Quantification of absolute and relative fitness in *Acinetobacter baylyi* under varying growth conditions

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ABSTRACT

Natural transformation is one of three ways for bacteria to acquire genetic material (DNA) horizontally. Several hypotheses exist for why some bacteria have this ability to take up DNA, and what the incoming DNA is used for. These include use of DNA for recombination, repair or as nutrients.

This master study focuses on the "food hypothesis". This hypothesis implies that the exogenous DNA is either used in the synthesis of new DNA and RNA molecules, or broken down to carbon, nitrogen and phosphate to use as energy and in synthesis of new nucleotides. The naturally transformable *Acinetobacter baylyi* is used as a model organism to test this hypothesis. By removing the *comFECB* operon from the wild type bacteria a non-transformable strain is produced. Comparisons of growth rates between these two strains in monocultures give the absolute fitness, whereas co-cultures give the relative fitness. If the *comFECB* operon has evolved as a means to use DNA as food, than the transformable wild type would have a fitness advantage over the non-transformable strain when DNA is the only nutrient in the growth media.

Although the addition of DNA to the growth media resulted in increased growth of *A. baylyi*, this effect was seen in both strains, independently of functional *com*-genes. This implies that DNA is in fact used as nutrients by this species, but not only by natural transformable bacteria with intact *comFECB* operons. The experiments done in this thesis do therefore not offer support for the "food hypothesis".

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1. INTRODUCTION

1.1 HORIZONTAL GENE TRANSFER IN BACTERIA

Bacterial populations grow by binary fission, each mother cell divides into two identical daughter cells. This is called vertical inheritance and the whole population will essentially have the same genes. Genetic alterations, the substrate for natural selection, will slowly accumulate through point mutations, deletions, and rearrangements. This is clearly different from eukaryotes that reproduce by meiotic sex, which leads to new genetic combinations in every generation. However, prokaryotes are not strictly asexual; bacteria may also acquire exogenous deoxyribonucleic acid (DNA) horizontally through horizontal gene transfer (HGT) (Lorenz & Wackernagel, 1994). HGT enables bacteria to transfer genes among closely and even distantly related species. Currently, three mechanisms of HGT have been described; conjugation, transduction and natural transformation.

1) Conjugation is the transfer of mobile genetic elements, such as transposons and plasmids via direct cell-to-cell contact. The elements themselves encode genes responsible for this cell-to-cell contact and transfer (Thomas & Nielsen, 2005). Some plasmids are incorporated in the host's chromosome. Later, when the plasmid leaves the chromosome to replicate, a part of the host chromosome can get torn off and incorporated into the plasmid, thereby getting transferred to the next cell.
The other two mechanisms of HGT do not depend on cell-to-cell contact. For transduction and transformation to occur, donor and recipient cells are not required to be in the same place, not even at the same time (Ochman et al., 2000).

2) *Transduction* is transfer of genes between bacterial cells through the infectious transfer of bacteriophages (bacterial viruses). When a phage infects the host cell and uses the machinery to produce new phage elements, parts of the host's own DNA may be packed in the new viruses by accident (generalized transduction). Alternatively, if the phage inserts in a specific place in the host chromosome, the flanking DNA will be packed in the new viruses and subsequently co-transferred with viral DNA to the recipient cell (specialised transduction) (Redfield, 2001).

From a bacterial perspective, novel genes acquired in HGT by conjugation and transduction are accidental side effects of the conjugative elements and phages' need for infectious transfer to be maintained as selfish genetic elements (Redfield, 1988; 2001). In this interpretation, conjugation and transduction did not evolve to provide new genes for their hosts, but rather to ensure infectious transfer and replication of the plasmids/phage. In the last known mechanism of HGT, natural transformation, the acquisition of DNA seems to be the main reason and not a side effect.

3) In *natural transformation* the bacteria does all the work itself to acquire new genetic material. The first report of natural transformation was described by Griffith in 1928. In this experiment he injected a mixture of heat-killed pathogenic S-form *Streptococcus pneumoniae* cells and living non-pathogenic R-form into mice. The mice died of infection, and only *S. pneumonia* S-form isolates were recovered from the dead animals. He concluded that the R-form had in some way transformed into the S-form in the infected mice, but did not know what was responsible for this transformation (Griffith, 1928). It was not until 1944 that Avery, MacLeod and McCarty determined that the transforming factor in this experiment was DNA (Avery et al., 1944). Today it is clear that the only thing necessary for initiation of transformation, beside the bacteria being able to take up DNA, is free DNA in the bacteria's environment (Lorenz and Wackernagel, 1994).

Competence

For the bacterium to be able to take up DNA it must be competent. Expression of genetic competence is regulated by several different *com*-genes in bacteria. Activation of these genes is called 'development of competence', and is the first step in natural transformation. The ability of being competent for natural transformation (also called naturally transformable) exists in a broad range of unrelated species. To date, more than 60 different species have been shown to be naturally transformable (Johnsborg et al., 2007). Although competence is widespread among species, these only amount to approximately 1% of all known bacterial species. Examples of natural transformable species are the Gram-negative *Acinetobacter baylyi, Haemophilus influenzae, Neisseria gonorrhoeae* and the Gram-positive *S. pneumoniae* and *Bacillus subtilis*. Most bacteria are competent only under certain conditions, which vary from species to species, and in limited time frames. *H. influenzae* and *N. gonorrhoeae* are known to express competence for transformation constitutively. There are also differences in

the proportion of the strain becoming competent; some have just a few competent cells, while in other strains the whole population can achieve competence (Thomas & Nielsen, 2005).

Mechanism of competence

As mentioned above, natural transformation was first reported in *S. pneumoniae*. Ever since, this species has been used as a model organism for studying the mechanisms and regulation of processes included in natural transformation in Gram-positive bacteria. The mechanism of competence can be described as a cascade of events in the cell. The gene *comC* is responsible for producing a precursor to the Competence Stimulating Peptide (CSP). CSP is further matured and exported out of the cell by the ComAB transporter. CSP works as a hormone-like substance in the environment, to coordinate competence development in the population. To induce competence in the bacterium, CSP binds to and activates ComD on the cell surface, which further activates ComE, the transcriptional regulator. ComE is responsible for activating approximately 20 genes, known as 'early competence genes'. The early competence genes are essential for the development of competence, and include the *comAB* and *comCDE* operons and *comX*. ComX controls the transcription of approximately 60 'late competence genes', some of which encode the DNA uptake and recombination machinery used in natural transformation. (Johnsborg & Håvarstein, 2009)

The model organism in this thesis is the Gram-negative *A. baylyi*. Less is known about the function of the *com*-genes in this species when compared to *S. pneumoniae*. Sixteen competence genes are known, organised in seven chromosomal loci; *comA, comEA, comP, drpA, (comB, comC, comE, comF), (comM, comN, comO, comL, comQ), (pilB, pilC, pilD)* (Averhoff & Graf, 2008). These seven loci are not physically linked and are distributed in approximately one-third of the genome. Studies of these *com*-genes, especially structural comparisons to related *com*-genes in other bacterial strains, have indicated possible functions of the genes. Table 1.1 below summarizes the possible functions of the *com*-genes in *A.baylyi* ADP-1. Mutant cells lacking either of *comA, comEA, comP, comC* or *comB* are non-competent, so these genes are absolute necessary for competence and transformation.

Table 1.1: Competence proteins of A. baylyi ADP-1

(Table adapted from Averhoff & Graf, 2008)

A. baylyi competence proteins	Potential function
ComA	Transport of DNA through the inner membrane
ComEA	DNA binding protein
DrpA	DNA processing protein
ComP	Structural subunit of the DNA translocator, transport of DNA through the periplasm
ComE	Structural subunit of the DNA translocator, transport of DNA through the periplasm
ComF	Structural subunit of the DNA translocator, transport of DNA through the periplasm
ComB	Structural subunit of the DNA translocator, transport of DNA through the periplasm
ComC	DNA binding and/or transport
PilD	Export and maturation of prepilins
PilC	Platform of the DNA translocator
PilB	Biogenesis of the DNA translocator
ComQ	Translocation of DNA through the outer membrane
ComL	Biogenesis or stabilization of ComQ multimers
ComM	Biogenesis of the DNA translocator
ComN	Biogenesis of the DNA translocator
ComO	Biogenesis of the DNA translocator

After competence has developed, there are several more steps involved in natural transformation. This includes binding of free DNA to the cell's surface and uptake into the cell. Once inside the cell the foreign DNA must be integrated into the cell's chromosome by recombination, or it will be destroyed in the cytoplasm (Redfield, 2001). The new integrated genes must be functionally expressed and be beneficial for the cell in some way for them to be selected for and to be passed on to further generations.

Uptake of DNA

DNA binds to the outside of the cell and becomes translocated into the cell as a single strand. Most species, such as *B. subtilis* and *A. baylyi*, are unselective in the DNA they take up. Some species however, only bind and take up DNA with specific recognition sequences. *H. influenzae* and other members of the *Pasteurellaceae* family require specific 'uptake signal sequences' (USS) present in the environmental DNA for it to be taken up by the cell (Redfield et al., 2006). The same is seen in *Neisseria* ssp. under another name, 'DNA uptake sequence'

(DUS) (Treangen et al, 2008). This way, these bacteria only take up DNA from closely related species, which contain these sequences.

