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Bacterial diversity of a marine electroactive biofilm

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*"Dive into the sea of thought and
find there pearls beyond price"*

- Moses ibn Ezra (1060 – 1139) -

A	Ampère
BCCM/LMG	Belgian Coordinated Collections of Microorganisms/ Laboratory of Microbiology (Ghent)
BUG	Benthic Unattended Generators
C	Cytosine
CFU	Colony forming units
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
EAB	Electroactive biofilm
EMBL	European Molecular Biology Laboratory, European Bioinformatics Institute, Cambridge, UK
EPS	Extracellular polymer substance
ERIC	Enterobacterial repetitive intergenic consensus sequences
FAME	Fatty acid methyl ester
G	Guanosine
ID	Identification
LMG-number	Reference number as deposited in the culture collection of the Laboratory of Microbiology
LM-UGent	Laboratory of Microbiology (Ghent)
MA	Marine agar
MFC	Microbial fuel cell
mV	Millivolt
PCR	Polymerase chain reaction
PYG	Peptone yeast extract glucose agar
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
R-number	Research number (as in the LM-UGent strain collection)
Rep	Repetitive sequence-based
REP	Repetitive extragenic palindromic sequences
TGGE	Temperature gradient gel electrophoresis
TSA	Trypticase soy agar
UPGMA	Unweighted pair-group method using arithmetic averages

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Lieve groetjes,

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21 mei 2008

PART IV: GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSION

PART I

INTRODUCTION, OBJECTIVES AND OUTLINE

"Energy is eternal delight"

- William Blake (1757-1827) -

1 Introduction

Currently, the world energy consumption is immense mainly due to an ever-increasing world population, technological progress and the industrial development of countries such as China and India. Furthermore, it is expected that the world energy consumption will increase by 71 percent from 2003 to 2030 (Energy Information Administration/ International Energy Outlook 2006).

At present, fossil fuels such as coal, petroleum and gas are the major and most often the only source of energy. In 2005, the Energy Information Administration estimated that 86 % of the primary energy consumption in the world came from the combustion of fossil fuels. Energy productions based on non-fossil fuels (such as geothermal, solar and wind energy) only constitute a minor part of the total energy generation; for instance, only 6.0 % of the energy originates from nuclear power plants.

The combustion of fossil fuels engenders high amounts of energy. Fossil fuels are hydrocarbon junctions that arose from plant and animal remains from the geological past of the Earth, mainly from the Carboniferous era. Also, the abiotic origin of fossil fuels was already claimed. Fossil fuels are non-renewable energy sources as their formation from the fossilized remains of dead plants and animals by exposure to heat and pressure in the Earth's crust takes millions of years.

Since centuries, fossil fuels have a major impact on human life. For instance, the wide-scale use of fossil fuels, coal at first and petroleum later, to fire steam engines drove the Industrial Revolution in the 18th and 19th centuries. In fact, the Industrial Revolution almost affected every aspect of the daily life of human society and marked a major turning point in human history. Since then, industrial development and the use of fossil fuels increased continuously, leading to high technological products.

The intensive use of fossil fuels causes depletion of stocks of fossil fuels much faster than that new ones being formed. In addition, the combustion of fossil fuels causes environmental problems as for instance approximately 21.3 gigatons of carbon dioxide per year are discharged while natural processes only absorb half of this amount, causing a net release of 11.6 gigatons of carbon dioxide per year. Carbon dioxide is a greenhouse gas that enhances radiative forcing and contributes to global warming. Also, other air pollutants are generated by the burning of fossil fuels such as nitrogen oxides, sulphur dioxide, volatile organic compounds and heavy metals which also exhibit harmfull environmental effects such as acid rain production and health problems (Holdgate, 1989, Montgomery, 2007). The predicted

depletion and the negative effects of fossil fuels on the environment drive forward the search for green and renewable energy sources.

Energy production by microorganisms through, for instance, bioethanol or hydrogen gas production can contribute to solve this problem (Buckley & Wall, 2006). Also, microbial fuel cells (MFCs) constitute an alternative energy source and the development and improvement of MFCs are currently investigated worldwide (e.g. Rabaey *et al.* (2005b)).

MFCs are not new. Potter (1912) was the first to generate electricity from *Escherichia coli* cultures, but this work received very little attention initially. Later, several attempts to generate electrical current with MFCs had variable degrees of success (Cohen, 1931, Delduca *et al.*, 1963). In the late seventies and eighties, several research groups studied electrical current production by means of MFCs and the current MFC concept was designed (Bennetto *et al.*, 1983, Karube *et al.*, 1977, Karube *et al.*, 1976).

At present, two major environments are investigated for MFC research namely the sea floor and wastewater. The significance of producing electrical current from the sea floor is for instance, to empower measuring devices at remote locations. On the other hand, electricity production from waste is promising as one of the major challenges of the next decades is the processing of waste and wastewater. In fact, the ideal scenario would be to generate energy out of waste which has been referred to as the 'from foul to fuel' principle. In general, microbial fuel cells are considered a very promising source of green and renewable energy.

2 Objectives of this work

External electron transport and the subsequent flow of electrons on metallic surfaces in marine environments attracted the attention of microbiologists since many years. This flow of electrons is caused by the presence of a biofilm, containing microorganisms, on the surface of metals and is referred to as 'microbial induced corrosion' or 'biocorrosion'. Therefore, the presence of an electroactive biofilm (EAB) on stainless steel electrodes was first described in the context of biocorrosion (Mollica & Trevis, 1976), although the composition of such biofilms remained largely unknown.

The present PhD study was performed in the context of a European project (EA-Biofilms-508866-NEST). A marine EAB was generated by placing a cathodically polarized electrode (-200 mV Ag/AgCl) in natural seawater at the harbour of Genoa, Italy. Electrical current was efficiently produced in less than 10 days and biofilm formation on the cathode was observed. Although the microbial populations of diverse types of MFCs had already been investigated, the bacterial community of this type of marine EAB was never analysed before.

The present study aimed at characterizing the microbial EAB community by culture dependent and culture independent analyses (Chapter 2). Furthermore, the composition of the bacterial population of the EAB was compared to that of the surrounding seawater in order to reveal if the bacterial population was enriched in specific bacterial groups (Chapter 2). Finally, the predominant bacterial groups were analysed in more detail in order to elucidate if novel bacterial taxa were present among the isolates (Chapter 3, Chapter 4).

3 Outline of the thesis

Part II gives an overview of the literature related to this thesis. In a first part (Chapter 1.1), a general introduction on biofilms is given considering the architecture and the formation of biofilms. In addition, negative effects but also possible industrial applications are discussed. A second part (Chapter 1.2) handles about microbial fuel cells and focuses on wastewater and benthic microbial fuel cells. Finally, a overview of the different methods used during the present research are discussed (Chapter 1.3)

Part III presents the experimental work that was performed during this thesis. In Chapter 2, the results of the study of the bacterial diversity of the marine electroactive biofilm are given. Chapter 3 describes a novel bacterial species within the *Gammaproteobacteria* that was isolated from the marine electroactive biofilm, namely *Alteromonas genovensis*. In chapter 4, the novel bacterial taxa within the *Roseobacter*-lineage that were obtained from the electroactive biofilm investigated are described.

Part IV comprises a general discussion, provides future perspectives of the present study and summarizes the results obtained during this thesis.

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*The most beautiful thing that we can experience
is the mysterious.
It is the source of all true art and science.*

- Albert Einstein (1879-1956) -

PART II

OVERVIEW OF THE LITERATURE

CHAPTER 1: Literature study

1.1 BIOFILMS

Biofilms are defined as structured communities of adherent microorganisms encased in a complex extracellular polymer substance (EPS) matrix (Hall-Stoodley & Stoodley, 2005). Biofilms develop on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial equipment, drinking water systems and natural aquatic systems (Donlan, 2002) and are made up of a single species or, more commonly, of multiple bacterial and fungal species (Nobile & Mitchell, 2007). Although biofilms are defined as adherent aggregates to a solid surface, they also exist as suspended flocs or as pellicles at air-liquid interfaces (Hall-Stoodley & Stoodley, 2005). Generally, bacteria in natural environments are mostly found in biofilms (Branda *et al.*, 2005). The idea of bacteria living in biofilms is not new: in 1684, Antoni Van Leeuwenhoek described the presence of "wee animalcules" in human tooth plaque that he observed with his primitive microscope (Costerton, 1999, Costerton *et al.*, 1999, Madigan *et al.*, 2000). Almost 300 years later, Zobell (1943) demonstrated that natural marine bacterial populations are attracted to surfaces to which they sometimes adhere in order to form sessile populations. Nowadays, biofilm research is a worldwide discipline spanning all areas of microbiology such as genomic and proteomic research, clinical and diagnostic microbiology and biocorrosion (Costerton, 1999).

1.1.1 Biofilm structure

During the last decades, the term biofilm is used to describe various structures ranging from patchy monolayers over very thick gelatinous masses to filamentous aggregates (Wimpenny *et al.*, 2000). Several new technologies, such as confocal laser scanning microscopy, differential interference contrast microscopy and microelectrode measurements advanced seriously our knowledge regarding biofilm structures.

A biofilm comprises several microcolonies and is often penetrated by channels (Costerton *et al.*, 1995). Futhermore, some microorganisms (such as *Pseudoalteromonas aeruginosa*) form mushroom-like structures under laboratory conditions (Sauer *et al.*, 2002). The microcolony is the basic unit of the biofilm, is enclosed in a dense slime and is attached to the surface. Microcolonies develop when adherent bacteria are present in a favourable environment where they produce EPSs (Costerton *et al.*, 1995). Convective flow and turbulence, caused by the roughness of the surface, represent a primitive circulatory system within the biofilm (Costerton *et al.*, 1995). In fact, there are three possible penetration ways for nutrients and cellular wastes in biofilms. First, advective transport and diffusion in biofilm channels facilitate the transport of molecules to the deeper parts of the biofilm. Second, a combination of diffusion and advective flow transports molecules through the smaller pores or conduits (advective flow in these structures is lower than in the larger channels). Moreover,

EPS may occupy the pore space and thus limit the advective flow. Third, no advection occurs within the microcolonies and thus, the transport is there only diffusion-driven. The diffusion efficiency depends on biofilm density (Costerton *et al.*, 1995).

A biofilm is characterized by physical and chemical microenvironments (Stewart & Franklin, 2008). For instance, dissolved oxygen values decrease in the deeper parts of the biofilm. Almost anoxic values are reached in the center of the microcolony and at the colonized surface, allowing the involvement of anaerobic bacteria (Costerton *et al.*, 1995, Jain *et al.*, 2007). Also, pH gradients are observed within a biofilm e.g. the pH decreases in cell-dense regions, mainly due to the presence of acid-producing bacteria. In general, spatial and chemical variation within the biofilm permits the involvement of many fastidious bacteria with a limited range of metabolic activities (Costerton *et al.*, 1995).

Biofilms are primarily composed of microbial cells and an EPS matrix (Jain *et al.*, 2007). EPSs are biopolymers of microbial origin in which microorganisms are embedded (Flemming *et al.*, 2007) and may account for 50 % to 95 % of the total organic carbon of biofilms (Jain *et al.*, 2007). EPS production is a general property of microorganisms in natural environments and occurs both in prokaryotic (*Bacteria*, *Archaea*) and in eukaryotic (*Algae*, *Fungi*) microorganisms. EPSs also contribute to the physicochemical and biological properties of biofilms (Flemming & Wingender, 2001). Polysaccharides and proteins are the main components of the EPS matrix (Branda *et al.*, 2005) but also extracellular DNA has been observed (Flemming *et al.*, 2007) (Table 1). In addition, noncellular materials such as mineral crystals, corrosion products, clay particles or blood components may be present in the biofilm matrix, depending on the environment in which the biofilm has developed (Donlan, 2002).

EPS polysaccharides vary in size (Sutherland, 1985) and are usually negatively charged (Allison, 1998) although regional variation in EPS charge has been observed (Costerton *et al.*, 1995). Most of the EPS polysaccharides contain sugars such as glucose, galactose, mannose, fructose and others. These sugars allow the association of divalent cations such as calcium that cross-link the polymer strands and thus provide a stronger binding in the matrix (Jain *et al.*, 2007). In general, a whole range of EPS polysaccharides has been observed within the biofilm matrix due to the presence of different bacterial strains (Branda *et al.*, 2005, Flemming *et al.*, 2007, Friedman & Kolter, 2004, Jain *et al.*, 2007, Matsukawa & Greenberg, 2004).

The second most abundant EPS components are proteins, which compose 1 to 60 % of the biofilm matrix (Table 1). Proteins in the EPS matrix are relatively small as their molecular masses range between 5,000 and 15,000 daltons (Flemming & Wingender, 2001). Examples of EPS proteins are amyloid adhesins (Larsen *et al.*, 2007, Larsen *et al.*, 2008) and curli, produced by members of the family *Enterobacteriaceae* (Barnhart & Chapman, 2006).

The presence of extracellular DNA in the EPS matrix was commonly considered a remnant of lysed cells. However, during the last years, a stabilizing role for extracellular DNA in the

biofilm matrix has been suggested (Yang *et al.,* 2007) as it appeared to be organized in distinct patterns, forming grid-like structures (Allesen-Holm *et al.,* 2006).

Component	% content of EPS
Polysaccharides	40 – 95 %
Proteins	< 1 – 60 %
Nucleic acids	< 1 – 40 %
Lipids	< 1 – 40 %

Table 1: Composition of the EPS matrix (Flemming *et al.*, 2007)

1.1.2 Biofilm formation

The extent of biofilm formation on surfaces is partially regulated by the amount of nutrients (Costerton *et al.*, 1995) as an increase of nutrients correlates with an increase of the number of attached bacterial cells (Jain *et al.*, 2007). Planktonic bacterial cells are attracted to organic nutrients that concentrate naturally at surfaces in aquatic systems, the so-called conditioning film (Costerton *et al.*, 1995, Jain *et al.*, 2007, Kumar & Anand, 1998). Other factors, including surface properties (e.g. roughness and hydrophobicity), physicochemical properties (e.g. pH, temperature, viscosity and flow rate) and the presence of other microorganisms also play an important role in biofilm development (Stewart & Franklin, 2008). Biofilm formation is characterized by five stages: reversible attachment, irreversible attachment, maturation stage-1 and stage-2 and detachment (Fig. 1). The biofilm cells, in each of these stages, exhibit a different phenotype compared to planktonic cells (Sauer *et al.*, 2002).
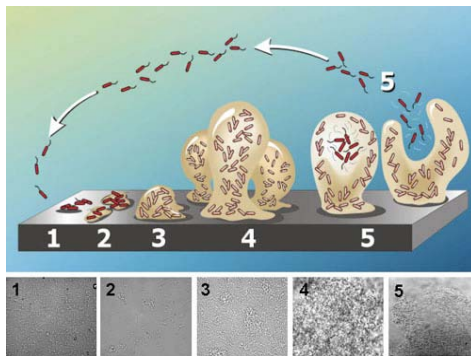
Figure 1: Biofilm formation.
Stage 1: reversible attachment. Stage 2: irreversible attachment. Stage 3: maturation
stage-1. Stage 4: maturation stage-2. Stage 5: detachment. The bottom panels represent
the 5 stages as observed by photomicroscopy of a *Pseudomonas aeruginosa* biofilm
(Stoodley *et al.*, 2002).

1.1.2.1 Reversible attachment

This initial attachment is a reversible process as some bacteria attach only briefly and many leave the surface to resume their planktonic lifestyle (Sauer *et al.*, 2002, Stoodley *et al.*, 2002). Flagella can be important in the initial attachment. For instance, a non-motile *Pseudomonas* strain shows a much lower adherence efficiency compared to the parental motile strain (Sauer *et al.*, 2002). In contrast, *Staphylococcus epidermidis* lacks flagella and thus must use different cell-to-surface attachment mechanisms, but these mechanisms have not yet been elucidated (Nobile & Mitchell, 2007).

An important question is how bacteria find the surface they will colonize. Costerton (1999) hypothesized that bacteria sense a surface by the excretion of protons and signalling molecules. Normally, these molecules diffuse radially away from the planktonic bacteria. When surfaces or interfaces are situated in the proximity of the bacteria, there is limited diffusion of the protons or signalling molecules and thus, a sharper gradient may be sensed by the bacterial cells. This signal may initiate the initial attachment of bacterial cells to a surface.

1.1.2.2 Irreversible attachment

This step is defined as the transition from a weak interaction of the cells with the substratum to a permanent binding (Stoodley *et al.*, 2002). During this stage, cell clusters develop and large amounts of EPS are produced (Sauer *et al.*, 2002, Stoodley *et al.*, 2002). EPS production

is regulated by quorum sensing in response to a variety of environmental factors such as nitrogen limitation and high osmolarity (Stoodley *et al.*, 2002). Bacteria that use quorum sensing produce and secrete certain signalling molecules, that allows the bacterial population to coordinate their gene expression and thus their collective behaviour, when a certain population density threshold is passed (Chen *et al.*, 2002). Quorum sensing can also determine the biofilm architecture as within a quorum sensing-deficient biofilm the EPS remained associated with the bacterial cells and did not fill the interstices between the microcolonies (Sauer *et al.*, 2002). Irreversible attachment is correlated with profound physiological changes. For instance, the expression of more than 30 genes was altered within 6 h following attachment in a *Pseudomonas aeruginosa* biofilm (Sauer & Camper, 2001). Whitely *et al.* (2001) assigned the differentially expressed genes to motility, attachment, translation, metabolism, transport and regulatory functions.

Irreversible attachment also induces a switch from flagella-based to type IV pili-based twitching motility (Sauer & Camper, 2001). This transition promotes the formation of microcolonies by strengthening the attachment (O'Toole & Kolter, 1998). In contrast, adherent *Staphylococcus epidermidis* cells produce a polysaccharide intercellular adhesin in order to form microcolonies (Gerke *et al.*, 1998).

1.1.2.3 Maturation stage-1

During this stage, the cell clusters become more layered with a thickness typically larger than 10 µm. The biofilm structure becomes more complex with channels, pores, redistribution of bacteria and an increased production of EPS (Davies *et al.*, 1998). Proteins involved in metabolism, phospholipids and lipopolysaccharide biosynthesis, membrane transport and secretion, adaptation and protective mechanisms were upregulated in a *P. aeruginosa* biofilm, compared to planktonic *Pseudomonas aeruginosa* cells (Hall-Stoodley *et al.*, 2004, Sauer *et al.*, 2002). The Arc proteins, involved in anaerobic processes, were also upregulated implying that oxygen becomes limited in some regions of the biofilm.

1.1.2.4 Maturation stage-2

The biofilm reaches its maximal thickness and the bacterial cells within the biofilm differ most from their planktonic counterparts as more than 50 % of detectable proteins in a *P. aeruginosa* biofilm were differentially expressed. In addition, profound variations in protein

expression between cells within the biofilms of maturation stage-1 and maturation stage-2 have been observed (Sauer *et al.*, 2002).

