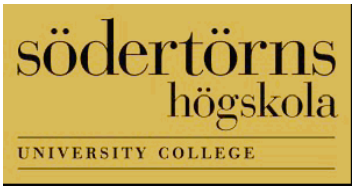
From the Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

and

School of Life Sciences
Södertörns högskola

Exploring the Metagenome of the Baltic Sea Sediment

Fredrik Hårdeman



Stockholm 2008

Published by Karolinska Institutet, Printed by Universitetsservice AB

ISBN: 978-91-7357-386-3

Abstract

Environmental microorganisms are fundamental to ecosystem function, acting as drivers in processes such as primary production, organic matter remineralisation, pollution remediation and global biogeochemical cycling. However, the study of the bacterial communities requires the application of advanced culture-independent methods considering that only a small fraction of the community is otherwise accessed.

The goal of this thesis was to investigate the bacterial community structures and functions of Baltic Sea coastal sediments. To assess the distribution and identity of metabolically active bacteria along a vertical redox gradient, a polyphasic method was applied including: reverse transcriptase-PCR (transcription) and bromodeoxyuridine immunocapture (replication) for 16S rRNA gene analyses through both clone library sequence analysis and terminal restriction fragment length polymorphism (T-RFLP). It was demonstrated that the bacterial communities were highly diverse and significantly different at different redox layers. Phylogenetic analysis identified several novel bacterial groups, some with potentially important ecological roles, notably the first genetic evidence of active anammox bacteria, demonstrating that the bacterial community of the Baltic Sea sediment includes several largely unexplored groups.

A metagenomic approach was used to access the bacterial diversity. Considering that the Baltic Sea sediment contained a diverse and largely unexplored bacterial community and also represent a permanently cold environment. This community is likely to harbor bacteria with enzymes adapted to low temperatures that would have a potential biotechnological value. The capacity of functional metagenomics for bioprospecting was demonstrated though the construction of a fosmid library of the prokaryotic genomic pool and expression screening, which enabled the identification of several novel lipolytical enzymes. A novel lipase, h1Lip1 (DQ118648) was isolated, overexpressed, purified and characterized for catalytic activity, substrate specificity, apparent temperature optimum and thermo-stability, demonstrating that the enzyme was low temperature active. 3D protein structure modelling of the lipase supported the presence of an alpha/beta-hydrolase fold, a catalytic triad and a lid structure, covering the active site. Comparative structure analyses and site directed-mutagenesis further showed the importance of a region within the N-terminal and lid for substrate affinity and thermal stability. In conclusion, these targeted molecular strategies demonstrate that the Baltic Sea sediments contain a highly diverse and unique bacterial community that also represents a useful source of biotechnologically interesting molecules.

List of publications

This thesis is based on the following publications and manuscripts, which are referred to by their Roman numerals:

I. Hårdeman, F. and Sjöling, S. 2007. Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultured bacteria of marine sediment. *FEMS Microbiol Ecol* **59:** 524-534.

II. Hårdeman, F., Perez-Bercoff Å. and Sjöling, S. Comparative modelling and mutational analysis of the low- temperature-active metagenomically derived lipase h1Lip1. Manuscript.

III. Edlund A., Hårdeman F., Jansson J.K. and Sjöling S. 2008. Active bacterial community structure along vertical redox gradients in Baltic Sea sediment. Environmental Microbiology. *Environmental Microbiology,* in press.

The papers are reprinted with permission from the respective publisher

Additional manuscript:

Hårdeman, F. and Sjöling, S. High Bacterial and low Archaeal diversity of a coastal Baltic Sea softbottom sediment. Manuscript.

List of abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
T-RFLP	Terminal Restriction Fragment Length Polymorphism
BrdU	Bromodeoxyuridine
16S subunit	16 Svedberg unit Ribosome subunit
16S rRNA gene	Gene encoding the 16S RNA
HMW	High Molecular Weight
kb	Kilo base
BAC	Bacterial Artificial Chromosome
PCR	Polymerase Chain Reaction
HSL	Hormone Sensitive Lipase
PDB	Protein Data Bank
k_{cat}	Reaction rate of a enzyme
K_m	Michaelis-Menten constant

Contents

Introduction

Microbial communities

All life on Earth is ultimately dependent on microbial life, present or past, and the foundation of the ecosystem is built on microbial activity. Fossil records hold that the first life was microbial, existing more than 3,85 billion years ago (Mojzsis *et al.*, 1996). The environmental microorganisms, which include bacteria, archaea, fungi, protozoa, algae and also viruses, are essential for the biogeochemical cycles of the key elements of life: carbon, nitrogen, sulphur, phosphorous and oxygen. The microbial contribution to the global primary production involves a complex flux of energy and matter through the ecosystem food web (Azam, 1998). The microbial part of the food web includes important processes of heterotrophic bacterial uptake of dissolved organic carbon but also predating protozoa that further transform the organic carbon (Azam *et al.*, 1983; Hagström *et al.*, 1988; Azam *et al.*, 1994; Azam, 1998). It also includes predation of bacteria by viruses and studies have suggested that viruses kill approximately 20% of the bacterial and archaeal oceanic biomass per day (Suttle, 2007). In the sea, which covers more than 70 % of the Earth's surface, the bacterial, archaeal and viral members of the microbial community have been shown to dominate the water column in both abundance and diversity (Hobbie *et al.*, 1977; Hagstrom *et al.*, 1979; Fuhrman and Azam, 1980; Azam and Malfatti, 2007). For example, one micro liter subsurface seawater has been estimated to contain thousands of different bacteria and archaea and ten thousands of different viruses (Azam and Malfatti, 2007). The collective genomes of these organisms have been termed the metagenome (Handelsman *et al.*, 1998). The bacterial and archaeal community structure and metabolic activity in marine sediments have been comparatively less studied. One gram of sediment may contain more than 10^{10} bacteria, and sediment has been estimated to contain up to 12000 different genomes, which is the highest for any environment (Torsvik *et al.*, 1996), other results suggest that these estimates are underestimated and that any microbial community may contain up to 10^{17} bacteria of 10^{7} different taxonomic groups (Curtis and Sloan, 2005). With large physical differences

compared to the water column, sediments represent an important environment for bacterial activity. The vertically stratified soft bottom sediments harbour bacteria with the ability to couple multiple redox reactions of organic and inorganic compounds in highly diverse catalytic biochemical reactions (Jorgensen and Boetius, 2007). In addition, cell surface bound and extracellular hydrolytic enzymes, such as lipases, proteases, glucosidases, phosphatases, nucleases and chitinases, are important in the bacterial carbon transformation (Hollibaugh and Azam, 1983; Kirchman and White, 1999; Azam and Malfatti, 2007). Hence, the marine sediment, containing highly diverse and abundant bacterial communities, has the potential to be an important source for the identification of novel genes and gene products for biotechnological purposes (DeLong, 2004). However, most of the marine bacteria and archaea have been shown to be difficult to culture, or have not yet been cultured (Amann *et al.*, 1995; Rappe and Giovannoni, 2003). Thus, the study of, and access to, the collective genomes of this 'hidden' diversity requires the application of molecular techniques. One new molecular tool which has become very powerful is metagenomics. In this study, Baltic Sea sediment bacteria were exploited by a metagenomic approach in order to identify novel low temperature active enzymes, based on the information that sediments represent permanently cold environments with high bacterial diversity. The bacterial diversity and community structure of Baltic Sea sediments was also investigated.

The metagenomics concept

Metagenomics is the study of, and the access to, the collective genomes of environmental microorganisms (Handelsman *et al.*, 1998; Riesenfeld *et al.*, 2004a). The power of metagenomics is the access, without prior sequence information, to the so far uncultured majority, which is estimated to be more than 99% of the prokaryotic organisms (Amann *et al.*, 1995; Rappe and Giovannoni, 2003). The metagenomic approach includes both functional and sequence-based analyses of DNA extracted directly from the environment. The DNA is often cloned into large clone libraries, allowing the access to novel genes, complete pathways and gene products through multiple screening possibilities (Handelsman *et al.*, 1998; Handelsman, 2004; Sjöling and Cowan, 2008).

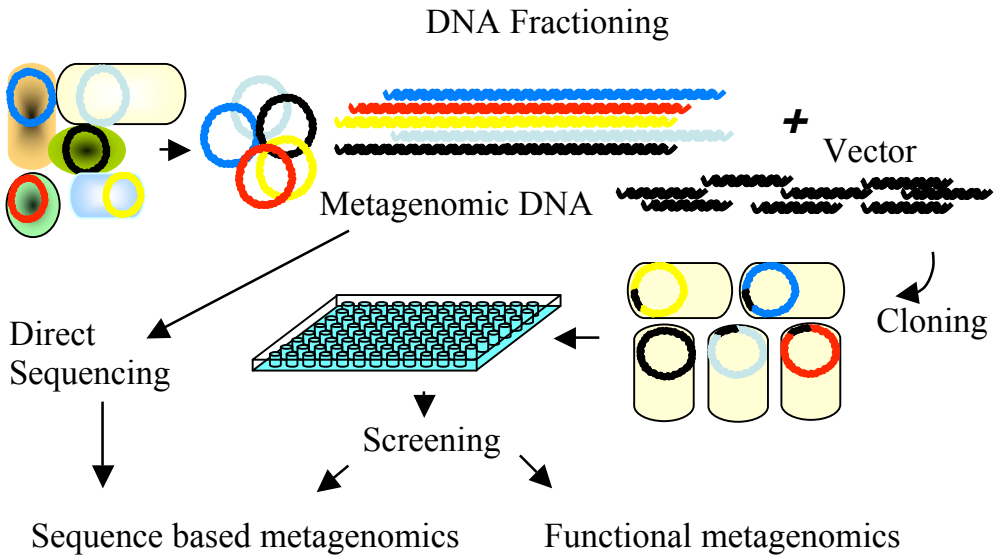


Figure 1.
Metagenomic approaches.

The metagenomic libraries can be constructed either from total environmental DNA (unselective) or from a specific fraction of the microbiota or the genome (enriched or targeted). The approach has been used for over a decade, however, the term metagenomics was first used in 1998 (Handelsman *et al.*, 1998). The approach has also been termed: environmental genomics (Beja, 2000), zoolibrary construction (Healy *et al.*, 1995), environmental DNA cloning (Stein *et al.*, 1996), eDNA cloning (Brady and Clardy, 2000), multigenomic cloning (Cowan, 2000), soil DNA cloning (MacNeil *et al.*, 2001), recombinant environmental cloning (Courtois *et al.*, 2003) and community genome analyses (Tyson *et al.*, 2004). Metagenomics is applied within many different research fields, particularly within microbial ecology, biodiversity and biotechnology (Handelsman *et al.*, 2002). Studies of bacterial and archaeal communities are most common, even though eukaryotic cDNA libraries have been produced (Grant *et al.*, 2006; Bailly *et al.*, 2007), mainly because of the more technically challenging methodology of RNA extraction and cDNA synthesis. Several reviews, that present broad overviews of the field of metagenomics, have highlighted the potentials and/or challenges of metagenomics (Daniel, 2004; Handelsman, 2004; Daniel, 2005; Galvao *et al.*, 2005; Sjöling *et al.*, 2006; Ward, 2006; Kowalchuk *et al.*, 2007; Leveau, 2007). There is a

consensus that metagenomics in the future will be regarded as one of the most important milestones in the field of microbiology. Metagenomics includes two general approaches, functional metagenomics and sequence-based metagenomics, which also includes shot-gun sequencing and comparative community metagenomics. Figure 1.

Functional metagenomics

In functional metagenomics, the goal is to identify novel bioactive compounds (Brady *et al.*, 2001; Gillespie *et al.*, 2002) or enzymes (Healy *et al.*, 1995; Henne *et al.*, 1999; Henne *et al.*, 2000; Uchiyama *et al.*, 2005) through heterologous expression screening of a metagenomic library (Schloss and Handelsman, 2003; Handelsman, 2004; Riesenfeld *et al.*, 2004a). The scope of the technology is the access to complete genes and pathways without any prior knowledge of sequence information of the target gene, enabling discoveries of novel and previously unknown genes and gene products (Handelsman, 2004). One major limitation in heterologous gene expression is that the host must have a compatible expression system for the cloned environmental DNA. Therefore, the frequency of detected activities is often low, which in turn requires the use of high throughput screening systems (Handelsman, 2004). However, the development of new vectors and expression hosts (Wang *et al.*, 2000; Courtois *et al.*, 2003) and the fact that DNA from bacteria of some phylogenetic groups is compatible with the most commonly used expression system, *E. coli* and thus expressed (Handelsman, 2004), further broadens the scope of the technology. Other limitations are data bias resulting from extraction inefficiency (difficulties in obtaining high quality DNA representative of the sampled community) and cloning inefficiency (sensitive to contaminants such as humic compounds) (Sjöling *et al.*, 2006). Discoveries made using functional metagenomics include various groups of novel enzymes, for example agarase (Voget *et al.*, 2003), amidase (Gabor *et al.*, 2004b), amylase (Rondon *et al.*, 2000), antibiotic resistance enzyme (Riesenfeld *et al.*, 2004b), chitinase (Cottrell *et al.*, 1999), cellulase (Healy *et al.*, 1995), DNAse (Rondon *et al.*, 2000), esterase/lipase (Henne *et al.*, 2000; Rondon *et al.*, 2000; Rhee *et al.*, 2005; Lee *et al.*, 2006b; Hårdeman and Sjöling, 2007), 4-hydroxybuturate dehydrogenase (Henne *et al.*, 1999), alcohol oxidoreductase (Knietsch *et al.*, 2003), oxygenase (van Hellemond *et al.*, 2007), degradative genes (Suenaga *et al.*,

2007), protease (Gupta *et al.*, 2002) and xylanase (Lee *et al.*, 2006a). Antibiotics and antimicrobial compounds have also been identified using functional screening (Wang *et al.*, 2000; Brady *et al.*, 2001; MacNeil *et al.*, 2001; Gillespie *et al.*, 2002; Courtois *et al.*, 2003).

Sequenced based metagenomics

Sequenced based screening identifies the gene, genomic fragment or complete genome of interest through direct sequencing or sequence homology, for example by hybridisation (Stein *et al.*, 1996) or PCR amplification (Vergin *et al.*, 1998). Sequencing may either be random (Rondon *et al.*, 2000) or targeted (Courtois, 2003 #165). Target genes, or pathways, may contain genetic information which is ecologically or biotechnologically interesting. For example, the identification of a phylogenetic marker gene (phylogenetic anchor) within a genomic fragment enables the linking of the sequence information, which could be a biologically interesting function, to a particular phyla (Stein *et al.*, 1996; Béjà *et al.*, 2000a; Rondon *et al.*, 2000; Quaiser *et al.*, 2002; Liles *et al.*, 2003). Other target genes may encode for biologically active molecules, such as the polyketide synthase cluster (PKS) (Courtois *et al.*, 2003). The more recent sequence-based metagenomic analyses bypass the cloning step and instead rely on direct sequencing of community DNA by whole community sequencing, sometimes following a whole genome amplification step (Abulencia *et al.*, 2006).

