Title sheet

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Front page illustration:	The picture on the front page illustrates the extracellular DNA (eDNA) content in the extracellular polymeric substances (EPS) of a pure culture of *Thermotoga neapolitana*. Two pictures were taken from the same microscopic field in the sample, but with different DNA stains, and merged together. A huge amount of eDNA, stained blue, surrounds the cell aggregate and single cells, stained green. The image was taken by a Confocal Laser Scanning Microscope (CLSM).

Abstract

DNA has previously been assumed as being an exclusively intracellular information storage material, but the discovery of extracellular DNA has changed this. Extracellular DNA is found to accomplish many functions like stabilizing the structure of a biofilm, provide protection against foreign DNA and function as nourishment for bacteria during periods of starvation etc. The production of eDNA has so far mostly been investigated in biofilms, however in this project the main focus was on eDNA in pure cultures of *Thermotoga neapolitana, Pseudomonas aeruginosa, Staphylococcus aureus, Tsukamurella spumae* and *Bacillus mycoides* and in complex environmental samples obtained from rocks inside two different streams.

The experiments performed during the making of this Master's thesis were organized into three parts where the first one consisted of experiments on the thermophile bacteria *T. neapolitana.* The second part included experiments on pure cultures and biofilms of *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides.* The third part concerned experiments on the complex environmental samples from streams mentioned above.

The presence and development of eDNA in the investigated samples was confirmed by imaging and genetic approaches and it was found that the production of eDNA in pure cultures were varying and depended on the growth phase of the culture and probably also was influenced by different stress factors.

The found eDNA was compared to genomic DNA extracted from the same cultures, using fragmentation with the restriction enzymes HaeIII and MspI. The results from these experiments were inconclusive since unfortunately no genomic DNA was detectable from the bacteria, making a comparison impossible. Another method of comparing eDNA and genomic DNA was tested using DNase I and ethidium momoazide bromide (EMA) to degrade and block the eDNA respectively so the genomic DNA that was released after cell lysis would be the only available genetic material. This method showed promising results, although it was not possible to amplify genomic DNA after cell lysis in all cases. The eDNA and genomic DNA was also compared for the presence of the three different housekeeping genes encoding 16S rRNA, *gyr*B and *rpo*B and not all primers gave the expected bands for four investigated bacteria types. However the general observation was that the eDNA bands were clearer than the bands originating from genomic DNA. Since the polymerase chain reaction (PCR) products from eDNA contained the same genetic information as that obtained from genomic DNA, this indicates that eDNA is most likely was a copy of the genomic DNA.

Resume

DNA har tidligere været betragtet som et udelukkende intracellulært materiale til lagring af genetisk information, men siden opdagelsen af ekstracellulært DNA har man været nødt til at revidere denne opfattelse. Ekstracellulært DNA har mange andre funktioner end lagring af genetisk information, eksempelvis i stabilisering af biofilm struktur, beskyttelse af bakterien mod fremmed DNA og som næringsstof for bakterierne i sulteperioder osv. Hidtil har produktionen af eDNA primært været undersøgt i biofilm, men i dette projekt fokuseres der i højere grad på eDNA fra renkulturer af *Thermotoga neapolitana, Pseudomonas aeruginosa, Staphylococcus aureus, Tsukamurella spumae* og *Bacillus mycoides* samt fra komplekse prøver fra sten i to forskellige åer.

Forsøgene der er blevet udført i forbindelse med dette specialeprojekt er opdelt i tre dele hvoraf den første består af eksperimenter på den termofile bakterie *T. neapolitana*. Den anden del indeholder eksperimenter på renkulturer og biofilm af *P. aeruginosa, S. aureus, T. spumae* og *B. mycoides*. Den tredje del omhandler eksperimenter på de komplekse prøver fra åer som nævnt overfor.

Udviklingen og tilstedeværelsen af eDNA i de undersøgte prøver blev bekræftet ved hjælp af en visuel samt en genetisk fremgangsmåde. Det blev fundet at produktionen af eDNA i renkulturer var varierende og afhængig af kulturens vækstfase og sandsynligvis også forskellige stressfaktorer.

Det fundne eDNA blev sammenlignet med genomisk DNA ekstraheret fra de samme kulturer ud fra fraktionsmønstre ved brug af restriktionsenzymerne HaeIII og MspI. Resultaterne fra disse forsøg var ikke fyldestgørende idet det genomiske DNA fra bakterierne desværre ikke var muligt at detektere hvilket gjorde en sammenligning umulig. En anden metode til at sammenligne eDNA og genomisk DNA blev prøvet, hvor Dnase I og ethidium momoazidbromid (EMA) blev brugt til henholdsvis at nedbryde og blokere eDNA således, at det genomiske DNA, som blev frigjort når cellerne blev lyseret, var den eneste tilgængelige DNA-type. Denne metode gav lovende resultater, selvom det ikke var muligt at amplificere genomisk DNA i alle tilfælde. Det genomiske DNA blev også sammenlignet med eDNA ud fra tilstedeværelsen af tre forskellige ”housekeeping” gener (16S rRNA, *gyr*B and *rpo*B) og ikke alle primerne gav det forventede bånd for de fire undersøgte bakterietyper. Generelt blev det dog observeret at bånd på agarose geler fra eDNA var klarere end bånd fra det genomiske DNA. Da polymerase kædereaktion (PCR) produkterne fra eDNA indeholdte samme genetiske information som det, der blev fundet for genomisk DNA, er det sandsynligt at eDNA er en kopi af det genomiske DNA.

Preface

This report is a Master's thesis by Rasmus Clemmensen. The thesis is submitted in fulfillment of the requirements for the degree of "Master of Science in Engineering, Biotechnology", at the Department of Biotechnology, Chemistry and Environmental Engineering at Aalborg University, Denmark. The work presented in this thesis has been carried out from October 2008 until December 2009. The laboratory work has taken place in the laboratories of the environmental biotechnology group at Aalborg University. The thesis has been carried out under the supervision of Professor Per Halkjær Nielsen, Associate Professor Jeppe Lund Nielsen and Ph.d.-stipendiat Dominik Marek Dominiak, from Aalborg University.

References are stated using the Harvard citation method i.e. [surname of first author, year of publication] and a full list of all references in alphabetical order is found in the list of references at the end of the thesis. Figures and tables are consecutively numbered. Abbreviations used in the thesis are listed and explained on the page after the preface. General abbreviations are not listed.

This report is mainly directed at persons with knowledge or special interest in the area of extracellular DNA.

Aalborg, December 4th 2009

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Rasmus Clemmensen

List of abbreviations

B. mycoides	*Bacillus mycoides*
bp	Base pair
CFA medium	Colonization factor antigen medium
CFTR	Cystic fibrosis transmembrane conductance regulator
CLSM	Confocal laser scanning microscopy
DAPI	4',6-diamidino-2-phenylindole
DDAO	Dodecyldimethylamine oxide
dH_2O	Deionised water
DNase	Deoxyribonuclease
DSM	65. Gym Streptomyces Medium
eDNA	Extracellular DNA
EDTA	Etylen-diamin-tetra-eddikesyre
EMA	Ethidium momoazide bromide
EPS	Extracellular polymeric substances
F primer	Forward primer
gDNA	Genomic DNA
HaeIII	Restriction enzyme
Kb	Kilo base pair
MspI	Restriction enzyme
MQ	Milli Q water
NH_4AC	Ammonium acetate
*Not*I	Restriction enzyme
OD_{600}	Optical density (600 nm)
PBS	Phosphate buffered saline
PCI	Phenolcloroformisoamylalcohol

PCR	Polymerase chain reaction
PI	Propidium iodide
R primer	Reverse primer
Rpm	Revolutions per minute
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
SYTO13	Green-fluorescent nucleic acid stains
Taq	*Thermus aquaticus*
ThT	Thioflavin T
T. neapolitana	*Thermotoga neapolitana*
Tris	Tris(hydroxymethyl)aminomethane
T. spumae	*Tsukamurella spumae*
v/v	Volume per volume

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1 Introduction

1.1 Bacterial biofilms

Most bacteria live close or attached to surfaces and establish a complex community of cells enclosed in a hydrated polymeric matrix, known as a biofilm (Sauer, 2003) and (Whitchurch, et al., 2002). The matrix is composed of polysaccharides, proteins and nucleic acids (Liu, et al., not published yet) and (Allesen-Holm, et al., 2006). Biofilms normally consist of many different bacteria and other microorganisms, but a biofilm can also be made from just a single bacterial species (Sauer, et al., 2007). A biofilm functions like a synergistic micro consortia, and bacteria have a prolonged life in a favorable environment when living in a biofilm (Böckelmann, et al., 2007).

Benefits and developing of biofilm life

There are three main reasons why bacteria adhere to surfaces and form biofilms: the bacteria are protected from environmental factors like bacteriophages, UV radiation, pH shifts, osmotic shock and antibiotics; the accessibility of nutrients and metabolic cooperation are better in a biofilm; and it is easier for bacteria to receive new genetic traits from other bacterial species (Tang, 2009).

The structural importance of the components depends on the age of the biofilm, the environmental conditions and on the particular strain (Allesen-Holm, et al., 2006). The extracellular polymeric substances in a matrix protect the cells within it from toxins and pollutants, and are important in developing the spatial structures (Sauer, et al., 2007). The structure of a biofilm is also affected by fimbria and polysaccharides, which are expressed during early biofilm development, and to control the development of a biofilm the developmental process from single cells to a thick biofilm must be understood in detail (Allesen-Holm, et al., 2006).

Quorum sensing is the ability of bacteria to communicate with each other in a population, to coordinate certain behaviors based on the local density of the bacterial population (Sauer, 2003). Quorum sensing is important in the formation and stabilization of a *Pseudomonas aeruginosa* biofilm and in resistance to antibiotics (Sauer, 2003) and (Allesen-Holm, et al., 2006). It seems that quorum sensing plays a role in *P. aeruginosa* initiating infection and persisting in a host (Sauer, 2003). In *P. aeruginosa* the expression of several virulence factors is controlled by quorum sensing (Allesen-Holm, et al., 2006).

Bacteria forming a biofilm are more resistant to antibiotics, biocides and the host immune response than free living bacteria of the same species (Sauer, 2003) and (Allesen-Holm, et al., 2006). According to Tang (2009) there are two main reasons why biofilms are more resistant to antimicrobial agents than normal bacterial cultures. The penetration of antibiotics could be delayed by the biofilm, and the growth rate of bacteria is different inside a biofilm. The efflux system is an active transport mechanism for unwanted toxins, like antibiotics, and this system can contribute to multi drug resistance and may be enhanced in biofilms (Tang, 2009). This resistance makes the biofilm a huge problem for the medical industry, especially when trying to treat a chronic bacterial infection (Sauer, 2003). The

resistance of a biofilm is much greater than for bacteria living in liquid cultures, and a biofilm can be up to 1,500 times more resistant to antibiotics and host immune responses (Sauer, et al., 2007). The resistance of biofilms makes them very undesirable in the food industry and hospitals etc. because they can cause chronic infections in both humans and animals (Allesen-Holm, et al., 2006).

The matrix also serves the purpose of retaining water and nutrients (Steinberger, et al., 2005). The supply of nutrients and fluids in a biofilm resembles the way a tissue functions. The ionic strength in the matrix is changed and by periodic contraction of the polymers in the matrix, the fluids and nutrients are pumped through water channels (Sauer, et al., 2007).

During the development of a biofilm, the genome of a bacteria changes. According to Sauer, et al. (2007) the change in the *P. aeruginosa* genome is 1 %, or 70 genes different in bacteria grown suspended in liquid culture compared to a 5 day old biofilm (Sauer, et al., 2007). Proteins that are expressed during biofilm development are differentially produced compared to the expression of protein from planktonic cells (Sauer, 2003).

There are contradictory suggestions about which compound is the primary cell to cell interconnecting compound in mature biofilms. Extracellular DNA is an abundant matrix material in *P. aeruginosa* biofilms, but the critical matrix compounds are found to be exopolysaccharides (Allesen-Holm, et al., 2006). Other experiments with DNase treatment of biofilms derived from different *P. aeruginosa* strains dissolved the biofilms, which suggests that eDNA is the primary matrix compound (Allesen-Holm, et al., 2006). Steinberger, et al. (2005) stated that eDNA is definitely important in the formation and stability of saturated biofilms (Steinberger, et al., 2005).

1.2 Extracellular DNA

Extracellular DNA is DNA released into the environment (Tavares, et al., 2001). Extracellular DNA originates either from active secretion or cell lysis (Steinberger, et al., 2005) and plays a key role in natural environments (Tavares, et al., 2001).

There is a general interest in eDNA because many molecular techniques measure DNA to estimate the amount of cells present in a sample. Overestimation of cells due to misinterpretation of DNA in many molecular methods is a big problem in molecular biology, because some of the DNA measured is eDNA, which is present outside the cell. In environmental samples the DNA is measured to estimate the amount of cells (Liebeskind, et al., 1994), but cell counts reveal that there is too much DNA compared to cells, and an overestimation of cell number due to misinterpretation of DNA of up to a factor of 10 is likely (Palmgren, et al., 1996). Corinaldesi, et al. (2005) estimated that the amount of eDNA is much greater than the amount of genomic DNA in marine sediments (Corinaldesi, et al., 2005).

Many different bacterial systems could have been overestimated through time, and much money could have been wasted by giving bacterial systems attention unnecessarily. If this is generalizable, then several molecular approaches using DNA should be reconsidered, because of potential overestimation of cell numbers due to DNA measurements (Corinaldesi, et al., 2005).

Some bacterial species produce a lot of eDNA and some produce less (Böckelmann, et al., 2006). Bacteria can produce different amounts of eDNA, which suggests that eDNA production is an adaption to the environment (Steinberger, et al., 2005). Extracellular DNA is released by *Streptococcus mutans, Enterococcus faecalis, Haemophilus influenza,* strain F8[1] and *Pseudomonas* species etc., but the amount of eDNA varies with different bacterial strains and growth conditions (Tang, 2009). Huge amounts of extracellular DNA were first discovered in biofilms of *P. aeruginosa,* and the pathogenic bacterium has become a model organism in biofilm research (Allesen-Holm, et al., 2006).

Today there is still very limited knowledge about eDNA's composition, universality and persistence. However, there is a lot of speculation, and investigations are ongoing (Steinberger, et al., 2005).

Origin of extracellular DNA

Finding the origin of eDNA could help in understanding the structural importance and other functions of eDNA. If eDNA is identical to genomic DNA then there would be good evidence that eDNA derives from cell lysis, but if eDNA is derived from active secretion there may be differences between eDNA and genomic DNA (Böckelmann, et al., 2006). The recent research on pure cultures has been contradictory about whether eDNA derives from lysed cells or from active secretion (Steinberger, et al., 2005). Species investigated so far suggest that eDNA most commonly originates from cell lysis, but that there is also an active excretion of eDNA in bacteria which is controlled by quorum sensing . DNA is an important component of the EPS and not just left over from lysed cells as believed earlier (Böckelmann, et al., 2006). According to Palmgren, et al. (1996) eDNA is actively excreted by bacteria, because no signs of cell lysis, such as decreasing cell numbers and activity of intracellular enzymes, were seen during their experiments (Palmgren, et al., 1996). In *P. aeruginosa* small vesicles from the outer membrane are suggested to release eDNA (Allesen-Holm, et al., 2006), and this is independent of cell lysis (Whitchurch, et al., 2002). The excretion of vesicles containing DNA inside or attached to the membrane is controlled by quorum sensing (Spoering, et al., 2006).

The study by Böckelmann, et al. (2006) is the only one which finds notable differences between eDNA and genomic DNA, from work with pure cultures of strain F8 (Böckelmann, et al., 2006). Extracellular DNA and genomic DNA have been compared by cleaving both with different restriction enzymes and analyzing the products using an agarose gel electrophoresis profile. The results show that the DNAs are of similar size, but not identical, and in general the eDNA is longer than the genomic DNA (Böckelmann, et al., 2006). 98 % similarity of eDNA and genomic DNA is found when performing a PCR with the universal primers 27F and 1492R (Böckelmann, et al., 2006). According to the random amplification of polymorphic DNA profile the eDNA and genomic DNA have some differences, but in general they have very common banding patterns (Böckelmann, et al., 2006). The eDNA could not be digested by *Not*I, which indicates that eDNA might be protected by methyl groups, because *Not*I is sensitive to CpG[2] methylation (Böckelmann, et al., 2006). S1 endonuclease specifically cleaves single

[1] An aquatic bacterium isolated from 'river snow' of the South Saskatchewan River, Canada.
[2] CpG stands for cytosine and guanine separated by a phosphate.

stranded DNA/ RNA and could not cleave eDNA. Extracellular DNA was cleaved by DNase I and the results from both nucleases suggest that eDNA is double stranded DNA (Allesen-Holm, et al., 2006). When the eDNA is not totally identical to genomic DNA, there must be some form of active transport from the bacteria (Böckelmann, et al., 2006). According to the primary sequence the eDNA and genomic DNA cannot be differentiated for some species, but results from the terminal restriction fragment length polymorphism profile differed considerably (Steinberger, et al., 2005). Whether eDNA is circular or linear, or if any proteins are associated with the secondary structure of eDNA, is not yet clear (Steinberger, et al., 2005).

Function of extracellular DNA

DNA has always been assumed to be a purely intracellular information storage material, but it also contributes to the formation and structure of a biofilm, nutrition, protection against foreign DNA and phosphate cycling etc. Tavares, et al. (2001) suggests that eDNA acts as a source of nucleotides for DNA replication (Tavares, et al., 2001). During starvation eDNA serves as a nutrient for bacteria, and eDNA also plays a structural role in stabilizing biofilms (Steinberger, et al., 2005). It is possible that eDNA takes part in other functions, which today are attributed to exopolysaccharides (Steinberger, et al., 2005).

Extracellular DNA as a sole source of phosphorous, nitrogen and carbon

DNA consists of approximately 10 % phosphorous, in a weight to weight ratio, which makes it the most prevalent element of nucleic acids and founds the basis for a function of DNA in phosphorous cycling (Dell'Anno, et al., 2005) and (Böckelmann, et al., 2007). Extracellular DNA is an organic phosphorus compound that is an alternative source of phosphorous in marine and freshwater environments (Pinchuk, et al., 2008). In freshwater and marine habitats phosphorus plays an important role, because it often limits the growth of several bacteria (Pinchuk, et al., 2008).

In marine sediments eDNA has been proven to have an important role in the phosphorous cycle (Dell'Anno, et al., 2004) and (Corinaldesi, et al., 2005). In Dell'Anno, et al. (2005) the fact that DNA is an important molecule in phosphorous cycling is studied, and in the deep-sea sediments, which have a very high content of DNA, it is found that more than 90 % of the DNA in the top of the sediment is eDNA (Dell'Anno, et al., 2005). There are huge amounts of eDNA in the deep-sea sediments compared to DNA in all benthic prokaryotes living in the top 10 cm of the marine sediments (Dell'Anno, et al., 2005). 17 % of the total organic phosphorous regeneration is due to the remineralisation of eDNA in the top 10 cm of the deep-sea sediments (Dell'Anno, et al., 2005) and 41 % of the daily bacterial requirement of phosphorous is provided by eDNA. However, the ecological role of eDNA is not yet fully clarified (Dell'Anno, et al., 2004).

Prokaryotes dominate the life in the deep-sea sediments. Carbon, nitrogen and phosphorous are all elements needed for maintaining life (Dell'Anno, et al., 2005). Extracellular DNA supplies 4 % of the

prokaryotic carbon demand, 7 % of the prokaryotic nitrogen demand and 47 % of the phosphorous demand, which indicates that eDNA is especially important in phosphorous cycling (Dell'Anno, et al., 2005), but that it also contributes to carbon and nitrogen cycling (Dell'Anno, et al., 2005), (Corinaldesi, et al., 2005), (Whitchurch, et al., 2002) and (Tavares, et al., 2001).

Metal-reducing bacteria, like the genus *Shewanella,* reside in phosphate limited environments and utilize eDNA as a sole source of phosphorous. Nitrogen and carbon is especially beneficial during nutrient cycling during both aerobic and anaerobic conditions (Pinchuk, et al., 2008).

