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Marja Tiirola

Phylogenetic Analysis of Bacterial Diversity Using Ribosomal RNA Gene Sequences

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UNIVERSITY OF JYVÄSKYLÄ

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Editors
Jukka Särkkä
Department of Biological and Environmental Science, University of Jyväskylä
Pekka Olsbo, Marja-Leena Tynkkynen
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ABSTRACT

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Yhteenveto: Ribosomaalisen RNA-geenin sekvenssien käyttö bakteeridiversiteetin fylogeneettisessä analyysissä
Diss.

Broad-range PCR amplification of ribosomal RNA (rRNA) gene sequences and community profiling methods were applied in studies of the microbiology of three different environmental habitats. Length heterogeneity analysis of PCR-amplified 16S rRNA gene sequences (LH-PCR) was developed for use in profiling bacterial diversity and for optimisation of the broad-range bacterial PCR procedure.

A bioremediation system of polychlorophenol-contaminated groundwater in Kärkölä, Finland, was investigated over a six-year period. It was dependent on a stable bacterial community with low species diversity. One of the dominant organisms was isolated and named *Novosphingobium* sp. MT1. Molecular analysis of polychlorophenol-degrading bacteria isolated from the groundwater showed that they represented a wide range of phylogenetic groups. Comparative analysis of 16S rRNA and *pcpB* gene sequences suggested that the *pcpB* gene, involved in the initiation of chlorophenol degradation, had been achieved in Kärkölä sphingomonads by a recent horizontal transfer.

Bacterial diversity profiles in a thermophilic aerobic biofilm process treating paper mill process water showed that the bacterial community was completely different in the biofilm and in the suspended biomass. The biofilm community was sensitive to pH alterations, but recovered after disturbances. The study showed that LH-PCR was a fast and reliable method for screening biotechnological processes, but the separation resolution between bacterial groups was hampered by overlapping sequence lengths.

A direct diagnostic procedure based on broad-range bacterial PCR and sequencing was applied for the first time for the fish diseases. Direct molecular analysis was shown to be a potential tool for improving and expediting the diagnosis of flavobacteriosis, which is a serious salmonid disease in Finland.

Key words: Bacterial diversity; chlorophenol degradation; fish diseases;
PCR amplification; profiling; ribosomal RNA; thermophilic aerobic bioreactor.

M. Tiirola, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FIN-40351 Jyväskylä, Finland

Author´s address Marja Tiirola
Department of Biological and Environmental Science University of Jyväskylä
P. O. Box 35
FIN-40351 Jyväskylä
Finland
e-mail: mtiirola@jyu.fi

Supervisor Professor Markku Kulomaa
Department of Biological and Environmental Science University of Jyväskylä
P. O. Box 35
FIN-40351 Jyväskylä
Finland

Reviewers Docent Kristina Lindström
Department of Applied Chemistry and Microbiology
University of Helsinki
P. O. Box 56
FIN-00014 Helsingin yliopisto
Finland

Professor Atte von Wright
Institute of Applied Biotechnology
University of Kuopio
P. O. Box 1627
FIN-70211 Kuopio
Finland

Opponent Associate professor Max Häggblom
Department of Biochemistry and Microbiology
Rutgers University
Cook Campus, 76 Lipman Drive
New Brunswick
NJ 08901-8525
USA

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following scientific articles, which will be referred to by their Roman numerals. In addition, some unpublished results are described.

I Männistö, M. K., Tiirola, M. A., Salkinoja-Salonen, M. S., Kulomaa, M. S. & Puhakka, J. A. 1999. Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater. Arch. Microbiol. 171(1): 189-197.

II Tiirola, M. A., Männistö, M. K., Puhakka, J. A. & Kulomaa, M. S. 2002. Isolation and characterization of *Novosphingobium* sp. MT1, a dominant polychlorophenol-degrading strain in a groundwater bioremediation system. Appl. Environ. Microbiol. 68(1): 173-180.

III Tiirola, M. A., Wang, H., Paulin, L. & Kulomaa, M. S. 2002. Natural horizontal transfer of the *pcpB* gene facilitates chlorophenol degradation in sphingomonads. Submitted.

IV Tiirola, M. A., Suvilampi, J. E., Kulomaa, M. S. & Rintala, J. 2002. Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR). Submitted.

V Tiirola, M. A., Valtonen, E. T., Rintamäki-Kinnunen, P. & Kulomaa, M. S. 2002. Diagnosis of flavobacteriosis by direct amplification of rRNA genes. Dis. Aquat. Org. In press.

RESPONSIBILITIES OF MARJA TIIROLA IN THE ARTICLES OF THIS THESIS

Article I: The study was planned by Dr. Minna Männistö. I made the molecular analyses of the isolates (RFLP, GC-content and phylogenetic analyses). Dr. Minna Männistö did most of the writing, but I wrote the results of molecular analyses.

Article II: I planned and performed the study, except for testing the chlorophenol degradation of the isolates, which was made by Dr. Minna Männistö. I wrote the article.

Article III: I planned the study. It was performed together with Mrs. Hong Wang, who made the sequencing of the *pcpB* gene homologues and also participated in the writing process. Mr. Lars Paulin participated in the sequencing of the 16S rRNA genes.

Article IV: I planned and performed the molecular study. The bioreactor was set up and samples were collected by Mr. Juhani Suvilampi. I wrote the article.

Article V: I planned the study and analyzed the samples collected and cultivated by Dr. Päivi Rintamäki-Kinnunen. I wrote the article.

All these work were carried out under supervision of Prof. Markku Kulomaa.

ABBREVIATIONS

ARISA	automated ribosomal intergenic spacer analysis
bp	base pair
2-D	2-dimensional
Da	Dalton
DGGE	denaturing gradient gel electrophoresis
dsDNA	double-stranded DNA
EMBL	European Molecular Biology Laboratory
FISH	fluorescence *in situ* hybridization
GC-rich	guanidine and cytosine rich
ISR	intergenic spacer region
kb	kilobase pair
LH-PCR	length heterogeneity analysis of amplified PCR product
Mb	megabase pair
PCP	pentachlorophenol
PCR	polymerase chain reaction
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RISA	ribosomal intergenic spacer analysis
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSCP	single-strand conformation polymorphism
TCP	trichlorophenol
TeCP	tetrachlorophenol
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
tRNA	transfer RNA
OTU	operational taxonomic unit
SCBP	suspended carrier biofilm process

1 INTRODUCTION

An important objective in many ecological studies is to understand the natural variability and changes in microbial communities in response to altered environmental conditions. However, our understanding of microbial processes is only as good as our methods. The limitations of traditional cultivation-based methods and the problem of non-culturable bacteria have been admitted in microbiology. It has been estimated that only 0.1 to 1% of the total bacterial fauna can be cultivated in environmental samples (Jannash & Jones 1959, Staley & Konopka 1985, Hopkins et al. 1993, Amann et al. 1995). The ability to determine the natural abundance and activity of individual strains in their natural environment without cultivation thus constitutes a major challenge.

The in-depth studies of Woese and his collaborators (Woese & Fox 1977, Fox et al. 1980, Woese 1987) on the comparison of the base sequences of 16S ribosomal RNA (rRNA) molecules laid the foundation for culture-independent methods. Since the mid-1980s, the use of 16S rRNA based techniques has facilitated the molecular identification of a wide variety of as yet uncultivated microorganisms and novel microbial groups in various environments. From a human point of view, the discovery of uncultivated pathogens (ehrlichiosis, Anderson et al. 1992; Whipple's disease, Wilson et al. 1991) has been especially interesting, as well as the characterization of the natural microflora of various intriguing environments (reviewed by Ward et al. 1990, Head et al. 1998, Hugenholtz et al. 1998). Sequences can be retrieved directly from a sample by polymerase chain reaction (PCR) using primers corresponding to conserved bacterial priming sites, and the products can be cloned for sequencing.

Although molecular methods have been widely used in microbial ecology during the past decade, the number of environments studied with culture-independent methods is limited (Hugenholtz et al. 1998), since the 'clone and sequence' approach to the task is slow and laborious. Therefore, several PCR-based community fingerprinting methods have been developed to enable faster analyses. Denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993), length heterogeneity analysis of PCR-amplified 16S rDNA (LH-PCR) (Suzuki et al. 1998), single-strand conformation polymorphism (SSCP) (Lee et al. 1996,

Widjojoatmodjo et al. 1995, Schwieger & Tebbe 1998), restriction fragment length polymorphism (RFLP) and ribosomal intergenic spacer region analysis (RISA) are very promising for the structural and serial analyses of microbial communities.

In the work done for this dissertation 16S rDNA methodology (DGGE, LH-PCR, RFLP and cloning) were applied to the investigation of three research themes, all connected with aquatic microbiology (Fig. 1). The choice of habitats was affected by the interests and current projects of the Department of Biological and Environmental Science. First, the goal of the Kärkölä project (I, II and III) was to study the potential of the chlorophenol-contaminated Kärkölä aquifer for bioremediation *in situ*. Such a method of treating wastewater would be an economical solution to the difficult environmental problem facing a number of sawmill locations in Finland. Second, thermophilic wastewater treatment, especially of pulp and paper mill waters, has also been a long-term project in the department. In the molecular study of the thermophilic biofilm process (IV) we extended the knowledge gained from numerous more practical studies to the microbiological level. And third, the applications in fish bacteriology (V) were developed to improve the diagnostics of flavobacteriosis, which has been one of the major diseases in the Finnish fish farming industry.

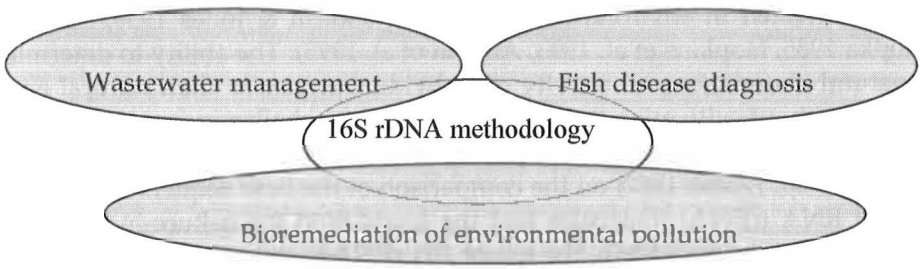

FIGURE 1 Research topics studied in this dissertation using 16S rDNA-based methods.

The three research environments can be considered as ecologically extreme, since limited species diversity was expected and later demonstrated in all of them. Therefore, these environments are excellent examples of cases where community fingerprinting methods are especially useful (II, IV and V). Our interest was also to isolate dominant species with an important role in the processes under study (II and V). The features and phylogenetic positions of chlorophenol-degrading organisms were investigated in detail in two additional studies (I and III). All of these projects and studies show conclusively the power of the 16S rDNA methodology and its applications in explaining the microbiological ecology and the phenomena taking place in it.

2 REVIEW OF THE LITERATURE

2.1 Bacterial diversity

2.1.2 Bacterial diversity in the environment

Microbes are a fundamentally important component of terrestrial and aquatic habitats, playing many key roles in geochemical processes, especially in nutrient cycling. Room or body temperature, the oxygen partial pressure of the atmosphere, neutral pH, and the abundance of nutrients have traditionally been considered to be "normal" conditions for the growth of microorganisms (Schlegel & Jannasch 1992). A characteristic feature of such 'normal' microbial communities has been their complexity, both in terms of numbers of organisms present and their genetic and functional diversity. On the basis of DNA-DNA reassociation analysis (Torsvik et al. 1990a) it has been shown that a gram of soil or sediment can contain 4 000 or as many as 10 000 different genomes of standard soil bacterial size (Torsvik et al. 1990b, Torsvik et al. 1998). Although it is not yet clear if each genome represents an individual species, the results indicate that the number of species in one environmental sample can exceed the number of species so far described.

The study of microbial communities can have two different goals that require different strategies: establishment of the diversity of species present and determination of the structure of the community (Liesack et al. 1997). Species richness is defined as the number of species present in a community. It depends on the sample size (Begon et al. 1990), which in microbiological terms is often used to describe the number of different species or different sequence types present in a habitat. The term diversity accounts for both abundance (or biomass) and species richness. Two common indexes of species diversity are the Shannon diversity index and Simpson's index (Begon et al. 1990). Community structure is a more demanding concept, since it involves quantitative information about the number of individuals in particular operational taxonomical units (OTUs). Most attempts to quantify microbial diversity to date have focused on species richness or taxonomic diversity at higher levels of the taxo-

nomic hierarchy such as genus, family, order, and phylum (O'Donnell et al. 1995). In culture-independent studies, numerical values of microbial diversity or similarity have very seldom been calculated (e.g. LaPara et al. 2001).

2.1.2 Species definition

Taxonomy is the theory of classification, which concerns the arrangement of organisms into groups or taxa. Identification is the mean by which unknown organisms are allocated to previously described taxa. All these terms pertain to systematics, which is the study of the diversity of organisms and their relationships (Austin & Priest 1986). Early classifications of bacteria were largely based on morphological information, as determined by light microscopy. Pure-culture techniques allowed the study of bacterial metabolism and physiology. More recently the term polyphasic taxonomy has been used to describe a classification where physiological information is supplement with data from chemical analyses and molecular biology (for reviews, see Busse et al. 1996, Vandamme et al. 1996). The phenetic classification of organisms concerns similarities shared by complete organisms (genotype and phenotype) with no reference to their evolutionary pathways, while phylogenetic classifications rely on rRNA, DNA or protein sequences and reflect the evolutional pathway of the organisms (Austin & Priest 1986).

A prokaryotic species can be defined as a collection of similar strains that differ sufficiently from other groups of strains to warrant recognition as a basic taxonomic unit (Staley & Krieg 1984). Species concept remains a difficult and controversial theme in bacterial systematics (Goodfellow & O'Donnell 1993, Young 1994). There are two main reasons for this: bacteria do not have enough sex; or they have too much sex (Young 1994). The first point is that bacteria reproduce by a binary fission, so that recombination is not a necessary part of their life cycle. The contrary argument is that horizontal gene flux can carry essentially any DNA from any bacterial species to any other (Young 1994). At the best, species classification in bacteriology is an artificial ranking, since bacteria form a species continuum. Traditional tests (morphological, biochemical, physiological and nutritional characterization) continue to be valuable as these phenotypic characters are important for delination of taxa (Busse et al. 1996). For the genetic classification and clear allocation of new bacterial species, whole genome DNA-DNA hybridization is thought to be the most favorable method, since it is not prone to errors caused by the horizontal transfer of a single gene. It is generally accepted that if two organisms have highly homologous DNA in a DNA-DNA hybridization analysis, they are genetically closely related. A relative DNA homology above 70% and a difference of 5°C or less between melting temperatures (ΔT_m) indicate a relationship at the species level (Wayne et al. 1987). DNA-similarity values between 20% and 60% give an indication as to genus (Johnson 1984). Values from 30% to 70% reflect a moderate degree of relationship (Stackebrandt 1991). There are several different methods to perform whole genome hybridizations (Grimont et al. 1988). The basis of the methods is

DNA reannealing: if a mixture of double-stranded DNA is heated and then cooled, homologous base sequences of will reassociate to form stable hybrids. Whole genome DNA reassociation techniques are rather time consuming and demanding, and only laboratories highly specialized in bacterial systematics can perform these analyses (Busse et al. 1996). The results may also depend on the technique used and therefore the analysis needs careful calibration.

Since the introduction of PCR and modern sequencing techniques, bacterial phylogenetic classification using rRNA sequences has become a standard. Despite some limitations (multiplication of genes, possibility of horizontal gene transfer) these sequences allow bacterial strains to be positioned in the species continuum and thus allow phylogenetic comparisons. In bacterial systematics, it is thought that organisms sharing more than 97% identity in their full-length 16S rRNA sequences might belong to one and the same species (Wayne et al. 1987). Stackebrandt and Goebel (1994) suggested that a 2.5% difference in 16S rRNA sequences should be sufficient to distinguish two sequences as belonging to two species. In comparative studies, strains with 16S rDNA sequence similarities below 97% never shared a DNA-DNA homology of more than 60% in whole genome hybridizations (Stackebrandt and Goebel 1994). In some cases, however, bacteria exhibiting more than 99% identity in their 16S rRNA sequences have been divided into two distinct species (e.g. many *Pseudomonas*).