Community sequencing

Community sequencing is random sequencing on a grand scale, which calls for enormous sequencing efforts, with the aim to access the entire genome complement of a given environmental sample (Venter *et al.*, 2004; Eisen, 2007; Kowalchuk *et al.*, 2007). The first large scale sequencing projects of environmental DNA were conducted using shotgun sequencing of the bacterio-plankton community of the Sargasso Sea (Venter *et al.*, 2004) and an acid mine drainage (Tyson *et al.*, 2004). More recently, metagenomic sequence information of the Earth´s oceans, from stations along a transect reaching around the world, the so called global ocean sampling (GOS) project, has been added to

the Sargasso Sea data (Rusch *et al.*, 2007; Yooseph *et al.*, 2007; Williamson *et al.*, 2008). The Sargasso Sea data included 148 new bacterial phylotypes, putatively 1800 genomic species and 1200 million unknown genes (Venter *et al.*, 2004). The GOS dataset included 7,7 milj new genes and 1 700 unique unknown protein families (Yooseph *et al.*, 2007). However, even if the sequencing effort was immense, very few genomes of the highly complex communities have been reconstructed (Venter *et al.*, 2004). The metagenome data of the much less complex microbial community, the acid mine drainage, consisting of very few species, was however possible to assemble into five genomes (Tyson *et al.*, 2004). The difficulties in assembly and annotation of large sequencing data sets have lead to the development of alternative ways of data analyses, focusing on what protein functions are over or under represented in a particular environment, enabling the comparison of community genomes of different environments (Tringe *et al.*, 2005). The obvious drawbacks of community sequencing are connected with the assembly and annotation of large sets of sequence data (Kowalchuk *et al.*, 2007). With the development of new bioinformatic tools and services, for example CAMERA (Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis) (Seshadri *et al.*, 2007), for the assembly, annotation, management and archiving of metagenomes (Markowitz *et al.*, 2006) and novel, efficient, low cost sequencing technologies, large scale metagenomic sequencing has become even more powerful, enabling the sequencing of whole microbial communities (Angly *et al.*, 2006; DeLong *et al.*, 2006; Edwards *et al.*, 2006; Woyke *et al.*, 2006; Rusch *et al.*, 2007; Yooseph *et al.*, 2007; Williamson *et al.*, 2008). The novel sequencing technologies include the pyrosequencing based 454 (454 Life Sciences, Roche) (Ronaghi *et al.*, 1998; Margulies *et al.*, 2005), Solexa (Solexa Ltd, Cambridge, UK) and SOLiD (Applied Biosystems, Foster City, CA, USA). Particularly, 454 sequencing, which currently generates 100 Mb per run read lengths up to 350 bp in a few hours (Genome Sequencer FLX), has been used in studies to generate environmental DNA reads (Angly *et al.*, 2006; Edwards *et al.*, 2006), (http://www.roche.com). The constant improvement of this technology, for example regarding error rate and read length will strengthen some of its observed drawbacks (Goldberg *et al.*, 2006). There is no doubt that mass sequencing will continue to be a useful tool for the microbial ecologist.

Marine metagenomics

Only over these past few years, metagenomics has played a very important role in marine sciences. The majority of the large scale sequencing efforts have been aimed at investigating bacterial communities of the marine water column (Béjà *et al.*, 2000b; Béjà *et al.*, 2000a; Beja *et al.*, 2002; Venter *et al.*, 2004; Angly *et al.*, 2006; DeLong *et al.*, 2006; Edwards *et al.*, 2006; Hallam *et al.*, 2006; Sogin *et al.*, 2006; Woyke *et al.*, 2006; Rusch *et al.*, 2007; Yooseph *et al.*, 2007). A few projects have been directed to the sediments (Hallam *et al.*, 2004; Abulencia *et al.*, 2006)(Paper I) and viral communities in the sediment (Breitbart *et al.*, 2004). One milestone was reached when the gene for bacterial rhodopsin, proteorhodopsin, was identified by random sequencing of a metagenomic library (Béjà *et al.*, 2000b). It could then be established that a new light harvesting function was present in a widely distributed marine environmental bacteria (Béjà *et al.*, 2000b) and over 700 proteorhodopsins, distributed globally, were identified (Venter *et al.*, 2004). Recent studies further support the widespread distribution of proteorhodopsin and discuss its potential importance for marine carbon transformation (Sabehi *et al.*, 2007). In addition to the information on bacterio-plankton from the GOS project, metagenomics driven discovery has increased the knowledge about marine archaea. In the Sargasso Sea shot-gun sequencing dataset, an archaeal scaffold was found with an ammonium monooxygenase (*amo*) gene, which was unexpected since oceanic nitrification had only been identified within the bacterial domain (Venter *et al.*, 2004). Regarding functional metagenomics, marine environments most certainly have more to offer, considering that sediments are typically unexplored low temperature environments (Morita, 1975), which harbour low temperature adapted bacteria with low temperature active enzymes (Russell and Hamamoto, 1998; Feller, 2003; D'Amico *et al.*, 2006a; Siddiqui and Cavicchioli, 2006).

Organisms at low-temperature

Low temperature environments harbour low temperature active bacteria with low temperature active enzymes (Russell and Hamamoto, 1998; Feller, 2003; D'Amico *et al.*, 2006a; Siddiqui and Cavicchioli, 2006). Typically organisms that live permanently at temperatures close to 0 °C are termed psychrophiles if they are unable to live above 20

°C, and psychrotolerant if they are able to live above 20 °C (Morita, 1975) in comparison to mesophilic organisms that usually have optimum growth temperature between 30-40 °C and above 40 °C respectively (Stetter, 1998). Inconsistent use of the terms psychrophilic, psychrotolerant and cold adapted (Finster, 2008) has lead to the suggestion of using the term "low temperature active" (the International Conference on Alpine and Polar Microbiology, 2006, Innsbruck, Austria) and hence I use that term in this thesis for both organisms and enzymes that are active at low temperature environments (below 20 °C).

The major problem with life at low temperature is the effect of temperature on biochemical reactions, which can be deduced from the Arrhenius equation: $k=Ae^{-Ea/RT}$ where A is the pre-exponential factor (related to steric factors and molecular collision frequency), Ea is the activation energy, R is the gas constant (8,314 J K^{-1} mol^{-1}) and T is the absolute temperature in Kelvin. According to the Arrhenius equation, a decrease in temperature will induce an exponential reduction in the reaction rate of any, including enzymatic, reaction (Lonhienne *et al.*, 2000). Typically, biological reactions of mesophilic organisms show approximately a 16- to 80-fold reduction in activity when the temperature is reduced from 37 °C to 0 °C (Collins *et al.*, 2008). Replicating bacteria have been identified at -20 °C and there are indications microbial of activity at even lower temperatures (Junge *et al.*, 2006). Bacteria that proliferate at subzero degrees but which are unable to live above 20 °C are often detected (Margesin and Schinner, 1994; Feller and Gerday, 2003; Somero, 2004; Siddiqui and Cavicchioli, 2006).

Organisms living at low temperatures are in thermal equilibrium with their environment and all cellular functions have to be adapted to circumvent the lack of available energy (Collins *et al.*, 2008). Adaptations and response mechanisms include cold-shock proteins (Wemekamp-Kamphuis *et al.*, 2002), lipid modification (Russell, 2008), increased enzyme production (Crawford and Powers, 1992) and expression of specific iso-enzymes adapted to different temperatures (Lin and Somero, 1995). An important aspect of low temperature adaptation is found at the protein level, enabling enzymes to be active at low temperature, which has been described in several excellent review articles (Smalås *et al.*, 2000; Feller, 2003; Hoyoux *et al.*, 2004; Siddiqui and Cavicchioli, 2006; Collins *et al.*, 2008). Each family of proteins has its own set of adaptations (Gianese *et al.*, 2002) and

several structural factors behind low temperature activity, such as increased protein flexibility, sometimes in a complex combination, have been suggested (Feller, 2003; Siddiqui and Cavicchioli, 2006; Collins *et al.*, 2008).

A low temperature active enzyme typically show: increased specific activity (k_{cat}) or catalytic efficiency (k_{cat}/K_m) at low to moderate temperatures and a shift in the apparent optimal temperature towards low temperatures, with a concomitant decrease in thermostability (Collins *et al.*, 2008). These adaptations are mediated by a range of structural changes of the protein, where the reduced protein stability is more of a side effect of increased flexibility (Siddiqui and Cavicchioli, 2006). To maintain a high reaction rate (k_{cat}) at low temperatures there is often a reduction of the activation enthalpy of the enzyme in which the disorder in the enzyme-substrate complex increases (Collins *et al.*, 2008). This may be generated by enhanced flexibility of the active site by lowering the number of enthalpy reactions that need to be broken during the formation of the enzyme-substrate transition state (Lonhienne *et al.*, 2000; Feller, 2003). The Michaelis-Menten constant (K_m), which is an indication of the substrate affinity of the enzyme, where a lower value indicates a higher affinity, tends to be lowest at, and hence best adapted to, the *in situ* temperature of the organism (Lonhienne *et al.*, 2000). The reasons behind these changes can be found at the level of enzyme structure and amino acid composition. The increased flexibility around the active site, often causes a larger and more accessible active site, can be achieved by the replacement of bulky side chain amino acids for those with smaller side chains (Russell *et al.*, 1998). Other low temperature adaptations that have been suggested are destabilization of the protein interior, mediated by a reduced core hydrophobicity, where interactions between hydrophobic groups based in weak Van der Waals forces would otherwise be stabilizing (Smalås *et al.*, 2000). A range of other changes has also been shown to be important such as, a higher proportion of hydrophobic residues at the surface of the protein, increased surface charge by charged amino acids, an increased number of proline residues in alpha-helices and the stacking of glycine residues making loops more flexible (Richardson and Richardson, 1988; Schrøder Leiros *et al.*, 1999; Fields, 2001; Feller, 2003; Saunders *et al.*, 2003; D'Amico *et al.*, 2006b; Siddiqui and Cavicchioli, 2006). Moreover, disruption of intramolecular, non-covalent, electrostatic interactions, that otherwise help to maintain

secondary and tertiary structure are mediated by a number of factors, for example, less hydrogen bonds (Alvarez *et al.*, 1998), less arginine-mediated interactions (Saunders *et al.*, 2003) and less aromatic interactions (Feller, 2003).

Enzyme biotechnology with low temperature enzymes

The marine environments, as other cold environments (polar, alpine and tundra regions), that are more or less permanently below 5 °C, constitute more than three-quarters of the Earth's surface (Hoyoux *et al.*, 2004). These environments are predicted to be a rich source for the identification of commercially interesting lipids, small molecules, proteins and particularly low temperature active enzymes (Podar and Reysenbach, 2006). Examples of enzymes with commercially applications that are also relevant for marine microorganisms are listed in table 1.

Enzymes are used in a variety of industrial applications and the global market sales were estimated to $2,3 billion in 2005 within the major sectors: detergents, food applications, agriculture/feed, textile processing, fine and bulk chemicals, paper/pulp, and pharmaceutical applications (Lorenz and Eck, 2005) and references therein.

Several review articles address the application of low temperature active enzymes as commercial products (Gerday *et al.*, 2000; Cavicchioli *et al.*, 2002; Hoyoux *et al.*, 2004; Antranikian *et al.*, 2005; Marx *et al.*, 2007). Except for a high catatlytic activity at low temperatures, some enzymes, for example esterases and lipases, are often stereo specific, which may be utilized in specific industrial processes (Cavicchioli *et al.*, 2002). The major economic benefit of these enzymes is in the form of energy saving by reduced reaction temperature or fewer heating steps.

In addition, using biocatalysists that function at lower temperatures, undesirable side reactions that occur at high temperatures may be avoided (Russell and Hamamoto, 1998; Gerday *et al.*, 2000; Cavicchioli *et al.*, 2002). Even the thermo instability, typical for a low temperature enzyme, can be an advantage in heat inactivation, which is important in food industry processes (Gerday *et al.*, 2000) and molecular biology (Kobori *et al.*, 1984).

Table 1

Enzymes from the marine environment that can be/ or have been suggested for potential low temperature biotechnological applications.

Enzyme with Marine relevance	**Application Low temperature**	**Reference**
Lipase and Esterase	Organic synthesis, food production (Antranikian *et al.*, 2005)	(Martinez *et al.*, 1996)
Protease	Food processing (Gerday *et al.*, 2000)	(Martinez *et al.*, 1996) (Cottrell *et al.*, 2005)
Glucosidase	Organic synthesis (Otto *et al.*, 1998)*	(Arrieta and Herndl, 2001)
Phosphatase	Heat inactivation (Kobori *et al.*, 1984) Enzyme immunoassay (Rossolini *et al.*, 1998)*	(Martinez *et al.*, 1996)
Amylase	Baking industry (Cavicchioli *et al.*, 2002)	(Martinez *et al.*, 1996)
Cellulase	Textile industry (Marx *et al.*, 2007)	(Cottrell *et al.*, 2005)
Xylanase	Food and feed processing (Marx *et al.*, 2007)	(Arrieta and Herndl, 2001)

* indicates not low temperature active application

There is also the possibility of genetically manipulating these enzymes, by protein engineering, to gain increased stability without loosing activity to better suit reaction conditions (Narinx *et al.*, 1997; Van den Burg *et al.*, 1998; Cavicchioli *et al.*, 2002). A few low-temperature active enzymes were isolated and characterised by functional metagenomics during the work of this thesis, nitrilases from the deep sea and polar regions (Robertson *et al.*, 2004), a cold active xylanase of waste water (Lee *et al.*, 2006a), an esterase from activated sludge (Roh and Villatte) and the h1Lip1 lipase (Paper I).

Lipases and esterases

A group of particularly biotechnologically relevant enzymes are the hydrolases (E.C. 3) acting on ester bonds (E.C. 3.1), for example the carboxylic ester hydrolases (E.C. 3.1.1) which include the triacylglycerol lipases (E.C. 3.1.1.3), referred to as lipases, and the carboxylesterases (E.C. 3.1.1.1), referred to as esterases. Esterases preferentially hydrolyse water soluble esters and triacylglycerols with fatty acids shorter than C_6, whereas lipases often hydrolyze water-insoluble substrates, typically triacylglycerols with medium to long-chain fatty acids (≥10 carbons atoms) (Jaeger *et al.*, 1999; Pandey *et al.*, 1999; Jaeger and Eggert, 2002). The ability of a ester hydrolase to hydrolyse triacylglycerols with fatty acids ≥10 carbons atoms are however the definition of a lipase (Jaeger *et al.*, 1999). Features such as enantio-/stereoselectivity (Reetz, 2001), a broad substrate specificity and activity in organic solvents (Gupta *et al.*, 2004) make lipases useful in synthetic organic chemistry and other industrial processes, such as the production of pharmaceuticals (Reetz, 2001). Lipases are also used in paper processing, food manufacturing, as food additatives (Jaeger *et al.*, 1999) and in the production of biofuel, catalysing the conversion of vegetable oil to methylalcohol ester (Jaeger and Eggert, 2002).

Both lipases and esterases share the highly conserved α/β-hydrolase protein fold (Ollis *et al.*, 1992). The majority of these enzymes share the conserved amino acid regions, which include a HG dipeptide within the oxyanion hole, the active site consisting of the motif GluXSerXGlu, where the nucleophilic serine residue acts within a catalytic triad together with Glu/Asp and His (Jaeger *et al.*, 1999). Another characteristic feature of lipases is the "lid" structure covering the active site, important in so called interfacial activation of hydrolytic activity upon contact with a lipid-water interface (Jaeger *et al.*, 1999). Lipases and esterases are commonly classified in subgroups by sequence homology (Arpigny and Jaeger, 1999) but other classification systems exist (the lipase engineering database) (Pleiss *et al.*, 2000). One group of lipases and esterases are the Hormone Sensitive Lipase (HSL) family (Hemilä *et al.*, 1994), classified as group IV (Arpigny and Jaeger, 1999). Interestingly, this group has been shown to include both low- and high-temperature active enzymes as well as mesophilic homologues (Arpigny and Jaeger, 1999). In this group of

enzymes, the N-terminal region that contains the lid has been suggested to be important for thermo-stability, but also for catalytic activity and substrate specificity (Mandrich *et al.*, 2004; Mandrich *et al.*, 2005; Foglia *et al.*, 2007).

