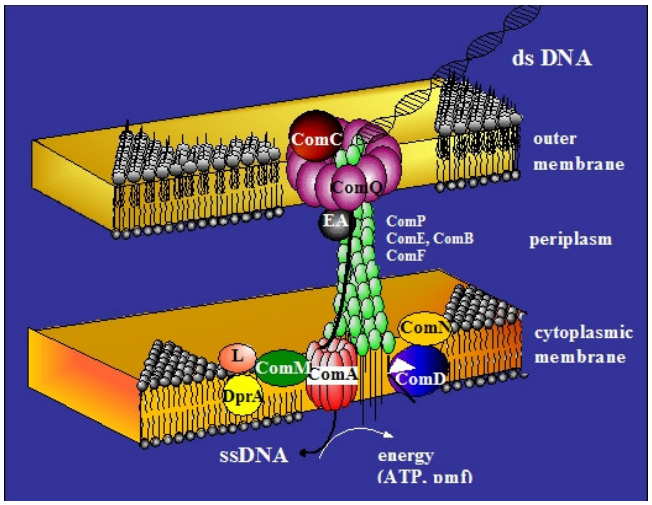
ANTIBIOTIC RESISTANCE PROBLEM IN AQUACULTURE: The role of *Com* gene products in DNA uptake as a nutrient source for bacteria



Master thesis in International Fisheries Management
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Cover picture:

Model of the transformation process in *Acinetobacter* sp. BD413

Source: http://cgi.server.unifrankfurt.de/fb15/mueller/averhoff/?html_content=research (08-04-08)

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ABSTRACT

Natural transformation is one of three mechanisms by which genetic material can be exchanged horizontally between bacteria and the only mechanism that the DNA is actively taken up by the recipient cell. It is also one of the mechanism by which bacteria acquire resistance against antibiotics and thus creating a growing concern worldwide for aquaculture industries. This study uses *Acinetobacter baylyi* BD413/wild strain to investigate the role of different *com* genes in DNA uptake as a nutrient source and thus strengthening the hypothesis that transformation evolved as a nutrient-uptake system. Since competition is induced by nutritional limitation in *Haemophilus influenzae* and *Bacillus subtilis*, model bacteria *Acinetobacter baylyi* BD413 were first grown in different concentrations of nitrogen (N) and phosphorus (P) to find out their nutritional limiting conditions. Afterwards, different concentrations of DNA were added into nutritionally limiting concentrations of N and P for these bacteria. The bacteria were then grown with and without DNA under these nutritional limiting conditions to see whether or not the DNA may be taken up by these bacteria as a nutrient source. *A. baylyi* was also grown without any carbon source in the medium and with DNA as a carbon source in the S2 medium to find out the role of DNA as the sole source of carbon and energy supporting microbial growth. *A. baylyi* growth was not found to be enhanced in phosphorus limiting conditions (below 5% P concentrations) but managed to grow even without any nitrogen sources in the medium. This suggests that *A. baylyi* could possibly get the nitrogen from dying cells during its growth phase. Adding different concentrations of DNA without any nitrogen sources in the media did not cause any significant increase in growth of *A. baylyi* suggesting that these bacteria do not take DNA as a nitrogen source. On the other side, adding DNA in phosphorus limited growth condition showed to induce significant increase in growth of *A. baylyi* suggesting that competence might be induced by phosphorus limited growth conditions for these bacteria. Like nitrogen, competence was not found to be induced by carbon limiting growth conditions since *A. baylyi* was not influenced by DNA and it may be grown even without any source of carbon.

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Mohammad Safayet Khan
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ABBREVIATIONS

bp	Base pairs
CFU	Colony forming units
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
HFIR	Homology - facilitated illegitimate recombination
HGT	Horizontal gene transfer
ssDNA	Single stranded DNA
NaCl	Sodium Chloride
S2	Minimal medium
S2L	S2 with lactate (20 mM)
LB	LB-Broth high salt
LBA	LB agar
S2-L	S2 without lactate
S2-P	S2 without phosphorus
S2-N	S2 without nitrogen
S2+DNA	S2 supplemented with DNA

Chapter One

INTRODUCTION

1.1. Antibiotic resistance as a problem in aquaculture

The major factors which influence the production and quality of seed in fish, prawn or shrimp hatcheries and their subsequent culture in grow-out systems are diseases and parasites affecting the stock. Aquaculture industries sometimes use antibiotics to control these fish diseases and parasites. Administration of low dose antibiotics for growth promotion has also led to the increased use of antibiotics in aquaculture. Types of antibiotics that are used varied form one control measure to another. In the UK four antibiotics are prescribed for aquaculture industries to treat fish diseases. In EU member states only four or five antibiotics are licensed for use in fin fish culture, and this restriction applies only to food fish not to companion (pet) or ornamental fishes. The extensive use of antibiotics in the late '1980's and early '1990's was due to the outbreak of bacterial diseases in intensive fish culture. The development of fish vaccines and the use of selected disease resistant stocks have greatly limited the utilization of antibiotics. The figure 1 shows the extreme reduction in the use of antibiotics in trout and salmon farming in Norway. Even though, the number of antibiotics may be higher through the so called 'prescription cascade'. In the USA, Canada and Norway the regulatory measures are stringent but this is not the case for other countries. There are actually no effective control on the use of antibiotics for food fish (or shell fish) in those countries (Alderman and Hastings 1998). Regulations for using them may be existed but not effectively enforced. There may also be no regimen for overlooking the use of antibiotics in the aquaculture practices. The potential consequences of the antibiotic use are the development of antibiotic-resistant microorganisms, multiple antibiotic resistance, resistance transfer to pathogenic bacteria, and reduced efficacy of antibiotic treatment for diseases caused by resistant pathogens (Frappaolo and Guest 1986).

Plasmid mediated resistance to antibiotics has been identified in *Aeromonas salmonicida*, *A. hydrophila, Vibrio arguillarum, Pseudomonas fluorescens, Pasteurella piscicida*, *Edwardsiella tarda* (Aoki 1988) and *Yersinia ruckeri* (DeGrandis and Stevenson 1985). Transferable R-plasmids have been found in *A. salmonicida* encoding resistance to chloarmphenicol, sulphonamide and streptomycin in Japan and to combinations of

sulphonamide, streptomycin, spectinomycin, trimethoprim and/or tetracycline in Ireland (Aoki 1997). Transferable resistance was also detected to combinations of oxytetracycline, streptomycin, sulphamethoxine, and/or trimethoprim. Laboratory studies with *A. salmonicida* have shown that resistance to oxolinic acid can readily be selected in the presence of antibiotics (Tsoumas et al. 1989).

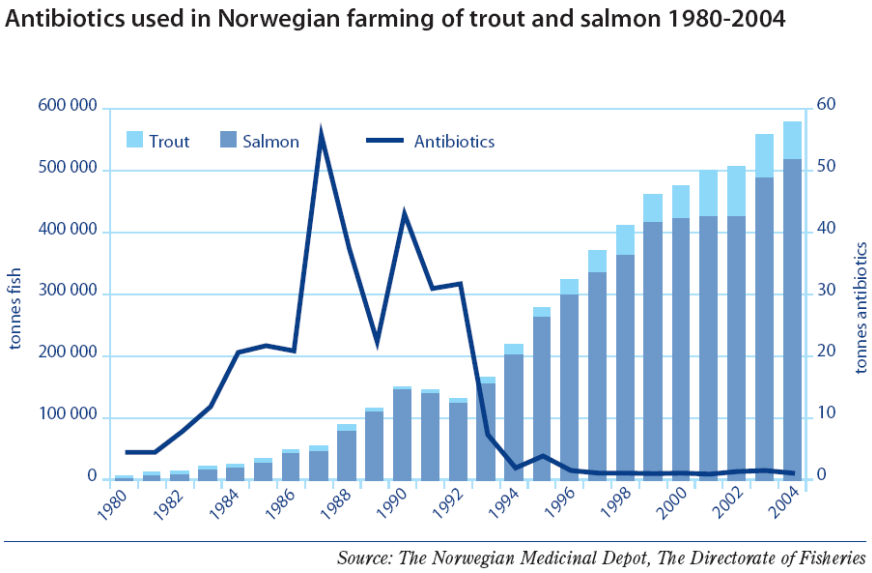

Figure 1: Antibiotics used in Norwegian farming of trout and salmon 1980-2004.

Resistance may arise because of gyr-A mutation (Oppegaard and Sørum 1994) or to the alterations in the outer membrane proteins that can be associated with cross-resistance to oxytetracycline (Barnes et al. 1990, Barnes et al. 1992).

1.2. Mechanisms of antibiotic resistance in bacteria

Antibiotics are generally designed to target chromosomally encoded proteins of single bacterial species which is responsible for causing anomaly in living system. A mutation in bacterial cell can develop antibiotic resistance to that targeted protein, which also renders the original protein to change its form and normal function. When no antibiotics are present in the environment, these resistant bacteria will be at a disadvantage and therefore will not spread or exist any longer. So, single chromosomal mutations alone are not the sole cause of bacterial resistance in the environment.

Unlike higher organisms bacteria can transfer all kinds of genes among closely and distantly related bacteria which is called Horizontal Gene Transfer (HGT). It is now generally believed that resistant genes are transferrable across major taxonomic divisions of bacteria and these genes travel not singly but in tandem arrays of several resistances to chemically distinct antibiotics (Summers et al. 1993).

1.3. Horizontal Gene Transfer (HGT) in bacteria

Horizontal Gene Transfer (HGT) is now considered as one of the main driving forces in microbial evolution and an important mechanism for the development of antibiotic resistances in Gram-positive and Gram-negative bacteria. Genetic exchange between bacteria can occur via 3 different HGT mechanisms: conjugation, transduction, and natural transformation. Conjugation is the transfer of mobile genetic elements (integrons, transposons, gene cassettes, etc.) on plasmids and requires specific genes and direct contact between donor and recipient cells. Transduction is viral-mediated gene transfer in which portions of the bacterial host chromosome are erroneously packaged into new virus particles when the virus enters the lytic phase; this bacterial DNA is transferred when the released virus particle infects a new host. Natural transformation is the simplest of the three mechanisms for genetic exchange and requires only naked/free DNA molecules and a competent recipient bacterium (Lorenz and Wackernagel 1994, Nielsen et al. 2001).