In the recent discussion it has been stated that the traditional classification which combines phenotypic and phylogenetic data is inconsistent (Lan & Reeves 2001). Some important phenotypic characteristics used in traditional bacterial systematics, such as virulence, may be captured by lateral (horizontal) transfer and easily lost during adaptation to a new niche. Therefore, it was suggested that whole bacterial species concept should be revised to be solely based on phylogenetic analysis of several core genes (housekeeping genes) present in the majority of the members of each species (Lan & Reeves 2001). In this model, a concept 'clone' was presented to accommodate and separate different niche-adapted strains within the species.

2.1.3 The bacterial genome

Among prokaryotic species the size of the genome ranges from 0.6 to 11.6 Mb (Fogel et al. 1999). Over 50 microbial genomes have been completely or partially sequenced (Wren 2000). By January 2002, 16 microbial genomes were completed (http://www.tigr.org/tdb/). Bacterial genome analyses have shown that about half of the predicted coding sequences are of unknown function, and around half of these unknown sequences seem to be unique to individual microorganisms (Wren 2000).

Examples of active horizontal gene transfer come from members of the genera which have a special capacity for the uptake and chromosomal incorporation of DNA (Smith et al. 1991). Although transformation of new genetic material can occur across species boundaries, it has been suggested that the interspecies passage of chromosomal genes is mostly limited to members of the same genus (Lorenz & Wackernagel 1994). It has also been shown that bacteria

differing in DNA sequence by up to 20% can and do exchange chromosomal DNA, but the exchange is usually local, often involving only a few hundred base pairs (Smith et al. 1991). Since bacterial genomes do not grow ever larger in size, acquisition of DNA must be counterbalanced by the loss of genes providing smaller overall selective benefits (Fig. 2). The fixation and long-term persistence of horizontally transferred genes suggests that they confer a selective advantage on the recipient organism (Koonin et al. 2001).

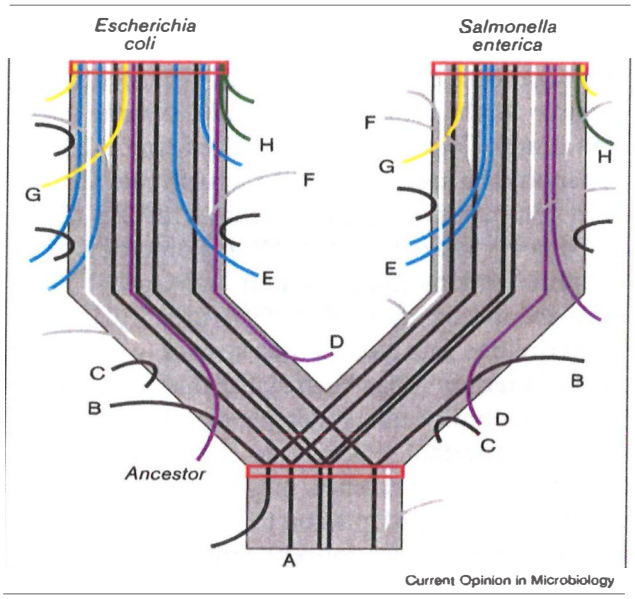

FIGURE 2 Evolution and molecular archaeology of bacterial chromosomes by gene loss and acquisition in *Escherichia coli* and *Salmonella enterica*. Although the bulk of the bacterial chromosome (red-outlined boxes) is inherited by vertical transmission (depicted in gray), large numbers of genes may be gained by horizontal transfer, including **A** acquired genes in both taxa present in a common ancestor, **B** acquireeed genes present in the common ancestor but lost in one lineage, **C** acquired genes that did not persist, **D** ancient acquired genes that catalyzed lineage differentiation (shown in magenta), E more recently acquired genes (shown in teal), **F** sequences lost by deletion (shown as gray arcs leaving the chromosome leaving white gaps behind), **G** and **H** transposons (yellow) and bacteriophage (green) DNA, which may have assisted the integration of useful DNA but is unlikely to confer selectable functions. (Taken from Lawrence 1999).

More information about horizontal gene transfer has been gained through comparative analysis of bacterial, archaeal, and eukaryotic genomes. Statistical analyses of bacterial genomes have shown that horizontal gene transfer events between major bacterial lineages explain a large proportion of bacterial genome; the estimates vary from 1.6% of the genes for *Mycobacterium genitalium* to 32.6%

in *Treponema pallidum* (Koonin et al. 2001). Acquisition of eukaryotic genes was detected in all the bacterial species studied (Koonin et al. 2001).

2.2 Phylogenetic chronometers in microbial classification

2.2.1 Phylogenetic chronometers

Phylogenetic chronometers are nucleotide or protein sequences that are valuable in analyzing the homology and evolutionary pathways of genes and organisms. A good phylogenetic chronometer for a taxonomic purpose should have a number of properties:

1) The molecule should be an essential constituent in all organisms.
2) Horizontal transfer of the gene has to be rare.
3) The evolutionary rate of the molecule should be high enough to separate closely related organisms.
4) Conserved regions allow alignment of the sequences derived from distantly related organisms and additionally allow a universal PCR amplification procedure for sequence determination.

The most widely used evolutionary chronometer in bacteriology is ribosomal RNA. What makes rRNA molecules so special? Amann et al. (1994) listed several reasons. First, rRNA molecules are composed of regions of higher and lower evolutionary conservation. Conserved regions enable researchers to align and compare homologous sequences and can serve as primer binding sites for *in vitro* amplification by PCR. For identification purposes the variable sequence regions provide sufficient data for researchers to reliably infer phylogenetic relationships. Variable sites are also appropriate targets for genus, species and sometimes even subspecies specific hybridization probes. The relatively high copy number (10^3 to 10^5) of rRNA molecules per cell allow visualization of individual microbial cells by fluorescently labeled rRNA-targeted oligonucleotide probes. Horizontal gene transfer of ribosomal genes had not been conclusively recorded in 1994. However, some evidence for the horizontal gene transfer of rRNA genes has been obtained recently (see next section). The practical reason for using rRNA and especially 16S rRNA sequences for phylogenetic studies is the availability of public databases that contain thousands of rRNA sequences for comparative analysis (Table 1).

TABLE 1 Some phylogenetic marker genes used in studying the genetic diversity of bacteria. Databases used: EMBL database (European Molecular Biology Laboratory, Heidelberg/Hinxton) release 23-Jun-2001 (3951820 sequences) and EMBLNEW database release 05-Jul-2001 (379181 sequences), sequence retrieval by the SRS program version 5.1.0 http://zeta.embl-heidelberg.de:8000/srs5/

Phylogenetic marker[a]	Sequences in the EMBL database	Search terms[b]
5S rRNA	697	[DE: 5S ! spacer] & [OC: Bacteria]
16S rRNA	39048	[DE: 16S ! spacer] & [OC: Bacteria]
23S rRNA	2127	[DE: 23S ! spacer] & [OC: Bacteria]
16S-23S ISR	2232	[DE: 16S & 23S] & [OC: Bacteria]
gyrB	605[c]	[DE: gyrB] & [OC: Bacteria]
nifH	775	[DE: nifH] & [OC: Bacteria]
recA	652	[DE: recA] & [OC: Bacteria]
rpoB	419	[DE: rpoB] & [OC: Bacteria]

[a]List of genes and abbreviations: ISR, internal spacer region; *gyrB*, DNA gyrase protein B; *nifH*, dinitrogenase reductase; recA, recombinase; *rpoB*, RNA polymerase beta-subunit
[b]DE, description; OC, organism. Multiple search terms are separated by '&' (and) and '!' (and not).
[c]830 *gyrB* sequences were available (10-Jul-2001) in the ICB database, Marine Biotechnology Institute, Iwate, Japan, http://seaweed.mbio.co.jp/icb/.

2.2.2 Ribosomal RNA molecules as phylogenetic chronometers

Ribosomal RNAs are functionally constant, universally distributed, and moderately well conserved molecules across broad phylogenetic distances (Brock et al. 1994). Ribosomes are sites of protein synthesis. In prokaryotes (domains Bacteria and Archaea) ribosomes consist of small (30S) and large (50S) subunits, yielding intact 70S ribosomes. S (Svedberg unit) refers to the sedimentation coefficient of ribosomal subunits or intact ribosomes when subjected to centrifugal force. The 30S subunit (small subunit or SSU) contains 16S rRNA and about about 21 proteins, while the 50S subunit (large subunit or LSU) contains 5S and 23S rRNA and 34 proteins (Brock et al. 1994).

Ribosomal RNA operons (*rrn* operons) have a peculiar and highly conserved anatomy in prokaryotes. As shown in Fig. 3, the common gene arrangement of the different ribosomal subunits within the *rrn* operon follows the order 16S-23S-5S (Gürtler & Stanisich 1996, Roth et al. 1998, Liao 2000). The primary structure of rRNAs is composed of regions showing different degrees of conservation, some being highly conserved whereas others are hypervariable (Ward et al. 1992, Fig. 4). The degree of similarity in ribosomal sequences between two organisms indicates their relative evolutionary relatedness (Fox et al. 1980, Woese 1987). As can be seen from Table 1, small subunit rRNA has become a widely used molecule in phylogenetical and environmental studies. In addition to 16S rRNA (around 1500 base pairs [bp]) the much smaller 5S rRNA (107 to 131 bp) has been used to study the phylogeny and evolution of bacteria (Hori & Osawa 1979, Stahl 1985). Although the calculated mean rate of substitu-

tion for 5S rRNA (1%/25 million years) is twice that for 16S rRNA (Ochman & Wilson 1987), the information value of 5S rRNA is limited due to the small size of the gene. After effective sequencing techniques have been developed, the use of 5S rRNA sequences has remained minor. The other large subunit RNA, 23S rRNA, (around 2900 bp) has larger information content than the small subunit rRNA molecule. This is not only because of its greater size but because large subunit RNA contains domains with higher mutation rates and rapid expansion. This has been revealed using eukaryotic counterparts of the molecules, 18S and 28S rRNA (Hassouna et al. 1984). Nonetheless, 16S rRNA has primarily been used in the work of developing the phylogeny of prokaryotes, because 16S rRNA sequences are experimentally more manageable than 23S rRNA (Madigan et al. 2000). With bi-directional sequencing, full-length 16S rRNA can be sequenced in a single reaction at best.

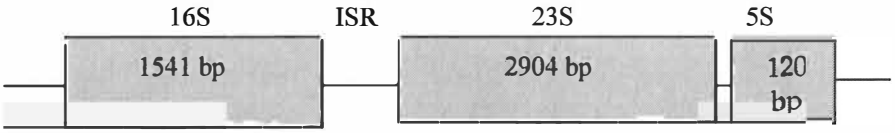

FIGURE 3 **Organization and lengths of ribosomal genes in the *Escherichia coli* genome. ISR is the internal spacer region between the ribosomal 16S and 23S genes.**

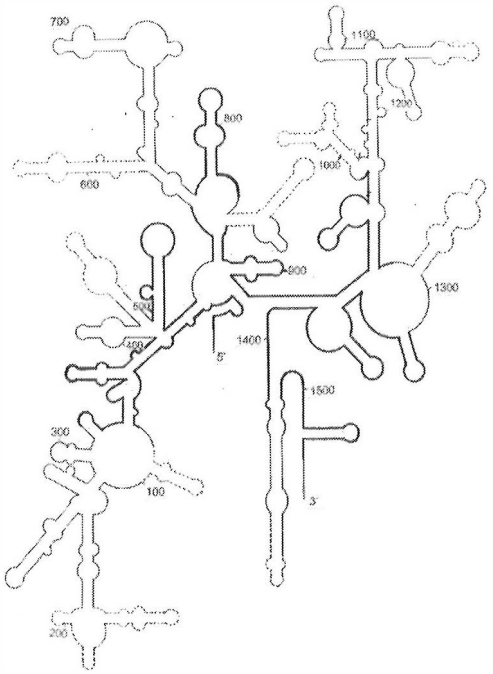

FIGURE 4 **Schematic of the *Escherichia coli* 16S rRNA secondary structure highlighting primary sequence domains of nearly universal conservation (thick lines), intermediate conservation (normal lines), or hypervariability (dashed lines). Nucleotides indicate positions of the nucleotides from the 5′ end, according to the *E. coli* 16S rRNA sequence (Brosius et al. 1978). (Taken from Ward et al. 1992.)**

The bacterial 16S rRNA gene sequence (16S rDNA) has proven to be a stable and specific phylogenetic marker. This marker and its eukaryotic counterpart, 18S rDNA, have been used to construct a universal tree of various microbial groups, plants and animals (Fig. 5). In this tree bacteria are separated into two phyloge-

netic Domains, termed Bacteria and Archaea, and the other microbial groups are grouped with the Plant and Animal Kingdoms in the Domain Eucarya.

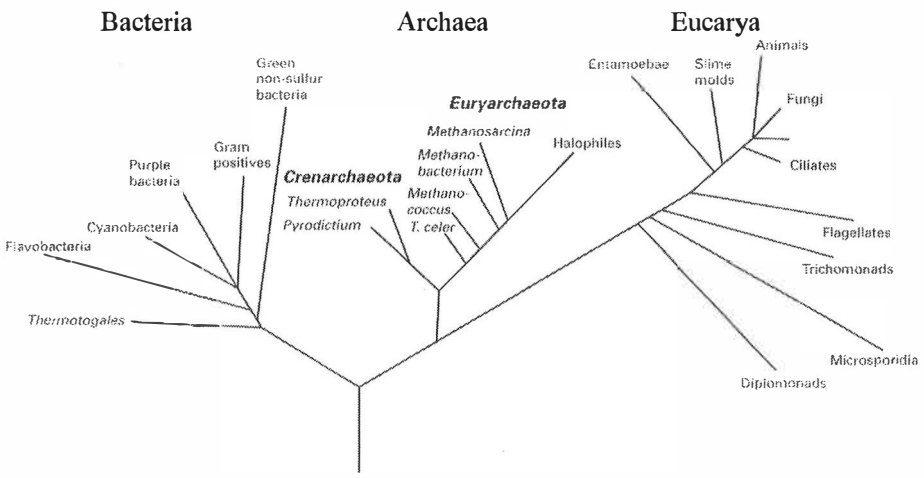

FIGURE 5 **The universal tree of life based on phylogenetic analysis of 16S and 18S rDNA of various microbial groups and plants and animals. (Taken from Woese 1994).**

One of the major pitfalls of the use of rRNA sequences is that the number of copies of rRNA operons vary from 1 to 15 among bacterial species (The Ribosomal RNA Operon Copy Number Database, http://rrndb.cme.msu.edu, 17-Jan-2002). Separate operons are dispersed throughout the bacterial genome (Fig. 6). The members of multigene families are subject to a homogenization process, which leads to concerted evolution (Ohta 1991). Gene conversion appears to play the major role in the sequence homogenization of bacterial rRNA genes (Liao 2000). The sequences of the rRNA gene have been found to be extremely homogeneous within a genome (Liao 2000), but recent findings also indicate the presence of some heterogeneity between different copies of 16S rDNA in some species, e.g. in *Escherichia coli* (Cilia et al. 1996) and in *Thermospora bispora* (Wang et al. 1997), as well as in a slowly growing mycobacteria resembling *Mycobacterium terrae* (Ninet et al. 1996) and *Mycobacterium celatum* (Reischl et al. 1998). The divergence between the two 16S rDNA sequences of *M. terrae* resembling strain was 1.2% (Ninet et al. 1996).