Studying the active prokaryotic communities

In this work, the diversity of metabolically active bacterial communities of the Baltic Sea sediment was also investigated. The diversity of bacterial and archaeal communities is typically studied by analysing the diversity of a phylogenetic "marker gene"(Woese *et al.*, 1975; Fox *et al.*, 1980), such as the 16S rRNA gene (Head *et al.*, 1998) but also hsp70 (Yap *et al.*, 1996), recA and EF2 have been used (Venter *et al.*, 2004). The marker gene is analysed using molecular techniques such as clone library and sequence analysis (Head *et al.*, 1998), Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu *et al.*, 1997), Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer *et al.*, 1993) and Temperature Gradient Gel Electrophoresis (TGGE) (Rosenbaum and Riesner, 1987).

Generally diversity studies are based on total environmental DNA extracts. Considering that 85% of the total bacterial community of some environmental samples are dormant or dead cells (Luna *et al.*, 2002a; Dell'Anno and Corinaldesi, 2004) and that extracellular DNA has been shown to be resistant to degradation and persist for long times in the environment (Romanowski *et al.*, 1993; England *et al.*, 2004) any obtained results would not show the diversity of the metabolically active and functionally important members. It is hence obvious that attempts to link results of the analysis of the bacterial community to the functional processes in the sampled environment would benefit from studies of the active fraction of the community. An active cell has been defined as; growing and having a metabolic activity, having an intact membrane with a membrane potential (Jansson and Prosser, 1997; Nebe-von-Caron *et al.*, 2000) or being able to replicate and repair DNA (Barer and Harwood, 1999). Some of the physiological states of a cell have been termed Viable But "nonculturable" (VBNC) and dormant. Dormant is defined as a reversible state of metabolic shutdown (Kaprelyants *et al.*, 1993) and has been suggested to be a strategic protection mechanism in response to harsh conditions (Kaprelyants *et al.*, 1993; Barer and Harwood, 1999). VBNC (Colwell *et al.*, 1985) relates to the viability of a cell.

Different techniques have been developed to study the metabolically active bacteria, for example Bromodeoxyuridine BrdU immunocapture (Urbach *et al.*, 1999) and RNA analysis (Schaechter *et al.*, 1958; Weller and Ward, 1989; Weller *et al.*, 1991). These techniques were used to examine the active bacterial community of the Baltic Sea sediments (Paper III).

The Baltic Sea sediments

The Baltic Sea is the second largest brackish sea on Earth, with a high input of freshwater from the surrounding landmasses and a narrow connection to the North Sea. This creates a saline gradient from the Southern Baltic Sea to the Northern Bothnian Bay (Rönnberg and Bonsdorff, 2004). Over 85 million people live within the drainage area (Rönnberg and Bonsdorff, 2004) and consequently, the Baltic Sea is subjected to eutrophication, high levels of nutrient input from antrophogenic sources, resulting in (toxic) cyanobacterial blooms (Elmgren, 1989; Wulff *et al.*, 1990; Rönnberg and Bonsdorff, 2004). The bacterial diversity and community structure are integral components of the structure of marine soft bottom sediments important for the functioning of the marine ecosystem, and hence research addressing the identification of the organisms involved in these processes is important.

Marine sediments are an important scene for biogeochemical cycling of which the microbial communities are major actors with enormous catalytic potential and ability to couple multiple redox reactions of organic or inorganic compounds (Jorgensen and Boetius, 2007). As described in (Fenchel and Finlay, 1995) biological respiration is a redox reaction with an electron donor and an electron acceptor. When several electron acceptors are available for the same substrate the most favourable (thermodynamically yields the most energy) occurs first. Oxygen is first utilized followed by nitrate and manganese, iron and sulfate (Froelich *et al.*, 1979; Sørensen *et al.*, 1979; Fenchel and Finlay, 1995). Oxygen diffuses from the water column to the sediments where it is rapidly consumed since the diffusion of oxygen is significantly slower in the sediments than in the water column (Gundersen and Jorgensen, 1990), with the effect that sediments are stratified in terms of available electron donors and respiration processes (Fenchel and

Finlay, 1995). Although it may vary, marine sediments are typically characterized by a thin oxic surface layer, an anoxic but oxidized zone, in which nitrate, manganese oxide and iron oxides are the main electron acceptors, and a sulfidic zone, in which sulfate reduction predominates (Fenchel and Finlay, 1995). In Baltic Sea sediments, with the high input of organic matter, the oxygen has been shown to be depleted within 2-4 mm of sediment (Conley *et al.*, 1997). Another important aspect is that small sediment particles (1-2) mm may maintain anoxic centers, harboring anoxic metabolizing organisms, even though the particles are located in an overall oxidized zone (Fenchel and Finlay, 1995). Notably, measurements of redox potential (by using a platinum electrode), as used in Paper III, must be interpreted with caution due to the lack of internal redox equilibrium in natural environments (Frevert, 1984).

The biogeochemical processes of sediments have been extensively studied, see for example (Sørensen *et al.*, 1979; Jorgensen, 1982; Canfield *et al.*, 1993; Thamdrup *et al.*, 1994) . However, few studies have investigated the bacterial communities with molecular techniques with potential correlation to biogeochemical processes (Urakawa *et al.*, 1999). Surprisingly, one study (Braker *et al.*, 2001) showed that there was no difference in bacterial community composition over a vertical profile, when analysing total community DNA. Nevertheless, studies of the actively metabolizing bacteria have detected differences (Mills *et al.*, 2004; Martinez *et al.*, 2006). These studies, further demonstrate the importance of analysing the active fraction of the bacterial community.

For the studies in this thesis, sediment samples were collected outside the Askö marine research station, situated on the Swedish coast of the Western part of the Baltic Proper. The Askö marine station has served as a base for marine research and nutrient monitoring since the 1960's. The area sampled during this study, nearby the station, was closely located to a reference station (B1) (Engqvist, 1996) in a large long term environmental monitoring program, Himmerfjärden eutrophication study (http://www2.ecology.su.se/dbHFJ/index.htm).

The present study

Objectives

The aim of this thesis was to clone the genomic pool of the prokaryotic community and to use an expression strategy for accessing novel bacterial genes and gene products. The aim was also to investigate the bacterial diversity and community structure of Baltic Sea sediments. A schematic presentation of the polyphasic approach that was used is shown in figure 2.

The particular objectives were to:

- Construct a metagenomic fosmid library for expression screening and identification of novel low temperature active enzymes, particularly esterases and lipases (Paper I).
- Investigate the potential protein structural factors behind low temperature protein adaptation of an isolated lipase (Paper II).
- Determine the distribution and composition of the actively metabolising bacterial communities in the sediments along a vertical redox gradient (Paper III).

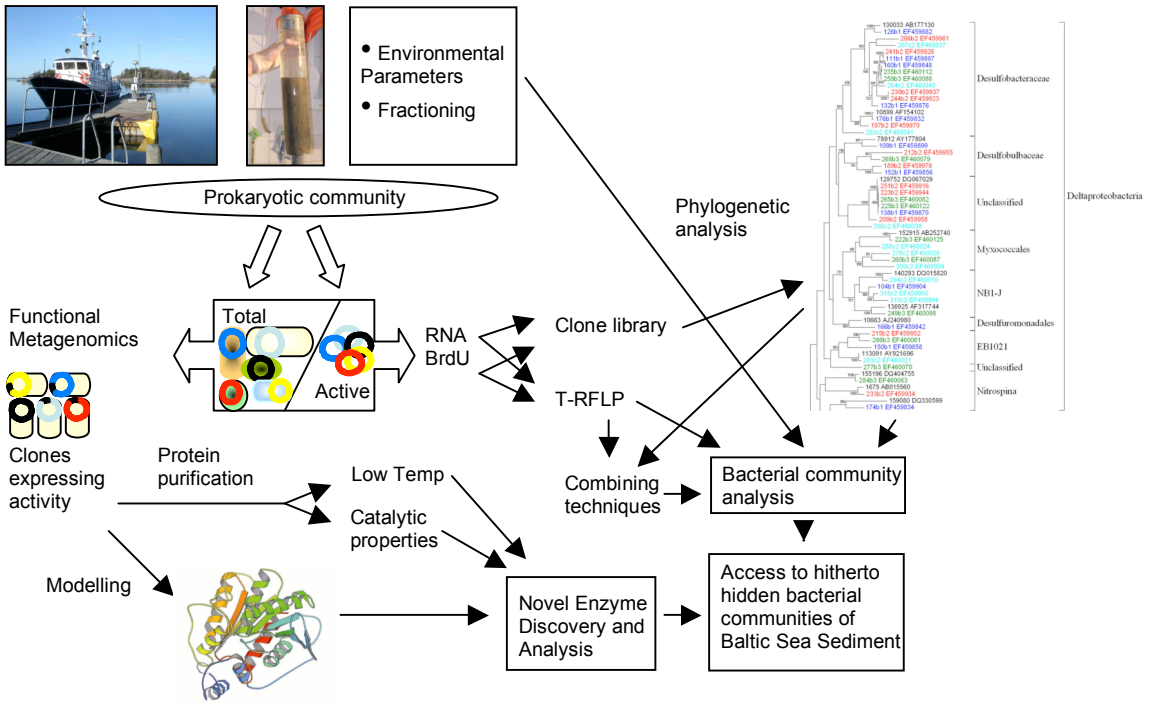


Figure 2.
Assessing and accessing the diversity and functions of the bacterial community of the Baltic Sea sediments in the work of this thesis, using a polyphasic approach.

Methods

Metagenomic cloning

Different strategies have been employed to construct metagenomic libraries depending on the purpose of the study. Typically, small DNA insert (less than 15 kb) libraries are constructed using plasmid vectors, whereas large DNA insert (over 30 kb) libraries are constructed using fosmid, cosmid or BAC (Bacterial Artificial Chromosome) vectors (Daniel, 2005). Large insert libraries are more technically demanding to produce since the quality of High Molecular Weight (HMW) DNA has to be high to prevent low cloning efficiency (Daniel, 2005). The advantages lie in the reduction of the number of clones necessary to cover a certain metagenome size (in Mb) and the possibility of accessing complete pathways, operons and clusters of genes (Daniel, 2005). It is also more likely to find a functional gene on the same DNA fragment as a phylogenetic marker gene, making it possible to link function to the identity of the organism from

which the genomic fragment originates from. Advantages with small insert libraries are that the plasmids are less sensitive to DNA contaminants, which may inhibit the cloning procedure, expression can be induced by the vector and not rely on host initialization of transcription and regulation (Gabor *et al.*, 2004a; Daniel, 2005). The disadvantages are that small inserts reduce the probability of encountering large and complex genes, and that it will be necessary to screen a large number of clones in order to cover a metagenome (Daniel, 2005).

In order to clone large fragments of the genomic pool of the prokaryotic community, I applied a HMW DNA extraction and cloning approach in this study. The fosmid vector pCC1FOS (CopyControl Fosmid Library Production Kit, Epicentre technologies) and the BAC vectors pBeloBAC11, pIndigoBac536 (Shizuya *et al.*, 1992) and superBAC1 (Handelsman *et al.*, 2002) were used. One of the advantages with the pCC1FOS and superBAC1 vectors is the possibility to induce to high copy numbers (Wild *et al.*, 2002). The pCC1FOS vector cloning is mediated by the lambda phage transfection methodology that generally results in a higher cloning efficiency compared with BAC, compensating for the limitation of the fosmid only accepting DNA fragments smaller than 40 Mb. In this work, the fosmid vector was found to be the most suitable vector in terms of cloning efficiency and coverage and was therefore used to construct a HMW DNA library of the prokaryotic community of Baltic Sea sediment.

HMW DNA extraction

Methods for extraction of environmental DNA are numerous and several have been developed particularly for metagenomic DNA extraction (Van Elsas and Smalla, 1995; Hurt *et al.*, 2001; Gabor *et al.*, 2003). There are two major approaches for DNA extraction, either direct extraction, which includes lysis of cells in the sample resulting in small DNA fragments of Low Molecular Weight (Ogram *et al.*, 1987), or indirect extraction, which includes dispersed cells that are isolated prior to lysis (Holben *et al.*, 1988; Bakken and Lindahl, 1995). Indirect extraction methods have been shown to be 10-100 times less efficient than direct extraction methods but the purity and quality, particularly the size of the DNA fragments are higher (Gabor *et al.*, 2003). In addition,

less eukaryotic DNA, which is incompatible with bacterial hosts for expression screening, is co-extracted (Gabor *et al.*, 2003). Thus, dispersed cells are an advantage when HMW DNA is required for creating large insert DNA libraries (Courtois *et al.*, 2001). Investigations of how large the portion of eukaryotic DNA is in direct extracted environmental DNA both show it to be abundant (Courtois *et al.*, 2001; Gabor *et al.*, 2003; Treusch *et al.*, 2004) and not significant (Courtois *et al.*, 2001), probably depending on what environment the sample originated from.

An indirect extraction methodology was applied in this work (Paper I) in order to extract HMW DNA of the sediment and to construct the metagenome library (Paper I). Indirect extraction techniques are often based in dispersing and separating the cells from the sediment or soil particles by blending in the presence of detergents, for example sodium dodecylsulfate (SDS) or hexadecyl trimethylammonium bromide (CTAB) (Bakken and Lindahl, 1995). Another common additative, polyvinylpyrrolidone pyrophosphate (PVPP) helps to remove humic acids (Daniel, 2005) that often co-extract with DNA (Steffan *et al.*, 1988; Tsai and Olson, 1992; Tebbe and Vahjen, 1993). Different centrifugation steps, sometime by density gradient centrifugation over a cushion of nycodenz or percoll can be used to isolate the prokaryotic cells (Bakken and Lindahl, 1995). Pulse Field Gel Electrophoresis (PFGE) is often used to purify and size separate the HMW DNA after lysis and protocols exist for including PVPP in the gel, further purifying the DNA (Quaiser *et al.*, 2002). In this work, both nycodenz density gradient centrifugation and a method to extract the prokaryotic community using low speed centrifugation (Bakken and Lindahl, 1995) were applied, however, the low-speed centrifugation method was found to generate a higher yield of cells/DNA and was therefore used to construct the sediment metagenomic library (Paper I).

Expression screening

In order to access novel lipases and esterases of the bacteria from the Baltic Sea sediment, the metagenome library was screened for fosmids expressing lipolytic activity. Several other studies have also successfully expression screened for lipolytic activities in either low- (Henne *et al.*, 1999; Henne *et al.*, 2000; Entcheva *et al.*, 2001) or high-

molecular weight DNA libraries (Lee *et al.*, 2004; Elend *et al.*, 2006; Lee *et al.*, 2006b; Elend *et al.*, 2007), possibly as a result of the potentially broad industrial use of lipases and esterases (Jaeger *et al.*, 1999; Gupta *et al.*, 2002; Jaeger and Eggert, 2002). A metagenomic library can be expression screened for lipolytic activity either by using agar plate assays or by using chromogenic substrates (Wilkinson, 2000). In plate screening for lipolytic activity, the degradation of the substrate glyceryl tributyrate, emulsified with gumarabic (Kok *et al.*, 1993), or olive-oil and rhodamine visualised by ultra violet light (Kouker and Jaeger, 1987) is investigated. Since glyceryl tributyrate is a triglyceride with three fatty acid acyl chains of four carbon atom length connected with an esterbond to the glycerol backbone, it can be hydrolysed by both esterases and lipases, whereas the olive-oil rhodamine is a strict lipase screening assay (Jaeger *et al.*, 1999). In this work, fosmids were screened for the expression of lipolytic activity at low temperatures using glyceryl tributyrate in order to isolate low temperature active lipases and esterases. The screening was successful and a very high frequency of hits was recorded (on average one positive fosmid out of a hundred screened) compared with other enzymatic assays in previous studies (Lorenz and Eck, 2005; Sjöling *et al.*, 2006). The high detection frequency of lipolytic enzymes in the Baltic Sea sediment metagenomic library could be explained by: an average fosmid insert was 30 kb, with 1 % active fosmids out of a total of 7000 (Paper I) this would correspond to one lipolytic gene per 3 Mbp environmental DNA, which is almost the size of a bacterial genome. The dominating group of the active community in the Baltic Sea sediments was *gamma-proteobacteria* (Paper III), and since the host of the metagenomic library was *E. coli*, a *gamma-proteobacteria,* the possibly expression of the heterologous DNA originating from *gamma-proteobacteria* would increase.

