PNP is moderate to highly toxic to some aquatic organisms in the environment. Human exposure to PNP comes from three main sources. The first is inhalation with maximum estimates of 60 ng mononitrophenols/kg body weight per day for ambient air and up to 0.12 ng PNP/kg body weight per hour in fog (Boehncke *et al.* 2000). Drinking water is another source and for PNP exposure and a maximum estimate is about 20 ng/kg body weight per day assuming 1 µg PNP/L drinking water. Dermal contact is the third main source of PNP exposure in humans, although it is limited mainly to workers in PNP or PNP related manufacturing plants.

Degradation of PNP has been observed in a number of bacterial species such as *Pseudomonas* (Munnecke and Hsieh 1976, Simpson and Evans 1953), *Moraxella* (Spain and Gibson 1991), *Flavobacterium* (Raymond and Alexander 1971) *Arthrobacter* and *Nocardia* (Hanne *et al.* 1993). Some of the bacterial species degrade PNP through a hydroquinone intermediate (Figure 1.1), while others through a 1,2,4-trihydroxybenzene intermediate (Figure 1.2). Both of these pathways result in the release of nitrite from the reaction and a cleavage of the aromatic ring. The pathway in Figure 1.1 is found prominently in Gram-negative species, while the pathway in Figure 1.2 is found mainly in Gram-positive bacteria (Zylstra *et al.* 2000).

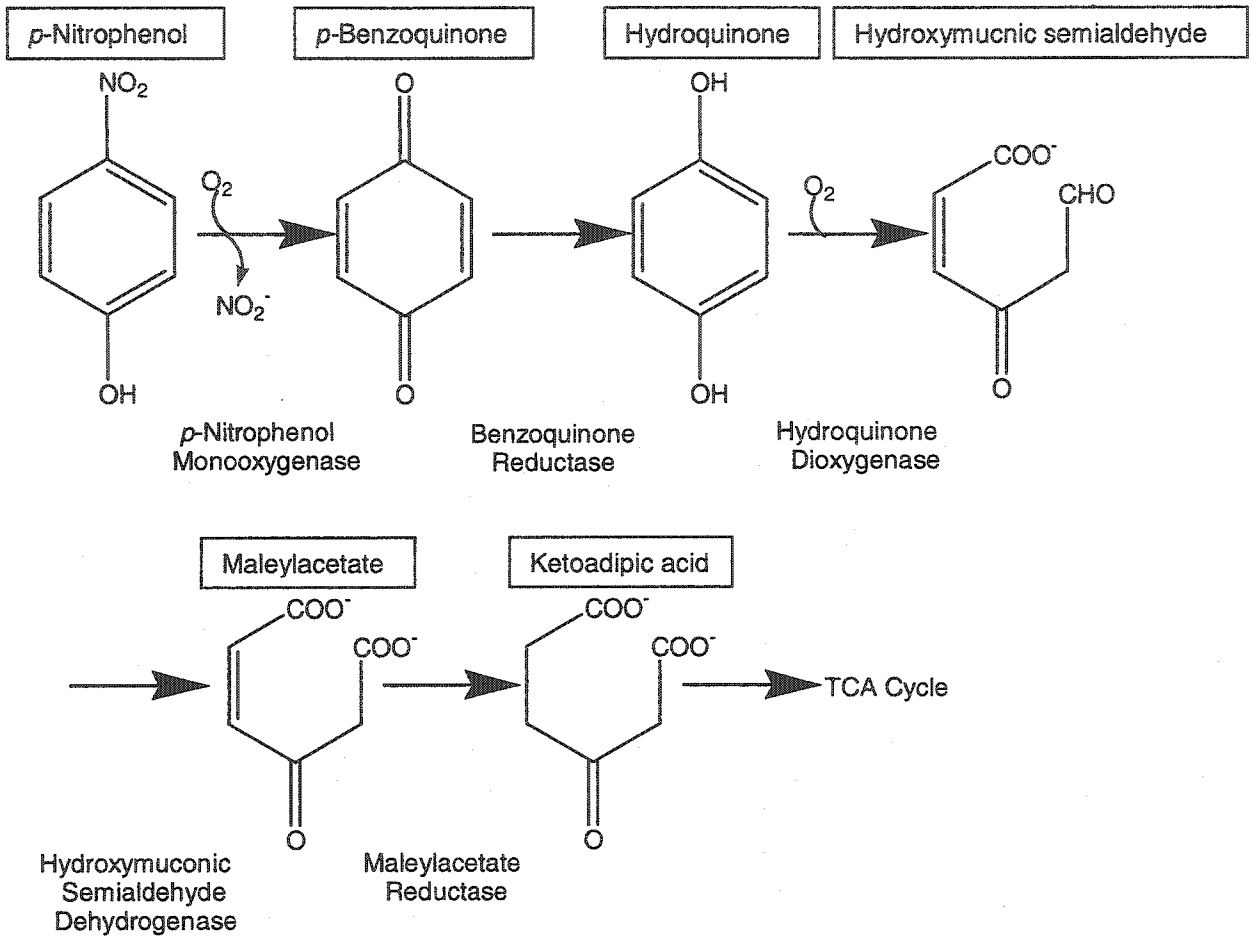

Figure 1.1. Metabolic pathway for the degradation of *p*-nitrophenol through the hydroquinone intermediate (Spain and Gibson 1991).

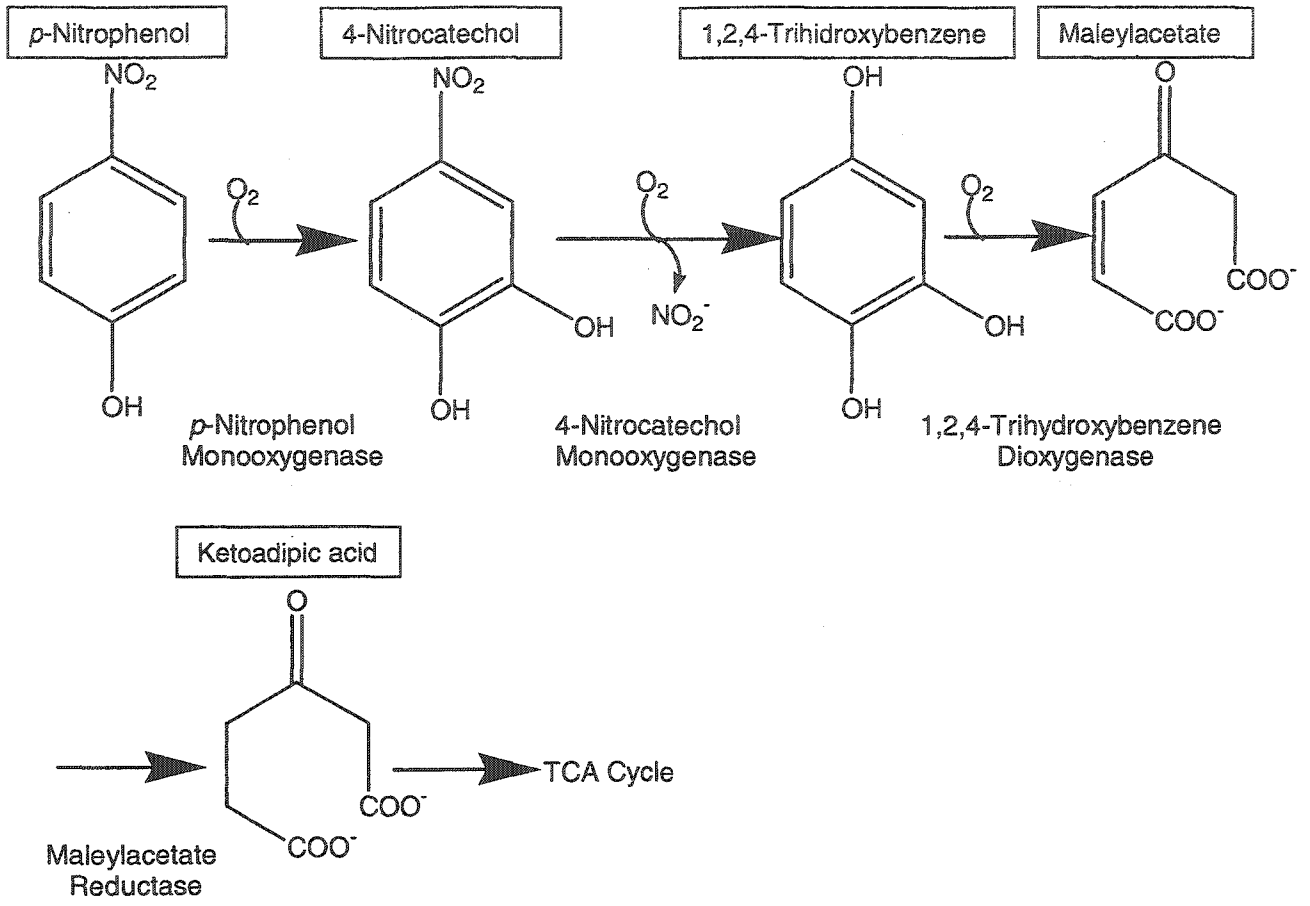

Figure 1.2. Metabolic pathway for the degradation of *p*-nitrophenol through the 1,2,4-trihydroxybenzene intermediate (Jain *et al.* 1994).

1.5 *Moraxella* species

The bacterium studied in this thesis is a *Moraxella* strain. *Moraxella* are ubiquitous in the environment and are present in both, soil and aquatic samples. They have been found in freshwater sediment and both sandy and loam soils (Carter *et al.* 1995), oil field samples (Rani *et al.* 1996), untreated drinking water in South Africa (Pavlov *et al.* 2004) and even in commercial rhizobial inoculants in Spain, though as a contaminant (Catroux *et al.* 2001). Some *Moraxella* species are known as human and animal pathogens. The most prominent pathogenic species is *Moraxella catarrhalis* and is associated with ear,

throat and nose infections (Verduin *et al.* 2002). In the environment, *Moraxella* sp. are important for nutrient cycling, a function of most environmental bacteria. Some *Moraxella* strains can degrade *o*-phthalate, salicylate, nitrophenols, benzoate, naphthalene-sulfonic acids and steroids (Madyastha and Shankar 1994, Rani *et al.* 1996, Sterjiades and Pelmont 1989, Wittich *et al.* 1988).

Jim Spain isolated a PNP degrading *Moraxella* strain from activated sludge (Spain and Gibson 1991). This strain of *Moraxella* has been the focus of several studies due to its ability to mineralize PNP to carbon dioxide, water and nitrite through the hydroquinone pathway in Figure 1.1 (Errampalli *et al.* 1999b, Leung *et al.* 1997a, Leung *et al.* 2000, Shimazu *et al.* 2001, Tresse *et al.* 1998). The intent of this study was to examine the abilities of this *Moraxella* sp. strain to survive and to breakdown PNP under different conditions.

1.6. Bioremediation

Bioremediation, the process of using living organisms to remediate a polluted area, is becoming a favourable and feasible choice in pollution clean up. One of the benefits of bioremediation is that it is a versatile technique that can be applied to many chemicals and types of environment. There are two main approaches for choosing microbial inocula to use in bioremediation, (i) to use a consortium of indigenous bacteria known to degrade the targeted pollutant or (ii) to use a characterized laboratory strain known to degrade the pollutant. The advantages to using the consortium of indigenous bacteria include the knowledge that the bacterial species of the consortium have survived within the environment being inoculated and also multiple species may be able to degrade the designated chemical or degradation by-products leading to a shorter remediation period

compared to pure cultures (Bouchez 1999). The disadvantages to using the indigenous consortium are that the portion of the total inoculum made up of the chemical degrading species may be low, there may be competition between different species for the available nutrients or the chemical degrading species may not degrade efficiently the pollutant. All these aspects could lead to slower degradation rates. The advantages of a characterized laboratory strain include using bacteria known to degrade the chemical effectively and possible knowledge of the optimal degradation conditions. Using single culture inocula also means potentially that all bacteria introduced will be involved in degradation. The main disadvantage to using a characterized laboratory strain is the possible unsuitability of the inoculum for the target environment (Watanabe and Hamamura 2003) and/or if there is a mixture of pollutants where some other chemicals may impede degradation (Bouchez 1999).

1.7. Approaches to monitoring inoculum success.

A distinguishable biomarker is usually required to observe the survival and function of microbial inocula in the environment. Biomarkers are needed because different bacteria can appear similar to each other under a microscope, on a nutrient agar plate or in a nutrient broth. Most biomarkers used to observe inocula are introduced to the organism or naturally present in the bacterium at the genetic level. A number of genes that have been isolated from various organisms facilitate a measurable or observable reaction to manifest the desired specimen. Some of the most prevalent marker genes are antibiotic resistance genes. These genes allow the inoculum bacteria to grow in the presence of specific antibiotics. The bacteria carrying the marker genes can then be isolated from a mixed sample by adding that antibiotic to the agar plate or broth. The use of antibiotic selection genes is common, but can only show growth or non-growth. Other

marker genes can distinguish the inoculum from the indigenous bacterial population as well as indicate the metabolic state of the inoculum. The most common marker genes with this capability include the *lacZ* gene from *E. coli*, the *lux* genes from the marine bacteria *Vibrio fischeri* or *V. harveyi*, the *luc* gene from fireflies and the *gfp* gene from the jelly-fish *Aequorea victoria* (Köhler *et al.* 2000). Bacteria that have incorporated these genes are referred to as labelled bacteria in this thesis.

The expression of the *lacZ* gene in labelled bacteria leads to production of β-galactosidase, an enzyme that allows the bacteria to hydrolyze β-glycosidic bonds. These bonds are found in some natural sugars such as the bond between glucose and galactose in a lactose molecule, or in some chromogenic chemicals such as X-Gal. X-Gal is synthesized to produce a blue coloured product upon hydrolysis by β-galactosidase and can be added to different media.

Use of the *luc* genes coding for the enzyme Luciferase allows researchers to observe bacteria with the addition of the substrate luciferin, a compound that reacts with oxygen and ATP in the presence of luciferase to produce light *(Lampinen et al. 1992)*. In contrast, the *lux* genes (*luxABCDE)* do not necessarily require the use of added substrate if completely inserted into the bacteria and can be used to directly measure the metabolic activity of individual cells. The *lux* genes use the cellular energy in the form of NADPH and produce a measurable amount of light *(Heitzer et al. 1998)*. Similarly there is no substrate needed if *gfp* genes are used as a marker. Green fluorescence protein is frequently chosen as a marker due to its stability within a cell and the feature of being visible under near UV or blue light (Errampalli *et al.* 1999a).

There can be drawbacks however to the methods used for generating labelled bacteria. Since insertion of marker genes is usually a random event, it is possible that critical or desired genes could be disrupted and cause loss of function in their gene products. Screening of labelled strains is therefore critical to ensure disruption of normal physiological functions has not occurred. although these disruptions may only be apparent under specific growth conditions.