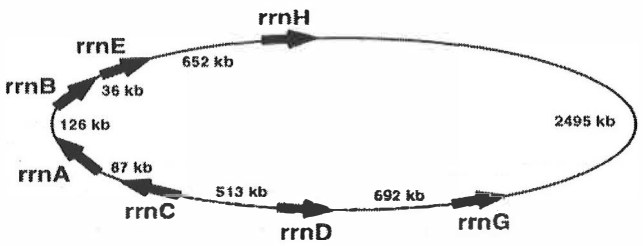

FIGURE 6 Genomic distribution of the rRNA genes in the 4639 kb *Escherichia coli* genome. The rRNA operons are depicted with arrows, and the distance (kb) between clusters is indicated. (Taken from Liao 2000).

With regard to the origin of divergent 16S rDNAs, horizontal gene transfer has been discussed in the halophilic archaebacterium *Haloarcula marismortui* (Mylvaganam & Dennis 1992). Horizontal transfer has also been shown in the thermophilic actinomycete genus *Thermomonospora* from *T. bispora*-like organism to *T. chromogena* (Yap et al. 1999), but has not yet revealed unsuspected relationships between different genera. Ribosomal genes have been extensively studied for over two decades, and if horizontal gene transfer had been a common event in bacterial evolution, this should have been reported more often. Essential genes, like those encoding ribosomal RNA, are unlikely to be transferred successfully, since recipient taxa would already bear functional orthologues (Lawrence 1999). These genes have experienced long-term coevolution with the rest of the cellular machinery and are unlikely to be displaced (Lawrence 1999).

2.2.3 Alternative phylogenetic chronometers

Although rDNA sequences have been extensively employed in bacterial identification at species level, this gene does not provide sufficient variability to resolve closely related strains. Another commonly used region for phylogenetic analysis is a stretch of DNA that lies between the 16S and 23S ribosomal genes, the 16S-23S **intergenic spacer region ISR** (for a review, see Garcia-Martinez et al. 1999), which is also called as intergenic transcribed spacer (ITS) or intergenic spacer (IGS). Analysis of the 16S-23S ISR has been applied to resolute strains within several species (Griffen et al. 1992, Gürtler & Stanisich, 1996, Wakefield 1998, Aakra et al. 1999, Rumpf et al. 1999). Some tRNA–encoding sequences can frequently be found inside the the ISR. The ISR can be amplified by PCR, using general bacterial 16S and 23S rRNA gene primers complementary to the conserved sequences flanking the ISR. In contrast to rRNA genes, ISR sequences are under minimal selective pressure during evolution, except the stretches of tRNA-coding sequences. According to Leblond-Bourget et al. (1996) the evolutionary rate of ISR is about 10 times greater than the evolutionary rate of the 16S rDNA. The length of the 16S-23S rRNA gene intergenic spacer region varies considerably between species (200-1500 bp) (Gürtler & Stanisich, 1996). However, the high level of sequence variation, insertions and deletions in the ISR

may bias the phylogenetic analysis of distantly related organisms. The method could be used as a tool to clarify controversial strain and species definition (Aakra et al. 1999). ISR sequences can be an excellent tool for species characterization, but in the case of multiple ribosomal operons and intercistronic heterogeneity (incomplete gene homogenization between operons), the mapping of phylogenetic relationships can be difficult (Boyer et al. 2001).

Theoretically any **protein-coding sequences** can be used in the phylogenetic analysis of organisms, if they have not spread horizontally between species. Protein-coding genes evolve fast, as synonymous substitutions do not induse changes in the amino acid sequences of their products. For protein-coding sequences, analyses can be performed based on either the nucleic acid or the amino acid sequence data. Although the analysis based on nucleic acid sequences would seem to be more informative, bases on the third codon positions are selectively neutral (random in nature) and their inclusion would lead to reduced certainty of the analysis in distantly related taxa (Gupta 1998). Differences in the genomic G + C content of species may make additional bias in the analysis, since two unrelated species with similar G + C content may have very similar bases in the third codon positions (Gupta 1998)

The gene encoding the type II topoisomerase DNA, B subunit (*gyrB*) is an example of the kind of genes used in taxonomical analysis (Yamamoto & Harayama 1996, Yamamoto & Harayama 1998). The average base-substitution rate of 16S rRNA genes is 1% per 50 million years, while that of *gyrB* is 0.7-0.8 % per one million years at synonymous sites (Yamamoto & Harayama 1996). When 20 *Pseudomonas* strains were compared by sequencing the genes for 16S rRNA, RNA polymerase σ^{70} factor (*rpoD*) and *gyrB*, the phylogenetic trees based on *gyrB* and *rpoD* were congruent, but the topology of the 16S rDNA tree was different. The topology of the 16S rDNA tree bore greater similarity to the other trees when the highly variable regions were excluded from the phylogenetic analysis, although the resolution contained to remain poor. Variable regions of the 16S rRNA did not correlate with the synonymous substitutions in the protein-coding genes, and thus it was suggested that highly variable regions should not be included in the calculation of genetic distances (Yamamoto & Harayama 1998).

Protein-coding genes could be an interesting alternative or addition to ribosomal sequences in taxonomical work as well as in community analysis. Differences between well resolved molecular phylogenies can be due to unrecognized gene duplication or to horizontal gene transfer (Gogarten et al. 1996). However, amplification of protein-coding sequences from phylogenetically diverse bacteria is difficult, since synonymous substitutions are equally located throughout the whole sequence and universal primers are difficult to design.

2.3 Community fingerprinting by broad-range bacterial PCR

2.3.1 The use of rRNA methods in microbial ecology

The first ecological applications of direct rRNA methods were based on RNA cloning/sequencing techniques and oligonucleotide hybridization (Stahl et al. 1985, Olsen et al. 1986, Pace et al. 1986, Giovannoni et al. 1988, Amann et al. 1990, Ward et al. 1990, Schmidt et al. 1991, Fuhrman et al. 1992). At the beginning of the 1990s these approaches were used in the analyses of hot spring environments, marine and freshwater environments, soil and rhizosphere, insect symbionts, pathogens in host environment, experimental bioreactors and in the food and copper leaching industry (for a review, see Ward et al. 1992). Analysis of microbial community composition and structure can be seen as a cycle of cloning and sequencing efforts and, on the basis of the sequences collected, development and use of probes for hybridization to confirm the occurrence of specific microorganisms or phylogenetic groups within the community (Fig. 7).

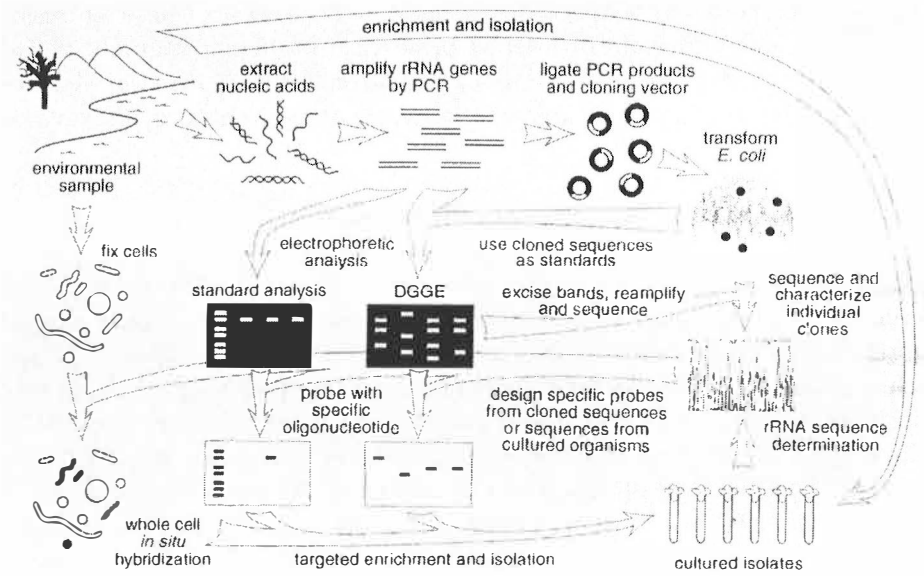

FIGURE 7 General approaches in the rRNA methods in microbial ecology. DGGE is described in chapter 2.3.2. (Taken from Head et al. 1998).

The starting point for sequence retrieval can be either rRNA molecules or the rRNA genes themselves (rDNA). The information retrieved from these two sources is different and can lead to different results (Griffiths et al. 2000). The rRNA gene pool should reflect the contributions of all the inhabitants of the community sampled, and thus should provide a measure of community composition. The abundance of the gene product, rRNA, theoretically provides both qualitative and quantitative information about the most actively growing mem-

bers of the community (Ward et al. 1992). Studies of a few well-characterized microorganisms have shown that ribosome content (and thus rRNA content) is correlated with growth rate (Srivastava & Schlessinger 1990).

In the case of many environmental samples and clone libraries, only partial 16S rDNA sequences have been determined. This has usually been the first 200-500 nucleotides from the 5′ terminus or the last 400 to the 3′ terminus of the 16S rDNA. It would seem that the first 500 nucleotides from the 5′ terminus of the 16S rDNA contain enough information to allow accurate assignment to one of the main lines of descent (bacterial phyla), and can thus be recommended as the region to concentrate on in future studies of this nature (Liesack et al. 1997). More complete sequence information can be derived from pure cultures isolated in parallel studies.

Nucleotide and protein sequences are available in the three databases belonging to the International Nucleotide Sequence Database Collaboration. These database are EMBL Nucleotide Sequence Database (United Kingdom), Genbank (United States of America) and DNA Data Bank of Japan, DDBJ (Japan). The databases interact with each other so that sequences submitted to one of them are soon included in the other databases, as well. In addition to these three major databases, several other specialized databases have been set up. These address a particular biological question of interest or serve a particular segment of the biological community. The reviewed databases are collectively listed in Molecular Biology Database Collection (http://www.nar.oupjournals.org) (Baxevanis 2001).

2.3.2 Analysis of broad-range bacterial PCR products

To analyze bacterial diversity without cultivation, ribosomal RNA genes can be amplified with broad-range bacterial primers. These primers can cover a large proportion of phylogenetically different bacterial sequences. This PCR approach is called universal bacterial PCR or broad-range bacterial PCR. In this dissertation the term broad-range bacterial PCR has been adopted, because of the limitations that the 'universal' amplification may have (section 2.3.3.2). After amplification, the PCR product must be analyzed. Because large numbers of samples are collected in surveys assessing the temporal and spatial dynamics of bacterial populations in natural ecosystems, the method used to conduct community profiling should be rapid and simple.

The first molecular profiling methods for the analysis of bacterial communities were based on direct analysis of the short 5S rRNA and tRNA molecules by high-resolution acrylamide gels (Höfle 1992, Höfle 1993, Bidle & Fletcher 1995). The size differences measured were, however, very limited. With the availability of the polymerase chain reaction (PCR) (Saiki et al. 1988), direct sequencing of ribosomal sequences rapidly became the method of choice. New methods for the separation of heterogeneous PCR products have been developed during the past decade (Fig. 8).

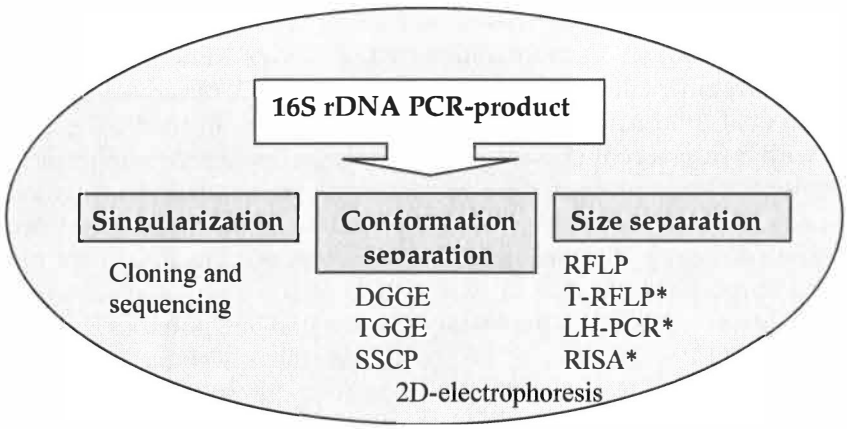

FIGURE 8 Possible methods for the separation of heterogeneous broad-range bacterial PCR products derived from multiple templates. Methods marked with an asterisk are automated and do not allow further processing of the interesting bands (sequencing). 2D-electrophoresis utilizes both conformation and size separation. Abbreviations are described in the text.

Cloning and sequencing. In the cloning-sequencing approach (e.g. Ward et al. 1990) a 16S rDNA or rRNA clone library is constructed from the PCR product amplified with universal or domain-specific primers. This is usually followed by sequencing tens or hundreds of clones, or by selecting different types of clones (OTUs, operational taxonomical units) before sequencing. Despite its many applications, the cloning and sequencing approach requires a lot of time and effort and hence cannot provide an immediate overview of the structure of whole bacterial assemblages.

RFLP analysis. Restriction fragment length polymorphism analysis of 16S rDNA (16S RFLP) (Avaniss-Aghajani et al. 1994), also known as amplified rDNA restriction analysis (ARDRA) (Vaneechoutte et al. 1992), has been used as a rapid method for the comparison of rDNA sequences. Briefly, rDNAs are obtained by PCR amplification using broad-range primers, and the product is digested with restriction enzymes, usually with a 4-bp recognition site. When a computer-simulated analysis of 10 tetrameric restriction enzymes was conducted, it was found that the mean number of restriction sites varied from 3.71 to 5.65 fragments per bacterial 16S rDNA sequence (Moyer et al. 1996). The analysis of the digestion products is performed by using high-resolution polyacrylamide gels or by using agarose gels with lower resolution. High-pressure capillary electrophoresis has also been suggested as a way of improving the analysis of the results (Moyer et al. 1996). Although the method has mainly been used in the analysis of pure cultures and clone libraries (e. g. Weidner et al. 1996, Weidner et al. 2000), it could also have some use in community analysis. In community fingerprinting, 16S RFLP could be used in demonstrating the dominance of specific phylogenetic groups or estimating species evenness or

richness. In samples with high species diversity multiple restriction fragments generated from one template make comparisons complicated.

T-RFLP analysis. A more automated system of RFLP analysis is terminal restriction fragment length polymorphism (T-RFLP) (Avaniss-Aghajani et al. 1996, Liu et al. 1997, Clement et al. 1998). At least one of the PCR primers is labeled with a fluorescent dye so that when the preparation is analyzed with an automated DNA sequencer, the size of the terminal restriction fragment is determined. A computer analysis of about 1600 bacterial sequences (Brunk et al. 1996) showed that in the majority (73%) of cases terminal fragment restriction length polymorphism is due to variation in indels (gaps and insertions), and only to a lesser extend it is the result of restriction site polymorphism. Simulations of T-RFLP patterns can be generated by a web-based research tool (http:// www.cme.msu.edu/RDP/html/analyses.html) (Marsh et al. 2000).

LH-PCR. Length heterogeneity analysis (LH-PCR) (Suzuki et al. 1998) of PCR amplified 16S rDNA is also an automated method that utilizes naturally occurring differences in the lengths of amplified gene fragments. The abbreviation "LH-PCR" has been used so far only in the analysis of 16S rDNA fragments. As a rule, strong length heterogeneity seems to be most common in the apical helices of ribosomal molecules, i.e. those ending in a hairpin loop (Van de Peer et al. 1996). The band sizes can be compared against 16S rDNA databases to specify bacterial groups that may correspond in size to the size of the band.