1.4. Molecular biology of natural transformation

In the natural transformation process, the recipient is the bacterium which takes up the free and naked DNA from the environment. The donor is the organism from which the DNA comes from. Neither physical contact nor proximity between the donor and recipient is required in natural transformation. The recipient bacteria must be exposed to environmental extracellular DNA molecules in the environment for natural transformation to takes place. DNA enters the environment through release from decomposing cells, disrupted cells of viral particles, or through excretion from living cells (Thomas and Nielsen 2005). A variety of genera of bacteria are also known to actively excrete DNA such as, *Acinetobacter*, *Alcaligenes*, *Aztobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Streptococcus* (Lorenz and Wackernagel 1994, Paget and Simonet 1994, Moscoso and Claverys 2004).

To date more than 40 bacterial species of very different phylogenetic and trophic groups have been shown their ability to take up free naked DNA via natural transformation (Lorenz and Wackernagel 1994).

Natural transformation in recipient bacteria is a sequential step-by-step process. Each step in the process represents a criterion that has to be met in order for the following steps to take place (Solomon et al. 1996, Dubnau 1999, Majewski 2001, Thomas and Nielsen 2005).

1.4.1. Development of competence

The first step in natural transformation is the development of competence. The transformable bacterium must be competent enough to be able to take up DNA. Competence expression in bacteria is a genetically-encoded physiological state that is regulated by many genes (e.g., *Com*-genes). These genes permit the bacteria to become competent to take up exogenous DNA into their cytoplasm (Lorenz and Wackernagel 1994, Dubnau 1999, Chen and Dubnau 2003). Many species only express competence genes and develop competence under specific conditions, often in response to stress, or in specific growth phases. Only *Niesseria gonorrhoeae* is found to be naturally competent regardless of environmental conditions (Thomas and Nielsen 2005). The development of competence has been found to involve approximately 20-50 genes.

1.4.2. DNA translocation into the cytoplasm

Competent bacteria have specific binding sites on the cell surface where extracellular DNA binds non-covalently (Thomas and Nielsen 2005). The number of binding sites varies from 30 to 80 in *Streptococcus pneumonia* and *Acinetobacter baylyi* respectively (Palmen and Hellingwerf 1997). When extracellular DNA binds to these binding sites on the cell surface of Gram-negative bacteria, it is translocated across the inner membrane. During translocation, the DNA is converted from double stranded (dsDNA) to single stranded (ssDNA) (Chen and Dubnau 2004). Most competent species take up DNA independent of its sequence, but some, for example *N. gonorrhoeae* and *Haemophilus influenzae* are selective in the DNA they translocate across the membrane (Lorenz and Wackernagel 1994).

The differences between species when it comes to both development of competence and uptake mechanism for DNA, suggest that possibly the functions of natural transformation differs between species (Palmen et al. 1993).

1.4.3. Integration of foreign DNA into host genome

Once the single stranded DNA has entered the cell it may invade the double stranded chromosome by homologous base-pairing, forming a heteroduplex molecule. The incoming DNA must contain regions of minimum 25-200 base pairs (bp) in length of high similarity to the recipient genome for the homologous base-pairing to occur (Thomas and Nielsen 2005). The stability of the heteroduplex molecule depends on the degree of similarity between donor and recipient DNA (Zawadski et al. 1995, Majewski and Cohan 1999). The formation of a heteroduplex molecule can result in integration of the foreign DNA via RecA- dependent recombination (Lorenz and Wackernagel 1994), which is a part of the machinery involved in DNA repair.

Integration of foreign DNA into the host chromosome can be occurred by three different recombination mechanisms. If the foreign DNA is identical to the host chromosome then it is called homogamic substitutive recombination (**Fig. 2a**). When a region of the donor DNA is similar but not exactly the same as the recipient and flanked by homologous regions, then it is called substitutive heterogamic recombination, allelic replacement (**Fig. 2b**). Additive integration is the mechanism which can occur when DNA present only in the donor is flanked by regions present in both donor and recipient (**Fig. 2c**). This can result in the recipient acquiring an antibiotic resistance gene. Additive integration can also happen when there is homology on one side of the invading DNA and random microhomology on the other, in a mechanism referred to as homology facilitated illegitimate recombination (HFIR) (de Vries and Wackernagel 2002) (**Fig. 2d**).

The fate of transforming chromosomal DNA depends on its ability to integrate into the host chromosome (Palmen et al. 1992, Palmen et al. 1993, Palmen and Hellingwerf 1997). As in other model bacteria such as *Escherichia coli* (Shen and Huang 1986), *Bacillus subtilis* (Harris-Warrick and Lederberg 1978, Majewski and Cohan 1999), *Streptococcus pneumoniae* (Majewski et al. 2000), and *Pseudomonas stutzeri* (Carlson et al. 1983, Lorenz et al. 1998, Sikorski et al. 2002) the frequency of recombination of transforming DNA into the host

chromosome is dependent upon the existence of DNA sequence similarity between donor and recipient in order for stable heteroduplex formation to occur.

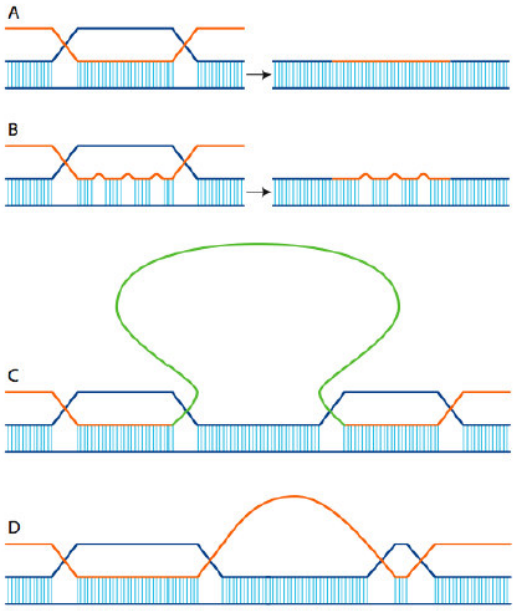

Figure 2. Various recombination types in bacteria. **(a)** Homogamic substitutive recombination in which donor (orange line) and recipient (blue line) DNA is sequence-identical, leading to no change in the recipient. **(b)** Heterogamic substitutive recombination occurs when some mismatches exist between donor and recipient. **(c)** Additive integration occurs when a unique DNA sequence (green line) in the donor is integrated into the recipient through homogamic/heterogamic recombination on each side of the unique DNA sequence. **(d)** HFIR, integration of donor DNA occurs by a stretch of homology on one side and random microhomology on the other side (figure by Jessica Louise Ray 2007).

1.4.4. Selection of integrated DNA

The integrated DNA can be neutral, deleterious or beneficial to the cell. For a transformation event to be maintained and propagated in a population of bacteria, it has to be positively selected for, in other words must somehow give the transformant an adaptive advantage over its non-transformed neighbors.

1.5. Relationships of resistance development to natural transformation

Natural transformation can occur with plasmids as well as chromosomal DNA fragments as donor DNA, however, transformation with plasmids is usually less efficient than transformation with chromosomal DNA (Nielsen et al. 1997).

Like chromosomal DNA fragments, plasmids are translocated across the membrane in a single stranded form and the uptake of plasmids requires complex steps to reassemble a circular duplex molecule in the recipient cytoplasm (Thomas and Nielsen 2005). In contrast with plasmid uptake, foreign DNA must be integrated into the recipient chromosome to survive in the cell. The integration is done by *rec/ruv* machinery in the cell.

The requirement of sequence similarity for homologous recombination to occur is a barrier to genetic exchange between distantly related species, however, numerous illegitimate means are available for integrating foreign DNA into the genome (Ochman et al. 2000). This mechanism facilitates to acquire antibiotic resistant genes from distantly related species such as, genetic exchange between bacteria and plant or fungi (Heineman and Sprague 1989, Garcia-Vallve, Romeu and Palau 2000).

1.6. Factors affecting the development of competence

Lack of sufficient competence is a key barrier to gene exchange by natural transformation in bacteria. The remaining barriers include DNA restriction in the cytoplasm, a limited ability of the incoming DNA to undergo heteroduplex formation with the recipient chromosome, stability of recombination intermediates, and finally, expression/stable maintenance of the transferred trait over generations.

The role of bacterial competence in long-term bacterial evolution and adaptation remains unclear. Expression of competence has been hypothesized to be beneficial due to the mechanisms contribution to DNA repair by providing undamaged alleles or repair/restoration of inactivated genes, uptake of DNA as a nutrient source and uptake of novel genetic variation and recombination of beneficial alleles. This thesis work will specifically examine the role of DNA as a nutrient source for bacteria growing under nutrient-limiting conditions.

1.7. *Acinetobacter baylyi* as a model organism to study HGT in bacteria

The genus *Acinetobacter* is a group of highly versatile, gram-negative bacteria which are ubiquitous in nature (Barbe et al. 2004). They are highly capable for adaptation and are found in different habitats, including water, soil, sewage, living organisms and as a part of the normal microflora on human skin. Several strains of the genus are capable of utilizing a variety of compounds (including hydrocarbons, aliphatic alcohols, glycols, carbohydrates and amino acids) as sole carbon and energy source. Bacteria of this group are strictly aerobic and possess a respiratory metabolism, oxidase-negative, catalase-positive, immobile and do not form spores. In the microscope they look like cocci (in stationary phase) or like short bacilli, they often appear in pairs or as longer chains (Barbe et al. 2004) (**Fig. 3**).