1.7.1. Tn5 (Transposon 5) random labelling of bacteria.

The method for labelling the bacteria in this thesis was Tn5 random gene insertion, a common approach for constructing labelled bacteria in the laboratory. In Tn5 random gene insertion, selected foreign genes are inserted into a bacterial chromosome through a transposon element or 'jumping gene'. The vector (i.e. plasmid) that carries the transposon element is constructed with an antibiotic resistance marker and two Tn5 inverted repeats (Figure 1.3). These inverted repeats are required for the insertion, or transposition, and the foreign genes are placed between them (Berg *et al.* 1989). The enzyme responsible for the transposition is transposase and the transposase genes are located outside the inverted repeats. This ensures that the transposase genes are not inserted into the chromosome (Alexeyev *et al.* 1995). Essentially, transposition happens only once, because the transposase genes are no longer available after transposition (De Lorezo *et al.* 1990). Additional features on the plasmid vector include a π-dependent origin of replication R6K (Kolter *et al.* 1978) and an RP4 origin of transfer (Miller and Mekalanos 1988). The implications of these additional features are that only certain types of bacteria can maintain and transfer the plasmid vector, such as *λpir* lysogen containing a RP4 gene. A *λpir* lysogen is a bacterium that is infected with a λ-

bacteriophage and the bacteriophage has inserted its genetic material into the bacterial chromosome.

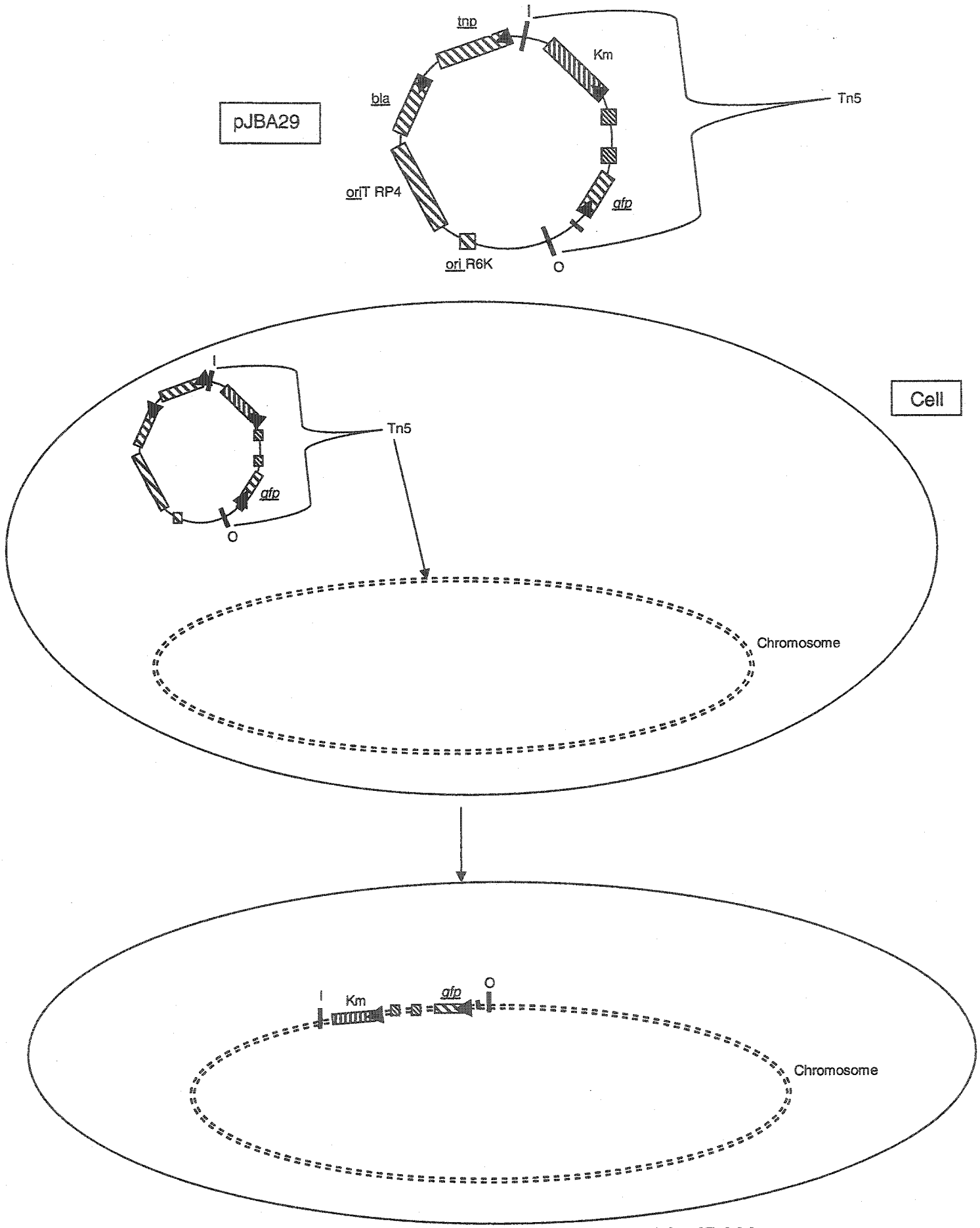

Figure 1.3 Example of Tn5 random labelling using plasmid pJBA29.
Km – kanamycin resistance gene, bla – ampicillin resistance gene.

1.8 Factors affecting inoculum survival.

Inocula face a number of challenges following introduction into the environment including competition and predation from indigenous micro-flora, low availability of nutrients, and abiotic environmental stresses (van Veen *et al.* 1997). Bacteria introduced into an environment are constantly under stress from these challenges and must overcome numerous obstacles to establish themselves. The average amount of bacteria in a gram of soil is between 4×10^{7} and 2×10^{9} cells (Paul and Clark 1989, Richter and Markewitz 1995). The large bacterial populations in the soil occupy many of the niches that the inoculum needs to establish itself in. With many niches unavailable, the inoculum would be more accessible to predating protozoa and be unable to obtain nutrients easily (Blumenroth and Wagner-Döbler 1998, van Veen *et al.* 1997).

As described previously, most nutrients are unavailable in the environment. Inocula must therefore function in nutrient deficient conditions and survive periods of starvation. Initially, inocula could be disadvantaged because bacteria are usually cultured in nutrient rich media to obtain high culture densities and the cellular machinery would be optimized for that environment. Abiotic stresses are also present in many environments. The typical abiotic stressors that inocula encounter are extreme temperatures, desiccation, fluctuations in pH or osmotic potential and exposure to damaging compounds such as peroxides (van Veen *et al.* 1997). Although not all of the described stressors would be encountered by inocula simultaneously, inocula can be exposed to a combination of stressors both biotic and abiotic stressors in the environment.

1.9 Objectives of the Thesis

The first objective of this thesis is to determine how carbon-starvation will affect the stress resistance response and survival of a *Moraxella* strain in both, river water and buffer. Milestones for achieving this objective include:

(i) Testing the response of *Moraxella* sp. to imposed stresses that mimic stresses found in the environment. (i.e. heat, osmotic and oxidative stress)

(ii) Construct a green fluorescent protein gene (*gfp*-) labelled derivative to study the stress-survival response of carbon-starved *Moraxella* sp. in a non-sterile river water sample.

(iii) Testing the survival of the *gfp*-labelled *Moraxella* in a non-sterile river water sample.

The second objective of this thesis is to determine whether carbon-starvation has an effect on PNP degradation by the *Moraxella* strain and whether this effect will manifest in both, a buffer system and a river water sample. The milestones to achieve this objective include:

(i) Examining the ability of *Moraxella* sp. to degrade PNP under starved and non-starved conditions.

(ii) Investigating possible factors that might influence PNP degradation in *Moraxella* sp.

(iii) Examining the survival of the *gfp*-labelled *Moraxella* in a PNP-spiked river water sample under starved and non-starved conditions.

2. Stress Survival Responses of a Carbon-Starved *p*-Nitrophenol-Mineralizing *Moraxella* Strain in River Water.

2.1. Introduction

Resistance to environmental stresses is an important factor affecting survival of bacteria in the environment. It has been shown that carbon-starvation provides a general cross-protection to bacterial cells in harsh conditions by enhancing certain characteristics: increased stress-resistance, slowed metabolism and an increased ability to degrade xenobiotics (Matin 1991, Roszak and Colwell 1987, Herman and Costerton 1993). A number of review articles have been published on related subjects, such as the effect of carbon-starvation on stress-resistance (Matin 1991, Hengge-Aronis 1993, Pichereau *et al.* 2000, McDougald *et al.* 2002, Kolter *et al.* 1993, Nyström 1999, Lazazzera 2000) and bacterial responses to starvation in the environment (Roszak and Colwell 1987, Matin *et al.* 1989, Matin 1996, Spector 1998, Wai *et al.* 1999, Takayama and Kjelleberg 2000, Gutierrez 2001). Although stress-survival responses of *Escherichia coli* (Jenkins *et al.* 1988, Jenkins *et al.* 1990, Jouper-Jaan *et al.* 1992), *Pseudomonas fluorescens* (van Overbeek *et al.* 1995), *Vibrio* sp. (Jouper-Jaan *et al.* 1992, Nyström *et al.* 1992), and *Staphylococcus aureus* (Watson *et al.* 1998) have been investigated to some extent, little is known about stress-responses of most environmental bacterial species.

Moraxella sp. is a bacterial species that possesses the ability to degrade various organic pollutants, such as nitrophenols, benzoate, naphthalene-sulfonic acids and steroids, and is found ubiquitously in the environment (Madyastha and Shankar 1994, Sterjiades and Pelmont 1989, Wittich *et al.* 1988). A *Moraxella* strain isolated from activated sludge by Spain and Gibson (1991) was able to mineralize *p*-nitrophenol (PNP), an anthropogenic by-product of parathion and acetaminophen production. Since PNP is found in many industrial waste sites and agricultural soil, the ability of this *Moraxella* strain to degrade PNP makes it favourable for use as a bioremediation organism.

Heat, osmotic and oxidative stresses were examined in this study because of their relatedness to stresses encountered by microbial remediation agents in the environment. Inocula face a number of challenges following introduction into the environment: competition from indigenous flora, predation by protozoa, low availability of nutrients, and environmental stresses such as pH, temperature fluctuations, hydrogen peroxide concentrations and osmotic pressure (van Veen *et al.* 1997). For bacteria to be used in a bioremediation setting, cross-protection from carbon-starvation could be beneficial. By understanding bacterial stress resistance responses to carbon-starvation, the effectiveness of bioremediation may be improved.

The aim for this study was to determine whether carbon-starvation (i) provides any cross-protection against heat, osmotic and oxidative stresses on the PNP-degrading *Moraxella* strain in buffer and non-sterile river water samples; and (ii) improves survival of *Moraxella* in river water. To conduct part of this study in non-sterile river water, a *gfp*-labelled *Moraxella* strain was constructed. This labelled *Moraxella* strain produced green fluorescent protein (GFP), which can be visualized with a transilluminator and makes the *Moraxella* distinguishable from indigenous bacterial populations.

2.2. Materials and methods

2.2.1. Bacterial strains

The PNP-mineralizing *Moraxella* strain used in this study is a Gram-negative rod-shaped bacterium isolated from an activated sludge (Spain and Gibson 1991). It was cultured in Tryptic Soy Broth (TSB; Becton Dickinson and Company, Sparks, MD, USA) and stored on Tryptic Soy Agar (TSA; Difco Detroit, MI, USA) plates at 4 °C.

A spontaneous rifampicin resistant (Rif^r) *Moraxella* strain was isolated by plating 0.1 mL of a 24-h old *Moraxella* culture with optical density (OD) at 600 nm of 1 onto TSA plates supplemented with 50 μg/mL rifampicin (Sigma-Aldrich Corp., St. Louis, MO, USA). The Rif^r *Moraxella* strain was repeatedly cultured on TSA and rifampicin-supplemented TSA to ensure stability of the Rif^r phenotype. To confirm the PNP-degrading capability of the Rif^r *Moraxella* strain, the Rif^r strain was cultured, harvested, and suspended in a sterile minimal salts medium (MSM, 1.249 mM KH_2PO_4, 3.73 mM K_2HPO_4, 0.4 mM $MgSO_4$-$7H_2O$, 0.02 mM $FeSO_4$-$7H_20$, 1.4 mM NH_4Cl, pH 7) supplemented with 0.025% yeast extract and 1440 μM PNP. Positive degradation was determined by monitoring the disappearance of the PNP using spectrophotometry at OD_{420nm} (Leung *et al.* 1997).

Escherichia coli S17-1λ was used as a host for the pJBA29, a Tn5 suicide-plasmid carrying the *gfp* gene (Tresse *et al.* 1998, Eberl *et al.* 1997). *E. coli* S17-1λ (pJBA29) was cultured in TSB supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin (Sigma-Aldrich Corp.) and stored on Luria-Bertani agar (LBA; Becton Dickinson and Company) plates supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin at 4 °C.

The *gfp*-labelled *Moraxella* strains were cultured in TSB supplemented with 50 μg/mL of kanamycin and rifampicin and stored on TSA supplemented with 50 μg/mL of kanamycin and rifampicin at 4 °C. All bacterial cultures were stored in 25% glycerol at – 80 °C until needed.

2.2.2. Generating the *gfp*-labelled *Moraxella* strains

A Rif^r PNP-mineralizing *Moraxella* strain was labelled chromosomally with the *gfp* gene by the Tn5-*gfp* suicide-plasmid pJBA29 (Eberl *et al.* 1997; Tresse *et al.* 1998). The

donor strain of the conjugation protocol was *E. coli* S17-1λ (pJBA29) and the Rif^r *Moraxella* strain was the recipient.

The conjugation protocol was adopted from De Bruijn and Rossbach (1994) with modifications. The *Moraxella* and *E. coli* strains were cultured overnight separately in antibiotic amended TSB to an OD_{600nm} of 1. The antibiotics used were 50 µg/mL rifampicin for the *Moraxella* strain and 100 µg/mL of ampicillin and kanamycin for the *E. coli* S17-1λ (pJBA29) strain. The cells were harvested by centrifugation (16,000 x g, 10 min), washed twice with sterile 10 mM $MgSO_4$ to remove the antibiotics, and suspended in sterile 10 mM $MgSO_4$ to an OD_{600nm} of 10. The donor and recipient cells were mixed in a 1:1 ratio and 50 µL of the cell mixture was added to a sterile polycarbonate membrane filter (0.22 µm average pore size, 25 mm diameter, Millipore, Billerica, MA, USA) placed on a TSA plate. Donor and recipient cultures were added to separate filters as controls. The samples were incubated overnight at 30 °C. To recover cells, each filter was vortexed in 5 mL of sterile 10 mM $MgSO_4$. The filters were removed and the cell suspensions were serially diluted and plated on TSA supplemented with 50 µg/mL of kanamycin and rifampicin (Kan/Rif TSA). There was no growth for the Rif^r *Moraxella* strain and there were less than 10 colonies for the *E. coli* S17-1λ (pJBA29) on the Kan/Rif TSA plates.

