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PHYSIOLOGICAL STATUS OF BACTERIA USED FOR ENVIRONMENTAL APPLICATIONS

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ABSTRACT

Several bacteria have properties of interest for biotechnological applications, such as bioremediation of pollutants and biocontrol of plant pathogens. In order to perform their intended tasks in the environment the cells need to remain viable and active. Therefore, the aim of this thesis was to use a combination of molecular approaches to determine the physiological status of specific bacterial populations in soil. Complementary experiments were done in pure cultures to gain a better understanding of specific physiological states, such as bacterial dormancy. In some studies, the bacteria were tagged with the following marker genes to enable them to be specifically detected in soil: *gfp* (encoding the green fluorescent protein, GFP), *luxAB* (encoding bacterial luciferase) or *luc* (encoding eukaryotic luciferase). Viability stains, 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) and propidium iodide (PI), were used to stain active and dead cells, respectively. The marker-gene tagged cells were incubated in soil under different conditions and the number of GFP fluorescent and stained cells was enumerated by flow cytometry at specified sampling periods. Luciferase activity was used to monitor metabolic activity of the population. In addition, the number of culturable cells was determined by selective plate counting and compared to the results obtained by flow cytometry. Finally, in one study, proteomics was used to elucidate which proteins were expressed under different nutrient conditions.

The physiological status of *Arthrobacter chlorophenolicus* A6 (a chlorophenol degrading bacterium) was investigated after introduction into soil incubated at different temperatures, 5 and 28 °C. The majority of the A6 population remained metabolically active after 20 days of incubation in soil at 5 °C. However, there was a fraction of the GFP-fluorescent A6 population that was not stained with CTC or PI, presumably indicating a subfraction of dormant cells that were alive but inactive. By contrast, after the same period of incubation at 28 °C, the majority of the cells died. The ability of *A. chlorophenolicus* A6 to enter a state of dormancy during incubation at cold temperatures, makes this strain a good candidate for treating chlorophenol contaminated soil in temperate climates.

Two *Pseudomonas fluorescens* strains, proposed for improving crop yields, were also studied. *Pseudomonas fluoresens* A506 is used to reduce frost damage to plants and *Pseudomonas fluorescens* SBW25 is a plant growth promoting bacterium. First, a GFP-tagged variant of the A506 strain was studied to determine whether GFP could be used to detect the cells when they were viable but non-culturable (VBNC). The results showed that GFP tagged cells could be detected even in a VBNC state as long as the cell membrane was intact. The SBW25 strain was studied in pure cultures and in soil to determine the physiological status of the cells under different nutritional conditions, using many of the approaches described above for A6. Most of the cells died after incubation for nine days in nutrient rich medium. By contrast when incubated under starvation conditions, most of the population was not stained with CTC or PI, indicating that most of the cells were presumably dormant. In soil, a subpopulation of the SBW25 cell population died. However, approximately 60% of the population in soil apparently entered a state of dormancy, similar to that observed under starvation conditions in pure cultures. Several differences were found in the proteins that were expressed when SBW25 was incubated under nutrient rich conditions compared to starvation conditions. These differences provide a clue as to what proteins enable SBW25 to survive starvation and dormant states.

LIST OF PUBLICATIONS

I. Lowder, M., Unge, A., Maraha, N., Jansson, J.K., Swigett, J., and Oliver, J.D. (2000). Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506. Applied and Environmental Microbiology. 66:3160-3165.

II. Backman, A., Maraha, N., and Jansson, J.K. (2004). Impact of temperature on the physiological status of a potential bioremediation inoculant, *Arthrobacter chlorophenolicus* A6. Applied and Environmental Microbiology; 70:2952-2958.

III. Maraha, N., Backman, A., and Jansson, J.K. (2004). Monitoring physiological status of GFP-tagged *Pseudomonas fluorescens* SBW25 under different nutrient conditions and in soil by flow cytometry. FEMS Microbial Ecology; 51:123-132.

IV. Maraha, N., VerBerkmoes, N.C., Spiers, A., Shah, M., Timms-Wilson, T., Goodall, T., Bailey, M., and Jansson J.K. (2006). Use of proteomics to study impact of nutrient status on *Pseudomonas fluorescens* SBW25. Manuscript

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1 INTRODUCTION

Microorganisms are integral parts of ecosystems. They can directly affect the health of other organisms by preventing or causing diseases, and are essential for the global cycling of nutrients. Specific roles of microorganisms in nature include biodegradation, bioremediation, carbon and nitrogen cycling, toxin production, pathogenesis and plant growth enhancement. A better understanding of the role and impact of microorganisms in nature requires greater knowledge of their physiology, specific population dynamics and of interactions between particular microbes and other organisms.

The use of microorganisms to control plant diseases or to clean up the environment from pollutants and chemical waste has been implemented frequently, as a consequence of increased global environmental awareness. For example, microbial biocontrol agents are good alternatives to chemical pesticides. Today, there are several strains of bacteria, fungi and yeasts that are registered and used in commercial applications to treat or reduce different kinds of plant diseases in the environment.

Risks associated with the use of microorganisms for environmental applications include the possible disruption of microbial processes that are essential to the general functioning of soil ecosystems, the production of toxic metabolites, and the potential for the released organism becoming an opportunistic human or animal pathogen. Therefore, it is important to be able to evaluate the results of using microbial inoculants in nature. In particular, risk assessment requires information about the potential fate and survival of released strains. Issues that we need to better understand include the physiology, behaviour, mode of action, long-term viability, and efficacy of microbes of interest. To be able to achieve these goals it is important to have the right tools for monitoring the cells and for assaying their activities.

One major obstacle to environmental monitoring has been the difficulty to detect and quantify specific microorganisms in complex natural settings. For example, soil consists of inorganic particles (sand, silt and clay), organic material (particulate plant, animal, and microbial debris, humic material etc.), a gas phase and a water-phase (Stotzky, 1982). Bacteria in natural environments are exposed to a wide variety of stressful conditions such as nutrient deprivation, extremes of temperature or pH, changes in oxygen tension, the presence of toxic compounds, etc (Mukamolova *et al.*, 2003).

Microorganisms constitute a majority of the organic fraction of soil, with one gram of soil containing up to 10^9 bacteria (Torsvik *et al.*, 1990; Torsvik *et al.*, 1996). However, the majority of the total bacterial biomass in soil is thought to be inactive most of the year (Morita, 1993).

Also, bacterial inoculants that can be cultivated in the laboratory often become stressed upon introduction into soil and are difficult to cultivate, although they may still be viable. Therefore, other methods are required to bypass this deficiency of cultivation-based approaches.

Recently a series of molecular tools have been introduced for specific monitoring of bacteria of interest in complex environments, such as soil, without the requirement for cultivation. Introduction of these new molecular techniques has also raised the prospect of developing new criteria and new definitions for different aspects of microbial physiology in the environment. These definitions and criteria in turn, complicate the decision-making process as to the risk assessment of release of microbial inoculants. In consequence, the need for precision and consensus in our use of the term “viability” as applied to bacteria and its practical implications is increasing. Therefore, this thesis has focused on the application of molecular tools to assess different physiological states of bacterial populations in order to better understand and define bacterial viability in natural ecosystems.

2 MODEL MICROORGANISMS

Different model bacterial strains were studied in this work. All of the strains are domesticated microorganisms that have been isolated from nature and cultured under laboratory conditions for many years. These domesticated strains all have properties of interest for environmental applications. Domesticated strains can become adapted to life in pure culture conditions, with constant supplies of nutrients and absence of competition from other organisms or predators. Since the aim is to eventually release these microorganisms into field conditions, the trick is to optimise their chance of survival, in harsh soil environments, long enough for them to perform their intended tasks. The three bacteria studied in the different papers of this thesis are the following: *Arthrobacter chlorophenolicus* A6, a chlorophenol degrading bacterium; *Pseudomonas fluorescens* A506, a plant frost protection bacterium; and *Pseudomonas fluorescens* SBW25, a plant growth promoting bacterium.

2.1 *ARTHROBACTER CHLOROPHENOLICUS* A6

Bioremediation exploits the catabolic diversity of microorganisms to transform chemical contaminants into less harmful compounds. In this process, one can either use the microorganisms already present at the site of contamination (intrinsic bioremediation) or inoculate with microorganisms having special catabolic properties (bioaugmentation) (Timmis *et al.*, 1999; Vogel, 1996; Westerberg *et al.*, 2000). However, bioaugmentation is not a straightforward technique as there are many parameters to consider to ensure inoculant survival and efficacy. Some of the abiotic variables impacting the survival of an inoculant used for bioaugmentation of a contaminated site are: soil humidity, temperature, chemical properties of the pollutant (contaminant) itself and its bioavailability (Liu *et al.*, 1993; van Veen *et al.*, 1997; Vogel, 1996).

Chlorophenolic compounds are examples of problematic soil pollutants that are targets for bioremediation. A model chlorophenol used for some studies in this thesis is 4-chlorophenol (4-CP). 4-CP is a toxic and recalcitrant compound that is formed, for example, from chlorination of waste water, in pulp mills, from breakdown of herbicides and from anaerobic degradation of more highly chlorinated phenols such as pentachlorophenol and 2,4,6-trichlorophenol (Madsen *et al.*, 1992; Pritchard *et al.*, 1987). There are several microorganisms that are able to degrade 4-CP, including some species of *Pseudomonas*, *Alcaligenes* and *Arthrobacter* (Häggblom, 1990; Häggblom *et al.*, 1995). The degradation capacity of these bacteria are usually limited to relatively low concentrations in the range of 20-100 ppm (0.2-0.8 mM), severely limiting the use of the strains mentioned above for bioremediation of higher concentrations of 4-CP.

By contrast, *Arthrobacter chlorophenolicus* A6 (A6) is capable of degradation of 4-CP up to concentrations of 350 ppm (Westerberg *et al.*, 2000). This microorganism has been extensively studied in our research

group and it has several properties of interest. For example, it can degrade different phenolic compounds in mixtures (Nordin et al., unpublished) and 4-CP at low temperatures of 5 °C (Backman *et al.*, 2003; Elväng *et al.*, 2001; Westerberg *et al.*, 2000). This bacterium was used as a model strain in Paper II of this thesis.

2.2 *PSEUDOMONAS FLUORESCENS* A506

Plant frost injury is a major problem in agriculture leading to large reductions in crop yields. In some cases, the degree of frost damage is related to ice nuclei produced by ice nucleation-active bacteria on plant surfaces (Gurian-Sherman *et al.*, 1993; Lindow *et al.*, 1988).Therefore reducing ice nucleation-active bacteria on plants can be used as a strategy to reduce frost damage on plants. Ice nucleation-active bacteria can be out-competed by using bacteria that can pre-emptively colonise plants (Lindow *et al.*, 1989). One bacterium identified as non ice-nucleation active is *Pseudomonas fluorescens* A506 (Lindemann *et al.*, 1987). Today, this bacterium has been approved by the United States Environmental Protection Agency as a biological frost protection agent. This bacterium was used as a model strain in Paper I of this thesis.

2.3 *PSEUDOMONAS FLUORESCENS* SBW25

Many plant diseases caused by fungi cause dramatic reductions in crop yields. One possible control means is to apply bacteria with antifungal activities as biocontrol agents as an alternative to chemical pesticides. Many bacteria with biocontrol properties produce compounds that inhibit growth of fungal pathogens. Bacterial antagonists of root pathogenic fungi use a variety of suppressive and inhibitory mechanisms including (a) competition for resources (e.g. iron), physical space, or nutrients supplied by seeds or roots, (b) production of one or more antibiotics or other fungistatic compounds, (c) degradation of pathogenic factors, (d) production of enzymes that degrade fungal cellular components, (e) induction of resistance in the target plant or (f) combinations of different mechanisms (Winding *et al.*, 2004).