DGGE and TGGE. Denaturing (DGGE) and temperature (TGGE) gradient gel electrophoreses are analyses that rely on sequence-dependent differences in the melting temperatures of relatively short DNA fragments. DGGE was introduced to microbial ecology by Muyzer et al. (1993). Later on, TGGE was taken from the medical sciences to study microbial communities (Heuer et al. 1997). In these analyses, DNA fragments move through a polyacrylamide gel containing a linear gradient of increasing DNA denaturant concentration (in DGGE) or temperature (TGGE). As the DNA molecule enters the increasing concentration of denaturant or the temperature at which the domain that has the lowest melting temperature melts, it forms a branched structure which has a lower mobility in the gel matrix. The stability of the melted PCR-product can be improved by attaching a GC-rich extension ('clamp') to the 5′ end of one of the PCR primers. DGGE has undoubtedly been one of the most popular screening methods in molecular microbial ecology.

SSCP. Single-strand conformation polymorphism is a technique that was introduced for studying bacterial community structures by Lee et al. (1996). Under nondenaturing conditions a PCR-amplified and denatured fragment of the 16S rRNA gene forms a folded structure. The electrophoretic mobility of the partially double-stranded DNA fragment in a gel is dependent on its molecular weight but also on the shape of the re-folded molecule.

RISA. Length heterogeneity analysis of the ribosomal intergenic spacer region (RISA) (Borneman & Triplet 1997) is analogous to LH-PCR. Length variation of the spacer region is considerable between different species (Condon et al. 1995). When the method is performed using at least one fluorescently la-

beled primer and an automated sequencing machine it is called ARISA (Fisher & Triplet 1999). The 16S-23S intergenic spacer analysis can also be used for community analysis as a fingerprinting tool. The main limitation of this technique derives from the unpredictability and random pattern of variations in spacer size (reviewed by García-Martínez et al. 1999). In the case of multiple ribosomal operons within a strain, the sizes and sequences of different 16S-23S PCR amplicons may vary considerably in a single bacterial cell (Condon et al. 1995). In community profiling procedures this means that a single species may contribute more than one peak to the community profile.

2-D electrophoresis. Two-dimensional electrophoresis has been used in genetic mutation analyses (Uitterlinden 1995), but could be used in microbial profiling as well. In current applications the sequence length analysis is done first in non-denaturing conditions and DGGE is the second dimension. Instruments for easy performance of the 2-D electrophoresis, also known as two-dimensional gene scanning (TDGS) are available. No reports on community fingerprinting by 2-D-electrophoresis have been found as yet in the literature.

2.3.3 Analytical difficulties with PCR methods

2.3.3.1 Sources of the bias: DNA extraction

Not only cultivation studies but also molecular investigations include critical steps that can induce a bias in the results. In PCR-based procedures these steps include efficient extraction of the community DNA, selection of PCR primers and PCR amplification itself. Extraction of the DNA from a laboratory-cultivated bacterial strain is relatively easy, but the extraction methods can seriously limit the analysis of bacterial communities, mainly owing to incomplete and variable cell lysis of different bacterial groups in difficult matrixes. Isolation of the DNA is based either on a direct lysis approach or on cell separation prior to lysis (Saano & Lindström 1995). Numerous reports (e.g. Ogram et al. 1987, Tsai and Olson 1991, Picard et al. 1992, Smalla et al. 1993, Moré et al. 1994, Porteous et al. 1997, Stach et al. 2001, Martin-Laurent et al. 2001, Niemi et al. 2001) and reviews (Liesack et al. 1997, von Wintzingerode 1997) have been published on DNA extraction methods through enzymatic, chemical and mechanical cell disruption. Mechanical cell lysis through homogenization with glass beads is an attractive alternative, since it is effective in disrupting even *Bacillus* endospores (Moré et al. 1994). The cell lysis step in the different DNA extraction methods has usually been evaluated by measuring the amount of the extracted DNA, but also by using direct microscopic examination, sample cultivation, and, more recently, community fingerprinting techniques such as 16S RFLP and RISA (Martin-Laurent et al. 2001) and DGGE (Niemi et al. 2001).

PCR amplification can be inhibited by sample contaminants, such as humic acids, phenolic compounds, blood, heparin, milk, bacterial debris, polysaccharides, buffers and detergents (reviewed by Rossen et al. 1992, Wilson 1997). Soil is one of the most difficult matrixes due to interference by humic acids or other soil components. To avoid interference by humic acids, laborious proto-

cols for DNA purification by cesium chloride gradient centrifugation (Steffan 1988), column purification (Miller 2001), magnetic/immunomagnetic capture separation (Jacobsen 1995) and gel electrophoresis (More et al. 1994) have been necessary. It may be possible to overcome the inhibition of PCR amplification by adding additives such as bovine serum albumin or T4 gene 32 protein (Kreader 1996, McGregor et al. 1995).

2.3.3.2 Choice of primers

The choice of PCR primers has a profound effect in the analysis of bacterial communities and can lead to a systematic PCR bias. The primers described by Lane (1991) have been widely used for purposes of sequencing bacterial isolates. Universal primer candidates have also been presented in several other studies (e.g. Giovannoni et al. 1990, Weisburg et al. 1991, Amann et al. 1995, Muyzer et al. 1993, Muyzer et al. 1996, Liu et al. 1997, Marchesi et al. 1998). Many primer and probe sequences are collected in the Molecular Probe Database (http://srs.ebi.ac.uk). Comparative analyses of broad-range bacterial primer combinations have been made by Weisburg et al. (1991), Brunk et al. (1996) and Liu et al. (1997). For the evaluation of primer (and probe) specificity a useful tool is the probe match utility of the Ribosomal Database Project (http://rdp.cme.msu.edu/html/analyses.html, Maidak et al. 2000). As more and more sequences have been generated, it has become clear that "universal" primers should be termed "general" primers, since variation exists even in the most highly conserved regions of the molecule. Mismatches between PCR primers and a template selectively reduce amplification efficiency, and therefore in "degenerate" primers degenerate nucleotides (mixtures of nucleotides) are often used in places with sequence heterogeneity. It has been reported that biases in template-to-product ratios occur in PCR with degenerate primers, i.e. templates containing GC-rich (guanine- and cytosine-rich) priming sites were preferentially amplified (Polz and Cavanaugh 1998). Introduction of deoxyinosine residues at positions where mismatches are frequently found has been suggested as a way of reducing this bias, although some inosine–containing primers did not function properly in the PCR amplification, probably owing to reduced annealing temperature (Watanabe et al. 2001). Another way of reducing PCR bias due to different primer binding energies is to use lower hybridization temperatures (45°C instead of 55°C) (Ishii & Fukui 2001).

Although theoretical testing is usually the starting point for primer design, empirical testing is also essential to confirm primer specificity. Systematic empirical testing of primers has shown that some primer pairs with more mismatches can more consistently amplify 16S rDNA from template DNA in a variety of organisms than primer pairs with fewer mismatches (Marchesi et al. 1998). Although the reason was not known, the authors suggested that this was caused by the formation of intramolecular duplexes within the primers, or a low hybridization potential in general.

2.3.3.3 Other criticism of broad-range bacterial PCR

The sensitivity of the PCR may be a negative aspect of broad-range bacterial PCR procedures, since one of the most commonly reported problem is the obtaining of false-positive results due to cross-contamination (Vaneechoutte & Van Eldere 1997). This problem is especially critical when handling clinical samples with few bacterial cells. Contamination of *Taq* DNA polymerase hindered the use of a TaqMan real-time PCR system for the detection of bacteria in clinical samples by the amplification of an approximately 100 bp 16S rDNA sequence with universal primers (Corless et al. 2000). Decontamination of the enzyme by UV-irradiation, 8-methoxypsoralen and DNase I treatment decreased the sensitivity of the PCR amplification (Corless et al. 2000).

Is the relative abundance of amplicons proportional to the actual abundance of organisms in the sample? Even in theory, the answer is no. The variation in rRNA operon copy numbers introduces bias in PCR-based screening methods, when the starting material is rDNA. Dependence of the amount of the amplification product on the number of ribosomal operons has been demonstrated (Farrelly et al. 1995), and could be a major limitation of quantitative rDNA-based community analysis. The number of rRNA genes correlates with the rate at which bacteria can respond to resource availability (Klappenbach et al. 2000); therefore the results may be underestimations of oligotrophic species. When using rRNA as a starting material the PCR product reflects the active pool of the bacterial flora.

Are standard PCR reactions then quantitative? Is the relative abundance of amplicons proportional to the abundance of genes in the starting mixture? It has been suggested that the relative amount of different amplicons obtained after PCR amplification may not be proportional to the relative content of the respective templates (Lipski et al. 2001). Systematic biases induced by the PCR procedure have been detected in several studies (reviewed by von Wintzingerode et al. 1997). When using degenerate primers overamplification of specific templates was observed; this was explained by different primer-binding energies, i.e. templates containing GC-rich priming sites were preferentially amplified (Polz & Cavannaugh 1998). Bias was reduced considerably by using high template concentrations, by performing fewer cycles, and by mixing replicate reaction preparations. It has been suggested that the PCR product could compete with the primers at higher product concentrations and cause a kinetic bias that favors the templates of minor original concentrations (Suzuki & Giovannoni 1996, Suzuki et al. 1998). On the other hand, different results have also been obtained. Fisher and Triplet (1999) examined the effect of the PCR cycle by ARISA with freshwater bacterial samples. They found that the patterns of the major amplification products were not significantly altered when the PCR cycle number was reduced from 30 to 15, but the minor products (near the limit of detection) were slightly sensitive to changes in cycling parameters.

PCR-generated artifacts (i.e. chimeric sequences, mutations, and heteroduplexes) can also lead to overestimation of community diversity (von Wintzingerode et al. 1997). The type of enzyme, number of PCR cycles, length of the

extension time and finally, species diversity in the sample have been shown to have a strong effect on the level of this bias (Qiu et al. 2001).

2.3.4 Competing molecular methodologies: advantages and disadvantages

Critics of PCR-based community studies and supporters of fluorescence *in situ* hybridization (FISH) techniques, in particular, have proposed that the PCR-based deduction of community composition may be biased. It has been suggested that if absolutely quantitative data on community diversity is required, the PCR-amplification step must be avoided and direct hybridization of the extracted nucleic acids (RNA or DNA) or whole cell (*in situ*) hybridization should be applied (Lipski et al. 2001). A fairly large number of oligonucleotide probes specific for higher microbial taxa are already available. Specific probes for metabolically interesting bacterial groups are also available, such as those for detecting microbial taxa involved in the formation of activated sludge, phospate removal, nitrification, the production and oxidation of methane, the reduction of sulfate, microbial leaching, and the production/spoilage of food or the contamination of drinking water (reviewed by Ward et al. 1992, Lipski et al. 2001). Specific hybridization of whole fixed microbial cells was first achieved by Giovannoni et al. (1988) using radiolabelled oligonucleotides in combination with microautoradiography. Fluorescent oligonucleotides can detect individual bacterial cells more rapidly (DeLong et al. 1989, Amann et al. 1990). However, in many environments fluorescent oligonucleotide probing can not currently be used due to the following obstacles (Ward et al. 1992, Amann 1994):

1) Surrounding material (e.g. animal or plant tissue) can form a highly fluorescent background.

2) Fixed microbial cells can show autofluorescence.

3) Microorganisms may contain too few ribosomes due to suboptimal environmental conditions.

Consequently, the hybridization signal may not be detected against background fluorescence (Hahn et al 1992). Some reports have shown that only 40 to 50 % of particles stained with DAPI were detected by hybridization (Glöckner et al. 1999).

Ideally, a fingerprinting technique should require no prior investment in terms of sequence analysis, primer synthesis, or characterization of DNA probes. Over the past ten years a number of such fingerprinting methods have been developed, including random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and arbitrarily primed PCR (AP-PCR) (Welch & McClelland 1990). Amplified fragment length polymorphism (AFLP) is also a new DNA fingerprinting technique that combines the RFLP technique and the PCR technique (Vos et al. 1995). These fingerprinting methods have the major disadvantage that they are very sensitive to reaction conditions, DNA quality and PCR tempera-

ture profiles (Riedy et al. 1992, Ellsworth et al. 1993). They are excellent tools to study pure cultures, but in samples with higher bacterial diversity they may generate patterns that are complex and difficult to interpret.

Nucleic acid microarrays look very promising and applications in cell and molecular biology. Large numbers of hybridizations can be performed simultaneously, which is a clear advantage. On glass slides, over 100 000 spots of DNA fragments can be accomodated per cm^2. By determining the bacterial genome relatedness using DNA microarrays some of the limitations of the traditional DNA-DNA hybridization analysis can be avoided (Cho & Tiedje 2001). Recent results show that microarray hybridization has potential as a tool for revealing gene composition in natural microbial communities; but the relative abundance of targeted genes in complex environmental samples is less clear, due to divergent target sequences (Wu et al. 2001). What the role of DNA microarrays will be in the microbial ecology in the future remains an open question.

2.4 Introduction to the research themes

2.4.1 Biodegradation of chlorophenols

2.4.1.1 Chlorophenol contamination and bioremediation in Kärkölä

Annual global production of 150 different organohalogen compounds containing chlorine, bromide, iodide and fluorine, has been estimated at approximately 20 million tons (Gribble 1996). Pentachlorophenol (PCP) and various chlorophenol mixtures have been extensively used in agriculture and in wood treatment against blue staining and soft rot fungi all over the world, and has thus led to long-lasting accumulation of the toxicant in a number of places. In addition to biocide use, chlorinated phenolics are produced during chlorine bleaching of pulp (Suntio et al. 1988, Jokela & Salkinoja-Salonen 1992). The chlorophenol contamination of soil and groundwater has been detected in many sawmill locations in Finland (Kitunen et al. 1987, Lampi et al. 1990). Studies on chlorophenol degradation in soil and groundwater have shown that these compounds are stable under environmental conditions (Valo et al. 1990).

A groundwater aquifer in Kärkölä, Finland, has been the target of a severe, long-term exposure to polychlorinated phenols. The adjacent sawmill used the fungicide KY-5, a mixture of 2,3,4,6-tetrachlorophenol (TeCP), 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) from the 1940s up to 1984, when the manufacture of this wood preservative for sawmill use was discontinued. The sawmill was accidentally destroyed by fire in 1976 and contamination of the surrounding groundwater was detected in 1987. Chlorophenol concentrations as high as 56-190 mg/l were measured in the groundwater between the drinking-water intake plant and the sawmill (Lampi et al. 1990). The groundwater has a low concentration of dissolved oxygen (<1 mg/l) and high

concentrations of ferrous iron (7-16 mg/l) (Salminen 1999). The narrow, heavily contaminated plume was 8-30 m subsurface (Valo et al. 1990). Commercial chlorophenol formulations also include some impurities, such as polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs) (Kitunen et al. 1985), but these have been shown to immobilize mainly in the top layer of the soil (Kitunen et al. 1987). During the last ten years, chlorophenol concentrations have not substantially decreased in the Kärkölä aquifer (Salminen 1999).

Bioremediation of chlorophenols has been successfully used in soil and groundwater decontamination (for reviews, see Häggblom & Valo 1995, Puhakka & Melin 1996, 1998). Early attempts to treat the Kärkölä groundwater included a batch process with immobilized *Mycobacterium chlorophenolicum* PCP-1 culture (Valo et al. 1990), an activated sludge process in the municipal treatment plant (Ettala et al. 1992), and activated carbon filtration (Järvinen et al. 1996). To achieve an efficient and a more economical viable solution, a fluidized-bed polychlorophenol bioremediation process at ambient groundwater temperature was developed (Järvinen & Puhakka 1994, Järvinen et al. 1994). A full-scale treatment of Kärkölä groundwater begin in January 1995, and its performance has been monitored ever since.