According to Dell'Anno, et al. (2005) the measurement of a high DNase activity, which catalyses the cleavage of the phosphodiester linkage in the DNA backbone is good evidence that eDNA contributes to phosphorous cycling (Dell'Anno, et al., 2005).

Exchanging genetics

It is well known that DNA release takes place in nature and that eDNA may contribute to genetic exchange by natural transformation, so the evolutionary role of eDNA cannot be disregarded (Tavares, et al., 2001). Vertical gene transfer is the normal way of inheriting genetic material, and this is when an organism inherits genetic material from its parents. Extracellular DNA is thought to play a role in horizontal gene transfer, where an organism incorporates a gene from an unrelated organism. Extracellular DNA is very persistent and when it is found in large amounts it may contribute to horizontal gene transfer (Corinaldesi, et al., 2005) and (Steinberger, et al., 2005). Extracellular DNA adsorbs to DNA surface receptors on bacteria to help bacteria to survive and spread their genetic information, which is suggested to proceed through a specialized protein complex (Liu, et al., not published yet).

Strains developed by horizontal gene transfer are being studied to find new techniques to promote gene transfer (Tavares, et al., 2001). The transport and growth of bacteria can be traced in natural environments by using eDNA, if the cryptic sequence of eDNA is identifiable (Steinberger, et al., 2005).

Network-like structures

Extracellular DNA has recently been found to form a spatial network-like structure in many pure cultures (Böckelmann, et al., 2006). The aquatic strain F8 produces a stable filamentous network of eDNA and microfilaments are only detected in F8 cultures between 4 to 7 days old (Böckelmann, et al., 2006). This is the only period of time that the network-like structure appears and eDNA is released in the liquid culture (Böckelmann, et al., 2006). In a pure culture the eDNA plays a very important structural role in the EPS (Böckelmann, et al., 2006).

The network-like structure consisting of eDNA is confirmed by specific nucleic acid stains, such as SYTO9, SYTO62, SYTO63, SYTOXGreen and DAPI. The network has also been tested for proteins,

polysaccharides and lipids by specific stains, but no positive results have been obtained (Böckelmann, et al., 2006). The presence of DNA was triple checked by measuring the absorbance at 260 nm and by treating the sample with DNase I, which removes all the extracellular material and verifies that the extracellular material consists of eDNA (Böckelmann, et al., 2006) and (Böckelmann, et al., 2007).

The structure in biofilms

Liu, et al. (2008) and Whitchurch, et al. (2002) conclude that eDNA plays an important structural role in bacterial aggregation, colony stability and biofilm formation (Liu, et al., 2008) and (Whitchurch, et al., 2002). The amount of eDNA plays a key role in industrial biofilms and it would be of great economic importance to know the origin of eDNA, to prevent biofilm formation or keep it controlled (Steinberger, et al., 2005).

The adsorption of DNA to a surface is always promoted by the divalent cation Mg^{2+}. The monovalent Na^{+} ion in high concentrations (100 mM) will also increase adsorption, but Na^{+} at low concentrations will decrease the adsorption. The adsorption is also dependant on the concentration of the eDNA, because other negatively charged components on the cell surface of the bacteria will inhibit the adsorption, because of electrostatic repulsion (Liu, et al., not published yet) and (Liu, et al., 2008). The retention of large DNA fragments is favored by the presence of large numbers of low-specificity binding sites (Steinberger, et al., 2005).

During alginate biosynthesis, which is required for initial biofilm formation, a large amount of eDNA is produced by *P. aeruginosa* (Böckelmann, et al., 2006) and (Böckelmann, et al., 2007). In the late log phase of *P. aeruginosa* large amounts of eDNA are released, but in the initial and mid log phase only a small amount of eDNA is released. This has been confirmed by both spectrophotometric and fluorometric measurements (Allesen-Holm, et al., 2006).

The production of eDNA in unsaturated biofilms is species dependent. *P. aeruginosa* and *P. putida* are both bacteria that produce a lot of eDNA , compared to biofilms consisting of *Rhodococcus erythropolis* and *Vairiovorax paradoxus* (Steinberger, et al., 2005). Fewer than 5 % of the total DNA in *Rhodococcus erythropolis* and *Vairiovorax paradoxus* biofilms was eDNA, but 17 % of the total DNA pool was eDNA in *P. aeruginosa* biofilms, and 32 % was eDNA in biofilms consisting of *P. putida* (Steinberger, et al., 2005). If two low eDNA producers form a multiple species biofilm, it can influence the amount of eDNA notably. This indicates that the mechanisms of a multi-species biofilm are complex and not yet fully understood (Steinberger, et al., 2005). All studies so far have been done in laboratories with pure cultures, but *in vivo* eDNA contributes to biofilm formation as well.

Biofilm formation can be inhibited by adding DNase I, but this depends on the age of the biofilm. If DNase I is present in the growth medium, then *P. aeruginosa* biofilm formation is prevented. Young biofilm is more susceptible to DNase I than an 84 hour old biofilm, which is so resistant that DNase I only affects the biofilm to a minor degree (Whitchurch, et al., 2002). The distribution of eDNA in a biofilm of *P. aeruginosa* changes during the development of the biofilm. When it is 2 days old, the

highest concentration of eDNA is on the surfaces of microcolonies. When the biofilm is 4 days old the highest concentration of eDNA is present on the substratum and in the stalk. The eDNA is present throughout the mushroom shaped structure when the biofilm is 6 days old (Allesen-Holm, et al., 2006). The DNase I treatment of *Enterococcus faecalis* reduces the biofilm formation when the biofilm is younger than 12 hours, but after 24 hours the DNase I only had a minor effect (Tang, 2009). In *Staphylococcus epidermidis* the DNase I treatment did not work after 12 hours, but it did reduce biofilm formation of the very young biofilms (Tang, 2009). When biofilm formation can be prevented or reduced by DNase I, it demonstrates that eDNA has an important structural role in biofilm formation, and the resistance to DNase I of older biofilms could be caused by other substances that strengthen the biofilm, such as the development of proteolytic enzymes that inactivate DNase I (Whitchurch, et al., 2002). The resistance to extracellular DNases was suggested by Böckelmann, et al. (2006) and Steinberger, et al. (2005) to be caused by complexes formed between DNA and proteins, sediments, clay or humic acids. In *Frankia strain R43 and CcI3,* which are nitrogen-fixing soil actinomycetes, evidence was found that eDNA was resistant to *Frankia* Dnases, but that it was degraded by DNase I (Böckelmann, et al., 2006) and (Steinberger, et al., 2005). Metal-ions keep the sludge flocs together and could be a physico-chemical protector of the DNA from degradation (Palmgren, et al., 1996). Also, the DNase treatment could suggest that young biofilms are held together by eDNA and that older biofilms are held together by other compounds (Allesen-Holm, et al., 2006).

More than 80 % of chronic inflammatory and bacterial infections, such as those involved in cystic fibrosis etc. are caused by biofilms (Sauer, et al., 2007). *P. aeruginosa* is a well studied model for biofilm formation and cystic fibrosis is aggravated by a chronic bacterial infection of *P. aeruginosa* (Whitchurch, et al., 2002).

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene are the cause of patients suffering from cystic fibrosis. The CFTR gene usually creates proteins that span the plasma membrane surrounding cells, which act primarily as channels for chloride ions. When the chloride ions are prevented from crossing the membrane, they are trapped in the skin, which is why cystic fibrosis patients have salty sweat, and thick mucus inside the airways. The respiratory tract of a cystic fibrosis patient is chronically and recurrently infected, typically by *P. aeruginosa*, which eventually leads to respiratory failure (Højby, 2006).

P. aeruginosa and other bacteria adapt to the host inflammatory responses by developing a mucoid biofilm, which has special characteristics that allow the formation of large colonies. The small airways of the lungs are in time blocked by the mucus, and the bacteria developing a biofilm within this mucus are difficult for both the host immune system and antibiotics to penetrate and eradicate (Højby, 2006).

Treatment of cystic fibrosis patients is still insufficient, but inhalation of DNase I can help degrade the mucous biofilm in the lungs. DNase I treatment is good at preventing biofilm formation, but has only a limited effect on a mature biofilm (Whitchurch, et al., 2002), so the treatment should be started as early as possible. The DNase I treatment may not eradiate the infection totally, but it seems that it helps the patients (Højby, 2006). The treatment of persistent infections like cystic fibrosis with DNase I

could be more successful if the structure of eDNA was elucidated (Steinberger, et al., 2005), and also the possible prevention of formation of biofilms (Højby, 2006).

1.3 Methods for detection and quantification of DNA

Extraction of extracellular DNA

It is very difficult to isolate eDNA without any contamination from genomic DNA. The DNA pool has to contain only eDNA when performing molecular work (Corinaldesi, et al., 2005).

According to Steinberger, et al. (2005) there are two different approaches to isolate DNA from a microbial community, direct extraction and indirect extraction (Steinberger, et al., 2005). The direct extraction method was developed by (Ogram, et al., 1987) and is generally an easy method, yielding more DNA with few steps, but it does not consider eDNA as a separate factor. The indirect extraction method was developed by (Holben, et al., 1988) and includes a washing step to remove eDNA (Steinberger, et al., 2005). The direct extraction method is commonly used because it is generally assumed that eDNA in natural and complex environments is of low abundance (Steinberger, et al., 2005). But there is evidence that eDNA in marine sediment etc. comprises more than 70 % of the total DNA amount, so the indirect extraction method should be reconsidered (Steinberger, et al., 2005).

In Corinaldesi, et al. (2005) there are some suggestions for the extraction procedure of eDNA in sediments, and those suggestions can also be used in the work with eDNA from pure cultures etc. (Corinaldesi, et al., 2005). The eDNA that is extracted should be representative of the total eDNA pool in the sample, and it has to be suitable for molecular work and quantification by stains etc. If the eDNA is to be compared to the intercellular DNA, then both extractions should be obtained from the same sample (Corinaldesi, et al., 2005).

The extraction step is very important in determination of the EPS composition and a good extraction procedure is characterized by minimal cell lysis (Frølund, et al., 1996). The separation of eDNA from genomic DNA is done by centrifugation, followed by filtering the supernatant, and finally eDNA precipitation (Corinaldesi, et al., 2005).

Staining techniques

Dodecyldimethylamine oxide [7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)] (DDAO) is an amphiphilic compound that exists in a neutral or protonated form, depending on the pH and ionic strength (Bonincontro, et al., 2003) and (Mel'nikova, et al., 2000). DDAO is too big to penetrate the cell membrane, which means that only the eDNA is stained. DDAO will bind to DNA because of electrostatic attraction. According to Mel'nikova, et al. (2000) the DDAO is fully protonated at around pH 2, and at a pH lower than 6 the DDAO surfactant becomes ionized (Mel'nikova, et al., 2000). The negatively charged phosphate groups of DNA may interact electrostatically with the ionized head group of DDAO, see Figure 1 (Bonincontro, et al., 2003).

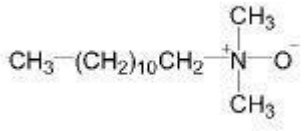

Figure 1: The chemical structure of DDAO. Picture is modified from (Goracci, et al., 2005).

SYTO13 dye is a cell permeable nucleic acid stain that can penetrate both Gram-positive and Gram-negative bacteria. SYTO13 does not only bind to DNA, but it shows a large amount of fluorescence when binding to all nucleic acids (Böckelmann, et al., 2006).

DAPI is a specific DNA binding stain which preferentially stains double stranded DNA (Palmgren, et al., 1996). DAPI is a cell permeable stain and can stain both live and fixed cells (www.invitrogen.com).

Propidium iodide (PI) is a specific DNA binding stain and it is impermeable to cell membranes (www.invitrogen.com).

The stains can be combined to provide specific information about the genomic and extracellular DNA from bacteria.

Use of ethidium monoazide bromide

DNA is an abundant molecule in biofilms and DNA is detected by different methods. DNA is sensitive to and degraded by DNases, which makes it possible to see if the samples contain DNA. Optical density can reveal DNA with a peak absorbance at 260 nm, and electrophoresis can also be used to detect DNA (Whitchurch, et al., 2002). Molecular methods based on measuring DNA often overestimate the amount of viable cells due to the problem of distinguishing between DNA from viable and dead cells (Wagner, et al., 2008), (Nocker, et al., 2006) and (Rudi, et al., 2005). Overestimation of viable cells is a serious problem in the food, water and pharmaceutical industries etc. (Wagner, et al., 2008), (Nocker, et al., 2006) and (Rudi, et al., 2005). To get around this problem, RNA can be measured. RNA based methods target the active part of a microorganism and are very clear indicators of viable cells, but working with the rapidly degrading RNA is expensive, very technically demanding and there is a high risk of contamination with RNA-degrading enzymes. Another possibility to distinguish between viable or dead cells is to use dyes for microscopic differentiation, but culture based techniques are dependent on both the temperature and type of medium. The limited number of bacteria used as the basis for extrapolating information from is another disadvantage, so it is generally preferable to work with the stable DNA instead (Nocker, et al., 2006).

Ethidium monoazide bromide could be a solution to these problems. It binds covalently to DNA, and results in sequences that cannot be PCR amplified (Nocker, et al., 2006) and (Rudi, et al., 2005). Due to the strong covalent binding of DNA and EMA, the blocking of DNA resists the hot temperatures during a PCR. The binding of EMA and DNA is activated by light exposure using visible light with a maximum absorbance of 460 nm (Nocker, et al., 2006) and free EMA is inactivated by light exposure (Rudi, et al., 2005) and reacts with water molecules (Nocker, et al., 2006), see Figure 2. EMA cannot penetrate the

cell membrane, or cell wall and it only blocks the DNA outside the cell, or inside cells which have a compromised cell wall (Nocker, et al., 2006). EMA binds very strongly and irreversibly, with a binding constant of 2 x 10^5 to 3 x 10^5 to DNA and RNA (Nocker, et al., 2006).

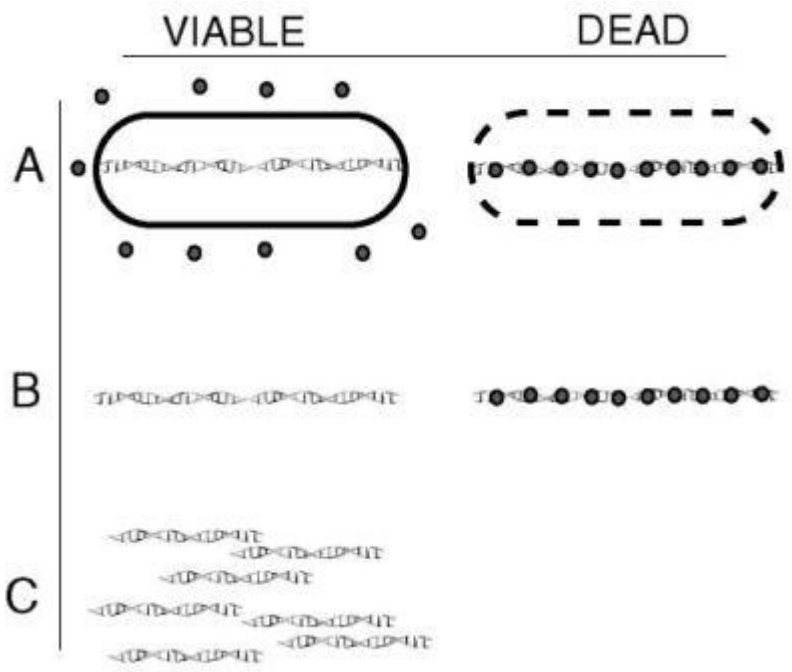

Figure 2: Schematic representation of EMA-PCR. (A) EMA is added to the test sample containing both viable and dead cells. EMA penetrates the dead cells and binds to the DNA. Light exposure for 1 min. leads to covalent binding and inactivation of free EMA. EMA does not enter viable cells. (B) There are two populations of DNA after purification. The DNA population from viable cells is unbound, while the DNA from the dead cells is covalently bound to EMA. (C) The unbound DNA from viable cells is PCR amplified, while the DNA from dead cells with bound EMA cannot be amplified. This is a modified picture from (Rudi, et al., 2005).

EMA blocks up to 75 % of the eDNA (Nocker, et al., 2006)**.** The blocked DNA cannot be amplified by PCR, and EMA consequently makes it possible to estimate more correctly the amount of viable cells by using a molecular method based on DNA measurements, but an overestimation of cells can still appear (Wagner, et al., 2008). During the extraction procedure the viable cells are lysed to measure the DNA from inside the cells and according to Nocker, et al. (2006) the inactivated EMA will not react with the genomic DNA released during lysis (Nocker, et al., 2006).

2 Aim of the project

The aims of the project are to found out if the thermophilic bacterium *Thermotoga neapolitana* produces eDNA; to confirm the presence of eDNA in pure cultures of *Pseudomonas aeruginosa, Staphylococcus aureus, Tsukamurella spumae* and *Bacillus mycoides;* and to confirm the presence of eDNA in complex environmental samples. The methods to be used include staining, extraction, amplification and agarose gel electrophoresis.

The development of eDNA in the pure cultures and biofilms of the bacteria mentioned above are to be investigated, to find out during which growth period the eDNA production occurs and to analyze the importance of eDNA in biofilm development, using imaging approaches.

Comparison of genomic DNA and eDNA of both pure and enzyme digested samples of *T. neapolitana, P. aeruginosa, S. aureus, T. spumae* and *B. mycoides* is to be performed in order to investigate similarities and differences of genomic versus extracellular DNA.

3 Materials and Methods

This chapter contains a description of the experimental methods used during this Master's thesis. If special equipment has been used it is noted in the following sections. All sterile laboratory work was conducted using a ClanLAF VFRS 1206 laminar air flow bench and handling and transport of samples were carried out on ice. Liquids and solids were sterilized by autoclaving at 121 °C for 30 minutes using a J. P. Selecta autoclave.

Amplification of gene fragments by PCR

To amplify fragments of DNA PCR was used. The reaction mixtures were prepared in a Biocap (Erlab, France) and 50 µl reaction mixture contained: 5 µl 10.2 % (v/v) Ampliqon 10 x Standard buffer (10 mM Tris-HCL pH 8.0, 75 mM KCl and 1.5 mM $MgCl_2$); 36.5 µl dH_2O; 5 µl 5 mM dNTP; 1 µl each of forward and reverse primer (10 pmol/µl); 0.5 µl *Thermus aquaticus (Taq)* polymerase (5,000 units/ml); and 1 µl of template DNA. The amount of dH_2O and template DNA were varied. A negative control containing no DNA and a positive control containing 2 µl of soil extract AAØ 4, 19/8 2005. PCR were performed using a Thermal Cycler (VWR, Belgium).

Agarose gel electrophoresis

Agarose gel electrophoresis was used to evaluate the size of eDNA. Gels were cast with 1 and 2 % agarose in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 2mM EDTA, pH 8). 20 % loading buffer (Sigma, Germany) was used in each well. Gels were run in 1xTAE buffer, applying 115-120 volts from a Standard Power Pack P25 (Biometra, Germany), for approximately 45 min. Gels were stained in 0.05 ‰ (v/v) SYBR GOLD in 1x TAE buffer for one hour and visualized using a High Performance Ultraviolet Transilluminator (Ultra-Violet Products, UK) equipped with a camera.

3.1 Media

Tryptic soy agar medium

40 g of tryptic soy agar (TSA) was suspended in 1 L of demineralized H_2O. The pH was adjusted to 7.3 before autoclaving, and the media was cooled down to 45°C before it was decanted into petri dishes.

10 % tryptone soya broth medium

3 g of tryptone soya broth (TSB) was suspended in 1 L of demineralized H_2O. The pH was adjusted to 7.3 before autoclaving.

Colonization factor antigen (CFA) medium

4 g Glucose, 4 g casein hydrolysate, 10 g malt extract and 1 L demineralized H_2O. The pH was adjusted to 7.2 before autoclaving.

65. Gym Streptomyces Medium (DSM 65)

4 g Glucose, 4 g casein hydrolysate, 10 g malt extract and 1 L demineralized H_2O. The pH was adjusted to 7.2 before autoclaving.

Glucose medium for *Thermotoga neapolitana*

See appendix "Glucose medium".