1.1.2.5 Detachment

Detachment is defined as the release of cells from the biofilm or from the substratum (Stoodley *et al.*, 2002), allowing the colonization of new niches (Sauer *et al.*, 2002) and can be caused by external perturbations such as increased fluid shear (Stoodley *et al.*, 2002), or by internal biofilm processes such as endogenous enzymatic degradation (Boyd & Chakrabarty, 1994, Kaplan *et al.*, 2003, Lee *et al.*, 1996). Detachment closes the biofilm cycle as bacteria return to their planktonic lifestyle. Dispersed biofilm cells are more similar to planktonic cells than to maturation stage-2 biofilm cells (Sauer *et al.*, 2004). In addition, gene expression levels are downregulated during detachment when compared to the other biofilm development stages. Three different detachment strategies can be discriminated: swarming/seeding dispersal, clumping dispersal and surface dispersal (Stoodley *et al.*, 2002). Swarming/seeding dispersal is characterized by microcolonies with an outer edge of stationary bacteria while the center of the microcolonies liquefies and motile bacteria swarm out of the biofilm (Hall-Stoodley *et al.*, 2004, Sauer *et al.*, 2002). Self-lysis in the microcolony enhances the ability of other cells to disperse from the biofilm (Mai-Prochnow *et al.*, 2006). Dispersed cells are metabolically more active and phenotypically more diverse, probably because they benefit from the nutrients released from the lysed cells (Webb *et al.*, 2003). Clumping dispersal is the shedding of whole aggregates surrounded by EPS. The cells within these clumps are physiologically more similar to attached cells than to planktonic cells (Hall-Stoodley *et al.*, 2004). Finally, surface dispersal is the movement of whole biofilms across a surface through shear-mediated transport, forming migratory ripple structures (Purevdorj *et al.*, 2002, Stoodley *et al.*, 1999).

1.1.3 Biotechnological applications of biofilms

Biofilms are of great interest in biotechnology and their applications are expanding in different industrial areas. Biofilms are used in bioremediation, which is a technology for the clean up of environmental pollutants, by using microorganisms. For instance, Macedo *et al.* (2005) demonstrated the efficient degradation of polychlorinated biphenyls by a mixed-culture biofilm. Biofilms have also been applied to remove natural toxins such as microcystins produced by cyanobacteria (Babica *et al.*, 2005). Biofilms are suitable for the treatment of recalcitrant compounds due to their high microbial biomass and their ability to immobilize compounds (Singh *et al.*, 2006). The increased exchange of genetic material in biofilms is also advantageous for bioremediation as the genes encoding for xenobiotic degradation are often located on plasmids. In addition, microbial biofilms can be implemented in the remediation of heavy metal pollution as metal ions can be entrapped within the biofilm matrix ("biosorption") by e.g. ion exchange and chelation. For instance, microorganisms produce a diverse array of metabolic end products, which can bind and/or react with metal species. Also, dead cells provide additional biosorptive sites (Harrison *et al.*, 2007).

Biofilms can also be used for chemical production via biosynthesis, fermentation or biotransformation (Li *et al.*, 2007) such as for instance poly-3-hydroxybutyrate biosynthesis in a *Cupriavidus necator* biofilm grown in a packed-bed reactor (Zhang *et al.*, 2004). The use of biofilms in fine-chemical production has been also suggested as biofilms inherently immobilize cells and so overcome the common obstacle in fine-chemical production processes (Li *et al.*, 2006). Fermentation processes established by bacterial biofilms, such as the production of ethanol by *Zymomonas mobilis* biofilms, resulted in a 15- to 100-fold higher yield compared to the planktonic cells (Kunduru & Pometto, 1996).

Biofilms are also applied in biofiltration. The latter process is used to eliminate nuisance chemical odors from industrial air streams. The contaminated air, containing e.g. hydrogen sulphide, methyl mercaptan or ammonia, is passed over a bed of media (such as wood chips, peat, compost or inorganic media) upon which biofilms are present. Bacteria within these biofilms oxidize odour constituents into harmless and odourless products (Shareefdeen *et al.*, 2003). Biofiltration has also been applied for the treatment of the effluents of aquaculture, thereby decreasing significantly the discharge of carbonaceous and nitrogenous matter in the environment (Yang *et al.*, 2001).

One of the most important applications of biofilms is their use in wastewater treatment for the degradation of the biological content of the sewage. A whole range of reactors in which biofilms are present and sewage passes through have already been designed (Nicolella *et al.*, 2000). Furthermore, the application of aerobic and anaerobic granular sludge has become important. Granular sludge is built up of spherical compact aggregates of microorganisms (mainly bacteria and EPS but also protozoa and fungi) and can be regarded as biofilms in suspension. Advantages of granular sludge are the high settle ability, high and stable metabolic

rates and its resistance to shocks and toxins (Weber *et al.*, 2007). In general, biofilms are often more efficient for wastewater treatment than activated sludge.

1.1.4 The downside of biofilms

More than 60 % of the bacterial infections in the developed world are considered to involve biofilm formation (Fux *et al.*, 2005). Pathogens that are present in biofilms more likely cause disease than their planktonic counterparts (Costerton *et al.*, 1999) and several reasons may account for this. First of all, biofilm formation results in an accumulation of bacterial cells. For instance, the infectious dose of *Vibrio cholerae* required to induce symptomatic cholera is reached within a biofilm. The detachment of parts of a biofilm leads to the dispersal of high doses of pathogens, sufficient to cause infections. Also, biofilms harbour phenotypically different bacteria and thus a virulent phenotype might easily survive and clonally expand within a biofilm. In addition, the high cell densities might stimulate quorum sensing networks which also can activate virulence mechanisms (Hall-Stoodley & Stoodley, 2005).

Several chronic infections are caused by biofilm-associated bacteria, for instance, native valve endocarditis, otitis media, periodontitis and chronic prostatitis. Also, indwelling medical devices or other devices constitute targets for biofilm colonization, e.g. *Enterococcus* sp. biofilms were observed on artifical hip prosthesis (Jain *et al.*, 2007).

Biofilm-associated bacteria release antigens that stimulate the antibody production of the host. As antibodies cannot penetrate the biofilm, bacteria within the biofilm are not or not efficiently killed. The formation of immune complexes (antigen-antibody) damages the surrounding tissue, leading to inflammation. For instance, the formation of *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients leads to inflammation and eventually to severe lung tissue damage. Biofilm infections typically show recurring symptoms until the biofilms are surgically removed from the body (Costerton *et al.*, 1999). Another example of a biofilm-associated infection is Legionnaires' disease, which is a severe pneumoniae caused by *Legionella pneumoniae*. This bacterium is found within biofilms in water systems worldwide, representing a major health risk (Berry *et al.*, 2006, Fields *et al.*, 2002). The formation and presence of biofilms on a food product or a contact surface lead to serious hygienic problems and economic losses due to the spoilage and the decreased shelf life of the product. Also, the persistence of foodborne pathogens such as *Campylobacter jejuni* causes severe public health risks (Kumar & Anand, 1998).

One of the major problems encountered when treating biofilm-associated infections is the inherent resistance of biofilms to antimicrobical compounds for which several mechanisms may account. First, the EPS matrix acts as a barrier that prevents the penetration of many antibiotics. Second, biofilm cells are metabolically less active than planktonic cells and thus

less vulnerable to antibiotics. Moreover, the transition from the active planktonic to the biofilm lifestyle is generally accompagnied by the upregulation of antibiotic-resistance genes. Third, the incoming antibiotics can be inactivated directly by the presence of antibiotic-degrading enzymes that are entrapped in the EPS matrix. Fourth, biofilm cells are phenotypically different from planktonic cells. For instance, up to 40 % of the cell wall proteins are altered which indicates that antibiotic targets might be modified. Fifth, reactive oxidants entrapped in the EPS matrix such as hypochlorite or H_2O_2 can deactivate antimicrobial agents in the outer layers of the biofilm. Sixth, as antibiotic-resistance genes are often present on transposable genetic elements and biofilms constitute an ideal environment for genetic exchange, a microbial population with an increased antibiotic resistance can be generated (Jain *et al.*, 2007). Also, biofilms are resistant to antibodies and bacteriophages (Costerton *et al.*, 1999). Apart from its role in infectious diseases, biofilm formation has a negative effect on several industries. For instance, biofilm formation in drinking water distribution systems leads to economical losses due to a decrease of the velocity, the clogging of pipes and an increase in energy utilization, leading to a general decrease in the operating efficiency of the system (Lechevallier *et al.*, 1987). Biofilm formation in heat exchangers and cooling towers greatly reduces the heat transfer and operating efficiency of the systems. In fact, submerged surfaces in all water environments are targets for biofilm formation. For instance, the fouling of ship hulls is mainly caused by biofilms consisting of algae, diatoms and bacteria. These biofilms increase the fluid resistance and fuel consumption (Lechevallier *et al.*, 1987).

Microbially induced corrosion or biocorrosion is the accelerated deterioration of metals owing to the presence of biofilms on their surfaces (Beech & Sunner, 2004). It is an electrochemical process involving the transfer of electrons through a series of oxidation and reduction reactions. The corrosion products accumulate at the surface of the metal and form one or more layers that serve as a diffusion barrier to reactants (Beech, 2003). Biocorrosion causes serious economic losses. For instance, Booth (1964) suggested that 50 % of the corrosion in pipelines are caused by bacteria and Flemming (1996) estimated that up to 20 % of all corrosion damage of metals is microbially driven. Several mechanisms were suggested to explain microbially induced corrosion. First, bacteria produce a wide range of enzymes including extracellular enzymes such as polysaccharidases, lipases, esterases and oxidoreductases. For instance, the activity of hydrogenase enzymes accelerated sulphate-reducing bacteria-influenced deterioration (Beech, 2003, Beech *et al.*, 2005). Also, biofilms influence biocorrosion through the binding of metal ions in the extracellular matrix and these complexes may participate in electron-transfers (Beech & Sunner, 2004, Beech *et al.*, 2005). The involvement of the EPS matrix in biocorrosion is not yet completely understood (Beech *et al.*, 2005), but the presence of acidic polysaccharides during biofilm formation may influence biocorrosion (Beech & Gaylarde, 1999). Numerous bacteria are known to promote corrosion of iron and its alloys through dissimilatory reduction reactions, driven by enzymes which results in the dissolution of the protective oxide/hydroxide films on metal surfaces. Thus, passive layers on steel surfaces can be lost or replaced by less stable, reduced metal films

that allow further corrosion. *Shewanella oneidensis* is one of the best-known organisms that drive biocorrosion through dissimilatory iron reduction (Beech *et al.*, 2005). Microorganisms that cause biocorrosion of metals, such as iron and stainless steel, are phylogenetically diverse, but are mainly sulphate-reducing, sulphur-oxidizing, iron-oxidizing/ reducing, manganese-oxidizing bacteria and bacteria secreting organic acids and slime (Beech & Gaylarde, 1999).

Ennoblement, the phenomenon in which the open-circuit potential (E_{corr}) is elevated to noble values (over 200 mV) has been observed in association with biocorrosion (Baker *et al.*, 2003). The open-circuit potential or free corrosion potential is the potential of a corroding surface (in the absence of a net electrical current) and is measured in relation to a reference electrode. Ennoblement is caused by biofilm formation and has mainly been observed for stainless steels in natural seawater (Baker *et al.*, 2003, Kolari *et al.*, 1998, Mansfeld, 2007, Mattilda *et al.*, 1997). The observation that one type of bacteria can shift E_{corr} of one metal in the positive direction while another type can shift E_{corr} of another metal in the negative direction suggests that it might be possible to construct a bacterial battery that has a larger cell voltage than the same battery that does not contain bacteria. This has lead to the construction of microbial fuel cells (MFCs) (Mansfeld, 2007).

1.2 MICROBIAL FUEL CELLS

A microbial fuel cell is a device that converts chemical energy into electricity with the aid of electrochemically active microorganisms (Lee *et al.*, 2003). Bacteria gain energy by the transfer of electrons from an electron donor (e.g. glucose) to an electron acceptor (e.g. oxygen, nitrate). The larger the difference in potential between the electron donor and the acceptor, the more energy that is generated and generally, the higher bacterial growth yield that is obtained. In a MFC, bacteria transfer their electrons towards an electrode i.e. anode, instead of towards their characteristic electron acceptor (Rabaey *et al.*, 2005b). On the other hand, microorganisms can also accept electrons from an electrode at low redox potentials (the cathode). For instance, *Geobacter* species accept electrons from the cathode for their anaerobic respiration (Gregory *et al.*, 2004). At present, the most-studied MFCs are those in which bacteria oxidize dissolved organic matter and transfer the obtained electrons towards the anode (Lovley, 2006a).

1.2.1 Architecture

A typical MFC consists of an anode receiving electrons generated by the bacterial metabolism and a cathode, accepting the electrons from the anode. Subsequently, the cathode transfers the electrons to an electron acceptor such as oxygen (Lovley, 2006a) or nitrate (Clauwaert *et al.*, 2007). Thus, electrons flow from the anode to the cathode through an external electrical connection over a resistor. The anodic compartment is typically maintained under anoxic conditions whereas the cathode is suspended in an aerated solution or exposed to air. The anodic and cathodic compartments are separated by a semipermeable membrane that allows the migration of protons released from the oxidation of the organic matter, from the anodic towards the cathodic compartment (Lovley, 2006a).
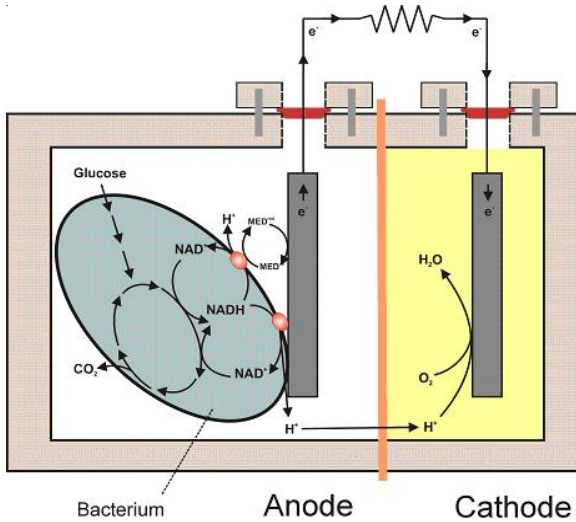
Figure 2: Schematic representation of a MFC comprising an anodic and cathodic compartment, separated by a proton exchange membrane (Rabaey & Verstraete, 2005).

Four different basic MFC configurations have been described in literature.

The first type is the uncoupled bioreactor MFC in which microbiological hydrogen or methane production takes place in one bioreactor, followed by a chemical fuel cell that converts hydrogen gas into electricity. This electricity production is not efficient due to the low bacterial efficiency of hydrogen production in the first cell and due to the high temperatures required for the oxidation of hydrogen gas in the second cell (Rabaey *et al.*, 2005b). The second type of MFC is the integrated bioreactor, which relies on the same principle as the previous one, except that hydrogen production and oxidation take place in the same bioreactor. In fact, these two types of MFC are often not regarded as real MFCs as they involve a chemical fuel cell to oxidize the generated gas (Rabaey *et al.*, 2005b).

In the third MFC type, electrons are transferred from the bacteria towards the anode. This transport can be achieved by direct electron transfer or, in a fourth type of MFC configuration, by an external electron shuttle. As bacteria act as catalysts in the electron transfer, the selection of a high performing microbial consortium is important (Rabaey *et al.*, 2005b).

Yet another type of biological fuel cell, but not regarded as a genuine MFC, is the enzymatic fuel cell in which enzymes (Rabaey *et al.*, 2005b) or cell extracts (Lovley, 2006a) are immobilised on the anode surface.

At present, four different designs of genuine MFCs have been presented in literature (Du *et al.*, 2007). The first type is the two-compartment MFC consisting of two chambers (the cathodic and anodic compartments) that are separated by a proton exchange membrane or a salt bridge. A major drawback is that flow conditions have to be maintained in both compartments, increasing significantly the energy costs (Du *et al.*, 2007).

The second MFC type is a single-compartment MFC that comprises a single chamber in which the anode and the cathode are placed, with the cathode being in direct contact with the atmosphere. As the cathode is directly exposed to air, the need for aeration is eliminated and thus the operation costs of the MFC decrease significantly (Du *et al.*, 2007, Rabaey *et al.*, 2005b). A third MFC type is the up-flow mode MFC that comprises a cylindrical shaped reactor in which the anode is placed at the bottom (covered by a layer of glass beads and glass wool) while the cathode is placed at the top. The feed stream is supplied continuously to the bottom of the reactor (anodic side) and passes towards the cathode (Du *et al.*, 2007, Jang *et al.*, 2004). The fourth design is the stacked MFC that comprises several MFCs, which are connected in series or in parallel in order to increase the power output (Aelterman *et al.*, 2006b).

The typical set-up of MFCs comprises "biological" anodes and "abiotic" cathodes. Anodes are considered to be biological when microorganisms act as catalysts for the reduction of the electrode with electrons derived from the oxidation of organic matter in the anodic compartment. On the other hand, cathodes abiotically transfer electrons to oxygen which functions as the terminal electron acceptor (He & Angenent, 2006). Generally, because of the slow oxygen reduction rate at the surface of the cathodes, artificial electron mediators are required (Shukla *et al.*, 2004) such as platinum or transition metal-based materials (e.g. cobalt tetramethylphenylporphyrin), although their application is limited due to the high costs (Cheng *et al.*, 2006, He & Angenent, 2006, Park & Zeikus, 2002, , 2003, Zhao *et al.*, 2005). In addition, the use of graphite granules as cathodic material significantly increases electrical current production (Freguia *et al.*, 2007).

On the other hand, microorganisms can also act as catalysts for the reduction of a terminal electron acceptor at the cathode surface. These types of cathodes are referred to as "biocathodes" (He & Angenent, 2006) and may present several advantages over the abiotic cathodes. With this type, the supplementation of electron mediators is omitted which lowers construction and operation costs of the MFCs and eliminates problems such as consumption and replacement of electron transfer mediators. Two types of biocathodes are distinguished depending on the terminal electron acceptor: aerobic biocathodes use oxygen as terminal electron acceptor while anaerobic biocathodes use alternative terminal electron acceptors such as nitrate, iron or manganese (He & Angenent, 2006). The most popular terminal electron acceptor is oxygen due to its high redox potential (He & Angenent, 2006). Rhoads *et al.* (2005) described a biocathode in which biological Mn (II) oxidation was coupled to oxygen reduction in a MFC, which increased the power output 40 times. Similar studies were performed in order to couple biological Fe (II) oxidation, conducted by *Acidothiobacillus ferrooxidans*, to oxygen reduction (He & Angenent, 2006, Nemati *et al.*, 1998). The formation of a biofilm on the cathode had a positive effect on the power output of the MFCs. For instance, Bergel *et al.* (2005) used an abiotic anode and a stainless steel cathode and recorded an increased power output due to biofilm formation on the cathode. In addition,

Hasvold *et al.* (1997) demonstrated that biofilm formation on the surface of the cathode of their "sea-water batteries" remarkably increased oxygen reduction rates. In the absence of oxygen, the electrons originating from the cathode reduce other compounds such as nitrate (Gregory *et al.*, 2004, Park *et al.*, 2005), iron or sulphate (He & Angenent, 2006). Clauwaert *et al.* (2007) were the first to couple biological oxidation of acetate at the anode with biological reduction of nitrate at the cathode.

1.2.2 Advantages of electricity production by MFCs

The direct transformation of energy to electricity results in a high conversion efficiency (Rabaey & Verstraete, 2005). For instance, Rabaey *et al.* (2004) reached up to 80 % glucose-to-power transition efficiency with a consortium originating from methanogenic sludge and mainly consisting of facultative anaerobes. Chaudhuri & Lovley (2003) recovered 80 % of the electrons, derived from sugar oxidation, as electricity by a pure culture of *Rhodoferax ferrireducens*. Moreover, MFCs operate at room temperature which lowers remarkably the heating costs e.g. as required for hydrogen gas oxidation (Rabaey *et al.*, 2005b, Rabaey & Verstraete, 2005). In addition, the oxidation of organic matter only releases fixed carbon into the atmosphere which avoids the treatment of off-gases (Lovley, 2006a, Rabaey *et al.*, 2005b). One of the major promising applications of MFCs is the generation of electricity from biowastes (e.g. wastewater) and organic matter (e.g. present in marine sediments) (Lovley, 2006a, Rabaey *et al.*, 2005b), and thus MFCs represent a source of green energy.