Integration of DNA

Once inside the cell, DNA can be degraded to single nucleotides for use in metabolism, used as templates for DNA repair, or it can be integrated to the chromosome by homologous recombination. For homologous recombination to occur, the DNA must have regions with high similarity in base pairs to the cell's chromosome. When the bacterium has successfully integrated the newly acquired DNA into the chromosome it is said to be 'transformed'. Thus there is a clear difference in bacteria being competent for transformation, i.e. being able to take up DNA, and the bacteria actually being transformed. A bacterium needs to be competent to be transformed, but being competent does not always lead to transformation.

If the incoming DNA does not have sequence similarity with the cells chromosome it can still be incorporated to the cell's genome, by a process called Homology facilitated illegitimate recombination (HFIR) (de Vries & Wackernagel, 2002). In this process foreign DNA gets linked to a piece of DNA that is homologous to the cell's chromosome (minimum 183 base pairs needed). This piece of homologous DNA serves as an anchor, bringing the foreign DNA close to the chromosome, so that illegitimate recombination can occur.

1.2 EVOLUTION AND MAINTENANCE OF NATURAL COMPETENCE FOR TRANSFORMATION

The uptake of complete genes and gene clusters can potentially give a selective advantage to bacterial populations. Particularly in niche expansion and when bacteria face lethal selective pressure, exemplified by HGT of antibiotic resistance (Johnsen et al., 2009b). Uptake of beneficial DNA may give a fitness advantage to a population over populations that do not take up this DNA. Fitness is defined as 'a genotypes contribution to the next generation, relative to the contributions of other genotypes' (Lawrence, 1995). However, incoming DNA may not always carry an advantage; it may also be dangerous for the cell. It is reasonable to assume that a large fraction of environmental free DNA comes from dead cells, with potential lethal mutations in their DNA (Redfield, 1988). It is also likely that an extra fitness cost is associated with the *com*-genes, as shown *in vitro* for *B. subtilis* (Johnsen et al., 2009). Unless beneficial DNA that gives advantages is frequently taken up by the competent cells, mutants

lacking *com*-genes would be more fit than cells with *com*-genes, and eventually, competence would be lost in populations. Still, it must be worth their while for the bacteria since these genes still exist after billions of years of evolution (Finkel & Kolter, 2001).
There are several hypotheses for why competence for natural transformation has evolved and still is being maintained in bacteria. The main hypotheses are described below.

1.2.1 Increased adaptation rates

This hypothesis has been called 'Transformation for recombination' (Johnsen et al., 2009), and is perhaps the hypothesis with the most supporters. By uptake of DNA from the environment, bacteria can get access to beneficial traits. By incorporating this DNA, the cell may have a better chance of surviving and will adapt to new environments faster. This way evolution of bacteria will 'speed up'; each individual will not have to wait for the slow processes of random mutations (including point mutations, inversions and deletions), but will benefit from the "work" done by generations before it (Johnsen et al., 2009). By acquiring specific traits from other species, bacteria may be able to explore new habitats and niches, and eventually new lineages in novel environments may give rise to new species.
The hypothesis that recombination increases the rate of adaptation has been addressed in experiments with several different bacterial species. Studies with *Escherichia coli* (Cooper, 2007) and *Helicobacter pylori* (Baltrus et al., 2008) have showed that cells capable of recombination adapt faster to new culture conditions than cells without the ability of recombination.
This hypothesis goes hand in hand with the DNA repair hypothesis described below, as many of the gene products for recombination and DNA repair are the same. The Rec-proteins have their name from when they first were discovered, as they were then thought to mainly function in recombination processes. Today it is clear that the primary function of this wide group of proteins is in DNA replication and repair, and recombination is a side effect to these primary functions (Redfield, 2001).

1.2.2 DNA repair

The DNA repair hypothesis was presented by Bernstein and colleagues in 1984. The hypothesis suggests that natural transformation provides bacteria with undamaged DNA that can be used as templates for repair of damaged DNA (Bernstein et al., 1984). This is especially the case if the incoming DNA comes from the same bacterial species; the DNA will be similar in sequence and thus easily incorporated by homologous recombination. Michod and colleagues investigated this hypothesis using Gram-positive *B. subtilis* as the model organism (Michod et al., 1987). They induced DNA damage in the cells by exposing them to UV radiation. Further, they added transforming DNA to the cells, and noted differences in frequencies of transformable and non-transformable cells. Transformable cells had a higher survival, and therefore the results in this experiment provided evidence in favour of the hypothesis. Later, Mongold tested the generality of these results by using Gram-negative *H. influenzae* (Mongold, 1992). She concluded that the addition of DNA to UV-induced damaged cells does in fact lead to better survival. However, she observed that also small fragments of DNA gave this effect, thereby questioning if the effect seen was due to templates for repair as the hypothesis states.

1.2.3 Food hypothesis

One of the most controversial hypotheses for the evolution and maintenance of natural competence for transformation is the "food hypothesis" (also called the nutrient hypothesis or gastronomy hypothesis). This hypothesis implies that DNA is used by bacteria as a source of nutrients (Redfield, 1993). The exogenous DNA is either used in the synthesis of new DNA and RNA molecules, or broken down to carbon, nitrogen and phosphate to use as energy and in synthesis of new nucleotides. According to this hypothesis, the last step included in natural transformation; the incorporation and functional expression of the newly acquired DNA, seems to be a secondary effect. When the exogenous DNA is used for food instead of recombination the term 'nutritional competence' (Palchevskiy & Finkel, 2009) may be more appropriate than natural transformation. In (Redfield, 2001) a "retrospective analysis" of the genes and processes involved in genetic exchange was presented. Here it is argued that the genes and regulation of competence and transformation all point to nutrient being the main reason for uptake of environmental DNA. This is based purely on the observations that many strains become competent in environments lacking nutrients, and that damage of the

chromosomal DNA does not seem to induce competence. Redfield also argues against the recombination hypothesis by mentioning that there has been no selection for genes that directly cause genetic exchange and recombination.

Not much work has been done in the laboratory to address this hypothesis, and the existing results are at best ambiguous. Although *E. coli* has not been shown to be naturally competent, homologues of proteins involved in natural transformation in other bacteria are used by *E. coli* to enable the bacteria to use DNA as a nutrient (Finkel & Kolter, 2001). Finkel and Kolter suggest that uptake of DNA in competent bacteria can have two non-mutually exclusive functions, acquiring genes for recombination AND nucleotides as a food source. Bacher and colleagues (Bacher et al., 2006) did a experimental study with *A. baylyi*, in which they noted that DNA in the medium did not give a growth benefit to competent cells, instead it actually had a negative effect on the growth rate, and so the study provides as evidence against the food hypothesis.

1.2.4 Episodic selection

Recently, Johnsen and colleagues presented a new hypothesis 'for the selective pressures responsible for maintaining natural competence and transformation' (Johnsen et al., 2009).

Episodic selection implies that two separate events work together to maintain competence in bacteria. First, a non-growing subpopulation will survive when the whole population experiences conditions that kill replicating cells, e.g. an antibiotic. The authors hypothesize that this may be true for environmental stressors that kill actively growing cells, such as toxins and possibly phage. And second, if this subpopulation is naturally transformable and has required a specific gene from environmental DNA which gives an advantage to the cells, these cells will survive and competence will be maintained.

It is clear that all of these hypotheses are plausible explanations for how natural transformation is maintained and possibly evolved, but they also have limitations, and none of them are mutually exclusive. Thus, a pluralistic model that combines several hypotheses may be necessary to understand the evolution and maintenance of competence and natural transformation (Cooper et al., 2004, Johnsen et al., 2009).

1.3 MODEL ORGANISM: ACINETOBACTER BAYLYI

Acinetobacter ssp, are bacteria that can utilize a broad range of carbon sources. They easily adapt to new habitats and are commonly found in water, soil and living organisms (Barbe et al., 2004). The specific strain used in this thesis was *A. baylyi* BD413/ADP1, first described by Juni & Janik (1969). It was first classified as a member of *Acinetobacter calcoaceticus*, but lately it has been included in the *A. baylyi* species (Vaneechoutte et al., 2006). BD413 and ADP1 are the European and American names of the same strain, respectively. This strain is naturally transformable and needs only fresh media to induce competence, unlike other bacterial species that require specific conditions to become competent, such as *B. subtilis* (Palmen et al., 1993). *A. baylyi* has a relative small genome, which has been fully sequenced by Barbe and colleagues (Barbe et al., 2004). *A. baylyi* does not discriminate between homologous and heterologous DNA and can take up DNA from various sources (Nielsen et al., 1997).

A. baylyi BD413/ADP1 is also a good "lab rat" in the sense that it may easily be constructed to other forms by genetic modification in the laboratory (Metzgar et al., 2004). In this thesis the competent wild type strain has been altered to a non-transformable strain by "knocking out" some of the *com*-genes responsible for competence (See Materials, chapter 2.1 for details). By comparing this *com*-gene knockout to the competent wild type, information about differences in fitness may be achieved.