2.4.1.2 Chlorophenol degradation at molecular level

Degradation of polychlorinated phenols by aerobic and anaerobic bacteria and fungi has been reported (for reviews, see e.g. Häggblom & Valo 1995, McAllister et al. 1996, Puhakka et al. 2000). Many of the well-known aerobic polychlorophenol-degrading bacteria belong to the genus *Sphingomonas* (Karlson et al. 1995, Nohynek et al. 1995, Nohynek et al. 1996, Cassidy et al. 1999). Recently, the genus *Sphingomonas* was divided into four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi et al. 2001). All these four genera together with their close relatives belong to the glycosphingolipid-containing α-4 subclass of the Proteobacteria cluster, referred to hence for the sphingomonads.

The pathway and the enzymes for microbial degradation of pentachlorophenol is known in detail in *Sphingobium chlorophenolicum* ATCC 39723 (Xun & Orser 1991, Orser & Lange 1993, Lange et al. 1996, Leung et al. 1999, Copley 2000) (Fig. 9). The rate-limiting step for PCP degradation in *S. chlorophenolicum* is the conversion of pentachlorophenol (PCP) to 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCH) (McCarthy et al. 1997). This step is catalyzed by the enzyme PCP-4-monooxygenase (molecular weight 60 kDa), encoded by the *pcpB* gene (Xun & Orser 1991a, Orser & Lange 1993). In addition to PCP, the PCP-4-monooxygenase enzyme can use many other halogenated phenols as a substrate (Xun et al. 1992a). The protein is a flavoprotein having two highly conserved domains which are thought to be involved in binding the flavin adenine dinucleotide (FAD) molecule (Orser & Lange 1994). The next step in the chlorophenol degradation pathway happens through reductive halogenation to tri-

and dichloro-p-hydroquinone (TrCH and DiCH) with gluthathione as the reducing agent by TeCH reductive dehalogenase encoded by the *pcpC* gene (Xun et al. 1992b, Xun et al. 1992c, Orser et al. 1993). The conversion of the dichlorohydroquinone to 2-chloromaleylacetate is catalyzed by a periplasmic protein 2,6-dichloro-*p*-hydroquinone 1,2-dioxygenase encoded by the *pcpA* gene (Xun & Orser 1991b, Lee & Xun 1997, Xun et al. 1999).

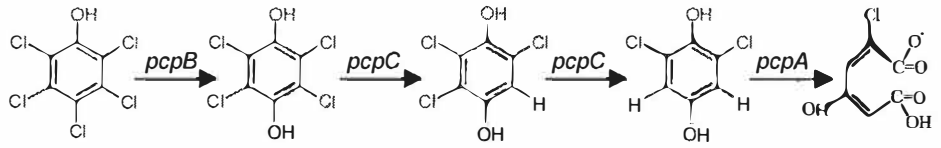

FIGURE 9 Pathway of PCP metabolism by *Sphingobium chlorophenolicum* ATCC 39723.

Sequence data acquired from the region downstream of *pcpB* has revealed two open reading frames (ORFs). The first of these (*pcpD*) bears high degree of similarity to the oxygenase reductases, and could be a protein involved in the electron transfer from NADPH to FAD, which is a prosthetic group of PCP-4-monooxygenase (Orser & Lange 1994). The second gene (*pcpR*) was very similar to members of the LysR family of transcriptional activators (Orser & Lange 1994).

In contrast to the *Sphingomonas* pathway, little is known about the enzymology recruited by other bacterial groups for the degradation of polychlorophenols. A chlorophenol-4-monooxygenase from *Burkholderia cepacia* has been isolated and characterized. This two-component protein (22 kDa and 58 kDa) was able to catalyze *para*-hydroxylation of 2,4,5-TCP, 2,4,6-TCP, 2,3,5,6-TeCP and even (although extremely slowly) PCP to corresponding chloro-*p*-hydroquinones (Xun 1996). Also a 2,4,6-trichlorophenol-4-monooxygenase has been purified from *Azotobacter* strain GP1 (Wieser et al. 1997). The enzyme (60 kDa) was shown to be a flavoprotein that formed homotetramers and also catalyzed *para*-hydroxylation of several other trichlorophenols and tetrachlorophenols, but not PCP (Wieser et al. 1997). Also the polychlorophenol metabolism in *Pseudomonas cepacia* AC1100 was initiated by *p*-hydroxylation by a flavin-containing monooxygenase (Tomasi et al. 1995). It has been shown that *'Rhodococcus chlorophenolicus'*, later reclassified as *Mycobacterium chlorophenolicum* (Häggblom et al. 1994), catalyzes the first degradation step, also a *para*-hydroxylation, by a cytochrome P450 enzyme (Uotila et al. 1991).

The enzymes for reductive dechlorination in anaerobic bacteria have not been characterized in detail. It is interesting that an anaerobic organism, *Desulfitobacterium frappieri*, PCP-1 was capable of dehalogenating at the *ortho*, *para* and *meta* positions a large variety of aromatic molecules with substituted hydroxyl or amino groups (Dennie et al. 1998). However, anaerobic degradation of chlorophenols is often partial and does not always lead to mineralization (Magar et al. 1999). Biodegradation of PCP by the white rot fungus *Phanerochaete*

chrysosporidium has been shown to be dependent on ligninases, but some other system may also be responsible for some of the PCP degradation (Mileski et al. 1988).

2.4.2 Aerobic thermophilic wastewater treatment

Many industrial wastewaters are hot (about 50-60°C), but biological processes are currently run under mesophilic conditions, at temperatures below 40°C. Biological treatment at high temperatures could be a preferred option in these cases, since it would reduce the operating and investment costs. Heat exchangers would not be needed and simpler process configurations could be applied. A key design criterion for thermophilic processes is biomass separation, since it is known that thermophilic bacteria may fail to aggregate (LaPara & Alleman 1999). Nonflocculating biomass resists conventional methods of cell separation from treated wastewater. In biofilm processes, such as the suspended carrier biofilm process (SCBP), biofilm is generated on the carrier surface and the biomass may thus escape washing. It has been suggested that thermophilic aerobic biofilm processes have stable performance, high loading rates and short hydraulic retention times (HRT's) (Barr et al. 1996, Malmqvist et al. 1996, Ragona & Hall 1998, Malmqvist et al. 1999).

The inefficient sludge separation in thermophilic bioreactors may a consequence of the lack of sludge–forming species, since cultivation studies have shown that these systems often carry predominantly *Bacillus* and *Bacillus*-like organisms (LaPara & Alleman 1999). However, the results obtained using cultivation may be biased due to selectivity of the procedure. Observations of quinone profiles in a solid-phase aerobic bioreactor treating high strength organic wastewater showed that the microbial diversity varied with temperature. At 50° and 60°C menaquinone-7 dominated (Lim et al. 2001), which is characteristic of *Bacillus* species. In a thermophilic aerobic wastewater reactor treating synthetic wastewater the community was mainly composed of *Bacillaceae* (Kurisu et al. 2002). Band-counting analyses of the PCR-DGGE results from an aerobic process treating pharmaceutical wastewater suggested that elevated reactor temperatures correlated with reduced species richness (LaPara et al. 2000). However, it is well known that several Gram-negative aerobes, including species of the genera *Hydrogenomonas*, *Thermomicrobium* and *Thermus*, can tolerate temperatures over 50°C (Tansey & Brock 1978).

2.4.3 Flavobacteriosis in the Finnish salmonid fish farming industry

Salmonid fish in Finnish fish farms are susceptible to several bacterial diseases. These include *Aeromonas salmonicida* (Rintamäki & Valtonen 1991), *Listonella anguillarum* (Pedersen et al. 1999), *Pseudomonas anguilliseptica* (Rintamäki & Koski 1987, Wiklund & Lönnström 1994), *Renibacterium salmoninarum* (Wiklund & Lönnström 1994), *Flavobacterium columnare* (Koski et al. 1993), *Flavobacterium*

psychrophilum (Wiklund et al. 1994), *Flavobacterium johnsoniae* (tentative) (Rintamäki-Kinnunen et al. 1997) and *Yersinia ruckeri* (Rintamäki et al. 1986, Valtonen et al. 1992) (Fig. 10). These diseases spread rapidly and cause economic losses if infections are not eliminated in time with antimicrobial drug treatment, such as antibiotics. Bacterial diseases are detected by microscopic examination and cultivation. During the past decade *Flavobacterium*-associated diseases have increased with serious outbreaks occurring on Finnish salmonid fish farms (E. Rimaila-Pärnänen and P. Vännerström, National Veterinary and Food Research Institute, personal communications). The reasons are unknown. Three flavobacterial pathogens have been reported in Finland: *Flavobacterium psychrophilum* (Wiklund et al. 1994) which causes either coldwater disease or rainbow trout fry syndrome (RTFS), and *Flavobacterium columnare* causing saddle-disease (Koski et al. 1993). A third type of flavobacteria-group (putative) pathogen found in fish hatcheries in the northern part of Finland was phenotypically similar to *Flavobacterium johnsoniae*. It was associated with erosion of the tail, gills, fins and jaws of rainbow trout (*Oncorhychus mykiss*) and Atlantic salmon (*Salmo salar* L.), as well as general inflammation and mortality (Rintamäki-Kinnunen et al. 1997). The diagnosis of flavobacteria-borne diseases is problematic due to difficulties in cultivating the causative pathogen, as has been shown in the study by Rintamäki-Kinnunen et al. (1997).

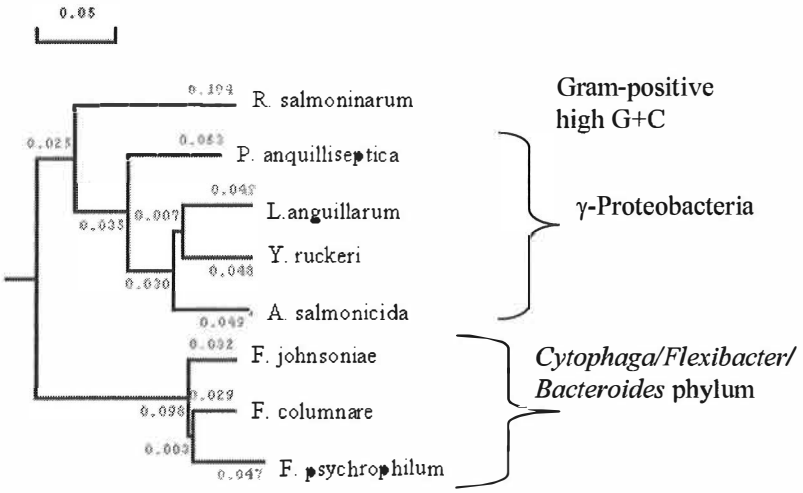

FIGURE 10 A neighbor-joining tree of the main fish pathogens on Finnish fish farms based on the 1200 bp fragment of 16S rDNA sequences (scale 0.05 changes per position). Sequences are derived from type strains of each pathogen species: *Renibacterium salmoninarum* (X51601), *Pseudomonas anguilliseptica* (AB021376), *Listonella anguillarum* (X16895), *Yersinia ruckeri* (Z75275), *Aeromonas salmonicida* (X60405), *Flavobacterium johnsoniae* (M59051), *Flavobacterium columnare* (AB010951) and *Flavobacterium psychrophilum* (AF090991). The numbers refer to lengths of the branches.

In the diagnostic field PCR-based techniques have so far been used to resolve specific problems with species-specific primers. Species-specific PCR answers

the question: "Does bacterium X occur in the sample?" This approach can answer only one question at a time. In broad-range bacterial PCR a set of universal primers can be used for all bacterial diseases. This approach answers the question: "Which is the bacterium that dominates in the sample?" Universal PCR and subsequent sequence analysis of the amplicons have not previously been applied to the diagnosis of fish diseases, although there has been several trials in the field of human clinical diagnostics (e.g. Wilson et al. 1990, Chen et al. 1994, Rantakokko-Jalava et al. 2000).

3 OBJECTIVES OF THE STUDY

The objective of this study was to analyze the diversity and structure of bacterial communities in three ecologically extreme environments with modern molecular tools. These extreme conditions were caused exogeneously by suboptimal temperature (either low or high temperatures), and/or toxic compounds (polychlorophenols) or endogeneously by unbalanced bacterial communities (fish skin disease). The general question is whether the system is limited or affected by a very low species diversity, and if so, which are the bacteria that dominate in these situations.

The specific objectives of the study were diverse:

A) Methods

1) Set up procedures for 16S RFLP, DGGE and sequencing of 16S rDNA in our laboratory.

2) Test LH-PCR analysis as a tool for separating microbial groups in diversity analysis. Develop the method and size standards for LH-PCR.

3) Test the effect of different DNA extraction procedures, template concentrations and selection of primers using LH-PCR.

B) Applications

4) Gain basic knowledge of species richness and phylogenetic diversity of chlorophenol-degrading bacteria in cold, long-term chlorophenol contaminated groundwater.

5) Reveal the abundance of the *pcpB* gene in phylogenetically different chlorophenol-degrading organisms and in sphingomonads in order to detect evidence of horizontal gene flux in the natural environment.

6) Evaluate the structure and the stability of the bacterial community in two different bioreactors: a chlorophenol-treating fluidized-bed process and aerobic thermophilic biofilm process treating pulp and paper-mill process water.

7) Isolate and characterize a dominant degrader from a fluidized-bed bioreactor treating chlorophenols.

8) Evaluate the feasibility of applying broad-range bacterial PCR and sequencing to the diagnosis of fish diseases.

4 MATERIALS AND METHODS

4.1 Sites for sample collection

Sites for sampling bacterial isolates and/or community DNA are indicated in Table 2. In addition, a large number of bacterial strains were used for comparison (described in II, III and V). The characteristics of the two bioreactors studied are presented in Table 3.

TABLE 2 Sampling sites and numbers of bacterial isolates and DNA samples studied in this research

Site for sample collection	Bacterial isolates	Community DNA samples	Description
Kärkölä groundwater	102	2	I, II
Kärkölä bioreactor	4	14	II
Thermophilic SCBP	-	28	IV
Konnevesi research station	5	4	V
Four commercial fish farms	11	23	V

TABLE 3 Characteristics of the two bioreactors studied

Character	Kärkölä fluidized-bed process	Thermophilic SCBP
Study period	6 years	37-day trial
Volume	5 m^3	1 m^3 in reactor R1 and 1 m^3 in reactor R2
Feed water	Groundwater contaminated with polychlorophenols (5-10 mg/l)	Pulp and paper mill process water (DOC 430 mg/l in average)
Hydraulic retention time (HRT)	2.5-2.7 h	8 to 3 h (in the two-stage process)
Operating temperature	5-8 °C	44-59°C
Carrier material	Coarse sand	Plastic carrier elements Flootek RF 438*
Aeration	Adjusted to 5-8 mg/l of dissolved oxygen O_2	Adjusted to 3-4 mg/l of dissolved oxygen O_2 in R1
Supplements added in the feed water	$(NH_4)_2HPO_4$	1. pH adjustment to 7.0 with 50% NaOH-solution 2. COD:N:P adjustment to 100:5:1 with technical urea and phosphoric acid
% Purification efficiency	>99% of total chlorophenols during stable operation	55 to 70 % of DOC in general

*Carrier elements were 36 mm in length and 44 mm in diameter, were made of polyethylene and had two internally crossed fins.

4.2 Methods and computer programs

The methods and computer programs used in this study are presented in Tables 4 and 5.

TABLE 4 The methods used in this study

Analysis	Description	Reference[a]
BACTERIAL ANALYSES		
Isolation of bacteria	I, II, V	
DNA extraction and purification	I, II, III, V	Wilson 1990
Analysis of DNA base composition	I	Johnson 1994
Whole cell fatty acid composition	I	Väisänen et al. 1994
Sequencing of 16S rDNA or other genes	I, II, III, IV, V	

(continues)

TABLE 4 (continues)

Analysis of bacterial growth rate	II	Koch 1994
Dot blot hybridization analysis	III	Boehringer Mannheim[b]
Southern blot hybridization analysis	II, III	Boehringer Mannheim[b]
Western blot analysis	III	
16S RFLP	I, II, V	
Chlorophenol toxicity test	I	
COMMUNITY ANALYSES AND FINGERPRINTING		
Community DNA extraction	II, IV, V	Wilson 1990
16S RFLP	II, V	
LH-PCR	II, IV	Suzuki et al. 1998, II[c]
DGGE	II	Muyzer et al. 1993
Direct 16S rDNA sequencing	V	
Cloning and sequencing of 16S rDNA	IV	
OTHER ANALYSES		
Chlorophenol analysis	I, II	

[a]Possible modifications are described in I-V.
[b]Protocols are described in detail in "The DIG User's Guide for Filter Hybridization", manufacturer's instructions for hybridization protocols.
[c]We developed the LH-PCR method and calculated the database analysis (IV) independently before the method was published by Suzuki et al. (1998).