Analyses of positive fosmids

With the goal of identifying the complete sequence of the gene, or genes, responsible for the lipolytic activity, further analysis of the active fosmids was necessary. Subcloning and transposon mediated knock-out mutagenesis are two suitable techniques and both approaches have been applied in this work (Paper I and unpublished). Trough subcloning, a small insert sub-clone library of the fosmid DNA fragment (40 kb) containing the expressed gene was constructed. Those sub-clones expressing lipolytic activity upon re-

screening were sequenced and sequences were assembled into a contig. The region containing the gene responsible as well as the open reading frame could be identified. Paper I further describes overexpression and purification of h1Lip1 by a fusion protein construct with a GST-tag, where the tag was cleaved off by a precission protease in the final purification step (Kaelin *et al.*, 1992).

Enzyme characterisation

The goal of characterising the identified enzyme, h1Lip1, was to; establish if it was a low temperature active enzyme; investigate the substrate specificity towards fatty acid monoester compounds; verify if the enzyme was a lipase or an esterase by using a discriminatory enzyme substrate. Kinetic investigations of the enzyme activity and stability are routine methods in order to establish whether an enzyme is low temperature active or not (Choo *et al.*, 1998; Rashid *et al.*, 2001; Alquati *et al.*, 2002; Kulakova *et al.*, 2004). Hydrolysis of the triglyceride derivative 1,2-di-O-lauryl-*rac*-glycero-3-glutaric acid 6'-methylresorufin ester (DGGR) can be used to distinguish between lipases and esterases (Jaeger *et al.*, 1999). In order to further classify h1Lip1, amino acid sequence comparisons were performed and h1Lip1 could be characterised as a group IV, a Hormone Sensitive Lipase (HSL) (Paper I). The secondary structure of h1Lip1 is shown in figure 3.

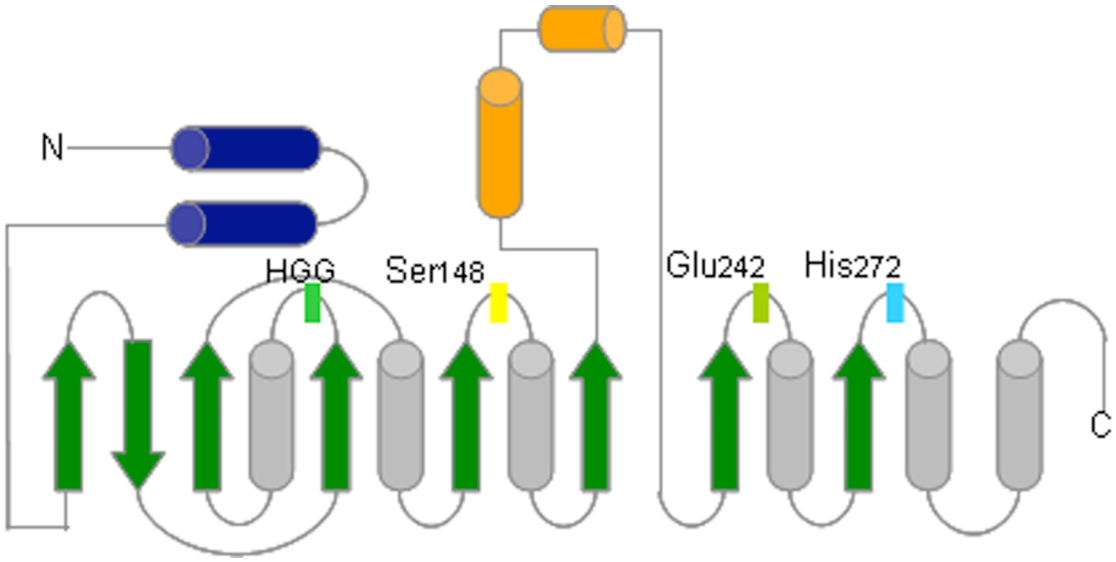


Figure 3.
Secondary structure prediction of the low temperature active lipase h1Lip1. beta-strands (green), helices (grey). The central (organge) and N-terminal (blue) helices form the 'lid' regions. The location of the oxyanion hole (HGG) and the members of the catalytic triad are indicated.

3D protein structure prediction and mutational analysis

With the aim to investigate the putative protein structure of the lipase h1Lip1, a theoretical three dimensional protein structure was predicted in Paper II. There are two ways of predicting the structure of a protein, *De Novo* prediction and comparative modelling (Wallner, 2005). In *De Novo* prediction the structure of a protein is predicted from the sequence alone based on the laws of physics (Wallner, 2005). In comparative modelling, the protein structure is predicted based on information from known protein structures from x-ray crystallography or nucleic magnetic resonance studies available in the Protein Data Bank (PDB) at http://www.rcsb.org. In this work, meta prediction at the Metaserver was used (http://meta.bioinfo.pl), (Ginalski *et al.*, 2003). Meta prediction uses the information from several different comparative modelling methods in order to predict the structure of a protein based on the assumption that if several predictors produce similar models it is a strong indication that the model is correct (Wallner, 2005). Based on results from the Meta server, and by using a suitable template structure (PDB structure

Id 1QZ3), the protein structure model of h1Lip1 was further built by using the program Pmodeller at http://www.sbc.su.se/~bjornw/ProQ/modeller.cgi (Wallner and Elofsson, 2003).

Once a putative protein structure has been predicted it is possible to compare it with other protein structures by superimposition. In order to investigate any potential low temperature adaptations of the putative h1Lip1 protein structure it was superimposed onto the template 1QZ3 revealing at least eight sites where the two proteins differed in three dimensional structure, of which one was located at the N-terminal, in the lid structure (Paper II). In order to further investigate any potential effect of the identified putative structural difference on enzyme activity or stability, site-directed mutagenesis was used to construct a mutant, h1Lip1-site1lid.

Bacterial community analyses

In this work, a combination of different molecular methods, including molecular finger printing techniques and clone library analyses, was used in order to investigate the bacterial community structure of the sediment. Generally, bacterial community analyses are based on total environmental DNA which includes all bacteria, alive, dead and dormant. However, the analysis of the active bacteria in a given environmental sample makes it potentially possible to determine who is responsible for the ongoing microbial processes in the sampled environment. Therefore, the fundamental questions like "who is active where?, what are they doing?" are probably best addressed by studying the active organisms of a community and in this work the following two approaches were applied:

Reverse transcriptase (rt) PCR

Analysis of reverse transcribed rRNA has been used in several studies for studying the active populations of bacterial communities (Weller *et al.*, 1991; Teske *et al.*, 1996; Nogales *et al.*, 1999; Mills *et al.*, 2005; Moeseneder *et al.*, 2005; Martinez *et al.*, 2006) since the RNA content of a bacterial cell reflects the expressed genes and hence can be related to cell growth (Schaechter *et al.*, 1958; Nomura *et al.*, 1984). In brief, the RNA is extracted directly and immediately after sampling and converted into cDNA by reverse

transcriptase and by using a universal bacterial primer, which anneals to the single stranded RNA. The cDNA is used as template in 16S rRNA gene PCR amplification and analysed for bacterial diversity and community structure. In Paper III, reverse transcribed 16S RNA from sediment samples was used in both clone library analysis and T-RFLP analysis. The advantage of using the rt-PCR approach when analysing active communities is the immediate extraction of RNA after sampling, without any incubation time *ex situ* where the communities may change during the incubation. The major limitation is the instability of the RNA, which therefore requires quick and strict handling.

Bromodeoxyuridine (BrdU) immunocapture

The second approach which was used in this work to analyse the active populations was Bromodeoxyuridine (BrdU) immunocapture. BrdU is a structural analogue of thymidine that cells may incorporate into the DNA during replication. This method has been used previously to detect actively replicating cells in a specific environment (Borneman, 1999, Urbach, 1999 #408). The methodology is based on the incubation of an environmental sample with BrdU *ex situ* followed by direct extraction of the BrdU-labelled DNA by immunocapture. The BrdU-labelled DNA can then be analysed by molecular phylogenetic methods in order to determine the active populations of the community (Urbach *et al.*, 1999; Edlund and Jansson, 2006). The limitations with this approach include the uncertainty of whether there are bacterial populations where BrdU can not be incorporated. In Paper III, BrdU-labelled DNA from three sediment depths was analysed using both 16S rRNA gene clone library and T-RFLP analysis.

Clone library analysis

Sequencing and phylogenetic analysis of a 16S rRNA gene clone library is a common approach to study the bacterial diversity of a given environment (Head *et al.*, 1998). From direct extracted environmental DNA the 16S rRNA genes are amplified by PCR using 16S rRNA gene specific primers (in this case bacterial). Using a proof-reading (low error rate) DNA-polymerase reduces the possibility of introducing experimental artefacts in the resulting PCR product (Wintzingerode *et al.*, 1997). Optimally, cloning of the PCR product and transformation into *E. coli* produces a library covering all the 16S rRNA

genes of the sampled environment. Sequencing of the cloned 16S rRNA genes can be analysed phylogenetically using several different methods, algorithms and databases. (Head *et al.*, 1998). In this work, the online automated handling work bench Greengenes was used (http://greengenes.lbl.gov). Greengenes is a database that contains only chimera-free sequences (DeSantis *et al.*, 2006a) with the aim of covering the entire range of 16S rRNA genes. The sequence taxonomy suggested by Hugenholtz (Hugenholtz, 2002) is used as well as other taxonomies. Greengenes is organised as pre-aligned sequences according to a 7682 character format by the Nearest Alignment Space Termination (NAST) algorithm (DeSantis *et al.*, 2006b). Greengenes supplies online alignment and chimera detection, Bellerophon III (DeSantis *et al.*, 2006a). The Greengenes database was used for taxonomic identification of the 16S rRNA gene sequences of the clone libraries in Paper III. After removal of putative chimeric sequences, selection of nearest neighbours in the Greengenes database and alignment, the phylogenetic analysis was performed using Maximum likelihood analysis with the PHYML program (Guindon and Gascuel, 2003).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The T-RFLP technique provides a community fingerprint of the dominant populations of the sampled environment and is therefore a suitable tool when comparing samples from different environments or along environmental gradients (Liu *et al.*, 1997). The PCR product, in this case the 16S rRNA gene, is labelled with a fluorescent tag and digested using different restriction enzymes. The different terminal restriction fragments (T-RFs) can thus be detected by a scanning laser by sequencing electrophoresis, either polyacrylamide or capillary electrophoresis, resulting in an electropherogram. The same species (16S rRNA sequence) will optimally produce T-RFs of the same length and the peak area of each individual T-RF can be used to estimate the relative abundance of the corresponding population. Multivariate statistical methods (Kitts, 2001; Edlund and Jansson, 2006; Edlund *et al.*, 2006) are used for analysing and interpreting the data and a couple of bioinformatics tools have been developed to identify the acquired T-RFs, e.g. the TAP-database and APLAUS (Edlund *et al.*, 2006). Some of the limitations with T-RFLP are incomplete restriction digestion and that potentially related species may result in T-RFs of the same length. In addition, the coverage of sequences deposited in the

databases is far from complete and since the diversity of environmental bacteria, particularly in sediments, is immense, the putative identification of T-RFs using available databases can be limited.

In paper III, both 16S rRNA gene clone library and T-RFLP analyses were applied in order to investigate the diversity of bacteria of the Baltic Sea sediments. With the aim to specifically study the active populations, the replicating and transcribing bacteria, and compare those of three redox depths (179 mV, -64 mV and -337 mV) reverse transcription (rt) of RNA and BrdU- labelling and immunocapture were used in both clone library and T-RFLP analysis.

Key findings

Metagenomics as a tool to access novel low temperature active enzymes

Mining of novel enzymes from the Baltic Sea sediments

In order to access novel lipase and esterase genes from the uncultured bacteria of the Baltic Sea sediments a functional metagenomic approach was applied. In Paper I, we demonstrated that by constructing a metagenomic fosmid library of sediment DNA and through expression screening, fosmids expressing lipolytic activity could be detected and low temperature active enzymes were identified. Approximately 1% of the clones were identified as lipolytically active, which was a high hit rate compared to other studies (Sjöling *et al.*, 2006).

A novel low temperature active lipase

Subcloning one of the lipolytically active fosmids enabled the identification of an open reading frame consisting of 978 bp encoding a 35.4 kDa lipase, h1Lip1 (DQ118648), with 54% amino acid similarity to a *Pseudomomas putida* esterase (BAD07370) (Paper I). Sequence motifs conserved in lipases were identified in h1Lip1, including the putative active site, GDSAG, a catalytic triad (Ser148, Glu242 and His272) and a HGG motif. The protein h1Lip1 was overexpressed and purified in order to be able to characterize the catalytic properties of the enzyme, that proved to be unique compared with previously identified lipases due to the apparent optimal temperature of 35 °C, the specific activity below 15 °C, and the low thermal stability at temperatures above 25 °C, resulting in enzyme inactivation at 40 °C with t½ <5 min (Paper I). Hydrolysis of the triglyceride derivative 1,2-di-O-lauryl-*rac*-glycero-3-glutaric acid 6'-methylresorufin ester (DGGR) confirmed that h1Lip1 was not an esterase, but a lipase. Therefore, results from the studies in Paper I demonstrate that h1Lip1 represents the first low temperature active lipase isolated by expression screening of a metagenomic library. Low temperature active

lipases and esterases have however been identified previously by conventional means (Choo *et al.*, 1998; Rashid *et al.*, 2001; Alquati *et al.*, 2002; Kulakova *et al.*, 2004) (Paper I). During and after the publication of Paper I additional low temperature active lipases and esterases have been identified in soil and activated sludge by metagenomic expressions screening (Kim *et al.*, 2006; Elend *et al.*, 2007; Roh and Villatte, 2008).

Hormone Sensitive Lipase

Amino acid sequence comparison showed that h1Lip1 is related to the group IV family of esterases/lipases containing the Hormone Sensitive Lipase (HSL) family, according to the classification suggested by Arpigny and Jaeger, 1999 (Arpigny and Jaeger, 1999). As reasoned in Paper I, the conserved active site, GDSAG, located close to the N-terminal and the HGG(G) motif immediately upstream, are characteristic of the group IV lipases (Jaeger *et al.*, 1999). This group consists of both low temperature and high temperature active lipases (Jaeger *et al.*, 1999). The h1Lip1 lipase is one of only a few metagenomically isolated lipases and esterases of the HSL family, all of which have been isolated from extreme environments, such as Indonesian thermal environment (Rhee *et al.*, 2005), Deep Sea hypersaline anoxic basins (Ferrer *et al.*, 2005) and low temperature soil (Kim *et al.*, 2006; Elend *et al.*, 2007).