1.4 AIMS OF STUDY

Aims of study:

To test the hypothesis that DNA can be taken up by bacteria for nutritional purposes, and to quantify fitness effects of the DNA uptake.

Main hypothesis:

The presence of functional com-genes increase the absolute and relative fitness of A. baylyi by providing access to DNA fragments as a nutrient source, under nutrient limiting conditions.

This hypothesis will be tested by comparisons of bacterial fitness between transformable wild type and a non-transformable *com*-gene knockout.

Specific objectives:

1. To identify the growth limiting concentrations of N, P, and C sources in liquid S2 medium.
2. To perform fitness assays using both the wild type and a *com*-gene knockout of *A.baylyi* BD413/ADP1 to quantify the effect of DNA uptake on absolute and relative bacterial fitness.

2. MATERIALS

2.1 BACTERIAL STRAINS

A. baylyi BD413 (Juni & Janik, 1969) and ADP-1200 are in principle the same strains, BD413 is used in Europe, whereas ADP-1 (which strain ADP-1200 is derived from, see Kok et. al., 1999) is used in the US. Strains were constructed by P. J. Johnsen as described in Bacher and colleagues (Bacher et al., 2006). Briefly, ADP-1200-2 has a complete *aphA3* gene in the *lipA* locus rendering the strain kanamycin resistant. ADP-1200 is isogenic to ADP-1200-2, except that the *aphA3* gene is incomplete (227 basepairs missing in the 3' end) and therefore the strain is not resistant to kanamycin. ADP-1200-2 DHFR-1 has the kanamycin resistance cassette (*aphA3*) inserted in the *lipA* locus. In addition, the *comFECB* locus was replaced by a trimetoprim resistance cassette (DHFR). The removal of the *comFECB* leaves the strain unable to bind and take up extracellular DNA and the strain is thus 'non-transformable'. The *comFECB* deletion is selectively neutral relative to the wild type (P. J. Johnsen, unpublished results).

Table 2.1: Bacterial strains used in this master study

Name of strain	Competence	Resistance	Source
A. baylyi BD413 (wild type)	+	-	Juni, 1969
A. baylyi ADP-1200	+	-	Kok, 1999
A. baylyi ADP-1200-2 DHFR-1 (*lipA::aphA3 ΔcomFECB :: DHFR-1*)	-	Kanamycin/ trimetoprim	Johnsen, unpublished
A.baylyi ADP-1200-2 (*lipA::aphA3)*	+	Kanamycin	Johnsen, unpublished
A. baylyi ADP-1200 DHFR-1 (*ΔcomFECB :: DHFR-1)*	-	Trimetoprim	Johnsen, unpublished

2.2 GROWTH MEDIA

The following growth media were used in this study:

- S2 minimal medium standard, with lactic acid as the carbon source (Juni, 1974)
- S2 minimal medium without carbon
- S2 minimal medium without nitrogen
- S2 minimal medium without phosphate
- S2 minimal medium with 4% phosphate
- S2 minimal medium with 5% phosphate
- S2 minimal medium without phosphate and with Tris-Cl as buffer
- S2 minimal medium with 4% phosphate and with Tris-Cl as buffer
- S2 minimal medium with 5% phosphate and with Tris-Cl as buffer

Media was prepared by dissolving the salts (see below) in 800 mL distilled water. Thereafter the solutions were added and the pH value adjusted to 6,7 for the phosphate buffered media, and pH 7 for the Tris-HCl buffered media. Tris-HCl is used as a buffer in the pH range of 7-9, according to the product homepage (www.merck.de). pH 7 was chosen so that the media were as similar as possible regarding pH values. The volume was adjusted to 1000 mL with distilled water, the pH adjusted, and the media was autoclaved at 121°C for 20 minutes. The partial or complete removal of phosphate would alter the buffer capacity of the media (see results section) and Tris-HCl was added to ensure the buffering capacity in these media.

- S2 minimal medium standard, with lactic acid as the carbon source
  1,5 g KH_2PO_4 (Merck, Germany)
  16,9 g Na_2HPO_4 x $2H_2O$ (Sigma-Aldrich, Germany/USA)
  0,2 g $MgSO_4$ x $7H_2O$ (Merck, Germany)
  2 g NH_4Cl (Merck, Germany)
  1 mL $CaCl_2$ (1% solution)
  0,5 mL $FeSO_4$ (0,1% solution)
  1,5 mL Lactic acid (Fluka Sigma-Aldrich, Germany)

- S2 minimal medium without carbon
  1,5 g KH_2PO_4
  16,9 g Na_2HPO_4 x $2H_2O$
  0,2 g $MgSO_4$ x $7H_2O$
  2 g NH_4Cl
  1 mL $CaCl_2$ (1% solution)
  0,5 mL $FeSO_4$ (0,1% solution)

- S2 minimal medium without nitrogen
  1,5 g KH_2PO_4
  16,9 g Na_2HPO_4 x $2H_2O$
  0,2 g $MgSO_4$ x $7H_2O$
  1 mL $CaCl_2$ (1% solution)
  0,5 mL $FeSO_4$ (0,1% solution)
  1,5 mL Lactic acid

- S2 minimal medium without phosphate
  0,2 g $MgSO_4$ x $7H_2O$
  2 g NH_4Cl
  1 mL $CaCl_2$ (1% solution)
  0,5 mL $FeSO_4$ (0,1% solution)
  1,5 mL Lactic acid

- S2 minimal medium with 4% phosphate
  0,06 g KH_2PO_4
  0,676 g Na_2HPO_4 x $2H_2O$
  0,2 g $MgSO_4$ x $7H_2O$
  2 g NH_4Cl
  1 mL $CaCl_2$ (1% solution)
  0,5 mL $FeSO_4$ (0,1% solution)
  1,5 mL Lactic acid

- S2 minimal medium with 5% phosphate
  0,075 g KH_2PO_4
  0,845 g Na_2HPO_4 x $2H_2O$
  0,2 g $MgSO_4$ x $7H_2O$
  2 g NH_4Cl
  1 mL $CaCl_2$ (1% solution)
  0,5 mL $FeSO_4$ (0,1% solution)
  1,5 mL Lactic acid

- S2 minimal medium without phosphate and with Tris-HCl as buffer

  As S2 minimal medium without phosphate.

  106 mL Tris-HCl pH 7 was added

- S2 minimal medium with 4% phosphate and with Tris-HCl as buffer

  As S2 minimal medium with 4% phosphate.

  106 mL Tris-HCl pH 7 was added

- S2 minimal medium with 5% phosphate and with Tris-HCl as buffer

  As S2 minimal medium with 5% phosphate.

  106 mL Tris-HCl pH 7 was added

2.3 DNA USED IN GROWTH EXPERIMENTS

- DNA from calf thymus (Sigma, USA). The DNA was dissolved at 1mg/mL in autoclaved water by gentle stirring at 4°C overnight. The concentration was determined to be 0,7µg/µL with the use of NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA). The DNA solution was divided into appropriate volumes in eppendorf tubes and stored at minus 20°C until use.

2.4 OTHER MATERIALS

- LB growth medium

  25 g of LB-broth, high salt (Fluka Sigma-Aldrich, Germany) was dissolved in 1L distilled water and the solution was autoclaved.

- 0,9 % NaCl solution (saline)

  9 g of NaCl (Merck, Germany) was dissolved in 1L distilled water and the solution was autoclaved.

- LB-agar plates

  12 g of agar (Merck, Germany) and 25 g of LB-broth, high salt was dissolved in 1L distilled water and the solution was autoclaved. Then plates were made.

- LB-agar plates with kanamycin

  Kanamycin stock was made by dissolving 0,5 g kanamycin sulphate (Fluka Sigma-Aldrich, Germany) in 10 mL milli-Q water and then sterile filtered. LB-agar was made as above. After sterilization of the agar, 1mL kanamycin stock was added to each litre of agar and plates with a kanamycin concentration of 50µg/mL agar were made.

- Tris-HCl buffer pH 7

  121,14 g trisbase, Tris(hydroxymethyl)-aminomethane (Merck, Germany) was dissolved in approximately 800mL distilled water. The pH was then adjusted to

7 with HCl (Merck, Germany), the volume adjusted to 1L, and the PH adjusted.

- 1% $CaCl_2$ solution

  1% $CaCl_2$ solution was made for the S2 growth media by dissolving 1,32g $CaCl_2$ x 2 H_2O (Merck, Germany) in 100 mL distilled water.

- 0,1% $FeSO_4$ solution

  0,1% $FeSO_4$ solution was made for the S2 growth media by dissolving 0,1g $FeSO_4$ x 7H_2O (Fluka Sigma-Aldrich, Germany) in 100 mL distilled water.