TABLE 5 Computer programs used in this study

Program	Description	Reference/source
CLUSTAL X	I, III, V	Thompson et al. 1997
GCG package	I, III	Genetic Computer Group, Madison, Wisc.
PHYLO_WIN	I	Galtier et al. 1996
BLAST	II, III, IV, V	Altschul et al. 1997
Seaview	I	Galtier et al. 1996
Treetool	I	Ribosomal database project, Univ. of Illinois, Urbana, Ill.
Treeview	III, IV, V	Page 1996
QuantityOne	II, IV	Bio-Rad Laboratories, Hercules, Calif.
DNAman, Version 4.11	II, III	Lynnon Biosoft, Vaudreil, Canada
SRS-Sequence Retrieval System	This dissertation	European Molecular Biology Laboratory, Heidelberg, Germany
Probe Match	This dissertation	Ribosomal database project, Univ. of Illinois, Urbana, Ill.

5 RESULTS AND DISCUSSION

5.1 Diversity of chlorophenol-degrading bacteria

5.1.1 Taxonomical study of chlorophenol-degrading isolates (I)

The Kärkölä aquifer offers an opportunity to study the response of a groundwater microbial community to a process of chlorophenol contamination that has continued for 25 years at least. The results showed that the ability to degrade polychlorophenols at low concentrations (this is, at the concentrations detected in the groundwater, < 10 mg/l) was found in organisms presenting many phylogenetic branches (I, Table 2). Of the 57 Kärkölä groundwater chlorophenol-degrading isolates, 17 representative isolates were selected for the phylogenetic analysis on the basis of different 16S RFLP patterns. Classification of these isolates was based on the partial 16S rDNA sequences (approximately 450 bp), whole cell fatty acid composition and genomic G + C content. They represented five different phylogenetic groups: α-, β- and γ-Proteobacteria, Gram-positives with high G+C content and the *Cytophaga/Flexibacter/Bacteroides* group. A polychlorophenol-degrading capacity was found in several phylogenetically different sphingomonads. Polychlorophenol degradation was also detected in several genera previously not known to contain chlorophenol degraders (*Nocardioides, Ralstonia, Flavobacterium* and *Caulobacter).* Several strains were also so unique that they could not be classified into current genera or species (I, Table 1). Except for the Gram-positives and strains from the *Cytophaga/Flexibacter/Bacteroides* group, most strains tolerated high amounts of PCP (IC_{50} > 100 mg/l).

The high diversity of the chlorophenol-degrading organisms in the contaminated groundwater suggests substantial microbial adaptation to this contamination. Chlorophenol-degrading organisms have also been isolated at Kärkölä outside the plume, as recently reported by Männistö et al. (2001a). The

proportion of cultivable bacteria capable of degrading at least tetrachlorophenol (the main contaminant) increased from 7% (n=88) outside the plume to 57% (n=102) inside the plume (Männistö et al. 2001a). Therefore it is evident that chlorophenol degraders exist in the Kärkölä aquifer. Why does this not lead to natural chlorophenol attenuation in the groundwater? The results from the laboratory and pilot-scale field experiments support the view that the aquifer harbors a chlorophenol-degrading microbial community which biodegrades chlorophenols under *in situ* conditions when oxygen is made available (Langwaldt et al. 1998, Puhakka et al. 2000 and references therein). Therefore, *in situ* biodegradation of chlorophenols seems to be limited only by oxygen availability. The oxygen concentrations measured in the aquifer have been very low or non-existing, which is also reflected in the high concentrations of ferrous iron (7-16 mg/l) (Salminen 1999). Iron oxidation and precipitation are problems that hinder *in situ* groundwater bioremediation efforts using aeration (Puhakka et al. 2000). Anaerobic degradation of polychlorophenols has been performed by mixed bacterial consortia (Mikesell & Boyd 1986) as well as by pure cultures (Dennie et al. 1998, Tartakovsky et al. 1999). However, this does not seem to happen in the Kärkölä aquifer.

The isolation and testing approach used in our study (I) may be one reason for the unexpectedly high diversity of chlorophenol-degrading microbes found. Groundwater isolates were isolated and cultivated systematically without chlorophenols so as not to lose the most sensitive organisms. Their chlorophenol-degrading capacity was studied later using low chlorophenol concentrations. This approach can also lead to the loss of some degraders, since catabolic properties are often unstable without selective pressure (Wyndham et al. 1994). The focus in performing isolations has usually been to trap those bacteria which tolerate/degrade higher chlorophenol concentrations. Therefore, most of the diversity in chlorophenol degraders may have remained unrecognized in previous studies. Furthermore, many of the chlorophenol-degrading strains in this study were not able to degrade PCP although they degraded 2,4,6-TCP and 2,3,4,6-TeCP. Studying only PCP-degradation would have ignored these bacteria. Degradation of 2,3,4,6-TeCP is of special interest in Finland, since the compound was the main component of the chlorophenol-containing fungiside KY-5 used in the country's sawmill industry (Häggblom & Valo 1995).

5.1.2 Horizontal gene transfer? Indications of bacterial adaptation mechanisms (III)

Genetic changes can happen via mutational processes or the introduction of novel sequences by horizontal transfer of DNA into various genetic backgrounds. The high phylogenetic diversity that was found in the chlorophenol degrading organisms led us to investigate whether horizontal gene transfer could have happened between the microorganisms in the Kärkölä groundwater. Of especial interest were the six different chlorophenol-degrading sphingomonads isolated from the groundwater (strains K6, K16, K39, K40, K74 and K101, I) and the sphingomonads isolated from the bioreactors treating the

Kärkölä effluent: *Novosphingobium subarcticum* KF1^T, KF3 and NKF1 (Puhakka et al. 1995, Nohynek et al. 1996) and *Novosphingobium* sp. MT1 (II). Other chlorophenol-degrading sphingomonads in the previous studies have always belonged to the species *Sphingobium chlorophenolicum* (Karlson et al. 1995, Nohynek et al. 1995, Ederer et al. 1997) or have been close to this species (Cassidy et al. 1999).

We studied the distribution of the *pcpB* gene encoding for PCP-4-monooxygenase in *Sphingobium chlorophenolicum* (Orser et al. 1993). We recorded three main observations:

1) All the chlorophenol-degrading Kärkölä sphingomonads were shown to carry highly similar *pcpB* gene homologues differing from the three *pcpB* gene variants previously sequenced from *S. chlorophenolicum* strains and from *Sphingobium* sp. UG30 (III, Fig. 2 and 3).

2) The DNA-hybridization analysis showed that non-degrading sphingomonads isolated outside the plume or obtained from the strain collections did not contain regions of similarity with the *pcpB* gene (III, Fig. 1).

3) The gene was absent from chlorophenol-degrading non-sphingo-monads isolated inside the plume.

These results and the comparative analysis of the 16S rDNA and *pcpB* gene trees suggested that recent horizontal transfer of the *pcpB* gene was involved in the evolution of the catabolic pathway in the Kärkölä sphingomonads. This supports the study by Ederer et al. (1997), which also pointed to the horizontal transfer of the *pcpB* gene. In the study of Saboo & Gealt (1998) *pcpB* gene homologues highly similar (98% identity) to the *S. chlorophenolicum* ATCC 39723 gene sequence were obtained from non-degrading β- and γ-proteobacterial strains isolated from soil samples from a PCP-contaminated wood treatment site in Virginia, USA.

Gene transfer in natural environments can occur via three major mechanisms: transformation, conjugation and transduction (reviewed by Lorenz & Wackernagel 1994, Yin & Stotzky 1997). Horizontal transfer events can be classified into the distinct categories of acquisition of new genes, acquisition of paralogs of existing genes and xenologous gene displacement whereby a gene is displaced by a horizontally transferred ortholog from another lineage (Koonin et al. 2001). Since the DNA-hybridization analysis showed that non-degrading sphingomonads did not contain regions of similarity with the the *pcpB* gene, it is likely that in the Kärkölä sphingomonads horizontal transfer facilitated the acquisition of totally new genes.

Our results support the view that the *pcpB* gene is also located in a mobile element, either a plasmid or transposon. Transposons are discrete DNA segments that are able to move in the absence of genetic homogeneity from a donor site to a target site. Currently, at least 25 catabolic plasmids and 16 transposons or transposon-like elements have been characterized (Tan 1999). An overview of these catabolic plasmids and transposons shows that they are often large, 70-

500 kb and 8.5-90 kb in length, respectively (Tan 1999). The *pcpB* gene was not found to be part of an extensive operon or to be present on the 100 kb endogeneous plasmid of *Sphingobium chlorophenolicum* ATCC 39723 (Orser & Lange 1994). We have not as yet obtained evidence of a plasmid location of the gene in the Kärkölä strains. The large size usually found in catabolic plasmids makes them difficult to be separated from the bacterial chromosome. To study the location of the *pcpB* gene, different plasmid isolation procedures, pulsed-field electrophoresis and complementation experiments need to be explored. In our preliminary laboratory tests, co-cultivation of a chlorophenol-degrading sphingomonad and a nondegrading sphingomonad led to the transfer of the degradation ability to the nondegrading sphingomonad (unpublished results).

The Kärkölä *pcpB* gene homologue consisted of 1632 nucleotides and the size of the protein (60 kDa) product, determined by SDS-PAGE (III, Fig. 4) corresponded to the size determined for the *Sphingomonas chlorophenolica* PCP-4-monooxygenases. The gene shared approximately 70% identity with the three *pcpB* genes earlier sequenced from sphingomonads. The similarity of the predicted amino acid sequences was also at the same level (70%). Structural and functional comparison of the Kärkölä PCP-4-monooxygenase is one of the topics of a forthcoming dissertation to be produced in our group.

5.2 Microbial diversity analyzed by the broad-range bacterial PCR method

5.2.1 Evaluation of the broad-range bacterial PCR method

DNA extraction. As cited in the review of literature, DNA extraction is the first phase in which a molecular study can be subject to bias. In the case of studying the Kärkölä groundwater samples, we found that when only freeze-thaw cycles or chemical/enzymatic lysis were used, the LH-PCR patterns were different from those obtained after mechanical lysis alone or after all the lysis methods combined (Fig. 11). Although many DNA extraction procedures have been developed to minimize the time spent on this step, the time used for efficient and complete cell disruption is worth investing when performing community analyses. Therefore in the community studies (II, IV and V) we extracted the DNA by combining chemical (detergent), enzymatic (proteinase) and mechanical (bead-milling) cell disruption.

PCR primers. Evaluation of the PCR primers used in this study for the broad-range bacterial PCR method (Table 6) shows that many of the primers have one or two mismatches with the prokaryotic sequences in the database. However, these mismatches were not usually located in the last three or four basepairs in the PCR primer, which could have more serious effect on the PCR amplification. Furthermore, prokaryotic sequences compared with the Probe Match program also included archaeal sequences, which may explain a small

fraction of the mismatches. A large proportion of the mismatches can be explained by sequencing errors, since the site of primer PRUN518, especially, often contained non-identified nucleotides. The experience from this research showed that the choice of primers was able to have a real effect on the result (see Fig. 13, chapter 5.2.2).

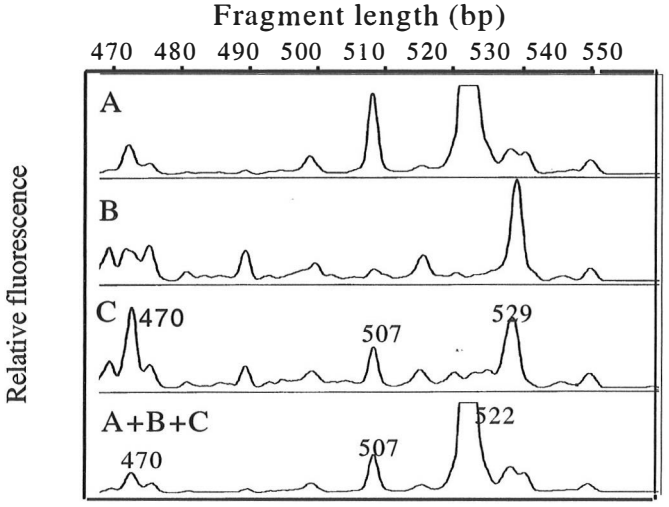

FIGURE 11 LH-PCR analysis shows that the method of cell disruption used has a strong effect on the resultant community profiles. Three cell disruption methods, separately (A, B and C) and in combination (A+B+C) were studied using the same Kärkölä groundwater samples. Methods: A, bead-milling with glass beads (1600 rpm, 3 min); B, three freeze-thaw cycles with boiling water and liquid nitrogen; C, cell lysis with 5 mg/ml lyzozyme at at 37°C for 1 h and with 1% SDS and 0.2 mg/l proteinase K at 37°C for 1 h. Otherwise the DNA extractions and LH-PCR procedure were performed as presented in III.

DNA concentration. The LH-PCR procedure was used to study the effect of DNA concentration on the PCR amplification (Fig. 12). The initial template concentration did not have a significant effect on the LH-PCR patterns across a broad range of DNA concentrations. The same peak lengths were detected independently of the initial DNA concentration in all the dilutions tested (10^0 to 10^6), but the total amount of PCR product and subsequently peaks in the LH-PCR were considerably lower in dilutions 10^4 to 10^6. The 10^6 template dilution (0.5 pg of template) was close to the sensitivity limit of the LH-PCR in study IV. However, the sensitivity of the PCR protocol can vary in different DNA samples due to PCR inhibition (chapter 2.3.3.1) and non-template DNA. The negative control (PCR amplification without template) remained close to the background level of the LH-PCR gel. In all our community studies, only sample series with duplicate negative controls (DNA extraction and PCR controls) were accepted. Although the DNA extractions and PCR amplifications were performed in the same laboratory, contamination of the samples did not pose a problem in our research.

TABLE 6 16S rDNA specific primers used for broad-range PCR amplification of bacterial genes in this study (II, IV, V).

Name	Target site[a]	Sequence (5'->3')	Specificity described in literature	Match, % of sequences[b]
fD1	8-27 (f)	AGAGTTTGAT CCTGGCTCAG	Matches most eubacteria, not some enterobacteria, Chlamydiae and *Borrelia* (Weisburg et al. 1991)	61/92/95
27f	8-27 (f)	AGAGTTTGAT CMTGGCTCAG	Matches most eubacteria including enterobacteria (Weisburg et al. 1991)	85/95/97
PRUN518	518-534 (r)	ATTACCGCGG CTGCTGG	Hybridizes with 99% of eubacterial sequences (Brunk et al. 1996)[c]	83/94/97
Com2-Ph	907-926 (r)	CCGTCAATTCC TTTGAGTTT	Matched 2306 of the 4332 prokaryotic sequences (Schwieger & Tebbe 1998)	49/86/97
rD1	1525-1541 (r)	AAGGAGGTGA TCCAGCC	Matches many eubacteria (Weisburg et al. 1991)	74/96/99

[a]The numbering refers to the *Escherichia coli* numbering (Brosius et al. 1978). F, forward primer; r, reverse primer.
[b]The match was calculated using the Probe Match program (http://rdp.cme.msu.edu/html/analyses.html, Maidak et al. 2000) for all the prokaryotic sequences (32 879 eubacterial and 1252 archaebacterial sequences) in the RDP (28-Dec-2001). Since the sequences in the database vary in length, the absolute number of sequences were compared to the number of sequences that had no more than 3 mismatches with the probe. The figures show the percentages of absolute matches (first value) and the the percentages allowing one and two mismatches (second and third value).
[c]Primer match was described for a primer which was identical in the region of primer PRUN518 but was two nucleotides longer.