3.2 Experiments on *Thermotoga neapolitana*

7 day old culture of *Thermotoga neapolitana* stained with DDAO and SYTO13

T. neapolitana was grown anaerobically in an 80°C incubator for 7 days. 240 µl was transferred to a sterile Eppendorf tube. One washing step was performed with 240 µl sterile filtered tap H_2O by centrifuging at 10,000 g for 4 min. 10 µl of DDAO stock solution (250 µM) was added. It was incubated for 1 hour without light. Two washing steps were then performed with 250 µl sterile filtered tap H_2O by centrifuging at 10,000 g for 4 min. The pellet was dissolved in 250 µl sterile filtered tap H_2O and 75 µl was transferred to another Eppendorf tube with 25 µl SYTO13 stock solution (5 µM). It was incubated for 15 min with no light. 50 µl of the sample was applied to gelatin coated Amann slides and air dried under a fume hood before looking at the samples in the LSM 510 META confocal laser scanning microscope (CLSM) (Zeiss, Germany).

Genomic versus extracellular DNA in *Thermotoga neapolitana*

9.5 ml of 0.2 µm sterile filtered 0.9 % NaCl in 10 mM EDTA was transferred to a 30 ml centrifuge tube and 0.5 ml *T. neapolitana* grown anaerobically in an 80°C incubator for 21 hours was added. The solution was centrifuged at 12,000 g for 30 min. at 4°C and the supernatant was decanted into a sterile 50 ml Greiner tube. The concentration of the supernatant was measured on a Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm and afterwards stored in the freezer at -20°C. The pellet fraction was thawed and transferred into a sterile Eppendorf tube which contained 500 µl 1M NaOH. The Eppendorf tube was then placed in a Thermomixer Comfort (Eppendorf, Germany) for one hour at 80°C. The sample was neutralized with 500 µl 1M HCl (Steinberger, et al., 2005). The sample was frozen at -20°C, thawed and 1000 µl was transferred into a plastic tube containing 4 sterile beads of 0.5 mm in diameter each. The sample was bead beaten for 40 sec. with a speed of 6.0 m/s in a FP 120 Fast Prep Cell Disruptor (BIO 101, USA and SAVANT, USA), (Nocker, et al., 2006). The sample was

transferred into a sterile Eppendorf tube and supernatant and pellet were separated by centrifuging at 13,000 g for 2 min. The concentration of the pellet was measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm. Double determination was done.

DNase I digestion

Both eDNA and genomic DNA from *T. neapolitana* were digested with DNase I. 3.5 µl reaction buffer, 16.5 µl sample and 15 µl DNase I (1 unit/µl) were mixed in a sterile Eppendorf tube very gently using a pipette. The samples were placed in a 37°C incubator for 30 min. The concentrations of the eDNA and genomic DNA digested with DNase I were measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm.

A sample of eDNA, eDNA digest, genomic DNA and genomic DNA digest were frozen at -80°C and placed in an Alpha 2-4 LO plus freeze-dryer (Martin Christ, Germany) over night. The concentrated samples were dissolved in 50 µl of dH_2O. Both concentrated and non concentrated samples of eDNA and genomic DNA that were digested and not digested were loaded on a 1 % agarose gel.

Cleavage with restriction enzymes

The growth conditions and handling of the sample was performed as in the section "Genomic versus extracellular DNA in *Thermotoga neapolitana*". The only difference was that 1000 µl of the treated pellet fraction was transferred into a plastic tube containing 0.655 g sterile beads of 0.1 mm in diameter. The concentrations of both genomic and extracellular DNA were measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm. Double determination was done.

The eDNA and genomic DNA fractions were both cut with the restrictions enzymes HaeIII and MspI with working concentrations of 50 unit. The reactants for solution 1 and 2 are described in Table 1.

Table 1: Reactants for restriction enzyme solutions 1 and 2.

Reactants	Solution 1	Solution 2
Buffer C	1.05 µl	1 µl
eDNA	7 µl	7 µl
dH_2O	1.75 µl	1 µl
HaeIII	0.7 µl	-
HaeIII+MspI	-	0.5 µl + 0.5 µl
Total	**10.5 µl**	**10 µl**

The reaction was run for 16 hours at 37°C and the reaction was stopped by placing the samples a Thermomixer Comfort (Eppendorf, Germany) at 80°C for 20 min. The samples were placed in the refrigerator overnight and 10 µl was loaded on a 1 % agarose gel.

Analyzing eDNA from *Thermotoga neapolitana* by agarose gel electrophoresis

5 ml of 0.2 µm sterile filtered 1.8 % NaCl in 10 mM EDTA was transferred to a 30 ml centrifuge tube and 5 ml *T. neapolitana* grown anaerobically in an 80°C incubator for 55 hours was added. The solution was centrifuged at 12,000 g for 30 min. at 4°C and the supernatant was decanted into a sterile 50 ml Greiner tube. The pellet was placed in the freezer at -20°C and the supernatant was placed in an Alpha 2-4 LO plus freeze-dryer (Martin Christ, Germany) over night. Six determinations were done.

The freeze-dried samples were diluted in 500 µl dH_2O and 50 µl from one sample was run on a 1 % agarose gel.

6 day old culture of *Thermotoga neapolitana*

The growth conditions and handling of the sample was performed as described in the section "Analyzing eDNA from *Thermotoga neapolitana* by agarose gel electrophoresis". The only difference was that 10 ml of a 6 day old culture was transferred to a 30 ml centrifugation tube that contained 10 ml of 0.2 µm sterile filtered 0.9 % NaCl in 10 mM EDTA and this sample was not concentrated. 10 µl of the sample was loaded on a 1 % agarose gel.

3.3 Experiments on *Tsukamurella spumae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus mycoides*

Tsukamurella spumae stained with DDAO and SYTO13

600 µl of *T. spumae* grown for 45 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a sterile Eppendorf tube. This was done with a pipette tip that was cut off to make sure that the biomass could come through the tip hole. One washing step was performed with 240 µl sterile filtered tap H_2O at 10,000 g for 4 min. 10 µl DDAO stock solution (250 µM) was added and it was incubated for 60 min. without light. Three washing steps were performed with sterile filtered tap water at 10,000 g for 4 min. The pellet was dissolved in 250 µl sterile filtered tap water and 75 µl was transferred to another Eppendorf tube with 25 µl SYTO13 stock solution (5 µM). It was incubated for 15 min. with no light. 50 µl was applied to gelatin coated Amann slides and air dried under a fume hood before looking at the samples in the LSM 510 META CLSM (Zeiss, Germany).

Time series of *Tsukamurella spumae* stained with DDAO and SYTO13

3 x 1 ml of *T. spumae* grown for 24 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to three sterile Eppendorf tubes. 1 ml of *T. spumae* grown for 48 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a sterile Eppendorf tube and 1 ml of *T. spumae* grown for 72 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a sterile Eppendorf tube. The transfer was performed with a pipette tip that was cut off to make sure that the biomass could come through the tip hole. One washing step was performed with 240 µl sterile filtered

tap H_2O at 10,000 g for 4 min. 10 µl DDAO stock solution (250 µM) was added to the 1 and 2 day old cultures, but 20 µl DDAO stock solution (250 µM) was added to the 3 day old culture. The samples were incubated for 60 min. without light exposure. One washing step was performed with 250 µl sterile filtered tap H_2O at 10,000 g for 4 min. The pellets were dissolved in 75 µl sterile filtered tap H_2O and 25 µl SYTO13 stock solution (5 µM) was added. The samples were incubated for 15 min. with no light exposure. 50 µl was applied to gelatin coated Amann slides and air dried under the fume hood. Samples from days 1 and 2 were saved in the freezer until the samples from day 3 were ready to be studied in the LSM 510 META CLSM (Zeiss, Germany).

Detection of amyloids performed by staining with thioflavin T (ThT)

1 ml of *T. spumae* samples grown for 24, 48 and 72 hours were transferred to sterile Eppendorf tubes and centrifuged at 10,000 g for 4 min. The supernatants from the 1 and 2 day old cultures were discarded and a washing step with 1 ml of phosphate buffered saline (PBS) was performed. The pellets were resuspended in 1 ml of PBS. The supernatant from day 3 was also discarded but the washing and resuspension steps were performed using 1 ml of sterile filtered tap H_2O. 4 µl of ThT stock (200µM) was added to each Eppendorf tube. These were then incubated in the dark for 15 min and washed once in PBS, for samples from day 1 and 2. The day 3 sample was incubated in the dark for 15 min and washed once in sterile filtered tap H_2O. The samples were examined in the LSM 510 META CLSM (Zeiss, Germany).

Analyzing eDNA from *Tsukamurella spumae* by agarose gel electrophoresis

9.5 ml of 0.2 µm sterile filtered 0.9 % NaCl in 10 mM EDTA was transferred to a 30 ml centrifuge tube and 0.5 ml *T. spumae* grown for 3 days in DSM 65 in an incubator at 28°C and 120 rpm was added. The solution was centrifuged at 12,000 g for 30 min. at 4°C and the supernatant decanted into a sterile 50 ml Greiner tube. Some of the supernatant was placed in an Alpha 2-4 LO plus freeze-dryer (Martin Christ, Germany) over night. Double determination was done. 50 µl sample, 50 µl of a diluted 1:10 sample and 50 µl of the concentrated sample was loaded on a 1 % agarose gel.

10 ml of the *T. spumae* culture was centrifuged without any salt solution when it was 6 days old. The separation and freeze-drying were handled as the 3 day old culture. 2 x 50 µl samples and 2 x 50 µl of the concentrated samples were loaded on a 1 % agarose gel.

Extracellular DNA from *Tsukamurella spumae* precipitated with ammonium acetate

30 ml of *T. spumae* which was grown for 24 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a 30 ml centrifugation tube and centrifuged at 3,000 g for 20 min. and again at 10,000 g for 10 min., all at 4°C. 5 ml supernatant was decanted into a sterile 50 ml Greiner tube. 2.5 ml 7.5 M NH_4Ac, 25 µl glycogen and 20 ml 96 % ethanol was added. The Greiner tube was placed in a -20 °C

freezer for 30 min. and then centrifuged at 9,500 g for 30 min. at 4°C. The pellet was marked with a pen and the supernatant was removed. Enough 70 % ethanol was poured into the Greiner tube to cover the entire pellet. The ethanol was removed quickly and the washing step was repeated. The pellet was diluted in 25 µl dH_2O. The whole protocol was performed 6 times to process the entire 30ml. The concentration was measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm.

Phase separation with Phenolcloroformisoamylalcohol (PCI)

150 µl from the ammonium acetate precipitation and 150 µl 25:24:1 PCI were added to a sterile Eppendorf tube which was vortexed for 1 min. The Eppendorf tube was centrifuged at 13,000 g for 5 min. at room temperature. The liquid separated into three different phases. The water phase was transferred to a sterile Eppendorf tube with a pipette. 10 µl of the water phase sample and 10 µl of water phase sample diluted 1:20 were run on a 1 % agarose gel.

Amplification of *16S* rRNA gene fragments from *Tsukamurella spumae* eDNA

The template was the supernatant obtained from *T. spumae* grown for 24 hours in DSM 65 in an incubator at 28°C and 120 rpm. The separation was performed by centrifuging at 12,000 g for 30 min. at 4°C. Three dilutions of the template were made. One PCR was performed with the 26F and 1390R primers and another PCR was performed with the 8F and 1492R primers. 1 µl template was used for each PCR and 10 µl of the PCR products were loaded on a 1 % agarose gel. PCR specifications are stated in Table 2.

Table 2: PCR specifications for the 30 cycle program used for amplification of bacterial *16S* rRNA genes.

PCR steps	Temperature [°C]	Time
Initial denaturation	93	10 min
Denaturation	92	30 sec.
Primer annealing	57	1 min.
Elongation	72	45 sec.
Final elongation	72	5 min
Cooling	4	-

Amplification of eDNA from Tsukamurella spumae with random hexamer primers

The PCR was performed with the random hexamer primers. 1 µl of eDNA extracted from the 24 hour old *T. spumae* culture mentioned above using the PowerSoil DNA Isolation Kit (MO BIO laboratories, Inc., USA) was used as template and 10 µl of the PCR products were loaded on a 1 % agarose gel. PCR specifications are stated in Table 3.

Table 3: PCR specifications for the 25 cycle PCR program used for amplification of bacterial eDNA with random hexamer primers.

PCR steps	Temperature [°C]	Time
Initial denaturation	93	10 min.
Denaturation	92	30 sec.
Primer annealing	55	1 min.
Elongation	72	45 sec.
Final elongation	72	5 min.
Cooling	4	-

Fragmentation of eDNA from *Tsukamurella spumae*

30 ml *T. spumae* which was grown for 19 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a 30 ml centrifugation tube and spun down at 3,000 g for 20 min. and at 10,000 g for 10 min., all at 4°C. The supernatant was filtered with a 0.2 µm sterile filter into a sterile 50 ml Greiner tube. The concentration was measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm. The protocol for fragmentation of genomic DNA was followed, see appendix "Fragmentation of genomic DNA".

Extraction of eDNA from *Tsukamurella spumae* using the PowerSoil DNA Isolation Kit

25 ml of *T. spumae* which was grown for 24 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a 30 ml centrifugation tube and centrifuged at 3,000 g for 20 min. and at 10,000 g for 10 min., all at 4°C. The supernatant was decanted to a sterile 50 ml sterile Greiner tube and the eDNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO laboratories, Inc., USA). 600 µl was loaded onto a Spin Filter and centrifuged at 10,000 g for 1 min. at room temperature. The flow through was discarded and another 600 µl was added and centrifuged again. This was done 4 times. 500 µl of Solution C5 was added to the Spin Filter and centrifuged at 10,000 g for 30 sec. at room temperature. The flow through was discarded. The Spin Filter was centrifuged at 10,000 g for 1 min. at room temperature. The Spin Filter was carefully placed in a clean 2 ml Collection Tube. 100 µl of Solution C6 was transferred to the center of the white filter membrane and centrifuged at 10,000 g for 30 sec. The DNA was collected in the Collection Tube and the Spin Filter was discarded. The concentration was measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm.

Cleavage of eDNA from *Tsukamurella spumae* with restriction enzymes

10 ml of *T. spumae* which was grown for 24 hours in DSM 65 in an incubator at 28°C and 120 rpm was transferred to a 30 ml centrifugation tube and centrifuged at 3,000 g for 20 min. and again at 10,000 g for 10 min., all at 4°C. The supernatant was decanted to a sterile 50 ml sterile Greiner tube.

The reactants in Table 4 were mixed and incubated for 3 hours at 37°C. The same amount of enzyme was added again and the samples were incubated at 37°C for 13 hours. The reaction was stopped by increasing the temperature to 65°C for 10 min. The products were stored in the refrigerator. The

working concentrations for both HaeIII and MspI were 50 units. 10 µl sample was loaded in each well on a 1 and 2 % agarose gel. The reactants for solution 1, 2 and 3 are described in Table 4.

Table 4: Reactants for restriction enzyme solutions 1, 2 and 3.

Reactants	Solution 1	Solution 2	Solution 3
Buffer C	2 µl	-	2 µl
Buffer Tango	-	2 µl	-
eDNA	10 µl	10 µl	10 µl
dH_2O	5.6 µl	6 µl	5.8 µl
HaeIII	2.4 µl	-	-
MspI	-	2 µl	-
HaeIII+MspI	-	-	1.2 µl + 1 µl
Total	**20 µl**	**20 µl**	**20 µl**

DNase I digestion of 1 and 5 day old pure cultures and a 5 day old biofilm

P. aeruginosa, *S. aureus, T. spumae* and *B. mycoides* were grown for 24 hours and for 5 days in CFA medium in an incubator at 28°C and 120 rpm. 4 x 1 ml of each culture was transferred to a sterile Eppendorf tube. One washing step was performed by centrifuging at 13,000 g for 3 min., discarding the supernatant and adding 1 ml of sterile filtered tap H_2O. 15 µl of reaction buffer and 15 µl of DNase I (1 unit/µl) were transferred to the first and second extract and incubated at 37°C for 1 hour.

Staining with DDAO and SYTO13

3 x 15 µl of the first and third samples were placed on a super frost microscope slide and dried at 46°C for about 15 min. 3 x 75 µl DDAO working solution (10 µM) was added to cover all biomass. The slides were placed under a tray on a table for 30 min. and H_2O was added to the table. The slides were tipped and the excess stain was collected in a container. One washing step was performed by quickly dipping the slides once into a beaker of autoclaved tap H_2O. The slides were dried in the fume hood. 3 x 75 µl of SYTO13 working solution (1.25 µM) was added and covered the biomass. The slides were placed under a tray on the table for 15 min. and water was added to the table. One washing step was performed by dipping the slides into a beaker of autoclaved tap H_2O. The slides were dried in the fume hood.

Staining with PI and DAPI

The second and fourth samples were stained similarly to the staining with DDAO and SYTO13, but with PI (5 µM working solution) and DAPI (0.05 mg/ml working solution) stains respectively. The handling of DAPI stain was carried out carefully inside a fume hood.

Exactly the same procedure was carried out on 5 day old cultures of *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides* grown in CFA medium in an incubator at 28°C and 120 rpm. One super frost

microscope slide was placed inside each flask of bacteria to grow up a 5 day old biofilm. The biofilms were stained with DDAO and SYTO13 and the staining procedure was the same as for the 24 hour old and 5 day old cultures, but no DNase I digestion was done. The slides were examined in the LSM 510 META CLSM (Zeiss, Germany).

Ethanol precipitation of eDNA

P. aeruginosa, S. aureus, T. spumae and *B. mycoides* were grown in CFA medium in an incubator at 28°C and 120 rpm. *P. aeruginosa* was grown for 27 hours, *S. aureus* was grown for 46 hours, *T. spumae* and *B. mycoides* were grown for 48 hours. 2 x 40 ml of each culture was centrifuged at 3,000 g for 30 min. and at 10,000 g for 20 min. *P. aeruginosa* and *S. aureus* were both centrifuged at room temperature and *T. spumae* and *B. mycoides* were both centrifuged at 4°C. The supernatants were filtered with a 0.2 µm sterile filter into a sterile 50 ml Greiner tube. The concentrations were measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm.

12.5 ml sterile filtered supernatant, 1.25 ml of 3 M NaAc pH 5.4, 0.25 ml TE- buffer and 5 µl glycogen was transferred to a sterile 50 ml Greiner tube and mixed. 25 ml cold 100 % ethanol was added and mixed well (vortexed). The Greiner tube was stored in the freezer at -20°C for 30 min. and centrifuged at 9,500 g for 30 min. at 4°C. The supernatant was carefully discarded. There was a little supernatant left which was used to dissolve the pellet. It was transferred to an Eppendorf tube which was centrifuged at 13,000 g for 15 min. at room temperature. The supernatant was discarded. The pellet was washed in 1 ml of 70 % ethanol. A folded napkin was used to suck up the last droplets and the Eppendorf tube was air dried for 20 min. The pellet was suspended in 50 µl of dH_2O and the concentration of eDNA was measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm. The ethanol precipitation was performed 3 times for each bacterium.

The three tubes containing the same bacteria were pooled together into one Eppendorf tube and placed in an Alpha 2-4 LO plus freeze-dryer (Martin Christ, Germany) over night. The concentrated samples were diluted in 50 µl TE buffer and the concentration was again measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm.

Fragmentation of eDNA

Fragmentation of eDNA from *T. spumae* was done according to the protocol for fragmentation of genomic DNA, see appendix "Fragmentation of genomic DNA". Steps 1-13 were followed and the concentration was measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm. 40 µl sample was loaded on a 2 % agarose gel.

Blocking of eDNA before PCR with universal and random hexamer primers

The supernatants used were extracted in section "Ethanol precipitation of eDNA". 10 µl filtered supernatant and 0.2 µl EMA stock (5000 µg/ ml) was added to a sterile Eppendorf tube and mixed well. The sample was incubated for 5 min. covered in aluminum foil and shaken every min. It was then exposed to light using a 100 w bulb (microscope bulb) for 2 min. (Nocker, et al., 2006).

PCR was prepared with both the universal primers 26F and 1390R and the random hexamer primers. The concentrations were measured on the Spectrophotometer ND 1000, (Nanodrop, UK) at 260 nm. 10 µl template was added in each PCR tube and 10 µl of the amplified samples were run on a 1 % agarose gel. PCR specifications are stated in Table 5.