Numerous colonies were observed on the Kan/Rif TSA plates with the 1:1 mixture of *E. coli* S17-1λ (pJBA29) and the Rif^r *Moraxella* strain. Fourteen labelled strains were selected and tested for *gfp* expression, oxidase production, and the stability of gene insertion. The information gained from the tests led to a refined selection of mutants to be tested for PNP degradation, and growth rate.

For *gfp* expression, each putatively labelled strain was examined under a UV transilluminator for the appearance of bright green fluorescent colonies. For oxidase production, an isolated colony was picked using a sterile inoculating loop and transferred to a piece of Whatman No. 1 filter paper (Whatman Inc., Clifton, NJ, USA) to which 1-2 drops of oxidase test reagent (N, N, N, N-tetramethyl-*p*-phenylenediamine dihydrochloride, 1%; Difco, Becton Dickinson and Company) were applied. Positive results were determined by the appearance of a dark purple colour in the region of the transferred culture. Both the wild-type and the Rif^r *Moraxella* strains were positive for the oxidase test. *E. coli* S17-1λ (pJBA29) was oxidase negative. To assess the stability of the *gfp* gene insertion, an isolated colony was selected and plated on four successive TSA plates before being re-plated on Kan/Rif TSA and screened for *gfp* expression.

For PNP degradation, eight *gfp*-labelled strains, chosen for the brightness of GFP fluorescence (designated as *Moraxella* strain M1, M2, M3, M6, M7, M8, M9, M10, and M12), were grown in TSB overnight to late-log phase (OD_{600nm} 0.7-0.9). Each strain was harvested, washed and suspended in MSM containing 0.025% yeast extract, to an OD_{600nm} of 0.5, to facilitate PNP degradation. This experiment was done in duplicate for each selected strain. PNP was added to each culture to a concentration of 1440 µM and the amount of PNP in solution was determined by a spectrophotometer at OD_{420nm} (Leung *et al.* 1997). Samples from the culture were taken at hourly intervals. One mL of culture was removed and centrifuged (16,000 x g, 10 min) at 22°C and 0.5 mL of supernatant was mixed with 0.5 mL of 0.5 M NaOH before measurement. The purpose of measuring the PNP degradation rates of the *gfp*-labelled strains was to ensure that any *gfp*-labelled *Moraxella* strain used in the experiments would not exhibit key characteristics different from the wild-type *Moraxella* strain.

The growth rates of three selected *gfp*-labelled strains (i.e. *Moraxella* strain M1, M3, M6) and the wild-type *Moraxella* strain were determined. This was done to allow the selection of a *gfp*-labelled strain with similar growth characteristics to the wild-type *Moraxella* strain. For this, the *Moraxella* strains were grown overnight in TSB to mid-log phase. Each culture was used to inoculate fresh TSB to an OD_{600nm} 0.1 and grown at 30 °C, 150 rpm. The optical density was measured at 1 h intervals until stationary phase was reached. Triplicate samples were performed on the four strains.

The *gfp*-labelled *Moraxella* M6 strain was chosen for the stress-survival study because it was not significantly different from the parent *Moraxella* strain based on growth rate and PNP degradation abilities.

2.2.3. Starvation of bacterial cells

Moraxella cells were cultured in TSB at 30 °C (150 rpm) to mid-log phase (OD_{600nm} of 0.5-0.7) before starvation. The cells (2 x 45mL) were harvested by centrifugation (3000 x g, 10 min, 20 °C) and washed twice with an equal volume of sterile MSM. *Moraxella* cell suspensions were prepared by mixing the cells with MSM to an OD_{600nm} 0.5 (approximately 2×10^8 CFU/mL). These cell suspensions were used immediately as the non-starved controls in subsequent stress experiments. A subsample of the cell suspension was starved in MSM at 30 °C with shaking (150 rpm). The carbon-starved cells were harvested after 1, 2 and 3 days of starvation and were used for the stress-survival experiments.

2.2.4. ATP levels during starvation

The *Moraxella* cells were prepared as previously described. ATP levels were determined with the Multi-Trace Water Test kit (Biotrace Ltd., The Science Park, Bridgend, UK) using the Multi-Light luminometer (Biotrace Ltd.). In brief, 100 µL of

bacterial culture were transferred to a cuvette, to lyse the cells and extract ATP, 100 µL of Extractant XM (Multi-Trace Water Test Kit) were added to the cuvette, gently vortexed for 2 s and allowed to stand at 22 °C for 2 min. To initiate the luminescence, 100 µL of reconstituted luciferin/luciferase reagent was added and gently vortexed for 2 s. The reaction was allowed to develop for 30 s. The sample was then placed in the Multi-Light luminometer and the amount of luminescence emitted was measured. The experiment was repeated for the 1-, 2-, and 3-day starvation cultures. The luminescence data were converted to ATP concentration by comparison to an ATP standard calibration curve.

2.2.5. Responses to various starvation and stress levels

A 0.5 mL of *Moraxella* cell suspension (about 1×10^8 CFU) was diluted into 4.5 mL MSM and exposed to various stress conditions (i.e., heat, osmotic and H_2O_2). After exposure to each stress challenge, 0.1 mL of stressed cell suspension was removed, serially diluted and plated on TSA. One-tenth mL of non-stressed *Moraxella* cell suspension was also removed before the stress challenges, serially diluted and plated on TSA. The same stress challenge procedures were repeated on the 1-, 2- and 3-day starvation cell cultures.

The stress tolerant responses of the starved and non-starved *Moraxella* cultures were examined. For the heat challenge, the cell samples were incubated at 40 °, 43.5 °, and 45 °C for 30 min. The cell densities of the starved and non-starved *Moraxella* under the stress conditions were determined by plate counting.

The starved and non-starved cells were suspended separately in sterile MSM supplemented with varying concentrations of NaCl for the osmotic challenge. The final NaCl concentrations were 0.15 M (physiological saline), 0.3 M, 0.45 M and 2.7 M. The stressed cells were removed for plate counting after 2 h incubation at 22 °C.

Oxidative stress was provided by incubating the cells in the presence of hydrogen peroxide. The final H_2O_2 concentrations in the starved and non-starved *Moraxella* cell suspensions were 300 µM, 400 µM and 500 µM. The stressed cells were removed for plate counting after 1 h incubation at 22 °C.

2.2.6. Monitoring stress tolerance of the *Moraxella* strain in buffer

To monitor the heat stress-response over an extended period, the starved and non-starved cell suspensions were incubated separately at 43.5 °C. Viability of the non-starved *Moraxella* cell suspension (about 1×10^8 CFU) was monitored at 20- then 40-min intervals for 4 h. The stress-challenged cell suspension was vortexed, serially diluted and plated on TSA as described earlier. The same procedures were repeated for 1- and 2-day starvation cell cultures. The same protocol was used to monitor the effect of carbon starvation on the stress resistance of *Moraxella* to 2.7 M NaCl and 500 µM H_2O_2 at 22 °C.

2.2.7. Monitoring stress tolerance of the *gfp*-labelled *Moraxella* strain in river water and buffer.

The river water samples were obtained from the Kaministiqua River in Thunder Bay, ON, Canada. This particular site was chosen because it is downstream from a pulp and paper mill and from agricultural sources. The physicochemical properties of the water sample were analysed by the Lakehead University Centre for Analytical Services. Dissolved organic carbon and various cation and anion concentrations were determined by a Skalar autoanalyser (Skalar, The Netherlands), an inductively coupled argon plasma spectrometer (Varion Inc., Mississauga, Ontario, Canada) and a Dionex ion chromatograph (Dionex Corporation, Oakville, Ontario, Canada), respectively, using standard methods (American Public Health Association, 1998). The data, averages of four separate samples, were as follows in mg/L: dissolved organic carbon, 18.4; NO_3,

0.14; PO_4, <0.001; SO_4, 13.4; Cl, 18.6; Al, 0.09; Ca, 11.6; Fe, 0.25; K, 1.0; Mg, 3.5; Na, 7.0; Cr, Cu and Ni <0.002; Cd and Zn <0.001; Co <0.01; pH 6.8. The water sample was collected in a sterile vessel. In triplicate, 1 mL of the river water was removed and plated pair-wise on TSA plates and TSA plates supplemented with 50 µg/mL kanamycin to estimate the number of background aerobic heterotrophic bacteria in the river water which ranged from 10^2 to 10^5 CFU/mL.

The stress-tolerance procedures were repeated on the 1-day starved and non-starved *gfp*-labelled *Moraxella* strain M6 in river water and sterile MSM buffer. The purpose of this experiment was to examine the stress tolerance behaviour of the carbon-starved *Moraxella* in a non-sterile river water system. TSA supplemented with 50 µg/mL kanamycin was used to determine the culturable cell density of the M6 strain. Colonies were counted under a UV transilluminator which also served to confirm the identity of the M6 cells by their green fluorescence. One modification to the stress-response procedures was to use 750 µM H_2O_2 in place of 500 µM H_2O_2. This change was necessary since M6 demonstrated a higher tolerance to H_2O_2 than the wild-type *Moraxella* in the river water (data not shown).

2.2.8. Long-term survival of starved and non-starved M6 in river water.

To prepare the river water microcosms, 7 mL of log-phase *Moraxella* M6 suspension (about 1.4×10^9 CFU) was pelleted by centrifugation (3000 x g, 10 min, 20 °C), washed twice with sterile ddH_2O and diluted into sterile 250 mL screw-cap bottles containing 70 mL of either non-sterile or autoclaved river water samples to an inoculum density of about 2×10^7 CFU/mL. Parallel sets of microcosms were set-up at both 10 ° and 22 °C to examine the effect of temperature on the survival of M6 in the river water samples. Samples were removed for plate counting at 0, 1, 2, 3, 7 and 14 days of

incubation. Viability of the M6 strain was estimated by plating the samples on TSA supplemented with 50 μg/mL kanamycin and counting them on a UV transilluminator as previously described. All treatments were carried out in triplicate. The same procedures were repeated for 1-day starved M6 cultures.

2.2.9. Statistics

All experiments in this study were performed in triplicates unless stated otherwise. Data were presented by using mean value of measurements and standard deviations were calculated by a SigmaPlot/SigmaStat software program (SPSS Inc., Chicago, Illinois, USA).

2.3. Results

2.3.1. Stress responses of *Moraxella* with various starvation periods

The ability of the wild-type *Moraxella* strain to survive specific physicochemical stresses following various durations of starvation was examined. For each of the three stresses examined (i.e., heat, osmotic, and oxidative), it was observed that carbon-starvation for 1 or 2 days increased the ability of the *Moraxella* strain to tolerate the damaging stress effects (Figure 2.1). In some cases, such as heat stress at 43.5°C and 45°C, the 3-day starvation cell cultures survived at similar and lower levels compared to the non-starved *Moraxella* cell cultures, respectively (Figure 2.1 A).

During an 0.5 h heat challenge, it was found that the 43.5°C stress demonstrated the best protection to the bacteria with about 2- and 3.5-fold increase in survival capability following 1 and 2 days of starvation, respectively (Figure 2.1 A). At 40°C, the heat was not sufficiently high to affect bacterial survival in 30 min. At 45°C, only 1-day and 2-day carbon starvation provided slight protection to the bacteria against the heat stress. For the osmotic stress at 2.7 M NaCl for 2 h, the 2-day carbon-starved cells survived about

100-fold better than the non-starved *Moraxella* cells (Figure 2.1 B). Other levels of osmotic stress did not demonstrate cross-protection by starvation as noticeably as 2.7 M NaCl. When exposed 500μM H_2O_2 for 1 h, the 2-day starved bacteria displayed a 9-fold increase in survival compared to the non-starved bacteria (Figure 2.1 C).

2.3.2. Stress tolerance of *Moraxella* in buffer

The stress tolerance of the wildtype *Moraxella* strain was monitored over a 4-h period with specific stress intensities. The carbon-starved cultures survived the stress challenges significantly better than non-starved cultures (Figure 2.2). During heat stress at 43.5 °C, the 1- and 2-day starvation cell cultures were significantly more tolerant than the non-starved *Moraxella* cells (Figure 2.2 A). The cell densities of the 1- and 2-day starved cultures were about 150-fold higher than the non-starved cultures at the end of the 4-h experiment.

When exposed to 2.7 M NaCl, an immediate difference was observed between the 1- and 2-day starvation cultures and the non-starved *Moraxella* cultures (Figure 2.2 B). An over 60-fold decrease in cell density was observed in the osmotic stressed non-starved cultures in the first 20 min, whereas the 1- and 2-day starvation cultures did not experience any significant drop during the same time period. As with the heat stress challenges, the 1- and 2-day starvation cultures survived about 200-fold better than the non-starved *Moraxella* cultures after a 4 h osmotic challenge.

In the oxidative stress challenge with 500 μM H_2O_2, the difference in stress tolerance between 1- and 2-day starvation cultures and non-starved *Moraxella* cultures was readily noticeable (Figure 2.2 C). The non-starved cell cultures experienced a 10-fold drop in cell density by about 140 min. However, the cell densities of the 1- and 2-day starvation cell cultures decreased by only about 0.3 log units by the end of the 4-h stress

challenge. At the end of the experiment, the cell densities of the 1- and 2-day starvation cell cultures were about 100-fold higher than the non-starved *Moraxella* cultures.

2.3.3. Effect of starvation on cellular ATP levels

To examine the energy level of the *Moraxella* cells during starvation, experiments were conducted to determine the amount of cellular ATP present. Following 1 day of starvation, there was a significant decrease in the total amount of cellular ATP from 8.5 to 4.7 fmoles/cell ($LSD_{\alpha=0.05}$ = 1.2 fmoles/cell). However, the ATP levels in the 1-, 2- and 3-day starvation cultures were maintained between 3.5 to 4.7 fmoles/cell and were not significantly different from each other.