3. METHODS

3.1 DETERMINATION OF GROWTH LIMITING CONDITIONS

To test the "food hypothesis", i.e. the ability of competent bacteria to use extracellular DNA as nutrients, a growth-limiting environment was required to ensure that DNA was the only nutrient available in the medium. Determination of growth limiting conditions for *A. baylyi* was also necessary for the subsequent work to reach the objectives regarding absolute and relative fitness.

A. baylyi BD413 was grown in media with different nutrient limiting conditions, media without carbon, nitrogen and phosphate, respectively. These conditions were obtained by removing lactic acid (carbon source), ammonium (nitrogen source) and phosphate salts, respectively. *A. baylyi* was also grown in media with different amounts of phosphate, as established by Khan (2008) (See Materials, chapter 2.2 for recepies).

The growth experiments were performed and measured over 96 hours in order to ensure sufficient time in stationary phase, and to create similar selective conditions as an ongoing experimental evolution project in our laboratory (Johnsen, Nielsen and Utnes, unpublished results).

Day 1. A single colony of *A. baylyi* BD413 was added to 3 mL S2 standard medium and incubated at 37°C with shaking over night.

Day 2. 1 mL of the overnight culture was transferred to an effendorf tube and centrifuged at 4°C and 13000 rounds per minute (rpm) for 3 minutes. The pellet was resuspended in 1 mL saline and the process was repeated. The bacterial culture was then diluted down to a 10^{-5} dilution, approximately 10^4 cells/mL. This was used as the start concentration for the growth test series.

300 µl of the 10^{-5} dilution was added to 2,7 mL of each of the growth media; S2 standard medium, medium without carbon, medium without nitrogen, medium without phosphate, medium with 4% phosphate, medium with 5% phosphate.

100 µL of each media was plated in two parallels on LB-agar plates to determine the start number of Colony Forming Units (CFU). The tubes were incubated at 37°C with shaking (200 rpm). The bacteria were left to grow for 96 hours, with dilution and plating on LB-plates every 24 hours.

Day 3. Counting after 24 hours. The media were diluted and plated on LB-plates to determine the CFU after 24 hours.

Day 4. Counting after 48 hours. The media were diluted and plated on LB-plates to determine the CFU after 48 hours.

Day 5. Counting after 72 hours. The media were diluted and plated on LB-plates to determine the CFU after 72 hours.

Day 6. Counting after 96 hours. The media were diluted and plated on LB-plates to determine the CFU after 96 hours.

The same procedure was performed with *A. baylyi* strains ADP-1200 and ADP-1200-2 DHFR-1, with the following modifications:

- Media with 0%, 4% and 5% phosphate were buffered with Tris-HCl (See section 3.2 for explanation).
- Experience showed that these strains grow slower than BD413, so a larger start concentration was achieved by using 10^{-4} dilution of overnight cultures instead of 10^{-5}. To determine the start CFU 100 µL of each media was diluted once (1:10) and plated in two parallels on LB-agar plates.

3.2 MEDIA BUFFERED WITH TRIS-HCL

S2 minimal medium without phosphate probably has limited buffering capacity. During the growth limiting tests with strain BD413, the pH in the media was measured using pH paper. These crude measurements indicated a slight decrease in pH values in media with reduced phosphate content. It is not clear how much this affects growth, so growth media without or with low content of phosphate were buffered using Tris-HCl to ensure stable pH in following experiments. This growth test was performed to confirm that the addition of a Tris buffer (Tris-HCl) to such media enables *A. baylyi* to grow.

A. baylyi ADP-1200 was grown for 96 hours in three different media. S2 standard medium was used as a control for maximum growth. Media with 0 % phosphate with and without tris-HCl were used to show the possible effect a buffer has on growth.

Day 1. A single colony of *A. baylyi* ADP-1200 was added to 3 mL S2 standard medium and incubated at 37°C with shaking over night.

Day 2. 1 mL of the overnight culture was transferred to an eppendorf tube and centrifuged at 4°C and 13000 rpm for 3 minutes. The pellet was resuspended in 1 mL saline and the process was repeated. The bacterial culture was then diluted down to 10^{-4} dilution; this was used as the start concentration for the test series.

300 µl of the 10^{-4} dilution was added to 2,7 mL of each of the growth media, S2 standard medium, medium with 0 % phosphate and medium with 0 % phosphate buffered with Tris-HCl. 100 µL of each media was diluted once (1:10) and plated in two parallels on LB-agar plates to determine the start CFU. The tubes were incubated at 37°C with shaking. The bacteria were left to grow for 96 hours, with dilution and plating on LB-plates every 24 hours.

Day 3. Counting after 24 hours. The media were diluted and plated on LB-plates to determine the CFU after 24 hours.

Day 4. Counting after 48 hours. The media were diluted and plated on LB-plates to determine the CFU after 48 hours.

Day 5. Counting after 72 hours. The media were diluted and plated on LB-plates to determine the CFU after 72 hours.

Day 6. Counting after 96 hours. The media were diluted and plated on LB-plates to determine the CFU after 96 hours.

3.3 THE EFFECT OF ADDED DNA ON GROWTH

Growth tests of a transformable and a non-transformable strain of *A. baylyi* were performed with and without added DNA. This was done to test the hypothesis that DNA is taken up as a source of nutrients by the transformable strain (ADP-1200), and thus contribute to increased absolute fitness of this strain.

A. baylyi ADP-1200 (transformable) and *A. baylyi* ADP-1200-2 DHFR (non-transformable) were grown for 96 hours in medium with 0 % phosphate + tris-HCl and a medium with 0 % phosphate + Tris-HCl with 25µL (0,7µg/µL, see Materials, chapter 2.3) added DNA. DNA concentration in the growth tubes is 5,8 µg per mL media as used by Khan (2008). S2 standard medium was used as a control. Except for different growth media and a different start concentration of bacteria (10^{-4} dilution instead of 10^{-5}) the experiments were performed as described in 3.1.

3.4 COMPETITION

According to the food hypothesis, transformable bacteria may take up DNA as food. In competition under nutrient limiting conditions this may give them a fitness advantage over non-transformable bacteria. The net growth of the transformable strain should therefore be larger than the growth of the non-transformable strain. In these experiments a medium with 0% phosphate is used as a nutrient limiting condition, as this has shown to support less growth than the other media tested previously in this thesis. Competition assays were performed to get information about the relative fitness of competent (transformable) *A. baylyi* ADP-1200-2 and the DNA uptake deficient mutant (non-transformable) ADP-1200 DHFR-1.

A. baylyi ADP1200-2 (transformable and kanamycin resistant) and *A. baylyi* ADP-1200 DHFR-1 (non-transformable and sensitive to kanamycin) were grown for 96 hours in medium with 0 % phosphate and tris-HCl as a buffer, with and without DNA. The two strains were grown in the same tube in direct 'head to head' competition. Kanamycin plates were used to select the competent strain. S2 standard medium was used as a control.

Day 1. A single colony of the two competing strains of *A. baylyi*, ADP-1200-2 and ADP-1200 DHFR-1 were added to 3 mL LB medium each and incubated at 37°C with shaking over night.

Day 2. 100 µL of the LB overnight cultures were diluted once (1:10). 50 µL of these dilutions were added to 3 mL S2 standard medium each and incubated at 37°C with shaking over night.

Day 3. 1 mL of the S2 overnight cultures was transferred to each their eppendorf tubes and centrifuged at 4°C and 13000 rpm for 3 minutes. The pellets were resuspended in 1 mL saline and the process was repeated. The bacterial cultures were then diluted down to 10^{-4} dilutions, which were used as the start concentration for the competition.

150 µL of each 10^{-4} dilution was added to 2,7 mL of each growth medium. 100 µL of each media was plated in two parallels on LB plates and LB-kanamycin (50µg/mL) plates to determine the start CFU. The tubes were incubated at 37°C with shaking. The bacteria were left to grow for 96 hours, with dilution and plating on LB-plates and LB-kanamycin plates every 24 hours.

Day 4. Counting after 24 hours. The media were diluted and plated on LB-plates and LB-kanamycin plates to determine the CFU after 24 hours.

Day 5. Counting after 48 hours. The media were diluted and plated on LB-plates and LB-kanamycin plates to determine the CFU after 48 hours.

Day 6. Counting after 72 hours. The media were diluted and plated on LB-plates and LB-kanamycin plates to determine the CFU after 72 hours.

Day 7. Counting after 96 hours. The media were diluted and plated on LB-plates and LB-kanamycin plates to determine the CFU after 96 hours.

To determine how the added DNA affects the relative fitness of the two competing strains, the Malthusian parameter (m) for each strain is calculated. These are further used to calculate the relative fitness parameter (w) (Lenski et al., 1991).

The following equations are used:

(1) $m = \ln (N_{96}/ N_0)$

(2) $w = m_1/m_2$

Where: m is the Malthusian parameter of each strain/population.

N_{96} is the final CFU in the direct competitions.

N_0 is the initial CFU in the direct competitions.

w is the relative fitness parameter.

4. RESULTS

4.1 DETERMINATION OF GROWTH LIMITING CONDITIONS – BD413 STRAIN

A starting point to this master thesis was to confirm and extend results made earlier by master student Mohammad Safayet Khan (2008). Determination of growth limiting conditions for *A. baylyi* was necessary for the subsequent work to reach the objectives regarding absolute and relative fitness. *A. baylyi* BD413 was chosen in these initial experiments to ensure identical experimental conditions as Khan (2008).