Pseudomonas fluorescens is a species of opportunistic Gram-negative bacteria that inhabits all major freshwater, marine and terrestrial ecosystems (Stanier *et al.*, 1966). Members of this species play a central role in the turnover of organic matters and are associated commonly with plant surfaces and tissues (Palleroni, 1984). *Pseudomonas fluorescens* SBW25 is a plant growth promoting strain, which has been widely studied due to its potential as an inoculum for improving crop yields. *P. fluorescens* SBW25 was originally isolated from the microflora of sugar beet (Bailey *et al.*, 1995). In the plant environment, *P. fluorescens* SBW25 is an aggressive colonizer of the rhizosphere (the region of soil surrounding and including the plant root) and the activities of certain isolates can enhance plant health and growth (Bailey *et al.*, 1995; Unge *et al.*, 2001). Despite the prevalence of *P. fluorescens* SBW25, and related strains, in natural environments, the causes of its ecological success (fitness) are yet not well understood. To

date the SBW25 strain is not known to produce any antifungal metabolites or any antimicrobial substances. Still, it has been shown to have fungal antagonistic properties, as demonstrated against *Pythium* (Ellis *et al.*, 2000; Naseby *et al.*, 2001; Thirup *et al.*, 2001; Winding *et al.*, 2004). A genetically modified strain of *P. fluorescens* SBW25 has been extensively studied in field trials and growth chamber studies of sugar beet and wheat in order to obtain information on the fate and environmental effects of genetically modified microorganisms released into the environment (Bailey *et al.*, 1995; Thompson *et al.*, 1995a; Thompson *et al.*, 1995b; Unge *et al.*, 2001). *P. fluorescens* SBW25 was used as a model strain in Papers III and IV of this thesis.

3 DEFINITIONS OF BACTERIAL PHYSIOLOGICAL STATUS

Diagnoses of clinical infections, analyses of food microbial contaminants, and environmental monitoring of pathogens and beneficial microbial strains are all currently primarily based on culture tests where viability is equated with culturability.

Some unresolved bacteriological public health problems have occurred where bacteria in a "nonculturable" state appear to be implicated. Principally, these concern aspects of the epidemiology and natural history of infectious diseases which cannot be reconciled with the sample pattern from which the known causal organisms can be isolated. Amongst the most studied epidemiological mysteries are cholera and campylobacteriosis where the failure to isolate *Vibrio cholera* and *Campylobacter jejuni* could be accounted to those organisms being present in a viable but "nonculturable" (VBNC) state (Colwell *et al.*, 1985; Colwell, 2000; Colwell *et al.*, 2000). Therefore, there is a need to have better methods for determination of microbial viability without reliance on culturability. Recently several culture independent approaches have been developed for assessment of microbial physiology. Several of these approaches have been used and further developed in this thesis. In particular, this thesis concentrates on the distinction between viable cells, active cells, dead cells and dormant cells.

3.1 VIABLE / ACTIVE CELLS

Reproductive growth is the most stringent proof for cell viability. Usually, the ability to be grown on culture medium is therefore used to define viability. However, it is known that cells that are viable are not always capable of growth in culture.

It is important to keep in mind while considering the ability to cultivate microorganisms in nature that most species have not yet been able to be cultivated under laboratory conditions. Difficulties with obtaining pure strains in culture can include slow or cell-density limited growth, and highly specific nutritional and/or environmental requirements. Therefore, one must take care when defining a microorganism as unculturable (or non-culturable) because it may be culturable given proper conditions for growth (Barer *et al.*, 1999; Button *et al.*, 1998). For example, bacteria that were difficult to culture from marine samples taken in Alaska were finally recovered when the samples were diluted to allow only one replicating bacterial unit per culture vessel. Under these dilute conditions the bacterial cells were able to grow with mean generation times in the range of 12-72 h. The resulting proportion of marine bacteria previously defined as "unculturable" decreased accordingly (Button *et al.*, 1993). Another example of bacteria with highly specific and exacting requirements for culturability include some species of the genus

Mycobacterium, that require the mycobacterial iron-chelating agent mycobactin in the culture medium in order to grow and divide (Barclay *et al.*, 1983; Wayne, 1994).

A number of additional criteria for cellular viability have been suggested; impermeability of the membrane to dyes such as propidium iodide (PI), the presence of metabolic activity as indicated by the production and retention of a fluorescent product from a nonfluorescent enzyme substrate or dye, and maintenance of a membrane potential (Nebe-von-Caron *et al.*, 2000; Shapiro, 2000). In this work, different viability stains (further discussed below) were used to define a viable active physiological state in the model bacterial population studies.

Active cells can be defined as cells with basal enzymatic activity, such as esterase activity or as respiring cells. Cell activity usually refers to parameters related to cell growth rate and membrane potential; whereas reproduction by for example the amount of DNA, RNA, cellular energy resources and proteins (Jansson *et al.*, 1997; Jansson *et al.*, 1999; Muttray *et al.*, 2000; Nebe-von-Caron *et al.*, 1998; Prosser, 1997). Cells with intact membranes are presumed capable of metabolic activity/repair and to be able to reproduce unless their DNA is damaged beyond repair or they cannot generate a positive energy balance (Nebe-von-Caron *et al.*, 2000). The cell activity can also be expressed as a cell's ability to carry out a specific process (Jansson *et al.*, 1997).

Luciferase activity has also been developed as a measure of metabolic activity of bacterial populations that are marked with *luxAB* or *luc* genes (see section 4.3.2). The relationship between metabolic activity, due to the availability of energy resources, and luciferase activity has been demonstrated in a number of studies. Luciferase activity was shown to be proportional to bacterial biomass when cells were growing in log phase, but to decrease when cells entered stationary phase or were starved, presumably due to low amounts of $FMNH_2$ present in nutrient limited cells (Duncan *et al.*, 1994; Meikle *et al.*, 1992; Meikle, 1994). The low luciferase activity in starved cells could also be due to higher rates of degradation of the luciferase protein compared to synthesis of the protein. Luciferase activity has also been shown to give similar results to other activity measurements such as measurement of cell respiration and direct viable counts, although the latter technique cannot discriminate specific cell populations in mixed communities (Duncan *et al.*, 1994; Meikle *et al.*, 1992; Meikle, 1994; Prosser, 1994).

Luciferase activity was used as a measure of metabolic activity for several of the bacterial strains studied in this work. For example, in Paper II, *luc*-tagged *A. chlorophenolicus* A6 bacteria showed a decrease in luciferase activity when incubated in Luria Bertrani medium (LB) at two different temperatures, 28 and 5 °C. The same trend was seen in soil at 28 °C. By contrast during incubation at 5 °C in soil the metabolic activity of the cell population decreased only slightly during the experimental period of 20 days. We interpreted these results to suggest that *A. chlorophenolicus* A6 survives better in soil at lower temperatures. According to several previous studies (Barer *et al.*, 1999; Barer *et al.*,

1993), stressed cells can become more resistant to adverse conditions and this could also be applicable for our study. In Paper III, the *luxAB*-tagged *P. fluorescens* SBW25 population showed an initial increase in luciferase activity followed by a decline when incubated at 28 °C in soil. In another study, the same strain was inoculated onto plants and found to express luciferase activity on all plant parts where the cells were localized (Unge *et al.*, 2001). By analysis of these results, our hypothesis is that this specific strain can maintain a metabolically active population (or sub-population) if it has access to nutrient resources (the specific sites on the wheat plant and embryo). However, extended maintenance under nutrient rich conditions in pure culture was eventually toxic to the same bacteria, resulting in a decline in luciferase activity (Paper III).

3.2 VBNC CELLS

Under some experimental conditions, for example starved cells (Backman *et al.*, 2004; Elväng *et al.*, 2001; Lowder *et al.*, 2000; Maraha *et al.*, 2004; Tombolini *et al.*, 1997; Unge *et al.*, 1999) and cells inoculated into soil (Backman *et al.*, 2004; Elväng *et al.*, 2001; Lowder *et al.*, 2000; Maraha *et al.*, 2004; Unge *et al.*, 2001), the number of specific marker-gene tagged cells (see details below) enumerated by flow cytometry were significantly greater than the number enumerated by plate counting. There are two possible explanations for these observed differences in cell numbers: either the flow cytometry counts consist of dead plus viable cells, or the flow cytometry counts reflect total viable cells including a fraction of the population that are dormant, or viable but non-culturable (VBNC) (Backman *et al.*, 2004; Möller *et al.*, 1994; Pinder *et al.*, 1993).

There is currently a great deal of confusion and contradiction regarding the definitions of VBNC and dormant physiological states of bacteria (Barer *et al.*, 1999; Colwell, 2000; Colwell *et al.*, 2000; Kell *et al.*, 1998; Kell *et al.*, 2000; Kjelleberg *et al.*, 1993; Nyström, 2001, 2003; Oliver, 1993; Oliver, 2000). Therefore, different current definitions in use for VBNC and dormancy are summarized below.

The first publication to present clear experimental evidence of a VBNC state, demonstrated that both *V. cholerae* and *E. coli*, following incubation in artificial seawater solutions, remained viable although they lost all ability to produce colonies on media routinely employed for their culture (Xu *et al.*, 1982).

The VBNC state can be divided into two categories: temporarily non-culturable (similar to starvation response) and permanently non-culturable. VBNC cells are usually characterized as uninjured cells that can retain activity but yet fail to grow on standard media. Injured cells may demonstrate a reduced culturability on inhibitory media compared with non-selective media. VBNC cells of pathogens may still be capable of expressing virulence factors such as toxins and invasions in response to exogenous stimuli (Rahman *et al.*, 1996). Cells entering a VBNC state

have been proposed to be analogous to the spore formation of differentiating bacteria (Roszak *et al.*, 1987a). Also, VBNC cells are usually defined as having the ability to recover, or resuscitate, should conditions again become favourable for growth. In some cases cells in the VBNC state do not appear to respond quickly to a reversal of the factor that initially induced nonculturability (Oliver, 1993; Roszak *et al.*, 1987b).

3.3 DORMANT CELLS

Dormancy has been defined as a reversible state of metabolic shutdown in a cell (Kaprelyants *et al.*, 1993a). This state reflects a state of low vitality or activity. Dormant bacterial cells are characteristically more resistant to environmental insults than cells in any other recognised physiological state. One example is, that for clinical purposes use of some antibiotics requires that the target organisms are growing, and that dormant (or at least non-growing) cells are thus resistant to the effects of those antibiotics (Kell *et al.*, 1998; Rahman *et al.*, 1996; Wayne, 1994). In other studies, dormant cells have been shown to have insufficient DNA or RNA replication activity to retain substantial enzyme activity (Wayne, 1994). A hypothesis is that many bacteria can remain in a low-activity state during harsh conditions, and later regain activity as the conditions become favourable again (Bar *et al.*, 2002; Kaprelyants *et al.*, 1993a; Oliver, 1993). This reversible state of low metabolic activity has been suggested to be a strategic way for bacteria to adapt to harsh conditions (Colwell, 2000; Kaprelyants *et al.*, 1993a).

One hypothesis is that a state of dormancy is a specific adaptive response resulting from programmed gene expression for cell survival under stress conditions (Colwell, 2000; Colwell *et al.*, 2000; Oliver, 1993). Another hypothesis is that dormancy is in fact a physiological state due to cell damage, resulting in a decline in the ability to be cultured leading eventually to cell death (Kolter *et al.*, 1993; Nyström, 2003). One recent description of a 17 kDa resuscitation promoting factor (Rpf) could be a strong evidence for identifying genes whose expression is regulated by this “bacterial cytokine” (Mukamolova *et al.*, 1998). This protein is necessary for the growth of viable *Micrococcus luteus* cells and loss of Rpf activity in the extracellular environment can account, at least in part, for the loss of culturability seen generally within the actinomycetes group (Kell *et al.*, 2000) and *Arthrobacter chlorophenolicus* falls into this category. Therefore, it would be of interest to look for Rpf-like factors in *A. chlorophenolicus*. Similar growth promoting factors are found within higher and differentiated organisms mediated “internally” by cyclins (Fisher *et al.*, 1995) and “externally” via hormones and growth factors (Fisher *et al.*, 1995; Heath, 1993; Shaywitz *et al.*, 1999).