2.3.4. Selection of a *gfp*-labelled *Moraxella* strain used for river water study.

Fourteen *gfp*–labelled *Moraxella* isolates were screened with the aim of identifying an eligible strain for use in a survival study in non-sterile river water (Table 2.1). All 14 selected strains exhibited bright green fluorescence when examined on a UV transilluminator (UVP, Inc., San Gabriel, CA, USA). Since all of the *gfp*-labelled strains were producing GFP, it was necessary to test the stability of the gene insertion. All 14 strains produced GFP and were resistant to rifampicin and kanamycin after 4 separate transfers on TSA plates. To confirm the identity of the labelled isolates, an oxidase test was chosen to screen the *gfp*-labelled isolates. The plasmid donor, *E. coli* S17-1λ (pJBA29), was oxidase negative and the Rif^r *Moraxella* recipient was oxidase positive. Seven of the oxidase positive *gfp*-labelled *Moraxella* isolates were then tested for their ability to degrade PNP. All seven *gfp*-labelled isolates displayed PNP degradation abilities similar to the parent *Moraxella* strain and three were subsequently tested for growth rates. Two *gfp*-labelled *Moraxella* isolates, M1 and M6, had degradation and growth rates similar to the wild-type *Moraxella* strains (Table 2.1). They were also starved in MSM buffer to ensure that their ability to survive under carbon-starvation was similar to

that of the parent *Moraxella*. Both of the labelled strains exhibited similar starvation-survival characteristics compared to the wild-type strain (data not shown). The *gfp*-labelled *Moraxella* strain M6 was selected for the stress-survival study in the non-sterile river water.

2.3.5. Stress tolerance of carbon-starved M6 in river water and buffer

Stress tolerance responses of the *gfp*-labelled *Moraxella* strain M6 were examined in both non-sterile river water and sterile MSM (Figure 2.3). When M6 was exposed to 43.5 °C in river water for 2 h, there was a 10-fold higher cell density for the 1-day starved M6 cultures over the non-starved cultures. In a comparable experiment carried out in MSM, a similar stress-tolerance response was observed (Figure 2.3 A).

When exposed to the 2.7M NaCl in river water, there was a difference of stress-tolerance abilities for the starved and non-starved M6 cultures in the first 40 min of exposure (Figure 2.3 B). After 4 hours, the density of the 1-day starved culture was 7-fold higher than the non-starved culture. In the MSM buffer, the C-starved M6 had a 32-fold higher cell density than the non-starved M6 cultures after 4 h exposure to 2.7 M NaCl.

When exposed to 750 μM H_2O_2 for 4 h in river water, the density of the 1-day starved M6 culture was 65-fold higher than the non-starved cultures (Figure 2.3 C). However, the 1-day starved M6 was only 18-fold higher than the non-starved culture when stressed in MSM for 4 h.

2.3.6. Long-term survival of carbon-starved M6 in river water

The level of physicochemical stresses used in this study resemble acute changes in the environment. To investigate the survival of the carbon-starved *Moraxella* strain in a natural river water sample, the persistence of starved and non-starved M6 was monitored in non-sterile river water incubated at 10 ° and 22 °C (Figure 2.4). At 10 °C the persistence of the starved M6 cultures was similar to the non-starved cells, and both

showed a significant decrease in cell density over 14 days (Figure 2.4 A). In contrast, the starved and non-starved M6 cultures in the 10 °C sterile river water microcosms showed similar cell densities and remained high for the whole incubation period. At 22 °C, the non-starved cell cultures survived better in natural river water than the 1-day starvation cultures, with about 8-fold higher cell density after 14-d incubation (Figure 2.4 B). In the sterile river water microcosms, the cell densities for both the starved and non-starved M6 cultures were similar and remained high for the duration of the incubation period.

Table 2.1. Biochemical screening tests of selected *gfp*-labelled *Moraxella* strains.

Moraxella Strain	Oxidase Functional	PNP Degradation[2] (μM/h)	Generation Time[4] (h)
w.t.[1]	+	45	2.22±0.3
M1	+	46	2.01±0.4
M3	+	54	1.83±0.3
M6	+	42	2.32±0.2
M7	+	35	n/t
M9	+	29	n/t
M10	+	29	n/t
M12	+	31	n/t
M13	+	n/t[3]	n/t
M14	+	n/t	n/t
M15	+	n/t	n/t
M16	+	n/t	n/t
M8	-	n/t	n/t
M2	-	n/t	n/t
M11	-	n/t	n/t

[1] w.t. – wild-type *Moraxella* strain.
[2] PNP degradation rate is expressed in μM per hour per initial cell density of OD_{600} of 0.5 and is an average of duplicate samples.
[3] n/t – not tested.
[4] Generation time is an average of triplicate determinations ± standard deviation

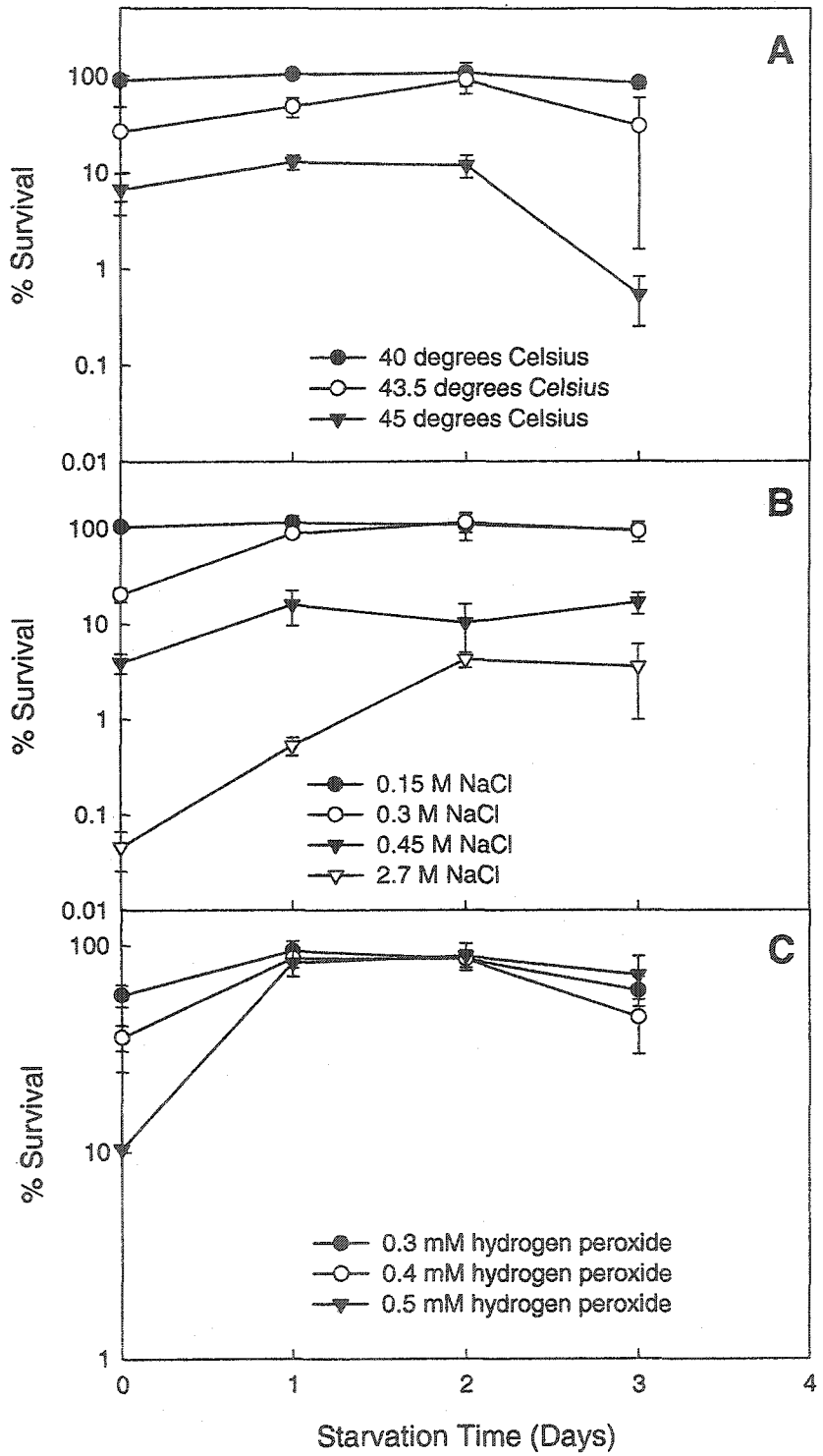

Figure 2.1. Percentage of survival of the starved and non-starved wildtype *Moraxella* strain when exposed to: (A) 0.5-h heat challenges, (B) 2-h osmotic challenges, and (C) 1-h oxidative challenges in MSM buffer. Values are the means of triplicate determinations ± standard deviation.

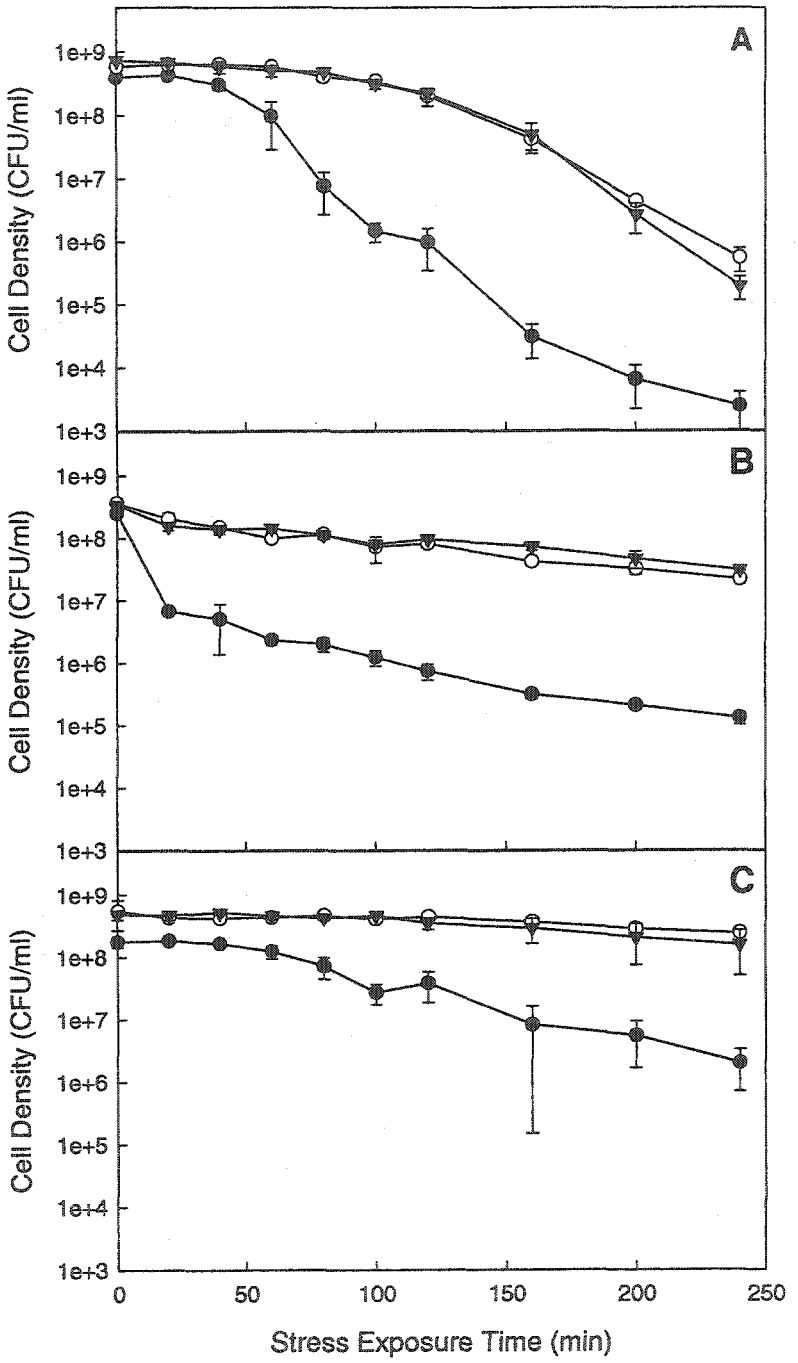

Figure 2.2. Survival of the starved and non-starved wildtype *Moraxella* strain in sterile MSM buffer at (A) 43.5°C, (B) 2.7 M NaCl, and (C) 500 μM H_2O_2. Symbols: •, non-starved *Moraxella*; o, 1-day starved *Moraxella*; ▼, 2-day starved *Moraxella*. Values are the means of triplicate determinations ± standard deviation.

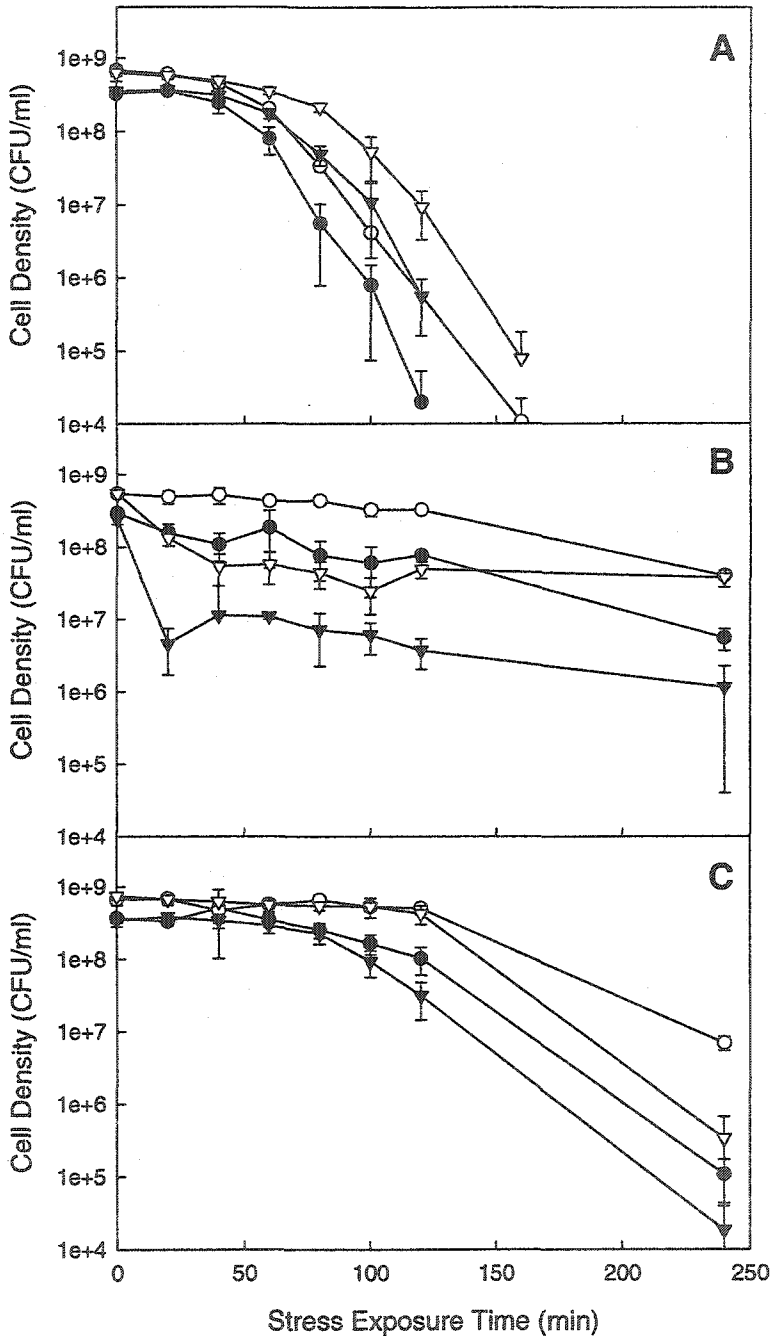

Figure 2.3. Survival of 1-day starved and non-starved *gfp*-labelled *Moraxella* M6 in MSM buffer and river water when exposed to (A) 43.5°C, (B) 2.7 M NaCl, and (C) 750 μM H_2O_2. Symbols: ∇, starved M6 in MSM; ▼, non-starved M6 in MSM; o, starved M6 in river water; •, non-starved M6 in river water. Values are the means of triplicate determinations ± standard deviation.