Previous results in our laboratory suggested that *A. baylyi* is able to grow without nitrogen and carbon in the media, but not at phosphate levels below 5%. It has also been shown that addition of DNA has little or no effect on the growth in media without nitrogen or carbon. In media low on phosphate, however, addition of DNA gave substantially improved growth in *A. baylyi* BD413 monocultures. It was concluded that media with low phosphate content provide growth limiting conditions, and that *A. baylyi* is able to use DNA as a source of nutrients in these media.

The initial experiment of this thesis, showed in the figure 4.1.1 below, supports Khan's findings. Growth is seen in media without nitrogen/carbon, but cell death is observed in medium without phosphate and in medium with 4% phosphate. In the medium with 5% phosphate, the growth slowly increases and reaches CFU numbers as high as in S2 standard media at 96 hours.

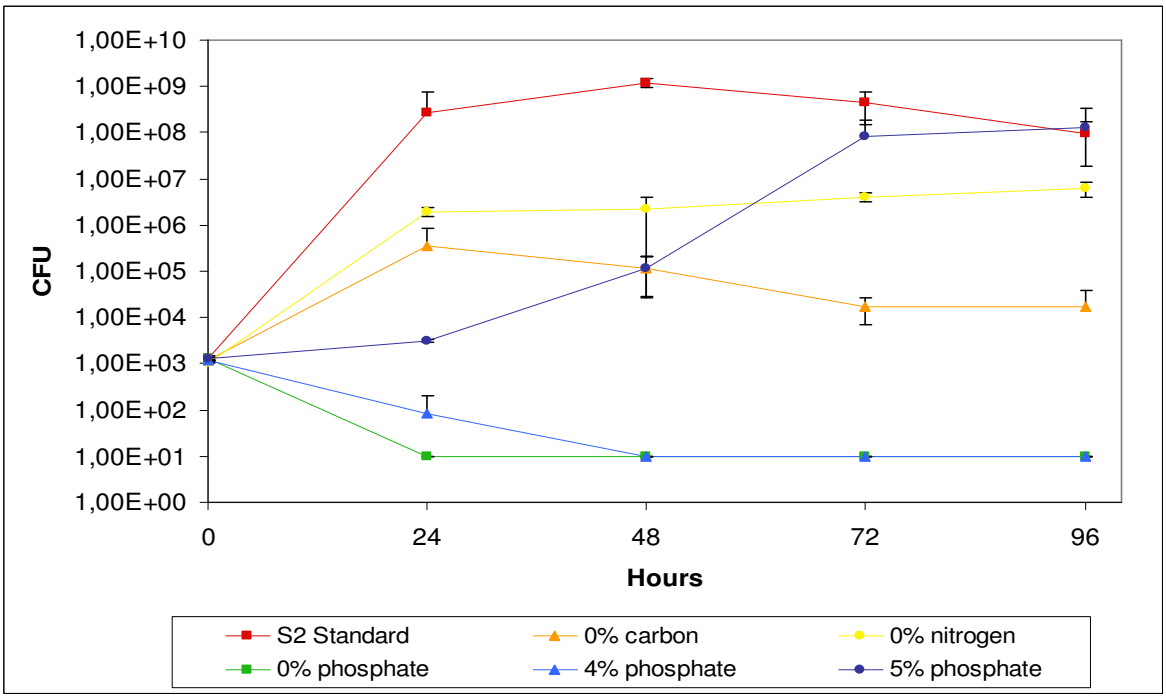

Figure 4.1.1: A. baylyi BD413 in different growth media. The data presented is the average of three independent parallels. Except for certain points which are the average of two parallels because of lacking data on the third parallel. This applies to the points 0% carbon 24 and 48 hours and 5% phosphate 24 and 72 hours. Error bars show standard deviation.

4.2 MEDIA BUFFERED WITH TRIS-HCl

Previous data from our laboratory suggested that low levels of phosphate in S2 minimal medium reduced growth, and that below 5% phosphate net cell death was observed (Khan, 2008). A possible explanation for the observed cell death in the media with 0% and 4% phosphate could be due to lack of buffering effect when the phosphate was removed from these media. Thus, S2 media with reduced phosphate content were buffered with Tris-HCL to test that assumption.

When Tris-HCl was added as a buffer to the medium without phosphate, the bacteria were able to grow, as seen in figure 4.2 (green line). These experiments strongly suggest that earlier observations of *A. baylyi* not being able to grow, both by Khan (2008) and the tests with strain BD413 in this thesis, is a pH issue, rather than a nutrient limiting issue. Because of this, the media with reduced phosphate contents (0%, 4% and 5%) will be buffered with Tris-HCl in the subsequent experiments.

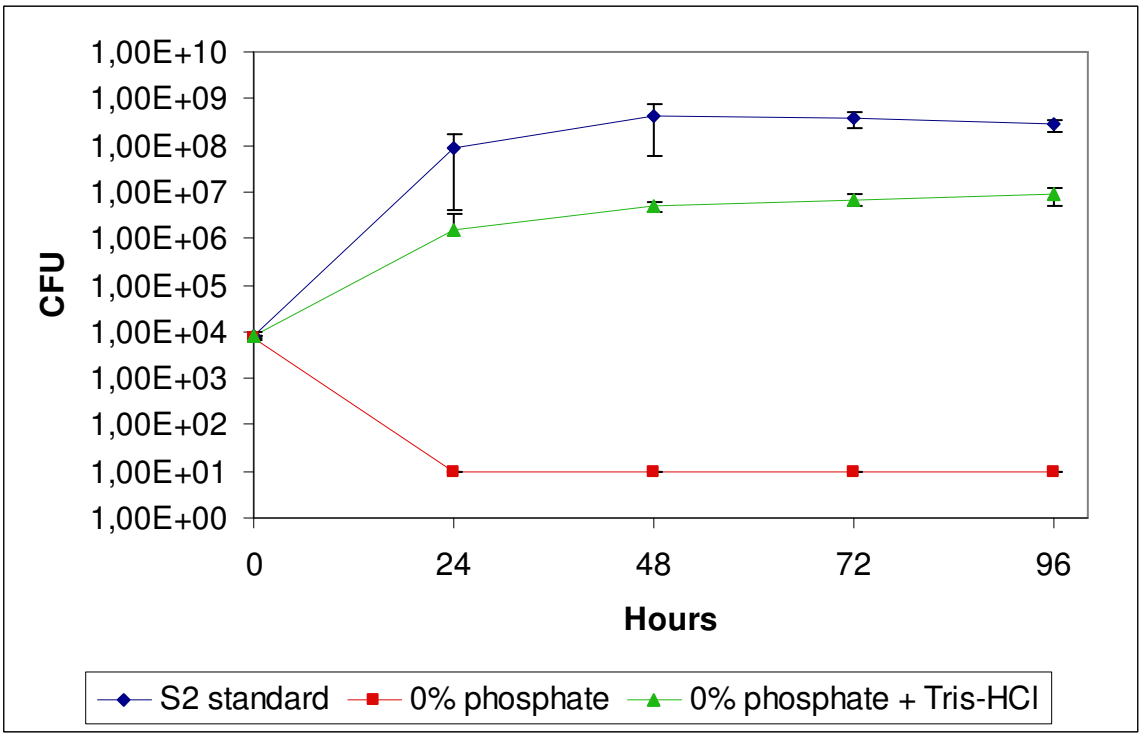

Figure 4.2: Growth curve A. baylyi ADP-1200*, media with 0 % phosphate, with/without Tris-HCl. The presented data is the average of three independent parallels. Error bars show standard deviation. A medium with 0% phosphate and without a buffer gives cell death (red line). When Tris-HCl is added to this medium (green line), growth is sustained, but CFU is lower than in S2 standard medium (blue line).*

4.1 DETERMINATION OF GROWTH LIMITING CONDITIONS – ADP1200 STRAINS

In the tests with *A. baylyi* strains ADP-1200 and ADP-1200-2 DHFR-1, the media with 0% phosphate, 4% phosphate and 5% phosphate were all buffered with Tris-HCl as mentioned above. The addition of this buffer allowed the bacteria to grow even without any phosphate in the medium, and the media with 4% phosphate and 5% phosphate gave CFU numbers as high as the S2 standard medium (See figures 4.1.2 and 4.1.3).

Ideally, ADP-1200 growth should also have been tested in non-buffered phosphate limited conditions. A single parallel of ADP-1200 in medium with 0% phosphate and without buffer gave no growth, the same as BD413 strain, but due to time limitations complete experiments were not performed.