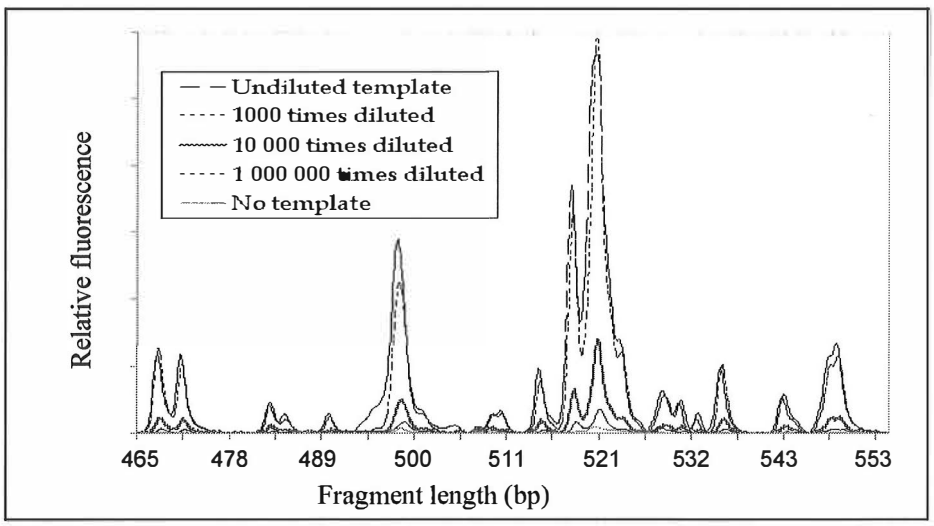

FIGURE 12 The effect of the DNA concentration was tested by serial dilution of the DNA template (biofilm sample from reactor R1, day 17, IV) before PCR amplification. LH-PCR patterns for template dilutions 10^0 (500 ng of DNA in 40 µl PCR reaction volume), 10^3, 10^4 and 10^6 are shown.

5.2.2 Microbial diversity in the fluidized–bed bioremediation process (II)

The Kärkölä fluidized-bed bioreactor is, to our knowledge, the only full-scale chlorophenol-bioremediation process on which reactor performance has been reported for a period of six years (II, Fig. 1). A level of chlorophenol removal of over 99% has been achieved for most of this time. Some reactor disturbances have, however, occurred after failures in the oxygen supply, which shows how dependent the process is on aerobic conditions. During the first year of operation, total chlorophenol concentrations of 10-23 mg/l were measured in the feed water. Since then, the concentration has been between 5-10 mg/l. No clear trend in the attenuation of chlorophenols in the Kärkölä aquifer can be identified. The water is pumped from several groundwater wells to the bioreactor, which may explain some of the variation in chlorophenol concentrations in the feed water.

The aim of the study (II) was to evaluate the structure and stability of the chlorophenol-degrading community in the Kärkölä fluidized-bed bioreactor. Broad-range bacterial PCR and three separation methods were used to analyze the PCR products: LH-PCR, RFLP and DGGE (II, Figures 2, 3 and 4). These analyses showed that the long-term operation had resulted in a stable microbial community. No shift in RFLP patterns was observed even after the process was disturbed in 1999. The choice of primers was found to have an effect on the PCR products (Fig. 13). It remained unclear why amplification with degenerate primers yielded fewer LH-PCR peaks than amplification with non-degenerate primers.

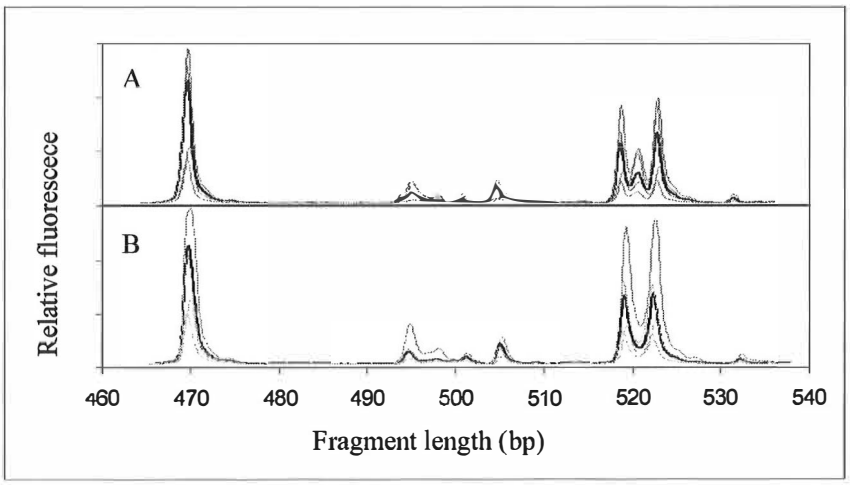

FIGURE 13 LH-PCR profiles of bioreactor samples (water samples 10-14, III, Fig. 1) amplified with primer pairs fD1/PRUN518 (A) and 27F/PRUN518 (B). Primer 27F is a degenerate version of the primer fD1. Sample 12 is highlighted to show an average band pattern.

In a parallel cultivation study, the organism possessing dominant molecular biomarkers in the bioreactor samples was isolated. This isolate was character-

ized using the 16S rDNA sequence and designated *Novosphingobium* sp. MT1. It was found that the degradation of polychlorophenol by the strain was dependent on the *pcpB* gene, since the mutant lacking the gene (II, Fig. 5) did not degrade chlorophenols (Chl$^-$). Other strains closely similar to MT1 were also isolated and found to carry the *pcpB* gene. The characteristics of strain MT1 (slow growth rate, single copy of rDNA operons) are well suited to an organism adapted to stable oligotrophic conditions. Other studies also showed that the strain possessed a high affinity for 2,3,4,6-TeCP and showed enhanced chlorophenol degradation at +8°C compared to that at room temperature (Männistö et al. 2001b).

As compared to the *Novosphingobium subarcticum* strains isolated earlier from a fluidized-bed bioreactor treating Kärkölä water (Puhakka et al. 1995, Nohynek et al. 1996), *Novosphingobium* sp. MT1 had several specific features. The *N. subarcticum* strains were not found to degrade pentachlorophenol, while MT1 did. It also seems that *N. subarcticum* has much faster growth rate. It forms colonies on TSA agar in 1 to 2 days (Nohynek et al. 1996), while it took 6 to 7 days before MT1 colonies could be seen on R2A agar plates (the strain did not grow on TSA agar). The ribosomal copy number in *N. subarcticum* is four (our unpublished data), as against one in MT1. *N. subarcticum* was isolated from a fluidized-bed system with 45-55 mg/l chlorophenol concentration in the feed water on a TGY agar plate supplemented with 10 mg/l of either 2,3,4,6-TeCP or PCP (Puhakka et al. 1995). In the Kärkölä fluidized-bed reactor from which MT1 was isolated, the chlorophenol feed concentration was below 10 mg/l, and the isolation was done without chlorophenols. In the high recycle flow bioreactor the chlorophenol concentration remains close to that of the effluent water, and not to that of the feed water. Therefore the reactor conditions were quite similar in the both bioreactors. It is interesting that although the isolated organisms were derived from the same genus and were isolated from similar environments, they had a significant difference in their ecological strategy: MT1 is more like an oligotrophic organism, while *Novosphingobium subarcticum* is a relatively fast-growing organism.

5.2.3 Microbial diversity in an aerobic thermophilic process (IV)

In order to study the performance and stability of the thermophilic SCBP, a two-stage process treating paper mill circulation water was set up on the mill premises. Since cultivation-based monitoring of the microbial changes in the reactor would have been both laborious and biased, the system was monitored using broad-range bacterial PCR. LH-PCR, cloning and sequencing was used in studying the microbial diversity present in the different compartments of the thermophilic SCBP. According to the molecular data, wide species diversity was revealed in the biofilm process at all the operating temperatures between 44 and 59°C. Sequences belonging to the β-Proteobacteria, γ-Proteobacteria and the *Cytophaga/Flexibacter/Bacteroides* group were assigned to the most prominent LH-PCR peak of the biofilm samples. Alkaline shock conditions, more than the

changes in temperature (within the range of 44-59°C), were problems that changed the microbial diversity in the biofilm community (IV, Fig. 2) as well as caused process upsets. When conditions were restored to neutral, the normal LH-PCR patterns of the microbial population in the biofilm recovered rapidly without further biomass inoculations.

The microbial diversity in the suspended biomass samples differed completely from that of the biofilm community, as judged from different LH-PCR patterns. Several Gram-negative sequences were associated with the most prominent peak size in the biofilm samples. The major peak sizes in the suspended biomass, however, represented lengths suggestive of *Bacillus*-like organisms. Temporal variation was very high among the LH-PCR patterns in the suspended samples, which may reflect the fact that bacterial populations developed in spurts. In previous microbial cultivation studies, only *Bacillus* and *Bacillus*-like organisms have been isolated from thermophilic aerobic wastewater treatment reactors (for a review, see LaPara and Alleman 1999). A recent molecular study has also shown that *Bacillaceae* is a major bacterial group in a thermophilic aerobic wastewater treatment bioreactor with a temperature fluctuating between 35 and 60°C (Kurisu et al. 2002). It is possible that in thermophilic conditions Gram-negative bacteria need a surface on which to thrive, but this is not needed by Gram-positive spore-formers.

In general, it seems to be easier to find changes in the population structure than to prove the similarity of two communities using the data obtained from LH-PCR patterns. As it was found in the biofilm samples and in the computational analysis (IV, Fig. 1), several bacterial groups may generate LH-PCR fragments of the same size. When used in combination with the database analysis, LH-PCR can provide some predictions regarding the microbial groups to which the observed band sizes may belong. So far, the method has been used in only three studies (Suzuki et al. 1998, Bernhard & Field 2000, Ritchie et al. 2000). Compared to DGGE, LH-PCR is more predictable and gives numerical results. Furthermore, LH-PCR analysis is easy to perform with present-day automated sequencing machines. This method could be a valuable tool in environmental biotechnology as well as in the food industry and other processes that need quick quality control systems to evaluate microbial community structures.

5.2.4 Broad-range bacterial PCR for direct diagnosis of fish diseases (V)

The aim of the study was to evaluate the use of broad-range bacterial PCR and direct sequencing in the diagnosis of flavobacteriosis from fish skin samples and to compare the results to those obtained by traditional cultivation (Fig. 14). For the study, 27 fish samples were collected and analyzed from outbreaks in August 2000 and January 2001. Flavobacterial cultivation as well as direct molecular analysis proved some difficulties. Ten of the samples were studied in detail using 16S RFLP and sequencing. On the basis of molecular analysis, *Flavobacterium columnare* dominated in six warm water samples and *F. psychrophi-*

lum in two cold water samples. In the parallel cultivation study that was performed using the traditional method of bacterial cultivation in fish parasitology (plate-streaking), *F. columnare* was cultivated only once (V, Fig. 2). Identification of the cultivated bacteria was performed using 16S rDNA, colony morphology and colour, Gram-stain and cell morphology. Additional phenotypic tests were performed for those isolates that did not clearly belong to any bacterial species (gliding motility, aerobic acid production from starch and esculin, cytochrome oxidase test, Congo-Red test). Instead of the organism that dominated in the direct molecular analysis, phylogenetically *F. hibernum*-like bacteria were usually cultivated. Although these bacteria might also be opportunistic pathogens, they were likely to be saprophytes growing in the presence of *F. columnare* –induced disease. The pathogenicity of *F. columnare* is very well known. In a previous large-scale monitoring of flavobacterial disease outbreaks (Rintamäki-Kinnunen et al. 1997) only (phenotypically) *F. johnsoniae*-like bacteria were isolated. These bacteria in fact shared the phenotypic characteristics with the saprophytic bacteria isolated in this study. It is thus possible that *F. columnare* was also masked by saprophytes in the large-scale study by Rintamäki-Kinnunen et al. (1997).

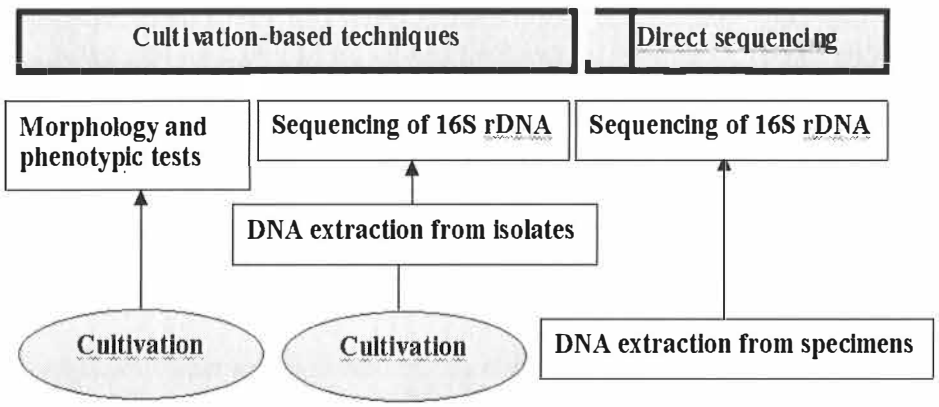

FIGURE 14 A schema of the cultivation-based and direct molecular approaches for the diagnosis of bacterial infections. The direct sequencing method omits the cultivation step totally.

The conflicting results from cultivation led us to test sample dilution before cultivation, and with this approach the dominant pathogens were obtained from the cold water samples. In one case natural fish skin bacteria were found to be effective in inhibiting the growth of other bacteria in the agar plate culture. *Pseudomonas* sp. MT5 was able to inhibit a thousand times more flavobacteria during primary cultivation (V, Fig. 3). Furthermore, the strain had antagonistic effects on a wide variety of gram-positive and gram-negative bacteria. Bacterial antagonism by several *Pseudomonas* strains has previously been reported (reviewed by Gatesoupe 1999). The inhibitory activity in this genus is apparently caused by competition for free iron by siderophores (Gram 1993, Smith &

Davey 1993, Gram et al. 1999). The mechanism of the antagonism of *Pseudomonas* sp. MT5 was not studied in this dissertation. The ecological significance of fluorescent pseudomonads in the bacterial communities and their possible protective role against pathogens requires more thorough-going studies.

The number of samples in this study was limited. Therefore we were only able to compare ten samples where both the cultivation and PCR analyses proved successful. The methodological problems encountered in the DNA extraction and/or PCR amplification may have been due to degradation of the DNA, since some flavobacteria produce large amounts of DNases (e.g. *F. columnare*) (Bernardet & Grimont 1989). It is also well known that although the PCR procedure can theoretically detect even a single bacterial cell in a sample, the detection limit can be much lower in real tissue samples. In an example dealing with fish samples (Gibello et al. 1999), the limit of PCR-detection of *Y. ruckeri* was $2x10^4$ cells per g of fish tissue.

Despite the small number of samples, the study opens up new avenues for fish disease diagnostics. It was noteworthy that the dominance of the pathogen in the external tissue samples was so strong that direct sequencing was possible. This methodology is much faster (diagnostics can be performed in 24 hours or even in one working day using capillary electrophoresis) than the current cultivation procedures and furthermore the same procedure can be used to detect all kinds of bacterial pathogens. Rapid diagnostics could help in minimizing and optimizing the use of antibacterial medication and preventing the spread of fish diseases on fish farms.