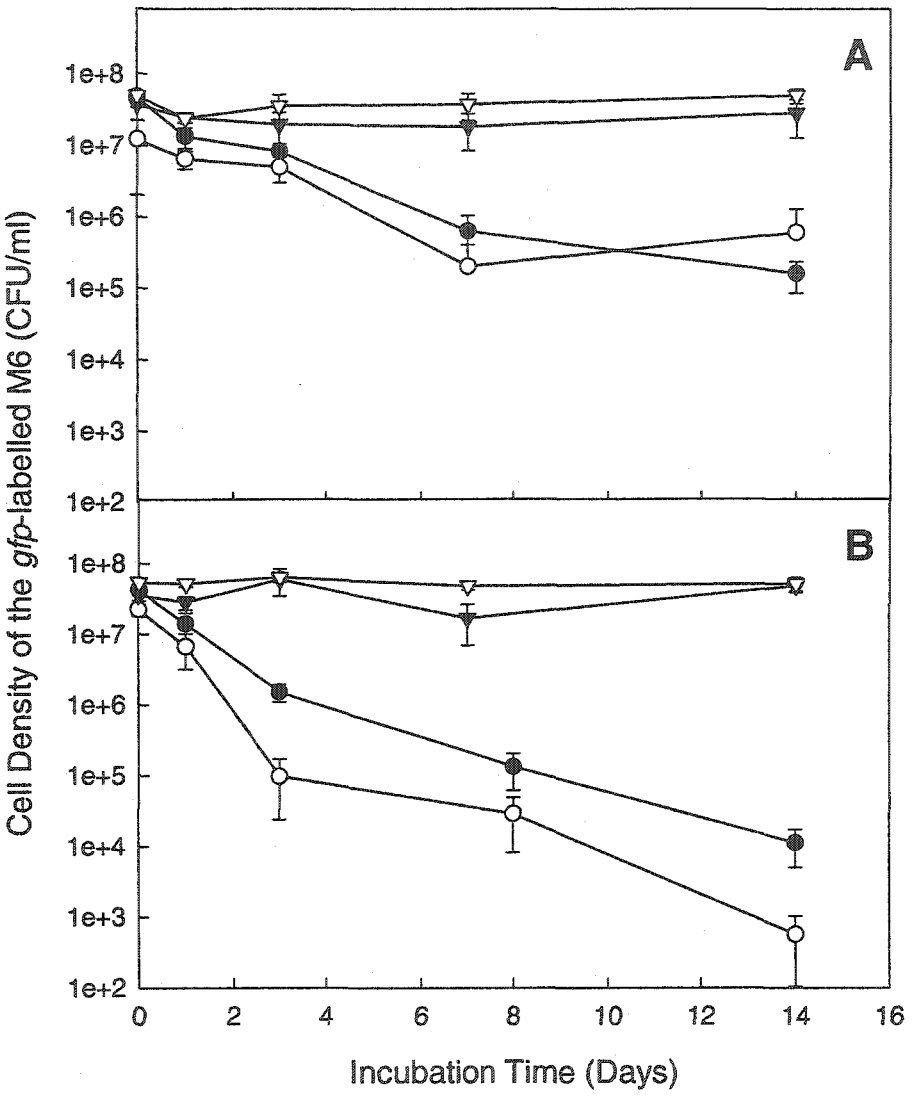

Figure 2.4. Survival of 1-day starved and non-starved *gfp*-labelled *Moraxella* M6 in river water microcosms incubated at (A) 10°C and (B) 22°C. Symbols: o, starved M6 in non-sterile river water; •, non-starved M6 in non-sterile river water; ∇, starved M6 in autoclaved river water; ▼, non-starved M6 in autoclaved river water. Values are the means of triplicate determinations ± standard deviation.

2.4. Discussion

Although members of the *Moraxella* genus are important environmental bacteria, the stress response of this species under C-starvation conditions has not been investigated. Based on the decrease in cellular ATP concentration in the *Moraxella* culture during C-starvation, the cells reached a starvation state after 1-day. This agrees with the findings that the stress tolerance responses of the bacteria increased following 1-day of C-starvation. Stress tolerance responses have also been observed in some other bacterial species (Jenkins *et al.* 1988, 1990; Jouper-Jaan *et al.* 1992; Nyström *et al.* 1992; van Overbeek *et al.* 1995; Watson *et al.* 1998).

When the stress-survival capabilities of the *Moraxella* strain were monitored over a 4-h stress exposure, the 1- and 2-day C-starved *Moraxella* cultures displayed superior tolerance to heat, osmotic and oxidative stresses over the non-starved cultures. Jouper-Jaan *et al.* (1992) had earlier shown that carbon-starvation for 22 and 48 h increased the stress tolerance of two *E. coli* strains (K165 and Sc122) and *Vibrio* sp. DW1. They also found that carbon-starvation for 9 days further enhanced the stress tolerance for the two *E. coli* strains against heat. A study by Nyström *et al.* (1992) showed that only 10 h of carbon-starvation was needed to provide maximum stress-tolerance for heat in *Vibrio* sp. S14. A study of *Pseudomonas fluorescens* (van Overbeek *et al.* 1995) showed that a starvation period of 24 h was optimal for providing heat and oxidative stress tolerance but 5 days of starvation was needed to provide optimal osmotic stress-tolerance. However, Jenkins *et al.* (1988, 1990) reported that only 4 h of carbon-starvation was needed to provide optimal protection against heat, osmotic and oxidative stress in *E. coli* K12. As an exception to the results found in our study and those previously described for other bacteria, a study by Watson *et al.* (1998) did not detect any increase in heat stress tolerance for *Staphylococcus aureus* after 7 days of glucose starvation, although they did

observe a 100-fold increase in tolerance for oxidative stress. It seems that the stress resistance responses of bacteria to carbon-starvation vary, depending on the type of environmental stress and the bacterial species involved.

A *gfp*-labelled *Moraxella* strain (M6) was engineered to perform the survival experiments in river water. Despite the fact that no significant difference was observed between the M6 strain and the wildtype *Moraexlla* strain in the screening tests, M6 seems to be more susceptible to the heat stress and more tolerant to H_2O_2 oxidation. It is not clear whether these minor differences are caused by energy burden imposed by the *gfp* marker or insertion mutation caused by the Tn5 transposition. However, when C-starved and non-starved M6 cells were compared for their tolerance to heat, osmotic and oxidative stresses in MSM, similar stress resistance responses were observed on the C-starved M6 as with the wildtype *Moraxella* strain.

In river water samples, *Moraxella* M6 showed superior stress tolerance to all three stresses tested (heat, osmotic and oxidative) following 1 day carbon-starvation. However, the river water seemed to have some impact on the tolerance of the bacteria to the heat, osmotic and oxidative stresses. The river water seemed to magnify the damaging effect of heat on the M6 strain. On the other hand, the adverse effect of osmotic and oxidative stresses on M6 was reduced in the river water samples in comparison to the MSM buffer. The presence of chelating and reducing substances in river water may decrease the effective concentration of NaCl and H_2O_2, respectively (Buffle, 1988).

When inoculated into river water samples at either 10 ° or 22 °C, the starved and non-starved M6 cultures exhibited similar survival patterns during 14 days of incubation. It seems that the stress-protection acquired through carbon-starvation did not give an advantage to the bacteria to survive better in the non-sterile river water microcosms. Our findings are similar to those of van Overbeek *et al.* (1995) that carbon-starvation did not

enhance the survival of *P. fluorescens* when introduced into two types of soil. A study on a phenol-degrading *Ralstonia eutropha* strain describes an increase in survival of a 2-day C-starved cell culture in a phenol-contaminated activated sludge compared to a non-starved cell culture (Watanabe *et al.* 2000). However, 7-day starvation cultures showed no survival advantage compared to non-starved cultures. The authors suggested that the 7-day starved *R. eutropha* culture had lost its phenol-oxidizing ability and therefore its competitive edge. Our study is different from the *R. eutropha* study in that no PNP was added to the river water microcosms. This may explain the discrepancy between the findings of Watanabe *et al.* (2000) and our study.

Our study showed that carbon-starvation increased the stress tolerance of the *Moraxella* strain in the stress challenges, but it did not improve the survival of the bacteria in river water microcosms. A possible explanation is that stresses encountered by bacteria in river water are more complex than the simple and singular stressors that were applied during the stress challenges. The bacteria introduced in the river water samples would have to overcome competition, changes in water chemical composition, and a difference in nutrient availability concurrently (Pritchard 1992). These combined stresses could overwhelm the bacterium's ability to survive in the microcosms. Although C-starvation did not benefit survival of the *Moraxella* strain in the river water microcosms, it may play an indirect but critical role on the survival of the bacteria in natural water systems. It has been shown in *Vibrio* and other bacteria that adhesion of the bacteria to substratum surfaces increases under C-starved conditions and hence facilitates biofilm formation (Costerton, 2000; Kjelleberg *et al.*, 1987). Therefore, C-starved bacteria may have a significant survival advantage in the environment through the formation of microbial biofilms.

3. Effect of Carbon Starvation on *p*-Nitrophenol Degradation by a *Moraxella* Strain in Buffer and River Water

3.1. Introduction

Carbon starvation is one of the most common stresses that bacteria encounter in the environment. Groat *et al.* (1986) showed that more than 50 starvation proteins were induced in *E. coli* under carbon starvation. Based on our current understanding of starvation stress responses of *E. coli* and a few bacterial models, the majority of these proteins are related to increase in stress resistance, nutrient scavenging or survival of bacteria in the environment (Matin *et al.* 1999). It is well documented that C-starved bacteria are more resistant to heat, osmotic, oxidative, pH and other environmental stresses (Hengge-Aronis 2000, Kolter *et al.* 1993, Matin 1996, Nyström *et al.* 1992, Roszak and Colwell 1987, Van Veen *et al.* 1997), but their ability to survive in environmental samples is less clear (Watanabe *et al.* 2000, Van Overbeek *et al.* 1995, Moore *et al.* 2004).

In general, the initial response of bacteria to carbon starvation is to up-regulate scavenging systems of potential nutrients. For instance, high-affinity mannitol, glucose and glutamate transport systems are expressed when certain marine bacteria are subjected to carbon starvation (Kjelleberg *et al.* 1987). Furthermore, other catabolic enzymes, such as hexokinase, lactate dehydrogenase, and many catabolic enzymes of the central catabolic pathways of bacteria, have also been shown to increase significantly under starvation conditions (Matin 1996). Despite the potential of exploiting the scavenging capability of C-starved bacteria for bioremediation, little is known about degradation of toxic pollutants by pre-starved microbial inocula (Watanabe *et al.* 2000, Herman and Costerton 1993).

p-Nitrophenol (PNP) is released in agricultural soil as a hydrolytic product of methyl parathion or parathion, which are commonly used as pesticides in agriculture (Munnecke and Hsieh 1976) PNP is also found in industrial wastes because it is a

precursor of pharmaceuticals like acetaminophen and 4-aminosalicylic acid and pesticides such as carbofuran, nitrofen, methyl parathion and parathion (Boehncke *et al.* 2000). Diesel fuel and gasoline exhaust also contain PNP which enters the environment through rain water (Boehncke *et al.* 2000, Tremp *et al.* 1993). Because of the wide-spread nature of PNP and other nitrophenols, they are considered as major pollutants by the USEPA (1976).

Several bacterial species have the ability to degrade PNP. These include *Nocardia* (Hanne *et al.* 1993), *Arthrobacter* (Jain *et al.* 1994), *Sphingomonas* (Leung *et al.* 1997a), *Flavobacterium* (Raymond and Alexander 1971), *Burkholderia* (Prakash *et al.* 1996), *Bacillus* (Kadiyala and Spain 1998), *Pseudomonas* (Munnecke and Hsieh 1976) and *Moraxella* (Spain and Gibson 1991). In general, aerobic degradation of PNP by Gram-negative bacteria is initiated by formation of p-benzoquinone, while the first intermediate of PNP degradation by Gram-positive bacteria is 4-nitrocatechol (Spain 1995).

The degradation pathway of PNP in a *Moraxella* strain, isolated from activated sludge, has been well studied. The initial reaction involves removal of the nitro group of PNP by a monooxygenase and the resulting *p*-benzoquinone is converted to hydroquinone before ring cleavage and complete degradation of the compound (Spain and Gibson 1991). Several studies have been conducted to explore the potential of the *Moraxella* strain as a bioremediation microorganism. Tresse *et al.* (1998) demonstrated that the *Moraxella* strain mineralized PNP efficiently at concentrations as high as 1440 μM. Others have shown that the bacteria survive in a PNP-spiked soil for more than 4 weeks (Errampalli *et al.* 1999b) and encapsulation in alginate further protects the bacteria from predation by protozoa (Leung *et al.* 2000). Shimazu *et al.* (2001) also genetically modified the *Moraxella* strain to break down some organophosphorus pesticides and PNP simultaneously.

Despite a general understanding of carbon starvation on nutrient scavenging of a few model bacterial strains, only a limited amount of information is available on the scavenging of pollutants by pollutant-degrading bacteria in the environment (Watanabe *et al.* 2000, Herman and Costerton 1993) and nothing is known about the effectiveness of PNP degradation by the *Moraxella* strain under C-starved conditions. The objectives of this study are to determine: (1) the effect of carbon starvation on PNP degradation by the *Moraxella* strain in a buffer system and (2) whether a C-starved *Moraxella* inoculum will improve the effectiveness of PNP degradation in non-sterile river water microcosms.