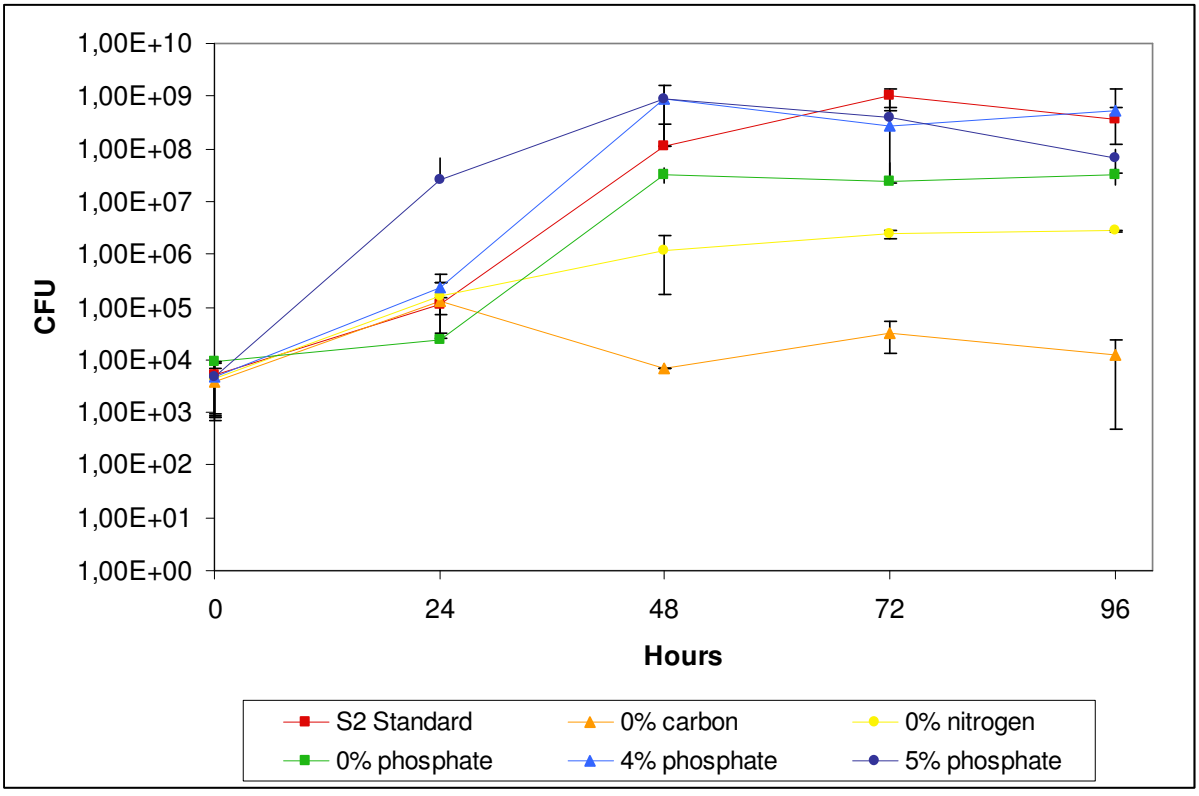

Figure 4.1.2: A. baylyi ADP-1200 in different growth media. The presented data is the average of three independent parallels, with the following exceptions that are based on two parallels: 0% phosphate, whole series (24 hours is a single experiment); S2 standard media 24 hours; 0% carbon 24 and 72 hours; 0% nitrogen 72 and 96 hours; 5% phosphate 96 hours. 0% carbon 48 hours is a single experiment only. Error bars show standard deviation.

ADP-1200-2 DHFR-1 is the non-transformable mutant that would be used against the transformable ADP-1200 in direct competition later. It was therefore important to do the same growth tests as ADP-1200 to make sure the two strains had the same growth profile. A comparison of figures 4.1.2 (ADP-1200) and 4.1.3 (ADP-1200-1 DHFR-1) show that the two strains have similar growth patterns in all media.

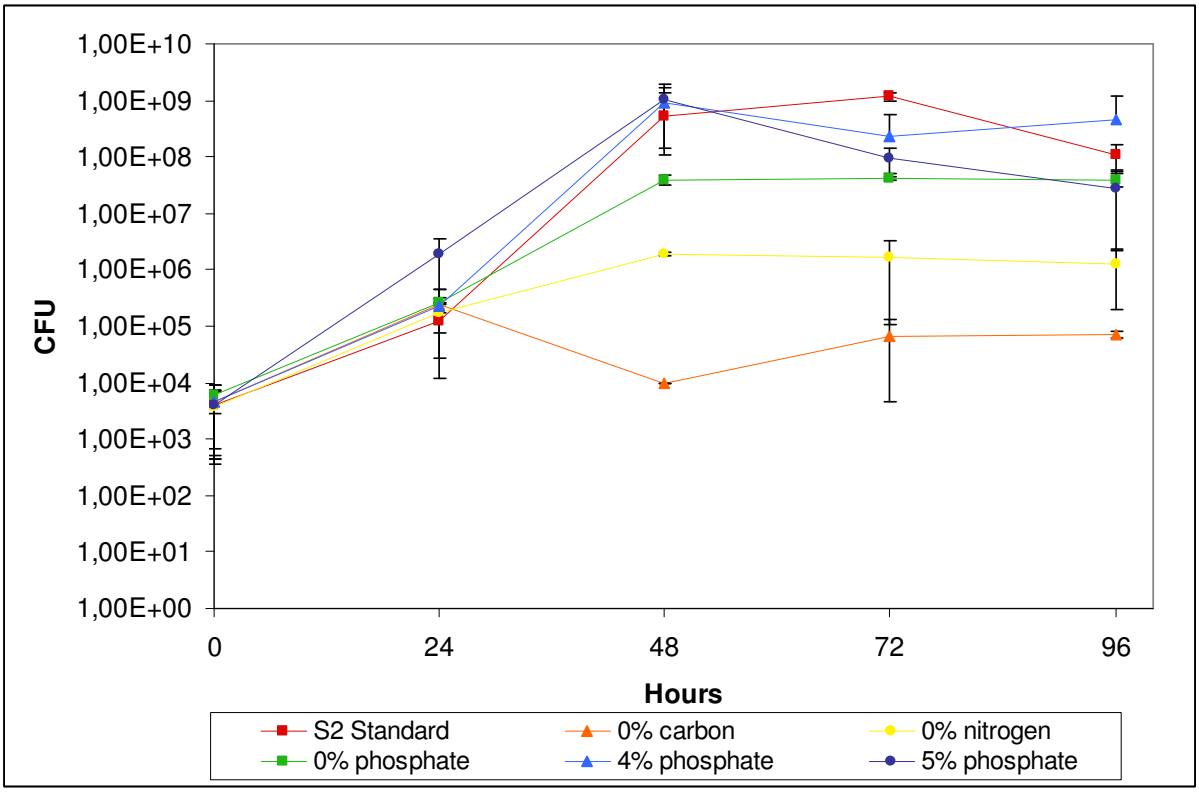

Figure 4.1.3: A. baylyi ADP-1200-2 DHFR-1 in different growth media. The presented data is the average of three independent parallels. The following points are based on two parallels: 0% phosphate, whole series (24 hours is a single experiment); 0% carbon 96 hours; 0% nitrogen 48 hours; 5% phosphate 72 hours. 0% carbon 48 hours is a single experiment. Error bars show standard deviation.

4.3 THE EFFECT OF ADDED DNA ON GROWTH

When DNA was added to the medium with 0% phosphate the growth increased in both the transformable strain and non-transformable strain. The blue line is ADP-1200 (transformable) without added DNA, growth increases with added DNA (green line). The same can be seen for ADP-1200-2 DHFR-1 (non-transformable); red line is without DNA, yellow line is with added DNA.