Also, MFCs have potential for widespread applications at remote locations or in developing countries (Rabaey & Verstraete, 2005). Lovley (2006a) suggested that MFCs, of which the anode is embedded in anaerobic marine sediments while the cathode is put in the overlying aerobic seawater (BUGs; Benthic Unattended Generators) can provide electricity to analytical tools at remote locations such as the ocean floor. Currently, the Naval Research Laboratory's Center for Bio/Molecular Science and Engineering (Washington, USA) is deploying a weather buoy in coastal waters, solely powered by BUGs (www.nrl.navy.mil/code6900/bug).

A MFC has no substantial intermediary processes as microorganisms directly transfer electrons from or towards the electrode. This makes MFCs the most efficient biological electricity producing process (Rabaey *et al.*, 2005b). However, the power output of MFCs is still low (Aelterman *et al.*, 2006a, Liu *et al.*, 2004, Logan, 2005, Rabaey *et al.*, 2005b). For instance, approximately 26 mW/m^2 (electrode surface) is obtained with a BUG (Tender *et al.*, 2002). On average, a MFC fed with wastewater produces 40 W/m^3 (reactor volume) (Pham *et al.*, 2006). Up to now, one of the highest power outputs (228-248 W/m^3) was reached by connecting stacked MFCs that were fed with wastewater (Aelterman *et al.*, 2006a). In contrast, conventional anaerobic digestion based bioconversion processes, which produce biogas, can yield power outputs twice as high (Pham *et al.*, 2006).

Microbial fuel cells may be used as a stand-alone power source but more and more, due to the limited power output, MFCs evolve towards more specific applications that combine electricity production with biological processes. For instance, the simultaneous treatment of wastewater and electricity production represents one of the major promising applications of MFCs (Aelterman *et al.*, 2006a, Logan, 2005). In addition, MFCs may also be used e.g. for biological denitrification (Clauwaert *et al.*, 2007, Jia *et al.*, in press), for sulphide removal out of wastewater (Rabaey *et al.*, 2006), for the removal of odors out of swine wastewater (Kim *et al.*, 2008) and to empower gastrobots (Wilkinson, 2000).

1.2.3 Extracellular electron transfer

Extracellular electron transfer is required to transfer the electrons towards or from the electrode. In fact, extracellular electron transport is necessary whenever an electron donor or acceptor cannot enter the cell, e.g. insoluble Fe (III) oxides in the sediment (Bretschger *et al.*, 2007, Nevin & Lovley, 2002, Rabaey *et al.*, 2007).

Indirect extracellular electron transfer can be achieved by the interaction of reduced metabolic products (such as hydrogen) with the anode where they are oxidized (Katz *et al.*, 2003, Lovley, 2006a). This type of electricity generation is inefficient because many fermentation products react very slowly if at all with the anode (Lovley, 2006a).

Artificial redox mediators can be added to a MFC in order to increase the efficiency of electron transfer (Park & Zeikus, 2003). Examples of redox mediators are neutral red (Park & Zeikus, 1999), phenazines and methylene blue (Katz *et al.*, 2003). Artificial mediators are typically redox molecules that are stable in both oxidized and reduced form (Park & Zeikus, 2003) and can either be taken up into the periplasm (Mckinlay & Zeikus, 2004) or directly be reduced at the bacterial surface. Although externally supplied mediators enhanced electron transport and electricity production, they are generally not suitable for large scale or long-term applications as they are too expensive, can exhibit toxic effects or can be degraded over longer time (Delaney *et al.*, 1984, Gil *et al.*, 2003).

Also, bacteria can produce their own mediator compounds to promote extracellular electron transport (Lies *et al.*, 2005, Rabaey *et al.*, 2004). For instance, *P. aeruginosa* produces phenazines in order to generate electricity in a MFC (Rabaey *et al.*, 2005a, Rabaey *et al.*, 2004). Moreover, the phenazines produced by a *Pseudomonas* strain enabled a Gram-positive bacterium, *Brevibacillus* sp., to perform extracellular electron transfer (Pham *et al.*, 2008). The synthesis of an electron shuttle is energetically expensive for the bacterium and therefore an electron shuttle must be recycled many times in order to recoup this energy investment (Madahevan *et al.*, 2006). It appears that in a batch system there is a selection towards microorganisms that produce their own mediators in order to transfer electrons to the electrode (Rabaey *et al.*, 2004). In contrast, there is no such selection in continuous flow systems as

the electron shuttle would be rapidly flushed away (Lovley, 2006b, Rabaey *et al.*, 2005c). The critical issue with mediated electron transfer is the diffusion of the shuttle out of the biofilm or the bacterial environment (Rabaey *et al.*, 2007). Redox shuttles tend to concentrate mainly in the biofilm (Bond & Lovley, 2005), but the underlying mechanism has not yet been elucidated. Rabaey *et al.* (2007) suggested that nearly all the shuttles accumulate close to the anode whereas the shuttle concentration at the top of the biofilm is almost negligible. This could create a duality between bacteria at the bottom of the biofilm, which have direct access to electron acceptors but are limited in electron donor availability and bacteria at the top of the biofilm that have direct access to the electron donor but are limited in electron acceptor availability.

In addition, the involvement of c-type cytochromes in extracellular electron transfer was claimed. Holmes *et al.* (2006) investigated the differential gene expression in *Geobacter sulfurreducens* during growth on electrodes versus growth on Fe (III) citrate. Expression levels of the gene encoding OmcS increased 19-fold when *G. sulfurreducens* was grown on electrodes instead of Fe (III) citrate. OmcS is an outer-membrane c-type cytochrome involved in the reduction of Fe (III) and Mn (IV) oxides (Mehta *et al.*, 2005). Also, the transcript levels of OmcT increased significantly when *G. sulfurreducens* grew on anodes. OmcT appears to be co-transcribed with OmcS, but is not required for the reduction of Fe (III) and Mn (IV) oxides (Mehta *et al.*, 2005). In addition, the *omcE* gene expression levels were also upregulated (Holmes *et al.*, 2006); the OmcE protein is known to be also involved in the reduction of Fe (III) and Mn (IV) oxides (Mehta *et al.*, 2005). Moreover, the genome sequence of *G. sulfurreducens* revealed more than 100 putative c-type cytochromes, suggesting the involvement of many c-type cytochromes in extracellular electron transport (Holmes *et al.*, 2006, Methé *et al.*, 2003).

Finally, a possible electron-transferring role was hypothized for pilus-like extensions, the so-called nanowires (Reguera *et al.*, 2005, Reguera *et al.*, 2006). These nanowires were observed during the reduction of Fe (III) oxides (Reguera *et al.*, 2005) and seemed to be especially important when thick biofilms are formed (Reguera *et al.*, 2006). In contrast, Holmes *et al.* (2006) observed no increase of the expression levels of the genes encoding pilus-like structures when *G. sulfurreducens* was grown on electrodes. In fact, it was hypothesized that nanowires are not absolutely required for electron transfer but are necessary for generating maximal levels of power (Reguera *et al.*, 2006).

1.2.4 Wastewater MFCs

One of the major challenges for the next years is the processing of waste and wastewater. In order to sustain an ever-growing world population, we will have to use the waste instead of disposing it (Angenent *et al.*, 2004). The ideal scenario is to produce energy from waste, the so-called 'from foul to fuel' principle (Ehrenman, 2004). Wastewaters, e.g. industrial wastewaters from food-processing industries and breweries or agricultural wastewaters from animal confinements, have a high energy value due to the high levels of degradable organic matter and thus constitute an ideal substrate for energy generation (Angenent *et al.*, 2004). Several biological processes may be applied to recover energy including methanogenic anaerobic digestion and biological hydrogen production. MCFs provide yet another energy harvesting strategy (Angenent *et al.*, 2004). The amount of sludge generated by MFCs is significantly lower than in traditional treatments as the bacterial growth yield is lower (Ghangrekar & Shinde, 2006, Rabaey *et al.*, 2005b) and aeration can be omitted as the cathode can be passively aerated by placing it in the open air (Rabaey & Verstraete, 2005). The elimination of aeration in MFCs is very important as traditional wastewater treatments are expensive due to the large amounts of energy needed for aeration (Liu *et al.*, 2004).

Liu *et al.* (2004) were the first to generate electrical current from domestic wastewater using a single-chamber MFC while simultaneously executing biological wastewater treatment i.e. the removal of the chemical oxygen demand. The chemical oxygen demand (COD) indirectly measures the organic compounds in water. In fact, it is the amount of oxygen needed to completely oxidize the organic compounds. Electricity generation by a MFC proved to be feasible as up to 80 % of the COD was removed and electricity was produced (Liu *et al.*, 2004). Since this study, the electrical current production and bacterial populations associated with the electrodes were studied for MFCs fed with different types of waste such as wastewater from a potato-processing factory and a hospital (Aelterman *et al.*, 2006a), artificial wastewater supplemented (or not) with electron donors (Jong *et al.*, 2006, Moon *et al.*, 2006, Phung *et al.*, 2004), sludge from wastewater treatment plants (Jung & Regan, 2007, Kim *et al.*, 2006, Kim *et al.*, 2007, Lee *et al.*, 2003), swine wastewater (Min *et al.*, 2005), urban waste (Rodrigo *et al.*, 2007) and organic waste (Kim *et al.*, 2004). In all cases, electricity was produced and was maintained for long periods e.g. ranging from two (Moon *et al.*, 2006) to five years (Kim *et al.*, 2004). Power production in waste water is coupled with the removal of COD; for instance, MFCs can remove up to 80 % (Liu *et al.*, 2004) or even up to 97 % (Kim *et al.*, 2004) of the COD. Not all the removed COD results in electrical current production as the efficiencies and thus the power output of the MFCs fed with wastes are still rather low (Aelterman *et al.*, 2006b, Logan, 2005). Several adaptations have been made in order to improve the power output such as the use of a single-chambered MFC (Kim *et al.*, 2007, Logan, 2005, Min *et al.*, 2005) which has a much lower internal resistance than two-chambered MFCs (Kim *et al.*, 2007). Biofilm formation was observed on the surface of the anodes (Kim *et al.*, 2004, Kim *et al.*, 2006, Lee *et al.*, 2003) and the biofilms comprised dense communities

of living Gram-positive and Gram-negative bacteria (Kim *et al.*, 2004). In some cases, bacterial clumps loosely associated with the biofilm were observed (Kim *et al.*, 2004, Kim *et al.*, 2006). These clumps are missing in MFCs fed with acetate and therefore, it was suggested that the bacteria within the clumps ferment the complex fuel (e.g. organic waste, starch-processing wastewater). Furthermore, the diversity of an anodic biofilm of a MFC fed with acetate was lower than that of a MFC fed with complex organic matter. Subsequently, the biofilm bacteria oxidize these fermentation products and transfer the electrons towards to the anode (Kim *et al.*, 2004). Kim *et al.* (2004) also reported the presence of nanobacteria-like particles in an anodic biofilm of a MFC fed with organic wastewater, of which the function remains unknown. The microbial population of the electrode-associated biofilms in wastewater MFCs differs significantly from the population of the initial inoculum, indicating an enrichment of a bacterial consortium that oxidizes the organic matter and transfer the electrons to the electrode (Jong *et al.*, 2006, Kim *et al.*, 2004, Kim *et al.*, 2006, Lee *et al.*, 2003). Also, the microbial diversity of the different parts of the MFC proved to be different (Kim *et al.*, 2006) as specific microbial communities were observed when the anodic biofilm, the bacterial clumps, the planktonic community and the ion-exchange membrane biofilm of the anodic compartment of a MFC (fed with artificial wastewater and inoculated with activated sludge) were investigated. Increased levels of *Proteobacteria*, especially the *Gamma-* and *Betaproteobacteria*, in the anodic biofilm and the bacterial clumps correlated with a decrease of *Bacteroidetes*. The higher degree of bacterial diversity in the anodic biofilm and bacterial clumps suggested that a complex microbial population generated energy on the surface of the electrode (Kim *et al.*, 2006). Moreover, various metabolites produced in the bacterial clumps, the anodic biofilm and on the anode surface can also function as electron donors or acceptors (Gregory *et al.*, 2004). In addition, Jong *et al.* (2006) observed that different microbial populations were observed on the anodes of a thermophilic MFC continuously fed with brewery wastewater when the anodes were put at different places in the anodic compartment. In contrast to mesophilic MFCs, no *Proteobacteria* were present among the microbial populations of the anodes. In general, the bacterial diversity of the anodic biofilms of a thermophilic wastewater MFC was much lower than that of the anodic biofilms of a mesophilic MFC (Jong *et al.*, 2006).

Overall, the microbial diversity of wastewater or sludge MFCs is much higher than that of benthic MFCs where *Deltaproteobacteria* dominate (Bond *et al.*, 2002). The low abundance of *Deltaproteobacteria* can be explained by the different microbiological ecology of the two systems (Kim *et al.*, 2004, Lee *et al.*, 2003). Also, the diffusion of small amounts of oxygen into the anodic compartment of a wastewater MFC can inhibit the anaerobic respiration of members of the family *Geobacteraceae*. Although *Geobacteraceae* species are much less abundant than in benthic MFCs, their presence has also been reported in MFCs inoculated with anaerobic sludge from a wastewater treatment plant (Jung & Regan, 2007). In that study, the microbial populations of MFCs supplemented with acetate, lactate or glucose were dominated by *Geobacteraceae* (*Geobacter sulfurreducens* and other *Geobacter*

species). The abundance of *G. sulfurreducens* in the lactate and glucose MFCs decreased while the presence of other *Geobacter* species increased, suggesting a competition between different members of the family *Geobacteraceae* and indicating a functional redundancy (Jung & Regan, 2007).

1.2.5 Benthic MFCs

1.2.5.1 Microbiology of marine environments

The microbial populations of different marine habitats such as Arctic and Antarctic seas, open oceans, sediments, coastal subsurface layers and microbial mats have already been characterized. The most intensive study, aimed to analyse the marine bacterial populations, was the genome shotgun sequencing of bacterial communities of the Sargasso Sea (Venter *et al.*, 2004). Using a 97 % sequence similarity cut-off value to delineate unique phylotypes, resulted in at least 1800 phylotypes of which 148 represented previously unknown bacteria. Members of the phylum *Proteobacteria* (mainly *Alpha-*, *Beta-* and *Gammaproteobacteria*) dominated the samples and members of the phyla *Firmicutes*, *Actinobacteria*, *Cyanobacteria* and *Bacteroidetes* were also abundant although to a lesser extent. The presence of these bacteria among a variety of marine environments (such as tidal flat ecosystems, coastal microlayers, shores and polar seas) was confirmed by other studies (Agogué *et al.*, 2005, Eilers *et al.*, 2001, Mergaert *et al.*, 2001, Stevens *et al.*, 2005). Mainly the same bacterial groups were retrieved from marine sediments and microbial mats (originating from a variety of environments such as hypersaline oil-polluted and Antarctic waters) including members of the phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* (Abed *et al.*, 2007, Köpke *et al.*, 2005, Van Trappen *et al.*, 2002). Although largely the same groups were found in different marine environments, some of the dominant microbial members are present in a vertically stratified way (Fuhrman *et al.*, 1997, Giovannoni *et al.*, 1996). It is likely that many of these bacterial groups are specialized in exploiting the vertical gradients in physical, chemical and biological factors (Giovannoni & Stingl, 2005). Also, the boundary between the photic and dark upper mesopelagic zone is reflected in the bacterial population composition, indicating that the upper mesopelagic community is specialized in using resources descending from the photic zone (Giovannoni & Stingl, 2005). In addition, the microbial plankton populations of coastal waters and ocean gyres differ significantly. This is explained by a higher productivity of continental shelves compared to ocean gyres because physical processes, such as updwelling and mixing, bring nutrients to the surface. In general, most of the bacterial groups found in gyres also occur in large numbers in coastal seas but a number

of clades, particular members of the *Betaproteobacteria* appear to be mainly restricted to coastal seas (Rappé *et al.*, 2000). One of the most abundant groups in marine environments is the "marine group I *Archaea*" which belong to the phylum *Crenarchaeota*. They constitute up to 40 % of the mesopelagic microbial community but are also present in the upper layers of cold waters (DeLong, 1992, Fuhrman *et al.*, 1992).

One of the most abundant groups in marine environments are members of the *Alphaproteobacteria*, constituting the *Roseobacter* lineage (Buchan *et al.*, 2005, Gonzàlez & Moran, 1997). All genera of this lineage are confined to marine environments except the genus *Ketogulonicigenium* of which members were isolated from a variety of soils (Urbance *et al.*, 2001).
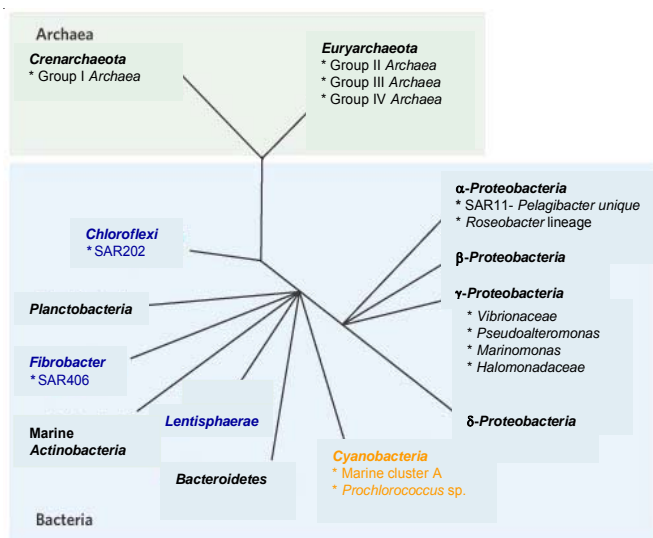
Figure 3: Representation of the phylogeny of the major bacterioplankton clades. Names in black characters indicate the abundant microbial groups in seawater; gold indicates the bacterial groups found in the photic zone; blue indicates the bacterial groups restricted to the mesopelagic and surface waters during polar winters. Redrafted from Giovannoni & Stingl (2005).

The *Roseobacter* lineage, also called the "marine alpha bacteria" (González *et al.*, 1997) is phylogenetically coherent (Buchan *et al.*, 2005, Wagner-Dobler & Biebl, 2006) and is classified within the family of the *Rhodobacteraceae* of the order *Rhodobacterales*. At present, the *Roseobacter* lineage comprises 82 validly published species within 35 genera (Table 2, Fig. 3). Shiba (1991) first created the genus *Roseobacter* in order to harbour *Roseobacter litoralis* and *Roseobacter denitrificans* which are aerobic, pink-pigmented bacteria containing bacteriochlorophyll a. Members of the *Roseobacter* lineage have been studied intensively which resulted in the description of many novel genera and species (22 novel genera were described within the last 5 years) and also in the reclassification of established roseobacters (Arahal *et al.*, 2005, Martens *et al.*, 2006, Pujalte *et al.*, 2005). Yet, the *Roseobacter* lineage

also includes a number of uncultured environmental bacteria of uncertain biological significance. Most of the latter roseobacters have no cultured neighbours.