The *Acinetobacter baylyi* strain used in this study is called BD413 or ADP1 (European and American names respectively). BD413/ADP-1 is a microencapsulated mutant of a strain called BD4 which was difficult to use in the laboratory due to formation of mucoid colonies (Juni and Janik 1969), however BD413 is still naturally transformable like BD4. It is a Gram-negative water- and soil-bacterium in the λ-proteobacteria. It is prototrophic, aerobic and grows in both rich and minimal salts medium. It grows optimally at 33-37^0 C, but also grows at room temperature and can survive at 4^0 C for several days (Palmen et al. 1994). *A. baylyi* is an ideal model organism because it shares most of the features that make *Escherichia coli* a desirable lab strain, but unlike *E. coli*, it is naturally transformable (Metzgar et al. 2004). *A. baylyi* is non pathogenic to humans, but a relative, *A. baumanii*, is a growing cause of infection in immune-compromised hosts.

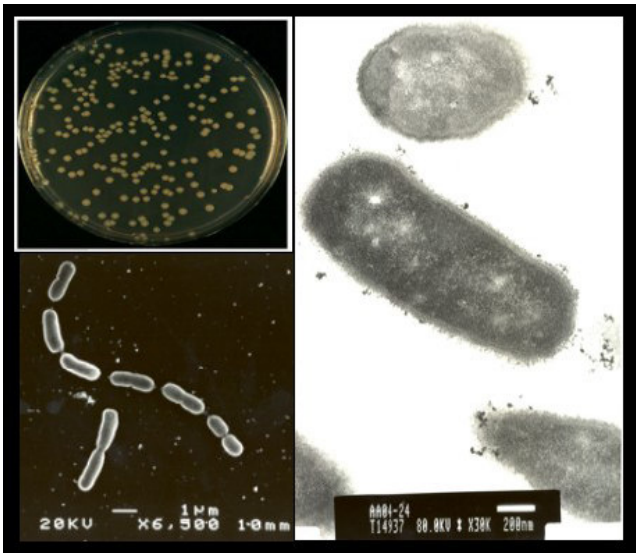

Figure 3. *Acinetobacter baylyi* strain BD413. Clockwise from top left: 2-day-old colonies on a LB plate, transmission electron micrograph, and scanning electron micrograph. (Photograph and micrographs by Kåre M. Nielsen).

Earlier studies of this strain demonstrated the development of natural genetic competence during exponential growth phase, with peak competence at early exponential phase (Juni and Janik 1969). Later studies have shown that BD413 is promiscuous for DNA uptake.

Because of its robust competence expression during exponential growth and its promiscuity with regard to DNA uptake (i.e. does not discriminate based on DNA source for the uptake over the cell membranes), BD413 is good model organism for studying the molecular barriers regulating the integration of foreign donor DNA after it has already entered the recipient cell. The *Acinetobacter baylyi* BD413 strain has between 20 and 50 genes involved in the natural transformation process. This thesis will examine the role of a selected subset of these genes and gene products as providers of DNA as a nutrient source under varying growth conditions.

1.8. Hypothesis

The testable hypothesis is that *Acinetobacter baylyi* BD413 with intact com genes will acquire DNA molecules from the nutrient limited growth medium and show a more rapid growth. Thus the com gene products role in ensuring an increased ability to utilize the DNA as nutrient sources present in the medium will be examined.

1.9. Aims and objectives

- To investigate the role of com gene products as providers of DNA as a nutrient source under nutritionally limited conditions.
- To perform a background study of the upcoming fitness assay experiment using both the wild type (BD413 strain) and com-gene knockouts to quantify the effect of com-genes on absolute and relative bacterial fitness.

Chapter Two

MATERIALS AND METHODS

2.1. Bacterial Strains

Acinetobacter baylyi BD413 (wild-type) is the bacterial strain used in this study, which was collected from the previously made freeze stock (at -70^{0}C) of this strain. It was originally isolated for a soil enrichment as a BD4 strain (Taylor and Juni 1961). Mutagenesis of this strain yielded a nonencapsulated mutant, called BD413 that does not form aggregates in culture (Juni and Janik 1969).

2.2. Growth Media

LB-Broth, high salt (LB) (Luria Bertani Bouillon, high salt) (Invitrogen, Germany/Fluka Sigma-Aldrich, India) was prepared by dissolving 20 g in 1 L distilled water followed by autoclaving (121^0 C for 20 minutes).

LB agar (LBA) was made by adding 12 g agar (Merck, Germany) to 25 g ***LB*** (Fluka Sigma-Aldrich, India) and then dissolving it in 1 L distilled water followed by autoclaving.

Minimal medium, ***S2*** was made with the following ingredients dissolved in 1 L distilled water and p^H adjusted to 6.7: 1.5 g of KH_2PO_4, 34 g of Na_2HPO_4 x 12 H_2O, 0.2 g of $MgSO_4$ x 7H_2O, 2 g of NH_4Cl, 1 mL of $CaCl_2$ 1% solution and 0.5 mL of $FeSO_4$ x 7 H_2O 1% solution. Carbon sources were added as appropriate: ***S2 with lactate*** **(*S2L*)** was made by adding 1.5 mL of lactic acid (20 mM) before autoclaving.

Minimal medium, S2 without phosphorus, ***S2-P*** was made with the following ingredients dissolved in 1 L distilled water and p^H adjusted to 6.7: 0.2 g of $MgSO_4$ x 7H_2O, 2 g of NH_4Cl, 1 mL of $CaCl_2$ 1% solution and 0.5 mL of $FeSO_4$ x 7 H_2O 1% solution. Carbon sources were added as appropriate: ***S2-P with lactate*** **(*S2L*)** was made by adding 1.5 mL of lactic acid (20 mM) before autoclaving.

Minimal Medium S2 without nitrogen, ***S2-N*** was made with the following ingredients dissolved in 1 L distilled water and p^H adjusted to 6.7: 1.5 g of KH_2PO_4, 34 g of Na_2HPO_4 x

12 H_2O, 0.2 g of $MgSO_4$ x $7H_2O$, 1 mL of $CaCl_2$ 1% solution and 0.5 mL of $FeSO_4$ x 7 H_2O 1% solution.

Carbon sources were added as appropriate: ***S2-N with lactate*** **(*S2L*)** was made by adding 1.5 mL of lactic acid (20 mM) before autoclaving.

Minimal Media S2 without carbon sources ***S2 -C*** was made with the following ingredients dissolved in 1 L milQ water and p^H adjusted to 6.7: 1.5 g of KH_2PO_4, 34 g of Na_2HPO_4 x 12 H_2O, 0.2 g of $MgSO_4$ x $7H_2O$, 1 mL of $CaCl_2$ 1% solution and 0.5 mL of $FeSO_4$ x 7 H_2O 1% solution. This media was then autoclaved (121^0 C for 20 minutes).

Minimal Media S2 supplemented with DNA ***S2+DNA*** was made with the following ingredients dissolved in 500 milQ water and P^H adjusted to 6.7: 1.5 g of KH_2PO_4, 34 g of Na_2HPO_4 x 12 H_2O, 0.2 g of $MgSO_4$ x $7H_2O$, 1 mL of $CaCl_2$ 1% solution, 0.5 mL of $FeSO_4$ x 7 H_2O 1% solution and 120 mL of DNA (300µg/mL).

2.3. Donor DNA

Deoxyribonucleic acid sodium salt from calf thymus (***DNA***) (Sigma Aldrich, Germany) was used in this study. This DNA is a high molecular weight DNA prepared from male and female calf thymus tissue. The molecular weight is reported to be between 10-15 million Daltons. It contains 41.9 mole % G-C and 58.1 mole % A-T (Marmur and Doty 1962). To prevent shearing of this large, genomic DNA, this was dissolved at 1 mg/mL of autoclaved (121^0 C for 20 minutes) distilled water with no sonication and stirring. Gently inversion overnight at 4^0C was done to completely solubilize the DNA.

2.4. Sterile Saline

0.9% NaCl was prepared by dissolving 9 g of NaCl in 1 L of distilled water and then autoclaved (121^0 C for 20 minutes).

2.5. 50% Glycerin

50% glycerin was prepared by dissolving 50 mL of 100% glycerine into 50 mL of distilled autoclaved (121^0C for 20 minutes) water.

2.6. Methods

Acinetobacter species are able to utilize a wide range of organic compounds as single sources of carbon, including aromatic compounds (benzoate, salicylate) and carboxylic acids, such as caproic (hexanoic) acid (Bauman et al. 1968).

The experiment was performed in S2-minimal medium supplied with one carbon source that is efficiently utilized (lactate, 0.1%) and N and P sources according to determined growth limiting concentrations. In addition the medium was supplemented with various concentrations of DNA. The culture medium was minimal S2 medium (Juni 1974) with growth-limiting concentrations of the carbon source (lactate, 0.1%).

The competition assays were performed over a 96 hour period. All the experiments were done in triplicate using 50 mL Falcon tubes and two LBA plates/tube for a total of 6 culture plates per treatment.

Day 1.

An overnight culture (ON culture) was made by inoculating 3 ml of LB media with one colony of bacteria in a 15 mL conical tube (NUNC, USA), and incubating it overnight at 37^0 C with shaking.

Day 2.

The tube was centrifuged in an Eppendorf 5810R bench top cooling centrifuge (Eppendorf, Germany) for 10 minutes, at 4000 rpm at 4^0 C. The supernatants were discarded. The pellets were resuspended in 3 mL sterile saline solution. This resuspended solution was then diluted 1:10, 1:100, 1:1000 and 1:100000 with sterile saline. Dilution series thus made from the $1:10^{-5}$ cells/sterile saline in 10-fold steps from 10^{-1} to 10^{-4} with sterile saline. To enumerate the initial bacterial colony, 0.1 mL of the 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution was plated on two LBA plates (FRAGILE, Norway) for each dilution.