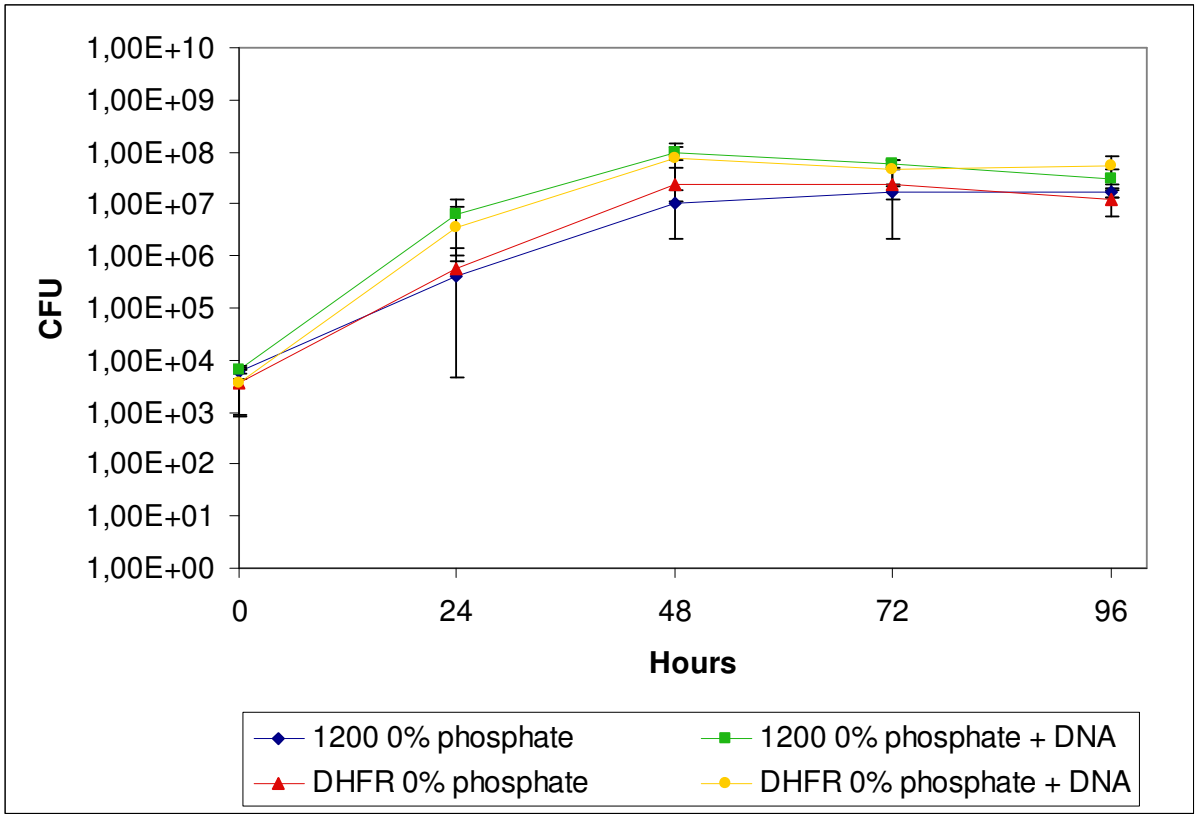

Figure 4.3: Growth curve, media with 0 % phosphate with/without DNA. The presented data is the average of three independent parallels. Blue and green lines show the transformable strain ADP-1200 without and with added DNA. Red and yellow lines show the non-transformable strain ADP-1200-2 DHFR-1 without and with added DNA. Error bars show standard deviation.

4.4 COMPETITION

The direct competition (relative fitness measurements) (figure 4.4.1 below) gave the same results as the DNA experiments above (absolute fitness experiments). Both the transformable (ADP-1200-2) and non-transformable (ADP-1200 DHFR-1) strain have an increased CFU when DNA is added. The red, orange and yellow lines show ADP-1200-2 in S2 standard medium, 0% phosphate and 0% phosphate with added DNA. The green, blue and purple lines show ADP-1200 DHFR-1 in S2 standard medium, 0% phosphate and 0% phosphate with added DNA.

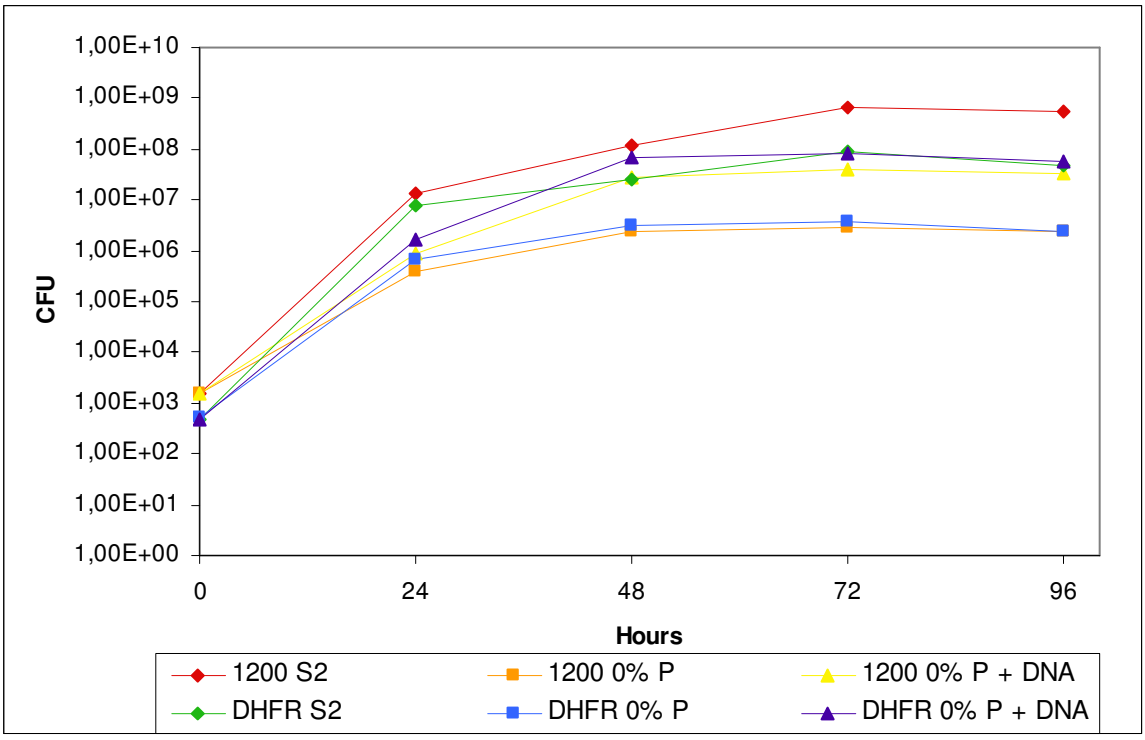

Figure 4.4.1: Growth curve, competition. The presented data is the average of six independent parallels, except for certain points. S2 standard medium 48 hours and 96 hours (both strains) are 5 parallels. S2 standard medium 72 hours (both strains) is 4 parallels. 0% phosphate 24, 48 and 96 hours (both strains) are 5 parallels. 0% phosphate 72 hours (both strains) is 4 parallels. Error bars show standard deviation.

Relative fitness was calculated, and the results are presented in table 4.4 and figure 4.4.2. Note that relative fitness (w) = 1,0 indicates no difference in relative fitness between the two competitors (neutrality). Relative fitness over 1,0 would indicate that the transformable strain (ADP-1200-2) has a higher relative fitness than the non-transformable (ADP-1200 DHFR-1). Relative fitness below 1,0 would indicate that the non-transformable strain (ADP-1200 DHFR-1) has a higher relative fitness than the transformable (ADP-1200-2).

The data presented here strongly suggest that the addition of DNA to the medium does not increase the relative fitness of the transformable wild type (ADP-1200-2) compared to the non-transformable strain (ADP-1200 DHFR-1) (Table 4.4, and Fig. 4.4.2). Interestingly, the absence of phosphate in the medium seems to reduce the relative fitness of wild type when compared to the non-transformable strain. The control experiment in S2 standard media suggested that the wild type (ADP-1200-2) was slightly more fit than the non-transformable strain (ADP1200-DHFR-1). This is in contrast to the previous results in our laboratory, which have suggested that these strains have a relative fitness close to 1 (Johnsen, unpublished). Additional statistical analyses and more experiments would bring us closer to resolving this issue.

Table 4.4: Relative fitness

Medium	m1200	m DHFR	w	SD
S2	12,61905	11,50264	1,096482	0,051063
- DNA	7,168403	8,148458	0,878125	0,097011
+ DNA	10,18026	11,70707	0,869178	0,117448

m is the Malthusian parameter of each strain/population.
w is the relative (Darwinian) fitness of each genotype.
SD is standard deviation of w.
S2 data is the average of 5 parallels, - DNA data is the average of 3 parallels and + DNA data is the average of 6 parallels.

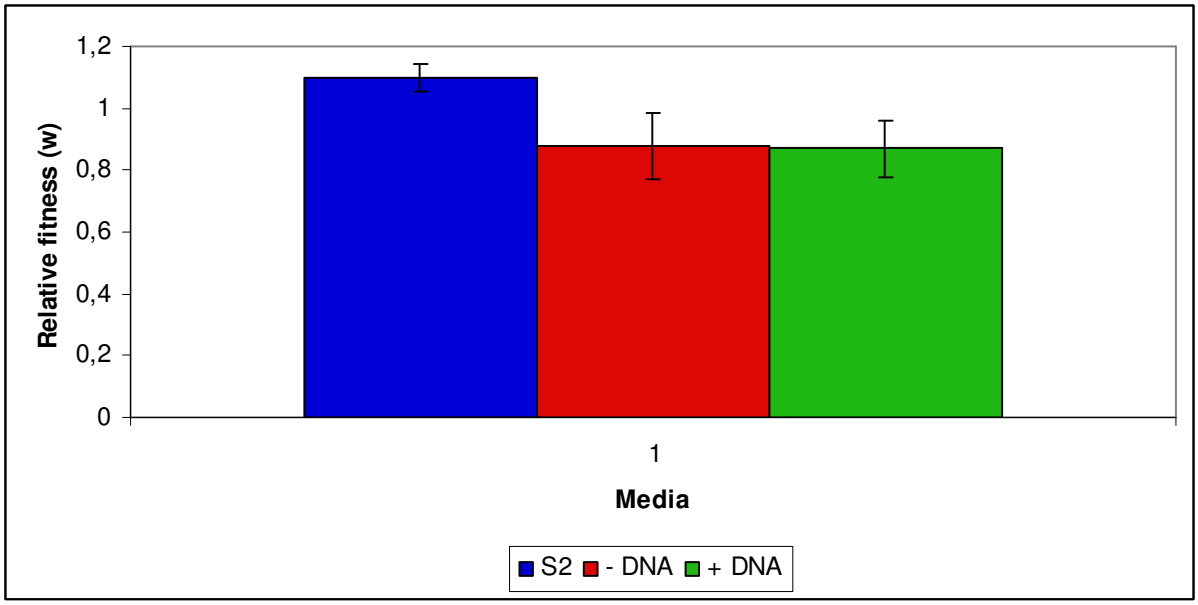

Figure 4.4.2: Relative fitness. S2 is estimated from 5 parallels, - DNA from 3 parallels and + DNA from 6 parallels. Error bars show 95% confidence interval. The red block shows relative fitness in medium without DNA, green block shows relative fitness in medium with added DNA. As a control, blue block shows relative fitness in S2 standard medium. The addition of DNA does not give a change in relative fitness between the groups.