3.2. Materials and Methods

3.2.1. Bacterial strains

The PNP-mineralizing *Moraxella* strain used in this study is a Gram-negative rod-shaped bacterium isolated from an activated sludge (Spain and Gibson 1991). It was cultured in Tryptic Soy Broth (TSB; Becton Dickinson and Company, Sparks, MD, USA) and stored on Tryptic Soy Agar (TSA; Difco, Detroit, MI, USA) plates at 4°C.

A green fluorescent protein gene (*gfp*)-labelled *Moraxella* strain (M6 strain) was constructed to examine the survival and PNP degradation of the bacteria in non-sterile river water samples. The wildtype *Moraxella* strain was labelled by a Tn5-*gfp* suicide plasmid and the M6 strain was selected based on the stability of the gene insertion and *gfp* expression. The growth rate and PNP degradation of the M6 strain were found to be identical to the wildtype *Moraxella* strain (Moore *et al.* 2004). The labelled *Moraxella* produced GFP that could be visualized with a transilluminator and the inoculum could easily be distinguished from the indigenous bacterial populations. The labelled bacterium was cultured in TSB supplemented with 50 μg/mL of kanamycin and rifampicin and stored

at 4°C on the same antibiotic agar medium. All bacterial cultures were stored in 25% glycerol at – 80°C until needed.

3.2.2. Starvation of bacterial cells

The *Moraxella* cells were cultured in TSB at 30 °C (150 rpm) to mid-log phase (OD_{600nm} 0.5-0.7) prior to starvation. The cells (2 x 45 mL) were harvested by centrifugation (3000 x g, 10 min, 20 °C), washed twice with an equal volume of sterile minimal salts solution (MSM, 1.249 mM KH_2PO_4, 3.73 mM K_2HPO_4, 0.4 mM $MgSO_4.7H_2O$, 0.02 mM $FeSO_4.7H_20$, 1.4 mM NH_4Cl, pH 7) and suspended in MSM to an optical density (OD_{600nm}) of 0.2 (approximately 3×10^8 CFU/mL). The cell suspension was used immediately as the non-starved experimental controls to examine PNP degradation by the bacteria. A subsample of the cell suspension was C-starved in MSM at 30 °C with shaking (150 rpm). The C-starved cells were harvested after 1, 2 and 3 days of starvation and their PNP-degrading ability was determined.

3.2.3. Effect of carbon starvation on PNP degradation

For the PNP degradation experiments, *Moraxella* cells were suspended in sterile MSM supplemented with 150 µM *p*-nitrophenol (PNP; Sigma, St.Louis, MO, USA) to an OD_{600nm} of 0.2 and samples were incubated at 30 °C (150 rpm). The concentrations of PNP in the cell suspensions were determined at 30 minute intervals as described previously (Leung *et al.* 1997a). One-mL samples were removed and centrifuged (12,000 x g, 10 min). One-half mL of the supernatant was mixed with 0.5 mL of 0.5 M NaOH and the concentration of PNP was measured spectrophotometrically at 420nm. The blank consisted of 0.5 mL of 0.5 M NaOH and 0.5 mL MSM.

3.2.4. PNP degradation by PNP-induced *Moraxella* cells

To prepare the PNP-induced cells, *Moraxella* sp. was cultured in TSB at 30 °C (150 rpm) to mid-log phase, washed twice with MSM and suspended in MSM to an OD_{600nm} of 0.2. The cell suspension was supplemented with PNP to a final concentration of 150 μM. The PNP-amended cell suspension was incubated at 30 °C with shaking (150 rpm). One hour after the disappearance of PNP, cells were harvested, washed twice with sterile MSM and suspended in MSM supplemented with 150 μM PNP, to OD_{600nm} of 0.2. PNP degradation was monitored at 15-minute intervals as previously described.

3.2.5. Bacterial growth in carbon-rich and -depleted media

Moraxella cells were grown in TSB at 30 °C with shaking (150 rpm), harvested at mid-log phase, washed twice with sterile MSM and suspended in MSM supplemented with 0, 0.1 or 1% yeast extract (Difco, Detroit, MI, USA). The samples were incubated at 30 °C with continuous shaking (150 rpm) and cell densities were determined by drop plating on TSA at 24-h intervals.

3.2.6. Effect of cell density on PNP degradation

Moraxella cell suspensions were prepared from a log-phase cell culture as described earlier. Their cell densities were adjusted to OD_{600nm} values of 0.2, 0.3 and 0.4 to determine the effect of cell density on PNP degradation. The *Moraxella* suspensions were amended with PNP to a final concentration of 150 μM and incubated at 30 °C and shaken at 150 rpm. PNP degradation was monitored hourly as previously described.

3.2.7. Effect of carbon starvation on cell size

Moraxella cultures were harvested, washed twice with sterile saline and diluted 10-fold with autoclaved double-distilled water. One-mL portions of the diluted cell suspensions were filtered onto sterile 0.22 μm pore size polycarbonate membrane filters

(Millipore, Billerica, MA, USA). The filters were air-dried in a biosafety hood for 2 h and the samples were coated with gold by an EffaCoater (Ernest F. Fullam Inc., Latham, NY, USA). The cells were examined under a JSM-5900LV scanning electron microscope at 11,000 x magnification (JEOL Inc., Peabody, MA, USA) and cell size was estimated using an ImagePro program (Media Cybernetics Inc., Silver Spring, MD, USA). Microscopic examinations of the *Moraxella* cells were performed at 0, 1 and 90 days of carbon starvation.

3.2.8. Effect of carbon starvation on PNP uptake

Moraxella cells were cultured in TSB at 30 °C (150 rpm) to mid-log phase (OD_{600nm} = 0.5), harvested, washed and suspended in sterile MSM to an OD_{600nm} of 1.0. A portion of the cell suspension was sampled for the PNP uptake assay. The rest of the sample was carbon-starved at 30°C and the PNP uptake of the starved *Moraxella* cells was determined after 24 h incubation. Heat-killed cell suspensions were used as negative experimental controls. [U-^{14}C]PNP was added to the starved and non-starved cell samples to achieve a final concentration and specific activity of 20 µM and 4.8 x 10^6 dpm/µmole PNP, respectively. At intervals between 30 to 60 s, PNP uptake was terminated by diluting a 200 µl sample into 2 ml of ice-cold 100 mM LiCl solution. The samples were immediately filtered through a 0.22 µm polycarbonate membrane filter. Each filter was washed once with 5 ml of ice-cold 100 mM LiCl solution and the radioactivity of the filter was determined by a liquid scintillation counter (Leung *et al.* 1997a).

Cellular protein concentration of the samples was determined by a standard Biorad protein assay (Biorad, Hercules, CA, USA). One-ml portion of the cell suspension was dispensed into a 2 mL screw-cap tube with 1 g of 100-212 µm silica beads (Sigma, St.

Louis, MO, USA). Cells were broken by a Bio101/Savant FastPrep FP120 Cell Disrupter set at 6 m/s beating for 45 s (Qbiogene Inc., Carlsbad, CA, USA). Complete cell disruption was confirmed by bright field microscopy. Cell debris was removed by centrifugation at 12,000x g for 5 min. Eight hundred µL of the lysate were mixed with 200 µL of the Biorad protein assay reagent and absorbance of the reaction product was measured spectrophotometrically at 595 nm. A protein calibration curve was constructed based on bovine serum albumin protein samples (Sigma Biochemicals, St. Louis, MO, USA) that ranged from 0 to 200 µg/L. All treatments were performed in triplicate.

3.2.9. PNP degradation by and survival of *gfp*-labelled *Moraxella* in river water

River water samples were obtained from the Kaministiqua River in Thunder Bay, ON, Canada. This particular site was chosen because it is downstream from a pulp and paper mill and from agricultural sources. The physicochemical properties of the water sample were analysed by the Lakehead University Centre for Analytical Services. Dissolved organic carbon and various cation and anion concentrations were determined by a Skalar autoanalyser (Skalar, The Netherlands), an inductively coupled argon plasma spectrometer (Varian Inc., Mississauga, Ontario, Canada) and a Dionex ion chromatograph (Dionex Corporation, Oakville, Ontario, Canada), respectively, using standard methods. The data, averages of four separate samples, are presented as follows in mg/L: dissolved organic carbon, 18.4; NO_3, 0.14; PO_4, <0.001; SO_4, 13.4; Cl, 18.6; Al, 0.09; Ca, 11.6; Fe, 0.25; K, 1.0; Mg, 3.5; Na, 7.0; Cr, Cu and Ni <0.002; Cd and Zn <0.001; Co <0.01; pH 6.8. The water sample was collected in a sterile vessel. In triplicate, 1 mL of the river water was removed and plated pair-wise on TSA plates and TSA plates supplemented with 50 µg/mL kanamycin to estimate the number of background aerobic heterotrophic bacteria in the river water, which ranged from 10^2 to 10^5 CFU/mL.

Sterile 250 mL polypropylene screw-cap centrifuge bottles were used to prepare the river water microcosms. Seven-mL portions of log-phase *Moraxella* M6 culture (OD_{600nm} 0.5) were pelleted by centrifugation (3000 x g, 10 min, 20 °C), washed twice with sterile double-distilled water and diluted into 63 mL of non-sterile river water samples to achieve an inoculum density of about 5×10^7 CFU/mL. The microcosms were spiked with PNP to final concentrations of 80, 200 and 360 µM. Uninoculated river water microcosms and non-spiked controls were also prepared. The microcosms were incubated at 22 °C with shaking (150 rpm). Degradation of PNP was monitored spectrophotometrically at 420 nm for 48 h as previously described. Microcosm samples were also removed for plate counting at 0, 1, 2, 3, and 7 days of incubation. Cell densities of the M6 strain in the samples were estimated by plating on TSA supplemented with 50 µg/mL kanamycin and counting on a UV transilluminator as previously described (Moore *et al.* 2004). All treatments were carried out in triplicate. The same procedures were repeated for 1-day starved M6 cultures.

3.2.10. Statistics

All experiments in this study were performed in triplicates unless stated otherwise. Data were presented by using mean value of measurements and standard deviations were calculated by a SigmaPlot/SigmaStat software program (SPSS Inc., Chicago, Illinois, USA).

3.3. Results

3.3.1. Effect of carbon starvation on PNP degradation

At 150 µM PNP, the non-starved *Moraxella* culture showed an induction time for PNP degradation of 6 h and degradation was completed by about 10 h (Figure 3.1). The 1-day starved *Moraxella* culture showed a faster onset of PNP degradation than the non-

starved culture. It had an induction time of 1 h and needed only 4 h to break down the PNP completely. However, induction time for PNP degradation increased as starvation time of the *Moraxella* cells extended beyond one day. The induction time for the 2-day C-starved cells was about 3 h and the culture required about 8 h for the total degradation of PNP. After three days of starvation, the PNP degradation pattern of the C-starved cells was almost the same as for the non-starved *Moraxella*. The degradation ability of *Moraxella* cells pre-exposed to PNP was examined and the induced *Moraxella* culture displayed no observable induction time prior to PNP degradation. In general, the PNP degradation rates of the induced and 1-day C-starved cultures were similar and they were faster than those of the non-starved, 2-day and 3-day C-starved cultures (Figure 3.1).

3.3.2. Survival of *Moraxella* strain in carbon-depleted and -rich media

Growth patterns of the *Moraxella* strain were examined in MSM supplemented with various amount of yeast extract for three days. The cell density of the cultures with 0.1 and 1 % yeast extract increased in the first 24 h from $3.8x10^8$ to $1.5x10^9$ and $3.8x10^9$ CFU/mL, respectively. However, their cell densities dropped to about $9x10^7$ CFU/mL after 3-days incubation. In the carbon-depleted medium, the density of the *Moraxella* cells increased from $3.8x10^8$ to $5.4x10^8$ CFU/mL within one day and continued to increase by 85% over 3 days (Figure 3.2). The *Moraxella* cells survived very well in the carbon-depleted medium, showing only a 15% decrease in cell density after 30-days incubation (data not shown).

3.3.3. Effect of carbon starvation on cell size

The effect of carbon starvation on the size of *Moraxella* cells was examined to determine if reductive division was responsible for the increase in cell density and hence improvement of PNP degradation in starved *Moraxella* cultures. No visible difference was observed between the non-starved and 1-day starved *Moraxella* cells (Figure 3.3).

Dimensions of both the non-starved and 1-day starved *Moraxella* cells were about 1 μm x 0.5 μm. Although not shown the 2-day and 3-day starved *Moraxella* cells were similar to that of the non-starved cells. After 90 days of C-starvation, the cells became coccoid in shape with a diameter of about 0.6 μm (Figure 3.3).

3.3.4. Effect of cell density on PNP degradation

The effect of inoculum density on the rate and onset of PNP degradation was examined to determine whether the increase in cell density observed during the initial starvation period was responsible for the decrease in induction time. Similar induction time was observed for the three densities of non-starved *Moraxella* cell samples (OD_{600nm} 0.2, 0.3, and 0.4). All three samples started degrading PNP after about 6 h of exposure and showed complete degradation of the pollutant by 9.5 h. However, at OD_{600nm} 0.3 and 0.4, the *Moraxella* samples showed a higher initial rate of PNP degradation than that of the OD_{600nm} 0.2 samples (Figure 3.4).

3.3.5. Effect of carbon starvation on PNP uptake

The 1-day starved *Moraxella* cells had a significantly higher rate of PNP uptake than non-starved *Moraxella* (Figure 3.5). Non-starved *Moraxella* culture showed a cellular concentration of 5 ηmol PNP/mg protein after 30 seconds of exposure to 20 μM [U-^{14}C] PNP. One-day starved *Moraxella* cells treated with the same concentration of [U-^{14}C] PNP exhibited a large initial uptake of 18 ηmol PNP/mg protein before dropping to 7 ηmol PNP /mg protein in 4 min. Interestingly, the heat-killed *Moraxella* cells showed a cellular concentration of 8 ηmol PNP/mg protein which was significantly higher than that of the living non-starved *Moraxella* cells (Figure 3.5).

3.3.6. Survival of starved and non-starved M6 in PNP-spiked river water.

Regardless of the concentration of PNP in the microcosms, the 1-day starved M6 cultures showed complete degradation of PNP significantly earlier than that of the non-

starved M6 cultures (Figure 3.6). At 80 μM PNP, PNP degradation started after 6 h for the C-starved *Moraxella* but after 9 h for the non-starved inoculum. The C-starved inoculum also completely degraded PNP 4 h faster than the non-starved inoculum. Similar trends of degradation were observed at 200 and 360 μM PNP. However, as the PNP concentration of the river water samples increased from 80 to 200 and 360 μM, the time for complete PNP degradation by the non-starved *Moraxella* cells increased from 19 h to 27 h and 33 h, respectively. It only took the C-starved inocula about 16 h to degrade the PNP completely regardless of PNP concentration in the water samples (Figure 3.6).