Table 5: : PCR specifications for the 30 cycle PCR program used for amplification of bacterial *16S* rRNA genes with 26F and 1390R and amplification of bacterial eDNA with random hexamer primers.

PCR steps	Temperature [°C]	Time
Initial denaturation	93	10 min.
Denaturation	92	30 sec.
Primer annealing	57	1 min.
Elongation	72	45 sec.
Final elongation	72	5 min.
Cooling	4	-

Genomic versus extracellular DNA

P. aeruginosa, S. aureus, T. spumae and *B. mycoides* were grown in CFA medium in an incubator at 28°C and 120 rpm. *P. aeruginosa* and *S. aureus* were grown for 21.5 hours, *T. spumae* for 20 hours and *B. mycoides* for 19.5 hours.

Genomic versus extracellular DNA with the use of EMA and lysozyme

Three x 500 µl was removed from each culture to sterile Eppendorf tubes. One tube was centrifuged at 3,000 g for 30 min. and at 10,000 g for 20 min. at room temperature. The supernatant was decanted into a sterile Eppendorf tube.

10 µl EMA stock solution (5000 µg/ml) was added to a second and a third Eppendorf tubes. They were mixed on the vortex and incubated for 5 min. covered in aluminum foil. During the incubation the samples were shaken every min. The Eppendorf tubes were placed 10 cm away from a 400 w bulb for 2 min. (Nocker, et al., 2006). The second sample was centrifuged like the first and 500 µl lysozyme solution (10 mg lysozyme, 50 µl 0.5 M EDTA, 50 µl 1 M Tris-HCl pH 7.4 and 400 µl Milli Q water (MQ) H_2O mixed using the vortex) was added to the third Eppendorf tube and reacted for 30 min. at 37°C and then centrifuged like the other samples. The supernatants were decanted into sterile Eppendorf tubes.

PCR was prepared with the universal primers 26F and 1390R. A 30 cycle PCR was performed with samples from the four different bacteria without EMA treatment, with EMA treatment and with EMA

and lysozyme treatment in 4 dilutions. 10 µl of template was used for each PCR and 10 µl of the amplified samples were run on a 1 % agarose gel. PCR specifications are stated in Table 6.

Table 6: PCR specifications for 30 cycle PCR program used for amplification of bacterial *16S* rRNA genes with EMA treatment, without EMA treatment and with EMA and lysozyme treatment.

PCR steps	Temperature [°C]	Time
Initial denaturation	93	10 min
Denaturation	92	30 sec.
Primer annealing	57	1 min.
Elongation	72	45 sec.
Final elongation	72	5 min
Cooling	4	-

Genomic versus extracellular DNA with the use of DNase I and a cell disruptor

560 µl of culture obtained from *P. aeruginaosa, S. aureus, T. spumae* and *B. mycoides* respectively were added to sterile Eppendorf tubes. 70 µl 10 x reaction buffer and 70 µl DNase I (1 unit/µl) were added to each Eppendorf tube and mixed gently before incubating for 15 min. at room temperature. 70 µl stop solution (50 mM EDTA) was added. Three washing steps were performed by centrifuging the samples for 3 min. at 13,000 g, discarding the supernatant and adding 560 µl autoclaved 0.2 µm sterile filtered tap H_2O. The samples were placed in a 70°C water bath for 10 min. The samples were transferred into plastic tubes containing 0.655 g sterile beads of 0.1 mm in diameter and the cells were bead beaten for 40 sec. at 6.0 m/s in a FP 120 Fast Prep Cell Disruptor (BIO 101, USA and SAVANT, USA), (Nocker, et al., 2006).

PCR was prepared with the universal primers 26F and 1390R. A 30 cycle PCR was performed on the samples from *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides* digested with DNase I and on pure eDNA. 10 µl of template was used for each PCR and 10 µl of the amplified samples were run on a 1 % agarose gel. PCR specifications are stated in Table 7.

Table 7: PCR specifications for 30 cycle PCR program used for amplification of bacterial *16S* rRNA genes.

PCR steps	Temperature [°C]	Time
Initial denaturation	93	10 min
Denaturation	92	30 sec.
Primer annealing	57	1 min.
Elongation	72	45 sec.
Final elongation	72	5 min
Cooling	4	-

PCR was prepared with the UP1Ei, UP2ri (*gyrB*) and 1698F, 2041R *(rpoB)*. A 35 cycle PCR was performed on the samples from *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides* digested with DNase I and on pure eDNA. 10 µl of template was used for each PCR and 10 µl of the amplified samples were loaded on a 1 % agarose gel. PCR specifications (Holmes, et al., 2004) are stated in Table 8.

Table 8: PCR specifications for 35 cycle PCR program used for amplification of bacterial DNA with *gyrB* and *rpoB* primers.

PCR steps	Temperature [°C]	Time
Initial denaturation	94	5 min.
Denaturation	94	45 sec.
Primer annealing	50	1 min.
Elongation	72	1 min.
Final elongation	72	10 min.
Cooling	4	-

3.4 Experiments on complex environmental samples

Complex bacterial sample from a natural environment

The samples were taken from rocks beneath the water surface in the two streams Kjærs Mølleå and Hvorup å. The sampling included rock imprints directly onto the agar plates and rocks scrubbed with toothpicks which were placed inside 10 % TSB medium. 2 x 3 rocks were investigated in both streams. Algae were avoided during sampling. All samples were placed on a yellow line basic vibrating table (Macherey-Nagel, Germany) at 120 rpm at room temperature for 3 days. Because of massive growth the samples were isolated on agar plates and grown for one day. This was done twice, and after the 2nd isolation, the plates were placed at room temperature for 24 hours and then stored in the refrigerator for two days before staining.

Staining with DDAO and SYTO13 in Eppendorf tubes

240 µl 0.2 µm sterile filtered tap H_2O was added to a sterile Eppendorf tube. Biomass was scrubbed off an isolated agar plate and added. One washing step was performed with 240 µl sterile filtered tap H_2O by centrifuging at 10,000 g for 4 min. 10 µl of DDAO stock solution (250 µM) was added. It was incubated for 1 hour without light. Two washing steps were then performed with 250 µl sterile filtered tap H_2O by centrifuging at 10,000 g for 4 min. The pellet was dissolved in 250 µl sterile filtered tap H_2O and 75 µl was transferred to another Eppendorf tube with 25 µl SYTO13 stock solution (5 µM). It was incubated for 15 min with no light. 50 µl of the sample was applied to gelatin coated Amann slides and air dried under a fume hood before looking at the samples in the LSM 510 META CLSM (Zeiss, Germany). This was made for all plates.

Staining with DDAO and SYTO13 directly on slides

One or more colonies from each sample of the 2nd agar plate isolation were placed inside a sterile test tube with 10 ml 10 % TSB medium. All test tubes were placed on a yellow line basic vibrating table (Macherey-Nagel, Germany) at 120 rpm at room temperature for 24 hours. 3 x 50 µl sample was transferred to each well of the 6 well Amann slides. The 6 well Amann slides were placed under a tray for 1 hour and H_2O was added to the table to prevent evaporation of the sample. The slides were dried under the fume hood. 50 µl of 10 µM DDAU solution was added to each well. The slides were placed

under a tray for 2 hours and H_2O was added to the table. The liquid was tipped off the slides into a test tube and the slides were carefully dipped into a beaker filled with demineralized H_2O. All the slides were placed in a rack and dipped into the container filled with demineralized H_2O for 1 hour and placed in a cabin to avoid light exposure. The slides were dried under the fume hood. A solution of 750 µl SYTO13 stock solution (5 µM) and 2250 µl demineralized H_2O was made and 50 µl of the SYTO13 working solution (1.25 µM) was placed in each well for 15 min. The liquid was tipped off the slides into a test tube and the slides were carefully dipped into a beaker filled with demineralized H_2O. The slides were dried under the fume hood for about 15 min. and stored in the refrigerator overnight. The samples were studied with a LSM 510 META CLSM (Zeiss, Germany) the next day.

4 Results

4.1 Experiments on *Thermotoga neapolitana*

In this section different cultures of *T. neapolitana* have been used to try and prove or disprove the existence of eDNA. This has been attempted using the following methods.

7 day old culture of *Thermotoga neapolitana* stained with DDAO and SYTO13

The culture of a 7 day old *T. neapolitana* produced a large amount of EPS, and samples including EPS were stained with DDAO and SYTO13 to provide evidence that eDNA was present. *T. neapolitana* was subjected to stress during growth, through exposure to sampling for other experiments, because of temperature differences and the anaerobic conditions during sampling.

A huge amount of eDNA was observed from the 7 day old culture of *T. neapolitana.* The eDNA resembled a sponge (blue), with the cells or cell aggregates (green) filling out the space inside the spongy eDNA. The eDNA contained cell-shaped black pores, which indicated that a cell had made all the surrounding eDNA and then decayed, leaving a cell-shaped pore. Therefore some of the eDNA present in this sample could have originated from active excretion or from cells that had been lysed long ago. If the blue patch was cell-shaped, then it probably originated from a dead cell. The eDNA was observed around both single cells and cell aggregates, see Figure 3.

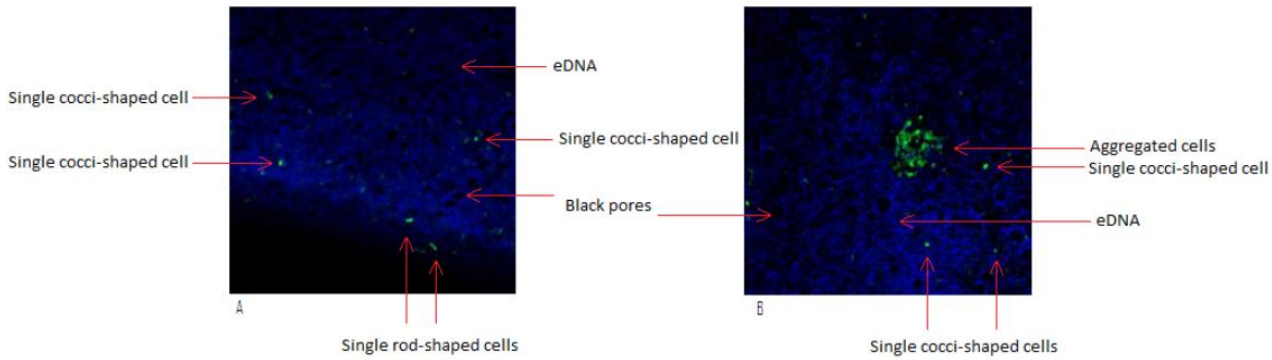

Figure 3: Both picture A and picture B show 7 day old cultures of *T. neapolitana* taken with the same magnification of 400. Picture A consists of two images taken of the same region of the sample, but with different channels, and merged into one picture. A few single viable cells, stained green, are seen surrounded by a huge amount of eDNA, stained blue. There were both cocci- and rod-shaped cells in the sample, the red arrows shows specifically what is seen on the picture. Picture B also consists of two images taken from the same region of the sample, but with different channels, and merged into one picture. A huge amount of eDNA, stained blue, is seen surrounding the cell aggregate, stained green. The red arrows indicate what is seen on the picture.

Genomic versus extracellular DNA in *Thermotoga neapolitana*

The concentrations of genomic DNA and extracellular DNA from a 21 hour old culture of *T. neapolitana* were compared by spectrophotometer measurements and the sizes were analyzed by agarose gel electrophoresis. The genomic DNA and extracellular DNA were digested with DNase I to find out if they could be cleaved.

Three different types of growth media for *T. neapolitana* were tested to find out if the media containing yeast extract or peptone were causing any background noise on the agarose gels. One glucose medium, see appendix "Glucose medium"; one glucose medium with casein instead of yeast extract; and one glucose medium with casein instead of both yeast extract and peptone were tested. There was no observable background noise from any of the three media (results not shown). The glucose medium was selected as the growth medium for further experiments because it had the best growth conditions for *T. neapolitana*.

The purity and concentrations of both eDNA and genomic DNA from *T. neapolitana*, both digested with DNase I, are shown in Table 9.

Table 9: Spectrophotometer ND 1000 measurement of eDNA and genomic DNA from *T. neapolitana*, both digested with DNase I.

Spectrophotometer ND 1000 measurements	**eDNA**	**Digested eDNA**	**Genomic DNA**	**Digested genomic DNA**
Average	28.4 ng/µl	16.0 ng/µl	230.6 ng/µl	223.6 ng/µl
260/280 ratio	1.02	0.97	1.31	1.24
260/230 ratio	0.60	0.52	0.92	1.10

It was possible to calculate the amount of DNA in the 500 µl samples that were transferred initially from the bacterial suspension by multiplying by the dilution factor of 1:20 for eDNA and 1:2 for genomic DNA. This gave an actual concentration for eDNA of 568 ng/µl and 461 ng/µl for genomic DNA.

Concentrated and non concentrated samples of eDNA and genomic DNA from the 21 hour old culture of *T. neapolitana* that were digested with DNase I and non digested were loaded on a 1 % agarose gel, but there were no detectable bands from the genomic DNA on the agarose gel. However, bands of eDNA and concentrated eDNA were visible above 10,000 bp and no bands were observed from the DNase I digested eDNA, as shown on Figure 4.

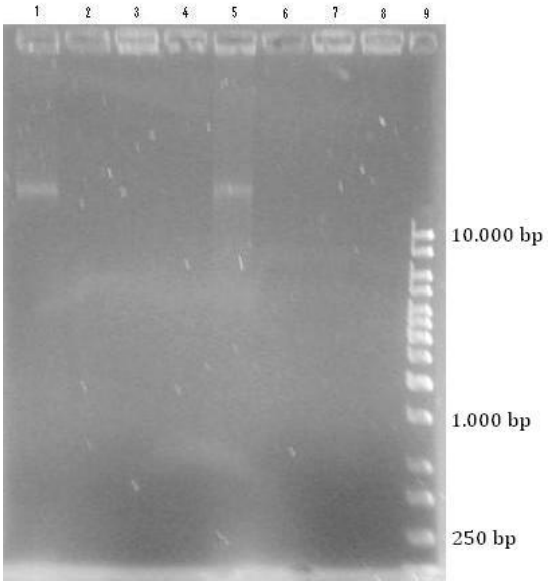

Figure 4: 1 % agarose gel. Lane 1: eDNA. Lane 2: eDNA digest. Lane 3: genomic DNA. Lane 4: genomic DNA digest. Lane 5: Concentrated eDNA. Lane 6: Concentrated eDNA digest. Lane 7: Concentrated genomic DNA. Lane 8: Concentrated genomic DNA digest. Lane 9: GeneRuler™ 1 Kb ladder (Fermentas, Germany).

Because the genomic DNA was not visible on the gel in Figure 4, a larger amount of smaller beat beads were used in the succeeding restriction enzyme experiments on another 21 hour old T. neapolitana culture.

The purity and concentrations of both eDNA and genomic DNA from the second 21 hour old *T. neapolitana* culture are shown in Table 10.

Table 10: Spectrophotometer ND 1000 measurements of eDNA and genomic DNA from *T. neapolitana*.

Spectrophotometer ND 1000 measurements	**eDNA**	**Genomic DNA**
Average	30 ng/µl	249 ng/µl
260/280 ratio	2.01	1.51
260/230 ratio	0.1	0.68

The actual concentrations of DNA in the 500 µl samples were calculated by multiplying with the dilution factor of 1:20 for eDNA and 1:2 for genomic DNA. This gave an actual concentration for eDNA of 572 ng/µl and 500 ng/µl for genomic DNA.

The samples treated with the restrictions enzymes HaeIII and MspI, to cleave the DNA into smaller fragments, did not reveal any bands from either genomic or eDNA, see Figure 5.

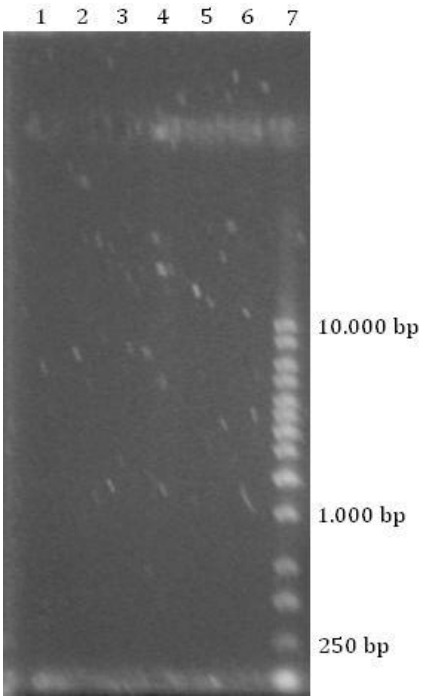

Figure 5: 1 % agarose gel. Lane 1: eDNA. Lane 2: eDNA (HaeIII). Lane 3: eDNA (HaeIII+MspI). Lane 4: Genomic DNA. Lane 5: Genomic DNA (HaeIII). Lane 6: Genomic DNA (HaeIII+MspI). Lane 7: GeneRuler™ 1 Kb ladder (Fermentas, Germany).

The cleavage experiment with the restriction enzymes was repeated, and this time 50 µl sample was loaded on the gel, instead of 10 µl. The increased amount of DNA did not reveal any visible bands, (results not shown).

Analyzing eDNA from *Thermotoga neapolitana* by agarose gel electrophoresis

The staining and DNase experiments provided convincing evidence that *T. neapolitana* produced large amounts of eDNA, but it was difficult to show both eDNA and genomic DNA using agarose gel electrophoresis. The volume of *T. neapolitana* could have been too small and the growth too short, so a 10 times larger volume of a 55 hour old *T. neapolitana* culture was grown, which was approximately double the growth period of the earlier experiments. The eDNA fraction was concentrated by freeze-drying.

A very large smear appeared throughout the gel. A larger concentration of NaCl (1.8 %) had been used, but the SYBR GOLD solution binds specifically to DNA and the smear was assumed to result from the eDNA, and not precipitated salt, see Figure 6.

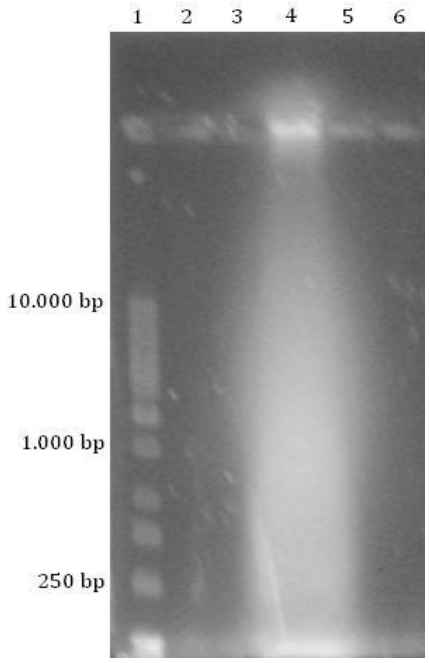

Figure 6: 1 % agarose gel. Lane 1: GeneRuler™ 1 Kb ladder (Fermentas, Germany). Lane 2 and 3: free. Lane 4: eDNA. Lane 5 and 6: free.

A 6 day old *T. neapolitana* culture was grown to see if the eDNA was still present after 6 days of growth. This time the eDNA from *T. neapolitana* was not concentrated by freeze-drying, because of the unwanted appearance of the gel in Figure 6. A very faint smear was visible, shown on Figure 7, from one of the samples of *T. neapolitana at* around 10,000 bp. The smear did not appear throughout the entire gel and there was less eDNA than in Figure 6.