5. DISCUSSION

5.1 GROWTH LIMITING CONDITIONS AND THE ADDITION OF TRIS-HCL.

The first objective of this master thesis was to identify the growth limiting concentrations of N, P, and C sources in liquid S2 medium.

This study has showed that *A. baylyi* is capable of growth in the absence of both carbon and nitrogen. Further, data presented in this thesis suggest that media reduced in phosphate may provide nutrient limiting conditions, and ultimately lead to cell death at phosphate concentrations below 5%. This supports previous experimental work in our laboratory (Khan, 2008). However, in the growth limiting tests using *A. baylyi* strain BD413, media with low concentrations or completely without phosphate may have had severely reduced buffering capacity. The addition of Tris-HCl to all media depleted in phosphate gave changed survival patterns; *A. baylyi* was now able to grow in these media.

Previous data has shown that the addition of DNA to phosphate limiting conditions lead to a positive effect on growth. The offered explanation for this was that DNA provided nutrients (phosphate) during *A. baylyi* growth (Khan, 2008). This master thesis provides an additional explanation for the observed cell death, other than the nutrient issue. As mentioned above, it is possible that media with low phosphate content does not have sufficient buffering capacity to hold the pH stable enough to support growth. Therefore, the addition of DNA could have a stabilizing effect on pH, giving a positive effect on growth.

5.2 THE EFFECT OF DNA ON GROWTH IN MONOCULTURES AND COMPETITION

The second objective of this thesis was to perform fitness assays using both the wild type and a *com*-gene knockout of *A. baylyi* BD413/ADP1 to quantify the effect of DNA uptake on absolute and relative bacterial fitness.

When DNA was added to the growth medium, an increase in CFU was observed. If this observation had only been seen in the wild type strain, this would have been in favour of the food hypothesis. This was however not the case, as there was increased CFU in both the transformable wild type and non-transformable *com*-gene knockout. A possible explanation for this observation would be that DNA was broken down to nucleotides in the medium and

then transferred into the bacteria, this way the non-transforming strain would also be able to benefit from the added DNA. For this explanation to be valid it must be assumed that the *comFECB* operon in *A.baylyi*, which is absent from the non-transforming strain, only assists in uptake of larger DNA fragments, and that single nucleotides are transferred into the cell by other means.
The competition assays (relative fitness) gave similar results as seen in the DNA experiments (absolute fitness). Both the transformable (ADP-1200-2) and non-transformable (ADP-1200 DHFR-1) strains grew to higher CFU when DNA was added to the medium. Relative fitness was calculated, and showed that in S2 standard medium the transformable strain had a slight fitness benefit. In the medium with 0% phosphate with/without added DNA however, the competent strain seemed to loose fitness. It was actually the non-transformable strain that had the highest relative fitness.

Bacher and colleagues (Bacher et al., 2006) presented similar experiments as in this thesis. These authors compared the evolution of wild type and non-competent lineages of *A. baylyi*. The strains they used were constructed in a similar way, with knockout of the *comFECB* operon. They performed fitness assays by direct competition of the two strains, with low-density (10^3 CFU/ml) and high density (10^8 CFU/ml) of cells. In the high density tests stationary phase is reached quickly, whereas in the low density tests the population spends longer time in exponential phase. In the high density tests the *com*-gene knockout were slightly more fit than the wild type, same as is seen in our study. Their next result however, gave the opposite results than observed in our study; when they added DNA to the medium the growth rate in mono cultures decreased, suggesting that addition of DNA gave reduced fitness. They therefore concluded that competence in *A. baylyi* is not maintained for nutrient acquisition.

The hypothesis stated in the aims of this study was:
The presence of functional com-genes increase the absolute and relative fitness of A. baylyi by providing access to DNA fragments as a nutrient source, under nutrient limiting conditions.

There has been some theoretical work done to address the food hypothesis, but relative few experiments in the laboratory. Redfield says that this hypothesis can be tested by observing the regulation of competence and transformation in bacteria (Redfield, 2001). If DNA is used as nutrients, then competence should be induced when nutrients are needed. This is seen to be

true for bacteria like *B. subtilis*, where competence is induced in starvation medium or nutrient limiting medium. In *A. baylyi,* on the other hand, competence is induced in the exponential phase, independent of media (Palmen et al., 1993).

There are some limitations to the food hypothesis. For example, only one of the DNA strands gets taken up by the cell, which would seem a "waste of good food" (Dubnau, 1999). As mentioned earlier, some bacterial species are selective in the DNA they take up. What is the point of being selective if the DNA only is meant to be used as nutrition? That said, even if natural transformation may have evolved for other reasons in these particular species, it does not exclude the gastronomy hypothesis completely, different species may be competent for different reasons and for several reasons.
The ability of competence may have started out for a single purpose in a common ancestor. During evolution of different species the processes of competence and natural transformation may themselves have evolved to an array of purposes, including recombination, DNA repair and nutrition. During the course of evolution, the mechanisms and regulation of competence would have changed. This is supported by the variety of competent species that exist today, who regulate and express competence in distinctive ways.

Finkel and Kolter discovered a mutant of *E. coli* that was unable to survive in competition with the wild type. Further investigation showed a mutation in a gene that had high homology to a *H. influenzae* competence gene. Even though *E. coli* is not known to be naturally transformable, the role of this specific gene in the acquisition of DNA as nutrient was addressed experimentally. Several different experiments gave evidence that this gene is important in the process of DNA uptake in *E. coli*. From this study they suggest that the DNA uptake function of a competence system can be used for nutrients rather than or in addition to new genetic material, thereby supporting the food hypothesis (Finkel & Kolter, 2001). They also mention the "problem" that cells only take up one strand of the double stranded DNA and thereby seem to waste their food. In Gram-negative bacteria this would be less of an issue, as the resulting single strand that does not enter the cytoplasm, stays in the periplasm and gets degraded to nucleotides which are subsequently moved to the cytoplasm for use (Finkel & Kolter, 2001).

It this master study it is clear that something happens to the fitness of the competent strain in media with low phosphate content, but what? Palmen and co-workers have done extensive

work to characterize the process of natural transformation in *A. baylyi*, including tests with different pH values. They showed that below a pH of 6,5 DNA uptake is inhibited (Palmen et al., 1993). There is a slight possibility that the tris-HCl buffer used in this master study did not give enough buffer effect, as it was used at its absolute lowest pH range (pH 7). If the pH in the medium was actually below 6,5, this could be an explanation to why the competent strain seemed to loose fitness. Ideally the pH should have been measured regularly in the media during the growth experiments, to ensure that the pH did not sink that low.

As mentioned above: the ability for competence and natural transformation may have evolved for different reasons in different species. The mechanisms and regulation of these processes would also have evolved and be maintained to tightly fit the purpose for DNA uptake. This would explain why there are so many plausible hypotheses regarding this issue.
In my opinion it is hardly a coincidence that the species known to be selective in DNA sequence also are the species known to be constituently competent. It is tempting to believe that the selectivity of these bacteria may serve as a "security valve" to ensure that only homologous DNA enters the cell. This would indicate a usage of incoming DNA for recombination or repair reasons, and the bacteria would avoid uptake of "useless" heterologous DNA. A variety of this could be when competence is induced by quorum sensing; the bacteria in a population become competent when they are surrounded by many cells of their own species. When so many related bacteria are in close proximity to each other, the DNA in the environment is more likely to be homologous than heterologous. However, a crowded space may also lead to lack of nutrients, and so quorum sensing may be a signal for the bacteria to induce competence for nutritional reasons.
In other species competence is induced in response to starvation, strongly suggesting that in these specific species DNA is used as nutrients. The model organism in this thesis, *A. baylyi*, has not yet been "characterised" to fit perfectly into any of the existing hypotheses. This could indicate that it is not specialized, but is competent for several reasons.

In this master study I have shown that addition of foreign DNA in the growth medium had a positive effect on growth. This effect was however shown in both strains independent of presence of functional *com*-genes. These results are therefore not according to the hypothesis, and do not offer evidence to support it. Even though my work has not been able to provide evidence for the food hypothesis by using *A. baylyi*, more accurate experiments in the future may give other results.

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