Dormant cells may also provide certain advantages to a bacterial population because such cells may continue to participate in nutrient cycling reactions or production of secondary metabolites that inhibit

other organisms. Loss of the capacity to replicate does not mean that an organism ceases to have a useful function in its population.

Dormant cells have been defined as metabolically inactive but capable of making a transition to a growing and active state upon entrance into more favourable conditions (Barer *et al.*, 1999; Kell *et al.*, 1998). Cells that have entered a dormant, or VBNC, state and that cannot grow on culture media have, in some studies, been demonstrated to regain this ability given appropriate conditions such as nutrient amendment (De Wit, 1995). For example, starved *M. luteus* bacteria, have been shown to enter a dormant state and these cells are then able to be resuscitated to an active state (Kaprelyants *et al.*, 1993a; Kaprelyants *et al.*, 1993b; Mukamolova *et al.*, 1998; Votyakova, 1994). In Paper II, we could also see that dormant *A. chlorophenolicus* could be resuscitated upon addition of yeast extract. The number of viable cells stained with CTC increased from 50% to almost 80%, while the total number of cells quantified remained constant. However, when the same bacterium was incubated under starvation conditions at 28 °C the cells could not be resuscitated to an active state by addition of yeast extract, indicating that most of the bacterial population was dying under those conditions (Paper II). In Paper III, a similar resuscitation experiment was performed on *P. fluorescens* SBW25. By adding yeast extract to the bacterial population incubated at 28 °C in phosphate buffered saline (PBS) after seven days, a 30% increase in the number of viable cells, stained by CTC, was observed, whereas the total number of cells remained constant (Maraha *et al.*, 2004).

Often, the definition of dormant cells and VBNC cells refer to the same physiological state. One possible way to distinguish between dormant and VBNC cells is by using the following definitions: dormant cells are viable cells that have low activity and usually are not able to be cultured, whereas VBNC cells are viable cells that cannot be cultured that may or may not be active. General properties expressed by dormant or VBNC cells are; decreased cell size and RNA content, condensed cytoplasm, the accumulation of specific proteins, resistance to environmental insults, and an inability to multiply as judged by traditional plating methods (Kaprelyants *et al.*, 1993a).

There may be different factors that cause a cell population to enter a dormant or VBNC state, depending on the cell type and the environmental conditions. For example, actinobacteria are more frequently defined as dormant, whereas vibrio and enterics tend to become VBNC when encountered with stress, and this could be due to differences in their specific stress responses. Bacterial populations in the environment are frequently exposed to stresses due to limitations and changes in nutrient availability (Albertson, 1990; Amy, 1983; Flärdh *et al.*, 1994; Maraha *et al.*, 2004; Xu *et al.*, 1982; Yamamoto *et al.*, 1996; Yamamoto, 2000), temperature (Backman *et al.*, 2004; Duncan *et al.*, 1994; Grimes *et al.*, 1986; Kaprelyants *et al.*, 1993b), salinity (Tholozan *et al.*, 1999), solar illumination and oxygen saturation. However, the survival of bacteria over extended periods is to a large extent determined by their ability to cope with these stresses. For example, a

change of temperature from warm to low incubation temperatures has been shown to induce a VBNC state within *V. vulnificus* (Kjelleberg *et al.*, 1993) and dormancy in *A. chlorophenolicus* A6 (Backman *et al.*, 2004). Another example is the case of *M. luteus* that was shown to enter a dormant state with reduced activity (Kaprelyants *et al.*, 1993b) or no activity in lactate-limited chemostat cultures at room temperature (Barer *et al.*, 1999).

For both cells defined as VBNC or dormant, proof of viability is often demonstrated by resuscitation of the cells to a culturable state, for example by addition of nutrients, as mentioned above.

3.4 DEAD CELLS

Ageing in bacteria may be defined as the process that occurs when growth ceases. This presumably leads to loss of replication functions, accumulation of cellular damage and decline in the resources available to repair those damages. These processes reduce the viability of non-growing bacterial cells, eventually leading to cell death (Barer *et al.*, 1999).

Another important criterion for defining dead cells is the lack of cell membrane integrity. Cells without an intact membrane cannot maintain or generate the electrochemical gradient which generates membrane potential and can be classified as dead cells. As their internal structures are freely exposed to the environment they will eventually decompose (Nebe-von-Caron *et al.*, 2000). In addition, the interior of a bacterial cell is typically 100-200 mV negative with respect to the exterior, representing the potential difference across this membrane that largely depends on energy metabolism. Unlike us, bacteria do not store large energy reserves and therefore when bacteria have an energy limitation, they can lose their membrane potentials (Shapiro, 1995). Depending on the cell type, these bacteria can either become dormant, or if membrane integrity is compromised, the cells can die. Without membrane integrity, the bacteria cannot maintain ion concentration gradients across the membrane and the consequence is usually cell death (Shapiro, 1995).

Cell death may also result from the activation of lysogenic phages or “suicide” genes such as *sok/hok* or autolysins that disrupt the cell membrane (Aizenman *et al.*, 1996; Franch *et al.*, 1996). The capacity for cells to self-destruct may confer a survival advantage on the microorganism at the population level, particularly when the population is large and confronted with starvation (Aizenman *et al.*, 1996; Nyström, 1998). The death of some cells in a population may permit the survival of others in the bacterial population, therefore, death should not necessarily be viewed as a passive reaction to lethal stress (Postgate, 1967). Under certain conditions, cell death can be regarded as a complex multi-step mechanism aimed at sacrificing some bacteria in the population so as to release nutrients permitting the survival of others (Mukamolova *et al.*, 2003).

4 METHODS FOR MONITORING BACTERIAL POPULATIONS IN DIFFERENT PHYSIOLOGICAL STATES

4.1 COLONY FORMING UNITS (CFU)

The classical method for counting the number of viable bacteria in a pure culture or in soil samples is cultivation on solid agar medium and counting of resulting colonies (CFU). Different bacteria have different nutrient requirements and therefore specific media are required for optimum growth of a given species or strain. Therefore, although previously growth was used as a criterion to define viable bacteria, today we know that many microorganisms are not always capable of growth on agar medium (see sections: 3.2 and 3.3). In this work the model strains were all monitored by counting the number of CFU that grew on agar medium, supplemented with antibiotics for selection. This enabled us to determine the culturable fraction of the cell population and in turn to distinguish which fraction was no longer culturable.

In all studies done in this thesis, we detected a distinct difference between the total numbers of cells enumerated by GFP fluorescence compared to the number of cultivated cells on agar plates. One hypothesis is that this difference in numbers is a consequence of the bacterial population becoming stressed and entering a dormant state that is no longer culturable. This hypothesis is supported by parallel experiments that showed a corresponding decrease in metabolic activity of the cell populations. This distinction in total counts compared to CFU counts has also been reported in other studies as well (Barer *et al.*, 1993; Colwell, 2000; Colwell *et al.*, 2000; Kaprelyants *et al.*, 1993a; Kaprelyants *et al.*, 1993b; Kell *et al.*, 1998; Kell *et al.*, 2000; Oliver, 1993; Oliver, 2000).

4.2 DIRECT COUNTS - MICROSCOPY

The total number of bacteria in environmental samples has traditionally been obtained by microscopic counting of cells that have been stained with fluorescent stains. This is the “direct count” approach. Most of the fluorescent stains used, bind specifically to nucleic acids, such as acridine orange (AO), 4’,6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC) (Bleom, 1995; Davey *et al.*, 1996). FITC has been shown to have less problems with background staining in soil than for instance DAPI, and less unspecific binding than AO (Bleom, 1995). Lately, other nucleic acid stains have appeared on the market, for instance from the SYTO series (Molecular Probes).

4.2.1 Kogure Method

In order to determine cell viability without reliance on cultivation, the "direct viable count" (DVC) method was developed during the late 1970s (Kogure *et al.*, 1979). This technique became one of the most commonly used methods to determine the VBNC state. In this procedure, small amounts of nutrients (typically yeast extract) are added together with nalidixic acid (to inhibit DNA synthesis) to a bacterial population under investigation and incubated several hours. If the cells are able to respond to the nutrient addition, they begin to elongate but since DNA synthesis and septation are tightly coupled, the cells are unable to divide. The result is cells that are significantly elongated, and detectable as viable cells by microscopy. The disadvantage of this method is that gram-positive bacteria are generally resistant to nalidixic acid, as are some gram-negative bacteria such as *Aeromonas salmonicida* (Morgan *et al.*, 1993).

4.2.2 Viability Stains

The uptake of, and efflux of; dyes, drugs and other reagents by and from bacterial cells are affected by the structure of the cell wall and the presence of pores and pumps in the membrane which may or may not be analogous to those found in mammalian cells. The outer membrane of gram-negative bacteria excludes most lipophilic or hydrophobic molecules, while gram-positive bacteria may take up a somewhat wider range of reagents without additional chemical treatment (Steen, 2000). These properties of cell membranes were exploited in this thesis by use of different stains that are dependent on cell membrane integrity for determination of cell viability.

The stains used in this work were PI (propidium iodide) which stains cells with damaged/leaky cell membranes and CTC (5-cyano-2,3-ditolyl-tetrazoliumchloride), which stains cells with an active electron transport. The PI dye binds to double-stranded nucleic acids causing the cell to fluoresce red (Davey *et al.*, 1996). PI has a propyl group with a quaternary ammonium as its *N*-alkyl group, and thus bears a double positive charge. This charge is generally believed to be membrane-impermeable; e.g. that the dye is excluded by prokaryotic and eukaryotic cells with intact cytoplasmic membranes (Shapiro, 2000). Cells which take up propidium iodide are usually considered to be non-viable or dead (Davey *et al.*, 1996; Nebe-von-Caron *et al.*, 2000; Veal *et al.*, 2000; Vesey *et al.*, 1994).

Other common dyes used for staining dead cells are; oxonols (such as Bis-oxonol/$DiBAC_4$), cyanine dyes or rhodamine-123 (Rho123) that bind to the cytoplasmic membrane or TO-PRO3, trypan blue, amino actinomycin (7-AAD) and ethidium bromide (EB) that attach to nucleic acids (Shapiro, 1995).

The viability stain CTC is a redox dye, which produces a fluorescent formazan (5-cyano-2,3-ditolyl-tetrazoliumformazan, CTF) product when it is chemically or biologically reduced. CTF is easily detected by its

red/orange fluorescence using microscopy or flow cytometry. The dye CTC has been used as an indicator of respiratory electron transport (Rodriguez *et al.*, 1992; Schaule *et al.*, 1993), as well as of membrane integrity of the cell (Davey *et al.*, 1996). CTC is considered to be an indicator of oxidative metabolism in bacteria. This stain was optimised as a viability stain for pseudomonads in pure culture (Lowder *et al.*, 2000), and in soil (Paper III).

Other examples of dyes used for staining viable cells are; fluorescein diacetate (FDA) that is hydrolysed and retained within intact cell membranes, calcein and chemichrome B, a commercial reagent. The later are cleaved like FDA but with slower reactions and are also used for the determination of bacterial viability (Pinder *et al.*, 1993; Shapiro, 1995).