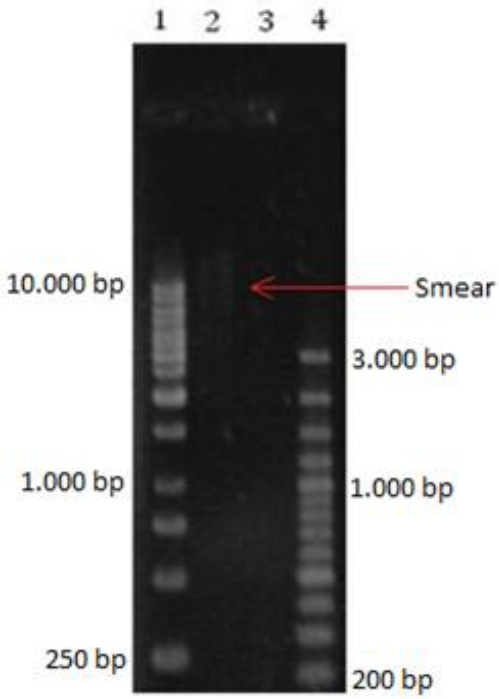

Figure 7: 1 % agarose gel. Lane 1: GeneRuler™ 1 Kb ladder (Fermentas, Germany). Lane 2 and 3: eDNA. Lane 4: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). The red arrow indicates the faint smear around 10,000 bp.

4.2 Experiments on *Tsukamurella spumae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus mycoides*

T. spumae was the most accessible bacterium, which was the reason that most experiments were carried out with this bacterium.

Tsukamurella spumae stained with DDAO and SYTO13

A 45 hour old *T. spumae* culture was stained with DDAO and SYTO13 to confirm the presence of eDNA in a culture of *T. spumae*. Overall there was not much eDNA present in the 45 hour old *T. spumae* culture, but eDNA was observed in some areas, as seen on Figure 8, which shows *T. spumae* single cells and small aggregates surrounded by eDNA. Some clouds of SYTO13 were observed, indicated by a red arrow. The SYTO13 and DDAO stains compete to stain eDNA, and the clouds are probably eDNA stained with both stains. The distinctly cell-shaped bright objects are living cells.

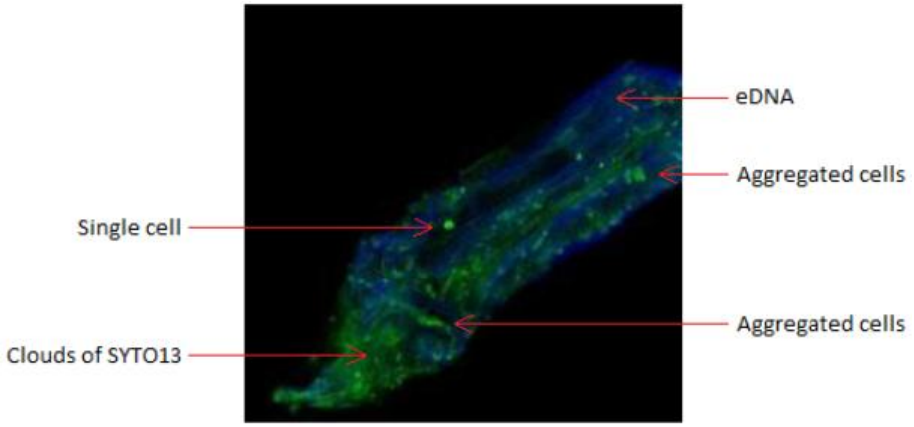

Figure 8: Two pictures were taken from the same region of a 45 hour old *T. spumae* sample, but with different channels, and merged into one picture. The magnification was 400. Extracellular DNA is represented in blue surrounding both single cells and cell aggregates, which appear green. The red arrows identify what is seen on the picture.

Time series of *Tsukamurella spumae* stained with DDAO and SYTO13

In this particular section, a time series of *T. spumae* stained with DDAO and SYTO13 was performed to try and find out when the maximum amount of eDNA was expressed.

Minimal eDNA was present in the 45 hour culture, and so a time series of *T. spumae* was made to find out when eDNA was present in large amounts. The *T. spumae* culture was investigated during a period of 1-3 days to follow the development of cells and eDNA, see Figure 9, Figure 10 and Figure 11 for days 1-3 respectively. Generally there was more eDNA present in the 3 day old *T. spumae* samples, and a correlation between eDNA and aggregates was observed, as these samples also contained huge aggregates compared to the samples from days 1 and 2. The samples from day 1 showed mostly single cells and small aggregates, but almost no production of eDNA, see Figure 9.

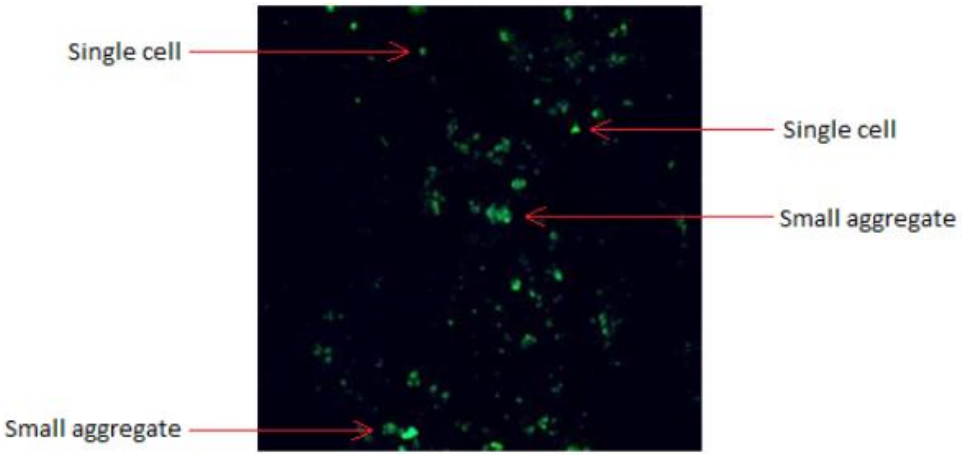

Figure 9: The day 1 sample showed almost no production of eDNA. The magnification was 400. There was an excess of single cells and a few very small aggregates were observed. Two pictures were taken from the same region of the *T. spumae* sample, but with different channels, and merged into one picture. The single cells and cell aggregates appear green and are illustrated by the red arrows.

The development of aggregates and eDNA was increased in the *T. spumae* samples from day 2. There were still a lot of single cells, but eDNA was clearly visible, mainly present in the proximity of the small cell aggregates, see Figure 10.

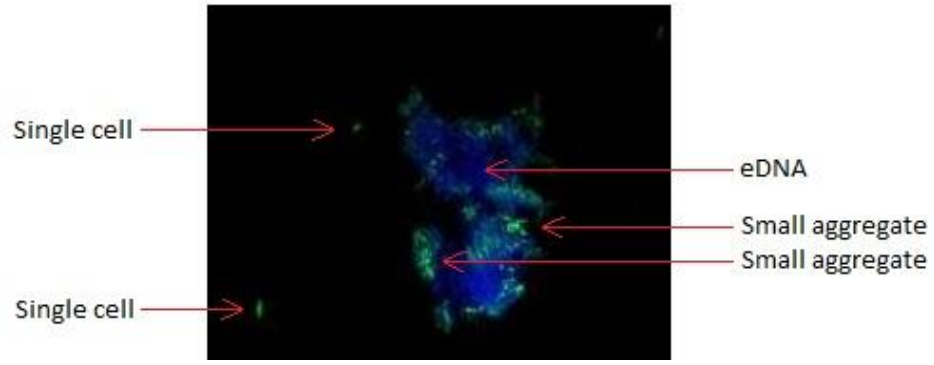

Figure 10: There was slightly more eDNA in the samples from day 2. There were fewer single cells and larger aggregates, which also indicated more eDNA. The magnification was 400. Two pictures were taken from the same region of the *T. spumae* sample, but with different channels, and merged into one picture. The single cells and cell aggregates appear green and the eDNA appears blue, which are illustrated with the red arrows.

The sample from day 3 resembled a sponge of eDNA, where the cell aggregates were filling out the space inside the spongy eDNA. A large network of eDNA was surrounding both single cells and cell aggregates, see Figure 11. It seemed that increased amounts of eDNA promoted aggregation, possibly due to a glue-like mechanism. The eDNA contained cell-shaped black pores, which indicated that a cell had made all the surrounding eDNA and then decayed, leaving a cell-shaped pore. Therefore some of the eDNA present in this sample could have originated from active excretion or from cells that had been lysed long ago. When the blue patch was cell-shaped, it had probably originated from a dead cell, which was not the case in this sample.

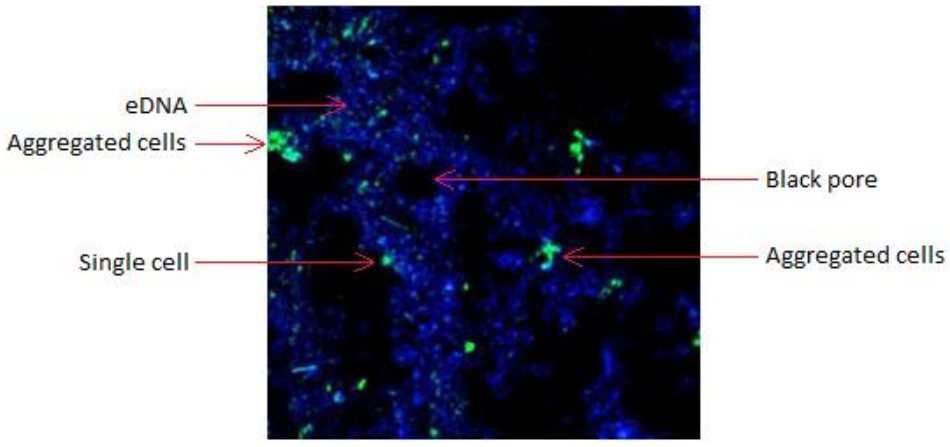

Figure 11: Day 3 samples showed more and larger aggregates, and eDNA was present in large amounts surrounding the aggregates. The magnification was 400. Two pictures were taken from the same region of the *T. spumae* sample, but with different channels, and merged into one picture. The single cells and cell aggregates appear green and the eDNA appears blue, indicated by the red arrows.

Detection of amyloids performed by staining with thioflavin T (ThT)

Amyloids attach to cell surfaces, like eDNA, and if amyloids were found in the culture of *T. spumae,* eDNA may have been present as well. There were plenty of cells that were ThT positive, which indicated that amyloids were present in the samples. The green channel showed how many of the cells were ThT positive, whilst the gray channel showed how many cells in total were present in the sample. In the sample from day 1 it was only the larger cells that were ThT positive; the sample from day 2 showed that both small and large cells were ThT positive; and the sample from day 3 showed that more of the smaller cells were ThT positive. The cells became more aggregated during the days. Samples from day 1 and 2 contained crystals, which made it difficult to observe exactly how many cells were present in the samples, but the green channel gave a clear ThT signal. The crystals were caused by precipitated salt from the PBS. The sample from day 3 did not contain any crystals because sterile filtered tap H_2O was used instead of PBS. The pictures from days 1 and 2 are not shown but on Figure 12 a sample from day 3 is shown.

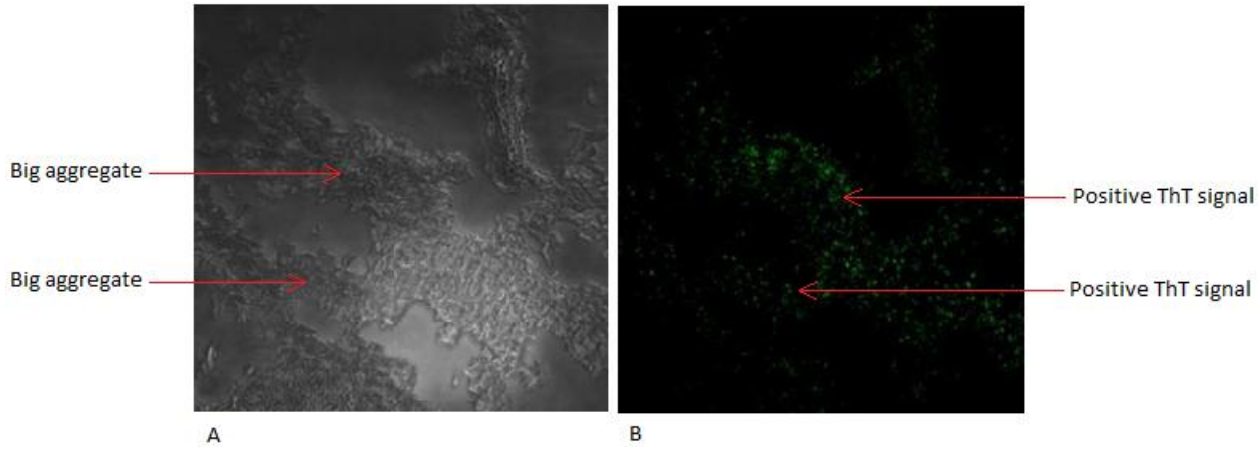

Figure 12: A 3 day old *T. spumae* sample stained with ThT. The magnification was 400. The two pictures were taken from the same region of the sample with different channels. Picture A shows many small cells together in aggregates and picture B shows the fraction of cells that are ThT positive. The red arrows identify what is seen on the pictures.

Analyzing eDNA from *Tsukamurella spumae* by agarose gel electrophoresis

A 3 day old sample of *T. spumae* was analyzed by agarose gel electrophoresis, to attempt to demonstrate that eDNA was present. As shown on Figure 11, the 3 day old *T. spumae* sample produced the most cell aggregates, and thereby the most eDNA. A light smear appeared from the concentrated eDNA, but no band was seen from either eDNA or eDNA diluted 1:10, (results not shown). The DNA ladder showed that the gel was of a very bad quality, because it was not clearly visible, as if it had not been stained for a sufficient amount of time, even though this was not the case.

A new agarose gel was made and a 6 day old culture of *T. spumae* was analyzed. There was a light smear all through the gel from the concentrated samples, but no bands or smears were observed from the pure eDNA samples. The concentrated samples were highly fluorescent in the area at the top of the gel, which suggested that some eDNA was trapped in the wells of the gel, see Figure 13.

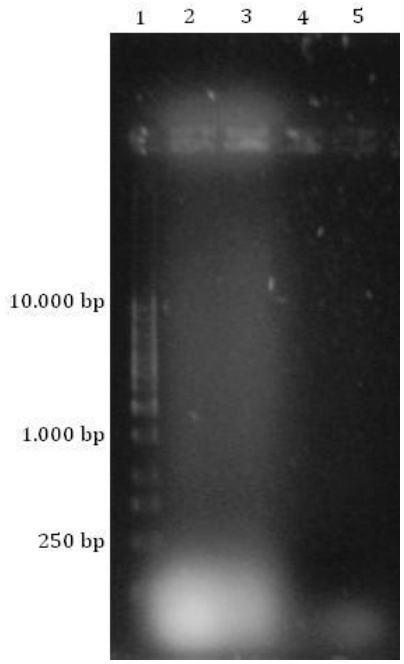

Figure 13: 1 % agarose gel. Lane 1: GeneRuler™ 1 Kb ladder (Fermentas, Germany). Lanes 2 and 3: Concentrated eDNA samples. Lanes 4: and 5: eDNA samples.

Extracellular DNA from *Tsukamurella spumae* precipitated with ammonium acetate

The eDNA from a 24 hour old culture of *T. spumae* was precipitated with ammonium acetate, to concentrate the eDNA. The concentration of eDNA was measured spectrophotometrically, see Table 11.

Table 11: Spectrophotometer ND 1000 measurements of eDNA precipitated with ammonium acetate from a 24 hour old *T. spumae* culture.

Spectrophotometer ND 1000 measurements	**eDNA**
Average	2870 ng/µl
260/280 ratio	2.03
260/230 ratio	1.51

The concentration of the eDNA precipitated with ammonium acetate seemed very high, and the sample was turbid, so it was suspected to contain some cell debris or proteins. A phase separation with PCI was made to remove any cell debris and proteins. The liquid was separated in three different phases: the bottom phase containing alcohol and proteins, the inter phase containing cell debris, and the topmost phase containing water and DNA. The water phase sample looked clearer and was run on an agarose gel, see Figure 14. A small narrow band above 10,000 bp was observed for the pure water phase sample, but a 1:20 dilution of the water phase sample did not reveal any band.

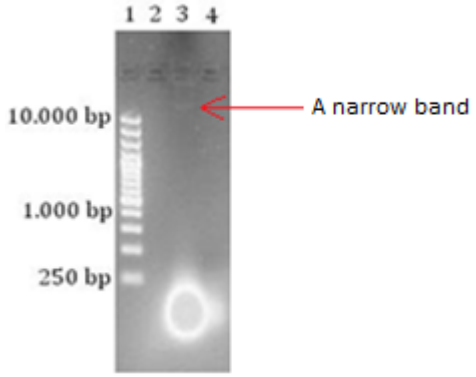

Figure 14: 1 % agarose gel. Lane 1: GeneRuler™ 1 Kb ladder (Fermentas, Germany). Lane 2: water phase sample diluted 1:20. Lane 3: Pure water phase sample. Lane 4: free. A narrow band above 10,000 bp is indicated by the red arrow.

Amplification of *16S* rRNA gene fragments from *Tsukamurella spumae* eDNA

A PCR was performed with the *16S* rRNA universal primers 26F/ 1390R and 8F/ 1492R on eDNA from a 24 hour old *T. spumae* culture. The PCR products were loaded on an agarose gel, see Figure 15, which shows light bands in lanes two and three at 1,364 bp, from the PCR with the 26F and 1390R primers, indicated by the red arrows. The positive control with the 26F and 1390R primers was clearer in contradistinction to the unclear positive control with the 8F and 1492R primers, where no bands were observed.

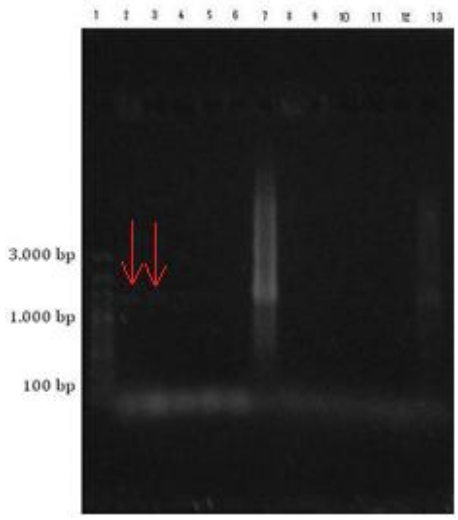

Figure 15: 1 % agarose gel containing PCR products. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2-7: PCR was performed with 26F and 1390R primers. Lane 2: eDNA. Lane 3: eDNA diluted 1:100. Lane 4: eDNA diluted 1:800. Lane 5: eDNA diluted 1:32,000. Lane 6: negative control. Lane 7: positive control (soil extract AAØ4, 19/8-2005). Lane 8-13: PCR was performed with 8F and 1492R primers. Lane 8: eDNA. Lane 9: eDNA diluted 1:100. Lane 10: eDNA diluted 1:800. Lane 11: eDNA diluted 1:32,000. Lane 12: negative control. Lane 13: positive control (soil extract AAØ4, 19/8-2005). Two bands at 1,364 bp are indicated by the red arrows and a smear from the two positive controls is seen.

Amplification of eDNA from *Tsukamurella spumae* with random hexamer primers

Another PCR was performed with random hexamer primers on the eDNA from the 24 hour old *T. spumae* culture mentioned above extracted using the PowerSoil DNA Isolation Kit. The PCR products were loaded on an agarose gel but did not show any bands from either the products or the positive control, which indicated that the PCR may not have functioned correctly, or that the amplified fragments were too scattered thoughtout the gel and could not form any visible band or smear, (results not shown).

Fragmentation of eDNA from *Tsukamurella spumae*

The fragmentation should make the eDNA more visible on an agarose gel, because all the eDNA can be gathered in smaller fragments, and at the end of the fragmentation experiment sequences can be obtained by using a Genome Analyzer II, (Illumina, USA). These would then be compared to the eDNA sequence from Macrogen inc. in Korea, where high throughput Applied Biosystems 3730XL sequencers are used. The concentration of the filtered eDNA from a 19 hour old *T. spumae* culture was measured on the Spectrophotometer ND 1000, see Table 12, to confirm that there was enough eDNA to continue the protocol for fragmentation of genomic DNA. According to the protocol the amount of DNA should be between 1-5 µg before starting.

Table 12: Spectrophotometer ND 1000 measurements of eDNA from a 19 hour old *T. spumae* culture.