Three dimensional protein structure of h1Lip1

In order to determine the location of the active site and the catalytic triad of h1lip1 in the three dimensional protein structure and to confirm the presence of a lid, a theoretical three dimensional protein structure model was constructed by homology modelling (Paper II). The goal was also to investigate whether there were any differences in the h1Lip1 protein structure compared to other known lipase/esterase structures with the aim to understand low temperature activity. Not surprisingly, the metaserver analysis showed that the enzyme which had the highest structural homology to h1Lip1 also belonged to the HSL family, however, this enzyme (PDB structure Id 1QZ3), the EST2 of *Alicyclobacillus acidocaldarius,* was from a thermophilic organism (De Simone *et al.*, 2000). The detailed prediction of the three dimensional protein structure of h1Lip1, together with the superimposition onto the thermophilic esterase template 1QZ3, confirmed that h1Lip1 consists of 10 alpha helices and 8 beta sheets, resulting in an

overall alpha-, beta-hydrolase fold, and showed the three dimensional distribution of the active site and the catalytic pocket. The results in Paper II further confirmed the presence of a typical lipase 'lid' at the N-terminal as suggested in Paper I. The existence of a lid further supported that h1Lip was a lipase (Jaeger *et al.*, 1999). Furthermore, a number of sites in the alpha carbon backbone of h1Lip1 were identified that differed from the template in three dimensional structure, thus suggesting sites with a potential role in low temperature adaptation.

The importance of the N-terminal

One of the sites that differed from the template in three dimensional structure was found to be within the putative lid. Considering that the lid and the N-terminal previously have been suggested to be important for modulating the catalytic efficiency and that changes in the N-terminal have caused changes in substrate affinity (K_m) and reduced thermo-stability (Mandrich *et al.*, 2005; Foglia *et al.*, 2007), this particular site was further analysed by site directed mutagenesis in Paper II. The amino acid residue at the identified site (Aspartate 24) was replaced with the corresponding residues (Tyrosine 22, Lysine 23 and Histidine 24) of the thermophilic EST2 (1QZ3). The mutation caused a slight (12%) reduction in catalytic activity, and a major (74%) increase in substrate affinity (K_m) at 25 °C. Importantly, the thermo-stability was significantly reduced, as demonstrated by a complete inactivation of the h1Lip1 mutant after <5 min incubation at 40 °C compared with a $t_{½}$ of <10 min at 25 °C for the wild type h1Lip1 (Paper II, figure 5). Even though the mutation did not render a more thermo-stable enzyme, the result strengthens what has been suggested for other lipolytic enzymes, that the N-terminal of a lipase is important for substrate affinity (K_m) and thermo-stability.

Recently, the crystal structure of a metagenomically isolated thermophilic esterase (Byun *et al.*, 2007) and a preliminary structure model of another esterase (Est25) (Kim *et al.*, 2007) were presented. h1Lip1 is, however, to date to my knowledge the only metagenomically isolated low temperature active enzyme that has been investigated by three dimensional protein structure homology modelling.

Active bacteria and their vertical distribution in sediment

In Paper III, I together with my colleagues specifically studied the diversity of the metabolically active bacteria in the Baltic Sea sediment by analysing the community structure along a vertical redox potential gradient. Both clone library analysis and T-RFLP using 16S rRNA gene analysis were applied. In addition, both BrdU immunocapture and rt-PCR approaches were used to determine the active populations, where the two different technologies represent two different aspects of cell growth, transcription (rt-PCR) and replication (BrdU). Paper III represents the first study, to our knowledge, in which two different methods were combined to study the active bacteria. The results demonstrated that the bacterial communities differed significantly between the different redox depths showing a vertical stratification. Interestingly, the dominating populations were not the same as the active populations, which is consistent with previous findings (Edlund and Jansson, 2006). This could be explained by the existence of a large portion of inactive, or dormant bacteria, implying that the fraction of metabolically active bacteria in sediment is small (Luna *et al*., 2002b). Furthermore, the bacterial community structure was most strongly correlated to organic carbon, followed by nitrogen and redox potential whereas there was no significant correlation to total phosphorous (Paper III). This is in agreement in what has been found by others (Wilms *et al.*, 2006).

Results also showed that the Baltic Sea sediment harbours a novel and unstudied bacterial community since obtained sequences showed very low sequence identities (<93%) to known sequences in GenBank. Generally, the bacterial communities varied considerably at the order level between different redox depths, while the major phylogenetic groups were similar for all redox depths. In other studies, the difference between bacterial communities of different sediment depths has been found both at the phyla level (Martinez *et al.*, 2006, Mills, 2004 #913) and in minor ribotypes (Urakawa *et al.*, 1999) highlighting the importance of studying active communities. Several community members belonging to less frequently observed divisions, for example OP3 and WS3 were identified. Interestingly, many members with known important ecological functions were also identified, for example: iron- and nitrate- reducers at reduced depths, indicating

that these processes were actively occurring at the occasion of sampling. Several *Planctomycetes,* that are known to be ubiquitous in the environment and to catalyze important transformations in global carbon and nitrogen cycles (Glockner *et al.*, 2003), were identified. Particularly, the identification of Anammoxales, which was only detected at the intermediate depth, was the first genetic indication of active "anammox" bacteria in the Baltic Sea sediments. Sequences clustering within the *Desulfobacteraceae* and *Desulfobulbaceae* families, belonging to the *Deltaproteobacterial* class were identified at all investigated redox depths. The results show that sulfate reducers are present at both the reduced and oxidized sediment depths. Whether sulfate reduction is actually occurring should be concluded from analyses of for example dissimilatory sulfitereductase expression. In summary, the results demonstrate the presence of a number of known ecologically relevant genera and a vast collection of unknown genera that evidently are active at the sampled redox depths.

Using a polyphasic approach it was possible to correlate the results obtained by the clone library analysis with those from T-RFLP analysis. For example, approximately 70% of the individual community member in the T-RFLP profiles were putatively identified by *in silico* restriction digestion of 16S rRNA gene sequences obtained from the clone library analysis of the same samples. By matching the T-RFs from BrdU and rt-PCR from the same depth, and also comparing with direct extracted DNA, we could show an expected correlation between the detected replicating and transcribing bacteria (Paper III).

Concluding remarks

At the beginning of my Ph. D. studies, the Baltic Sea sediment bacterial communities were largely unexplored by molecular approaches and the field of metagenomics had just started to develop. I was interested in applying functional metagenomics in order to identify novel interesting genes and enzymes. In particular, no low temperature active enzyme had been mined from a metagenomic library. The bacterial diversity of the Baltic Sea sediments was considered to be high, but required confirmation. The goal was therefore to investigate the diversity of the bacteria in the sediment, how the communities were structured and what bacteria were active. Particularly, a highly diverse bacterial community would potentially reflect a high diversity of metabolic enzyme genes. Out of a biotechnological perspective, these enzymes, including the knowledge derived from their structures and functions, could be valuable in industrial applications, making the marine sediments suitable for enzyme mining. Therefore, a polyphasic approach was used in this project to assess and access the diversity of Baltic Sea sediment bacteria, as shown in figure 2.

Potential commercial importance of the bacterial diversity of Baltic Sea sediment

Given that the marine sediments largely represent low temperature environments, the presence of low temperature adapted bacteria with low temperature active enzymes was expected. This work demonstrated that a functional metagenomic approach was successful in accessing lipolytically active enzymes from uncultured bacteria and gives promises for further identification of novel enzymes in the future. The low temperature active enzyme h1Lip1 (Paper I) and the other detected enzymes which have not been published, represent a reservoir of enzymes with potential use in biotechnology. Examples of biotechnological applications of low temperature active esterases and lipases could be the selection between specific enantiomers such as (R,S) of solketal acetate (Ferrer *et al.*, 2005), ketoprofen ethyl ester processes (Kim *et al.*, 2006; Yoon *et al.*, 2007), ibuprofen production (Elend *et al.*, 2007). Other suitable applications could be as detergent additative, reducing temperature and thus reducing energy costs, an area where actually several patents have been filed (Marx *et al.*, 2007).

Ecological importance of mined enzymes

Ultimately, enzymes identified by metagenomic expression screening, in this case with lipolytic activity (Paper I), primarily have an important function in the marine ecosystem. For example, in Paper III, it was shown that sulfate reducers are abundant and active in the Baltic Sea sediment. These bacteria may use volatile fatty acids as electron donors together with hydrogen in sulfate reduction (Sorensen *et al.*, 1981). Lipolytic enzymes, releasing fatty acids, could be important in generating electron donors for sulfate reduction, perhaps with interactions with fermenting organisms. The fact that several active lipase/esterase fosmids were detected out of which one low temperature active, h1Lip1, was characterized in detail (Paper I), indicates that lipases are active at the *in situ* temperature in the sediment. Either sulfate reducing bacteria themselves may contain extracellular lipases/esterases releasing fatty acids or other organisms could be contributing in a synergistic relationship. Given a diverse input of fatty acids in the form of triglycerides or phospholipids, the microbial community would benefit from sharing the burden of synthesizing an array of lipolytic enzymes with different optimum substrates and catalytic properties.

Ecological importance of the active bacterial community

Interestingly, the Baltic Sea water column has recently been suggested to be heavily impacted by typical freshwater planctonic bacterial phylotypes due to the high input of freshwater and the overall brackish appearance of the Baltic Sea (Riemann *et al.*, 2008). If this is true for the Baltic Sea sediments is not yet known but would be interesting to investigate. For further analyses of the ecological roles of the bacteria in the Baltic Sea sediment, Stable Isotope Probing (SIP) (Radajewski *et al.*, 2000) could be used. SIP is another approach with the aim of identifying metabolically active populations that utilise specific substrates (Dumont and Murrell, 2005). A microbial community is incubated with the substrate of interest, which is synthesised to contain ^{13}C, whereupon DNA and RNA of metabolically active organisms will be enriched with ^{13}C in relation to ^{12}C. Considering that many members of the active bacteria in sediments off the Askö archipleago have been identified, SIP could be a useful tool to further investigate the ecological roles of Baltic Sea sediment bacteria.

The major findings in this thesis can be summarized as follows:

- A fosmid library was constructed from metagenomic HMW DNA of the prokaryotic community of the Baltic Sea sediment and it was demonstrated by expression screening that a high number, approximately 1%, of the fosmids expressed lipolytic activity (Paper I).
- The isolation, overexpression and characterisation of a novel low temperature active lipase, h1Lip1, belonging to the hormone sensitive lipases, by functional metagenomics (Paper I) demonstrated that the Baltic Sea sediments represent a useful reservoir of novel enzyme genes suitable for bioprospecting.
- Theoretical protein structure modelling of h1Lip1 enabled the identification of an N-terminal lid and amino acid residues potentially involved in low temperature adaptation. Amino acid residues within the N-terminal/lid were important for enzyme thermo stability, as shown by site-directed mutagenesis (Paper II).
- The highly diverse active bacterial community of the Baltic Sea sediments was shown to be significantly different at different redox depths and the community structure was mainly impacted by organic carbon, nitrogen and redox potential (Paper III).
- The active bacterial community members are not necessarily the most abundant community members (Paper III).
- Bacterial community members were identified, by sequencing and phylogenetic analysis, and several unstudied and novel groups were detected, most notably there was the first genetic evidence of active anammox bacteria in the Baltic Sea sediment, indicating that the bacterial diversity of Baltic Sea sediments is largely unexplored (Paper III).

Future perspectives

Future studies should be aimed at evaluating more aspects of metagenomics of the Baltic Sea sediments. First and foremost, the literature suggests a range of assays available that were not used in this study, for example other enzymatic activities, like phosphatases,

amylases, proteases, nitrilases etc, but also antibiotic resistance and functional antibiotic screens to detect betalactamases or other enzymes connected with antibiotics, for example from the in this study identified *Actinobacteria*. More of the lipolytically active fosmids are currently further investigated and they represent an existing pool of potentially novel lipases and esterases. The diversity and functions of the Baltic Sea sediment bacterial community can also be further investigated by exposing the fosmid library to complete sequencing, with the aim of further identifying the metagenome of the Baltic Sea. Such studies could be combined with functional approaches, like SIP or gene expression microarray analysis for the search for active and functional genes. One particularly interesting project would be to combine the analyses of DNA of active microorganisms with community sequencing, perhaps aided by genome amplification to investigate the sediment community. The existing Metagenomic library could also be screened for phylogenetic anchors related to the functional groups detected, like potential sulphate reducers and nitrogen cyclers. The results and experiences gained from this thesis should be useful for further microbial studies of both Baltic Sea sediments and the study of environmental microbial communities.

Acknowledgements

While spending all these years, first as an undergraduate, and then as a PhD student, at Södertörn and KI the numbers of people that have aided and taken part of the journey is impossible to count.
First and foremost I would like to thank Docent Sara Sjöling my main supervisor for accepting me as a PhD student! Thanks for always being supportive and for always having time to listen to my ideas. Thanks for introducing me to Metagenomics! Thanks for an introduction to Africa and South Africa in particular, I will remember that period as an enormously positive experience forever.
Professor Gunnar Sandström, my KI-supervisor, thanks for being interested in my research and for all good ideas about interdisciplinary projects. Thanks for your support.
Professor Janet Jansson, my co-supervisor, thanks for introducing me to Microbial Ecology, I will never forget the thrill of isolating toxic chemical degraders from my aquarium.
Professor Don Cowan, in the Cape, thanks for letting me be in your lab and for always offering developing discussions.
I want to thank the people at the Clinical Microbiology department and former Clinical Bacteriology for attending my seminars and giving useful suggestions about my research even though the subject was a bit far from the most of yours. Especially, Professor Andrej Weintaub, Professor Carl Erik Nord, Professor Bengt Wretlind.
I have a lot to thank the old and new group members of the JJA and SSJ groups. I have certainly enjoyed the science-discussions and every-day stuff. Anna, it was indeed always great fun to share all those years from undergraduate to PhD student with you! Thanks for all the fun times shared and for all the challenging discussions about science and life. Hope to see more of you in the future!
Maria, thanks for joining us as Post-Doc, being more than one working with the same techniques in the lab can really help. Karolina, your teaching skills are excellent and I suspect that you don´t like to be called this, but you ARE the Corrector of Maniatis! Cia, you have always been inspiring! I have never seen you tired! How do you do it? Ninwe, you are a wonderful person and I have enjoyed every piece of foodstuffs you have brought to the coffee-hours. Agneta, thanks for showing me how to be organized, how to have fun and how to change! Kersti, thanks for the “kulmalen cellulosa” and for always being genuinely friendly. Johan, thanks for good teaching cooperation! Åsa PB, thanks for help and suggestions about structure modelling, hope to see you soon again! Petter, I am so glad to see you come through as a PhD student! Keep up the good work and it will be allright! Diana, thanks for all your efforts during the autumn and for keeping up with a tired supervisor with too much to do at times. Anna-Ida, Misiu, Katrin, Oras, Shiva, thanks for your efforts and for the time spent! Elisabeth, Veronica, Karin and he rest of the guys that moved to Uppsala, I wish you all well!
Stefan Hallgren! I would not have survived without your friendship! Thanks for sharing all the years at Södertörn! Its wonderful to see your family grow! I need my daily dose of Rugby-discussions and Fish hormone evaluations.

Benjamin and Sonja, thanks for becoming friends and for your constant support, there is always time for a beer!