4.3 MARKER GENES

The presence of large numbers of indigenous microorganisms and the particulate soil matrix complicate the ability to monitor a specific bacterium in soil. Also, as discussed above, it is known that microbial inoculants grown in the laboratory may lose their ability to be cultured on agar medium after long-term incubation in the environment, thereby limiting the applicability of culture-based detection techniques (Oliver, 2000). One way to overcome these limitations is to incorporate a marker gene, into the genome of the inoculant cells, allowing those cells to be specifically detected without the need for cultivation (Jansson *et al.*, 1999; Jansson *et al.*, 2000). Marker genes are tools used for specific detection of cells of interest. Marker genes are usually expressed from constitutive promoters, resulting in a detectable phenotype that can be used to specifically identify the marked cells. These can be compared to “reporter genes” that also produce a detectable phenotype, but are usually fused to an inducible promoter. Several reviews have described the advances in reporter- and marker-gene constructs and their applications (Jansson *et al.*, 2000; Jansson, 2003; Leveau *et al.*, 2002).

Some of the most useful marker genes for monitoring purposes available to date are genes encoding bioluminescent proteins, such as the *luc* gene, encoding firefly luciferase (Cebolla *et al.*, 1993; Möller *et al.*, 1994; Tombolini *et al.*, 1999) and the *gfp* gene, encoding green fluorescent protein (GFP) (Unge *et al.*, 1999; Unge *et al.*, 2001). Bacteria tagged with these genes can be detected based on the respective phenotypes: bioluminescence or fluorescence.

4.3.1 GREEN FLUORESCENT PROTEIN (GFP)

The green fluorescent protein (GFP) was originally isolated and cloned from the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994). In *A. victoria*, GFP emits green light as a result of an energy transfer from the Ca^{2+} activated photoprotein aequorin to GFP, when illuminated with blue light. The *A. victoria* GFP is small, consisting of 238 amino acids. The GFP chromophore is formed by the amino acids 65-67: Ser-Tyr-Gly (Prasher,

1992). Chromophore formation requires oxygen, without any additional requirements of exogenous substrates or cofactors. The protein can function as a reporter under conditions that are not growth dependent.

Wild type GFP emits green light at 508 nm upon excitation with blue light at 396 nm. One problem with wild type GFP is that excitation at 396 nm leads to loss of fluorescence (photo bleaching), due to photoisomerisation. Therefore GFP mutants have been created with shifted excitation and emission wavelengths to get higher fluorescence intensities, better solubility, faster protein folding and extended protein half-lives. For example, the GFP mutant P11 is a red-shifted mutant, where amino acid 167 is mutated from Ile to Thr resulting in a change of the maximum excitation wavelength from 396 nm to 471 nm. P11 emits light at 502 nm when it is excited at 471 nm (Heim *et al.*, 1994). This GFP variant has been used for chromosomal tagging of the model organisms used in Papers I-III of this thesis. GFP constructs are normally introduced into the host cell, either on a plasmid or integrated into the chromosome. The fluorescence emitted by the cells can then be observed by eye as fluorescent colonies or by fluorescence microscopy. GFP-expressing single cells can also be identified and quantified by flow cytometry (Norman *et al.*, 2006).

GFP is frequently used as a marker and reporter system to assess the fate and activity of microbial strains and the dynamics of bacterial populations in soils (Errampalli *et al.*, 1999a; Errampalli *et al.*, 1999b; Norman *et al.*, 2006; Tresse *et al.*, 1998), aqueous systems (Banning *et al.*, 2002; Webster *et al.*, 1985), rhizospheres (Thompson *et al.*, 1992; Tombolini *et al.*, 1997), activated-sludge (Wallner *et al.*, 1997; Watanabe *et al.*, 2000) and biofilms (Davey *et al.*, 2000; Skillman *et al.*, 1998). Furthermore, GFP can be used to visualize gene expression (Cubitt *et al.*, 1995) and horizontal gene transfer (Sorensen *et al.*, 2003). One recent example of studies using GFP as reporter gene, is a study in which a whole-cell biosensor was developed based on induction of the SOS-response, which allows GFP-based detection of genotoxic substances in soil (Norman *et al.*, 2006).

The major advantage of using *gfp* as a marker gene is that GFP does not require any addition of exogenous substrates. One potential disadvantage of the use of the *gfp* gene as a reporter of gene expression is the extreme stability of the GFP protein. Whereas stability of the protein is advantageous for the tracking of cells, it can be problematic in studies of temporal changes in gene expression using marker genes, since, once the reporter protein is synthesized, it will persist (Jansson, 2003). On the other hand, GFP stability can be an advantage for detection and enumeration of the total number of cells in a population, independent of their physiological status. This property was taken advantage of for several of the experiments described later in this work.

In paper I, the model strain, *P. fluorescens* A506 was chromosomally tagged with two tandem copies of the mutant *gfp* gene, P11 (Heim *et al.*, 1994), resulting in the tagged strain designated *P. fluorescens*

A506::gfp2. In papers II and III, one copy of *gfp* was used as a marker gene integrated into the chromosomes of the model strains *A. chlorophenolicus* A6 and *P. fluorescens* SBW25, respectively. In the case of SBW25, the *gfp* gene was combined with *luxAB* gene markers on a dual marker gene cassette (Unge *et al.*, 1999).

4.3.1.1 GFP in combination with Viability Stains

In this work, by gating GFP-fluorescing cells that stained with either CTC or PI, it was possible to quantify the fraction of active and dying/dead GFP-fluorescent cells, respectively. Flow cytometry data obtained from soil samples was treated by subtracting the data from uninoculated soil to account for background particulate matter in the gated region otherwise containing GFP-fluorescent cells.

Examples of flow cytometry output by a FACSCalibur flow cytometer used to distinguish different physiological states of bacteria in pure culture and in soil are shown in figure1.

Figure 1.

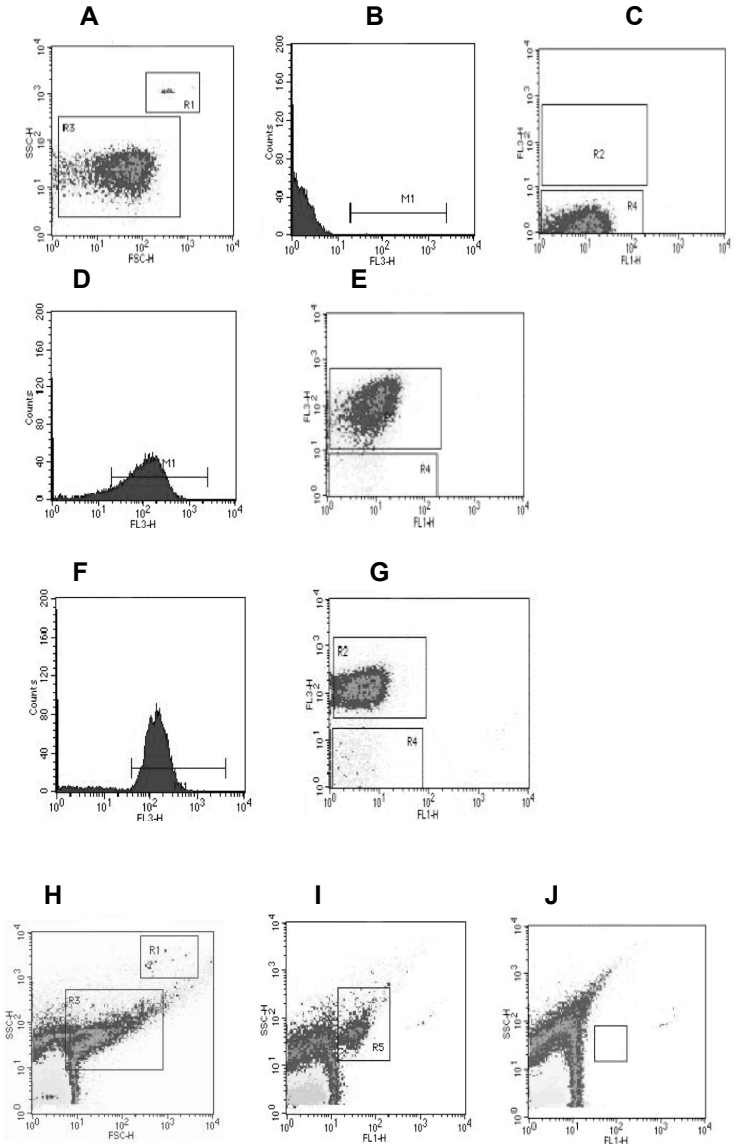

Figure 1. Examples of flow cytometry output for different physiological states of *Arthrobacter chlorophenolicus* A6

Panels A-G: Cells incubated in nutrient-rich conditions, LB, overnight at 28 °C. In (A), the parameters are forward scatter (FSC) and side scatter (SSC). In (B), red/orange fluorescence properties (FL3) are shown and in (C) green fluorescence properties (FL1). R1 is the area containing microscopic beads added as an internal standard for quantification purposes. R3 is the area containing the whole bacterial population, R4 is the area containing the GFP-fluorescent cells, and R2 is the area for stained cells with red fluorescence properties. In (D), the cells were stained with CTC and red fluorescence properties (FL3) are shown. In (E), the CTC stained cells are shown in the gated area R2. In (F-G), the cells were lysed using a mixture of lysozyme and sodium dodecyl sulfate (SDS) and thereafter stained with PI to verify and to quantify the number of dead cells. In (F), the peak red fluorescence properties (FL3) are shown. In (G), the PI-stained cells area shown in the gated area R2 the R4 square contains GFP fluorescent cells that were not stained with PI.
Panels H-J, detection of GFP-tagged bacterial cells in non-sterile soil samples, incubated overnight at 28 °C. In (H) the soil was inoculated with GFP-tagged cells and the cells are distinguished by FSC and SSC properties; whereas in (I) the same population is distinguished from soil background (J) by GFP fluorescence using FL1. In these panels, R1 is the area containing microscopic beads added as an internal standard for quantification purposes. R3 is the area containing the whole bacterial population and R5 is the area containing the GFP-fluorescent cells.

4.3.1.2 Detection of GFP-tagged cells by Flow cytometry

Flow cytometry is used today as a rapid and very sensitive method for the enumeration of bacterial cells. It is possible to quantify the number of microorganisms in a sample using a flow cytometer if the volume of the analysed sample is known. It is also possible to calculate the concentration of the cells within the sample by adding an internal standard, such as microscopic beads at a known concentration to the sample (Tombolini *et al.*, 1997).

In Papers I, II and III, the treated cell suspensions from soil samples or pure cultures were injected into a flow cytometer equipped with an argon ion laser excitation light source (488 nm). Air-cooled argon ion lasers containing a mixture of argon and krypton can emit light at any of the argon or krypton wavelengths from the blue to the far red. GFP fluorescence was detected in the range of 525 to 550 nm with a fluorescence detector (FL1). Red fluorescence in the range of 605 to 650 nm, from PI or CTC was detected by a different fluorescence detector (FL3). Forward scatter (FSC) and side scatter (SSC) data were also collected. These parameters are dependent on the size and the internal granularity/shape of the cells, respectively. One of the disadvantages of flow cytometry is the necessity of the sample to be in the form of solution or some kind of suspension. This requirement is problematic for soil samples that require cell extraction prior to analysis. In addition, flow cytometry measurements are limited by background optical noise, which can reduce detection capacities and raise detection limits (Steen, 2000).

In this work, flow cytometry was used in combination with two viability stains (see sections: 4.2.2 and 4.3.1.1) to quantify the active and dying/dead fractions of GFP-tagged bacterial populations in soil (Papers II and III) and in pure cultures (Papers I, II and III).