Spectrophotometer ND 1000 measurements	**eDNA**
Average	276 ng/µl
260/280 ratio	1.34
260/230 ratio	0.54

The eDNA concentration was high enough to proceed, taking into account the volume of eDNA. In the protocol for fragmentation of genomic DNA there is a section where the fragmented sample is loaded on a 2 % agarose gel and the visible bands are cut out and processed according to the protocol. In this case there was no visible band, so the fragmentation protocol could not be continued, (results not shown).

It appeared that a higher concentration of eDNA was necessary, so an extraction of eDNA from a 24 hour old *T. spumae* culture using the PowerSoil DNA Isolation Kit was performed to obtain a sufficiently high concentration of eDNA to proceed with the fragmentation. The concentration of eDNA was measured on the Spectrophotometer ND 1000 and there was approximately 0.8 µg eDNA, which was not enough to continue with the genomic DNA fragmentation protocol.

Cleavage of eDNA from *Tsukamurella spumae* with restriction enzymes

The eDNA from a 24 hour old culture of *T. spumae* was cleaved with the restriction enzymes HaeIII and MspI to obtain smaller bands of eDNA. According to the protocol for fragmentation of genomic DNA, the products should be loaded on a 2 % agarose gel, so the cleaved products were loaded on a 1 and 2 % agarose gel to demonstrate a band or a smear from eDNA. No band or smear appeared from either cleavage, which suggests that the amount of eDNA was not enough to be visible on an agarose gel, or that the eDNA was cleaved into small pieces of eDNA and run out of the gel, (results not shown).

DNase I digestion of 1 and 5 day old pure cultures and a 5 day old biofilm

Pictures of 24 hour old cultures and 5 day old cultures of *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides,* both untreated and treated with DNase I, were taken with a CLSM. Two similar staining methods were carried out, DDAO/ SYTO13 and PI/ DAPI to visualize the eDNA and genomic DNA from the four bacteria samples. Pictures of 5 day old biofilms, stained with DDAO and SYTO13 were also taken with a CLSM.

Pictures of the 24 hour old cultures and 5 day old cultures generally showed no eDNA. In general the pictures from the 24 hour old cultures showed small cells with very few aggregates, and the 5 day old cultures showed more aggregates and a tiny amount of eDNA present in cultures of *P. aeruginosa* and *T. spumae*. No eDNA was observed in cultures of *S. aureus* and *B. mycoides*. The young cultures had smaller cells and appeared less green, because they had only one copy of the genome. Because there was too little eDNA present in both the young and the old samples, no notable difference between samples treated with and without DNase I was observed. The DDAO/ SYTO13 staining was more distinct than the PI/ DAPI staining. DDAO and SYTO13 stains are a good DNA stain combination because they counterbalance each other well. PI and DAPI are not that well balanced, DAPI has a high affinity to DNA and can overpower the PI stain, which has a low fluorescent intensity, (results not shown).

The biofilms were stained with DDAO and SYTO13. There was very little biofilm established from *S. aureus* and *T. spumae* cultures, and no eDNA was observed. The biofilms of *P. aeruginosa* and *B. mycoides* cultures showed some clouds of SYTO13 that were observed at the same positions as the eDNA. Those clouds could have been eDNA initially stained with DDAO and afterwards bound by SYTO13, because the stains compete to bind the free eDNA, see Figure 16 which shows the 5 day old biofilm of a *B. mycoides* culture.

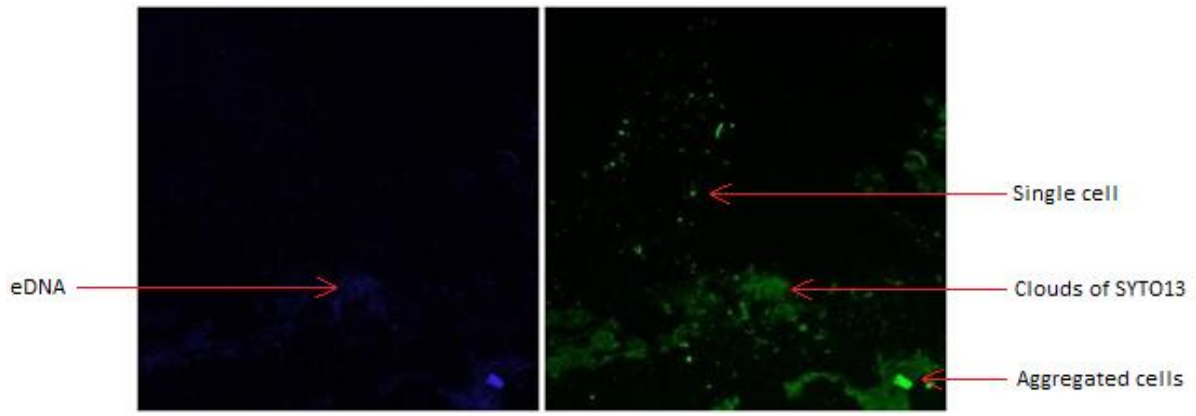

Figure 16: Biofilm of *B. mycoides* stained with DDAO and SYTO13. The magnification was 400. The two pictures were taken from the same region of the sample, but with different channels. The left picture shows the DDAO channel which reveals the eDNA, and the picture to the right shows the genomic DNA stained by SYTO13. The red arrows indicate what is seen on the pictures.

Ethanol precipitation of eDNA

The concentrations of eDNA from 27-48 hour old cultures of *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides* were measured on the Spectrophotometer ND 1000 to estimate the amount of eDNA in the samples to see whether there was enough eDNA to begin fragmentation of eDNA according to the protocol for fragmentation of genomic DNA. The OD_{600} was also measured to reveal the exponential growth phase of the bacteria, see Table 13.

Table 13: Average of OD_{600} and concentration of eDNA samples after separation and filtration.

Samples	OD_{600}	eDNA	260/280	260/230
P. aeruginosa	0.48	100 ng/µl	0.90	0.21
S. aureus	0.31	153 ng/µl	0.93	0.28
T. spumae	0.67	127 ng/µl	0.88	0.22
B. mycoides	0.69	155 ng/µl	0.88	0.27

The purity of the eDNA was not good for any of the bacteria, so an ethanol precipitation was performed to collect purer eDNA. After the ethanol precipitation there was a visible pellet from *S. aureus, T. spumae* and *B. mycoides*, but no pellet was observed from *P. aeruginosa*. The concentrations were measured on the Spectrophotometer ND 1000 after ethanol precipitation and the concentration of *P. aeruginosa* was very low, which seemed to match the fact that there was no observable pellet, see Table 14.

Table 14: Spectrophotometer ND 1000 measurements of eDNA after ethanol precipitation.

Samples	eDNA	260/280	260/230
P. aeruginosa	3 ng/µl	2.30	0.30
S. aureus	35 ng/µl	1.52	0.71
T. spumae	40 ng/µl	1.40	0.68
B. mycoides	52 ng/µl	1.38	0.53

The purity of eDNA was better after the ethanol precipitation. The three Greiner tubes containing the same bacteria were pooled together and freeze-dried over night, because the concentrations were too low to continue the fragmentation experiment.

After freeze-drying there was a little white powder on the side of the Eppendorf tube from *P. aeruginosa*. *S. aureus* had a little white/ yellow powder on the side of the Eppendorf tube. *T. spumae* had the most white/yellow powder and it was situated in the bottom of the Eppendorf tube. *B. mycoides* had some white/yellow powder, also at the bottom of the Eppendorf tube. The residue from each bacterium was dissolved in 50 µl TE buffer, because the fragmentation protocol required dilution of the DNA in TE buffer, before running on a 2 % agarose gel. The concentration was measured again on the Spectrophotometer ND 1000 to see if the concentration of eDNA was high enough to continue with the fragmentation experiment, see Table 15.

Table 15: Spectrophotometer ND 1000 measurements of eDNA after freeze-drying.

Samples	eDNA	260/280	260/230
P. aeruginosa	11 ng/µl	1.78	0.36
S. aureus	91 ng/µl	1.48	1.05
T. spumae	116 ng/µl	1.51	0.74
B. mycoides	103 ng/µl	1.42	0.60

The fragmentation of eDNA was performed according to the protocol for fragmentation of genomic DNA on the *T. spumae* sample, because *T. spumae* had the highest concentration after freeze-drying, see Table 15.

The fragmented eDNA was loaded on an agarose gel, but no band or smear appeared, (results not shown). The concentration of the fragmented eDNA was measured on a Spectrophotometer ND 1000 to see how the concentration and purity had changed after fragmentation, see Table 16.

Table 16: Spectrophotometer ND 1000 measurements of eDNA from *T. spumae* after fragmentation.

Sample	eDNA	260/280	260/230
T. spumae	37 ng/µl	1.39	0.30

Blocking of eDNA before PCR with universal and random hexamer primers

In order to investigate the similarities and difference of genomic versus extracellular DNA, the EMA treatment of eDNA was tested.

The OD_{600} and the concentration of eDNA from *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides* used in the following experiments are shown in Table 13. There were no bands visible from the PCR samples with the random hexamer primers, only a small smear from the positive control was observed. The PCR samples with the 26F and 1390R primers that were not blocked by EMA showed visible bands at 1,364 bp for all bacteria, see Figure 17. A faint narrow band at 1,364 bp was observed from the negative control with 26F and 1390R primers, but it was likely overflow from the positive control in the lane next to it. Because no bands appeared from the PCR samples with random hexamer

primers, it was impossible to state if the eDNA was blocked by EMA or not. From all bacteria a band at 1,364 bp was revealed by the PCR samples with 26F and 1390R primers, and the eDNA was blocked by EMA, as no bands appeared after treatment with EMA.

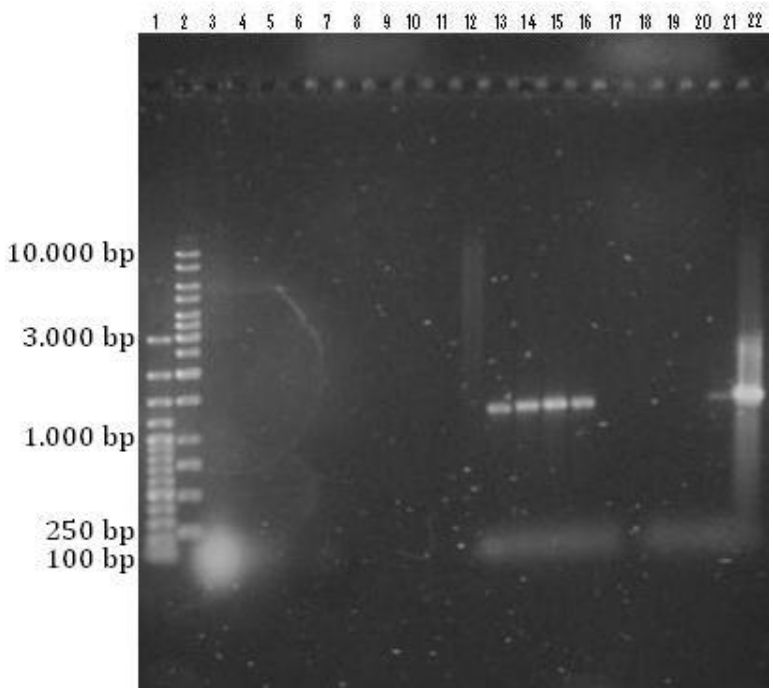

Figure 17: 1 % agarose gel. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2: GeneRuler™ 1 Kb ladder (Fermentas, Germany). Lane 3: *T. spumae*, random hexamer primers. Lane 4: *B. mycoides*, random hexamer primers. Lane 5: *S. aureus*, random hexamer primers. Lane 6: *P. aeruginosa*, random hexamer primers. Lane 7: *T. spumae*, random hexamer primers (EMA). Lane 8: *B. mycoides*, random hexamer primers (EMA). Lane 9: *S. aureus*, random hexamer primers (EMA). Lane 10: *P. aeruginosa*, random hexamer primers (EMA).Lane 11: Negative control, random hexamer primers. Lane 12: Positive control (Soil extract AAØ4 19/8-2005), random hexamer primers. Lane 13: *T. spumae*, 26F and 1390R primers. Lane 14: *B. mycoides*, 26F and 1390R primers. Lane 15: *S. aureus*, 26F and 1390R primers. Lane 16: *P. aeruginosa*, 26F and 1390R primers. Lane 17: *T. spumae*, 26F and 1390R primers (EMA). Lane 18: *B. mycoides*, 26F and 1390R primers (EMA). Lane 19: *S. aureus*, 26F and 1390R primers (EMA). Lane 20: *P. aeruginosa*, 26F and 1390R primers (EMA). Lane 21: Negative control, 26F and 1390R primers. Lane 22: Positive control (Soil extract AAØ4 19/8-2005), 26F and 1390R primers.

Genomic versus extracellular DNA with the use of EMA and lysozyme

Figure 17 showed that EMA treatment did block the eDNA from PCR samples with 26F and 1390R primers and in this experiment the EMA treatment was performed directly in cultures of the four different bacteria. After the eDNA had been blocked, the cultures were treated with a lysozyme solution to damage the cell walls and expose the genomic DNA. A comparison of eDNA and genomic DNA was made by agarose gel electrophoresis.

A 30 cycle PCR was performed with the universal primers 26F and 1390R on samples from *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides* without EMA treatment, with EMA treatment and with EMA and lysozyme treatment in 4 dilutions.

The PCR products from *P. aeruginosa* were loaded on an agarose gel. A band at 1,364 bp was observed for eDNA from *P. aeruginosa* with no EMA treatment and for the positive control. No bands were visible for the EMA treated sample and for the EMA and lysozyme treated samples, see Figure 18.

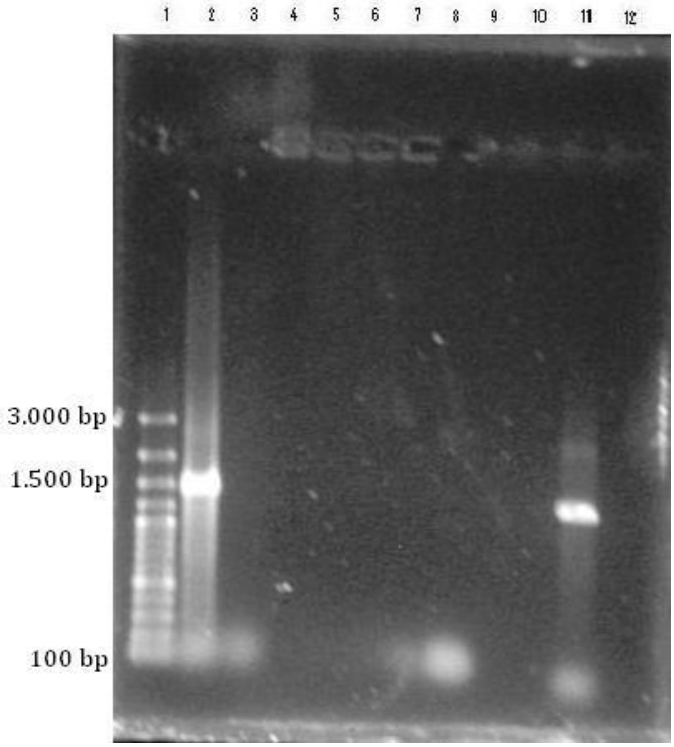

Figure 18: 1 % agarose gel containing *P. aeruginosa* samples. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2: eDNA. Lane 3: EMA treated eDNA. Lane 4: EMA + lysozyme treated eDNA. Lane 5: EMA + lysozyme treated eDNA diluted 1:20. Lane 6: EMA + lysozyme treated eDNA diluted 1:40. Lane 7: EMA + lysozyme treated eDNA diluted 1:100. Lane 8: Negative control. Lane 9 and 10: free. Lane 11: Positive control (Soil extract AAØ4 19/8-2005). Lane 12: free.

The PCR products from *S. aureus and B. mycoides* were loaded on an agarose gel. A band at 1,364 bp was observed for eDNA from *S. aureus and B. mycoides* with no EMA treatment, and for the positive control. No bands were visible for the EMA treated samples or for the EMA and lysozyme treated samples, see Figure 19.

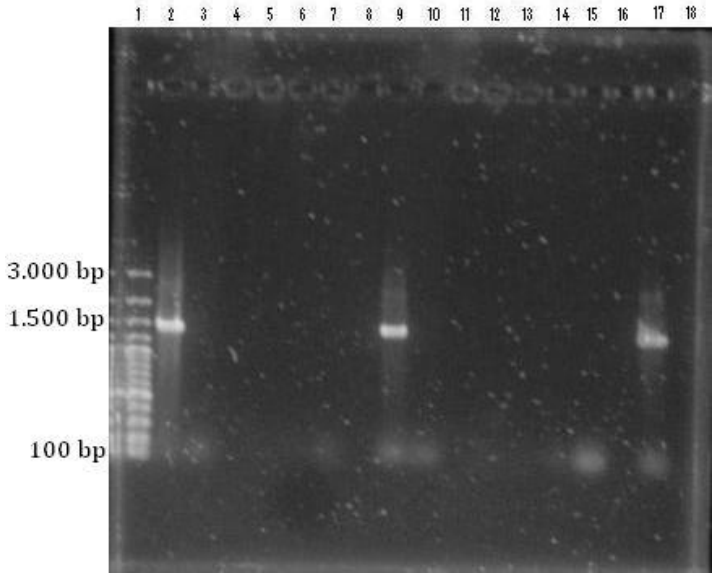

Figure 19: 1 % agarose gel containing *S. aureus* and *B. mycoides* samples. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2: eDNA (*S. aureus)*. Lane 3: EMA treated eDNA (*S. aureus)*. Lane 4: EMA + lysozyme treated eDNA (*S. aureus)*. Lane 5: EMA + lysozyme treated eDNA diluted 1:20 (*S. aureus)*. Lane 6: EMA + lysozyme treated eDNA diluted 1:40 (*S. aureus)*. Lane 7: EMA + lysozyme treated eDNA diluted 1:100 (*S. aureus)*. Lane 8: free. Lane 9: eDNA (*B. mycoides)*. Lane 10: EMA treated eDNA (*B. mycoides)*. Lane 11: EMA + lysozyme treated eDNA (*B. mycoides)*. Lane 12: EMA + lysozyme treated eDNA diluted 1:20 (*B. mycoides)*. Lane 13: EMA + lysozyme treated eDNA diluted 1:40 (*B. mycoides)*. Lane 14: EMA + lysozyme treated eDNA diluted 1:100 (*B. mycoides)*. Lane 15: Negative control. Lane 16: free. Lane 17: Positive control (Soil extract AAØ4 19/8-2005). Lane 18: free.

The PCR products from *T. spumae* were loaded on an agarose gel. A band at 1,364 bp was observed for eDNA from *T. spumae* with no EMA treatment and for the positive control. No bands were visible for the EMA treated sample or for the EMA and lysozyme treated samples. Overflow occurred into the lanes on both sides of the positive control, see Figure 20.

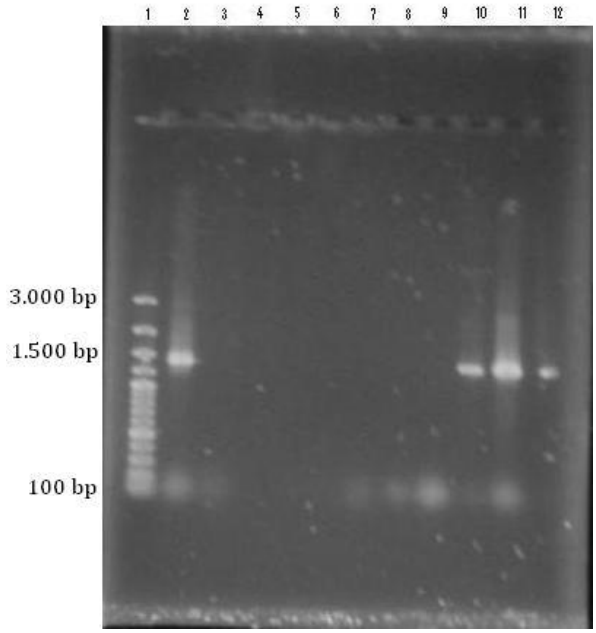

Figure 20: 1 % agarose gel containing *T. spumae* samples. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2: eDNA. Lane 3: EMA treated eDNA. Lane 4: EMA + lysozyme treated eDNA. Lane 5: EMA + lysozyme treated eDNA diluted 1:20. Lane 6: EMA + lysozyme treated eDNA diluted 1:40. Lane 7: EMA + lysozyme treated eDNA diluted 1:100. Lane 8: free. Lane 9: Negative control. Lane 10: free. Lane 11: Positive control (Soil extract AAØ4 19/8-2005). Lane 12: free.