0.1 mL of the $1:10^{-5}$ cells/sterile saline was then inoculated with 5 mL of different concentrations (50%, 5%, 2.5%, 1.25%, 0.75% and 0%) of nitrogen and (50%, 5%, and 4%) of phosphorus in 50 mL Falcon tubes (SASTEDT, Germany). These cells were also inoculated with varying amounts of DNA in previously determined nitrogen (6, 30, 60 and 300 µg/mL of DNA with 0% N) and phosphorus (only 6 µg/mL with 4% P) limiting concentrations at this stage. These tubes were then incubated at 37^0 C with shaking (120 rpm)

for 24, 48, 72 and 96 hours. The lids on the Falcon tubes were left loose to avoid development of anaerobic conditions.

Day 3.

Initial bacterial colony was counted. To see the bacterial growth after 24 hours of incubation, in different nutrient with and without varying DNA concentrations, 0.1 mL of the cells/ media was then diluted in 10-fold steps from 10^{-1} to 10^{-7} with sterile saline. To enumerate bacterial colony after exponential phase 0.1 mL of the different dilutions (10^{-6} and 10^{-7} for 50% N; 10^{-5} and 10^{-6} for 5% N; 10^{-5} and 10^{-6} for 2.5% N; 10^{-4} and 10^{-5} for 1.25% N; 10^{-4} and 10^{-5} for 0.75% N and 10^{-3} and 10^{-4} for 0% N; 10^{-5} and 10^{-6} for 50% P; 10^{-6} and 10^{-7} for 5% P; undiluted and 10^{-1} for 4% P; 10^{-3} and 10^{-4} for 0% N with DNA;10^{-1} and 10^{-2} for 4% P with DNA) were plated on two LBA plates (FRAGILE, Norway) for each dilution.

Day 4.

Bacterial colonies were counted after 24 hours of incubation. To see the bacterial growth in different nutrient and DNA concentrations after 48 hours of incubation 0.1 mL of the cells/ media was then diluted in 10-fold steps from 10^{-1} to 10^{-7} with sterile saline. To enumerate bacterial colony at the end of the exponential phase 0.1 mL of the different dilutions (10^{-6} and 10^{-7} for 50% N; 10^{-5} and 10^{-6} for 5% N; 10^{-5} and 10^{-6} for 2.5% N; 10^{-4} and 10^{-5} for 1.25% N; 10^{-4} and 10^{-5} for 0.75% N and 10^{-3} and 10^{-4} for 0% N; 10^{-6} and 10^{-7} for 50% P; 10^{-5} and 10^{-6} for 5% P; undiluted and 10^{-1} for 4% P; 10^{-3} and 10^{-4} for 0% N with DNA;10^{-6} and 10^{-7} for 4% P with DNA) was plated on two LBA plates (FRAGILE, Norway) for each dilution.

Day 5.

Bacterial colonies were counted after 48 hours of incubation. To see the bacterial growth in different nutrient and DNA concentrations after 72 hours of incubation 0.1 mL of the cells/ media was then diluted in 10-fold steps from 10^{-1} to 10^{-7} with sterile saline. To enumerate bacterial colony at the stationary phase 0.1 mL of the different dilution (10^{-6} and 10^{-7} for 50% N; 10^{-5} and 10^{-6} for 5% N; 10^{-5} and 10^{-6} for 2.5% N; 10^{-4} and 10^{-5} for 1.25% N; 10^{-4} and 10^{-5} for 0.75% N and 10^{-3} and 10^{-4} for 0% N; 10^{-5} and 10^{-6} for 50% P; 10^{-4} and 10^{-5} for 5% P; 10^{-3} and 10^{-4} for 0% N with DNA;10^{-5} and 10^{-6} for 4% P with DNA) was plated on two LBA plates (FRAGILE, Norway) for each dilution.

Day 6.

Bacterial colonies were counted after 72 hours of incubation. To see the bacterial growth in different nutrient and DNA concentrations after 96 hours of incubation,0.1 mL of the cells/different concentrations of nitrogen and phosphorus media was diluted in 10-fold steps from 10^{-1} to 10^{-7} with sterile saline. To enumerate bacterial colony at the end of the stationary phase 0.1 mL of the different dilution (10^{-5} and 10^{-6} for 50% N; 10^{-5} and 10^{-6} for 5% N; 10^{-5} and 10^{-6} for 2.5% N; 10^{-4} and 10^{-5} for 1.25% N; 10^{-4} and 10^{-5} for 0.75% N and 10^{-3} and 10^{-4} for 0% N; 10^{-5} and 10^{-6} for 50% P; 10^{-5} and 10^{-6} for 5% P; 10^{-3} and 10^{-4} for 0% N with DNA;10^{-5} and 10^{-6} for 4% P with DNA) was plated on two LBA plates (FRAGILE, Norway) for each dilution.

Day 7.

Bacterial colonies were counted after 96 hours of incubation. All the data was collected by growing the bacteria for each concentration of media in triplicate (in three 15mL Falcon tubes) and plated the overnight culture of bacteria at the media in duplicate LBA plates (2 LB agar plates for each Falcon tubes. The data was plotted in Microsoft Excel spreadsheet software and growth curve was generated.

2.7. Statistical Analysis

First CFU raw data were logarithmically transformed (Microsoft Excel). The normality of the log- transformed data distribution was then verified using the Shapiro -Wilk test (Systat 11), and the homogeneity of variances by Hartley's F-max test (Microsoft Excel). Log-transformed data were analyzed by ANOVA (Systat 11). *Post Hoc* comparisons were performed by Tukey's procedure (Systat 11). All data sets containing only two groups (e.g. phosphorus with and without DNA and carbon growth experiment) were analyzed by two-sample T-test with pooled variances (Systat 11). Data were expressed as mean ±95% confidence interval (CI), and statistical differences were accepted as significant when $P<0.005$.

Chapter Three
RESULTS

3.1. Nitrogen growth curve for *Acinetobacter baylyi* BD413

To study the effect of variable nitrogen concentration on bacterial growth, the N concentration in S2 media was adjusted from 50% to 5% . The growth was measured over 4 days and the results are shown in figure 4a. The precise number of CFU used to start the experiment was 20600 (± 1,018 x 10^3) CFU/mL.

At the end of the exponential phase after 24 hours of incubation in 50% N medium, the viable cell counts of *A. baylyi* BD413 reached ~164 x 10^6 (± 1,63 x 10^8) CFU/mL. In 5%, 2.5%, 1.25%, 0.75% and 0% N medium it was ~ 44 x 10^6 (± 7,080 x 10^7), ~ 175 x 10^5 (± 2,92 x 10^7), ~ 89 x 10^5 (± 1,46 x 10^7) , ~ 60 x 10^5 (± 6,67 x 10^6) and ~ 31 x 10^4 (± 4,10 x 10^5) CFU/mL respectively. There was overall differences in CFU/mL among different groups (F=684,459; df = 5; P<0,001). Lowest growth was recorded in 0% N and highest was in 50% N. CFU/mL progressively increases with increasing concentrations of nitrogen.

After 48 hours of incubation the bacteria enters into a stationary phase and in 50%, 5%, 2.5%, 1.25%, 0.75% and 0% N medium, the viable cell counts of *A. baylyi* BD413 was ~ 156 x 10^6 (± 1,40 x 10^8), ~ 59 x 10^6 (± 8,85 x 10^7), ~ 58 x 10^5 (± 1,25 x 10^7), 35 x 10^5 (± 5,96 x 10^6), 290 x 10^4 (± 4,59 x 10^6), and ~266 x 10^3 (± 2,04 x 10^5) CFU/mL respectively. General differences were found among different groups (F = 872,597; df = 5; P<0,001). In particular highest growth was found in 50% N which is different (P<0,001) from all others. 0,75% N and 1,25% N are not significantly different. The lowest growth was in 0% N which was also significantly different than the others (P<0,001).

After 72 hours of incubation in 50%, 5%, 2.5%, 1.25%, 0.75% and 0% N medium, the viable cell counts of *A. baylyi* BD413 was ~72 x 10^6 (± 1,43 x 10^8) CFU/mL, ~56 x 10^6 (± 8,15 x 10^7) CFU/mL, ~60 x 10^5 (± 1,23 x 10^7) CFU/mL, ~48 x 10^5 (± 6,51 x 10^6) CFU/mL, ~34 x 10^5 (± 6,06 x 10^6) CFU/mL and ~268 x 10^3 (± 3,11 x 10^5) CFU/mL respectively.

Significant differences were found (F = 493,114; df = 5; P<0,001) after 72 hours among different groups. 50% N and 5% N were not significantly different in terms of CFU/mL and thus no change in growth was found with increasing nitrogen concentrations. 0% N was always different than the others (P<0,001). Lowest growth was constantly in evidence at 0% N.There was no differences between 0,75% N, 1,25% N and 2,5% N but 2,5% N was significantly different from 5% N and 50% N.

After 96 hours of incubation in 50%, 5%, 2.5%, 1.25%, 0.75% and 0% N medium, the viable cell counts of *A. baylyi* BD413 was 209 x 10^5 (± 1,32 x 10^7) CFU/mL, 35 x 10^6 (± 8,81 x 10^7) CFU/mL, ~71 x 10^5 (± 1,07 x 10^7) CFU/mL, ~291 x 10^4 (± 6,82 x 10^6) CFU/mL, ~212 x 10^4 (± 1,96 x 10^6) CFU/mL and ~245 x 10^3 (±6,23 x 10^5) CFU/mL respectively. There was overall differences in CFU/mL among different groups (F= 268,996; df = 5; P<0,001). Lowest growth was in 0% N followed by 0,75% N. Significant diffrences were found between 0% N and other groups (P<0,001). No differences were observed between 0,75% N and 1,25% N. Differences in growth was also found in 2,5% N, 5% N and 50% N (P<0,001). Equally higher CFU/mL values were found in 5% N and 50% N.

