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Exploring the Metagenome of the Baltic Sea Sediment

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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:


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


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Additional manuscript:

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<th>Definition</th>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Terminal Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>16S subunit</td>
<td>16 Svedberg unit Ribosome subunit</td>
</tr>
<tr>
<td>16S rRNA gene</td>
<td>Gene encoding the 16S RNA</td>
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<tr>
<td>HMW</td>
<td>High Molecular Weight</td>
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<tr>
<td>kb</td>
<td>Kilo base</td>
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<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>HSL</td>
<td>Hormone Sensitive Lipase</td>
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<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Reaction rate of a enzyme</td>
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<td>$K_m$</td>
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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, nuclease 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).
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 shotgun 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 Hellemont *et al.*, 2007), degradative genes (Suenaga *et al.*, 2003), and others.
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: 

\[ k = A e^{-\frac{E_a}{RT}} \]

where \( A \) is the pre-exponential factor (related to steric factors and molecular collision frequency), \( E_a \) 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; Schröder 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 catalytic 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 biocatalysts 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.

<table>
<thead>
<tr>
<th>Enzyme with Marine relevance</th>
<th>Application Low temperature</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Lipase and Esterase</td>
<td>Organic synthesis, food production (Antranikian et al., 2005)</td>
<td>(Martinez et al., 1996)</td>
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</table>

* indicates not low temperature active application

There is also the possibility of genetically manipulating these enzymes, by protein engineering, to gain increased stability without losing 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 C₆, 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 additives (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 anthropogenic 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
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 additivite, 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 glycercylyl 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 glycercylyl 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 glycercylyl 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). Through 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 precision 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.
Secondary structure prediction of the low temperature active lipase h1Lip1. beta-strands (green), helices (grey). The central (orange) 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](http://www.rcsb.org). In this work, meta prediction at the Metaserver was used (http://meta.bioinfo.pl, Ginalska *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](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 fingerprinting 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 Pseudomonas 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 thermostability (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 $t_{1/2}$ 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 additivative, 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 archipelago 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.
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