4.3.1.3 Fluorescence Activated Cell Sorting (FACS)

Flow cytometry can be coupled to cell sorting resulting in the process of fluorescence activated cell sorting (FACS). Most commercial FACS machines available to date were optimized for the study of eukaryotic cells. Considerable refinements are required for sorting of prokaryotic cells and this is still difficult. Recent developments in FACS include mutliple detectors able to detect up to 12 different colors within (Hi-D FACS), in addition to size and shape of the cells (Herzenberg *et al.*, 2002).

FACS has been used in combination with GFP to study horizontal gene transfer among bacteria in natural environments. This study was done in pure cultures, using *Escherichia coli* and *Pseudomonas putida* strains. The *gfp* marker gene was inserted on a conjugative plasmid (PKJK10) and fused to a synthetic *lac* promotor. The *lac* gene was introduced into the chromosome of the donor bacteria in order to facilitate repression of

GFP expression in the donor cells. Green fluorescence was induced in the donor cells by addition of isopropyl-thio-β-D-galactoside (IPTG). After transfer of the plasmid to recipient bacteria lacking the chromosomally encoded repressor, the GFP was expressed in the recipient cells and therefore could be detected by their green fluorescence. The cells were detected and quantified by flow cytometry and the transconjugant cells were isolated by (FACS) (Sorensen *et al.*, 2003).

One aim of this work was to sort the sub-fractions of the bacterial populations seen in paper II, III and IV (i.e. viable, dead and dormant sub-fractions), in order to better understand their physiological status, for example by studying differences in their protein expression patterns using proteomics (section 4.4). Bacteria were incubated under starvation conditions that were previously detected by flow cytometry measurements in combination with the viability dyes to result in a large fraction of dormant cells (section 4.3.1.1 and 5.2.2).

In order to be successful at sorting sub-fractions of bacterial populations it is necessary to have a FACS that can rapidly sort cells within an acceptable working time without excessive dilution of the sample. For example, for proteomics experiments (see below) approximately 10^6 cells are required per experiment. In addition, sorting needs to be performed quickly, i.e. within 12 h, to maintain the physiological status of the cells. Different FACS machines were tested in this work.

Cell sorting was first tested at Professor Mark Bailey's laboratory with help of Dr. Andrew Whiteley at the Center of Ecology and Hydrology (CEH) in Oxford, UK. The flow cytometer used was a FACSCalibur (Becton Dickinson) with some updated features such as: a fluidic system that can circulate up to 20L (normally 4L) and a filter collector for sorting samples. Sorting was performed so that the cells were collected on an isopore membrane filter (Millipore, Ireland) with a size of 0.2μm (type GTBP). Once the cells were sorted on the filter, the filter was removed to a microcentrifuge tube. To collect the cells from the filter, 300μl of PBS were added to the filter and it was vortexed for 5 min. The cells were then collected by centrifugation for 2 min. at 14,000 rpm on a microcentrifuge. The settings used for sorting were: FSC E01, SSC 520V, FL1 999V, FL3 922V. The settings for normal cell counts were: FSC E01, SSC 450V, FL1 999V, and FL3 813V. All settings were in log-scale. Unfortunately, the sorting capacity of this equipment (50 cells/second) was too slow to sort enough cells. To sort 10^6 cells, it took about 10 hours and the volume of buffer needed was 20L. However, we did find that the easiest cells to be sorted were the active, CTC-stained cells. The dead, PI-stained cells had a weaker fluorescence signal and the dormant, unstained cells were the most difficult to sort.

An updated FACSVantage, or "FACSDiVA" (Becton Dickinson) was also tested. This equipment was located at the Center for Biomedical Microbiology, Technical University of Denmark (DTU), in Lyngby, Denmark, under the guidance of Professor Søren Molin and Dr. Claus

Sternberg. The FACSDiVA sorts cells using pressure, by comparison to the FACSCalibur that uses capillary attraction to sort cells. The pressure applied to sort cells in the FACSDiVA is 2 PSI. The fluid stream is separated into different tubes according to gated fractions in order to collect the sorted cells and to differentiate from waste. This machine is equipped with three different lasers: laser 1, an argon laser with excitation of 488nm (Coherent Innova 90C); laser 2 an argon/krypton laser with excitation of 647 nm (Coherent Innova Spectrum 70C); laser 3, an argon laser with the excitation of 363 nm (Coherent Innova 90C). It has the capacity to sort 30,000 cells/second and to simultaneously sort four different populations or fractions of a population. However, in order to use the FACSDiVA it has to be calibrated every time before use and the calibration takes up to 1h.

The first problem we faced when trying to sort unstained cells (presumably dormant cells) of SBW25 from CTC and PI-stained (live and dead cells, respectively) was that the FACSDiVA could not differentiate the starved cells from the background noise. The cells were too small to be detected clearly. This problem was solved by adjusting to the settings used for the FACSCalibur machine in previous experiments (Papers II and III). Secondly we had problems detecting a signal from the stained cells. This problem was solved by adjustment according to internal standard beads used for the FACSDiVA. In this machine there is no need to count fluorescent beads, since the exact number of cells sorted into the tube are enumerated. These are the settings of the FACSDiVA that we at last were confident with and used for sorting: FSC 543V, SSC 335V, FITC-A (FL1) 742V, PE (FL2) 835V, PerCp-Cy5-5-A (FL3) 830V, UV1 (FL4) 800V, APC (FL6) 819V and all were in log-scale.

Due to time constraints, we did not succeed in sorting out the fractions we were interested in, but we did succeed in developing a useful protocol that could work out for sorting bacterial cells in future experiments (Maraha et al., unpublished). Below, in Figure 2, are some examples of the flow cytometry output used for sorting using the FACSDiVA to distinguish different physiological states of *Pseudomonas fluorescens* SBW25 in pure cultures of starved and unstarved cells.

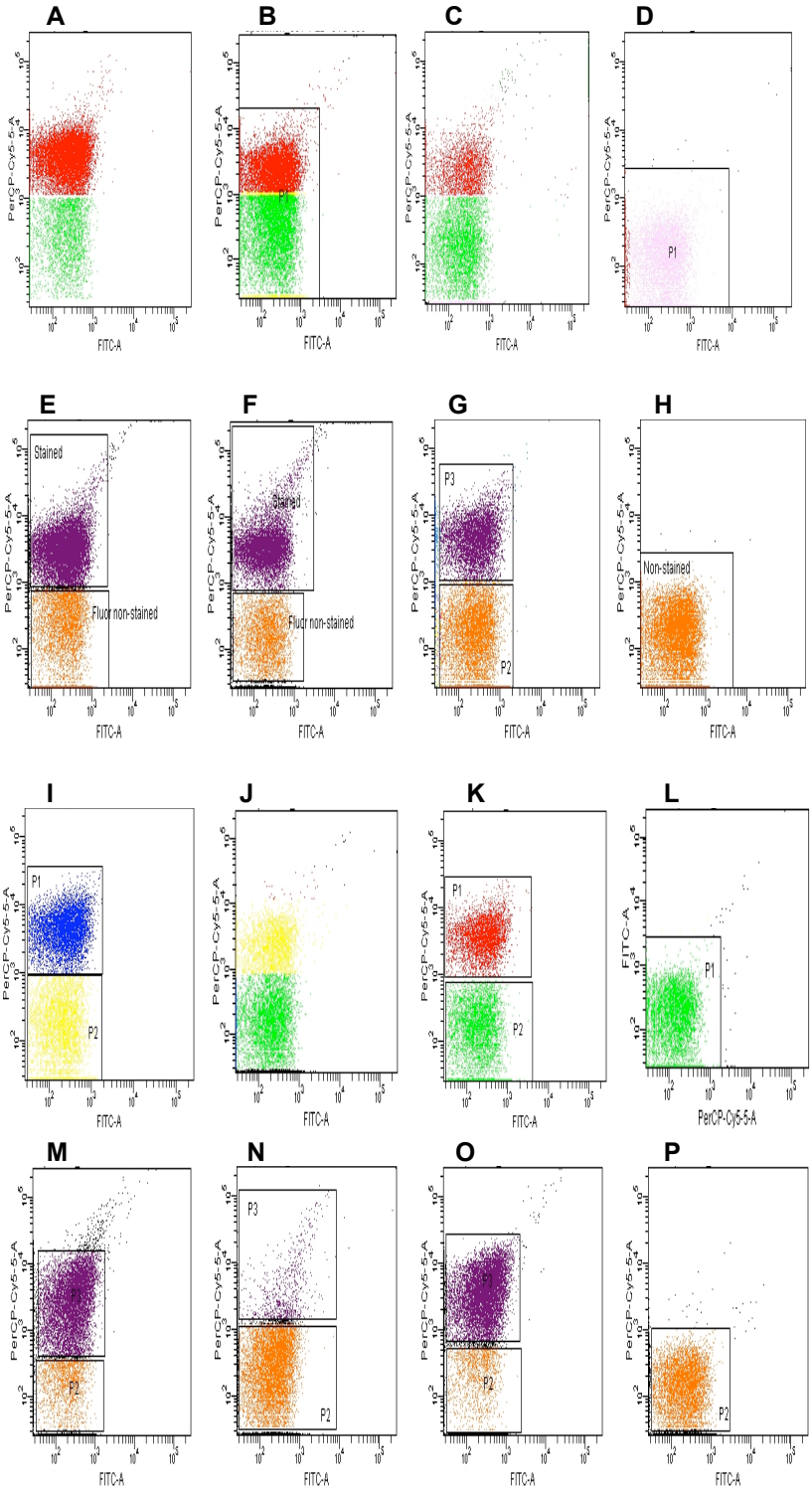
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
PerCP-Cy5-5-A
FITC-A
Stained
Fluor non-stained
Non-stained
P1
P2
P3

Figure 2. Examples of flow cytometry output for different physiological states of *Pseudomonas fluorescens SBW25*

Panels A-H: Cells incubated in nutrient-rich conditions, LB overnight, were pre-starved for 48h in PBS, at 28 °C. In (A-D), the cells were taken after being pre-starved for 48h and transferred to fresh LB medium. The red/orange fluorescence properties are shown and the parameters are FITC-A (FL1) and PerCP-Cy5-5-A (FL3). The stained cells are the fraction seen in red while the non-stained fraction is seen in green. In (A), the cells were stained with CTC and PI. In (B), the cells were stained with only CTC. In (C), the cells were stained with only PI. In (D), the unstained bacterial population is seen in light purple. In (E-H), the cells were sampled two days after incubation in LB, at 28 °C. The stained cells are the fraction seen in purple while the unstained fraction is seen in orange. In (E), the cells were stained with CTC and PI. In (F), the cells were stained with only CTC. In (G), the cells were stained with only PI. In (H), the unstained bacterial population is shown. Panels I-P, the cells were grown in LB overnight, pre-starved in PBS for 48h and transferred to fresh PBS and incubated at 28 °C. In (I-L), the cells were taken after being pre-starved for 48h and transferred to fresh PBS medium. In (I), the cells were stained with CTC and PI (in blue and unstained population in yellow). In (J), the cells were stained with only CTC (in yellow). In (K), the cells were stained with only PI (in red). In (L), non-stained bacterial population is seen in green. In (M-P), the cells were sampled two days after incubation in LB, at 28 °C. The stained cells are the fraction seen in purple while the non-stained fraction is seen in orange. The stained cells are shown in purple and non-stained in orange. In (M), the cells were stained with CTC and PI. In (N), the cells were stained with only CTC. In (O), the cells were stained with only PI. In (H), shown is non-stained bacterial population.