The key ovservations of the reduced N-levels indicates that *A. baylyi* still manage to grow at reduced rate even without any nitrogen source in the medium.

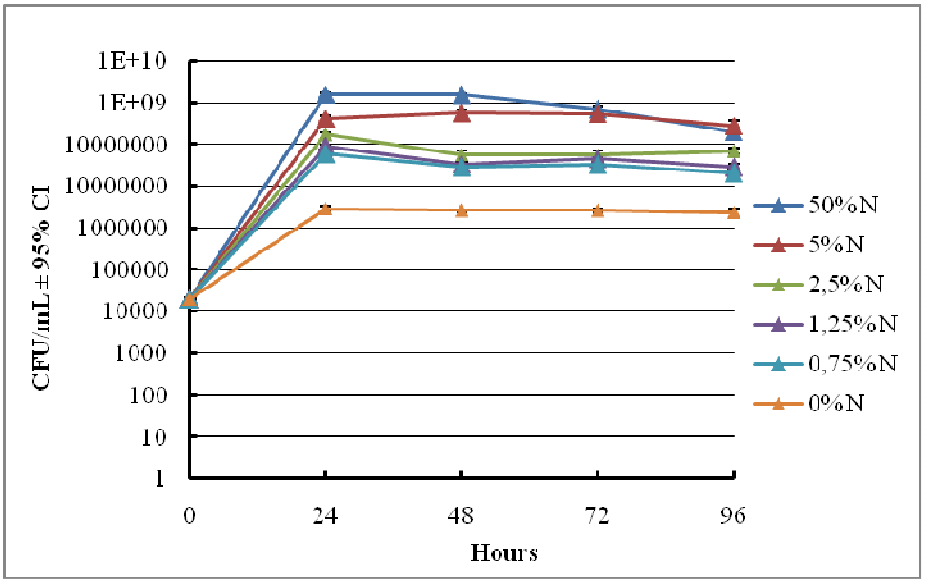

Figure 4a. *Acinetobacter baylyi* BD413 growth curve with varying concentrations of nitrogen (t_0 = 20600 CFU/mL).

3.2. Nitrogen growth curve for *Acinetobacter baylyi* BD413 with and without DNA

To study the effect of DNA on nitrogen limiting growth medium for *A. baylyi*, 6 µg/mL of DNA was added in 0% N. The growth was measured over 4 days and the results are shown in figure 4b. The precise number of CFU used to start the experiment was 28300 (± 1,13 x 10^2) CFU/mL.

At the end of the exponential phase after 24 hours of incubation in 5% N medium, the viable cell counts of *A. baylyi* BD413 was reached to ~269 x 10^5 (± 2,44 x 10^7) CFU/mL and in 0% N medium the viable cell was 123 x 10^3 (± 5,25 x 10^4) CFU/mL. 5% of Nitrogen was used in this experiment as a positive control and 0% N was considered negative control though bacterial growth was still evidence at 0% N concentration level. In 0% N concentration with 6 µg/mL of DNA the viable cell was ~166 x 10^3 (± 1,32 x 10^5) CFU/mL at the end of the exponential phase. There was overall differences in CFU/mL among different groups (F=6070,102; df = 2; P<0,001). Growth in 0% N was significantly different than others (P<0,001). Again growth in 0% N with DNA was also different than the other two growth media (P<0,001). Lowest growth was recorded in 0% N and highest was in 5% N.

After 48 hours of incubation the bacteria enters into a stationary phase and in 5%, 0% and 0% N with DNA the viable cell counts of *A. baylyi* BD413 was ~35 x 10^6 (± 2,12 x 10^7) CFU/mL, ~103 x 10^3 (± 6,41 x 10^4) CFU/mL, and ~165 x 10^3 (± 1,39 x 10^5) CFU/mL respectively. General differences were found among different groups (F = 7628,004; df = 2; P<0,001). In particular highest growth was found in 5% N which is different (P<0,001) from all others. The lowest growth was in 0% N which was also different (P<0,001) than the other two groups.

After 72 hours of incubation in 5%, 0% and 0% N with DNA the viable cell counts of *A. baylyi* BD413 was 32 x 10^6 (± 1,25 x 10^7) CFU/mL, ~113 x 10^3 (± 1,31 x 10^5) CFU/mL and ~177 x 10^3 (± 3,20 x 10^5) CFU/mL respectively. Significant differences were found among different groups (F=2607,083; df = 2; P<0,001) after 72 hours. 5% N and 0% N were significantly different (P<0,001) and increased in growth was found with increasing nitrogen concentrations.

0% N was always different (P<0,001) than the other groups. Lowest growth was constantly in evidence at 0% N. Adding DNA had a significant increase (P<0,001) in growth than without DNA.

After 96 hours of incubation in 5%, 0% and 0% N with DNA the viable cell counts of *A. baylyi* BD413 was ~166 x 10^5 (± 9,64 x 10^6) CFU/mL, ~89 x 10^3 (± 1,02 x 10^5) CFU/mL and ~185 x 10^3 (± 1,58 x 10^5) CFU/mL. There was overall differences in CFU/mL between different groups (F=3621,481; df = 2; P<0,001). Lowest growth was in 0% N followed by 0% N with DNA. Significant diffrences were found between 0% N and others (P<0,001). Differences in growth (P<0,001) was also found in 5% N and 0% N with DNA

The key ovservations of the DNA on reduced N-levels here is that adding a certain amount (6 µg/mL) of DNA cause significant increase (P<0,001) of growth of *A. baylyi*.

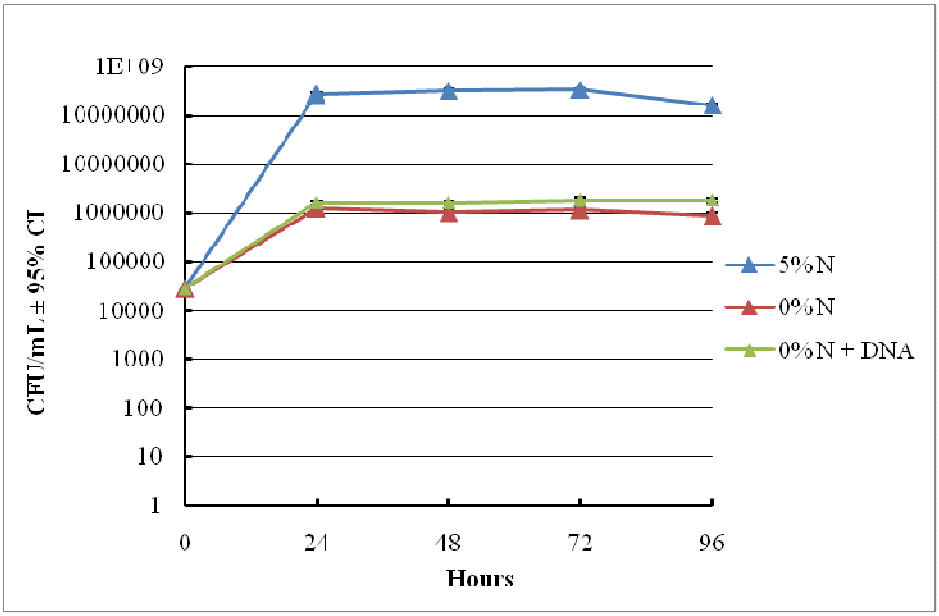

Figure 4b: *Acinetobacter baylyi* BD413 grown in nitrogen with and without DNA (t_0 =28300 CFU/mL).

3.3. Nitrogen growth curve for *Acinetobacter baylyi* BD413 with different concentrations of DNA and without DNA

To study the effect of variable DNA concentrations on bacterial growth, the DNA concentration in S2-N media was adjusted to 30 µg/mL, 60 µg/mL and 300 µg/mL. The growth was measured over 4 days and the results are shown in figure 4c. The precise number of CFU used to start the experiment was 16000 (± 9,05 x 10^2) CFU/mL.

At the end of the exponential phase after 24 hours of incubation in 5% N medium the viable cell counts of *A. baylyi* BD413 was reached to ~247 x 10^5 (± 5,17 x 10^7) CFU/mL and in 0% N medium the viable cell was 65 x 10^3 (± 1,57 x 10^5) CFU/mL. 5% of Nitrogen was used in this experiment as a positive control and 0% N was considered negative control though bacterial growth was still evidence at 0% N concentration level. In 0% N concentration level varying amount of DNA was added. After 24 hours in 0% N with 1, 5, 10 and 50 times 6 µg/mL of DNA the viable cell was ~169 x 10^3 (± 2,95 x 10^5) CFU/mL, ~123 x 10^3 (± 7,72 x 10^4) CFU/mL and ~283 x 10^3 (± 2,03 x 10^6) CFU/ mL respectively. Overall differences in CFU/mL were observed among different groups (F = 180,902; df = 4; P<0,001). No differences were found between 0% N and 0% N with 5 times DNA. However growth in 0% N was significantly different (P< 0,001) than 0% N with 10 and 50 times DNA and also with 5% N. Growth in 0% N with 5 times DNA does not show any differences with the growth in 0% N with 10 times and 50 times of DNA but significantly different (P<0,001) than the 5% N. No differences were observed in 0% N with 10 times DNA and 0% N with 50 times DNA, however, the last one was significantly different (P<0,001) than 5% N.

After 48 hours of incubation the bacteria enter into a stationary phase and in 5% N medium the viable cell counts was reached to ~42 x 10^6 (± 7,70 x 10^7) CFU/mL and in 0% N medium the viable cell was ~100 x 10^3 (± 1,63 x 10^5) CFU/mL . In 0% N with 5, 10 and 50 times 6 µg/mL of DNA the viable cell was ~187 x 10^3 (± 4,15 x 10^5) CFU/mL, ~130 x 10^3 (± 3,33 x 10^4) CFU/mL and ~242 x 10^3 (± 1,93 x 10^6) CFU/mL respectively. Overall differences in CFU/mL were observed among different groups (F = 172,497; df = 4; P<0,001). But, no significant differences were found between groups. However growth in 5% N was significantly different (P<0,001) than other groups.