Roseobacters typically comprise up to 20 % of coastal and 15 % of mixed-layer ocean bacterioplankton and are most abundant in coastal zones and polar oceans (González *et al.*, 1997, González *et al.*, 2000, Selje *et al.*, 2004, Suzuki *et al.*, 2001). They have been found across diverse marine habitats including open oceans, coastal waters, sea ice, sea floor, hypersaline microbial mats and coastal biofilms (Buchan *et al.*, 2005). They are often associated with marine organisms such as sponges (Taylor *et al.*, 2004), cephalopods (Barbieri *et al.*, 2001), scallop larvae (Sandaa *et al.*, 2003), sea grasses (Weidner *et al.*, 2000) and diseased corals (Cooney *et al.*, 2002).

Genus	Type species	No[1]	Reference
Antarctobacter	*Antarctobacter heliothermus*	1	Labrenz *et al.* (1998)
Citreimonas	*Citreimonas salinaria*	1	Choi & Cho (2006a)
Dinoroseobacter	*Dinoroseobacter shibae*	1	Biebl *et al.* (2005b)
Donghicola	*Donghicola eburneus*	1	Yoon *et al.* (2007a)
Jannaschia	*Jannaschia helgolandensis*	5	Wagner-Dobler *et al.* (2003)
Ketogulonicigenium	*Ketogulonicigenium vulgare*	2	Urbance *et al.* (2001)
Leisingera	*Leisingera methylohalidivorans*	1	Schaefer *et al.* (2002)
Loktanella	*Loktanella salsilacus*	9	Van Trappen *et al.* (2004)
Maribius	*Maribius salinus*	2	Choi *et al.* (2007)
Maritimibacter	*Maritimibacter alkaliphilus*	1	Lee *et al.* (2007a)
Marinovum	*Marinovum algicola*	1	Martens *et al.* (2006)
Nereida	*Nereida ignava*	1	Pujalte *et al.* (2005)
Oceanibulbus	*Oceanibulbus indolifex*	1	Wagner-Döbler *et al (2004)*
Oceanicola	*Oceanicola granulosus*	4	Cho & Giovannoni (2004)
Octadecabacter	*Octadecabacter arcticus*	2	Gosink *et al.* (1997)
Pelagibaca	*Pelagibaca bermudensis*	1	Cho & Giovannoni (2006)
Phaeobacter	*Phaeobacter gallaeciensis*	3	Martens *et al.* (2006)
Pseudoruegeria	*Pseudoruegeria aquimaris*	1	Yoon *et al.* (2007b)
Rhodobacter	*Rhodobacter capsulatus*	7	Imhoff *et al.* (1984)
Roseicyclus	*Roseicyclus mahoneyensis*	1	Rathgeber *et al.* (2005)
Roseisalinus	*Roseisalinus antarcticus*	1	Labrenz *et al.* (2005)
Roseovivax	*Roseovivax halodurans*	2	Suzuki *et al.* (1999)
Roseobacter	*Roseobacter litoralis*	2	Shiba (1991)
Roseovarius	*Roseovarius tolerans*	4	Labrenz *et al.* (1999)
Rubellimicrobium	*Rubellimicrobium thermophilum*	1	Denner *et al.* (2006)
Ruegeria	*Ruegeria atlantica*	5	Uchino *et al.* (1998)
Sagittula	*Sagitulla stellata*	1	González *et al.* (1997)
Salipiger	*Salipiger mucescens*	1	Martínez-Cánovas *et al.* (2004)
Shimia	*Shimia marina*	1	Choi & Cho (2006b)
Sulfitobacter	*Sulfitobacter pontiacus*	10	Sorokin (1995)
Thalassobacter	*Thalassobacter stenotrophicus*	1	Mácian *et al.* (2005)
Thalassobius	*Thalassobius mediterraneus*	3	Arahal *et al.* (2005)
Thalassococcus	*Thalassococcus halodurans*	1	Lee *et al.* (2007b)
Wenxinia	*Wenxinia marina*	1	Ying *et al.* (2007)
Yangia	*Yangia pacifica*	1	Dai *et al.* (2006)

[1]: Number of species within the genus

Table 2: List of the genera of the *Roseobacter* lineage (as per February 2008). The type species of every genus and the number of species within the genus are given.

1%

Rubellimicrobium thermophilum CCUG 51817[T] (AJ844281)
Rhodobacter massiliensis CCUG 47968[T] (DQ342309)
Rhodobacter capsulatus ATCC 11166[T] (D16428)
Rhodobacter blasticus NCIMB 11576[T] (D13478)
Maribius salinus CL-SP27[T] (AY906863)
Maribius pelagius B5-6 (DQ514326)
Dinoroseobacter shibae DFL 12[T] (AJ534211)
Wenxinia marina HY34[T] (DQ640643)
Roseicyclus mahoneyensis ML6[T] (AJ315682)
Ketogulonicigenium robustum LMG 21292[T] (AF136850)
Ketogulonicigenium vulgare DSM 4025[T] (AF136849)
Roseisalinus antarcticus EL-88[T] (AJ605747)
Oceanicola granulosus HTCC 2516[T] (AY424896)
Octadecabacter arcticus 238[T] (U73725)
Octadecabacter antarcticus 307 (U14583)
Jannaschia donghaensis DSW-17[T] (EF202612)
Jannaschia seosinensis JCM 13035[T] (AY906862)
Jannaschia rubra CECT 5088[T] (AJ748747)
Jannaschia helgolandensis Hel 10[T] (AJ438157)
Loktanella fryxellensis LMG 22007[T] (AJ582225)
Loktanella atrilutea IAM 15450[T] (AB246747)
Loktanella salsilacus R-8904[T] (AJ440997)
Loktanella vestfoldensis LMG 22003[T] (AJ582226)
Loktanella agnita R-10 SW5[T] (AY682198)
Maritimibacter alkaliphilus HTCC 2654[T] (DQ915443)
Oceanicola batsensis HTCC 2597[T] (AY424898)
Oceanicola marinus AZO-C[T] (DQ822569)
Nereida ignava DSM 16309[T] (AJ748748)
Roseobacter denitrificans OCh 114[T] (M96746)
Roseobacter litoralis ATCC 49566[T] (X78312)
Oceanibulbus indolifex HEL-45[T] (AJ550939)
Sulfitobacter guttiformis EL-38[T] (Y16427)
Sulfitobacter donghicola JCM 14565[T] (EF202614)
Sulfitobacter mediterraneus CH-B427[T] (Y17387)
Sulfitobacter pontiacus LMG 19752[T] (Y17388)
Sulfitobacter marinus JCM 13602[T] (DQ683726)
Roseovarius crassostreae DSM 16950[T] (AF114484)
Thalassobacter stenotrophicus CECT 5294[T] (AJ631302)
Thalossobius gelatinovorans IAM 12617[T] (D88523)
Thalassobius mediterraneus CECT 5383[T] (AJ878874)
Thalassobius aestuarii JC2049[T] (AY442178)
Shimia marina JCM 13038[T] (AY962292)
Pseudoruegeria aquimaris KCTC 12737[T] (DQ675021)
Ruegeria pomeroyi DSS-3[T] (AF098491)
Ruegeria lacuscaerulensis ITI-1157 (U77644)
Ruegeria atlantica IAM 14463[T] (D88526)
Ruegeria pelagia HTCC 2663 (DQ916141)
Ruegeria mobilis CIP 109181[T] (AB255401)
Roseovarius nubinhibens DSM 15170[T] (AF098495)
Roseovarius mucosus DSM 17069[T] (AJ534215)
Roseovarius tolerans EL-172[T] (Y11551)
Thalassococcus halodurans UST 050418-052[T] (DQ397336)
Marinovum algicola ATCC 51440[T] (X78315)
Phaeobacter daeponensis TF-218 (DQ981486)
Leisingera methylohalidivorans MB2[T] (AY005463)
Phaeobacter gallaeciensis BS107[T] (Y13244)
Phaeobacter inhibens LMG 22475[T] (AY177712)
Donghicola eburneus JCM 13604[T] (DQ667965)
Yangia pacifica JCM 12573[T] (AJ877265)
Salipiger mucescens LMG 22090[T] (AY527274)
Pelagibaca bermudensis HTCC 2601[T] (DQ178660)
Citreimonas salinaria JCM 13036[T] (AY962295)
Roseivivax halodurans Och 239[T] (D85829)
Roseivivax halotolerans Och 210[T] (D85831)
Sagittula stellata E-37[T] (U58356)
Antarctobacter heliothermus EL-219[T] (Y11552)
Sinorhizobium meliloti LMG 6133[T] (X67222)

Figure 4: Dendrogram depicting the 16S rRNA gene sequence similarities among the members of the genera of the *Roseobacter* lineage (as per February 2008). Bootstrap values (1000 replicates) above 70 % are given.

The abundant availability of cultured representatives of the *Roseobacter* lineage entailed a range of physiological studies. Their morphology is adapted to the marine environment as, for instance gas vacuoles, holdfasts, poly−β−hydroxybutyrate inclusion bodies and toga-like structures were observed (Buchan *et al.*, 2005). Furthermore, roseobacters exhibit a large range of metabolic characteristics that are advantageous for living in marine environments. Some members of the *Roseobacter* lineage, such as *Roseobacter denitrificans* (Shiba, 1991), *Roseobacter litoralis* (Shiba, 1991) and *Roseovarius mucosus* (Biebl *et al.*, 2005a) are able to perform aerobic anoxygenic photosynthesis. This means that these bacteriochlorophyll a-containing bacteria are able to derive energy from light without the generation of oxygen (Allgaier *et al.*, 2003, Buchan *et al.*, 2005, Wagner-Dobler & Biebl, 2006). Aerobic anoxygenic phototrophy enhances the growth yield in surface waters, probably providing an advantage for the organism in a carbon-limited environment (Buchan *et al.*, 2005, Wagner-Dobler & Biebl, 2006). In addition, it is possible that aerobic anoxygenic photosynthesis affects the magnitude and dynamics of the carbon reservoir in the ocean (Buchan *et al.*, 2005).

The ability to perform sulphur transformations was also observed among several roseobacters (Buchan *et al.*, 2005). Especially the capacity to degrade dimethylsulfoniopropionate showed to be abundantly present (González *et al.*, 1999). Dimethylsulfoniopropionate is an osmolyte that is produced in high concentrations by micro- and macroalgae and halophytic plants (Wagner-Dobler & Biebl, 2006). Roseobacters can break down dimethylsulfoniopropionate in two ways i.e. cleavage by a lyase enzyme or the demethylation/demethiolation pathway (González *et al.*, 2003, Wagner-Dobler & Biebl, 2006). In general, bacteria only possess one of these pathways but several members of the *Roseobacter* lineage have both (González *et al.*, 2003, González *et al.*, 1999). In addition, several roseobacters transform inorganic forms of sulphur such as elemental sulphur, sulphide, sulphite and thiosulphate (Buchan *et al.*, 2005). For instance, *Sulfitobacter pontiacus* (Sorokin, 1995) and *Sulfitobacter mediterraneus* (Pukall *et al.*, 1999) oxidize sulphite.

The ability to oxidize carbon monoxide has also been observed among members of the *Roseobacter* lineage, e.g. *Ruegeria pomeroyi* (Buchan *et al.*, 2005, Moran *et al.*, 2004). This pathway can be used as an energy supplement when organic matter is limited but carbon monoxide is ubiquitous (Moran *et al.*, 2007). Representatives of the *Roseobacter* lineage degrade aromatic compounds such as lignin (Buchan *et al.*, 2005, González *et al.*, 1997) by means of the β−ketoadipate pathway (Buchan *et al.*, 2000, Buchan *et al.*, 2001, Buchan *et al.*, 2004). Moreover genomic analyses of three roseobacter genomes showed the presence of at least six pathways for the catabolism of aromatic compounds such as phenolic metabolites from marine plankton or lignin derivates from coastal marshes (Moran *et al.*, 2007).

Roseobacters produce antibiotics against various marine organisms (Martens *et al.*, 2007) e.g. production of tropodithietic acid (Brinkhoff *et al.*, 2004) by *Phaeobacter inhibens* (Martens *et al.*, 2006), okadaic acid by *Marinovum algicola* (Lafay *et al.*, 1995) and trypthantrin by *Oceanibulbus indolifex* (Wagner-Döbler *et al.*, 2004). The production of antibiotics can be

important in biofilm formation in order to outcompete other bacterial strains (Wagner-Dobler & Biebl, 2006). In general, roseobacters are good biofilm formers, are predominant in the early colonization of surfaces in marine environments (Dang & Lovell, 2000) and produce efficiently extracellular compounds (Bruhn *et al.*, 2006, Rao *et al.*, 2005). In addition, it was demonstrated that *Roseobacter* strain 27-4 only produced its antibacterial compound when growing in a biofilm (Bruhn *et al.*, 2006) in order to outcompete other bacteria (Wagner-Dobler & Biebl, 2006). Also, Rao *et al.* (2005) showed that *Phaeobacter gallaeciensis* is a superior competitor in a mixed-species biofilm.

As some roseobacters produce antibacterial compounds and are often associated with marine algae, the potential of roseobacters as probiotics in aquaculture was proposed. Algae are commonly used as living feed for the aquaculture and thus may serve as vehicles for the probiotic roseobacters (Bruhn *et al.*, 2005, Ruiz-Ponte *et al.*, 1999).

1.2.5.2 Benthic MFC

A benthic MFC is an MFC in which the anode is embedded in anoxic marine sediments while the cathode is placed in the overlying aerobic seawater (Lovley, 2006a). More recently, such MFCs are referred to as Benthic Unattended Generators (BUGs) (Fig. 5). These systems rely on the natural voltage gradient between the sediments and the overlying seawater (DeLong & Chandler, 2002, Tender *et al.*, 2002). This gradient is created by microbial oxidation of subseafloor organic carbon, which results in the generation of electron-rich reductants such as Fe^{2+} or HS^-. In fact, the organic matter stored in anoxic subseafloor sediments constitutes a large potential of energy (Bond *et al.*, 2002, DeLong & Chandler, 2002, Tender *et al.*, 2002). This sediment-associated organic carbon represents 2 % of the dry weight of marine sediments along continental margins (DeLong & Chandler, 2002) and primarily results from the sedimentation of phytoplankton detritus (Tender *et al.*, 2002). Reimers *et al.* (2001) were among the first to describe this type of electricity production. Killing the microorganisms in the sediment (of a laboratory MFC) resulted in a rapid decrease of electricity production, indicating that power is generated by the action of bacteria (Reimers *et al.*, 2001).
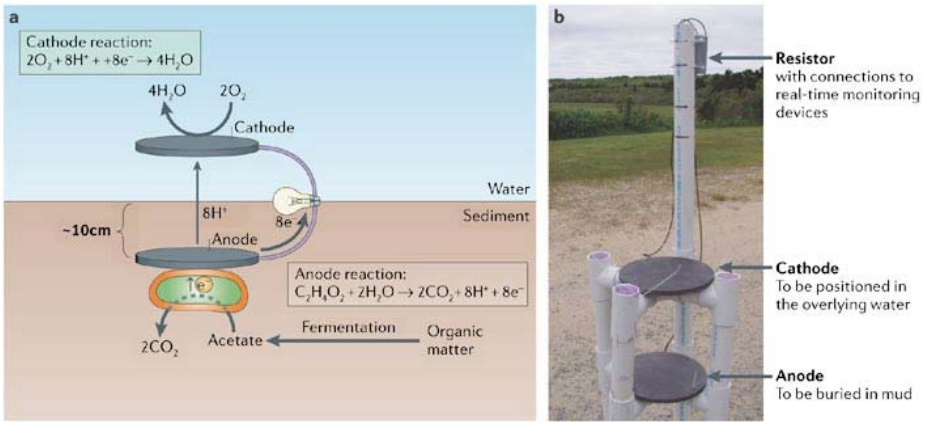
Figure 5: (a) A benthic MFC with the anode is buried in anoxic sediments while the cathode is placed in the aerobic seawater. Members of the family *Geobacteraceae* oxidize acetate and other fermentation products and transfer the obtained electrons towards the anode. The electrons migrate, due to the difference in potential, to the cathode where oxygen is reduced.
(b) An actual benthic MFC before deployment is shown (Lovley, 2006a).

Electricity production was demonstrated in laboratory sediment batteries and the microbial communities were attached to the anodic surface, forming a biofilm (Bond *et al.*, 2002). Microbial analysis of this anodic community revealed an enrichment of *Deltaproteobacteria*. Moreover, 70 % of the deltaproteobacterial sequences were assigned to the family *Geobacteraceae* (Bond *et al.*, 2002). *Desulfuromonas acetooxidans*-like bacteria were enriched a 100-fold compared to the non-current harvesting anodes. A pure culture of *D. acetooxidans* was able to oxidize acetate in order to produce electrical current and killing of this bacterium resulted in a rapid inhibition of current production (Bond *et al.*, 2002).

Tender *et al.* (2002) confirmed the enrichment of *Deltaproteobacteria* (*Geobacteraceae*, *Desulfobulbus* and *Desulfocapsa* species) on the anode of a benthic MFC. Two anode reactions were suggested to generate electrical current, namely the oxidation of sediment sulphide and the oxidation of sedimentary organic matter catalyzed by the microorganisms attached to the anode (Tender *et al.*, 2002). Although the oxidation of hydrogen sulphide initially enhanced power production, over the long-term the deposition of elemental sulphur slowly deactivated the anode surfaces (Reimers *et al.*, 2006).

Holmes *et al.* (2004) investigated the microbial communities on current harvesting electrodes from different aquatic sediments. The microbial diversity was much lower on the current harvesting anodes than on the control anodes which were not connected to the cathode. This was mainly due to an enrichment of *Deltaproteobacteria* of which the majority belonged

to the family *Geobacteraceae* except for the estuarine sediments where *Desulfobulbus* and *Desulfocapsa* accounted for the majority of the *Deltaproteobacteria*. Similarly, the microbial diversity on the cathodes connected to the anodes was much lower that on the controls, but the enrichment on the cathodes was completely dependent on the environment. For instance, cathodes from laboratory marine sediment fuel cells were enriched in certain *Gammaproteobacteria* (i.e. *Cycloclasticus/* type I methanotroph cluster) whereas the majority of the sequences retrieved from salt marsh MFCs belonged to the certain *Alphaproteobacteria* (i.e. the family *Rhodobacteraceae*) (Holmes *et al.*, 2004).

The predominance of *Deltaproteobacteria* in benthic MFC was also confirmed by other studies (Reimers *et al.*, 2006, Ryckelynck *et al.*, 2005). In addition, Reimers *et al.* (2006) demonstrated that the microbial diversity of biofilms on the anodes increased with the sediment depth. Bacteria at the top of the anode belonged to the genus *Desulfuromonas* whereas those on the middle part of the anode showed an equal representation of *Epsilonproteobacteria*, *Desulfocapsa* and *Desulfuromonas* species. A diverse range of species was present on the lower part on the anode, including mainly the phylum *Epsilonproteobacteria*, the genus *Syntrophus* of the phylum *Deltaproteobacteria,* the genus *Cytophaga* and Candidate Division OP1 and OP11 bacteria.

All these MFCs operate at intermediate temperatures (below 50°C), although extremophiles could serve as more efficient catalysts of electrical current production due to e.g. higher activity, greater stability, extended lifetime and the capacity of utilizing a broader range of fuels (Mathis *et al.*, 2008). In the latter study, electricity production by thermophilic bacteria (at 60°C) was 10 times higher than by bacteria at 20°C. Microbial analysis of the latter anodic biofilm revealed the predominance of *Thermincola carboxydophila*, which is an anaerobic carboxydotrophic, hydrogenogenic bacterium first isolated from a hot spring in the lake Baikal (Sokolova *et al.*, 2005).