A big thanks to Amir for always being a nice guy!
Thanks for great valuable help and encouragement: Ulrika thanks for the help with the ÄKTA-machine and also for help with protein purification. Magnus should have mentioning here as well! When in to equipment, a big thanks to the TBU, PSW groups and Kurslab for always being positive about lending equipment. A special mentioning to Daniela and Maria, you helped everyone including me a lot when you were here, thank you.
Thanks to the teachers who gave me an excellent education in biology and chemistry, Inger, Odd, Mats, Einar, Anthony, Thomas and Micke.
To all people that I have had time to share my days at SH with, Peter Swoboda, Thanks for putting my knowledge in Swedish for a test. Lunch discussion group: Tessan, Christian, Andrea, nothing is uninteresting!
The office and the office next door, Håkan, Emma, Lars, Anna-Karin, Oskar, Petra, Magnus, thanks for your company and: Where also Maria, Ulrika, Elin, Sofia, Asim and Linda should be mentioned. Monica, thanks for always sharing and for always having time for a coffee at odd hours.
Lab next door: Andreas, Kalle, thanks for always being helpful.
Gunnar, Galina, Rosa, Hossein, Ivo, thanks for help with the sequencing-gel and nice discussions anytime.
Lots of additional people should have a mentioning, Kristina, Gaby*2, Schaun, Håkan, Parthena, Bengt, Anders, Tord, Julian, Anneli, Gilbert, Barbara, Yann, Mikael, Jan-Erik, Elinor, Micke W, Wessam, Robert, Karin, Anna Anthony Izabela and everyone in the administration. I have probably forgot many now… Sorry, I like you a lot anyway!
Actually, since spending well over 35 weeks, teaching at one of the courses, microbial ecology 10 p, over the years. I have to thank all the students. You have all been a great challenge to my intellect, getting from the lab to “the real world” is something I always have enjoyed.
My friends Patrik, Andreas it was great fun growing up with you! We have been scarce lately, let’s try to change that!
Stefan and Marcus, it was great fun growing up with you as well! I certainly enjoy seeing your growing families! Hope to see more of each others.
My friend, Emil, every day spent with you is great! What can I say more, All my other friends in Attila RG, My “uncles” Sören, Mange, Laxen and Ryder how much haven’t I learned from you. Every day is different, but always similar, in the best Rugby-club in the world! I will get back to practice and games soon. Cissi, my Idol, the best researcher and Rugby-player ever, hope you get back soon.
I would also like to acknowledge my parents, Lars and Ally-Marie and my brother Hampus for always supporting me. I would be nothing without you. Big thanks to my Gandmother Ingrid and my uncles Ulf and Ulf on my two sides with their families. Big thanks to all my other relatives and friends aswell! To the entire family of my Sara, You are too many to count now :) I like you all very much.

The last things are always the best; Sara, My LOVE ! I cannot thank you enough for all the evenings and weekends I have spent in the lab, without you ever being really angry with me. (A special sorry for being generally absent when writing this thesis) You give unconditional love and encouragement, always. Thanks for being who you are, Thanks for coming home again from abroad. I love you and I hope that I show it.

References

Abulencia, C.B., Wyborski, D.L. et al. (2006) Environmental Whole-Genome Amplification To Access Microbial Populations in Contaminated Sediments. *Applied and Environmental Microbiology* **72**: 3291-3301.

Alquati, C., De Gioia, L. et al. (2002) The cold-active lipase of Pseudomonas fragi. Heterologous expression, biochemical characterization and molecular modeling. *European Journal of Biochemistry* **269**: 3321-3328.

Alvarez, M., Zeelen, J.P. et al. (1998) Triose-phosphate Isomerase (TIM) of the Psychrophilic Bacterium Vibrio marinus. . *Journal of Biological Chemistry* **273**: 2199-2206.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**: 143-169.

Angly, F.E., Felts, B. et al. (2006) The Marine Viromes of Four Oceanic Regions. *PLoS Biology* **4**: e368.

Antranikian, G., Vorgias, C.E., and Bertoldo, C. (2005) Extreme environments as a resource for microorganisms and novel biocatalysts. In *Marine biotechnology I*. LeGal, Y., and Ulber, R. (eds). Berlin: Springer, pp. 219-262.

Arpigny, J.L., and Jaeger, K.E. (1999) Bacterial lipolytic enzymes: classification and properties. *Biochemical Journal* **343**: 177-183.

Arrieta, J.M., and Herndl, G.J. (2001) Assessing the diversity of marine bacterial beta-glucosidases by capillary electrophoresis zymography. *Applied and Environmental Microbiology* **67**: 4896-4900.

Azam, F. (1998) Microbial control of oceanic carbon flux: the plot thickens. *Science* **280**: 694-696.

Azam, F., and Malfatti, F. (2007) Microbial structuring of marine ecosystems. *Nature Reviews Microbiology* **5**: 782-791.

Azam, F., Smith, D.C., Steward, G.F., and Hagström, Å. (1994) Bacteria - Organic-matter coupling and its signifincance for oceanic carbon cycling. *Microbial Ecology* **28**: 167-179.

Azam, F., Fencel, T. et al. (1983) The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series* **10**: 257-263.

Bailly, J., Fraissinet-Tachet, L. et al. (2007) Soil eukaryotic functional diversity, a metatranscriptomic approach. *ISME Journal* **1**: 632-642.

Bakken, L.R., and Lindahl, V. (1995) Recovery of bacterial cells from soil. In *Nucleic acids in the environment: Methods and applications*. Van Elsas, J.D., and Trevors, J.T. (eds). Heidelberg, Germany.: Springer-Verlag, pp. 9-27.

Barer, M.R., and Harwood, C.R. (1999) Bacterial viability and culturability. *Advances in Microbial Physiology* **41**: 93-137.

Beja, O., Suzuki, M.T. et al. (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. *Nature* **415**: 630-633.

Béjà, O., Suzuki, M.T. et al. (2000a) Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. *Environmental Microbiology* **2**: 516-529.

Béjà, O., Aravind, L. et al. (2000b) Bacterial rhodopsin: Evidence for a new type of phototrophy in the sea. *Science* **289**: 1902-1906.

Borneman, J. (1999) Culture-independent identification of microorganisms that respond to specified stimuli. *Applied and Environmental Microbiology* **65**: 3398-3400.

Brady, S.F., and Clardy, J. (2000) Long-Chain N-Acyl Amino Acid Antibiotics Isolated from Heterologously Expressed Environmental DNA. *Journal of American Chemistry Society* **122**: 12903 - 12904.

Brady, S.F., Chao, C.J., Handelsman, J., and Clardy, J. (2001) Cloning and heterologus expression of a natural product biosynthetic gene cluster from eDNA. *Organic Letters* **3**: 1981-1984.

Braker, G., Ayala-del-Rio, H.L., Devol, A.H., Fesefeldt, A., and Tiedje, J.M. (2001) Community structure of denitrifiers, Bacteria and Archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. *Applied and Environmental Microbiology* **67**: 1893-1901.

Breitbart, M., Felts, B. et al. (2004) Diversity and population structure of a near-shore marine-sediment viral community. *Proceedings of the Royal Society of London Series B-Biological Sciences* **271**: 565-574.

Byun, J.-S., Rhee, J.-K. et al. (2007) Crystal structure of hyperthermophilic esterase EstE1 and the relationship between its dimerization and thermostability properties. *BMC Structural Biology* **7**.
Canfield, D.E., Jorgensen, B.B. et al. (1993) Pathways of organic carbon oxidation in three continental margin sediments. *Marine Geology* **113**: 27-40.
Cavicchioli, R., Siddiqui, K.S., Andrews, D., and Sowers, K.R. (2002) Low-temperature extremophiles and their applications. *Current Opinion in Biotechnology* **13**: 253-261.
Choo, D.W., Kurihara, T., Suzuki, T., Soda, K., and Esaki, N. (1998) A cold-adapted lipase of an Alaskan psychrotroph, Pseudomonas sp. strain B11-1: Gene cloning and enzyme purification and characterization. *Applied and Environmental Microbiology* **64**: 486-491.
Collins, T., Roulling, F. et al. (2008) Fundamentals of cold-adapted enzymes. In *Psychrophiles: from biodiversity to biotechnology*. Margesin, R., Schinner, F., Marx, J., and Gerday, G. (eds). Berlin Heidelberg: Springer-Verlag, pp. 211-227.
Colwell, R.R., Brayton, P.R. et al. (1985) Viable but Non-Culturable Vibrio cholerae and Related Pathogens in the Environment: Implications for Release of Genetically Engineered Microorganisms. *Nature Biotechnology* **3**: 817-820.
Conley, D.J., Stockenberg, A. et al. (1997) Sediment-water Nutrient Fluxes in the Gulf of Finland, Baltic Sea. *Estuarine, Coastal and Shelf Science* **45**: 591-598.
Cottrell, M.T., Moore, J.A., and Kirchman, D.L. (1999) Chitinases from uncultured marine microorganisms. *Applied and Environmental Microbiology* **65**: 2553-2557.
Cottrell, M.T., Yu, L.Y., and Kirchman, D.L. (2005) Sequence and expression analyses of Cytophaga-like hydrolases in a Western arctic metagenornic library and the Sargasso seat. *Applied and Environmental Microbiology* **71**: 8506-8513.
Courtois, S., Frostegård, Å. et al. (2001) Quantification of bacterial subgroups in soil: comparision of DNA extracted directly from soil or from cells previously released by density gradient centrifugation. *Environmental Microbiology* **3**: 431-439.
Courtois, S., Cappellano, C.M. et al. (2003) Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Applied and Environmental Microbiology* **69**: 49-55.
Cowan, D. (2000) Microbial genomes-the untapped resource. *Trends in Biotechnology* **18**: 14-16.
Crawford, D.L., and Powers, D.A. (1992) Evolutionary adaptation to different thermal environments via transcriptional regulation. *Molecular Biology and Evolution* **9**: 806-813.
Curtis, T.P., and Sloan, W.T. (2005) Exploring Microbial Diversity--A Vast Below. *Science* **309**: 1331-1333.
D'Amico, S., Sohier, J.S., and Feller, G. (2006a) Kinetics and Energetics of Ligand Binding Determined by Microcalorimetry: Insights into Active Site Mobility in a Psychrophilic [alpha]-Amylase. *Journal of Molecular Biology* **358**: 1296.
D'Amico, S., Collins, T., Marx, J.C., Feller, G., and Gerday, C. (2006b) Psychrophilic microorganisms: challenges for life. *EMBO Reports* **7**: 385-389.
Daniel, R. (2004) The soil metagenome - a rich resource for the discovery of novel natural products. *Current Opinion in Biotechnology* **15**: 199-204.
Daniel, R. (2005) The Metagenomics of soil. *Nature Reviews Microbiology* **3**: 470-478.
De Simone, G., Galdiero, S. et al. (2000) A snapshot of a transition state analogue of a novel thermophilic esterase belonging to the subfamily of mammalian hormone-sensitive lipase. *Journal of Molecular Biology* **303**: 761-771.
Dell'Anno, A., and Corinaldesi, C. (2004) Degradation and Turnover of Extracellular DNA in Marine Sediments: Ecological and Methodological Considerations. *Applied and Environmental Microbiology* **70**: 4384-4386.
DeLong, E.F. (2004) Microbial population genomics and ecology: the road ahead. *Environmental Microbiology* **6**: 875-878.
DeLong, E.F., Preston, C.M. et al. (2006) Community genomics among stratified microbial assemblages in the ocean's interior. *Science* **311**: 496-503.
DeSantis, T.Z., Hugenholtz, P. et al. (2006a) Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology* **72**: 5069-5072.
DeSantis, T.Z., Jr., Hugenholtz, P. et al. (2006b) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research* **34**: W394-399.
Dumont, M.G., and Murrell, J.C. (2005) Stable isotope probing [mdash] linking microbial identity to function. *Nature Reviews Microbiology* **3**: 499-504.

Edlund, A., and Jansson, J.K. (2006) Changes in Active Bacterial Communities before and after Dredging of Highly Polluted Baltic Sea Sediments. *Applied and Environmental Microbiology* **72**: 6800-6807.
Edlund, A., Soule, T., Sjöling, S., and Jansson, J.K. (2006) Microbial community structure in polluted Baltic Sea sediments. *Environmental Microbiology* **8**: 223-232.
Edwards, R., Rodriguez-Brito, B. et al. (2006) Using pyrosequencing to shed light on deep mine microbial ecology. *BMC Genomics* **7**: 57.
Eisen, J.A. (2007) Environmental Shotgun Sequencing: Its Potential and Challenges for Studying the Hidden World of Microbes. *PLoS Biology* **5**: e82.
Elend, C., Schmeisser, C., Hoebenreich, H., Steele, H.L., and Streit, W.R. (2007) Isolation and characterization of a metagenome-derived and cold-active lipase with high stereospecificity for (R)-ibuprofen esters. *Journal of Biotechnology* **130**: 370-377.
Elend, C., Schmeisser, C. et al. (2006) Isolation and biochemical characterization of two novel metagenome-derived esterases. *Applied and Environmental Microbiology* **72**: 3637-3645.
Elmgren, R. (1989) Man 's Impact on the Ecosystem of the Baltic Sea: Energy Flows Today and at the Turn of the Century *AMBIO AMBOCX* **18**: 326-332.
England, L.S., Vincent, M.L., Trevors, J.T., and Holmes, S.B. (2004) Extraction, detection and persistence of extracellular DNA in forest litter microcosms. *Molecular and Cellular Probes* **18**: 313-319.
Engqvist, A. (1996) Long-term Nutrient Balances in the Eutrophication of the Himmerfjarden Estuary. *Estuarine, Coastal and Shelf Science* **42**: 483-507.
Entcheva, P., Liebl, W., Johann, A., Hartsch, T., and Streit, W. (2001) Direct cloning from enrichment cultures, a reliable strategy for isolation of complete operons and genes from microbial consortia. *Applied and Environmental Microbiology* **67**: 89-99.
Feller, G. (2003) Molecular adaptations to cold in psychrophilic enzymes. *Cellular and Molecular Life Sciences* **60**: 648-662.
Feller, G., and Gerday, C. (2003) Psychrophilic enzymes: hot topics in cold adaptation. *Nat Rev Micro* **1**: 200-208.
Fenchel, T., and Finlay, B.J. (1995) *Ecology and evolution of anoxic worlds.* New York: Oxford University Press Inc.
Ferrer, M., Golyshina, O.V. et al. (2005) Microbial Enzymes Mined from the Urania Deep-Sea Hypersaline Anoxic Basin. *Chemistry & Biology* **12**: 895-904.
Fields, P.A. (2001) Review: Protein function at thermal extremes: balancing stability and flexibility. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **129**: 417-431.
Finster, K. (2008) Anaerobic Bacteria and Archaea in Cold Ecosystems. In *Psychrophiles: from Biodiversity to Biotechnology*, pp. 103-119.
Foglia, F., Mandrich, L. et al. (2007) Role of the N-terminal region for the conformational stability of esterase 2 from Alicyclobacillus acidocaldarius. *Biophysical Chemistry* **127**: 113-122.
Fox, G.E., Stackebrandt, E. et al. (1980) The phylogeny of prokaryotes. *Science* **209**: 457-463.
Frevert, T. (1984) Can the redox conditions in natural waters be predicted by a single parameter? *Aquatic Sciences - Research Across Boundaries* **46**: 269-290.
Froelich, P.N., Klinkhammer, G.P. et al. (1979) Early oxidation of organic matter in pelagic sediments of the eastern equatorial Atlantic: suboxic diagenesis. *Geochimica et Cosmochimica Acta* **43**: 1075-1090.
Fuhrman, J.A., and Azam, F. (1980) Bacterioplankton Secondary Production Estimates for Coastal Waters of British Columbia, Antarctica, and California. *Applied and Environmental Microbiology* **39**: 1085-1095.
Gabor, E.M., de Vries, E.J., and Janssen, D.B. (2003) Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiology Ecology* **44**: 153-163.
Gabor, E.M., Alkema, W.B.L., and Janssen, D.B. (2004a) Quantifying the accessibility of the metagenome by random expression cloning techniques. *Environmental Microbiology* **6**: 879-886.
Gabor, E.M., de Vries, E.J., and Janssen, D.B. (2004b) Construction, characterization, and use of small-insert gene banks of DNA isolated from soil and enrichment cultures for the recovery of novel amidases. *Environmental Microbiology* **6**: 948-958.
Galvao, T.C., Mohn, W.W., and de Lorenzo, V. (2005) Exploring the microbial biodegradation and biotransformation gene pool. *Trends in Biotechnology* **23**: 497-506.
Gerday, C., Aittaleb, M. et al. (2000) Cold-adapted enzymes: from fundamentals to biotechnology. *Trends in Biotechnology* **18**: 103-107.