After 72 hours of incubation in 5% N medium the viable cell counts was reached to ~38 x 10^6 (± 6,91 x 10^7) CFU/mL and in 0% N medium the viable cell was ~80 x 10^3 (± 2,69 x 10^5) CFU/mL respectively. In 0% N with 5, 10 and 50 times 6 µg/mL of DNA the viable cell was ~147 x 10^3 (± 4,79 x 10^5) CFU/mL, ~115 x 10^3 (± 1,29 x 10^5) CFU/mL and 89 x 10^3 (± 5,47 x 10^5) CFU/mL respectively. Overall differences were observed among different groups (F = 206,135; df = 4; P<0,001). But, no significant differences were found between groups. However growth in 5% N was significantly different (P<0,001) than other groups.

After 96 hours of incubation in 5% N medium the viable cell count reached ~266 x 10^5 (± 5,87 x 10^7) CFU/mL and in 0% N medium the viable cell was ~73 x 10^3 (± 7,27 x 10^4) CFU/mL. In 0% N with 5, 10 and 50 times 6 µg/mL of DNA the viable cell was ~223 x 10^3(± 5,14 x 10^5) CFU/mL, ~112 x 10^3 (± 7,47 x 10^4) CFU/mL and ~94 x 10^3 (± 6,11 x 10^5) CFU/mL respectively. Overall differences in CFU/mL were observed among different groups (F = 262,575; df = 4; P<0,001). Growth was not significantly different in 0% N and 0% N with 5 times DNA and 50 times DNA but different (P<0,001) in 0% N and 0% N with 10 times DNA and in 5% N. Again growth in 0% N with 5 times DNA is not different with 50 times DNA but significantly different (P<0,05) in 0% N with 10 times and and also highly different (P<0,001) with 5% N. Differences in growth (P<0,01) was observed in 0% N with 10 times DNA with 0% N with 50 times DNA. Growth in 0% N with 50 times of DNA was also significantly different (P<0,001) than 5% N.

The key ovservations of the varying DNA concentrations on reduced N-levels here is that adding higher concentrations of DNA does not cause a significant increase in the growth of *A. baylyi*.

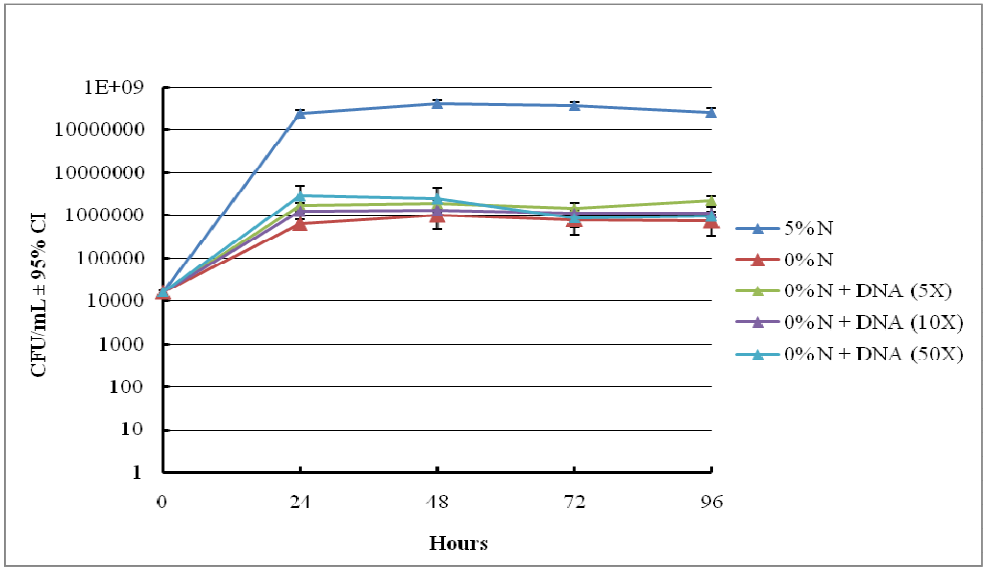

Figure 4c: *Acinetobacter baylyi* BD413 grown in nitrogen with different concentrations of DNA and without DNA (t_0 =16000 CFU/mL).

3.4. Nitrogen growth curve for *Acinetobacter baylyi* BD413 with different concentrations of DNA and without DNA in milQ water

To study the effect of variable DNA concentrations on bacterial growth in S2-N media prepared with milQ water instead of distilled water, the DNA concentration was adjusted to 6 µg/mL, 30 µg/mL, 60 µg/mL and 300 µg/mL as before. The growth was measured over 4 days and the results are shown in figure 4d. The precise number of CFU used to start the experiment was ~13900 (± 1,75 x 10^3) CFU/mL.

At the end of the exponential phase after 24 hours of incubation in 0% N the viable cell count was 54 x 10^3 (± 1,06 x 10^5) CFU/mL. 0% N was considered negative control though bacterial growth was still evidence at 0% N concentration level. In 0% N concentration level varying amount of DNA were added. After 24 hours in 0% N with 1, 5, 10 and 50 times of 6 µg/mL of DNA, the viable cell was 69 x 10^3 (± 1,45 x 10^5) CFU/mL, ~64 x 10^{-3} (± 2,04 x 10^5) CFU/mL, 75 x 10^{-3} (± 2.15 x 10^5) CFU/mL, and ~67x 10^{-3} (± 2,53 x 10^5) CFU/mL respectively. No significant differences were found among groups.

After 48 hours of incubation the bacteria enters into a stationary phase and in 0% N medium the viable cell was ~62 x 10^3 (± 2,67 x 10^5) CFU/mL. In 0% N with 1, 5, 10 and 50 times of 6 µg/mL of DNA the viable cell was ~63 x 10^3 (± 1,73 x 10^5) CFU/mL, ~84 x 10^3(± 1,46 x 10^5) CFU/mL, ~87 x 10^3 (± 2,49 x 10^5) CFU/mL and ~107 x 10^3 (± 8,70 x 10^5) CFU/ mL respectively. No significant differences were also found among groups after 48 hours.

After 72 hours of incubation in 0% N medium the viable cell was ~44 x 10^3 (± 1,41 x 10^5) CFU/mL and in 0% N with 1, 5, 10 and 50 times of 6 µg/mL of DNA the viable cell was ~48 x 10^3 (± 1,01 x 10^5) CFU/mL, ~75 x 10^3 (± 3,26 x 10^5) CFU/mL, ~83 x 10^3 (± 2,17 x 10^5) CFU/mL and ~52 x 10^3 (± 7,94 x 10^4) CFU/mL respectively. Significant differences were observed among different groups (F=3,610; df = 4; P<0,05). Growth in 0% N and 0% N with 5 times DNA were significantly different (P<0,05). However no differences in growth were observed in other groups..

After 96 hours of incubation in 0% N medium the viable cell was ~51 x 10^3 (± 5,78 x 10^4) CFU/mL and in 0% N with 1, 5, 10 and 50 times 6 µg/mL of DNA the viable cell was ~92 x 10^3 (± 8,55 x 10^4) CFU/mL, ~122 x 10^3 (± 4,43 x 10^5) CFU/mL, ~85 x 10^3 (± 2,45 x 10^5) CFU/mL and ~33 x 10^{-3} (± 3,33 x 10^4) CFU/mL respectively. Overall differences in CFU/mL were observed among different groups (F = 17,17; df = 4; P<0,001). Growth was significantly different (P<0,05) in 0% N and 0% N with 1 times DNA, 5 times DNA and highly different (P<0,01) in 10 times DNA but not different in 0% N with 50 times DNA. No differences in growth were observed in between other groups.

The key ovservations of the varying DNA concentrations on S2-N media prepared with milQ water is that adding higher concentrations of DNA does not necessarily cause any significant increase of growth of *A. baylyi* even in nitrogen limiting conditions. Different water sources also do not affect growth under limiting N concentrations.

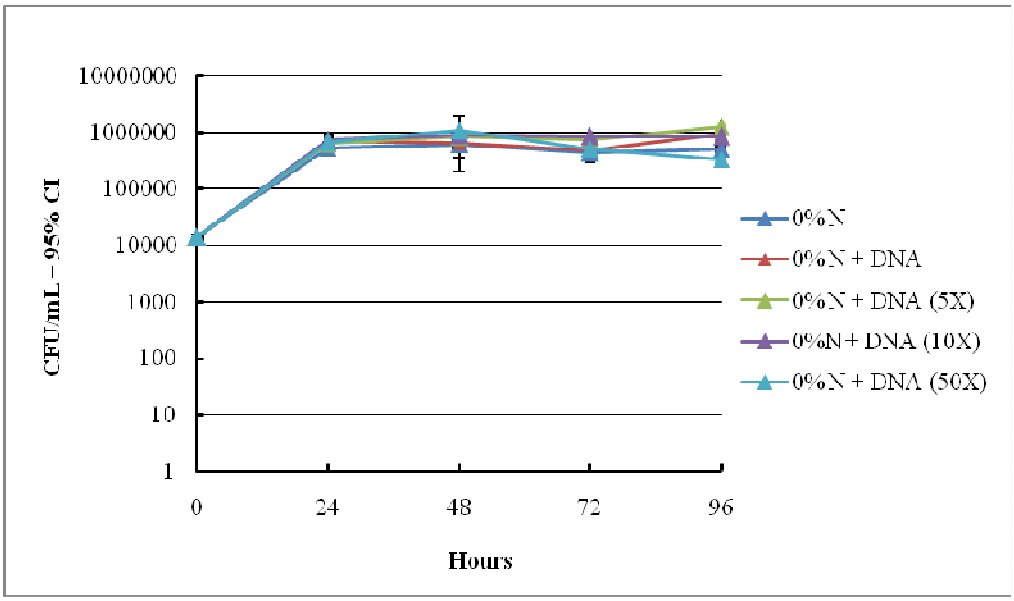

Figure 4d: *Acinetobacter baylyi* BD413 grown in nitrogen with different concentrations of DNA and without DNA in milQ water (t_0 =13900 CFU/mL).