Although the majority of the aquatic MFC research focuses on benthic MFCs, other habitats have also been explored. For instance, Reimers *et al.* (2007) filled the anodic compartment of a MFC with marine plankton and observed that approximately 80 % of the initial organic carbon was removed mainly due to sulphate reduction coupled to organic carbon oxidation, resulting in electricity generation. Members of the classes *Delta-*, *Epsilon-* and *Gammaproteobacteria*, the phyla *Bacteroidetes* and *Fusobacteria* were detected, showing that the microbial population of the latter MFCs was phylogenetically diverse (Reimers *et al.*, 2007).

1.3 TOOLS FOR STUDYING DIVERSITY AND TAXONOMY OF BACTERIA

Before the 1980s, bacterial diversity was studied by culture dependent methods but gradually it became clear that only a part of the bacterial population was recovered by cultivation (Amman *et al.*, 1995, Fry, 2000). This was referred to as 'the great plate count anomaly' i.e. the observation of a vast discrepancy between the number of cells in a sample and the number of colonies they produce on traditional microbiological media (Staley & Konopka, 1985). This has lead to a shift from analysing a microbial population through cultivation to characterization by the application of molecular techniques directly on the sample (Suzuki *et al.*, 1997). Bacterial numbers estimated by direct counts (e.g. epifluorescence) are orders of magnitude higher than estimated with plate counts (Suzuki *et al.*, 1997). In fact, plate counts of bacteria from natural habitats such as soil, freshwater and marine environments demonstrated that less of 1 % of these bacteria are culturable (Fry, 2000). In general, it was suggested that 99.9 % of all bacteria are unculturable (Rothschild, 2006). Several reasons may account for this high degree of unculturability. Culture conditions provide a poor environmental mimic and are generally much too simple and too homogenous to isolate environmentally relevant bacteria (Nichols, 2007, Ritz, 2007). The physical, chemical and biological complexities of the environment are likely to be all involved in the behaviour of a bacterium, also including division, growth and metabolism (Nichols, 2007). In addition, bacteria in their natural environment grow and develop as members of a community which interact with each other. These interactions, negative or positive, are often necessary for the development of a certain bacterium. In cultivation systems, the community only comprises bacteria that grow in the conditions used and is a poor reflection of the original community. In other words, a new 'local ecology' is developed within the culture system (Ritz, 2007). Also, culturable bacteria may enter a non-culturable state, referred to as the 'viable but non-culturable' behaviour (Ritz, 2007). Finally, a part of the bacterial communities is composed of unknown species that cannot easily be cultivated on traditional microbiological growth media (Suzuki *et al.*, 1997). In fact, the detection of novel bacterial taxa in every environment investigated indicates that many bacteria have not yet been detected or studied.

Although cultivation was inferior for a long time, a renewed interest in cultivation has been observed during the last years (Nichols, 2007, Ritz, 2007). Pure cultures are indispensable for the determination of biochemical, physiological and genetic traits of bacteria (Ritz, 2007) as, for instance, required for the description of novel bacterial taxa (Kämpfer *et al.*, 2003). Also, the screening and application of microorganisms in biotechnological processes demand the *in vitro* growth of the bacterium (Ritz, 2007). Finally, microbial cultivation provides a frame in which the theoretical results of metagenomic analyses can be verified due to a more direct access to the bacterial genome. For instance, typically 40 % or more of the protein genes retrieved by metagenomic studies encoded unknown functions (Ritz, 2007) and genetic experiments on pure cultures can help to resolve these issues. The integration of metagenomic data and classical cultivation methods can be used to study how bacteria

are adapted to particular ecosystems (Bertin *et al.*, 2008).

The barrier of culturability remains a major problem and challenge in cultivation-based studies. Several attempts have been made to overcome the cultivation restrictions by mimicking as much as possible the natural growth conditions. For instance, the use of natural growth media (e.g. seawater and soil), synthetic media with low nutrient concentrations (mimicking natural oligotrophic environments), extinction and diluting culturing and the addition of cell-to-cell signalling compounds to culture media have yielded an increase in microbial *in vitro* recovery both in terms of percent and diversity (Nichols, 2007). In general, these new approaches demonstrated that the barriers of the 'great plate count anomaly' might not be insurmountable.

In conclusion, both culture dependent and independent analyses are necessary to generate as much as possible a complete overview of the microbial diversity in a sample. In addition, the use of both types of procedures has shown that the bacterial diversity remains largely unknown and is often underestimated.

1.3.1 Culture dependent methods

During the present study, isolates were obtained from three different growth media (Chapter 2). Subsequently, they were analysed by a polyphasic taxonomic approach including a variety of genotypic and phenotypic techniques in order to identify them and to describe novel bacterial taxa. Polyphasic taxonomy, introduced in 1971 by Colwell, integrates the different kinds of information on microorganisms i.e. genotypic, phenotypic and phylogenetic data (Vandamme *et al.*, 1996).

In polyphasic identification, different methods are used in a stepwise approach. Only some fast analyses are performed on all isolates. The results obtained from such initial screening studies allow to select representative isolates which are further examined. The final identification results can then be extrapolated to the entire initial collection of isolates. In the present study, we used whole cell fatty acid methyl ester (FAME) analysis and rep-PCR fingerprinting as initial screening tools while we analysed representative strains only by means of 16S rRNA gene sequence analysis, DNA-DNA hybridisation experiments, determination of the mol % G + C and phenotypic tests. These methods are discussed below.

1.3.1.1 Whole cell fatty acid methyl ester analysis

Chemotaxonomy is defined as the application of analytical methods to collect information on various chemical constituents of the cell in order to classify bacteria e.g. whole cell fatty acid methyl ester (FAME) analysis (Vandamme *et al.*, 1996). Fatty acids vary in chain length, double-bond position and substituent groups and are intensively used in taxonomic studies (Vandamme *et al.*, 1996, Welch, 1991). More than 300 different chemical structures of fatty acids have been investigated (Vandamme *et al.*, 1996). In general, cellular fatty acids are defined as any cellular lipids that have a carbon chain length of 9 to 20 carbon atoms. This includes the majority of fatty acids located in the cell membrane (glycolipids and phospholipids) and the fatty acid constituents of lipopolysaccharides but not the long-chain (24 to 90 carbon atoms) mycolic acids or the isoprenoid quinones (Welch, 1991).

In order to compare their FAME profiles, bacterial cells must be grown in standardized conditions (28°C on Trypticase Soy Agar). After exactly 24h of incubation, cells are harvested and lysed by saponification in order to liberate the fatty acids from the cellular lipids. Subsequently, the fatty acids are converted to their corresponding methyl esters. Afterwards, the cellular fatty acid fractions of the isolates are separated by gas-liquid chromatography. The system consists of a gas chromatograph equipped by a flame ionization detector, a 5 % methylphenyl silicone fused-silica capillary column (25 m by 0.2 mm), an automatic sampler, an integrator and a computer. The samples are injected into the column and subsequently, a computer-controlled program begins at 170°C and is gradually increased to 270°C at 5°C/min. The fatty acids, contained in a sample, are separated because of different retention times under conditions of increasing temperature.

The obtained FAME profiles are analysed by numerical analysis and similar FAME profiles are grouped. Typically, isolates within such a FAME cluster belong to closely related species. FAME analysis provides a suitable method for rapidly grouping a large number of isolates into clusters of closely related isolates. These clusters form the basis for the selection of representative isolates that will be incorporated in more expensive analyses such as 16S rRNA gene sequence analysis (Van Trappen *et al.*, 2002).

In addition, the comparison of FAME profiles to a commercial database (MIDI, Newark) enables a tentative identification (Vandamme *et al.*, 1996). Although FAME analysis is a rapid, easy and relative inexpensive way to cluster a large set of isolates, the taxonomic resolution level may be different for different bacterial groups (Vandamme *et al.*, 1996).

1.3.1.2 Rep-PCR fingerprinting

DNA-based typing methods are techniques that allow the determination of the genetic diversity within a species or within a group of isolates which may exihibit similar phenotypic chemotaxonomic characteristics (e.g. a FAME cluster). Repetitive-sequence-based (rep)-PCR genomic fingerprinting relies on the use of DNA primers complementary to naturally occurring highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski & Weinstock, 1992). Examples are the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence (Versalovic *et al.*, 1991), the 35-40 bp repetitive extragenic palindromic (REP) sequence (Versalovic *et al.*, 1991), the 154 bp BOX element (Versalovic *et al.*, 1994) and the polytrinucleotide $(GTG)_5$ sequence (Versalovic *et al.*, 1994). These sequences appear to be located in distinct, intergenic positions in the genome and may be present in both orientations. Oligonucleotide primers were designed to prime these repetitive sequences and the use of these primers and PCR lead to the selective amplification of distinct genomic regions located between these sequences. The amplified fragments are subsequently separated on the basis of their length by electrophoresis on an agarose gel, yielding a specific genomic fingerprint for a given isolate (Versalovic *et al.*, 1994). Afterwards, these fingerprints are *in silico* analysed and permit differentiation at the species, subspecies or strain levels. In addition, isolates with highly similar rep-PCR profiles are phylogenetically closely related (Rademaker *et al.*, 2000).

These rep-PCR fingerprinting techniques proved to be reliable, reproducible, fast and highly discriminatory and are successfully used to investigate intraspecific diversity (Versalovic *et al.*, 1994). In addition, they allow the selection of representative strains to incorporate in other (more time-consuming) methods such as the determination of mol % G + C and DNA-DNA hybridisation experiments.

1.3.1.3 16S rRNA gene sequence analysis

Ribosomal DNA is one of the best targets for phylogenetic studies because it is universally present, functionally constant and has a mosaic structure of highly conserved and more variable domains (Woese, 1987). Therefore, direct sequencing of the rRNA genes (mostly the 16S rRNA gene) provides a phylogenetic framework, functioning as the backbone for modern microbial taxonomy (Vandamme *et al.*, 1996). Currently, it is part of the standard description of novel bacterial species (Kämpfer *et al.*, 2003). Identification of the isolate examined is obtained by comparing its 16S rRNA gene sequence to international public

sequence databases such as EMBL and GenBank. However, the resolution of 16S rRNA gene sequence analysis between closely related organisms is generally low and there is no threshold value of 16S rRNA gene similarity for species delineation. Therefore, 16S rRNA gene sequence comparison only yields a tentative identification if high (> 97 %) similarity values are found. In contrast, organisms with less than 97 % 16S rRNA gene sequence similarity will not result in DNA-DNA hybridisation values of more than 60 % and therefore, it was suggested that 16S rRNA gene sequence analysis can replace DNA-DNA hybridisation studies but simply and solely if the rRNA gene sequence similarity is below 97 % and if the sequence data of all related taxa are publicly available for comparision (Stackebrandt & Goebel, 1994).

More recently, Stackebrandt & Ebers (2006) suggested to increase the level of 16S rRNA gene sequence similarity for species 'recognition' to 98.7 – 99 % as they revealed that DNA-DNA hybrisation values were below 70 % if 16S rRNA gene similarities were lower than 98.7 – 99 %. In contrast, up to 5 % 16S rRNA gene sequence diversity was observed within some species of the *Epsilonproteobacteria* (Harington & On, 1999).

1.3.1.4 DNA-DNA hybridisation experiments

The percent DNA-DNA hybridisation is an indirect parameter of the similarity between two entire bacterial genomes (Vandamme *et al.*, 1996) and is used as the gold standard to delineate bacterial species (Wayne *et al.*, 1987). A species is the basic unit of bacterial taxonomy and is "defined as a group of strains, including the type strain, sharing at least 70 % DNA-DNA relatedness with 5°C or less ΔT_m" (T_m is the melting temperature of the hybrid as determined by stepwise denaturation; ΔT_m is the difference in T_m (°C) between the homologous and heterologous hybrids formed under standard conditions) (Wayne *et al.*, 1987).

DNA-DNA hybridisation experiments rely on the renaturation of denatured DNA strands. Heating double stranded native DNA results into single stranded molecules (denaturation). The melting or denaturation temperature T_m is the temperature at which half of the double stranded DNA molecules are denatured. Upon slow cooling, complementary single stranded DNA will reassociate to their original conformation (renaturation). When single stranded DNA of different strains are mixed, heteroduplexes can be formed and the extent of this formation reflects the similarity between the two strains. The percent DNA-DNA hybridisation between different strains is expressed as the percentage of the homologous duplex formation (Goris *et al.*, 1998). As the number of sequenced bacterial genomes continuously increases, the direct determination of the similarity between entire genome sequences may replace DNA-DNA hybridisation experiments over time (Goris *et al.*, 2007).

1.3.1.5 Determination of the DNA G + C content

The DNA base composition (moles percent guanosine + cytosine) is compulsory for the description of novel bacterial taxa (Kämpfer *et al.*, 2003). Generally, the range of the mol % G + C in a bacterial species is narrow (1 – 3 %) and does not differ by more than 10 % within a well-defined bacterial genus (Stackebrandt & Liesack, 1993). The DNA G + C content ranges from 25 mol % to 80 mol % in the bacterial world (Tamaoka, 1993).

1.3.1.6 Phenotypic methods

These methods comprise morphological, physiological and biochemical analyses and are used in the identification schemes in the majority of microbiology laboratories, constituting a part of the formal description of species and subspecies (Kämpfer *et al.*, 2003, Vandamme *et al.*, 1996). In addition, phenotypical consistency to the genotypic data is required to generate useful classification systems (Vandamme *et al.*, 1996). The morphological analysis includes cellular (e.g. shape, endospore, flagella and Gram staining) and colonial (e.g. color and dimensions) characteristics. Physiological and biochemical features comprise growth in different conditions (e.g. temperature, pH and salt concentrations), antimicrobial susceptibility, presence of different enzymes etc. Miniaturized systems, such as API that contain a set of dehydrated reagents, are frequently used to examine phenotypic characteristics in a fast and easy way (Vandamme *et al.*, 1996).

1.3.2 Culture independent methods

As discussed above, state-of-the-art biodiversity studies also include culture independent analyses for which a range of methods is currently available. The most commonly used techniques are briefly discussed below.

1.3.2.1 Population fingerprinting

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) of PCR-amplified DNA fragments are sequence-based fingerprinting techniques (Muyzer *et al.*, 1993, Muyzer & Smalla, 1998). First, bulk DNA is extracted directly from a sample (e.g. a biofilm) and subsequently, specific regions are amplified by a whole-population PCR using universal primers. The primers mostly target the hypervariable regions of the 16S rRNA gene such as the V_3 region, but also primers targeting other genes (e.g. *rpoB*) have been used (Mollet *et al.*, 1997). As the V_3 region is one of the hypervariable regions of the 16S rRNA gene, it is found to be suitable to distinguish phylotypes from their relatives. Using DGGE, the amplicons are electrophoretically separated on a polyacrylamide gel containing an increasing gradient of denaturants (formamide and urea) while using TGGE, the amplicons are separated by a temperature gradient across the gel. This results in a partial melting of the double stranded PCR amplicons and the degree of melting depends on their DNA sequence. This way, fragments of the same length are separated based on their sequence divergence which results in a banding pattern on the gel. In general, DGGE resolves the differences in the bacterial populations of different samples in a fast way. In addition, taxonomic information can be deduced from DGGE analysis, for instance, by comparing the obtained bands with those of reference strains and sequencing selected bands.

One of the major limitations of DGGE analysis is that only the predominant community members, typically comprising more than 1 % of the entire bacterial population, are detected. However, the use of group-specific primers can enhance the detection sensitivity (Heilig *et al.*, 2002). Also, comigration of different bands (Sekiguchi *et al.*, 2001) and 16S rRNA gene heterogeneity complicate the interpretation of DGGE gels (Meroth *et al.*, 2003).

Single-strand conformation analysis is based on the same principle as DGGE namely sequence-dependent electrophoresis. The single-stranded DNA fragments, originating from PCR amplicons, differentially fold depending on their sequence and thus their speed of migration is altered. These fragments are subsequently separated using a non-denaturing electrophoresis to maintain their secondary structure. So, a banding pattern representing the differential intra-molecular folding is obtained (Rolfs *et al.*, 1992).

Another example of a community fingerprinting method is terminal restriction fragment length polymorphism. First, a specific region of the 16S rRNA genes (or other genes) of the bulk population DNA is amplified by PCR using fluorescently labelled primers. These amplicons are then digested by restriction endonucleases and are subsequently electrophoretically separated. Only the end-labelled fragments are detected during the analysis. Terminal restriction fragment length polymorphism can be automated which significantly increases the throughput but on the other hand, its use is limited by the choice of primers and endonucleases (Kitts, 2001, Marsh, 1999)

1.3.2.2 Construction of a clone library

The construction of 16S rRNA gene libraries enables to make an inventory of the bacterial groups present in the environment investigated independent of their cultivability (Kemp & Aller, 2004). Bulk DNA is extracted from the bacterial population and the 16S rRNA genes are amplified via PCR using universal primers that target parts of or the entire 16S rRNA gene. In addition, other genes can be used in order to construct a clone library. The purified amplicons are ligated within commercially available vectors. Subsequently, the vectors are used to transform competent *E. coli* cells. Sometimes, the clones are presorted before sequencing in order to reduce the number of sequencing reactions. For instance, restriction length polymorphism and DGGE analysis can be used to group similar clones in clusters of which representative members are then selected for sequencing experiments (Kemp & Aller, 2004). The cloned 16S rRNA genes are identified by comparing their sequences with those stored in public sequence databases. Thus, the microbial diversity present in the sample investigated is determined. However, diversity estimates based on the types and the relative proportions of sequences in 16S rRNA gene libraries are prone to biases (e.g. PCR) and methodological artefacts that may alter the frequency of a particular phylotype in the clone library (Kemp & Aller, 2004).

Coverage of the clone library is defined as the fraction of phylotypes of the environment that are present in the clone library and is expressed by means of coefficients (Kemp & Aller, 2004). For instance, Good's coverage coefficient is defined as (Good, 1953):

$$C = 1 - \frac{n_1}{N}$$

With n_1 representing the number of unique phylotypes in the library and N the library size. In addition, accumulation curves are also used to assess whether the population has been characterized completely. These curves plot the cumulative number of phylotypes identified against the number of clones analysed. Strongly asymptotic plots imply that very few additional

phylotypes will be recovered if more clones are being identified. Such types of curves indicate that the clone library can be considered identified and that it 'covers' completely the population investigated (Kemp & Aller, 2004). In addition, the phylotype richness, the number of different phylotypes within an environment, can be determined by clone library analysis.

1.3.2.3 Microarrays

DNA microarrays are typically glass surfaces spotted with thousands of probes to which DNA, originating from a community or from a pure culture, is hybridized (Greene & Voordouw, 2003). Microarrays were primarily developed and used for gene expression profiling of pure cultures of individual organisms, but during the last years, major advances have been made in their application to environmental samples (Gentry *et al.*, 2006). Currently, at least five types of microarrays can be discriminated. Phylogenetic oligonucleotide arrays are designed based on a conservative marker (e.g. 16S rRNA gene) and are used to compare the phylogenetic relatedness of communities in different environments. Functional gene arrays are developed to detect key functional genes that encode proteins, which catalyze various biogeochemical processes such as carbon and nitrogen cycles. These arrays may also provide information on the microbial populations that control these biogeochemical processes. Community genome arrays contain the whole genomic DNA of cultured organisms and allow the determination of the composition of a population investigated. Metagenomic arrays are spotted with probes that originate directly from environmental DNA and can be applied without prior sequence knowledge of the community. Whole-genome open reading frame arrays consist of probes that target all open reading frames in one or multiple genomes. Traditionally, these arrays have been used for functional genomic analyses of individual organisms, but they can also be used for comparative genomic analyses or to investigate the interactions of multiple organisms at the transcriptional level (Gentry *et al.*, 2006). In general, DNA microarrays can be used to examine the structure, function and dynamics of microbial ecosystems. In addition, microarrays also allow a high throughput screening (Wagner *et al.*, 2007).