Gianese, G., Bossa, F., and Pascarella, S. (2002) Comparative structural analysis of psychrophilic and meso- and thermophilic enzymes. *Proteins-Structure Function and Genetics* **47**: 236-249.
Gillespie, D.E., Brady, S.F. et al. (2002) Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. *Applied and Environmental Microbiology* **68**: 4301-4306.
Ginalski, K., Elofsson, A., Fischer, D., and Rychlewski, L. (2003) 3D-Jury: a simple approach to improve protein structure predictions. *Bioinformatics* **19**: 1015-1018.
Glockner, F.O., Kube, M. et al. (2003) Complete genome sequence of the marine planctomycete Pirellula sp. strain 1. *PNAS* **100**: 8298-8303.
Goldberg, S.M.D., Johnson, J. et al. (2006) A Sanger/pyrosequencing hybrid approach for the generation of high-quality draft assemblies of marine microbial genomes. *PNAS* **103**: 11240-11245.
Grant, S., Grant, W.D. et al. (2006) Identification of Eukaryotic Open Reading Frames in Metagenomic cDNA Libraries Made from Environmental Samples. *Applied and Environmental Microbiology* **72**: 135-143.
Guindon, S., and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696-704.
Gundersen, J.K., and Jorgensen, B.B. (1990) Microstructure of diffusive boundary layers and the oxygen uptake of the sea floor. *Nature* **345**: 604-607.
Gupta, R., Beg, Q.K., and Lorenz, P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology* **59**: 15-32.
Gupta, R., Gupta, N., and Rathi, P. (2004) Bacterial lipases: an overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology* **64**: 763-781.
Hagstrom, Larsson, U., Horstedt, P., and Normark, S. (1979) Frequency of Dividing Cells, a New Approach to the Determination of Bacterial Growth Rates in Aquatic Environments. *Applied and Environmental Microbiology* **37**: 805-812.
Hagström, A., Azam, F., Andersson, A., Wikner, J., and Rassoulzadegan, F. (1988) Microbial Loop in an Oligotrophic Pelagic Marine Ecosystem - Possible Roles of Cyanobacteria and Nanoflagellates in the Organic Fluxes. *Marine Ecology-Progress Series* **49**: 171-178.
Hallam, S.J., Putnam, N. et al. (2004) Reverse Methanogenesis: Testing the Hypothesis with Environmental Genomics. *Science* **305**: 1457-1462.
Hallam, S.J., Mincer, T.J. et al. (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. *Plos Biology* **4**: 520-536.
Handelsman, J. (2004) Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* **68**: 669-+.
Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J., and Goodman, R.M. (1998) Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. *Chemistry & Biology* **5**: R245-R249.
Handelsman, J., Liles, M., Mann, D., Riesenfeld, C., and Goodman, R.M. (2002) Cloning the metagenome: culture-independent access to the diversity and functions of the uncultivated microbial world. In *Methods in Microbiology, Functional Microbial Genomics*. Wren, B., and Dorrell, N. (eds). New York.: Academic Press.
Head, I.M., Saunders, J.R., and Pickup, R.W. (1998) Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microbial Ecology* **35**: 1-21.
Healy, F.G., Ray, R.M. et al. (1995) Direct Isolation of Functional Genes Encoding Cellulases from the Microbial Consortia in a Thermophilic, Anaerobic Digester Maintained on Lignocellulose. *Applied Microbiology and Biotechnology* **43**: 667-674.
Hemilä, H., Koivula, T.T., and Palva, I. (1994) Hormone-sensitive lipase is closely related to several bacterial proteins, and distantly related to acetylcholinesterase and lipoprotein lipase: Identification of a superfamily of esterases and lipases. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1210**: 249-253.
Henne, A., Daniel, R., Schmitz, R.A., and Gottschalk, G. (1999) Construction of environmental DNA libraries in Escherichia coli and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. *Applied and Environmental Microbiology* **65**: 3901-3907.
Henne, A., Schmitz, R.A., Bomeke, M., Gottschalk, G., and Daniel, R. (2000) Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli. *Applied and Environmental Microbiology* **66**: 3113-3116.
Hobbie, J.E., Daley, R.J., and Jasper, S. (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology* **33**: 1225-1228.

Holben, W.E., Jansson, J.K., Chelm, B.K., and Tiedje, J.M. (1988) DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Applied and Environmental Microbiology* **54**: 703-711.

Hollibaugh, J., and Azam, F. (1983) Microbial degradation of dissolved proteins in seawater. *Limnology and Oceanography* **28**: 1104-1116.

Hoyoux, A., Blaise, V. et al. (2004) Extreme catalysts from low-temperature environments. *Journal of Bioscience and Bioengineering* **98**: 317.

Hugenholtz, P. (2002) Exploring prokaryotic diversity in the genomic era. *Genome Biology* **3**: reviews0003.0001 - reviews0003.0008.

Hurt, R.A., Qiu, X. et al. (2001) Simultaneous Recovery of RNA and DNA from Soils and Sediments. *Applied and Environmental Microbiology* **67**: 4495-4503.

Hårdeman, F., and Sjöling, S. (2007) Metagenomic approach for the isolation of a novel low-temperature-active lipase from uncultured bacteria of marine sediment. *FEMS Microbiology Ecology* **59**: 524-534.

Jaeger, K.E., and Eggert, T. (2002) Lipases for biotechnology. *Current Opinion in Biotechnology* **13**: 390-397.

Jaeger, K.E., Dijkstra, B.W., and Reetz, M.T. (1999) Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology* **53**: 315-351.

Jansson, J.K., and Prosser, J.I. (1997) Quantification of the presence and activity of specific microorganisms in nature. *Molecular Biotechnology* **7**: 103-120.

Jorgensen, B.B. (1982) Mineralization of organic matter in the sea bed[mdash]the role of sulphate reduction. *Nature* **296**: 643-645.

Jorgensen, B.B., and Boetius, A. (2007) Feast and famine -- microbial life in the deep-sea bed. *Nat Rev Micro* **5**: 770-781.

Junge, K., Eicken, H., Swanson, B.D., and Deming, J.W. (2006) Bacterial incorporation of leucine into protein down to -20 °C with evidence for potential activity in sub-eutectic saline ice formations. *Cryobiology* **52**: 417-429.

Kaelin, J., William G., Krek, W. et al. (1992) Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell* **70**: 351-364.

Kaprelyants, A.S., Gottschal, J.C., and Kell, D.B. (1993) Dormancy in non-sporulating bacteria. *FEMS Microbiology Letters* **104**: 271-286.

Kim, S., Joo, S. et al. (2007) Purification, crystallization and preliminary crystallographic analysis of Est25: a ketoprofenspecific hormone-sensitive lipase. *Acta Crystallographica* **63**: 579-581.

Kim, Y.-J., Choi, G.-S. et al. (2006) Screening and characterization of a novel esterase from a metagenomic library. *Protein Expression and Purification* **45**: 315-323.

Kirchman, D.L., and White, J. (1999) Hydrolysis and mineralization of chitin in the delaware estuary. *Aquatic Microbial Ecology* **18**: 187-196.

Kitts, C.L. (2001) Terminal Restriction Fragment Patterns: A Tool for Comparing Microbial Communities and Assessing Community Dynamics *Current Issues in Intestinal Microbiology* **2**: 17-25.

Knietsch, A., Waschkowitz, T., Bowien, S., Henne, A., and Daniel, R. (2003) Metagenomes of complex microbial consortia derived from different soils as sources for novel genes conferring formation of carbonyls from short-chain polyols on Escherichia coli. *Journal of Molecular Microbiology and Biotechnology* **5**: 46-56.

Kobori, H., Sullivan, C.W., and Shizuya, H. (1984) Heat-Labile Alkaline Phosphatase from Antarctic Bacteria: Rapid 5' End-Labeling of Nucleic Acids. *PNAS* **81**: 6691-6695.

Kok, R.G., Christoffels, V.M., Vosman, B., and Hellingwerf, K.J. (1993) Growth-Phase-Dependent Expression of the Lipolytic System of Acinetobacter-Calcoaceticus Bd413 - Cloning of a Gene Encoding One of the Esterases. *Journal of General Microbiology* **139**: 2329-2342.

Kouker, G., and Jaeger, K.E. (1987) Specific and sensitive plate assay for bacterial lipases. *Applied and Environmental Microbiology* **53**: 211-213.

Kowalchuk, G., Speksnijder, A., Zhang, K., Goodman, R., and van Veen, J. (2007) Finding the Needles in the Metagenome Haystack. *Microbial Ecology* **53**: 475-485.

Kulakova, L., Galkin, A., Nakayama, T., Nishino, T., and Esaki, N. (2004) Cold-active esterase from Psychrobacter sp. Ant300: gene cloning, characterization, and the effects of Gly-->Pro substitution near the active site on its catalytic activity and stability. *Biochimica et Biophysica Acta - Proteins & Proteomics* **1696**: 59-65.

Lee, C., Kibblewhite-Accinelli, R., Wagschal, K., Robertson, G., and Wong, D. (2006a) Cloning and characterization of a cold-active xylanase enzyme from an environmental DNA library. *Extremophiles* **10**: 295-300.

Lee, M.-H., Lee, C.-H., Oh, T.-K., Song, J.K., and Yoon, J.-H. (2006b) Isolation and Characterization of a Novel Lipase from a Metagenomic Library of Tidal Flat Sediments: Evidence for a New Family of Bacterial Lipases. *Applied and Environmental Microbiology* **72**: 7406-7409.

Lee, S.W., Won, K. et al. (2004) Screening for novel lipolytic enzymes from uncultured soil microorganisms. *Applied Microbiology and Biotechnology* **65**: 720-726.

Leveau, J. (2007) The magic and menace of metagenomics: prospects for the study of plant growth-promoting rhizobacteria. *European Journal of Plant Pathology* **119**: 279-300.

Liles, M.R., Manske, B.F., Bintrim, S.B., Handelsman, J., and Goodman, R.M. (2003) A census of rRNA genes and linked genomic sequences within a soil metagenomic library. *Applied and Environmental Microbiology* **69**: 2684-2691.

Lin, J.J., and Somero, G. (1995) Thermal adaptation of cytoplasmic malate dehydrogenases of eastern Pacific barracuda (Sphyraena spp): the role of differential isoenzyme expression. *The Journal of Experimental Biology* **198**: 551-560.

Liu, W., Marsh, T., Cheng, H., and Forney, L. (1997) Characterization of microbial diversity by determining Terminal Restriction Fragment Length Polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63**: 4516-4522.

Lonhienne, T., Gerday, C., and Feller, G. (2000) Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1543**: 1-10.

Lorenz, P., and Eck, J. (2005) Metagenomics and industrial applications. *Nat Rev Micro* **3**: 510-516.

Luna, G.M., Manini, E., and Danovaro, R. (2002a) Large Fraction of Dead and Inactive Bacteria in Coastal Marine Sediments: Comparison of Protocols for Determination and Ecological Significance. *Applied and Environmental Microbiology* **68**: 3509-3513.

Luna, G.M., Manini, E., and Danovaro, R. (2002b) Large fraction of dead and inactive bacteria in coastal marine sediments: comparison of protocols for determination and ecological significance. *Applied and Environmental Microbiology* **68**: 3509-3513.

MacNeil, I.A., Tiong, C.L. et al. (2001) Expression and isolation of antimicrobial small molecules from soil DNA libraries. *Journal of Molecular Microbiology and Biotechnology* **3**: 301-308.

Mandrich, L., Pezzullo, M. et al. (2004) Analysis of Thermal Adaptation in the HSL Enzyme Family. *Journal of Molecular Biology* **335**: 357-369.

Mandrich, L., Merone, L. et al. (2005) Role of the N terminus in enzyme activity, stability and specificity in thermophilic esterases belonging to the HSL family. *Journal of Molecular Biology* **345**: 501-512.

Margesin, R., and Schinner, F. (1994) Properties of Cold-Adapted Microorganisms and Their Potential Role in Biotechnology. *Journal of Biotechnology* **33**: 1-14.

Margulies, M., Egholm, M. et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **31**: 31.

Markowitz, V.M., Ivanova, N. et al. (2006) An experimental metagenome data management and analysis system. *Bioinformatics* **22**: e359-367.

Martinez, J., Smith, D.C., Steward, G.F., and Azam, F. (1996) Variability in ectohydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea. *Aquatic Microbial Ecology* **10**: 223-230.

Martinez, R.J., Mills, H.J., Story, S., and Sobecky, P.A. (2006) Prokaryotic diversity and metabolically active microbial populations in sediments from an active mud volcano in the Gulf of Mexico.*Environmental Microbiology* **8**: 1783-1796.

Marx, J.C., Collins, T., D'Amico, S., Feller, G., and Gerday, C. (2007) Cold-Adapted Enzymes from Marine Antarctic Microorganisms. *Marine Biotechnology* **9**: 293.

Mills, H.J., Martinez, R.J., Story, S., and Sobecky, P.A. (2004) Identification of Members of the Metabolically Active Microbial Populations Associated with Beggiatoa Species Mat Communities from Gulf of Mexico Cold-Seep Sediments. *Applied and Environmental Microbiology* **70**: 5447-5458.

Mills, H.J., Martinez, R.J., Story, S., and Sobecky, P.A. (2005) Characterization of microbial community structure in Gulf of Mexico gas hydrates: Comparative analysis of DNA- and RNA-derived clone libraries. *Applied and Environmental Microbiology* **71**: 3235-3247.

Moeseneder, M.M., Arrieta, J.M., and Herndl, G.J. (2005) A comparison of DNA- and RNA-based clone libraries from the same marine bacterioplankton community. *FEMS Microbiology Ecology* **51**: 341-352.

Mojzsis, S.J., Arrhenius, G. et al. (1996) Evidence for life on Earth before 3,800 million years ago. *Nature* **384**: 55-59.

Morita, R.Y. (1975) Psychrophilic bacteria. *Bacteriol Rev* **39**: 144-167.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.

Narinx, E., Baise, E., and Gerday, C. (1997) Subtilisin from psychrophilic antarctic bacteria: characterization and site-directed mutagenesis of residues possibly involved in the adaptation to cold. *Protein Engineering* **10**: 1271-1279.

Nebe-von-Caron, G., Stephens, P.J., Hewitt, C.J., Powell, J.R., and Badley, R.A. (2000) Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *Journal of Microbiological Methods* **42**: 97-114.