3.5. Phosphorus growth curve for *Acinetobacter baylyi* BD413

To study the effect of variable phosphorus concentration on bacterial growth, the P concentration in S2 media was adjusted from 50% to 4% . The growth was measured over 4 days and the results are shown in figure 5a. The precise number of CFU used to start the experiment was 21400 (± 5,65 x 10^2) CFU/mL.

At the end of the exponential phase after 24 hours of incubation in 50% P medium, the viable cell counts of *A. baylyi* BD413 reached ~80 x 10^5 (± 8,68 x 10^6) CFU/mL. In 5% and 4% P medium it was ~155 x 10^6 (± 5,24 x 10^8) CFU/mL and only ~107 (± 24,61) CFU/mL respectively. There was overall differences in CFU/mL among different groups (F = 3894; df = 2; P<0,001). Growth in 5% P was significantly different (P<0,001) than others. Again growth in 50% P was also different than the other two growth media (P<0,001). Lowest growth was recorded in 4% P and highest was in 5% P.

After 48 hours of incubation the bacteria enters into a stationary phase and in 50% and 5% P medium the viable cell count was ~115 x 10^6 (± 1,02 x 10^8) CFU/mL and ~70 x 10^{-5} (± 6,42 x 10^7) CFU/mL. No growth was evident in 4% P concentration level after 48 hours. CFU values were higher in 5% P than in 50%P (t=-6.462; df =10; P<0.001).

After 72 hours of incubation in 50% and 5% P media the viable cell count was ~154 x 10^5 (± 1,43 x 10^7) CFU/mL and ~39 x 10^5 (± 2,12 x 10^7) CFU/mL. CFU values were higher in 50% P than in 5%P (t=-5.059; *df* =10; *P*<0.001).

After 96 hours of incubation in 50% and 5% P media the viable cell count was ~108 x 10^5 (± 3,09 x 10^7) CFU/mL and ~36 x 10^5 (± 2,24 x 10^7) CFU/mL respectively. CFU values were higher in 50% P than in 5%P (t=-3.919; *df*=10; *P* = 0.003).

All this shows that after an initial higher CFU value recorded in 5% P in the first 24h of culture, the highest values are then obtained in 50% P at all times. *A. baylyi* does not manage to grow below 4% P concentration level.

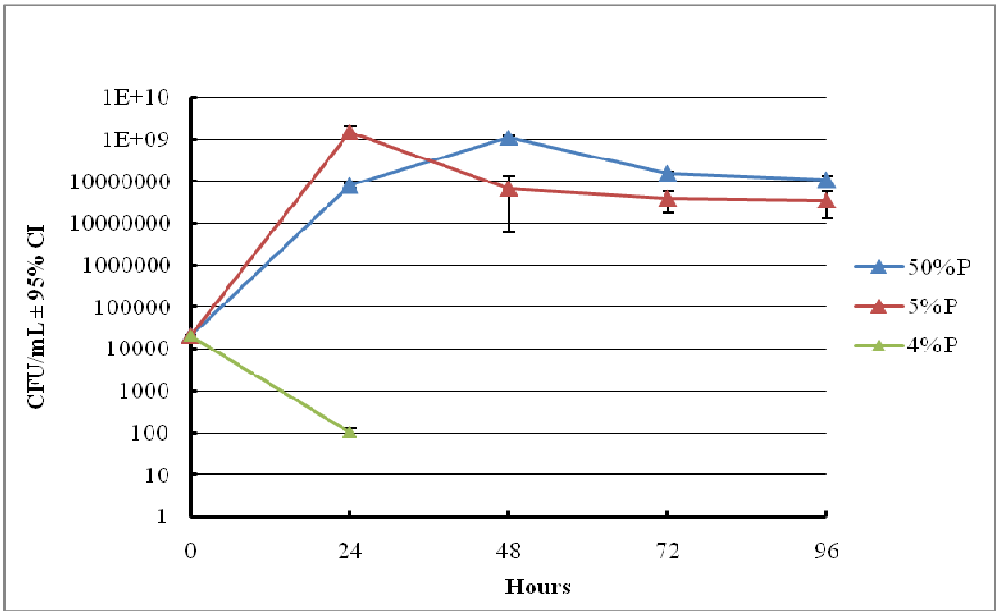

Figure 5a: *Acinetobacter baylyi* BD413 growth curve with varying concentrations of phosphorus (t_0 = 21400 CFU/mL).

3.6. Phosphorus growth curve for *Acinetobacter baylyi* BD413 with and without DNA

6 µg/mL of DNA was added in 4% P to study the effect of DNA on phosphorus limiting growth medium for *A. baylyi*. The growth was measured over 4 days and the results are shown in figure 4b. The precise number of CFU used to start the experiment was 28300 (± 1,13 x 10^2) CFU/mL.

At the end of the exponential phase after 24 hours of incubation in 5% P medium the viable cell counts of *A. baylyi* BD413 was reached to ~65 x10^6 (± 1,19 x 10^8) CFU/mL and in 4% P medium the viable cell was only 110 (±13,78) CFU/mL. 5% P was used in this experiment as a positive control and 4% P was considered negative control since bacterial growth was not evident at 4%P concentration level after 48 hours. In 4% P concentration with 6 µg/mL of DNA the viable cell was ~335 x 10^2 (± 6,56 x 10^4) CFU/mL at the end of the exponential phase. There is overall differences in CFU/mL among different groups (F = 7978,598; df = 2; P<0,001). Growth in 4% P with DNA was significantly different (P<0,001) than others. Again growth in 5% P was also different (P<0,001) than the other two growth media. Lowest growth was recorded in 4% P and highest was in 5% P.

After 48 hours of incubation the bacteria enters into a stationary phase and in 5% P, and 4% P with DNA the viable cell counts of *A. baylyi* BD413 was ~203 x 10^3 (± 2,40 x 10^7) CFU/mL, 86 x 10^6 (± 1,90 x 10^8) CFU/mL. CFU values were higher in 4% P with DNA than in 5%P (t=10.385; df=8; P<0.001).

After 72 hours of incubation in 5% and 4% P media wth DNA the viable cell count was 181 x 10^4 (± 1,51 x 10^6) CFU/mL and ~215 x 10^5 (± 6,70 x 10^7) CFU/mL. CFU values were higher in 4% P with DNA than in 5%P (t=15.750; df=10; P<0.001).

After 96 hours of incubation in 5% and 4% P media with DNA the viable cell count was ~191 x 10^4 (± 1,56 x 10^6) CFU/mL and ~33 x 10^5 (± 9,88 x 10^6) CFU/mL respectively. CFU values were higher in 4% P with DNA than in 5%P (t=2.990; df=10; P = 0.014).

This indicates that after an initial higher CFU value recorded in 5% P in the first 24h of culture, the highest values are then obtained in 4% P with DNA at all times. The results also indicate that DNA uptake and utilization as a food source might require more than 24h.

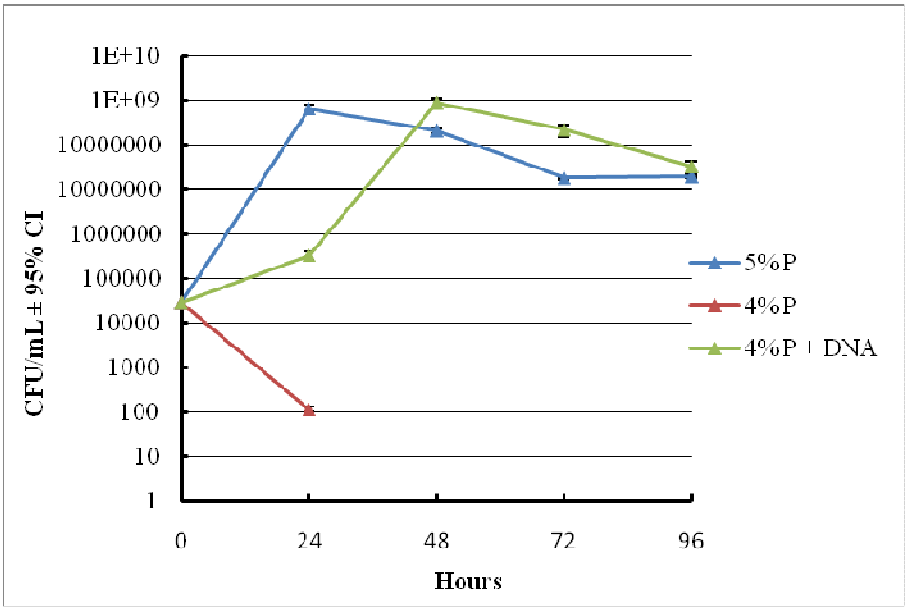

Figure 5b: *Acinetobacter baylyi* BD413 grown in phosphorus with and without DNA (t_0=28300 CFU/mL).

3.7. Carbon source growth curve for *Acinetobacter baylyi* BD413

To study the use of DNA as a carbon source 300 µg/mL of DNA was used instead of lactic acid. In one experimental set up *A.baylyi* was grown in S2 media without any sorts of carbon source and in another it was grown with S2 media with DNA as a carbon source. The growth was measured over 4 days and the results are shown in figure 5. The precise number of CFU used to start the experiment was 22200 CFU/mL.

At the end of the exponential phase after 24 hours of incubation in S2 medium without any sorts of carbon source (i.e., without lactic acid and DNA) the viable cell counts of *A. baylyi* BD413 was reached to ~223 x 10^3 (± 1,34 x 10^6) CFU/mL and in S2 medium with DNA as a carbon source the viable cell was ~6 x 10^5 (± 4,72 x 10^6) CFU/mL. CFU values were similar in S2 with DNA than in S2 without DNA (T-test).