Survival patterns of the C-starved and non-starved *Moraxella* M6 in the river water samples were similar regardless of the PNP concentration (Figure 3.7). In the absence of PNP, cell densities of both starved and non-starved inocula decreased continuously from about $4x10^7$ to $1x10^5$ CFU/mL in 7 days. At 80 μM PNP, the decrease in inoculum densities was slower in the first two days of incubation than the latter part of incubation. As the PNP concentration of the water samples increased to 200 and 360 μM, the *Moraxella* cell densities doubled in the first two days of incubation before declining steadily in the microcosms (Figure 3.7).

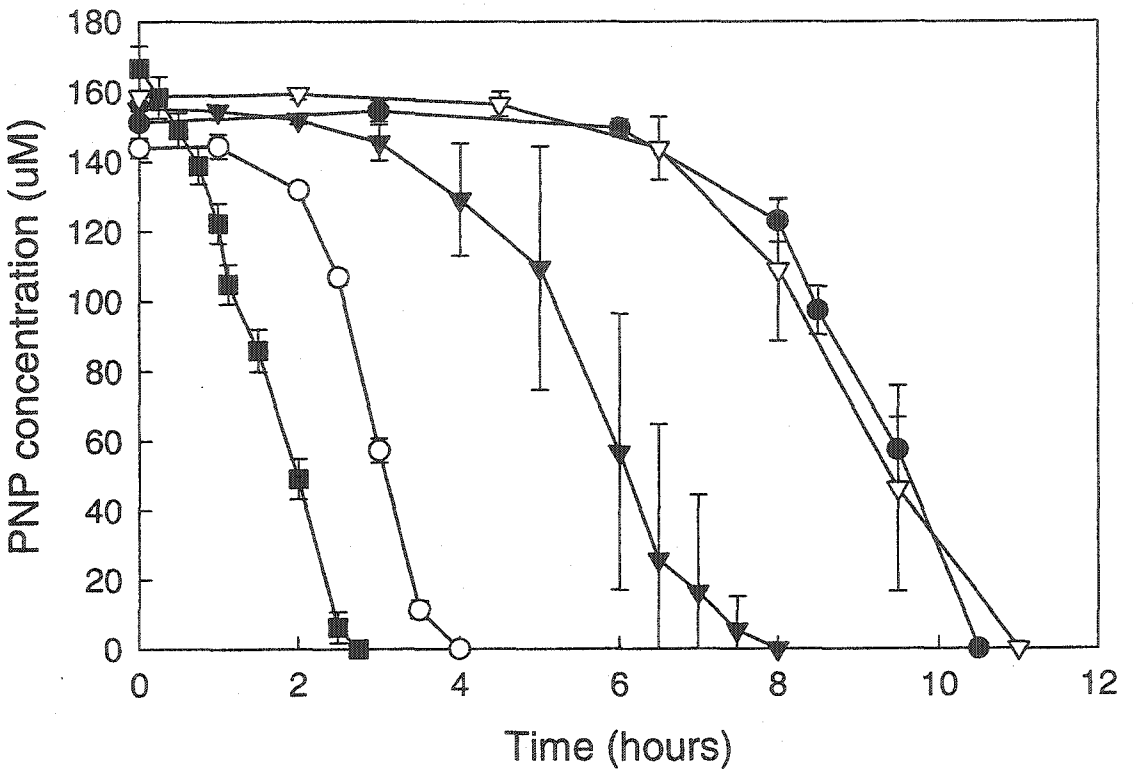

Figure 3.1. PNP degradation by the *Moraxella* strain in MSM. Symbols: •, non-starved cells; ○, 1-day starved cells; ▼, 2-day starved cells; ▽, 3-day starved cells; ■, PNP-induced cells. Values are the means of triplicate determinations ± standard deviation.

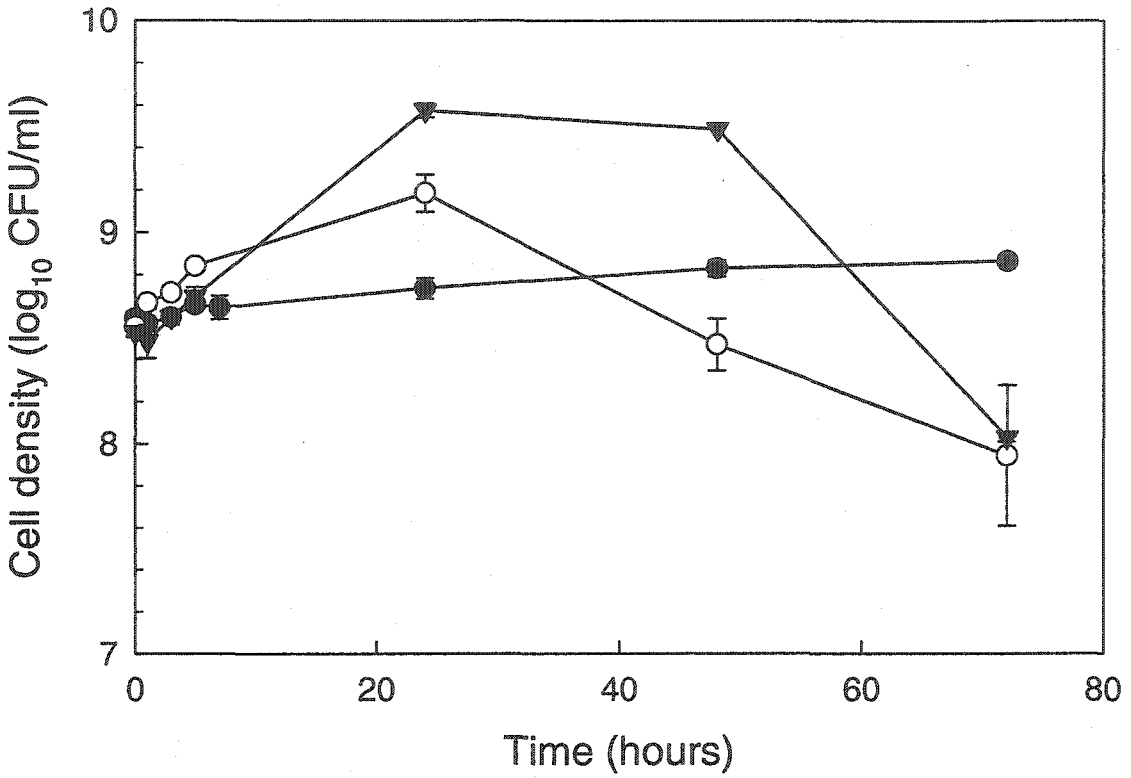

Figure 3.2. Growth dynamics of the *Moraxella* strain at various carbon nutrient levels. Symbols: •, carbon-depleted MSM; ○, MSM supplemented with 0.1 % yeast extract; ▼, MSM supplemented with 1 % yeast extract. Values are the means of triplicate determinations ± standard deviation.

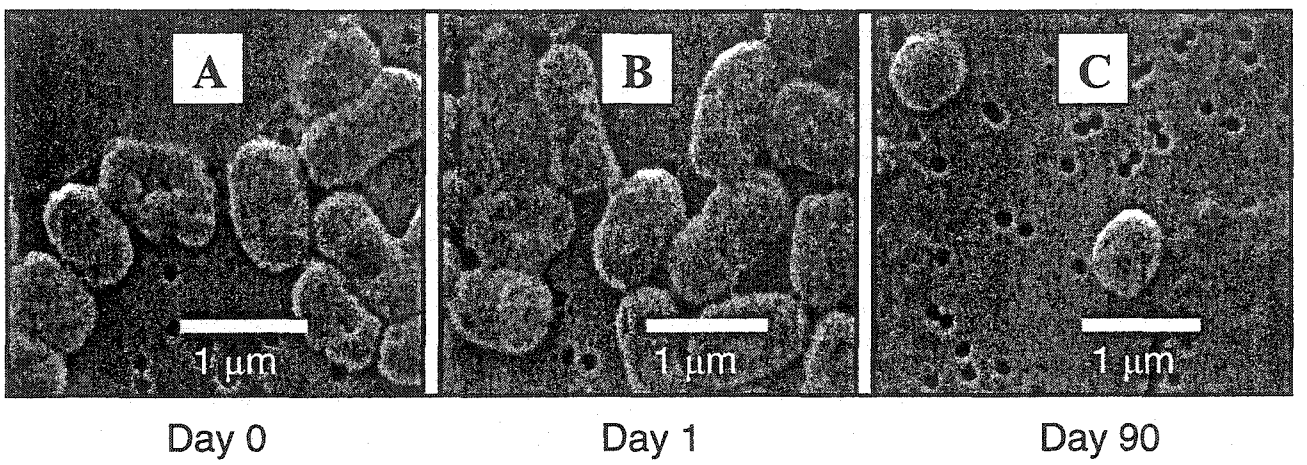

Figure 3.3. Scanning electron micrographs of the *Moraxella* cells. A, Non-starved cells; B, 1-day starved cells; C, 90-day starved cells.

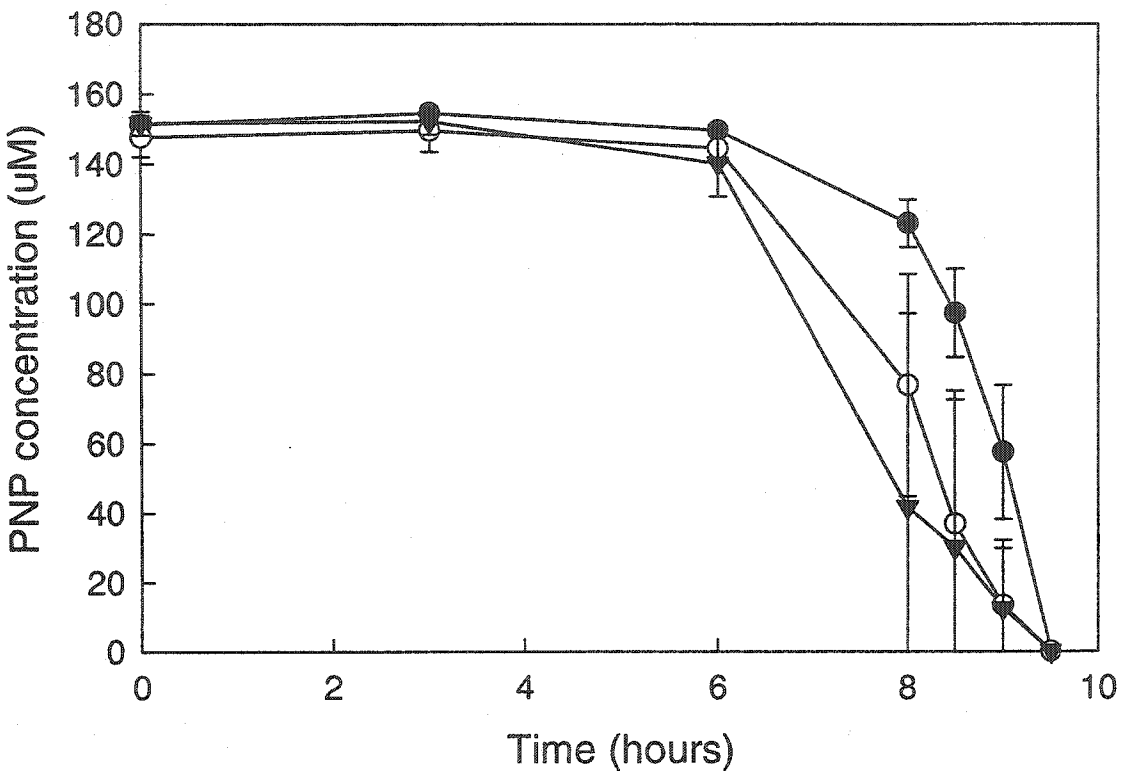

Figure 3.4. Effect of *Moraxella* cell density on PNP degradation. Symbols: ●, OD_{600nm} of 0.2; ○, OD_{600nm} of 0.3; ▼, OD_{600nm} of 0.4. Values are the means of triplicate determinations ± standard deviation.

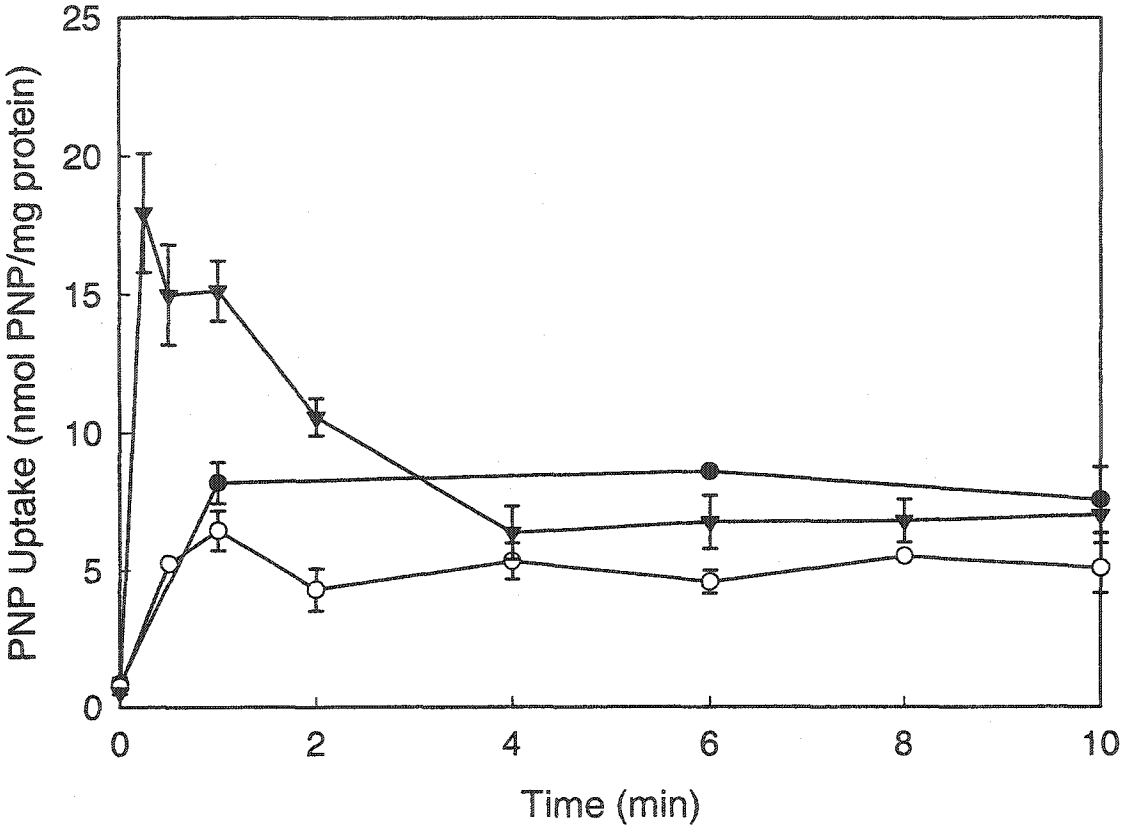

Figure 3.5. PNP uptake by C-starved and non-starved *Moraxella* cells. Symbols: ▼, 1-day starved cells; ○, non-starved cells; ●, heat-killed cells. Values are the means of triplicate determinations ± standard deviation.