5.3 Molecular techniques used for separation of heterogeneous PCR products

Species-specific PCR can answer only a single question at a time. Broad-range bacterial PCR, instead, offers several possibilities for subsequent characterization of the PCR product based on the length and configuration separation (Fig. 8, Chapter 2.3.2). In order to maximize the amount of information about the whole bacterial community, the product separation technique has to be rationally chosen. Direct sequencing of the PCR product may only be possible in extreme conditions, for example in pathogenic situations (V). RFLP analysis produces several bands of one template, and therefore the technique is also restricted to samples with limited species diversity. Otherwise it is quite difficult to interpret the banding patterns. Of the techniques available, the cloning and sequencing approach is suitable for the most complex systems (Fig. 15). The costs and labor intensity of the technique limits its approach to unique samples, and therefore it is not suitable for time and space series. Furthermore, it contains more steps that are susceptible to bias (cloning step in addition to DNA extraction, PCR and sequencing).

LH-PCR, RISA, DGGE/TGGE, SSCP, T-RFLP and 2-D electrophoresis are all feasible methods for profiling bacterial diversity. LH-PCR, RISA and T-RFLP have the advantage that they give reproducible results which can be standardized. Numerical LH-PCR and T-RFLP results can be compared against the large 16S rRNA gene databases. In the LH-PCR analysis, sequence lengths are quite conserved in each bacterial genus, although the phylogenetic group of bacteria can not be reliable predicted from the fragment lengths. In the T-RFLP and especially RISA analysis different sequence lengths can be obtained from closely similar strains, which can be an advantage or disadvantage in different cases. In T-RFLP, the analysis can be done using a large set of restriction enzymes, which increases the resolution of the analysis. Because of the relatively small number of available ISR sequences, sequence lengths in RISA are difficult to connect to cultivated organisms or bacterial groups. LH-PCR, (A)RISA and T-RFLP are techniques that can be done using automated sequencing machines. Automation of the methods has several advantages (speed, data in digital form), but hampers further analyses, such as the sequencing of interesting bands.

The advantage of DGGE, TGGE, SSCP and 2D-electrophoresis is that they are usually performed in normal polyacrylamide gels and therefore offer the possibility to directly excise and sequence the band (or spot) of interest. However, these analyses do not produce numerical results and therefore it is difficult to compare the results obtained from separate electrophoresis runs and in different laboratories. Although the theoretical mobility differences in DGGE, TGGE and SSCP may have more variety, the actual band separation resolution may not be as high as in LH-PCR, T-RFLP or automated RISA, which are performed in long and high-resolution sequencing gels or capillary electrophoresis. 2-D electrophoresis might increase the separation resolution of diverse bacterial communities, but there is not much experience of its use in the community profiling. Furthermore, in 2-D electrophoresis only one sample could be applied in each electrophoresis gel. DGGE has been one of the mostly used community screening methods, but automated analyses such as LH-PCR, T-RFLP and ARISA would be faster techniques for comparing large sample series.

In addition to its scientific applications, broad-range bacterial PCR together with its supporting methodology could prove useful in routine biotechnology and in the diagnostic field. Such uses include, for instance, daily process follow-ups in environmental biotechnology, quality control in the food processing industry and rapid diagnosis of sepsis in human clinical samples. It is obvious that bacterial cultivation can not be totally discontinued, since the virulence, antibiotic resistance and toxicity of bacteria can not be determined solely on the basis of the molecular results. However, if some of the resources allocated to the numerous traditional microbiological laboratories were designated for developing automated molecular methods, this could enhance and rationalize the work. For example, in the Finnish National Veterinary and Food Research Institute, 200 different microbiological media are in use and 200 000 agar plates are casted every year (http://www.eela.fi/5keskuslaboratorio/bak.htm, 15-Jan-2002). Maintenance of such an arsenal of research methods and the skilled personal headed to analyze the results is also costly. Fear of the problems associated with

achieving uniform PCR amplification and lack of staff familiar with this modern technology may limit the development and use of broad-range bacterial PCR. However, combined with public databases, it offers one of the most universal and straightforward research tools in bacteriology.

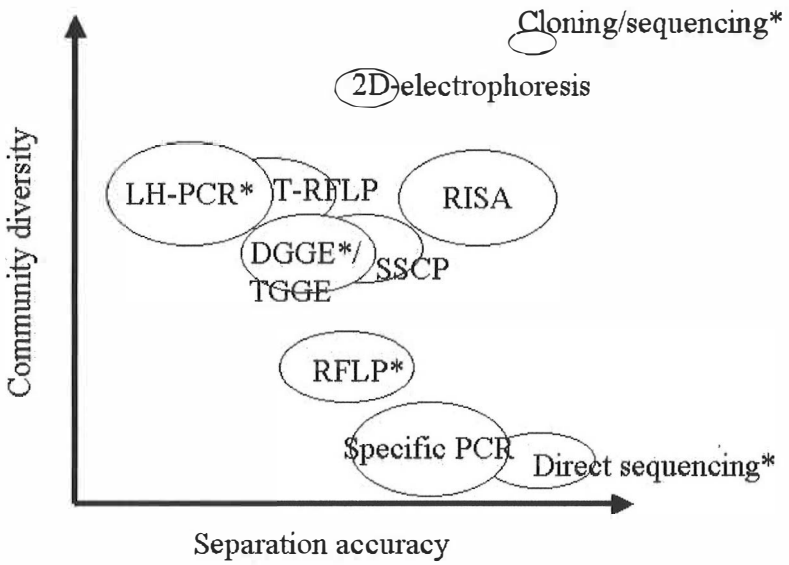

FIGURE 15 Schematic diagram of the usefulness of the existing 16S rDNA-based molecular profiling methods showing their respective separation accuracy in samples with different microbial diversity. The size of the oval indicates the simplicity of the technique: simpler methods are marked with bigger ovals. The methods used in this dissertation are marked with asterisks.

6 CONCLUSIONS

Ribosomal RNA gene sequences can be analyzed directly from environmental nucleic acid extracts circumventing the need to culture prior to phylogenetic identification. The biases included in the broad-range bacterial PCR approach are potential variability in rDNA copy number, inequality in cell lysis and DNA extraction, and problems with achieving uniform PCR amplification. In spite of these limitations, PCR is a powerful method with which to study the differences in microbial environments in terms of community structure across temporal and spatial gradients.

There was two dimensions to the current research. First, we tested and optimized the broad-range bacterial PCR procedure for the use of community profiling. Second, we examined three different ecosystems with these community profiling methods. The lowest bacterial diversity was found in the fish samples, in which only one bacterial species dominated at a time. The foundation for the work done in all of the studies was the amplification of 16S rDNA sequences. The main conclusions are:

1) Broad-range bacterial PCR is a versatile method that can be applied in many kinds of bacterial samples. Once an appropriate technique for the separation of the heterogeneous PCR products has been chosen, the technical optimization of the procedure as well as characterization of diverse microbial communities and large sample series can be carried out with a good measure of success.

2) The study on the Kärkölä fluidized-bed bioreactor and the thermophilic SCBP demonstrated that LH-PCR is a useful tool in the serial and spatial analysis of microbial communities. However, overlapping sequence lengths in several bacterial phyla were found to exist in theory as well as in practice. This makes interpretation of the results more difficult.

3) Long-term contamination of the Kärkölä groundwater has led to a microbial community with numerous chlorophenol-degrading bacterial groups. Many of these bacteria represented genera that were not previously known to degrade chlorophenols.

4) The chlorophenol-degrading sphingomonads isolated from the Kärkölä source all carried highly similar *pcpB* gene homologues, probably achieved by recent horizontal transfer.

5) Other chlorophenol-degraders (non-sphingomonads) from the same site may have some other pathway for chlorophenol degradation, since they did not carry *pcpB* gene homologues.

6) The Kärkölä fluidized-bed bioreactor was characterized by a stable bacterial community with low species diversity. One of the dominant organisms, *Novosphingobium* sp. MT1, was isolated. This polychlorophenol-degrading strain had features commonly found in slow-growing oligotrophic organisms.

7) The microbial community of the attached biofilm in the thermophilic SCBP included several types of Gram-negative bacteria, and the community structure differed clearly from that of the suspended biomass.

8) Broad-range bacterial PCR and sequencing was used for the first time in the diagnosis of fish diseases. In this study, the method was used to reveal the dominating bacterium in flavobacteriosis. The research showed that the results obtained from molecular and cultivation-based studies often differ in their outcome, which was explained by difficulties in the cultivation of external samples. Serial sample dilution before cultivation was recommended to improve the cultivation procedure.

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YHTEENVETO

Ribosomaalisen RNA-geenin sekvenssien käyttö bakteeridiversiteetin fylogeneettisessä analyysissä

Bakteereiden tunnistamisessa hyödynnetään usein ribosomaalisen 16S rRNA-geenin sekvenssejä. Tämä geeni soveltuu suhteellisen hitaan muutosnopeutensa ansiosta bakteereiden fylogeneettisten suhteiden määrittämiseen, ja geenialueen horisontaalista siirtymistä lajien välillä on tavattu hyvin harvoin. Lisäksi geenin sisällä olevat erittäin konservoituneet alueet sopivat polymeraasiketjureaktiossa (PCR, polymerase chain reaction) tarvittavien alukkeiden sitoutumiskohdiksi. Tässä työssä on kehitetty ja hyödynnetty menetelmiä, joiden avulla voidaan erotella ja tutkia useista erilaisista bakteereista samanaikaisesti monistettuja 16S rRNA-geenin fragmentteja. Menetelmät mahdollistavat bakteereiden tunnistamisen ja bakteeriyhteisön diversiteetin, monimuotoisuuden, määrittämisen suoraan ympäristönäytteistä ilman bakteeriviljelyä. Tämä on selvä etu, sillä bakteeriviljely on työläs ja valikoiva menetelmä, eikä se usein anna oikeaa kuvaa ympäristönäytteiden bakteeriyhteisöistä.

Tutkimuksessa tarkasteltiin kolmea erilaista mikrobiyhteisöä. Näistä ensimmäinen oli Kärkölän kloorifenoleilla saastunutta pohjavettä puhdistavan bioreaktorin mikrobiyhteisö, jota seurattiin kuuden vuoden ajan. Riittävän hapetuksen vallitessa reaktori puhdisti yli 99% polyklooratuista fenoleista. Reaktorin bakteeridiversiteetti oli vakaa ja koostui vain muutamista lajeista. Yksi suorassa molekyylibiologisessa analyysissä dominoivista bakteereista eristettiin, ja tälle bakteerikannalle annettiin nimi *Novosphingobium* sp. MT1. Kanta on esimerkki alhaisissa lämpötiloissa (5-8°C) viihtyvistä, niukkaravinteisiin olosuhteisiin sopeutuneista kloorifenolia hyödyntävistä bakteereista. Polyklooratttujen fenoleiden (2,4,6-trikloorifenoli, 2,3,4,6-tetra-kloorifenoli ja pentakloorifenoli) hajotuksessa bakteerikanta käytti hajotusreittiä, jonka aloittavaa entsyymiä, pentakloorifenoli-4-mono-oksygenaasia, koodaa *pcpB*-geeni. Kärkölän *pcpB*-sekvenssi eroaa nukleiinihappojärjestykseltään n. 30% aikaisemmin tunnetuista, viljellyistä bakteereista eristetyistä *pcpB*-sekvensseistä. Reaktoriin tulevasta pohjavedestä eristettiin myös 59 polykloorattuja fenoleita hajottavaa bakteeria, joiden lajidiversiteetti analysoitiin 16S rRNA geenin sekvenssien avulla. Kloorifenoleita hajottavien bakteereiden todettiin edustavan hyvin erilaisia fylogeneettisiä bakteeriryhmiä (α-, β- ja γ-proteobakteerit, korkean genomisen G+C pitoisuuden omaavat Gram-positiiviset bakteerit sekä *Cytophaga/Flexibacter/Bacteroides* -ryhmään kuuluvat bakteerit). Suuri osa eristetyistä α-proteobakteereista kuului merkittävään hajottajabakteerien ryhmään, sphingomonadeihin. Sphingomonadeilla on jo aiemmin havaittu olevan monipuolisia ominaisuuksia hajottaa toksisia ja hitaasti hajoavia yhdisteitä, kuten kloorifenoleita, polyaromaattisia hiilivetyjä, dioksiineja ja furaaneita. Kaikkien

Kärkölän kloorifenoleita hajottavien sphingomonadien todettiin olevan *pcpB*-geenin kantajia. Kun bakteereiden 16S rRNA ja *pcpB* geenit sekvensoitiin, viittasivat tulokset vahvasti siihen, että kloorifenoleiden hajotuksessa toimivat geenit ovat siirtyneet horisontaalisesti sphingomonadeihin tai tähän ryhmään kuuluvien bakteerikantojen välillä. Sen sijaan muissa kloorifenoleita hajottavissa bakteeriryhmissä *pcpB*-geeniä ei havaittu.

Toinen tutkituista mikrobiympäristöistä oli paperitehtaan kiertoveden termofiilinen puhdistusprosessi. Termofiilisen aerobisen prosessin ongelmana on ollut syntyvän biomassan huono laskeutuvuus. Tavoitteena oli kehittää ja seurata biofilmiprosessia, jossa mikrobiyhteisö on kiinnittyneenä kantajamateriaaliin, ja välttyy näin huuhtoutumiselta. Työssä tutkittiin erityisesti LH-PCR –menetelmän (length heterogeneity analysis of amplified PCR product) käyttöä mikrobiyhteisön analysoinnissa. Käytetyllä alukkeiden yhdistelmällä erilaisista bakteereista syntyvien PCR-tuotteiden pituus voi teoreettisesti vaihdella välillä 465-563 nukleotidia. Fylogeneettisten bakteeriryhmien sekvenssipituudet ovat osittain päällekkäiset, ja vain harvoin bakteeriryhmä voidaan suoraan ennustaa PCR-tuotteen pituuden perusteella. Nopeutensa ja toistettavuutensa vuoksi LH-PCR soveltui kuitenkin muutoin hyvin prosessin mikrobiologian seurantaan. Biofilmiin kiinnittyvän ja suspensiossa lisääntyvän mikrobiyhteisön todettiin eroavan selkeasti toisistaan. Biofilmin monipuolinen mikrobiyhteisö muuttui suspensiossa lisääntyvien bakteereiden suuntaan, kun prosessin pH-olosuhteissa tapahtui voimakkaita muutoksia, mutta alkuperäisen kaltainen LH-PCR –profiili palautui pH-olosuhteiden palauduttua neutraaleiksi.

Erityisen alhainen bakteeridiversiteetti havaittiin flavobakterioosiin sairastuneiden kirjolohen ja Atlantin lohen poikasten ihon haavaumissa. Diversiteetti-analyysin ja suoran 16S rRNA-geenin sekvensoinnin perusteella *Flavobacterium*-sukuun kuuluvat patogeenit dominoivat voimakkaasti näissä näytteissä. Flavobakteerien viljelyssä esiintyvät ongelmat aiheuttivat sen, että patogeeneja saatiin eristettyä vain niistä näytteistä, joissa viljely suoritettiin laimennussarjan kautta. Suoraa sekvensointia ei ole tietääksemme aiemmin käytetty kalatautien määrityksessä. Sekvensointimenetelmien kehityttyä nykyiselle tasolleen suora sekvensointi tarjoaa nopean ja yksinkertaisen diagnoosimenetelmän erityisesti tapauksissa, joissa bakteeriviljelyn onnistuminen on epävarmaa.

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ORIGINAL PAPERS

I

Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater

by

Männistö, M. K., Tiirola, M. A., Salkinoja-Salonen, M. S., Kulomaa, M. S. & Puhakka, J. A. 1999.

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II

Isolation and characterization of *Novosphingobium* sp. MT1, a dominant polychlorophenol-degrading strain in a groundwater bioremediation system

by

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III

Natural horizontal transfer of the *pcpB* gene facilitates chlorophenol degradation in sphingomonads

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IV

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V

Diagnosis of flavobacteriosis by direct amplification of rRNA genes

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