4.3.2 Luciferase Marker genes

Luciferase marker/reporter genes such as eukaryotic luciferase (*luc*) and bacterial luciferase (*luxAB*) enable tagged cells to be detected based on light production (bioluminescence). These reporter genes have been used to identify environmentally responsive genes in a number of microorganisms, including cyanobacteria (Möller *et al.*, 1994; Wolk, 1991), *Pseudomonas* spp. (Blackburn *et al.*, 1994; Meikle *et al.*, 1992; Unge *et al.*, 1999), *Streptomyces* spp. (Sohaskey *et al.*, 1992a; Sohaskey *et al.*, 1992b), and rhizobia (Milcamps *et al.*, 1998).

Bacterial luciferase catalyses the reaction:
$RCHO + O_2 + FMNH_2 \rightarrow RCOOH + FMN + H_2O$ + light (around 490 nm), where RCHO is a long chain aldehyde, such as decanal.

Bacterial luciferase itself consists of a heterodimer of an α and β subunit encoded by the *luxA* and *luxB* genes, respectively. Only the *luxAB* genes are required for expression of the luminescent phenotype, provided the aldehyde substrate is exogenously added. Alternatively, the entire *lux* operon (*luxCDABE*) can be used as a marker with no need for substrate addition since *luxCDE* genes encode the natural substrate for the luciferase reaction. The luciferase enzyme has been determined to have a half-life in *Synechocystis sp.* cells of approximately 8 hours (Kunert *et al.*, 2000). In Paper III, a strain of *P. fluorescens* SBW25 was used that was previously chromosomally tagged with a dual *gfp*/*lux*AB marker system, encoding GFP and bacterial luciferase (Unge *et al.*, 1999; Unge *et al.*, 2001). The resultant strain was designated *P. fluorescens* SBW25::*gfp/lux* (Unge *et al.*, 1999).

Eukaryotic luciferase, encoded by the *luc* gene from the firefly, *Photinus pyralis*, and the click beetle, *Pyrophorus plagiophtalamus*, can also be used as a marker gene for bacterial detection.

Firefly (eukaryotic) luciferase carries out the reaction:
luciferin+ATP+$O_2 \rightarrow$ oxyluciferin+AMP+PP_i+CO_2+light (562 nm).

The eukaryotic luciferase reaction is highly efficient (approximately 10 times higher light yield than the bacterial reaction (Lampinen *et al.*, 1992), which increases the sensitivity of detection compared to bacterial luciferase. The eukaryotic luciferase enzyme was previously determined to have a half-life of approximately 3-4 hours in mammalian cells (Lampinen *et al.*, 1992; Leclerc *et al.*, 2000; Thompson *et al.*, 1991). The disadvantage of using eukaryotic luciferase genes is that the liquid luciferin substrate can be hard to administer to some sample types if cells are to be detected *in situ*, compared to the decanal vapor used as a substrate for bacterial luciferase (Möller *et al.*, 1994). In Paper II, *A. chlorophenolicus* A6 was chromosomally marked with the firefly luciferase gene (strain abbreviated A6L).

The luciferase marker genes have several advantages over most other marker systems owing to the sensitivity and quantitative potential of the assays. A major advantage of luciferase marker genes is that light

output can be detected without disruption of the cells. Disadvantages of the luciferase marker genes are their dependence on substrate addition and cellular energy reserves for expression, sometimes limiting their utility in environmental settings. However, this can also be an advantageous property, since cells will only emit light if they are metabolically active. Therefore, luciferase activity can be used to monitor metabolic activity of microbial populations, as discussed in more detail later in this work and applied in Papers II and III. Luciferase activity resulting in light emission (bioluminescence) has been used as an assay for metabolically active *luxAB* or *luc*-tagged cells (Jansson *et al.*, 1999; Jansson *et al.*, 2000; Prosser, 1994). The bioluminescent phenotype is dependent on the energy reserves of the cells, $FMNH_2$ or ATP for bacterial or eukaryotic luciferase expression, respectively (see section 3.2 above). Cells that are dormant (or VBNC) may or may not still express luciferase activity.

Bioluminescent cells may be detected by several methods, for example as luminescent colonies in a dark room, by exposure to X-ray film, by charged coupled device (CCD) camera image enhancement or by microscopy (Jansson, 1995; Prosser, 1994). The average bioluminescence resulting from a population of cells can be quantified by using a luminometer. In such an instrument, photons are collected and amplified by a sensitive photomultiplier tube. The output is then given as quanta/s or relative light units (RLU). Luminometers are very sensitive, being able to detect *E.coli* cells bearing plasmid-encoded bacterial luciferase at cell concentrations as low as 1-10 and 10-100 cells/ml in liquid culture (Möller *et al.*, 1994; Rattray *et al.*, 1995). The most important advantage of luminescence-based measurement of activity is selectivity. In terrestrial and freshwater environments, background luminescence is negligible. In this work, luminometry was used to estimate the metabolic activity of *luc*-tagged *A. chlorophenolicus* A6 and *luxAB*-tagged *P. fluorescens* SBW25 populations (Papers II and III).

4.4 PROTEOMICS

Proteomics can be employed to compare changes in the expression levels of many proteins under particular genetic and environmental conditions. Unlike transcriptomics that focuses on gene expression, proteomics examines the levels of proteins and their changes in response to different genotypes and conditions. Proteomics is a natural extension of genomics, the characterization of the gene products (i.e. proteins). This method can be employed to study the entire suite of proteins in a cell; collectively called the “proteome”. While the genome is static, the proteome is dynamic. The genome generally contains a set number of copies of every gene; however, proteins in the proteome can be expressed in a wide range, varying from only a few copies per cell for regulatory proteins to many thousands per cell for ribosomal subunits. Furthermore, proteins can be highly decorated with any number of post-translational modifications (PTMs); thus far over 300 have been recorded.

These modifications can be static or dynamic, and may be present in multiple locations on a given protein (James, 2001; VerBerkmoes *et al.*, 2004b).

The studies of proteomes under well-defined conditions can provide a better understanding of complex biological processes and may allow inference of unknown protein functions. Proteomic approaches can also provide information about posttranslational modifications that cannot be obtained from mRNA expression profiles. These approaches have proven critical to our understanding of physiological protein function, translocation, and sub-cellular localization. Microorganisms once subjected to environmental perturbations usually undergo fundamental changes in cellular physiology and/or morphology, as reflected and directed by changes in the global gene and protein expression patterns. For example, up- and down-regulation of specific protein sets is seen in *E.coli* in response to a number of chemical and physical stresses, such as heat, oxidative agents, hyperosmotic shock, temperature changes and nutrient input. These responses are thought to act as protective mechanisms leading to elimination of the stress agent and/or repair of cellular damage. However, the cellular responses, as reflected by the proteome, can differ widely according to the stresses imposed and the microorganism in question (Han *et al.*, 2006). Several proteomics studies have previously shown that the responses of an organism to an environmental stimulus are not simply the sum of independent responses of individual genes but a coordinated series of linked events leading to cross-adaptation among the stress responses.

Although the first proteomic analyses were conducted 30 years ago, renewed interest in this field has been fueled by several recent advances, including the availability of public genome and protein databases, the development of database search engines capable of exploiting these databases, and the introduction of high-sensitivity easy-to-use mass spectrometry (MS) techniques. Other important recent advances include improved two-dimensional gel electrophoresis (2D-gels), computer programs for analysis of the 2D-gel images, protocols for proteolytic digestion of proteins in excised gel pieces, and low-flow chromatography methods. Recently in order to reduce complexity and detect low-abundance proteins, proteomics researchers have become aware of non-gel-based technologies combined with sub-cellular fractionation by n-dimensional chromatographies (Han *et al.*, 2006).

The experimental approach for the measurement of a bacterial proteome can be broken down into four general steps. The first step of sample preparation is cell growth, the second step is protein fractionation or separation followed by the third step characterization of the protein, leading to the final step of computational analysis of the data (bioinformatics). The typical procedure for proteome study is that the protein mixtures are first separated by 2D-gels and protein spots are

excised from the gel. The proteins contained in the gel pieces are digested using a sequence-specific protease, such as trypsin, and the resulting peptides are analyzed by mass spectrometry. Another technique is to use shotgun proteomics and to bypass the gel separation. Both of these techniques are described below.

4.4.1 2D PAGE

Two dimensional polyacrylamide gel electrophoresis (2D PAGE) employs 2D gel electrophoresis to separate and visualize proteins. Individual spots can be excised from the gel, digested with suitable protease enzymes, and then characterized by mass spectrometry (MS). The development of 2D separations of intact proteins by isoelectric focusing in the first dimension based on isoelectric point (p*I*), followed by separation based on molecular mass, has allowed for visualization of over 1000 protein spots from a single organism on a single gel (Gatlin *et al.*, 1998). 2D-gels are currently the most widely used proteomic approach for analyzing the protein composition of cells, tissues or biofluids and might even be called “classic”. Klose in 1975 developed the technique that was first used for analyzing basic proteins. The later use of an immobilized pH gradient (IPG) gel instead of the carrier ampholyte method allowed researchers to apply 2D-gels for easier and more reproducible proteome analyses (Sickmann *et al.*, 2001). The current use of commercially available 18-cm IPG strips (pH, 3 -10) along with high-sensitivity staining is generally able to resolve up to 1000-1500 protein spots depending on the organism. By using narrow-pH-range IPG strips, the main benefit is that the total number of protein spots per pH unit that can be separated increases due to higher spatial resolution.

As another strategy for enhancing the separation capacity of 2D-gels, different sample pre-fractionation methods have been applied for example; sequential extractions, subcellular fractionation, selective removal of most abundant protein components, preparative isoelectric focusing (IEF) separations, and chromatographic fractionation of sample mixtures (Molloy *et al.*, 2000). These strategies offer the benefits of high protein-loading capability along with the ability to discriminate two or more proteins migrating together. The combination of liquid chromatography (LC), 2D-gels, and MS/MS detection has expanded the upper limits of protein visibility typically obtained by gel-based approaches, but this method has higher costs in terms of price, labor and time. A number of general protein detection methods have been developed using organic dyes, silver staining, radiolabeling, reverse staining, fluorescent staining and chemiluminescent staining. Typically, the most widely used stains are Coomassie brilliant blue and silver staining for protein detection, but these stains have low sensitivity. Fluorescent dyes, e.g. SYPRO Ruby protein gel stain, provide great sensitivity and broad dynamic responses compared to the other stains. These dyes are easier to handle, have long

shelf lives, minimal disposal issues but on the other are more expensive. Thus, fluorescence-based protein detection has become a more common practice in recent years (Han *et al.*, 2006; VerBerkmoes *et al.*, 2004b).

The fluorescent dye, SYPRO Ruby Protein Gel stain (Molecular Probes, Eugene, OR 97402, USA) was tested in this work (Maraha et al., unpublished). SYPRO Ruby dye is a permanent stain comprised of ruthenium as part of an organic complex that interacts non-covalently with proteins. SYPRO Ruby Protein Gel Stain provides a sensitive, gentle, fluorescence-based method for detecting proteins in one-dimensional and two-dimensional sodium dodecyl sulfate-polyacrylamide gels. Proteins are fixed, stained from 3h to overnight and then rinsed in deionized water or dilute methanol/acetic acid solution for 30 min. The stain can be visualized using a wide range of excitation sources, such as UV to visualize the stained 2D gels.

These gel-based techniques are and will remain highly useful tools for assessing differential protein expression (Han *et al.*, 2006). More protein spots will be identified as advanced MS technologies such as MALDI (matrix-assisted laser desorption/ionization)-TOF(time of flight)-MS, electrospray ionization (ESI)-MS, and MS/MS are paired with functional genomic studies based on the complete genome sequence.

4.4.2 Shotgun Proteomics

Currently there are two major methods for analyzing proteins by mass spectrometry. The top-down method involves measuring intact proteins as well as interrogating the MS/MS of these intact proteins. In the bottom-up (shotgun method) intact proteins are digested with a protease such as trypsin, Glu-C or cyanogens bromide (CNBr), and the resulting peptide mixtures are analyzed by MS or MS/MS. Thus, 2D-PAGE followed by in-gel digestion and MS analysis is also considered a bottom-up method (see above section).