Genomic versus extracellular DNA with the use of DNase I and a cell disruptor

The EMA and lysozyme treated samples were expected to reveal visible bands from genomic DNA, but no band appeared. Instead of blocking the eDNA with EMA, the eDNA was digested with DNase I in this experiment and the genomic DNA was obtained by breaking the cells mechanically with an FP 120 Fast Prep Cell Disruptor.

A 30 cycle PCR was performed with the 26F and 1390R primers on the samples from *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides* digested with DNase I, and on pure eDNA from the section "Genomic versus extracellular DNA with the use of EMA and lysozyme". A 35 cycle PCR was performed with UP1Ei/ UP2ri (*gyrB*) and 1698F/ 2041R *(rpoB)* primers on the same samples.

A clear band at 1,364 bp was observed from the PCR products with the 26F and 1390R primers, using both eDNA and genomic DNA from *P. aeruginosa*. No bands were observed from the PCR products with UP1Ei and UP2ri using both eDNA and genomic DNA from *P. aeruginosa*. A very faint narrow band at 343 bp was observed from the PCR products with the 1698F and 2041R primers on the eDNA from *P. aeruginosa*, but no band from the genomic DNA was observed with these primers, see Figure 21.

A very clear band at 1,364 bp was observed from the PCR products with the 26F and 1390R primers on eDNA and a more unclear and ill-defined band was observed from the genomic DNA from *S. aureus*. A well defined band at 1,180 bp was observed from the PCR products with UP1Ei and UP2ri primers on eDNA from *S. aureus*. No band was observed from the PCR products with UP1Ei and UP2ri on genomic DNA from *S. aureus*. Bands at 343 bp and 900 bp were observed from the PCR products with 1698F

and 2041R primers on eDNA from *S. aureus*, whilst the genomic DNA from the PCR products with 1698F and 2041R revealed only a band at 343 bp, see Figure 21.

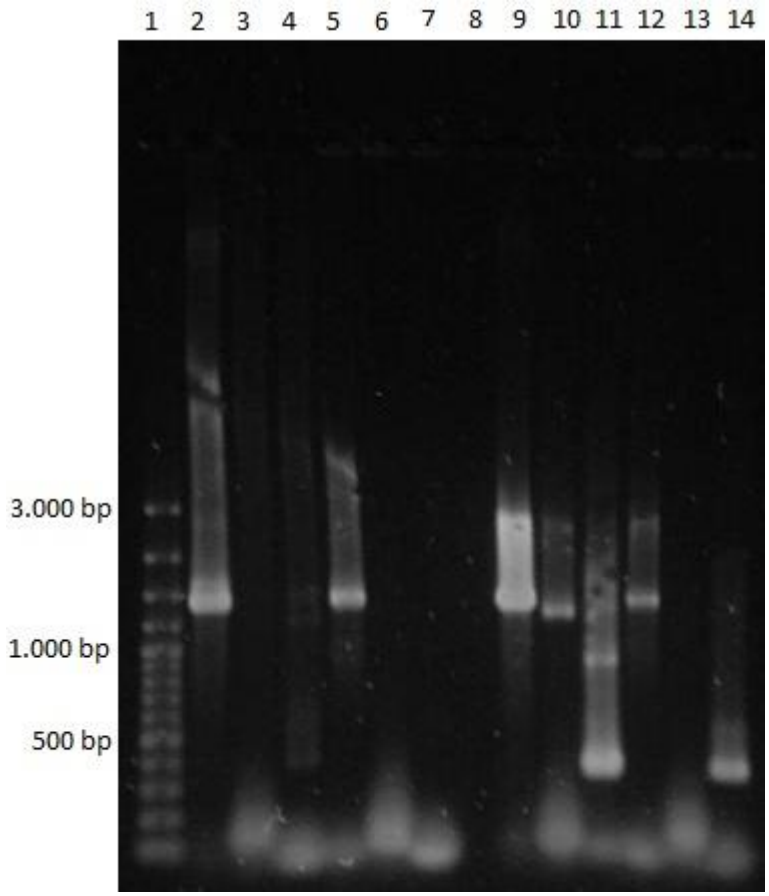

Figure 21: 1 % agarose gel containing PCR products from three different housekeeping genes from *P. aeruginosa* and *S. aureus*. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2: eDNA, *P. aeruginosa* (*16S*). Lane 3: eDNA, *P. aeruginosa* (*gyrB*). Lane 4: eDNA, *P. aeruginosa* (*rpoB*). Lane 5: gDNA, *P. aeruginosa* (*16S*). Lane 6: gDNA, *P. aeruginosa* (*gyrB*). Lane 7: gDNA, *P. aeruginosa* (*rpoB*). Lane 8: free. Lane 9: eDNA, *S. aureus* (*16S*). Lane 10: eDNA, *S. aureus* (*gyrB*). Lane 11: eDNA, *S. aureus* (*rpoB*). Lane 12: gDNA, *S. aureus* (*16S*). Lane 13: gDNA, *S. aureus* (*gyrB*). Lane 14: gDNA, *S. aureus* (*rpoB*).

A band at 1,364 bp was observed from the PCR products with 26F and 1390R primers on both eDNA and genomic DNA from *T. spumae*, where the band from eDNA was clearer than the genomic band. No bands were observed from the PCR products with UP1Ei and UP2ri on both eDNA and genomic DNA from *T. spumae*. A band at 343 bp was observed from the PCR products with 1698F and 2041R primers on both eDNA and genomic DNA from *T. spumae*, see Figure 22.

A band at 1,364 bp was observed from the PCR products with 26F and 1390R primers on eDNA from *B. mycoides*, but no band appeared from the genomic DNA. No bands were observed from the PCR products with UP1Ei and UP2ri on both eDNA and genomic DNA from *B. mycoides*. A band at 343 bp was observed from the PCR products with 1698F and 2041R primers on both eDNA and genomic DNA from *B. mycoides*, see Figure 22.

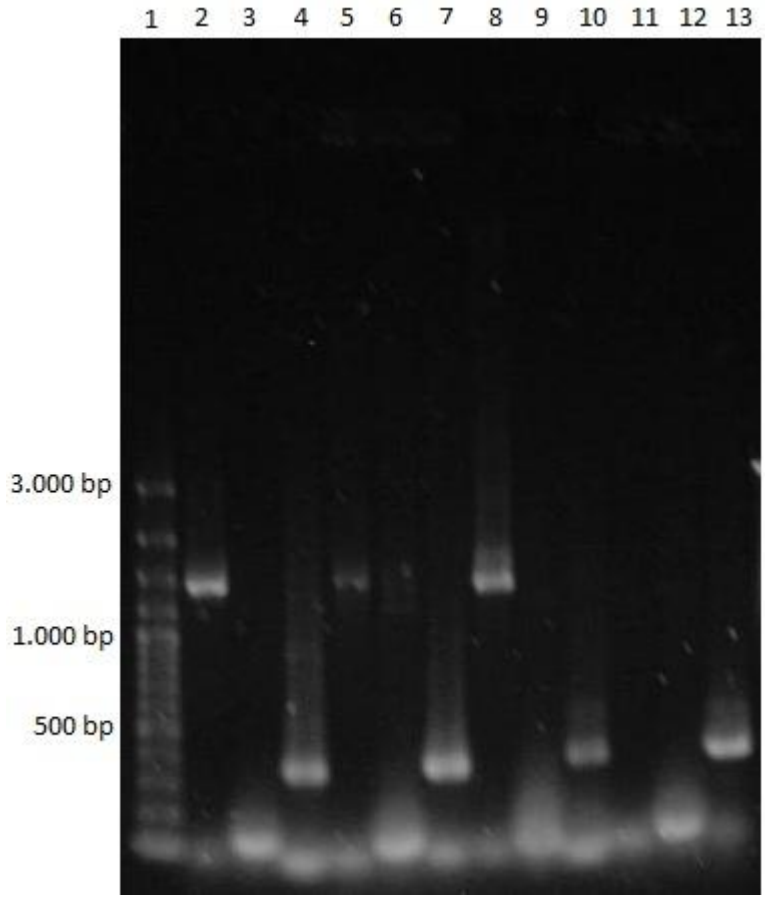

Figure 22: 1 % agarose gel containing PCR products from three different housekeeping genes from *T. spumae* and *B. mycoides*. Lane 1: GeneRuler™ 100 bp plus DNA ladder (Fermentas, Germany). Lane 2: eDNA, *T. spumae* (*16S*). Lane 3: eDNA, *T. spumae* (*gyrB*). Lane 4: eDNA, *T. spumae* (*rpoB*). Lane 5: gDNA, *T. spumae* (*16S*). Lane 6: gDNA, *T. spumae* (*gyrB*). Lane 7: gDNA, *T. spumae* (*rpoB*). Lane 8: eDNA, *B. mycoides* (*16S*). Lane 9: eDNA, *B. mycoides* (*gyrB*). Lane 10: eDNA, *B. mycoides* (*rpoB*). Lane 11: gDNA, *B. mycoides* (*16S*). Lane 12: gDNA, *B. mycoides* (*gyrB*). Lane 13: gDNA, *B. mycoides* (*rpoB*).

4.3 Experiments on complex environmental samples

In this section the complex environmental samples obtained from rocks inside two different streams have been used to try and prove or disprove the existence of eDNA. This has been attempted by staining the eDNA and genomic DNA with DDAO and SYTO13 respectively to confirm the presence of eDNA in complex environmental samples. The presence of eDNA was investigated in complex environmental samples by direct rock imprints on agar plates and by toothpick scrubs from rocks grown in 10 % TSB medium.

Complex bacterial sample from a natural environment

In general there was a lot of growth on the plates from both direct rock imprints and toothpick scrubs, which were taken in the two streams Kjærs Mølleå and Hvorup å. Pictures from both staining in Eppendorf tubes and directly on slides generally showed that the cells were doing well and that some

were forming aggregates. A weak signal of eDNA in the direct rock imprint samples stained in Eppendorf tubes from Hvorup å was observed. The signal appeared cell-shaped and was not concentrated around living cells, so it was assumed that it was eDNA from remaining cells, see Figure 23.

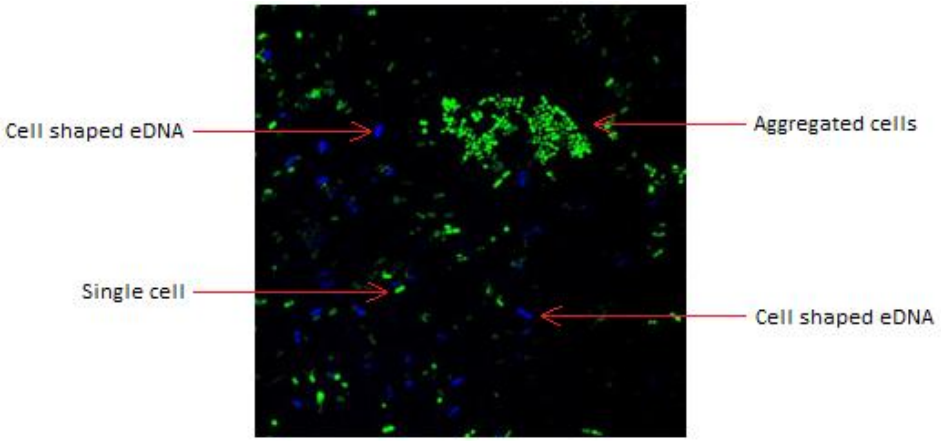

Figure 23: Complex sample from direct rock imprint from Hvorup å stained with DDAO and SYTO13 in Eppendorf tubes. The magnification was 400. Two pictures were taken from the same region of the sample, but with different channels, and merged into one picture. The genomic DNA appears green and the eDNA appears blue. The red arrows indicate what is seen on the picture.

No eDNA was observed in the direct rock imprint samples from Kjærs Mølleå, and in toothpick scrub samples from Hvorup å and Kjærs Mølleå, from both stains. The cells formed large aggregates with clouds of SYTO13 in between the cells that could have been eDNA stained green, because of the competition between the two stains, see Figure 24.

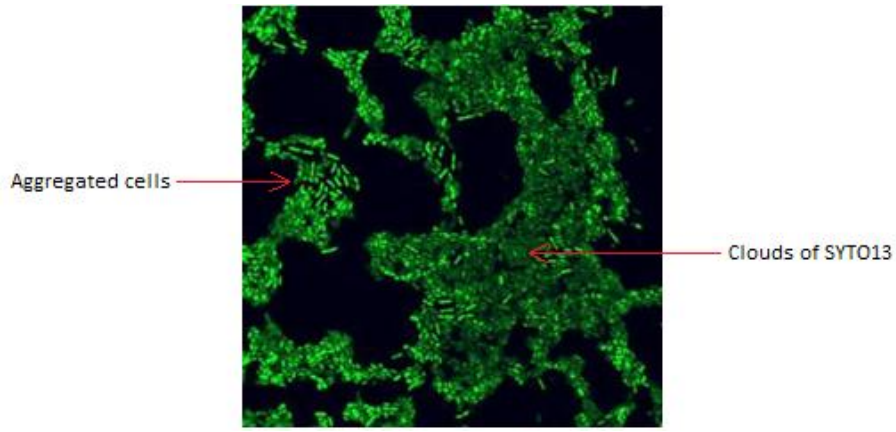

Figure 24: Complex sample from direct rock imprint from Kjærs Mølleå stained with DDAO and SYTO13 directly on slides. The magnification was 400. Two pictures were taken from the same region of the sample, but with different channels, and merged into one picture. The clouds of SYTO13 and aggregated cells appear green. No blue staining was observed. The red arrows indicate what is seen on the picture.

5 Discussion

5.1 Experiments on *Thermotoga neapolitana*

T. neapolitana has never been identified as an eDNA producing bacteria and as a consequence there is no previous published data on eDNA derived from *T. neapolitana*. The experiments on *T. neapolitana* included an imaging approach and a genetic approach, which will be discussed and compared to similar published experiments performed on other bacteria.

Confocal microscopic investigations of a 7 day old culture of *T. neapolitana* stained with DDAO and SYTO13 strongly suggested that eDNA was present in the samples, as seen on Figure 3. A large amount of the DDAO stained eDNA was found inside the EPS from the *T. neapolitana* culture surrounding the single cells and cell aggregates. The staining experiments did not reveal information about the origin of the stained eDNA, i.e. whether it originated from lysed cells or from an active secretion. However, the pictures may indirectly indicate that some of the eDNA was actively secreted, since the amount of eDNA was so large that it seems unlikely that it all originated from lysed cells. Furthermore the shape of the stained eDNA appeared as blurred and cloudy, see Figure 3, which also supports the hypothesis about active secretion, because eDNA from lysis would appear as a cell-shaped patch, if lysis had recently occurred. Some of the black pores inside the EPS without DDAO stained eDNA were cell-shaped, which could indicate that the eDNA was synthesized by a cell and therefore surrounded this cell, which had subsequently decayed. The age of the culture could affect this phenomenon, and should be considered, because it is highly likely that some of the bacteria would begin lysis after 7 days of growth, and the blurred eDNA patches could be caused by lysis that occurred some time before staining.

Corinaldesi, et al. (2005) claimed they developed an efficient procedure for isolating eDNA from the genomic DNA originating from the same sample, avoiding any DNA release from cell lysis during the extraction procedure. The separation of eDNA from genomic DNA in *T. neapolitana* was performed by 30 min. centrifugation at 12,000 g, which was in the same range as suggested in Corinaldesi et al. (2005), but the filtration of the supernatant containing eDNA suggested by Corinaldesi, et al. (2005) was not performed. This approach was chosen because the goal of the filtration is to remove contaminants like viruses, soil granules and bacterial cells, which may be problematic for complex samples such as sediment samples, but are not expected in the *T. neapolitana* pure culture. Also the filtration step involves a large risk of breaking cell walls, due to the high shear forces during filtration of any bacterial cells that may have been present in the eDNA-rich supernatant, which would result in release and contamination of genomic DNA.

The genetic approaches for *T. neapolitana* include *T. neapolitana* cultures which had grown for 21 hours, 55 hours and 6 days. The eDNA from the 21 hour old culture was extracted according to the method described by Corinaldesi, et al. (2005), with the above mentioned modification, to observe any differences or similarities between eDNA and genomic DNA. If the eDNA is not totally identical to genomic DNA, this supports the hypothesis that some form of active transport from the bacteria must

occur (Böckelmann, et al., 2006). On the agarose gel, Figure 4, no band or smear from the genomic DNA was observed, even though the DNA extraction included base/acid treatment, heating, bead beating and the freezing steps, as suggested by (Steinberger, et al., 2005), to make sure that the cells were lysed and that the genomic DNA would show on the gel. The agarose gel revealed two defined bands of eDNA above 10,000 bp, but since no bands appeared from the genomic DNA the comparison could not be accomplished, and therefore it was not possible to determine whether eDNA occurs as a result of active secretion. Following treatment of eDNA with DNase I, which is an endonuclease that nonspecifically cleaves DNA to release di-, tri- and oligonucleotide products, revealed no visible bands on the agarose gel, which indicates that the bands seen on Figure 4 were in fact eDNA and not a high molecular weight polymeric artifact. These findings are consistent with the results from Allesen-Holm et al. (2006), who found that DNase I cleaved the eDNA.

The concentrations of both eDNA and genomic DNA from the 21 hour old culture of *T. neapolitana* were measured spectrophotometrically, and the concentrations of eDNA and genomic DNA from a second 21 hour old culture of *T. neapolitana,* with the same growth conditions, were also measured spectrophotometrically. The concentrations were rather similar, see Table 9 and Table 10. The eDNA from the second culture was cleaved with the restriction enzymes HaeII and MspI. These should cut the DNA into smaller fragments, which can then be visualized on an agarose gel, but no bands were observed from eDNA or genomic DNA. Because of the similar concentrations of the two cultures, at least one band of eDNA was expected to be visible on Figure 5, which was not the case. The missing bands from genomic DNA could be a result of an incomplete cell disruption, but the time, speed and type of beads should have been satisfactory according to Nocker, et al. (2006). Since DNA has a negative net charge it may have the ability to stick to surfaces (Tavares, et al., 2001), and the surface of the beads may have attracted some of the genomic DNA, but it seems unlikely that the entire genomic DNA was stuck to the beads. When the dilution factor was taken into account the real concentration of eDNA was surprisingly higher than the concentration of genomic DNA obtained from the two cultures of *T. neapolitana*, with concentrations of 568-572 ng/µl for eDNA and of 461-500 ng/µl for genomic DNA.

A 55 hour old culture of *T. neapolitana* resulted in a large smear throughout the gel, and a 6 day old culture showed a smaller smear around 10,000 bp. This suggests that eDNA was present in higher concentrations in the younger culture, which agrees with the findings of Allsen-Holm et al, 2006. There is no reason to believe that the eDNA functioned as nourishment for the older culture, as it is proposed to do in sediments and similar environments where periods of starvation and feast alternate. In an artificial medium such as the one used for the cultures, nutrients are plentiful and bacteria have no reason to store nutrients in eDNA, because they are so readily accessible from the medium.

5.2 Experiments on *Tsukamurella spumae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus mycoides*

The experiments on *T. spumae* included a microscopic approach and a genetic approach. A time series of *T. spumae* cultures stained with DDAO and SYTO13 from 1-3 days was investigated using the CLSM, and the samples went from mostly single cells or small aggregates, with almost no or a very small production of eDNA, to large aggregates producing significant amounts of eDNA. There was generally not much eDNA present in the samples from day 1, which probably results from the young age of the culture where the bacteria cannot manage to produce detectable amounts of eDNA. In contrast to this, the cells were smaller and more aggregated in the samples from day 3, and a remarkable difference in the produced amount of eDNA was observed in samples from day 2 to day 3.

A time series of the *T. spumae* culture was stained with the amyloid specific ThT from 1-3 days and investigated using the CLSM. This staining experiment showed the same tendency for bacterial aggregation as was seen for the staining experiment with DDAO and SYTO13. The ThT stain indicated that many amyloids were present, but in order to be sure of this an antibody assay could be used.