1.3.2.4 Metagenomics

Metagenomics is the analysis of whole genomes extracted from bacterial communities without the need for cultivation (Chen & Pachter, 2005). The aim of metagenomic studies is to identify and characterize the genetic material of a bacterial community in order to fully understand the genetic diversity, population structure and community ecology (Handelsman, 2004). The DNA sample can be cloned into bacterial artificial chromosome vectors creating a metagenomic library. This library is then analysed by a sequence-driven approach i.e. analysing gene sequences (e.g. house keeping genes) or by a function-driven approach i.e. revealing which proteins are encoded by the cloned sequences. Additionaly, the entire library can be sequenced by whole-genome shotgun sequencing. In conclusion, metagenomic analysis generates very useful information but still encounters some problems, for instance, genome assembly appears to be difficult due to genomic heterogeneity within a species (Handelsman, 2004, Nichols, 2007).

1.3.2.5 Metaproteomics

Metaproteomics or community proteomics is the analysis of the whole microbial community proteome (Ram *et al.*, 2005) and aims at identifying as many proteins as possible with high accuracy (Denef *et al.*, 2007). In nature, microbial cells rarely exist as individual colonies but interact with other microbes in a community and with their environment, creating an ecosystem. Although the development and use of DNA-based molecular techniques (e.g. clone library, DGGE and metagenomics) provide information about the bacterial diversity of a given population, linking the diversity and functionality of a microbial community is still a hard task. The study of the entire protein profile (e.g. by mass spectrometry analyses) complements and extends DNA-based studies as it permits to identify key proteins directly involved in different biological processes, to analyze changes associated with specific processing and to observe differences in protein expression levels in response to environmental changes (Ansong *et al.*, 2008, Ram *et al.*, 2005). Although sequencing costs are steadily decreasing, it would be impractical to perform metagenomic and metaproteomic analyses on every sample investigated. Therefore, it is important to determine the extent to which genomic data sets can be used for proteomics of organisms for which no protein sequence data exist and vice versa (Denef *et al.*, 2007, Lo *et al.*, 2007).

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PART III

EXPERIMENTAL WORK

CHAPTER 2: Bacterial diversity of a marine electroactive biofilm

Bacterial diversity of a marine electroactive biofilm grown on a cathodically polarized electrode

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SUMMARY

Stainless steel electrodes were cathodically polarized in natural seawater in order to produce electrical current. The current increased and stabilized at 0.5 A / m^2 in less than 10 days. The microbial population of the biofilm formed on the surface of the current harvesting cathodes was examined by culture dependent and culture independent analyses.

Three hundred fifty six isolates were obtained and subsequently identified by whole cell fatty acid methyl ester analysis and 16S rRNA gene sequencing. The results showed that the isolates represented different phylogenetic groups including members of the *Alpha-* and *Gammaproteobacteria*, the phylum *Firmicutes*, the family *Flavobacteriaceae* and the phylum *Actinobacteria*.

Culture independent analysis, through the construction of a clone library confirmed the predominance of *Alpha-* and *Gammaproteobacteria* and largely the same phylogenetic groups of bacteria were detected by both culture dependent and culture independent analyses. Denaturing gradient gel electrophoresis demonstrated that the microbial population of the biofilm formed on the cathode and of the surrounding seawater comprised the same dominant members.

This study demonstrated that the microbial population of the marine electroactive biofilm is phylogenetically highly diverse.

INTRODUCTION

Due to the predicted depletion of fossil fuels and the concerns about global warming, the development of sustainable green energy sources is a major international research topic. Energy production by microorganisms through, for instance, bioethanol or hydrogen gas can contribute to solve this problem (Buckley & Wall, 2006). Also microbial fuel cells (MFCs) constitute an alternative energy source and the development and improvement of MFCs are currently investigated worldwide (e.g. Rabaey *et al.* (2005)).

Microbial fuel cells are devices in which microorganisms convert organic matter into electricity (Lovley, 2006). Bacteria in MFCs, inoculated with different environmental samples such as e.g. marine sediments (Bond *et al.*, 2002, Reimers *et al.*, 2006) or waste water (Angenent *et al.*, 2004, Min *et al.*, 2005), oxidise the dissolved organic matter and transfer the generated electrons towards the electrode. These electrons move from the anode over a resistor towards a cathode and in this way, current is produced. The electron transfer from the bacterial metabolism towards the electrode is possible if external mediators (e.g. neutral red) are supplied which function as electron shuttles in case of the MFC with mediated electron transfer (Park & Zeikus, 2000). Alternatively, electron transfer can be achieved through direct contact between the bacteria and the electrodes i.e. through biofilm formation on the electrode surface (Bond & Lovley, 2003) as observed in mediator-less MFCs (Rabaey *et al.*, 2004) The presence of nanowires has also been suggested to facilitate extracellular electron transport (Gorby *et al.*, 2006, Reguera *et al.*, 2005).

Research on the microbial composition of marine MFCs is mainly focussing on benthic MFCs in which the anode is embedded in anoxic marine sediments while the cathode is placed in the overlying aerobic seawater (Tender *et al.*, 2002). These types of MFCs generate electricity in response to the natural difference in potential between the anoxic sediments and the overlying oxic seawater (Ryckelynck *et al.*, 2005). Several studies already investigated the diversity of the microbial populations grown on the surface of such anodes (Bond *et al.*, 2002, Lovley, 2006, Reimers *et al.*, 2006). For instance, Holmes *et al.* (2004) characterized, by means of 16S rRNA gene sequencing, the microbial diversity of biofilms formed on the surface of electrodes that were placed in marine environments (marine, estuarine, salt marsh and fresh water) on different locations. They observed that members of the family *Geobacteraceae* accounted for the majority of the obtained sequences except in the clone libraries from the biofilms originating from the estuarine sediments in which members of the *Desulfobulbaceae* predominated. In contrast, a significant portion of sequences retrieved from the fresh water anodes were identified as *Geothrix fermentans*. In addition, the bacterial population present on current harvesting cathodes suspended in marine and salt marsh waters was also studied. Members of the *Gammaproteobacteria* (namely the *Cycloclasticus* / type I methanotroph cluster) predominated on marine sediments cathodes while *Alphaproteobacteria* (namely *Rhodobacteraceae*) accounted for the majority of sequences

obtained from the salt marsh sediment electrodes. These results demonstrated that microorganisms involved in electricity production may vary according to the environment and that the bacterial communities present on cathodes are phylogenetically diverse.

Although the majority of marine MFC research is focussing on anodes and benthic MFCs, cathodes and aerobic seawater MFCs are also being studied. For instance, Dumas *et al.* (2007) studied the use of stainless steel as anodic and cathodic material and Bergel *et al.* (2005) investigated the catalysis of oxygen reduction by a seawater biofilm formed on cathodically polarized electrodes.

The present study investigated the microbial composition of a marine biofilm that was formed on the surface of cathodically polarized stainless steel electrodes by means of a polyphasic taxonomic approach (EA-BIOFILMS-508866 (NEST)).

EXPERIMENTAL PROCEDURES

Biofilm generation

A cathodically polarized electrode was placed in natural aerated seawater as described elsewhere (Bergel *et al.*, 2005, Faimali *et al.*, in press). Current production on all polarized samples was regularly measured and recorded. The current increased and stabilized at 0.5 A / m^2 in less than 10 days. Microscopic analysis using an Olympus BX41 epifluorescence microscope coupled with an UV filter block for DAPI showed biofilm formation on the cathode.

Isolation procedures

The biofilm was removed from the stainless steel cathode by sonication (Branson™ 3200) (90 s) in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. These cell suspensions were used for the construction of a clone library (see below) and for cultivation experiments. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated onto 3 different isolation media in order to isolate the heterotrophic cultivable microbial fraction of the marine biofilms: Marine Agar (MA) (Difco™ 2216), R$_2$A (Difco™) and Trypticase™ Soy Agar (TSA) (BBL™). The inoculated media were incubated aerobically at 20 °C for several days. Pure cultures were obtained from all 3 different growth media and isolates were stored at – 80 °C using MicroBank™ vials.

FAME analysis

All isolates were grouped and tentatively identified using whole cell fatty acid methyl ester analysis (FAME) as described by Mergaert *et al.* (2001). The isolates were grown routinely on TSA for 24 h at 28 °C or on MA for 48 h at 20 °C if they failed to grow in the former conditions. The obtained FAME profiles were grouped by the unweighted pair-group method

using arithmetic averages (UPGMA) and relying on the Bray-Curtis coefficient using the BioNumerics 4.61 (Applied Maths, Belgium) software. The Bray-Curtis coefficient is defined as follows (Bray & Curtis, 1957, Clarke *et al.*, 2006):

$$D_{BC(j,k)} = \frac{\sum\limits_{i=1}^{n} | x_{ij} - x_{jk} |}{\sum\limits_{i=1}^{n} | x_{ij} + x_{jk} |}$$

i is the relative proportion of a fatty acid which is compared across samples *j* and *k*. Clusters were delineated on the basis of at least 70 % profile similarity. The isolates were tentatively identified by comparing their FAME profiles to a commercial database (MIS, Newark, USA).

16S rRNA gene sequence analysis
16S rRNA gene sequence analysis was performed on selected representatives of each of the FAME clusters to elucidate their exact taxonomic position. Almost-complete sequences were obtained using the universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). For some isolates, only partial 16S rRNA gene sequences were generated using the universal primers pA and pD (5' GTA TTA CCG CGG CTG CTG 3') as described by Coenye *et al.* (1999). The FASTA software was used to find the most similar sequences in public databases. The 16S rRNA gene sequences were aligned using the CLUSTAL_X (Thompson *et al.*, 1997). Finally, neighbour-joining dendrograms (Saitou & Nei, 1987) were constructed by the BioNumerics 4.61 software.

Construction of a clone library
A clone library of the 16S rRNA genes was constructed in order to investigate the microbial diversity by means of culture independent analyses.
Cell suspensions (see above) were centrifuged at 13 000 rpm for 15 min. The resulting cell pellets were suspended in 750 µl of TNE (100 mM of Tris-HCl pH 8.0; 50 mM of NaCl, 50 mM of EDTA pH 8.0) and 50 µl of 10 mg / ml lysozyme solution was added. After 10 min incubation at room temperature, 0.5 g of glass beads was added and cells were lysed by bead beating (3 times 30 s at 1.25 rpm) (Retsch™). Subsequently, 100 µl of SDS and 100 µl of sarkosyl were added to the tube. Proteinase K (50 µl of 20 mg / ml solution) was added followed by incubation at 50 °C for 1 hour. Afterwards, 1 volume of phenol:chloroform:isoamylalcohol (25:24:1) was added, mixed thoroughly and centrifuged for 10 min at 13 000 rpm. Subsequently, the supernatant was transferred to a new eppendorf tube and 1 volume of chloroform:isoamylalcohol was added. The suspension was mixed thoroughly and centrifuged

for 10 min at 13 000 rpm. The DNA present in the aqueous phase was precipitated by addition of 0.8 volumes of isopropanol followed by centrifugation at 13 000 rpm. The DNA pellet was resuspended in 50 µl of water and stored at – 80 °C.

Bulk 16S rRNA genes were amplified using the primers F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and S17 (5'-GTTACCTTGTTACGACTT-3'). The PCR mixture (25 µl) consisted of 2.5 µl of 10 X PCR buffer, 0.5 µl of 10 mM dNTPs, 0.4 µl of 10 µM forward primer (F27), 0.4 µl of 10 µM reverse primer (S17), 1 U of DNA polymerase (Sigma) and 2 µl of DNA solution. The thermal cycle used was: 1 cycle at 95 °C for 5 min, 35 cycles at 95 °C for 30 sec, 53 °C for 30 sec and 72 °C for 1.75 min followed by a final extension step of 10 min at 72 °C. The integrity of the PCR products was verified by electrophoresis on a 1 % agarose gel. Bands of 1.5 kb were purified from the gel using the Qioquick gel extraction kit (Qiagen) and finally eluted in 45 µl of TE-buffer (1 M of Tris HCl pH 8, 500 mM of EDTA pH 8).

The 16S rRNA gene fragments were ligated in a pCR®-XL-TOPO® vector using the Topo XL cloning kit (Invitrogen) according to the manufacturer's instructions. The clones were stored at - 80 °C using DMSO as cryoprotectant. Ten percent of the clones were randomly selected for 16S rRNA gene sequencing. In order to check if the clone library was fully identified, the number of operational taxonomic units (OTUs) was plotted against the number of clones. An OTU was defined as a group of clones sharing at least 98.0 % sequence similarity.

All 16S rRNA gene sequences obtained from the isolates and the clones were used to construct a neighbour-joining dendrogram.

Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified bulk 16S rRNA genes
DNA was extracted as described above and the V_3 region of the bulk 16S rRNA genes was amplified by PCR as described by Muyzer *et al.* (1993). The obtained PCR amplicons were separated by electrophoresis on a polyacrylamide gel with an increasing gradient of denaturants (Muyzer *et al.*, 1993). Following staining with SYBR® Gold (Invitrogen) and visualisation with UV illumination, the DGGE fingerprint gel was numerically analysed using the BioNumerics 4.61 software. The DGGE fingerprints were clustered using UPGMA and relying on Pearson product-moment correlation coefficient.

RESULTS AND DISCUSSION

Of the three growth media tested, MA yielded the largest number of isolates (3.6 x 10^5 cfu/ml) and the highest diversity of morphotypes. Growth was also observed on TSA (2.0 x 10^3 cfu/ml) and R_2A (1.7 x 10^3 cfu/ml) and was more uniform compared to MA. In total, 356 isolates, representing the morphological diversity of all primary isolation plates, were picked up: 77 % from the MA isolation medium, 14 % from TSA and 9 % from R_2A.

Whole cell fatty acid analysis was first used to group isolates with similar FAME profiles and as an approximate identification approach (Vandamme *et al.*, 1996). Numerical analysis of the obtained FAME profiles grouped the 356 isolates in twenty FAME clusters sharing at least 70 % profile similarity. Fourteen isolates had unique FAME profiles and clustered separately at the 70 % similarity level. The FAME clusters comprised 3 to 91 isolates (Table 2.1). The majority of the isolates were identified as Gram-negative bacteria representing 11 of the 20 FAME clusters (60.8 % of all isolates), while 9 FAME clusters represented Gram-positive bacteria (35.3 % of the isolates). In addition, 5 of the 14 isolates with unique fingerprints were identified as Gram-negative bacteria and 6 isolates as Gram-positive bacteria. Three isolates gave no match to the commercial database but Gram-staining demonstrated that all were Gram-positive bacteria (Table 2.1).

Comparison of the obtained FAME profiles to a commercial database yielded identification scores that were relatively high for the isolates of two FAME clusters: FAME cluster VII with identification scores of 0.723 ± 0.204 and FAME cluster IX with identification scores of 0.747 ± 0.157. Isolates of both FAME clusters were identified as *Bacillus* strains. In contrast, the FAME identification scores were low (typically less than 0.550) for isolates of all the other FAME clusters (Table 2.1).

The isolates belonged to different phylogenetic groups including the *Alphaproteobacteria* (FAME cluster IV), *Gammaproteobacteria* (FAME clusters I, II, XIV, XVI – XX), *Firmicutes* (FAME clusters III, VI – IX), *Actinobacteria* (FAME clusters V, X, XI) and *Flavobacteriaceae* (FAME cluster XII). Of the isolates that grouped separately, four were tentatively identified as *Gammaproteobacteria*, three as *Firmicutes*, three as *Actinobacteria* and one as *Flavobacteriaceae*. Finally, isolates belonging to FAME clusters XIII and XV and four isolates with unique positions remained unidentified (Table 2.1).

In order to obtain a more accurate identification at least one representative isolate per FAME cluster was selected for 16S rRNA gene sequence analysis (Table 2.1, Fig. 2.1). Comparison of the obtained 16S rRNA gene sequences with public databases confirmed that the diversity among the isolates was high and generally also confirmed FAME based identification.

The 16S rRNA gene sequence similarities between representative isolates and the type strains of their closest phylogenetic neighbours ranged between 96.6 % and 100 %, which suggested that several isolates represented novel bacterial species (Stackebrandt & Ebers, 2006). FAME clusters I and IV isolates have been investigated in detailed taxonomic studies and indeed comprised several bacterial taxa (Vandecandelaere *et al.*, in press and unpublished results).

FAME clusters XIII and XV isolates, which could not be identified by FAME analysis, were identified as *Winogradskyella poriferorum* with 99.5 % sequence similarity (Lau *et al.*, 2005) and as *Exiguobacterium* sp. with 98.9 % sequence similarity (Collins *et al.*, 1983), respectively (Table 2.1).

Genus level identification was the same in both analyses for strains representing 8 FAME clusters, comprising 96 of the 342 isolates (FAME clusters II, III, VI, VIII, IX, XIV, XIX and XX). For strains of the remaining 12 FAME clusters (I, IV, V, VIII, X, XI, XII, XIII, XV, XVI, XVII and XVIII) representing 245 of the 342 isolates, the identification results only correlated at the higher taxonomic levels. Several reasons may account for this discrepancy. Many of the isolates represented environmental bacteria or novel species for which there are no reference profiles in the commercial databases. In addition, many of the isolates failed to grow in the standard conditions for use of the MIS database. Finally, it is generally known that FAME analysis often yields only approximate identification results (Vandamme *et al.*, 1996).

In general, when low (< 0.4) identification scores were obtained the tentative species identification results proved unreliable as revealed by 16S rRNA sequence analysis. In contrast, when high (> 0.4) FAME identification scores were obtained, the results of FAME analysis were confirmed by 16S rRNA gene sequence analysis.