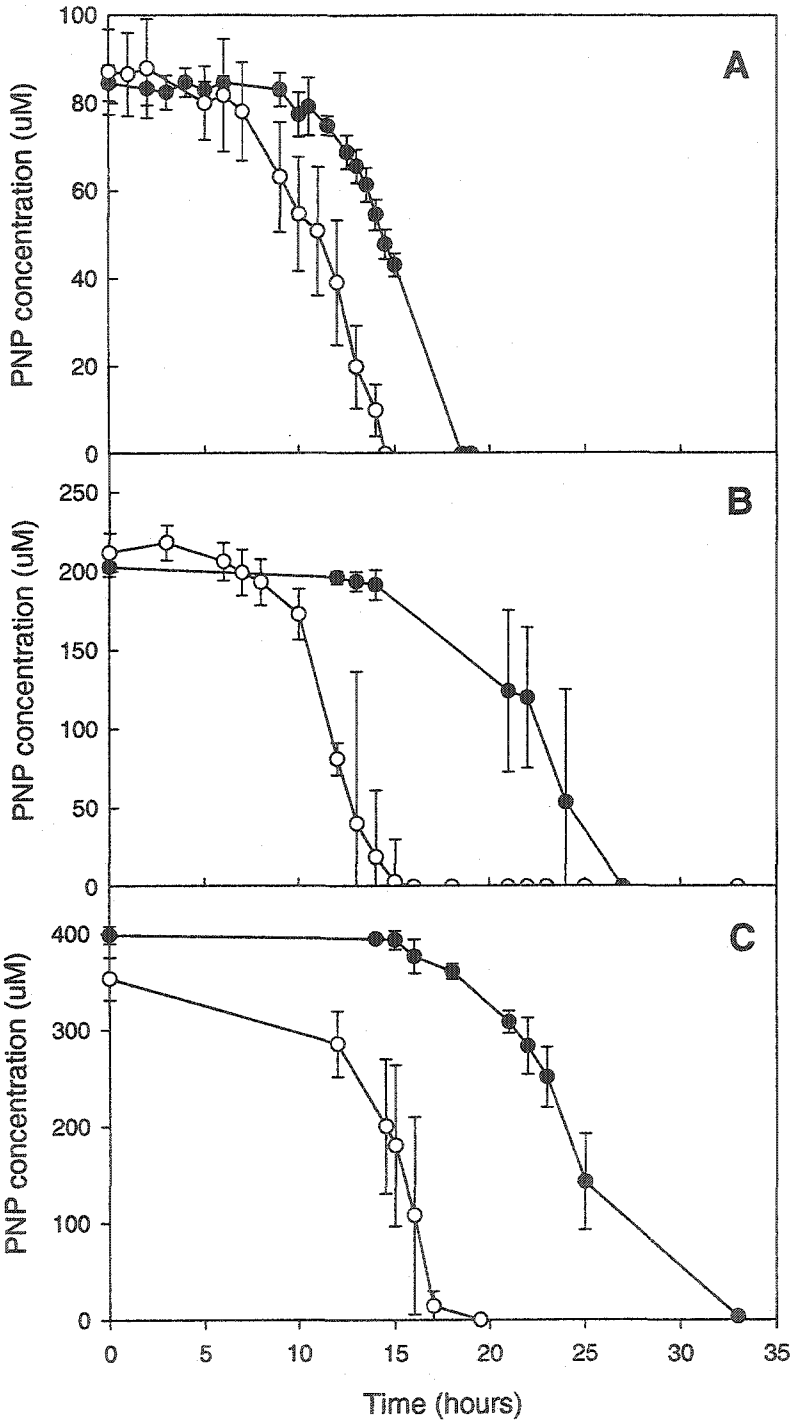

Figure 3.6. PNP degradation by 1-day C-starved (○) and non-starved (●) M6 *gfp*-labelled *Moraxella* cells in river water microcosms spiked with (A) 80 µM, (B) 200 µM, and (C) 360 µM PNP. Values are the means of triplicate determinations ± standard deviation.

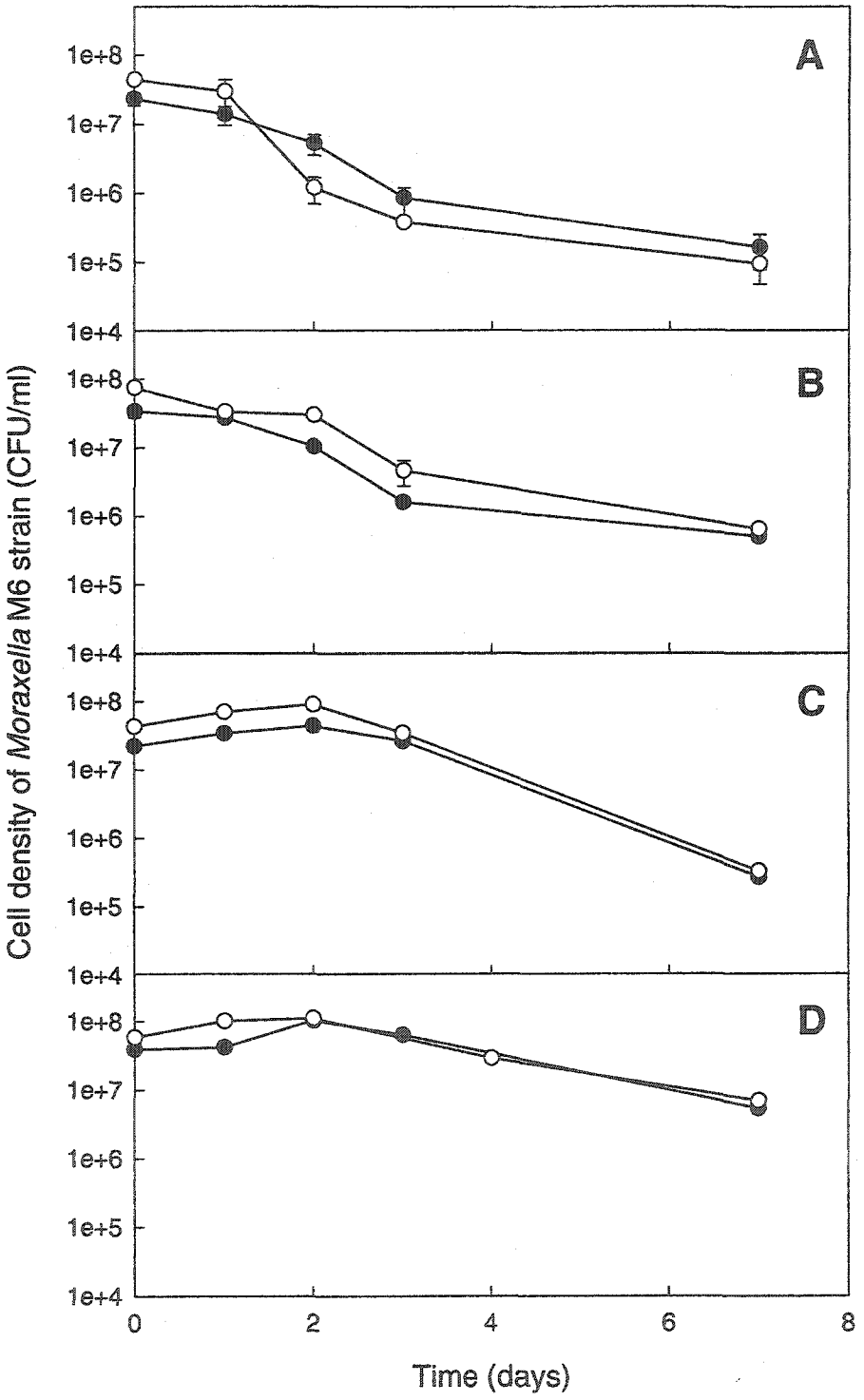

Figure 3.7. Survival of 1-day C-starved (○) and non-starved (●) M6 *gfp*-labelled *Moraxella* cells in river water microcosms spiked with (A) 0, (B) 80 μM, (C) 200 μM, and (D) 360 μM PNP. Values are the means of triplicate determinations ± standard deviation.

3.4. Discussion

One common C-starvation response of non-differentiating Gram-negative bacteria is an increase in their ability to catabolize and scavenge nutrients from the environment (Matin *et al.* 1999). In some instances, carbon starvation induces expression of catabolic enzymes, such as β-galactosidase (LacZ) and the peptide catabolic enzyme (CstA) of *E. coli*, even in the absence of their respective inducers (Matin 1996). However, it is not known if catabolic genes of organic pollutants are up-regulated in the absence of the inducer substrates during C-starvation. PNP degradation genes have recently been identified (Zylstra *et al.* 2000) but little is known about the effect of C-starvation on the catabolic activity of PNP-degrading bacteria.

Herman and Costerton (1993) showed that the PNP mineralization rate of a PNP-degrading actinomycetes increased significantly when the bacterial samples were C-starved prior to the PNP mineralization experiment. Because the actinomycetes was cultured in a PNP-supplemented growth medium, it was not known if the induction of PNP-degrading enzymes was affected by C-starvation. In our study, we showed that not only did the PNP degradation rate of the 1-day C-starved *Moraxella* cells increase consistently, but there was also a significant reduction of induction time of PNP degradation from 6 to 1 h. However, C-starvation did not totally eliminate the induction time of PNP degradation of the *Moraxella* cells, suggesting that C-starvation of the cells alone did not induce the expression of the PNP-degrading genes. Interestingly, the effect of C-starvation on the decrease of induction time of PNP degradation was transient for the *Moraxella* cells. It has been shown that PNP degradation genes are harboured in a plasmid of a *Pseudomonas* strain (Prakash *et al.* 1996). It is not known whether the PNP degradation genes of the *Moraxella* strain are plasmid-borne and if some *Moraxella* cells have lost their PNP-degrading plasmid during a prolonged C-starvation.

Carbon starvation of some bacterial species causes cell fragmentation (i.e., reductive division), which results in increased cell density (Kolter *et al.* 1993, Kjelleberg *et al.* 1983, Novitsky and Morita 1977). However, cell fragmentation was not observed in this study over the first three days of C-starvation and the *Moraxella* cells experienced a size reduction only after 90-day starvation in the MSM. Since most of the *Moraxella* cells harvested at the mid-log growth phase were likely to be in the process of cell division or committed to DNA replication, this could be the cause of the increase in cell density during the early stage of C-starvation. Because of the 40% increase in cell density in the 1-day C-starved *Moraxella* culture (Figure 3.2), the increase in the *Moraxella* cell density could contribute to the decrease of induction time and increase in catabolic rate of PNP degradation. However, our data showed that increase in *Moraxella* cell density from OD_{600nm} of 0.2 to 0.4 was associated only with an increase in the initial rate of PNP degradation, but not the induction time of the degradation process.

Canosa *et al.* (1999) showed that the regulatory gene of alkane degradation (*alkS*) of the OCT plasmid was controlled by the C-starvation (or stationary phase) sigma factor, RpoS. Under C-starvation conditions, RpoS induced the production of AlkS, which inturn induced the promoter (*PalkB*) of the alkane-degrading operon in the presence of inducer substrates. Similarly, in conjunction with the regulatory protein (XylS) of the meta-operon of the xylene degradation genes of the TOL plasmid, RpoS is also responsible for maintaining a high level of expression from the promoter (*Pm*) of the meta-operon of the *xyl* genes under C-starvation (Ramos *et al.* 1997). In addition to RpoS, inducer substrates are required for both the AlkS and XylS to activate the *PalkB* and *Pm* promoters, respectively. In Gram-negative PNP-degrading bacteria, *pnpA* and *pnpB* encode PNP monooxygenase and benzoquinone reductase, the first two enzymes of the PNP degradation pathway, respectively. The ring cleaving enzyme of PNP is encoded by

pnpDEC (Zylstra *et al.* 2000). Similar to the *alk* and *xyl* operons, it has been suggested that a PNP regulatory gene (*pnpR*) is necessary to induce the expression of *pnpA* and *pnpB*. It is possible that the C-starvation sigma factor (RpoS) up-regulates the *pnpR* gene of the C-starved *Moraxella* cells as in the *alkS* and *xylS* systems and hence increases the expression of the PNP degradation genes under carbon starvation. We also showed that there was an initial surge of PNP uptake by C-starved *Moraxella* cells. This agrees with our hypothesis that the increase in cellular PNP together with the PnpR regulatory protein will initiate an early induction of the PNP degradation genes, causing a decrease of induction time for PNP degradation.

A *gfp*-labelled PNP-degrading *Moraxella* strain M6 (Moore *et al.* 2004) was constructed to examine the PNP scavenging ability and persistence of C-starved *Moraxella* cells in non-sterile river water samples. Because of poor survival of microbial inocula under biological and physicochemical stresses in the environment, the inocula often fail to stimulate degradation of pollutants (Alexander 1999, Leung *et al.* 1997b). Our data showed that log-phase *Moraxella* cells degraded various concentrations of PNP (i.e., 80-360 μM) in the river water samples and the time required for the removal of the pollutant increased as the concentration of the PNP was raised in the river water. More importantly, we demonstrated that the 1-day carbon starved *Moraxella* cells consistently showed a shorter induction time for PNP degradation than non-starved inocula in natural river water samples. Unlike the non-starved *Moraxella*, the C-starved inocula completely degraded the PNP in about 16 h regardless of the PNP concentration in the water samples. All inocula showed better survival in the PNP-contaminated river water samples than the uncontaminated water samples over the first two days of incubation. This agrees with the observation that PNP was completely metabolized in the first 34 h and the

depletion of the PNP substrate caused a decrease in *Moraxella* cell density after 2 days of incubation.

In conclusion, carbon starvation for one day significantly decreased the induction time of PNP degradation of the *Moraxella* strain in both sterile buffer and non-sterile river water. Our data showed that inoculum cell density alone did not affect the induction time for PNP degradation. However, PNP uptake was significantly higher in the carbon-starved *Moraxella* cells and this may initiate an early expression of the PNP catabolic genes.

4. Acknowledgements.

This research was supported by a Natural Science and Engineering Research Council of Canada (NSERC) Discovery Grant granted to K.T. Leung. We thank Dr. J.C. Spain for providing the *Moraxella* strain and D. Goulet for technical assistance.

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