Nogales, B., Moore, E.R.B., Abraham, W.-R., and Timmis, K.N. (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environmental Microbiology* **1**: 199-212.

Nomura, M., Gourse, R., and Baughman, G. (1984) Regulation of the Synthesis of Ribosomes and Ribosomal Components. *Annual Review of Biochemistry* **53**: 75-117.

Ogram, A., Sayler, G.S., and Barkay, T. (1987) The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods* **7**: 57-66.

Ollis, D., Cheah, E. et al. (1992) The alpha/beta hydrolase fold. *Protein Engineering* **5**: 197-211.

Otto, R.T., Bornscheuer, U.T., Syldatk, C., and Schmid, R.D. (1998) Synthesis of aromatic n-alkyl-glucoside esters in a coupled β-glucosidase and lipase reaction. *Biotechnology Letters* **20**: 437-440.

Pandey, A., Benjamin, S. et al. (1999) The realm of microbial lipases in biotechnology. *Biotechnology and Applied Biochemistry* **29**: 119-131.

Pleiss, J., Fischer, M., Peiker, M., Thiele, C., and Schmid, R.D. (2000) Lipase engineering database: Understanding and exploiting sequence-structure-function relationships. *Journal of Molecular Catalysis B: Enzymatic* **10**: 491-508.

Podar, M., and Reysenbach, A.L. (2006) New opportunities revealed by biotechnological explorations of extremophiles. *Current Opinion in Biotechnology* **17**: 250-255.

Quaiser, A., Ochsenreiter, T. et al. (2002) First insight into the genome of an uncultivated crenarchaeote from soil. *Environmental Microbiology* **4**: 603-611.

Radajewski, S., Ineson, P., Parekh, N.R., and Murrell, J.C. (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* **403**: 646-649.

Rappe, M.S., and Giovannoni, S.J. (2003) The uncultured microbial majority. *Annual Review of Microbiology* **57**: 369-394.

Rashid, N., Shimada, Y., Ezaki, S., Atomi, H., and Imanaka, T. (2001) Low-Temperature Lipase from Psychrotrophic Pseudomonas sp. Strain KB700A. *Applied and Environmental Microbiology* **67**: 4064-4069.

Reetz, M.T. (2001) Combinatorial and Evolution-Based Methods in the Creation of Enantioselective Catalysts. *Angewandte Chemie International Edition* **40**: 284-310.

Rhee, J.K., Ahn, D.G., Kim, Y.G., and Oh, J.W. (2005) New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Applied and Environmental Microbiology* **71**: 817-825.

Richardson, J.S., and Richardson, D.C. (1988) Amino acid preferences for specific locations at the ends of alpha helices. *Science* **240**: 1648-1652.

Riemann, L., Leitet, C. et al. (2008) The Native Bacterioplankton Community in the Central Baltic Sea Is Influenced by Freshwater Bacterial Species. *Applied and Environmental Microbiology* **74**: 503-515.

Riesenfeld, C.S., Schloss, P.D., and Handelsman, J. (2004a) Metagenomics: Genomic analysis of microbial communities. *Annual Review of Genetics* **38**: 525-552.

Riesenfeld, C.S., Goodman, R.M., and Handelsman, J. (2004b) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environmental Microbiology* **6**: 981-989.

Robertson, D.E., Chaplin, J.A. et al. (2004) Exploring Nitrilase Sequence Space for Enantioselective Catalysis. *Applied and Environmental Microbiology* **70**: 2429-2436.

Roh, C., and Villatte, F. (2008) Isolation of a low-temperature adapted lipolytic enzyme from uncultivated micro-organism. *Journal of Applied Microbiology* **0**: advance online publication 31 January 2008; doi:2010.1111/j.1365-2672.2007.03717.x.

Romanowski, G., Lorenz, M.G., and Wackernagel, W. (1993) Use of polymerase chain reaction and electroporation of Escherichia coli to monitor the persistence of extracellular plasmid DNA introduced into natural soils. *Applied and Environmental Microbiology* **59**: 3438-3446.

Ronaghi, M., Uhlén, M., and Nyrén, a.P. (1998) DNA SEQUENCING: A Sequencing Method Based on Real-Time Pyrophosphate. *Science* **281**: 363-365.

Rondon, M.R., August, P.R. et al. (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Applied and Environmental Microbiology* **66**: 2541-2547.

Rosenbaum, V., and Riesner, D. (1987) Temperature-gradient gel electrophoresis : Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts. *Biophysical Chemistry* **26**: 235-246.

Rossolini, G.M., Schippa, S. et al. (1998) Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. *Cellular and Molecular Life Sciences (CMLS)* **54**: 833-850.

Rusch, D.B., Halpern, A.L. et al. (2007) The Sorcerer II Global Ocean Sampling Expedition: Northwest Atlantic through Eastern Tropical Pacific. *PLoS Biology* **5**: e77.

Russell, N.J. (2008) Membrane Components and Cold Sensing. In *Psychrophiles: from Biodiversity to Biotechnology*, pp. 177-190.

Russell, N.J., and Hamamoto, T. (1998) Psychrophiles. In *Extremophiles: Microbial Life in Extreme Environments*. Horikoshi, K., and Grant, W.D. (eds): Wiley-Liss, pp. 25-45.

Russell, R.J., Gerike, U., Danson, M.J., Hough, D.W., and Taylor, G.L. (1998) Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. *Structure* **6**: 351-361.

Rönnberg, C., and Bonsdorff, E. (2004) Baltic Sea eutrophication: area-specific ecological consequences. *Hydrobiologia* **514**: 227-241.

Sabehi, G., Kirkup, B.C. et al. (2007) Adaptation and spectral tuning in divergent marine proteorhodopsins from the eastern Mediterranean and the Sargasso Seas. *ISME J* **1**: 48-55.

Saunders, N.F.W., Thomas, T. et al. (2003) Mechanisms of Thermal Adaptation Revealed From the Genomes of the Antarctic Archaea Methanogenium frigidum and Methanococcoides burtonii. *Genome Research* **13**: 1580-1588.

Schaechter, M., Maaloe, O., and Kjeldegaard, N.O. (1958) Dependency on medium and temperature of cell size and chemical composition during balanced grown of Salmonella typhimurium. *Journal of General Microbiology* **19**: 592-606.

Schloss, P.D., and Handelsman, J. (2003) Biotechnological prospects from metagenomics. *Current Opinion in Biotechnology* **14**: 303-310.

Schrøder Leiros, H.K., Willassen, N.P., and Smalås, A.O. (1999) Residue determinants and sequence analysis of cold-adapted trypsins. *Extremophiles* **3**: 205-219.

Seshadri, R., Kravitz, S.A., Smarr, L., Gilna, P., and Frazier, M. (2007) CAMERA: A Community Resource for Metagenomics. *PLoS Biology* **5**: e75.

Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., and Slepak, T. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. *Proceedings of the National Academy of Science, USA* **89**: 8794-8797.

Siddiqui, K.S., and Cavicchioli, R. (2006) Cold-Adapted Enzymes. *Annual Review of Biochemistry* **75**: 403-433.

Sjöling, S., and Cowan, D. (2008) Metagenomics: microbial community genomes revealed. In *Psychrophiles: from biodiversity to biotechnology*. Margesin, R., Schinner, F., Marx, J., and Gerday, G. (eds). Berlin Heidelberg: Springer-Verlag, pp. 313-330.

Sjöling, S., Stafford, W., and Cowan, D.A. (2006) Soil metagenomics: exploring and exploiting the soil gene pool. In *Modern Soil Microbiology*. Van Elsas, J.D., Trevors, J.T., and Jansson, J.K. (eds). Boca Raton London New York: CRC Press, Taylor and Francis Group, pp. 409-434.

Smalås, A.O., Schrøder Leiros, H.-K., Os, V., and Willassen, N.P. (2000) Cold Adapted Enzymes. In *Biotechnology Annual Rewiev*. El-Gewely, M.R. (ed): Eslewier Science.

Sogin, M.L., Morrison, H.G. et al. (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings in National Academy of Science, USA* **103**: 12115-12120.

Somero, G.N. (2004) Adaptation of enzymes to temperature: searching for basic "strategies". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **139**: 321-333.
Sorensen, J., Christensen, D., and Jorgensen, B.B. (1981) Volatile Fatty Acids and Hydrogen as Substrates for Sulfate-Reducing Bacteria in Anaerobic Marine Sediment. *Applied and Environmental Microbiology* **42**: 5-11.
Steffan, R.J., Goksoyr, J., Bej, A.K., and Atlas, R.M. (1988) Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology* **54**: 2908-2915.
Stein, J.L., Marsh, T.L., Wu, K.Y., Shizuya, H., and DeLong, E.F. (1996) Characterization of uncultivated prokaryotes: Isolation and analysis of a 40-kilobase-pair genome fragment front a planktonic marine archaeon. *Journal of Bacteriology* **178**: 591-599.
Stetter, K.O. (1998) Hyperthermophiles: isolation, classification, and properties. In *Extremophiles*. Horikoshi, K., and Grant, W.D. (eds). New York, USA: Wiley-Liss.
Suenaga, H., Ohnuki, T., and Miyazaki, K. (2007) Functional screening of a metagenomic library for genes involved in microbial degradation of aromatic compounds. *Environmental Microbiology* **9**: 2289-2297.
Suttle, C.A. (2007) Marine viruses -- major players in the global ecosystem. *Nat Rev Micro* **5**: 801-812.
Sørensen, J., Jørgensen, B.B., and Revsbech, N.P. (1979) A comparison of oxygen, nitrate, and sulfate respiration in coastal marine sediments. *Microbial Ecology* **5**: 105-115.
Tebbe, C.C., and Vahjen, W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Applied and Environmental Microbiology* **59**: 2657-2665.
Teske, A., Wawer, C., Muyzer, G., and Ramsing, N.B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology* **62**: 1405-1415.
Thamdrup, B., Fossing, H., and Jørgensen, B.B. (1994) Manganese, iron and sulfur cycling in a coastal marine sediment, Aarhus bay, Denmark. *Geochimica et Cosmochimica Acta* **58**: 5115-5129.
Torsvik, V., Sørheim, R., and Goksøyr, J. (1996) Total bacterial diversity in soil and sediment communities-A review. *Journal of Industrial Microbiology* **17**: 170-178.
Treusch, A.H., Kletzin, A. et al. (2004) Characterization of large-insert DNA libraries from soil for environmental genomic studies of Archaea. *Environmental Microbiology* **6**: 970-980.
Tringe, S.G., von Mering, C. et al. (2005) Comparative metagenomics of microbial communities. *Science* **308**: 554-557.
Tsai, Y.L., and Olson, B.H. (1992) Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Applied and Environmental Microbiology* **58**: 754-757.
Tyson, G.W., Chapman, J. et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**: 37-43.
Uchiyama, T., Abe, T., Ikemura, T., and Watanabe, K. (2005) Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. *Nature Biotechnology* **23**: 88-93.
Urakawa, H., Kita-Tsukamoto, K., and Ohwada, K. (1999) Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology* **145**: 3305-3315.
Urbach, E., Vergin, K.L., and Giovannoni, S.J. (1999) Immunochemical detection and isolation of DNA from metabolically active bacteria. *Applied and Environmental Microbiology* **65**: 1207-1213.
Wallner, A. (2005) Protein structure prediction: model building and quality assesment. In: Thesis: Stockholm University.
Wallner, B., and Elofsson, A. (2003) Can correct protein models be identified? *Protein Science* **12**: 1073-1086.
Van den Burg, B., Vriend, G., Veltman, O.R., Venema, G., and Eijsink, V.G.H. (1998) Engineering an enzyme to resist boiling. *Proceedings in National Academy of Science, USA* **95**: 2056-2060.
Van Elsas, J.D., and Smalla, K. (1995) Extraction of microbial community DNA from soils In *Molecular microbial ecology manual*. Akkermans, A.D.L., van Elsas, J.D., and de Bruijn, F.J. (eds). Dordrecht, The Netherlands: Kluywer Academic Publishers pp. 1,3,3/1-1,3,3/11.
van Hellemond, E.W., Janssen, D.B., and Fraaije, M.W. (2007) Discovery of a Novel Styrene Monooxygenase Originating from the Metagenome. *Applied and Environmental Microbiology* **73**: 5832-5839.

Wang, G.Y.S., Graziani, E. et al. (2000) Novel natural products from soil DNA libraries in a streptomycete host. *Organic Letters* **2**: 2401-2404.
Ward, N. (2006) New directions and interactions in metagenomics research. *FEMS Microbiology Ecology* **55**: 331-338.
Weller, R., and Ward, D.M. (1989) Selective Recovery of 16S rRNA Sequences from Natural Microbial Communities in the Form of cDNA. *Applied and Environmental Microbiology* **55**: 1818-1822.
Weller, R., Weller, J.W., and Ward, D.M. (1991) 16S rRNA sequences of uncultivated hot spring cyanobacterial mat inhabitants retrieved as randomly primed cDNA. *Applied and Environmental Microbiology* **57**: 1146-1151.
Wemekamp-Kamphuis, H.H., Karatzas, A.K., Wouters, J.A., and Abee, T. (2002) Enhanced Levels of Cold Shock Proteins in Listeria monocytogenes LO28 upon Exposure to Low Temperature and High Hydrostatic Pressure. *Applied and Environmental Microbiology* **68**: 456-463.
Venter, J.C., Remington, K. et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**: 66-74.
Vergin, K.L., Urbach, E. et al. (1998) Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order Planctomycetales. *Applied and Environmental Microbiology* **64**: 3075-3078.
Wild, J., Hradecna, Z., and Szybalski, W. (2002) Conditionally amplifiable BACs: Switching from single-copy to high-copy vectors and genomic clones. *Genome Research* **12**: 1434-1444.
Wilkinson, D.E. (2000) Accessing genes from environmental DNA libraries. In *Department of Biochemistry and Molecular Biology*. London: University College London.
Williamson, S.J., Rusch, D.B. et al. (2008) The Sorcerer II Global Ocean Sampling Expedition: Metagenomic Characterization of Viruses within Aquatic Microbial Samples. *PLoS ONE* **3**: e1456.
Wilms, R., Sass, H. et al. (2006) Specific bacterial, archaeal, and eukaryotic communities in tidal-flat sediments along a vertical profile of several meters. *Applied and Environmental Microbiology* **72**: 2756-2764.
Wintzingerode, F.V., Gobel, U.B., and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews* **21**: 213-229.
Woese, C.R., Fox, G.E. et al. (1975) Conservation of primary structure in 16S ribosomal RNA. *Nature* **254**: 83-86.
Voget, S., Leggewie, C. et al. (2003) Prospecting for novel biocatalysts in a soil metagenome. *Applied and Environmental Microbiology* **69**: 6235-6242.
Woyke, T., Teeling, H. et al. (2006) Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature* **443**: 950-955.
Wulff, F., Stigenbrandt, A., and Rahm, L. (1990) Nutrient dynamics of the Baltic Sea. *Ambio* **19**: 126-133.
Yap, W.H., Li, X., Soong, T.W., and Davies, J.E. (1996) Genetic diversity of soil microorganisms assessed by analysis ofhsp70 (dnaK) sequences. *Journal of Industrial Microbiology and Biotechnology* **17**: 179-184.
Yoon, S., Kim, S., Ryu, Y., and Kim, T.D. (2007) Identification and characterization of a novel (S)-ketoprofen-specific esterase. *International Journal of Biological Macromolecules* **41**: 1-7.
Yooseph, S., Sutton, G. et al. (2007) The Sorcerer II Global Ocean Sampling Expedition: Expanding the Universe of Protein Families. *PLoS Biology* **5**: e16.