FAME cluster	FAME identification	16S rRNA gene sequence identification	Accession numbers°
I (n = 68)*	Gammaproteobacteria (0.368 ± 0.190)§	LMG 24078^T (1501 bp, 97.9 %, *Alteromonas macleodii* LMG 2843^T)¶	AM885866 / X82145
		LMG 24081 (1426 bp, 99.5 %, *A. macleodii* LMG 2843^T)	AM885867 / X82145
		LMG 24080 (1377 bp, 99.6 %, *A. macleodii* LMG 2843^T)	AM885868 / X82145
		LMG 24083 (1470 bp, 99.5 %, *A. macleodii* LMG 2843^T)	AM887685 / X82145
		LMG 24082 (1425 bp, 99.7 %, *A. macleodii* LMG 2843^T)	AM885870 / X82145
		R-25514 (1517 bp, 99.4 %, *Pseudoalteromonas spongiae* JCM 12884^T)	AM944021/ AY769918
		R-25603 (1520 bp, 98.6 %, *Pseudoalteromonas ruthenica* LMG 19699^T)	AM944022 / AF316891
		R-26197 (1028 bp, 97.7 % to *Pseudoalteromonas aliena* LMG 22059^T)	AM944023/ AY387858
II (n = 5)	Gammaproteobacteria (0.441 ± 0.221)	R-25580 (1472 bp, 99.7 %, *Acinetobacter johnsonii* DSM 6963^T)	AM944024 / Z93440
III (n = 4)	Firmicutes (0.204 ± 0.093)	R-25575 (1061 bp, 99 %, *Bacillus drentensis* LMG 21831^T)	AM944025 / AJ542506
IV (n = 91)	Alphaproteobacteria (0.316 ± 0.132)	LMG 24366^T (1396 bp, 98.9 %, *Leisingera methylohalidivorans* LMG 23656^T)	AM900415 / AY005463
		LMG 24365^T (1426 bp, 97.1 %, *Phaeobacter gallaeciensis* LMG 23163^T)	AM904562 / Y13244
		LMG 24367^T (1398 bp, 96.6 %, *Ruegeria atlantica* LMG 23161^T)	AM905330 / AB255399
		LMG 24372 (1343 bp, 99.9 %, *Ruegeria mobilis* CIP 109181^T)	AM905333 / AB255401
		LMG 24369^T (1343 bp, 98.1 %, *Phaeobacter daeponensis* LMG 24139^T)	AM943630 / DQ981486
		R-26162 (234 bp, 99.1 %, *Ruegeria pelagia* HTCC 2662^T)	AM944026 / DQ916141
		R-26145 (255 bp, 99.2 %, *R. pelagia* HTCC 2662^T)	AM944027 / DQ916141
		R-26160 (410 bp, 97.3 %, *P. gallaeciensis* LMG 23163^T)	AM944028 / Y132244
V (n = 5)	Actinobacteria (0.588 ± 0.207)	R-25598 (331 bp, 99.7 %, *Arthrobacter oxydans* ATCC 14358^T)	AM944029 / X83408
VI (n = 29)	Firmicutes (0.666 ± 0.148)	R-25657 (1488 bp, 99.0 %, *Staphylococcus cohnii* CCUG 7322^T)	AM944030 / AY688045
VII (n = 3)	Firmicutes (0.723 ± 0.204)	R-25600 (464 bp, 99.6 %, *Bacillus firmus* LMG 7125^T)	AM944031 / D16268
VIII (n = 5)	Firmicutes (0.633 ± 0.154)	R-25542 (1504 bp, 99.0 %, *Bacillus pumilus* LMG 7132^T)	AM944032 / AM237370
IX (n = 28)	Firmicutes (0.747 ± 0.157)	R-28766 (466 bp, 99.8 %, *B. pumilus* LMG 7132^T)	AM944033 / AM237370
X (n = 30)	Actinobacteria (0.508 ± 0.256)	R-25593 (1474 bp, 99.0 %, *Frigoribacterium faeni* KMM 3907^T)	AM944034 / AM410686
XI (n = 15)	Actinobacteria (0.697 ± 0.210)	R-25670 (425 bp, 99.5 %, *Arthrobacter agilis* LMG 17244^T)	AM944035 / X80748
XII (n = 4)	Flavobacteriaceae (0.400 ± 0.113)	R-28796 (1360 bp, 99.8 %, *Maribacter dokdoensis* DSM 17201^T)	AM944036 / AY960749
XIII (n = 6)	NO MATCH	R-26150 (458 bp, 99.5 %, *Winogradskyella poriferorum* JCM 12885^T)	AM944037 / AY848823
		R-26154 (462 bp, 99.6 %, *W. poriferorum* JCM 12885^T)	AM944038 / AY848823
XIV (n = 23)	Gammaproteobacteria (0.336 ± 0.150)	R-28056 (437 bp, 100 %, *Marinobacter hydrocarbonoclasticus* DSM 8798^T)	AM944039 / X67022
XV (n = 7)	NO MATCH	R-25570 (477 bp, 98.9 %, *Exiguobacterium aurantiacum* CCUG 44910^T)	AM944040 / X70316
		R-25587 (464 bp, 98.9 %, *Exiguobacterium acetylicum* CCUG 32630^T)	AM944041 / X70313
		R-25588 (455 bp, 98.9 %, *E. aurantiacum* CCUG 44910^T)	AM944042 / X70316
XVI (n = 6)	Gammaproteobacteria (0.186 ± 0.111)	R-28040 (469 bp, 99.8 %, *Idiomarina loihiensis* DSM 15497^T)	AM944043 / AF288370
XVII (n = 4)	Gammaproteobacteria (0.074 ± 0.055)	R-28770 (1507 bp, 99.8 %, *Marinobacter koreensis* DSM 17924^T)	AM944523 / DQ325514
		R-28768 (1506 bp, 99.8 % *M. koreensis* DSM 17924^T)	AM944524 / DQ325514
XVIII (n = 3)	Gammaproteobacteria (0.140 ± 0.047)	R-26152 (346 bp, 98.8 %, *A. macleodii* LMG 2843^T)	AM944044 / X82145
XIX (n = 4)	Gammaproteobacteria (0.382 ± 0.105)	R-28020 (613 bp, 98.9 %, *Pseudoalteromonas* sp. FR 1302^T)	AM944045 / DQ011614
XX (n = 2)	Gammaproteobacteria (0.324 ± 0.042)	R-28817 (1510 bp, 99.4 %, *Halomonas aquamarina* LMG 2853^T)	AM944046 / M93352
UC‡ (n = 14)			

Table 2.1: Summary of the FAME and 16S rRNA gene sequence identification results of the 356 marine isolates.

°: Accession numbers of the isolate and of the type strain are given, respectively
*: Number of isolates of this FAME cluster
§: Average identification score and standard deviation for all isolates within a FAME cluster
¶: The strain number of the isolate for which the 16S rRNA gene sequence was determined, the length of the 16S rRNA gene sequence and the percent of 16S rRNA gene sequence similarity between the isolate and the phylogenetic nearest type strain of an established species
‡: UC: unclustered isolates

Three hundred and eighty clones of the clone library were picked up and randomly 10 percent (40 clones) were selected for 16S rRNA gene sequence based identification (Table 2.2, Fig. 2.2). Comparison of the obtained 16S rRNA gene sequences with public databases demonstrated that the clones represented *Gammaproteobacteria* (19 clones), *Alphaproteobacteria* (14 clones), *Flavobacteriaceae* (3 clones) and *Firmicutes* (4 clones). The alphaproteobacterial fraction of the clone sequences represented bacteria belonging to the *Roseobacter* lineage whereas the majority of the gammaproteobacterial clone sequences were identified as uncultured *Gammaproteobacteria* or as members of the genus *Alteromonas*.

10%

Kordia algicida OT-1[T] (AY195836)
100 clone S19 (AM943981)
100 clone S18 (AM943983)
R-26150 (AM944037)
100 *Winogradskyella thalassocola* KMM 3907[T] (AY521223)
100 *Frigoribacterium faeni* 801[T] (Y18807)
100 R-25593 (AM944034)
100 R-26170 (AM944035)
97 *Arthrobacter globiformis* DSM 20124[T] (X80736)
89 R-25598 (AM944029)
100 R-25587 (AM944041)
98 *Exiguobacterium aurantiacum* NCDO 2321[T] (X70316)
98 R-25657 (AM944030)
100 *Staphylococcus aureus* subsp. *aureus* ATCC 12600[T] (D83357)
55 R-25542 (AM944032)
Bacillus subtilis DSM 10[T] (AJ276351)
100 76 R-28766 (AM944033)
100 clone 71 (AM943984)
94 clone 115 (AM943986)
uncultured gammaproteobacterium Arctic96AD-9 (AF354607)
90 clone S34 (AM943994)
98 clone S23 (AM943992)
100 clone S27 (AM943996)
57 clone S33 (AM943997)
clone S30 (AM943998)
94 clone S28 (AM943400)
R-25580 (AM944024)
100 *Acinetobacter calcoaceticus* DSM 30006[T] (X81661)
100 *Halomonas elongata* ATCC 33173[T] (X67023)
100 R-28817 (AM944046)
Alteromonas macleodii ATCC 27126[T] (X82145)
72 R-26152 (AM944044)
100 *Alteromonas genovensis* sp. nov. LMG 24078[T] (AM885866)
67 clone S14 (AM943403)
60 clone S10 (AM943402)
R-28040 (AM944043)
100 *Idiomarina abyssalis* KMM 227[T] (AF052740)
97 R-28020 (AM944045)
100 *Pseudoalteromonas haloplanktis* ATCC 14393[T] (X67024)
Ruegeria atlantica IAM 14463[T] (D88526)
Ruegeria mobilis LMG 24372[T] (AM905333)
100 *Nautella italica* gen. nov. sp. nov. LMG 24365[T] (AM904562)
Phaeobacter gallaeciensis BS107[T] (Y13244)
clone S7 (AM943407)
Phaeobacter caerulea sp. nov. LMG 24369[T] (AM943630)
50 *Leisingera methylohalidivorans* MB2[T] (AY005463)
98 *Leisingera aquimarina* sp. nov. LMG 24366[T] (AM900415)
Ruegeria scottomollicae sp. nov. LMG 24367[T] (AM905330)
clone S1 (AM943416)
89 clone S3 (AM943413)
61 clone S15 (AM943410)
clone S9 (AM943417)
clone S12 (AM943409)
Arcobacter nitrofigilis CCUG 15893[T] (L14627)

100

90

99

99

97

100

100

100

Figure 2.1: Neighbour-joining dendrogram showing the phylogenetic relationships based on 16S rRNA gene sequence similarities, of selected isolates, selected clones and their phylogenetic nearest neighbours. Bootstrap values (1000 replicates) above 50 % are shown. Bar represents 10 % sequence diversity.

OTU cluster#[*]	Clone number	16S rRNA gene identification	Accesion numbers°
I (n = 3)[*]	Clone S19[‡]	753 bp, 97.2 %, *Kordia algicida* NBRC 100336 §	AM943981 / AY195836
	Clone S2	942 bp, 98.2 %, *K. algicida* NBRC 100336^T	AM943982 / AY195836
	Clone S18	766 bp, 97.5 %, *K. algicida* NBRC 100336^T	AM943983/ AY195836
II (n = 4)	Clone 71	463 bp, 98.6 %, *Bacillus pumilus* LMG 7132^T	AM943984 / AM237370
	Clone 57	422 bp, 98.6 %, *B. pumilus* LMG 7132^T	AM943985 / AM237370
	Clone 115	463 bp, 98.6 %, *B. pumilus* LMG 7132^T	AM943986 / AM237370
	Clone 61	422 bp, 98.6 %, *B. pumilus* LMG 7132^T	AM943987 / AM237370
III (n = 7)	Clone S24	754 bp, 98.8 %, uncultured gammaproteobacterium Arctic96AD-3	AM943988 / AF354607
	Clone S35	771 bp, 97.9 %, uncultured gammaproteobacterium Arctic96AD-3	AM943989 / AF354607
	Clone 25	769 bp, 98.7 %, uncultured gammaproteobacterium Arctic96AD-9	AM943990 / AF354608
	Clone S26	767 bp, 99.5 %, uncultured gammaproteobacterium Arctic96AD-3	AM943991 / AF354607
	Clone S23	778 bp, 98.4 %, uncultured gammaproteobacterium Arctic96AD-9	AM943992 / AF354608
	Clone S22	770 bp, 99.0 %, uncultured gammaproteobacterium Arctic96AD-9	AM943993 / AF354608
	Clone S34	757 bp, 99.6 %, uncultured gammaproteobacterium Arctic96AD-3	AM943994 / AF354607
IV (n = 7)	Clone S32	770 bp, 96.5 %, uncultured gammaproteobacterium Arctic96AD-3	AM943995 / AF354607
	Clone S27	768 bp, 97.0 %, uncultured gammaproteobacterium Arctic96AD-3	AM943996 / AF354607
	Clone S33	756 bp, 95.2 %, uncultured gammaproteobacterium Arctic96AD-3	AM943997 / AF354607
	Clone S30	721 bp, 93.6 %, uncultured *Thiomicrospira* ML-1	AM943998 / AJ237769
	Clone S29	793 bp, 96.0 %, uncultured gammaproteobacterium Arctic96AD-3	AM943999 / AF354607
	Clone S28	757 bp, 96.7 %, uncultured gammaproteobacterium Arctic96AD-3	AM943400 / AF354607
	Clone S31	774 bp, 95.9 %, uncultured gammaproteobacterium Arctic96AD-3	AM943401 / AF354607
V (n = 2)	Clone S10	950 bp, 97.7 %, uncultured *Alteromonas* sp. clone JL-SCS-L21	AM943402 / AY663978
	Clone S14	1013 bp, 95.6 %, uncultured *Alteromonas* sp. clone JL-SCS-L21	AM943403 / AY663978
VI (n = 3)	Clone S20	782 bp, 97.9 %, *Phaeobacter gallaeciensis* LMG 23163^T	AM943404 / Y13244
	Clone S17	775 bp, 98.0 %, *P. gallaeciensis* LMG 23163^T	AM943405 / Y13244
	Clone S36	766 bp, 97.9 %, *Phaeobacter inhibens* LMG 22475^T	AM943406 / AY177712
VII (n = 5)	Clone S7	800 bp, 96.8 %, *P. gallaeciensis* LMG 23163^T	AM943407 / Y13244
	Clone S6	1020 bp, 94.4 %, *P. gallaeciensis* LMG 23163^T	AM943408 / Y13244
	Clone S12	900 bp, 99.4 %, *Ruegeria atlantica* LMG 23161^T	AM943409 / AB255399
	Clone S15	1018 bp, 96.4 %, *R. atlantica* LMG 23161^T	AM943410 / AB255399
	Clone S11	850 bp, 99.4 %, *R. atlantica* LMG 23161^T	AM943411 / AB255399
VIII (n = 6)	Clone S5	879 bp, 98.2 %, *R. atlantica* LMG 23161^T	AM943412 / AB255399
	Clone S3	1039 bp, 97.2 %, *R. atlantica* LMG 23161^T	AM943413/ AB255399
	Clone S4	1029 bp, 98.2 %, *R. atlantica* LMG 23161^T	AM943414 / AB255399
	Clone S8	991 bp, 98.2 %, *R. atlantica* LMG 23161^T	AM943415 / AB255399
	Clone S1	989 bp, 99.1 %, *R. atlantica* LMG 23161^T	AM943416 / AB255399
	Clone S9	900 bp, 99.0 %, *R. atlantica* LMG 23161^T	AM943417 / AB255399
IX (n = 1)	Clone S16	778 bp, 88.7 %, *Methylococcus* sp. JB140	AM943418 / X72769
X (n = 1)	Clone S21	774 bp, 91.1 %, uncultured gammaproteobacterium JTB254	AM943419 / AB015253
XI (n = 1)	Clone S13	700 bp, 84.0 %, uncultured *Alteromonas* sp. clone JL-SCS-L21	AM943420 / AY663978

Table 2.2: Summary of the identification results of the 16S rRNA gene sequence analysis of the clone library.

#: An OTU is defined as a group of clones which share at least 98 % 16S rRNA gene sequence similarity
°: Accession numbers of the clone and the highest match
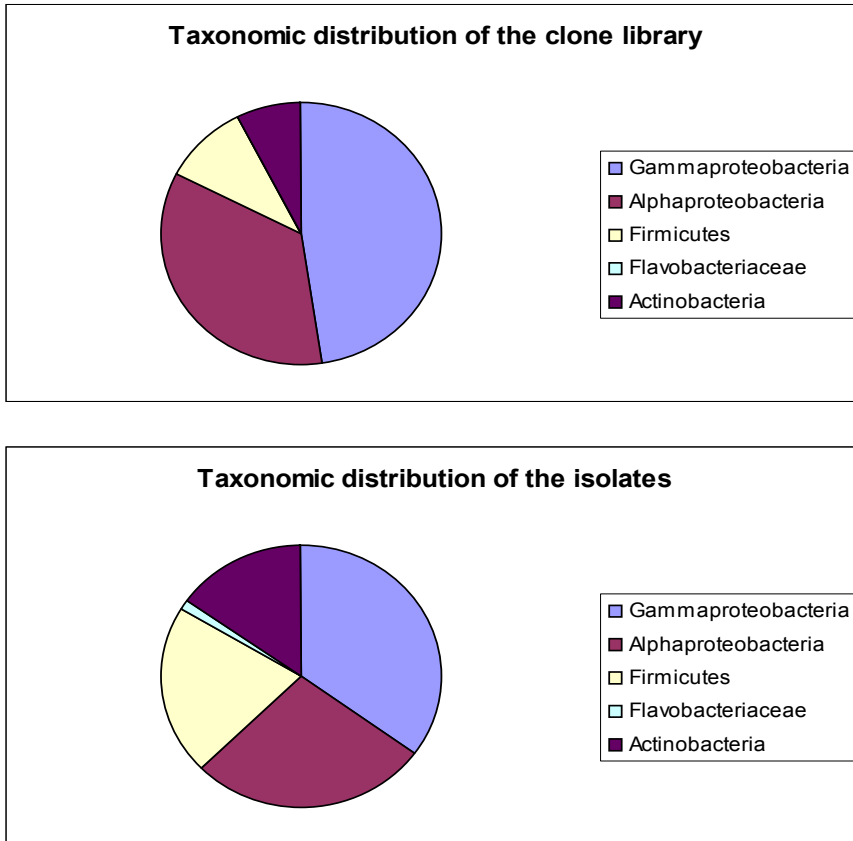*: Number of isolates within this OTU cluster
¶: Clone number
§: The length of the 16S rRNA gene sequence and the percent of 16S rRNA gene sequence similarity between the clone and its highest match

Although a relatively small number of clones was sequenced, the number of OTUs stopped increasing with additional clones sequenced and therefore the clone library was considered identified (Fig. 2.2).

OTUs vs number of clones
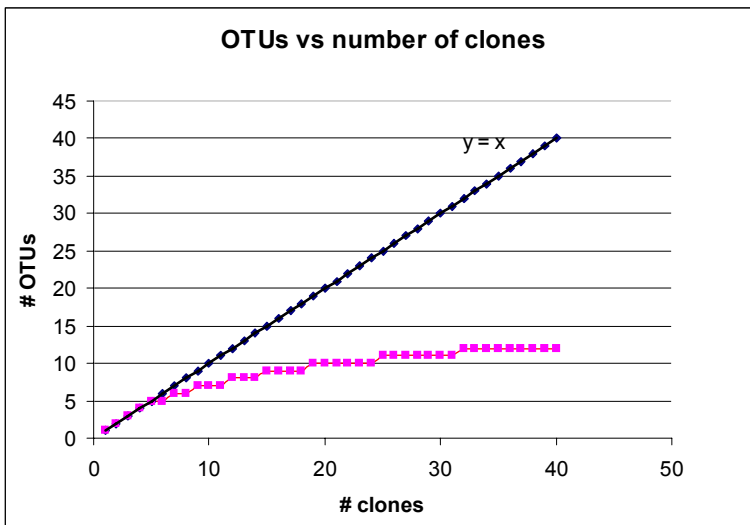
Figure 2.2: Graph showing the number of OTUs versus the number of clones. The pink line represents the number of OTUs versus the number of clones. The blue line is hypothetical and represents a clone library in which every clone belongs to a different OTU.

Taxonomic distribution of the clone library

Legend:
- ■ Gammaproteobacteria
- ■ Alphaproteobacteria
- □ Firmicutes
- □ Flavobacteriaceae
- ■ Actinobacteria

Taxonomic distribution of the isolates

Legend:
- ■ Gammaproteobacteria
- ■ Alphaproteobacteria
- □ Firmicutes
- □ Flavobacteriaceae
- ■ Actinobacteria

Figure 2.3: Taxonomic distribution of the different phylogenetic groups among the isolates and the clones.