After 48 hours of incubation the bacteria enters into a stationary phase and in S2 medium without any carbon source and S2 medium with DNA as a carbon source, the viable cell

counts of *A. baylyi* BD413 was ~158 x 10^3 (± 8,92 x 10^5) CFU/mL, and ~188 x 10^3 (± 1,42 x 10^5) CFU/mL. CFU values were similar in S2 with DNA than in S2 without DNA (T-test).

After 72 hours of incubation in S2 medium without any carbon source and S2 medium with DNA as a carbon source, the viable cell count was 240 x 10^3 (± 2,17 x 10^6) CFU/mL and ~288 x 10^2 (± 1,57 x 10^5) CFU/mL. CFU values were higher in S2 without DNA than in S2 with DNA (t=3.559; *df*=8; *P*=0.007).

After 96 hours of incubation in S2 medium without any carbon source and S2 medium with DNA as a carbon source, the viable cell count was ~61 x 10^3 (± 2,05 x 10^5) CFU/mL and ~115 x 10^2 (± 8,55 x 10^4) CFU/mL respectively. CFU values were higher in S2 without DNA than in S2 with DNA (t=3.906; *df*=8; *P*=0.005).

The results indicate that in the first 48h, CFU values do not differ in the two treatments. After this time, S2 without DNA seems to enhance bacterial growth. The results also indicate that the growth of *A. baylyi* is not influenced by DNA.

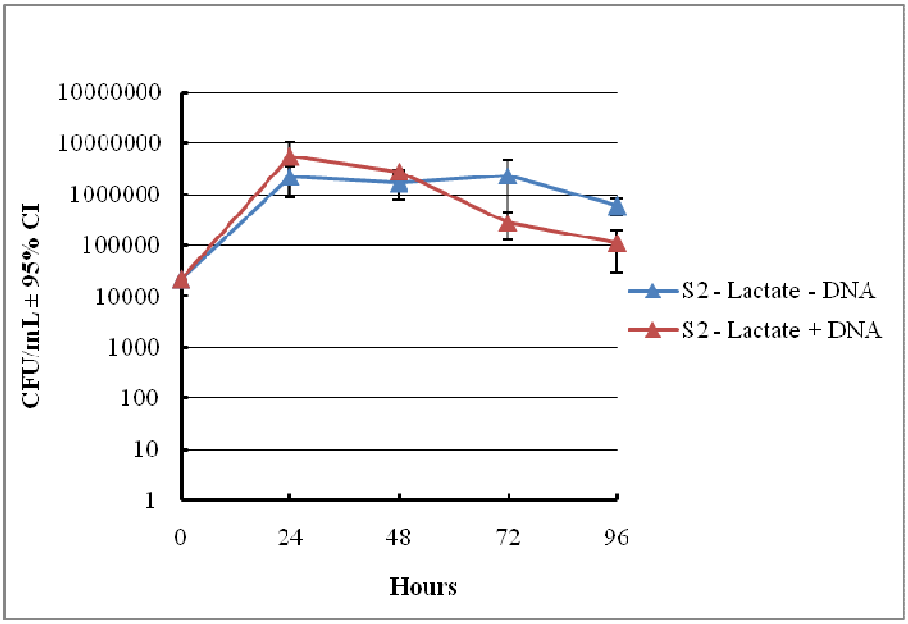

Figure 6: *Acinetobacter baylyi* BD413 grown in S2 media without lactic acid and S2 media with DNA (t_0 = 22200 CFU/mL).

Chapter Four
DISCUSSION

Most naturally transformable bacteria become competent only in response to certain environmental conditions, and can then efficiently bind and take up double-stranded DNA fragments. It has also been shown that, competence (i.e. the ability to take up DNA) is induced by nutritional limitation in both *Haemophilus influenzae* and *Bacillus subtilis* (Redfield 1993). In this work, this is found to be true for *Acinetobacter baylyi* grown in phosphorus limited growth condition but, not for nitrogen and carbon limiting growth conditions. The key ovservations of the reduced N-level indicates that *A. baylyi* is able to grow even without any nitrogen source in the medium and the addition of variable amounts of DNA does not induce any significant increase of growth of *A. baylyi*.

DNA concentrations were progressively increased and added to reduced N-levels media (0% N) without any apparent change in growth. The higher concentrations of DNA provided higher amount of ntrogen to the *A.baylyi* but this was not utilized by the cells. Which is sharply in contrast with the nutritional hypothesis of competence induction for gram negative bacteria. Some assumptions have been put forward for its apparent growth in absence of nitrogen sources as it was found that *A.baylyi* may manage to obtain nitrogen without any viable nitrogen sources. One assumption is that it could get the nitrogen from the distilled water that was used to prepare the media . The other assumption is that it could most likely get the nitrogen from the already dying cells during its growth phases. For this reason, S2-N media was prepared with milQ water instead of distilled water to see any affect of growth without any nitrogen source in water. But, the growth of *A.baylyi* does not reflect any change. Therefore, the remaining hypothesis stands out to be true in this context. *A. baylyi* can also found to be grown even without any sorts of carbon source and it can use DNA as a carbon source. In both cases the growth of *A.baylyi* did not vary. The findings of the use of exogenous DNA to use as a source of carbon by *A. baylyi* is in agreement with the results obtained in *E. coli* showing that this is able to take up and utilize exogenous DNA as a carbon source for growth (Finkel and Kolter 2001). On the other hand, the growth of *A. baylyi* is also found not to be influenced by DNA.

In contrary with nitrogen and carbon limiting conditions, *A. baylyi* does not grow in phosphorus limiting conditions (below 4% P) (**Fig. 5a**). In addition, the key ovservations of the DNA on reduced P-levels was that adding a certain amount of DNA cause significant increase in growth of *A. baylyi* which allows us to think the role of phosphorus in competence induction. This also supports the nutritional role of competence by this bacteria indicating possible induction of competence to take up free DNA for the sole purpose of phosphorus.

Bacteria inhabit a wide variety of niches, and within many of these environments extracellular DNA may be available. Estimates of extracellular DNA concentrations in various marine and aquatic environments range from 0.2 to 44 μg/liter (Lorenz and Wackernagel 1994). DNA has also been shown to be quite stable when complexed with various clays and soil minerals, binding at concentrations in the microgram to milligram range per gram of material (Lorenz et al. 1981, Lorenz and Wackernagel 1994). Naturally transformable bacteria such as *Bacillus subtilis* and *Pseudomonas stutzeri* retain small but significant amount of DNA when other nutrients have been washed away from the soil and sediment environments (Redfield 1993). With an abundance of DNA available in the environment and primarily of heterologous origin, it is unlikely that much of the DNA would be suitable for incorporation into the bacterial chromosome by homologous recombination, hence reducing its potential as a source of genetic diversity (Finkel and Kolter 2001). It was proposed that competence evolved to permit the uptake of DNA as a food supply and macromolecular DNA is indeed a good source of nutrition/food for hungry bacteria (Redfield 1993). Food is particularly a much stronger selective force than either recombination or DNA repair and the carbon, phosphates and nitrogenous bases of DNA are taken up by the bacteria as a nutrient source (Redfield 1993, Redfield et al. 1997). There is also a possibility of having nutrient functions of competence under certain conditions in certain organisms at some point of their evolutionary history (Dubnau 1999). This proposition is not inconsistent with the fact that in some organisms (*H. influenzae*), competence is induced by stationary phase and/or by nutrient depletion signals. However, it is not clear whether competent organisms may utilize DNA as a nutrient source or that they could generate enough energy from the uptaken DNA to result in significant macromolecular synthesis or growth.

It has been noted in naturally transformable bacteria, such as *B. subtilis*, *Streptococcus pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*, that only a single strand enters the cytoplasm during transformation (with the other strand being degraded), this mechanism lacks efficiency as a nutrient acquisition system (Dubnau 1999). However, in *Acinetobacter baylyi* like other gram-negative organisms, the degraded DNA which does not enter into the cytoplasm may take place in the periplasm (Dubnau 1999), thus allowing the retention and possible transport of the resulting nucleotides into the cytoplasm for catabolic use. The differences between species when it comes to both development of competence and uptake mechanism for DNA, suggest that possibly the functions of natural transformation differs between species (Palmen et al. 1993).

In most organisms, the acquisition of natural genetic competence is regulated, meaning that the proteins comprising the DNA uptake machinery are not expressed all the time and different conditions have to be met to express these proteins for DNA uptaking. Competence in most naturally transformable bacteria is regulated by nutrition functions. Several of the genes that affect competence development in *B. subtilis* are known to have other functions in nutrient acquisition or processing (Dubnau 1991). Competence is also shown to be inducted by a starvation medium, MIV which lacks a carbon source and the nutrient source needed for DNA replication and cell division (Redfield 1993).Some organisms increase the number of competent cells during some stage of their growth cycle. Some organisms are constitutively competent (*N. gonorrhoeae*), whereas some others (e.g. *H. influenzae),* the development of competence seems to be cell-autonomous. A. baylyi becomes was found to be competent in early or late exponential growth phases.

Certain uses of antibiotics in aquaculture (e.g. for disease treatment or for growth promotion) can lead to antibiotic resistance bacteria, and this resistance can then be transmitted to the general population, causing treatment-resistant organisms. The uses of antibiotics can also create antibiotic resistance in non-pathogenic bacteria. The indiscriminate use of antibiotics for veterinary purposes has increasingly become a matter of public concern, and legal requirements are being reinforced in many countries. Regulatory authorities should license antibiotics for use if the agents meet scientific criteria for quality, efficacy and safety. The authorities have to consider safety in relation to the treated animal, to the consumer and to the individuals handling the product during treatment.

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