Shotgun proteomics basically allows for identification of the protein components directly from a mixture, by first digesting all of the proteins in the mixture and ending up with peptides. These peptides are put through high-pressure liquid chromatography, LC or LC/LC for separations and thereafter sequenced by use of MS/MS analysis. The MS data are used to query a peptide database from the specific organism to identify the protein components of the original mixture. This method usually gives much more proteome information than when using 2D-PAGE techniques.

Looking at 2D-PAGE gel analysis, the detection level is usually high but the number of identified proteins is usually much lower (about 30-40%). The shotgun technique is more sensitive compared to 2D-PAGE gels, with much less sample loss. In addition the shotgun methodology is a

more recent technique exploiting the capabilities of liquid-based (LC-MS) methodologies, enabling any protein that can be subjected to either chemical or enzymatic digestion to be analyzed, including membrane proteins, proteins of high and low p*I* values, and proteins of high and low molecular mass. LC-MS is also a more time saving technique compared to 2D-PAGE gels, in terms of sample throughput including how fast samples are analyzed and how well they can be characterized in a short period of time (McDonald *et al.*, 2002).

The shotgun approach is also excellent for determining protein identities since a complex protein mixture (from cell lysate or protein complexes) is analyzed by generating peptides using different enzymes. The most commonly used protease today for digestion of the protein mixture is trypsin. The reasons are due to its specificity, cost, and the fact that it produces a positively charged residue on the C-terminus (lysine or arginine), which aids ionization and peptide sequencing by MS/MS. The peptides are much easier to handle, separate, and analyze than intact proteins. Each averaged size protein, after proteolytic digestion, generates about 20 peptide fragments. This means that a complex sample containing 1000 proteins will generate a new sample containing ~20,000 peptides. However, even very complex peptide mixtures are taken care of by the developed and robust liquid chromatography and mass spectrometry techniques. The peptides are separated physically over a period of time by their hydrophobicity or net charge, and are sequentially injected into the mass spectrometer. The key to forming gas-phase ions from these larger molecules, a prerequisite for MS analysis, lies in using electronspray ionization (ESI). With these ionization techniques, MS can be used as a new type of high-resolution readout for many existing biologic and biochemical procedures, providing biologic information in the form of molecular mass. Furthermore, tandem MS (MS/MS) enables full or partial sequencing of the proteolytic peptides (VerBerkmoes *et al.*, 2004b).

The mass spectrometer records the overall intensity as a function of time, obtaining a Total Ion Chromatogram (TIC) that gives a description of the fragmented ions and the mass of the precursor ions. The precursor masses and the fragmentation patterns are then submitted to search algorithms such as MASCOT and SEQUEST, which can query thousands of MS/MS spectra against protein or nucleotide databases (McDonald *et al.*, 2002; VerBerkmoes *et al.*, 2004a). The result is filtered and sorted to extract positive identifications. The protein identifications can then be compiled into the Kyoto Encyclopedia of Genes and Genomes (KEGG) maps and functional categories for rapid viewing of metabolic and signaling pathways that are activated (VerBerkmoes *et al.*, 2004a). Figure 3, shows a flow chart of the shotgun method shortly described in this chapter (4.4.2.).

The progress in the field of shotgun proteomics has made it possible today to measure approximately 1000-2000 proteins from a microbe under a given growth condition in a period of 1-3 days, depending on the technology used. Furthermore, if enough mass spectrometers are assembled, this analysis can be rapidly repeated for protein identification for an organism under a variety of different growth conditions (VerBerkmoes *et al.*, 2004a; VerBerkmoes *et al.*, 2006). One recent example is the use of shotgun proteomics to study the proteome of *Rhodopseudomonas palustris*, under six different metabolic conditions (classified into two major categories: aerobic growth in dark and anaerobic growth in light). The qualitative analysis revealed over 311 proteins exhibiting marked differences between conditions, many of those were hypothetical or conserved hypothetical proteins showing strong correlations with different metabolic mode (VerBerkmoes *et al.*, 2006). In our study, Paper IV, we studied proteomics of *Pseudomonas fluorescens* SBW25 under different nutritional growth conditions using the shotgun technique. We identified a total number of 1768 proteins in cells incubated in LB and 1887 proteins in starved cells (incubated in PBS). A total of 112 proteins showed a 2X (or greater) spectral difference when comparing cells incubated under the different conditions: 59 proteins were up-regulated in LB and 53 proteins were up-regulated in PBS (starvation).

Figure 3.

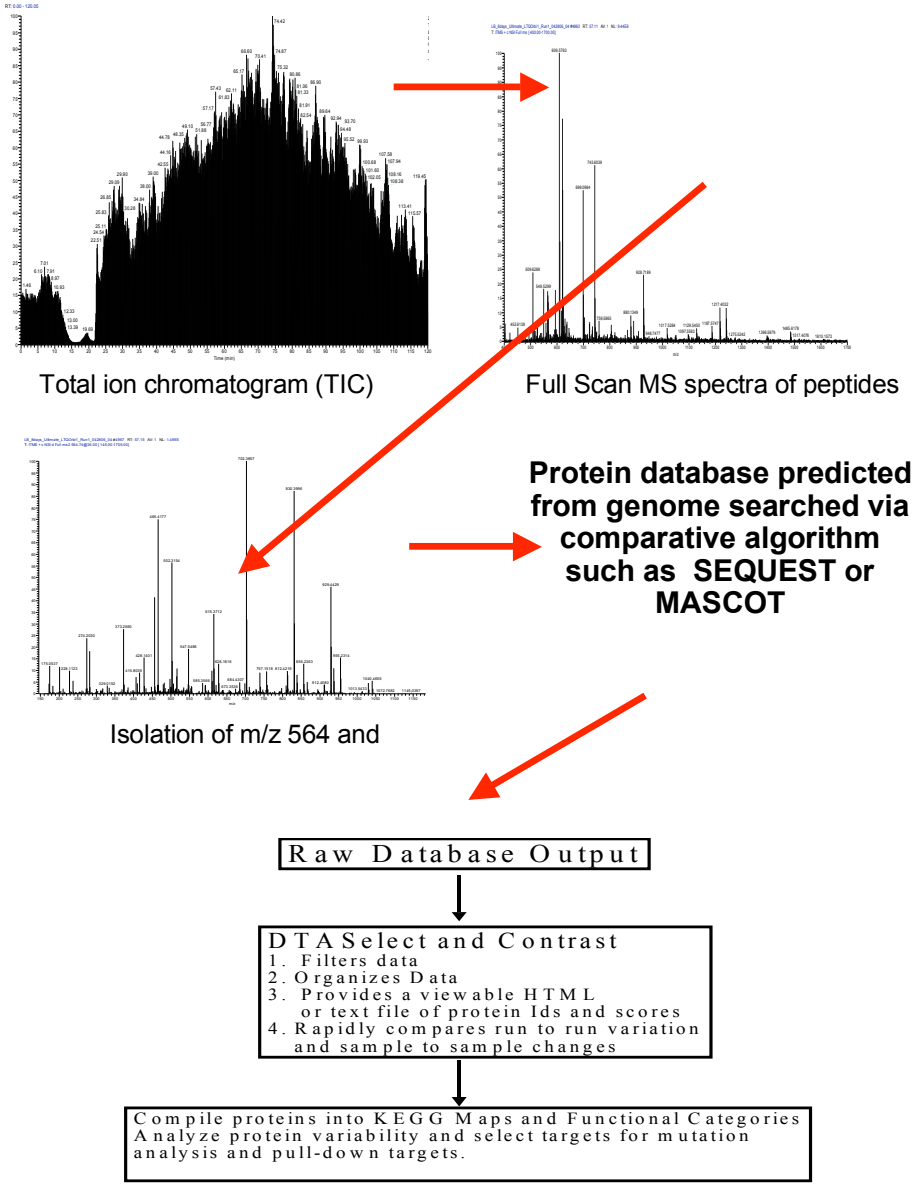

Figure 3. Example of "Shotgun" proteomics procedure (courtesy of Nathan VerBerkmoes)

5 PHYSIOLOGICAL STATUS OF SPECIFIC MICROBIAL POPULATIONS UNDER DIFFERENT CONDITIONS

We used different methods for detection of marker gene-tagged bacteria in pure culture and microcosm (soil) studies. A polyphasic approach was *used including growth on selective media, detection of luminescent luc/luxAB*-tagged cells by luminometry, and detection of *gfp*-tagged cells by flow cytometry (Figure 4).

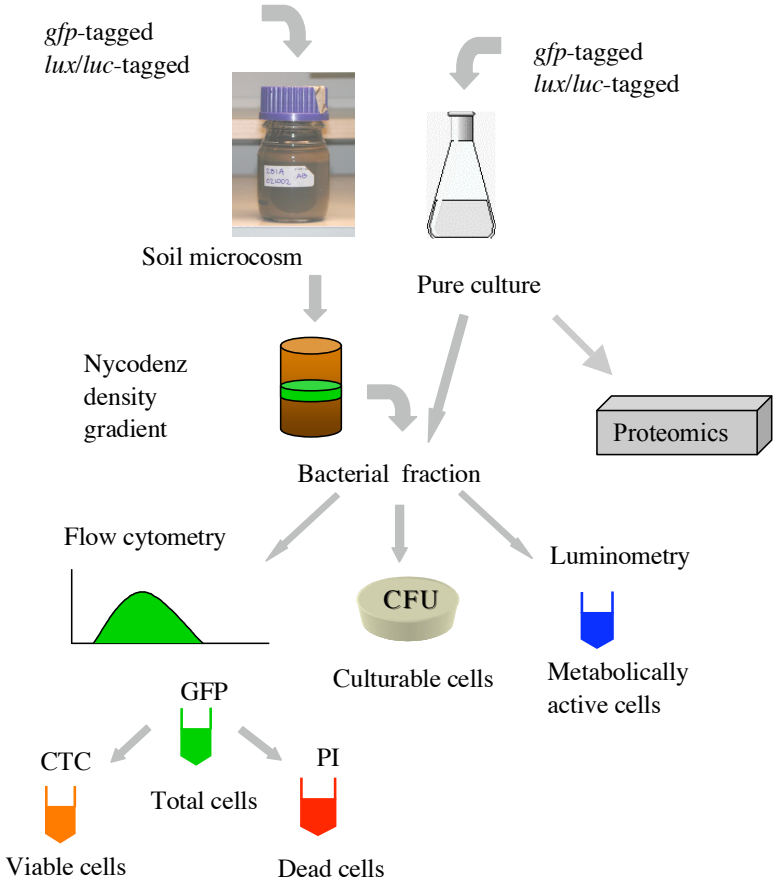

Figure 4. Experimental design; elements of which were used in Papers II, III and IV

5.1 NUTRIENT STATUS

The physiological status of specific microbial populations was investigated under different conditions in this thesis. In order to investigate the impact of nutrients on physiology of growing populations and during long-term incubations, we used Luria Bertrani medium (LB) as a nutrient rich growth medium. To study the different bacteria under carbon and nitrogen starvation conditions, phosphate-buffered saline (PBS) was used.

5.1.1 Nutrient rich conditions

Our observations were that when *A. chlorophenolicus* A6 (Paper II) and *P. fluorescens* SBW25 (Paper III) were grown under nutrient rich conditions the culturability and the viability of the populations declined after 9 or 20 days incubation, respectively. During this period the physical characteristics of the cells also changed, with the cells becoming smaller in size and more heterogenous as also observed previously by both flow cytometry and fluorescence microscopy (Davey *et al.*, 1996; Diaper *et al.*, 1994; Lopez-Amoros *et al.*, 1997; Unge *et al.*, 2001).