The agarose gel electrophoresis part included *T. spumae* grown for 3 and 6 days, representing the late exponential and stationary growth phases respectively. Smears were observed from the concentrated eDNA from both cultures. The concentrated sample from the 6 day old culture looked very dense in the area at the top of the gel, which suggests that some eDNA was trapped in the wells of the gel. The SYBR GOLD solution binds specifically to DNA and the result thus suggests that the smears were derived from eDNA.

The concentration of eDNA from a 24 hour old culture of *T. spumae* precipitated with ammonium acetate was very high at 2,870 ng/µl, and the 260/280 ratio was 2.03, which is higher than the 1.8 expected with pure DNA. This high ratio indicates that RNA is also present in the samples (www.nanodrop.com). The 260/230 ratio is a secondary measure of nucleic acid purity and the expected range is 1.8 - 2.2, which frequently is higher than the 260/280 value (www.nanodrop.com). The 260/230 ratio was 1.51 from eDNA precipitated with ammonium acetate, which indicates a contamination with phenols, carbohydrates or EDTA. A phase separation with PCI was performed to separate the DNA and the contaminants, e.g. proteins and cell debris, especially because the sample looked slightly unclear. The water phase, including eDNA, was run on an agarose gel, which revealed a small band above 10,000 bp. This indicated that eDNA was present, although it was not pure. The universal primers 26F/ 1390R and 8F/ 1492R were used to amplify the 16S rRNA gene fragment from eDNA from the 24 hour old *T. spumae* culture. The 8F and 1492R primers did not reveal any bands, but the 26F and 1390R primers revealed a band of around the correct size at 1,364 bp. Another PCR with random hexamer primers was performed on eDNA extracted by the PowerSoil DNA Isolation Kit from a similar culture of *T. spumae.* This did not reveal any bands or smears on the agarose gel, which indicates an unsuccessful PCR possibly caused by the wrong temperature or time settings of the PCR, because the eDNA was amplified in many different very small fragments that may have run out of the gel during electrophoresis. There is a risk that the eDNA sticks to the filters when using the PowerSoil

DNA Isolation Kit, even though the eDNA should be washed off. According to Steinberger, et al. (2005) the bacterial DNA is very persistent outside the cell and can be amplified by PCR for months, as opposed to eukaryote DNA which is rapidly degraded by nucleases (Steinberger, et al., 2005), so if eDNA was present it should have been possible to amplify from the samples.

The fragmentation experiment of eDNA from a 19 hour old *T. spumae* culture representing the exponential growth phase did not reveal any bands on the 2 % agarose gel. This could have been caused by the fact that the sample contained some impurities, as shown on the concentration measurement, see Table 12, or the bacteria did not make a sufficient amount of eDNA in 19 hours. The protocol for fragmentation of genomic DNA is very long and there is a risk that the eDNA was lost during the execution of the protocol. If an older culture of *T. spumae* had been used the concentration of eDNA would have been higher, as shown in the microscopy results obtained from the time series of *T. spumae,* and thus enabled amplification. However it was important to use cultures from the exponential growth phase to eliminate or reduce the problem of genomic DNA from lysed cells.

The eDNA from a 24 hour old *T. spumae* culture was cleaved with the restriction enzymes HaeIII, which cleaves DNA in the 5'-G G^C C-3' sequence, and MspI, which cleaves DNA in the 5'-C^C G G-3' sequence. The sequence of eDNA from *T. spumae* is unknown but it is very likely that the eDNA would be cleaved by these restriction enzymes, however, no band or smear appeared on the gel. There is also the possibility that the binding site for these restriction enzymes is too frequently occurring and thus the eDNA would have been cleaved into very small eDNA fragments which would have run out of the gel.

Pictures of 24 hour old cultures (exponential growth phase) and 5 day old cultures (late stationary growth phase) of *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides* generally revealed no, or very little, eDNA production. In the section "Time series of *Tsukamurella spumae* stained with DDAO and SYTO13" a huge development of eDNA was seen in the stationary growth phase (3 day old) samples, and it was expected that eDNA would be observed in the late stationary growth phase at least from the *T. spumae* culture. A different media had been used for the time series of *T. spumae* compared to the media used for this experiment and it may have influenced the production of eDNA, even though the bacteria grew equally well in both media. As previously mentioned it has been hypothesized that eDNA serves as a nutrient for bacteria during starvation, which could explain the missing eDNA in the later growth phases. The production of eDNA varies according to the growth conditions (for example it has been shown to be higher among cells living in biofilms compared to planktonic cells), and it is possible that eDNA was present in between early growth phases and then disappeared. According to Sauer (2003) the proteins that are expressed during biofilm development are differentially produced compared to the expression of protein from planktonic cells. 5 day old biofilms in the late stationary phase were grown and stained for eDNA to see if a different amount of eDNA was detected from a biofilm compared to a pure culture of the same bacteria. Steinberger, et al. (2005) found that 17 % of the total DNA pool was eDNA in *P. aeruginosa* biofilms, so the biofilm of *P. aeruginosa* was expected to produce large amounts of eDNA. Generally the investigated biofilms were small and contained only small amounts of eDNA, which could be due to the growth conditions, since bacteria that have

sufficient nutrients and are not exposed to any stress factors have no reason to produce a biofilm and eDNA. The formation of biofilms by bacteria is based on survival strategies, and the matrix of a biofilm including eDNA provides protection from hostile environments, including temperature factors, pH, antibiotics etc. The biofilm could have been washed away during the washing steps in the staining experiments, but biofilms normally adhere very strongly to surfaces, and this potential source of error is therefore not considered to have influenced the experiments to a significant extent. Another possibility could be the fact that a bacterium expresses each gene differentially at all times and genes that are involved in the expression of eDNA could have been turned on and off due to different types of unforeseen stress factors.

In order to investigate the similarities and differences of genomic and extracellular DNA, EMA treatment was tested. The EMA treatment results in binding of the EMA molecules to eDNA, which did block the eDNA from the polymerase used in the PCR, and therefore gave no PCR products with the 26F and 1390R primers. This was the case for eDNA from *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides* grown for 27-48 hours, which all gave the expected bands for PCR on eDNA that had not been treated with EMA. For PCR using random hexamer primers no bands were found for both EMA treated and untreated eDNA, which made it impossible to state if the eDNA was blocked or not. The random hexamer primers showed a faint smear for the positive control, and the concentration of eDNA in the bacterial cultures was expected to be much smaller than the concentration of the positive control, so a very faint band from the four bacteria could be present but difficult to visualize, see Figure 17. It was also possible that the PCR settings were not optimal for the amplification with random hexamer primers. The EMA treatment procedure used in this project varied slightly from the recommendations published by Nocker et al. (2006). Here it is stated that for the EMA treatment the samples should be light exposed for 1 min. using a 650 W halogen light source placed 20 cm from the sample tubes. In the experiments performed in this project the samples were exposed to light using a 100 W halogen lamp at 5 cm for 2 min. The blocked amplification by PCR after EMA treatment suggests that this approach did work successfully.

In order to compare the amplified PCR products from eDNA with the PCR products amplified from genomic DNA, the samples were treated with lysozyme after EMA treatment, in order to obtain genomic DNA from lysed cells which would not be blocked by EMA. The PCR products that were amplified using the universal 26F and 1390R primers on eDNA from *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides* and the positive control all showed bands corresponding to a size of 1,364 bp. The samples treated with EMA did not show a band, as expected, but the samples treated with both EMA and lysozyme also did not result in a band on the agarose gels, Figure 18, Figure 19 and Figure 20. The EMA should block the eDNA present in the samples and the lysozyme should break down the cell membranes and expose the genomic DNA, which should then be amplified. A possible explanation of the lack of bands could be that the cells were still intact after the lysozyme treatment, or that the genomic DNA was spun down with the cell debris during preparation, and therefore was not present in the samples used for PCR. However, it seems unlikely that the entire mass of genomic DNA should be removed from the supernatant by centrifugation. Another possibility could be that the EMA was still active during and after the lysozyme treatment and thereby blocked the genomic DNA from

amplification, but according to Rudi, et al. (2005) and Nocker, et al. (2006), free EMA is inactivated by light exposure and reacts with water molecules in the absence DNA molecules, see Figure 2.

Since the combination of EMA and lysozyme did not function as expected, the removal of eDNA by using DNase I was tried instead, followed by mechanical breaking the cells to obtain genomic DNA. PCR was performed with the 26F/ 1390R, UP1Ei/ UP2ri (*gyrB*) and 1698F/ 2041R *(rpoB)* primers on samples from *P. aeruginosa, S. aureus, T. spumae* and *B. mycoides,* on both eDNA and genomic DNA. Not all primers gave the expected bands for all bacteria types, but in general the eDNA bands were clearer than the bands originating from amplification of genomic DNA. For PCR with the 26F and 1390R primers on eDNA and genomic DNA a band appeared on the agarose gels with a size of 1,364 bp for all investigated bacteria, except for *B. mycoides,* which did not show a band from the genomic DNA. Since PCR amplification of genomic DNA isolated from this organism was possible using the 1698F and 2041R primers, it indicates that the genomic DNA was available for amplification and that it should have been 16S amplifiable. It therefore seems probable that a mistake was made during the handling, which caused the negative result of the PCR amplification with the 26F and 1390R primers on *B. mycoides* genomic DNA.

PCR using the UP1Ei and UP2ri primers on DNA from *P. aeruginosa, T. spumae and B. mycoides* did not result in visible bands on the agarose gel, see Figure 21 and Figure 22. The expected band at 1,180 bp was observed only for eDNA from the *S. aureus* culture. It was strange that no band from the genomic DNA of *S. aureus* was detectable and may indicate that there was a difference between eDNA and genomic DNA from *S. aureus*. The results of the PCR with the 1698F and 2041R primers on the same samples of *S. aureus* revealed a band at 343 bp for genomic DNA and bands at 343 bp and 900 bp for the eDNA, which suggested an unspecific product was amplified along the correct product. The PCR products from *P. aeruginosa* with the 1698F and 2041R primers showed a faint band around 343 bp from eDNA and no band from the genomic DNA. The PCR products from *T. spumae and B. mycoides* with the 1698F and 2041R primers showed a band at 343 bp for both eDNA and genomic DNA, which was in agreement with the expected size. The genomic and extracellular DNA from *P. aeruginosa*, *S. aureus, T. spumae* and *B. mycoides* in the exponential growth phase was investigated by PCR with three different housekeeping genes and some differences were observed, see Figure 21 and Figure 22, but more experiments have to be done to be sure of these differences. Allesen-Holm, et al. (2006) found no significant differences between eDNA and genomic DNA by using the methods PCR, RAPD and Southern blot analysis on *P.aeruginosa* in the late exponential growth phase. However minor differences among which individual genes are expressed might be the case (Allesen-Holm, et al., 2006).

5.3 Experiments on complex environmental samples

Environmental samples obtained from the two streams Kjærs Mølleå and Hvorup å were stained with DDAO and SYTO13 in Eppendorf tubes and directly on slides. The staining procedures were similar to each other and no considerable differences were noticed. Aggregates should be a good place to look for eDNA, because eDNA is thought to have a structural role in keeping the aggregates together (Steinberger, et al., 2005). The complex environmental samples were found to contain both single cells

and cell aggregates, but only a weak signal of eDNA was observed, see Figure 23. The signal appeared to be cell-shaped, which indicated that it was eDNA originating from cells with compromised cell membranes. Actively secreted eDNA was expected to surround the aggregates and keep the aggregates together, but this scenario was not exhibited on the pictures taken of the stained complex environmental samples, see Figure 23 and Figure 24. The production of eDNA mainly occurs under nutrient limiting conditions and excretion of eDNA must have a purpose, because it is a valuable compound for the organism. If this hypothesis is true, the findings in this project may indicate that the investigated complex environmental samples provide sufficient nutrients for the bacteria, since bacteria that are not exposed to starvation and other stress factors may not produce eDNA. Also factors such as temperature and electron acceptor accessibility etc. in suitable ranges in the streams from which the samples originated may contribute to the low eDNA production. The biofilm was fully developed and in most cases published eDNA in biofilms derive from young biofilm cultures and it is often reported to disappear as the biofilm matures and produces other exopolymeric substances.

5.4 General discussion

According to Corinaldesi, et al. (2005) the eDNA and genomic DNA has to originate from the same sample in order for comparisons to be made. When the genomic DNA was compared to eDNA in this thesis they both appeared to contain the same functional genes. It could be problematic to compare the results from culture to culture, even if they have had the same growth conditions and contain the same bacteria, because unknown variations could occur. The separation of eDNA from genomic DNA was performed by centrifugation, and the eDNA content may have been underestimated a little compared to the actual content in the samples, because some of the eDNA would stick to the cells and become part of the genomic fraction during the separation.

Böckelmann, et al. (2006) hypothesised that it could be possible to differentiate between eDNA obtained by cell lysis and by active secretion by studying the sequence of the different DNA types. This is based on an assumption that if the eDNA originates from cell lysis there is a good chance that the eDNA and genomic DNA would be identical, whereas there probably would be differences between the DNA types if the eDNA originated from active secretion. Böckelmann, et al. (2006) found small differences between eDNA and genomic DNA when they were compared by cleaving with 4 different restriction enzymes, and they found that eDNA was generally longer than the genomic DNA. In this thesis the experiments with restriction enzymes did not show any bands, either for eDNA or genomic DNA. The restriction enzymes and bacteria used in experiments performed for this thesis were different to those used by Böckelmann, et al. (2006), but it was nevertheless expected that some bands would be visible after treatment with restriction enzymes, which was not the case.

Steinberger, et al. (2005) found that genomic DNA and eDNA appeared to be identical for *P.aeruginosa*, *P. putida, Rhodococcus erythropolis* and *Vairiovorax paradoxus* biofilms when analysed by random amplified polymorphic DNA analysis. But when they compared genomic and eDNA by using 16S rRNA genes, the eDNA showed a slight divergence compared to genomic DNA (Steinberger, et al., 2005). The 16S rRNA amplification experiments in this thesis gave positive results for both the eDNA

fraction and the genomic DNA, and thus indicate that the bacteria investigated excrete the 16S rRNA genes, except for *B. mycoides,* which did not reveal a band from the genomic DNA.

DDAO is much more fluorescent when bound to DNA, but it still emits some fluorescent signal when it is not bound to DNA, and therefore it is necessary to wash the unbound stain away to prevent background noise from DDAO. DDAO cannot penetrate the cell membrane and therefore only stains the extracellular DNA, or DNA from cells with a comprised cell membrane. SYTO13 fluoresces very brightly when it is bound to DNA, and only faintly when it is unbound. This is why a washing step after staining with SYTO13 is not necessary. The SYTO dyes do not act exclusively as nuclear stains in live cells and should thus not be equated with DNA-specific stains such as DAPI or Hoechst 33342. DDAO and SYTO13 are a good DNA stain combination, because they counterbalance each other well, but competition between the two stains might still take place even though the stains were added in a specific order. This could occur when DDAO bound to DNA will prevent binding of SYTO13 and vice versa.

6 Conclusion

The aim of the project has been achieved by finding eDNA in the thermophilic bacteria *Thermotoga neapolitana,* using imaging and genetic approaches. The presence of eDNA in pure cultures of *Pseudomonas aeruginosa, Staphylococcus aureus, Tsukamurella spumae* and *Bacillus mycoides* was confirmed as well as in complex environmental samples from water streams.

The production of eDNA in pure cultures varied, depending on the growth phase of the culture, but it was probably also influenced by other stress factors. At present no method exists for the purification of eDNA which does not contain genomic DNA from cell lysis. The way to avoid faulty results is therefore to take great care not only during purification of eDNA, but also in the interpretation of the obtained results. In this thesis a very gentle centrifugation procedure was applied to avoid major contamination of the eDNA fraction. For biofilms the production of eDNA was different from that in plantonic cultures as a separate experiment showed that biofilms of *S. aureus* and *T. spumae* were very small and no production of eDNA was observed. The biofilms of *P. aeruginosa* and *B. mycoides* showed some clouds of SYTO13 which could be eDNA, but these biofilms were also very small and because of the small amount of biofilm and eDNA a correlation of biofilm and eDNA development was not found.

The comparison of genomic DNA and eDNA for *T. neapolitana* could not be accomplished, as no genomic DNA was visualised on the agarose gels. However differences were observed between genomic DNA and eDNA for *P. aeruginosa, S. aureus* and *B. mycoides,* when the DNA types were compared for the presence of 3 different housekeeping genes on an agarose gel. Contrastive to these findings were the results for *T. spumae* which revealed equal size bands for both genomic DNA and eDNA. Most of the investigated genes were present in both the eDNA and the genomic DNA fractions but in general the housekeeping genes from eDNA appeared as clearer bands on agarose gels than the bands originating from genomic DNA. The results obtained in this thesis thus may indicate that eDNA contains the same genetic information as genomic DNA and is therefore most likely a genomic copy.

7 Perspective

There are a lot of advantages for bacteria to form and live in biofilms. The extracellular DNA plays a structural role in the formation of a biofilm; it glues the bacteria together, and provides protection. The more eDNA that is present in a culture, the more aggregation will occur (Liu, et al., 2008). Chronic inflammation and other bacterial infections are most often caused by biofilms, and treatment of those could likely be more successful if the structure and role of eDNA was further elucidated (Steinberger, et al., 2005).

It is thus essential to determine the origin of eDNA and find out if it derives from some sort of active secretion, or by simple cell lysis. By comparing the genomic DNA and eDNA based on PCR results it was difficult to know if the signal appeared from contamination, from genomic DNA, or eDNA. Therefore, the results should be confirmed by PCR independent methods, for example by fragmentation with restriction enzymes or by quantitative PCR, which reveals information about the distribution of genes. There should also be a focus on how to separate eDNA from genomic DNA, and a method where no eDNA contamination of the genomic DNA occurs, and vice versa, should be used in every experiment, if at all possible.

Time series with shorter intervals should be performed to investigate how the production of eDNA develops, and a production curve for eDNA should be constructed for many different bacteria to find out if there is a general pattern, and if there is a dependency on the growth conditions of the bacteria. The effect of different stress factors, such as temperature changes, access to nourishment and stirring etc. should be investigated. These results could most likely be obtained by the application of different staining procedures (using gene-specific as well as general DNA specific stains) during biofilm development. Gene specific staining can probably be possible through enzymatic amplification of the fluorescent signals, or by the use of multiple and stronger fluorochrome markers.

If the sequence of eDNA was found, research into the formation of biofilm-related disease would have a new angle, which could possibly lead to the prevention of biofilm formation, and weakening or even removal of biofilms could take place.

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9 Appendix

9.1 Glucose medium

The glucose medium and the glucose solution were mixed after autoclaving. The concentration of the chemicals and the amount used to make 0.5 l of each solution are stated in Table 17 and Table 18.

Table 17: Chemicals and amount used to make 0.5 l of the glucose medium.

Glucose medium	Conc. [g/l]	Weighing out	Unit
NH_4Cl	1.00	0.50	G
K_2HPO_4	6.90	3.45	G
KH_2PO_4	0.90	0.45	G
NaCl	20.00	10.00	G
KCl	0.10	0.05	G
Cystein HCl	1.00	0.50	G
Yeast extract	2.00	1.00	G
Peptone	2.00	1.00	G
Biotin (2 mg/L)	0.00002	100.00	µl
Trace elements (DSMZ medium 141)	10.00	5	Ml
Rezasurin	1.00	0.5	Ml
Demineraliseret vand	-	0.25	L
Adjust pH (NaOH (1M))	8.00	8	

Table 18: Chemicals and amount used to make 0.5 l of the glucose solution.

Glucose solution	Conc. [g/l]	Weighing out	Unit
$MgCl_2 * 6H_2O$	0.20	0.10	G
$CaCl_2 * 2H_2O$	0.01	0.005	G
Glucose	5.00	2.50	G
Demineraliseret vand	-	0.25	L
Adjust pH (NaOH (1M))	8.00	8	

9.2 Fragmentation of genomic DNA

The fragmentation of eDNA was executed according the manufactures' description of fragmentation of genomic DNA. The protocol "Preparing Samples for Sequencing Genomic DNA" from Illumina Inc. 2007 was followed, see (www.illumina.com).