According to our results in Papers II and III the metabolic activity of the populations also decreased during long-term incubations under nutrient rich conditions. Since a majority of the populations were stained with propidium iodide (PI) and showed a decrease in average GFP fluorescence intensity/cell by the end of these incubations, the combined data indicate that the populations were dying. In addition, the cells were not possible to resuscitate by adding yeast extract (Paper III). These results are not surprising since it has been previously shown that bacterial populations incubated in nutrient-rich conditions for extended periods can eventually succumb to the build up of toxic byproducts, leading to cell death (Edwards, 2000; Postgate *et al.*, 1963).

Using 2D gel proteomics to study *P. fluorescens* SBW25, several proteins were only found to be differentially expressed under nutrient rich (LB) conditions compared to starvation conditions (PBS). Peptide sequences from 2D-gels were compared for cells incubated for 2 days under starvation conditions (PBS) or nutrient rich conditions (LB). A total of 58 proteins increased in abundance and 22 proteins decreased in abundance in LB compared to PBS. In addition, some proteins were synthesized only during one of the incubation conditions.

Six proteins were excised from the 2D-gels from cultures incubated in LB and sequenced. The protein identities were scored by comparing sequences from BLAST searches to scores of the SBW25 database genome at Oxford University, UK with similar results in both cases. Five of the 6 proteins identified from the 2D gels were identified as ABC transporters involved in transport of amino acids into the cell (Table 1). These data suggest that amino acids were available and utilized as nutrients after prolonged incubation in nutrient rich conditions.

The cells were in late stationary phase at the time of the sampling and therefore, also experiencing stress, although of a different kind than that encountered under starvation conditions. For example, the cells were probably exposed to a build up of toxic metabolites and also resorting in cryptic growth under prolonged nutrient rich conditions (Maraha et al., unpublished). However, using the 2D-gel was relatively labor intensive, compared to the shotgun technique where we were able to rapidly identify thousands of proteins under both growth conditions (see following section).

Table 1. Identities and functions of peptides that were identified as upregulated in LB compared to PBS after 2 days incubation using 2D gel proteomics

Locus	Spectral Average LB*	Spectral Average PBS*	Blast Score	ORNL number	Mass (kDa)	COG	Predicted function	Gene Name	COG Category
PFLU0826	18	16	0.0	Pfl0815	60.3	E	ABC-type dipeptide transport system periplasmic component	DdpA	Amino acid transport and metabolism
PFLU1000	18	39	0.0	Pfl0973	36.8	T	ABC-type amino acid transport/signal transduction systems periplasmic component/domain	HisJ	Amino acid transport and metabolism / Signal transduction mechanisms
PFLU1311	4.5	15	1.00E-119	Pfl1210	27.8	T	ABC-type amino acid transport/signal transduction systems periplasmic component/domain	HisJ	Amino acid transport and metabolism / Signal transduction mechanisms
PFLU1346	67	55	1.00E-178	Pfl1248	39.6	E	ABC-type branched-chain amino acid ransport system periplasmic component	LivK	Amino acid transport and metabolism
PFLU1812	0	1	1.00E-145	Pfl1606	30.7	E	ABC-type proline/glycine betaine transport systems periplasmic components	ProX	Amino acid transport and metabolism
PFLU5483	213	320	1.00E-101	Pfl5029	24.6	M	Outer membrane protein W	OmpW	Cell envelope biogenesis outer membrane

*Spectral averages of LB and PBS sample day2 from shotgun method.

5.1.2 Starvation

We also studied the impact of starvation conditions on the physiological status of specific populations. Our hypothesis was that starvation conditions in culture would be similar to the conditions encountered by microbial inoculants that are stressed in soil. For these experiments, the cells were suspended in phosphate-buffered saline, PBS, lacking any carbon, nitrogen and energy source. We observed that the bacterial populations survived better during long-term incubation under starvation conditions, compared to nutrient rich conditions discussed above (Papers II and III). In addition, in contrast to nutrient rich conditions where the majority of the cells died, the majority of the cells incubated under starvation conditions entered a dormant state. This hypothesis is supported by our findings that the total number of dead and viable cells did not add up to the total number of GFP-tagged cells enumerated by flow cytometry. We attributed the difference to a sub-fraction of the population that became dormant. In addition, the cells decreased in size and changed shape; growing more in groups and clumps. This starvation behaviour has been observed for several other microorganisms (Kaprelyants *et al.*, 1993a; Kaprelyants *et al.*, 1993b; Lopez-Amoros *et al.*, 1997; Oliver, 1993; Watanabe *et al.*, 2000; Yamamoto *et al.*, 1996).

It has previously been found that microorganisms first have relatively rapid responses to starvation conditions, followed by different long-term strategies, such as a lowering of metabolic activity, and if this becomes sufficiently low, dormancy may ensue (Kell *et al.*, 1998). From other studies, it is known that starvation starts a regulatory cascade leading to production of stress responsive proteins. For example, at the onset of the stationary phase *E.coli* cells undergo a global modification of their protein expression pattern, leading to a resistance to complex stresses such as increased cell density, presence of toxic byproducts, and nutrient limitation.

In Paper IV, we specifically studied the proteome of starved SBW25 cells and compared it to the proteome of SBW25 grown obtained under nutrient rich conditions.When studying the SBW25 proteome, we found that specific sets of proteins were upregulated only during starvation (PBS) compared to nutrient rich (LB) conditions. The COG categories of the upregulated proteins in PBS suggested that the expressed proteins represented proteins involved in DNA replication, recombination and repair, cell motility, chemotaxis and secretion/signal transduction systems. Chemotaxis is regarded as a stress avoidance mechanism (Mukamolova *et al.*, 2003). This suggests that the cells were stressed and were expressing chemotactic responses, including motility, in response to the lack of nutrients in PBS. Therefore, the cells responded in a predictable manner by producing proteins that would enable them to respond to nutrients should they become available. This may also be a mechanism used for survival in stressed environments in nature. In this study, the cells were incubated in an aqueous solution, so motility is a feasible option to use when searching for nutrients. This might not be

the same mechanism used for dormant cells in soil when motility options are much more limited. In addition, at least a subpopulation of the cells incubated under starvation conditions retained activity, as it is known that chemotaxis and motility are energy requiring processes.

5.2 Soil

Most studies today suggest that in plant and animal communities competitive interaction is the key determinant of species abundance and diversity, but little is known about the driving forces that determine the structure of more complex natural microbial communities, especially those in soil. Fluctuations in environmental conditions, which can lead to more evenness in populations, are not a prominent factor in soils, especially below the surface. Water-filled pores exist at the surface only immediately following rainfall; thereafter water paths come more tortuous and water activity decreases, reducing both microbe and solute movement. This may be one reason for the maintenance of high microbial diversity in soil (Zhou *et al.*, 2002).

It has been proposed that the normal dynamics of the soil microbial biomass is an oscillation between active and dormant physiological states, while significant growth occurs only at substantial substrate amendment. Usually, in soil only a minor part of the microbial biomass is generally metabolically active (Olsen *et al.*, 1987; Stenberg *et al.*, 1998; Stenström *et al.*, 2001). This proposed hypothesis is in agreement with our studies of bacterial populations in soil. Supporting this thesis, a study was done on substrate-induced respiration by either adding the nutrient glucose to soil samples. The observed reactions were that substrate-induced respiration reflected the response of two microbial groups in different physiological states in soil. One group of organisms immediately started to grow as a response to nutrient addition, whereas the other group only increasing their CO_2 production rate to a constant level (Stenström *et al.*, 1998; Stenström *et al.*, 2001).

In this work, we studied the physiological status of specific microbial populations in non-sterile soil (Papers II and III). Both *A. chlorophenolicus* A6 (Paper II) and *P. fluorescens* SBW25 (Paper III), showed similar survival trends after incubation in soil at 28 °C, as observed by a decrease in culturability and metabolic activity during the experimental period. However, we also observed that not all cells died, but that a sub-fraction of the population entered a dormant state in soil. When *A. chlorophenolicus* A6 was incubated in soil at 5°C, the situation was quite different. The cells survived much better in soil at this incubation temperature and also formed a large dormant fraction compared to incubation at 28 °C (Figure 5).

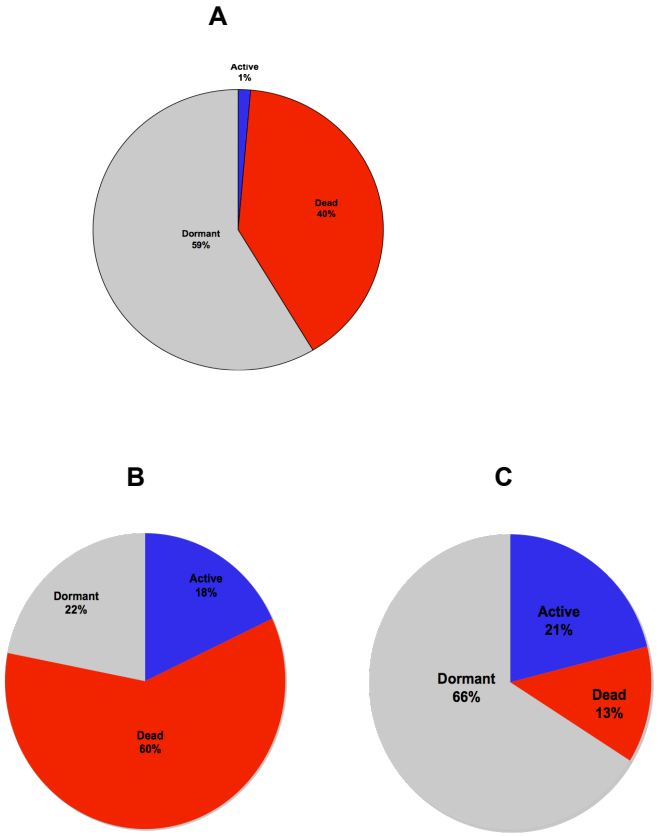

Figure 5. Representation of the physiological status of specific bacteria in soil as determined by flow cytometry in combination with viability stains. Blue: viable/active, CTC-stained cells; Red: dead, PI-stained cells; Grey: dormant, unstained cells (A) *P.fluorescens* SBW25, 28°C, (B) *A.chlorophenolicus* A6, 28°C, (C) *A.chlorophenolicus* A6, 5°C.

6 CONCLUSIONS

Flow cytometry in combination with GFP-tagging and viability staining enabled the physiological status of specific bacterial populations (gram positive and gram negative) to be monitored under different conditions in pure cultures and in non-sterile soil.

GFP-tagged cells *Pseudomonas fluorescens* A605 cells remain fluorescent following starvation and entrance into the VBNC state, but fluorescence is reduced when the cells die, presumably because of a deficiency in membrane integrity.

The majority of the *Pseudomonas fluorescens* SBW25 cell population died when the cells were incubated in nutrient rich medium. By contrast, a greater fraction of the cell population entered a “dormant state” when incubated under starvation conditions.

In soil, the *Pseudomonas fluorescens* SBW25 cell population exhibited physiological states reminiscent of those observed in both pure culture conditions. A large fraction of the population died, but a proportion of the cells also became presumably dormant.

Arthrobacter chlorophenolicus A6 survived in soil and in pure cultures at 5 °C in either an active state or in a presumable state of dormancy. The cells had poorer survival under nutrient rich conditions and in soil incubated at 28 °C.

Shotgun proteomics elucidated the composition of the baseline proteome of *Pseudomonas fluorescens* SBW25.

Several *P. fluorescens* SBW25 proteins were differentially upregulated according to incubation conditions and the identities of these proteins provide a clue as to the mechanisms that this microorganism uses to cope with stress due to starvation.

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