Largely the same phylogenetic groups of bacteria were detected in both approaches (Table 2.1, Table 2.2 and Fig. 2.1), except for the absence of actinobacterial sequences among the clones. Several reasons may explain this incongruence. *Actinobacteria* may constitute only a minor fraction of the microbial biofilm population, which was not picked up from the clone library. Alternatively, the techniques used may be less suitable for the construction of a clone library with DNA extracted from *Actinobacteria*. Finally, cultivation conditions may have favoured the growth of actinobacterial isolates leading to an overestimation of the presence of *Actinobacteria* in the microbial population.

The predominance of representatives of the *Gamma-* and *Alphaproteobacteria* among the isolates and the clones was remarkable. Holmes *et al.* (2004) investigated the microbial diversity of electroactive biofilms formed on cathodes that were suspended in seawater

whereas the anodes were placed in the underlying anoxic aquatic sediments. *Gammaproteobacteria* were enriched on the cathode of marine sediment fuel cells while *Alphaproteobacteria* (primarily *Paracoccus* and *Roseobacter* species) predominated on cathodes from salt marsh sediments. Our study confirmed the predominance of *Gamma*- and *Alphaproteobacteria* in an electrochemically active biofilm formed on the cathode that was placed in aerobic seawater.

The presence of members of the *Firmicutes*, *Actinobacteria* and *Flavobacteriaceae* in the present electrochemically active biofilm may reflect their common occurrence in marine environments (Gontang *et al.*, 2007, Ivanova *et al.*, 1999, Montalvo *et al.*, 2005, Van Trappen *et al.*, 2004, Vela *et al.*, 2007, Yoshpe-Purer & Golderman, 1987). To verify this hypothesis, DGGE analysis was performed in order to compare the dominant microbial populations of the electroactive biofilms and of the surrounding seawater. DGGE indeed revealed that the predominant populations were highly similar, demonstrating that the present electroactive biofilm community consists primarily of common marine organisms (Fig. 2.4).

Pearson correlation (Opt:9.30%) [0.0%-100.0%]
DGGE EAB

DGGE EAB

EAB
seawater
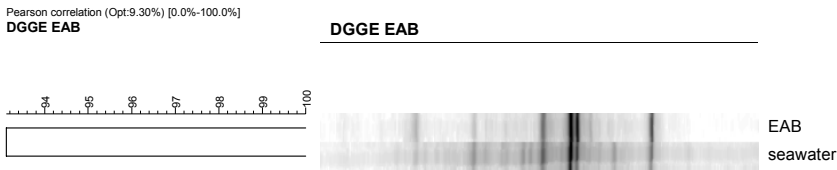
Figure 2.4: DGGE profiles of the predominant members of the EAB and of the surrounding seawater populations.

It is unclear which of these bacteria contribute to the electrochemical activity. In a first study, FAME cluster IV isolates (identified as members of the *Roseobacter* lineage of the *Alphaproteobacteria*) and one FAME cluster XX isolate (*Halomonas aquamarina*), representing six different species, were selected to test their electroactivity by means of cyclic voltammetry (Parot *et al.*, in press). All isolates exhibited high efficiency towards the catalysis of oxygen reduction. These results indicate that these bacteria might be all involved in electroactivity (Parot *et al.*, in press). These data also suggest that multiple species, possibly representing a large fraction of the present biofilm community, are responsible for electrochemical features of the marine biofilm.

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CHAPTER 3: Novel species within the *Gammaproteobacteria*, *Alteromonas genovensis* sp. nov.

Preamble

The bacterial diversity of a marine electroactive biofim was investigated by means of a polyphasic approach. First, all the isolates were screened with FAME analysis (Chapter 2). Two FAME clusters (FAME cluster I and IV) were predominant among the isolates but also constituted the predominant morphotypes on primary isolation plates. Both FAME clusters were screened by means of BOX-PCR fingerprinting and the isolates were grouped on the basis of their similar BOX-PCR profiles.

Eleven BOX-PCR clusters were delineated in FAME cluster I of which five represent isolates that belong to the genus *Alteromonas* although their identification was only tentative (Addendum 1, Fig. 5.1). These 5 BOX-PCR clusters were investigated in more detail by DNA-DNA hybridisation experiments and phenotypic testing.

Alteromonas genovensis sp. nov., isolated from a marine electroactive biofilm and emended description of *Alteromonas macleodii* Baumann *et al.* 1972 (Approved Lists 1980)

Ilse Vandecandelaere, Olivier Nercessian, Eveline Segaert, Wafa Achouak, Alfonso Mollica, Marco Faimali, Paul De Vos, Peter Vandamme

SUMMARY

Thirty-five isolates were obtained from a marine electroactive biofilm grown on a stainless steel cathode (Genoa, Italy) and were investigated by a polyphasic taxonomic approach. Whole cell fatty acid methyl ester analysis indicated that the isolates belong to the class *Gammaproteobacteria*, closely related or belonging to the genus *Alteromonas*. Genomic fingerprinting, using the BOX-PCR primer, delineated 5 clusters of isolates with similar BOX-PCR fingerprints.

Our study demonstrated that isolates from 4 BOX-PCR clusters belonged to *Alteromonas macleodii* and that the 14 isolates representing BOX-PCR cluster 1 constituted a novel species, which shared 98.4 % of 16S rRNA sequence similarity with its nearest phylogenetic neighbour, *Alteromonas hispanica*. Both phenotypic and genotypic analyses allowed to differentiate established *Alteromonas* species from this novel species for which the name *Alteromonas genovensis* sp. nov. is proposed. Its DNA G + C content is 44.5 mol%. The type strain of *Alteromonas genovensis* sp. nov. is LMG 24078^T = CCUG 55340^T.

The genus *Alteromonas* was created by Baumann *et al.* (1972, 1984) to accommodate Gram-negative, strictly aerobic, heterotrophic rods which are motile by a single polar flagellum. Gauthier *et al.* (1995) restricted the genus *Alteromonas* to the type species, *Alteromonas macleodii*, and transferred all remaining species into the genus *Pseudoalteromonas*. Several marine bacteria were since then classified as novel *Alteromonas* species and currently, the genus comprises 8 valid species: *Alteromonas macleodii* (Baumann *et al.*, 1972), *Alteromonas marina* (Yoon *et al.*, 2003), *Alteromonas stellipolaris* (Van Trappen *et al.*, 2004), *Alteromonas litorea* (Yoon *et al.*, 2004), *Alteromonas addita* (Ivanova *et al.*, 2005), *Alteromonas hispanica* (Martinez-Checa *et al.*, 2005), *Alteromonas tagae* (Chiu *et al.*, 2007) and *Alteromonas simiduii* (Chiu *et al.*, 2007).

The genus *Alteromonas* has gained increasing interest because of potential industrial applications (Hayase *et al.*, 2003, Kodama *et al.*, 1993, Martinez-Checa *et al.*, 2005, Raguenes *et al.*, 2003, Raguenes *et al.*, 1996, Shiozawa *et al.*, 1993).

Van Trappen *et al.* (2004) emended the description of the genus *Alteromonas* by reporting the formation of prosthecae and buds in several species when grown for 3 or more days at low temperatures. The presence of prosthecae and buds enhances the bacterial surface: volume ratio that facilitates substrate uptake in oligotrophic marine environments (Van Gemerden & Kuenen, 1984). Marine prosthecate bacteria also play a role in the corrosion of metals as they catalyse biologically the ennoblement of stainless steel surfaces (Baker *et al.*, 2003).

The present study was part of an analysis of the microbial diversity of a marine electroactive biofilm, grown on a stainless steel cathode (EA-BIOFILMS-508866 (NEST)) exposed to natural marine water at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy. Stainless steel samples (25 x 10 mm), cut from a UNS S 31254 plate 1 mm in thickness, drilled, screwed, and then treated with emery papers up to P1200, were immersed in a tank of about 100 litres of natural seawater at ambient temperature. The seawater was continuously renewed at a rate of about 1.5 - 2 litres per minute with water directly pumped from the sea. The stainless steel electrode was cathodically polarized at -200 mV Ag/AgCl (e.g. Faimali *et al.* (in press)). Current flowing from the nth polarised sample is calculated from the ohmic drop "Vn measured across the resistor Rn, whose value was chosen in such a way that a "Vn less than 10 mV was measured. During the tests the cathodic current on all polarised samples was regularly measured and recorded.

The current increased and stabilized at 0.5 A / m^2 in less than 10 days and microscopic analysis (400x magnification using an Olympus BX41 epifluorescence microscope coupled with an UV filter block for DAPI) showed biofilm formation on the cathode. The biofilm was removed from the stainless steel cathode by sonication (Branson™ 3200) (90 s) in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated on Marine Agar (MA) (Difco™ 2216) and incubated aerobically at 20 °C for several days.

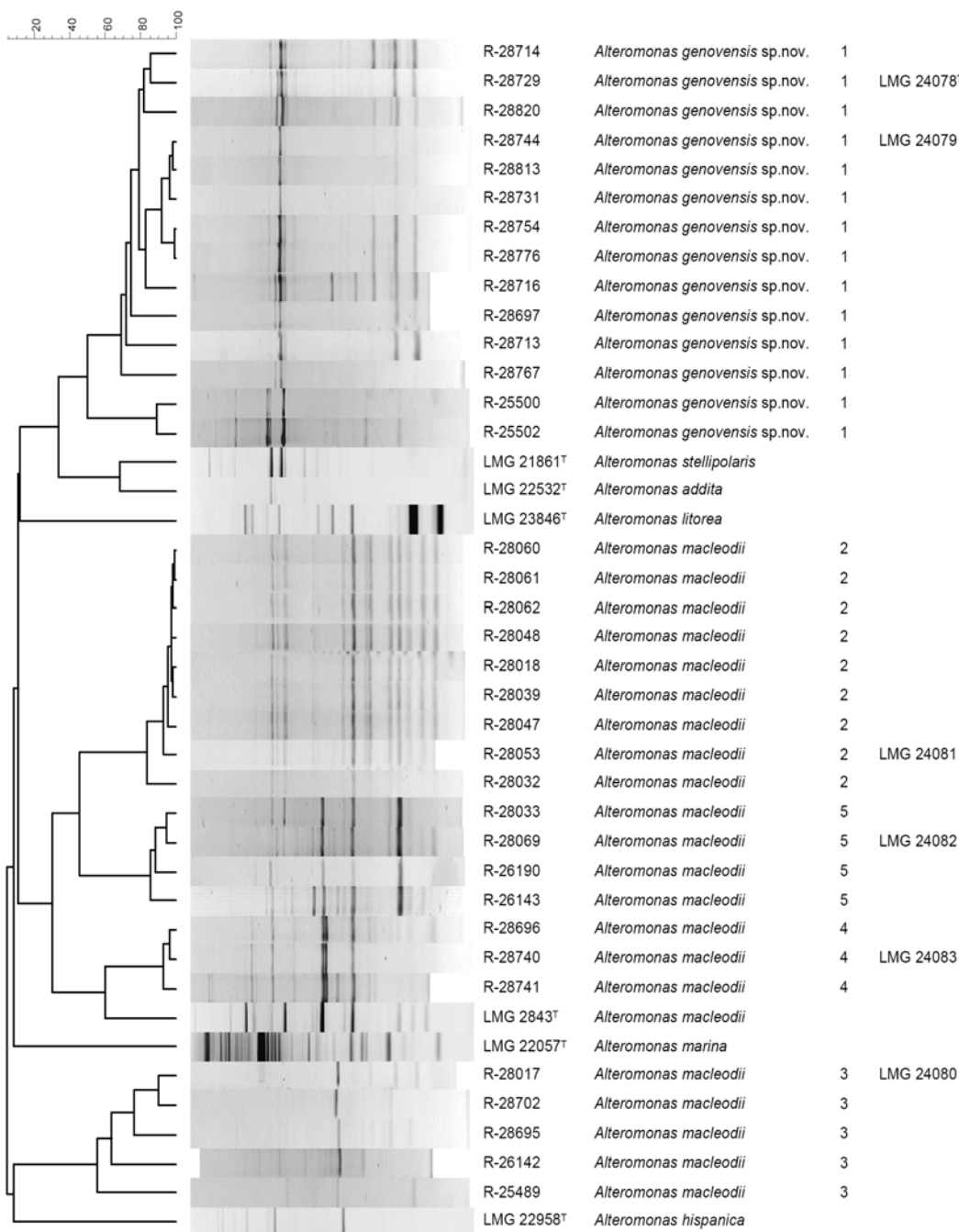Pure cultures were obtained and isolates were stored at –20°C or –80°C using MicroBank™ vials.

All isolates were tentatively identified using whole cell fatty acid methyl ester (FAME) analysis as described by Mergaert *et al.* (2001). For thirty-five isolates, comparison of the FAME profiles to a commercial database (MIS) indicated that they belonged to the class *Gammaproteobacteria* and that they were closely related or belonged to the genus *Alteromonas*. These data correlated with *Alteromonas*-like bacteria constituting considerable fractions of attached marine microbial populations (Acinas *et al.*, 1999, Dang & Lovell, 2000). In addition, *Alteromonas*-like bacteria are also frequent producers of extracellular polymer substances (EPS), which is important in the context of biofilm formation (Branda *et al.*, 2005, Flemming & Wingender, 2001). The dominant fatty acids of the 35 isolates were $C_{10:0}$3-OH (2.2 ± 0.8 %), $C_{11:0}$3-OH (1.4 ± 0.5 %), $C_{12:0}$ (3.3 ± 1.1 %), $C_{12:0}$3-OH (2.2 ± 1.2 %), $C_{14:0}$ (3.4 ± 0.9 %), $C_{15:1\omega8c}$ (1.2 ± 0.6 %), $C_{16:0}$ (19.4 ± 2.3 %), $C_{17:0}$ (2.6 ± 0.7 %), $C_{17:1\omega8c}$ (5.7 ± 1.9 %), $C_{18:0}$ (1.1 ± 0.5 %), $C_{18:1\omega7c}$ (13.6 ± 3.4 %), SUMMED FEATURE 2 (which comprises any combination of $C_{12:0}$ aldehyde, an unknown fatty acid of equivalent chain length 10.928, $C_{16:1}$ iso I and $C_{14:0}$3-OH) (4.5 ± 1.7 %) and SUMMED FEATURE 3 ($C_{16:1\omega7c}$ and/or iso-$C_{15:0}$ 2-OH) (30.5 ± 3.0 %); the remaining fatty acids constituted minor fractions only (< 1 %). These results correlated with previous fatty acid analyses of the genus *Alteromonas* (Ivanova *et al.*, 2000).

DNA was extracted according to Pitcher *et al.* (1989). The genetic diversity among the 35 isolates and the *Alteromonas* type strains was investigated by repetitive DNA-PCR (rep-PCR) fingerprinting using the BOX-A1R-primer (5' CTA CGG CAA GGC GAC GCT GAC G 3') (Versalovic *et al.*, 1994) as described by Rademaker & De Bruijn (1997) and Rademaker *et al.* (2000). Numerical analysis of the obtained DNA profiles using the BioNumerics 4.61 software (Applied Maths, St-Martens-Latem, Belgium), revealed 5 clusters of isolates with similar BOX-PCR profiles (at least 55.2 % intra-cluster profile similarity) (Fig. 3.1): BOX-PCR cluster 1 (14 isolates), BOX-PCR cluster 2 (9 isolates), BOX-PCR cluster 3 (5 isolates), BOX-PCR cluster 4 (3 isolates) and BOX-PCR cluster 5 (4 isolates). The BOX-PCR profiles of these isolates also differed from those of the *Alteromonas* type strains. Each of the clusters comprised some isolates with virtually indistinguishable profiles suggesting that they represented isolates of the same strains. Yet, each cluster also comprised isolates with marked differences in their PCR fingerprints indicating the presence of multiple strains.
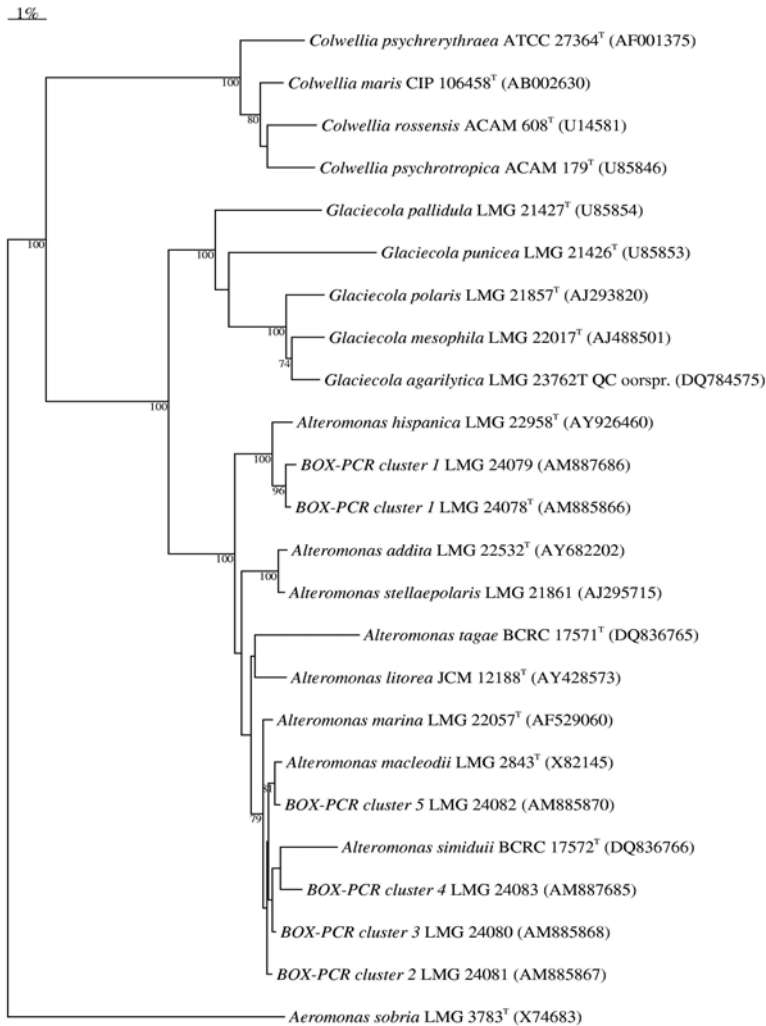
Pearson correlation (Opt:0.74%) [0.0%-100.0%]
BOX-PCR   **BOX-PCR**

Strain	Species	Cluster	LMG
R-28714	*Alteromonas genovensis* sp.nov.	1	
R-28729	*Alteromonas genovensis* sp.nov.	1	LMG 24078[T]
R-28820	*Alteromonas genovensis* sp.nov.	1	
R-28744	*Alteromonas genovensis* sp.nov.	1	LMG 24079
R-28813	*Alteromonas genovensis* sp.nov.	1	
R-28731	*Alteromonas genovensis* sp.nov.	1	
R-28754	*Alteromonas genovensis* sp.nov.	1	
R-28776	*Alteromonas genovensis* sp.nov.	1	
R-28716	*Alteromonas genovensis* sp.nov.	1	
R-28697	*Alteromonas genovensis* sp.nov.	1	
R-28713	*Alteromonas genovensis* sp.nov.	1	
R-28767	*Alteromonas genovensis* sp.nov.	1	
R-25500	*Alteromonas genovensis* sp.nov.	1	
R-25502	*Alteromonas genovensis* sp.nov.	1	
LMG 21861[T]	*Alteromonas stellipolaris*		
LMG 22532[T]	*Alteromonas addita*		
LMG 23846[T]	*Alteromonas litorea*		
R-28060	*Alteromonas macleodii*	2	
R-28061	*Alteromonas macleodii*	2	
R-28062	*Alteromonas macleodii*	2	
R-28048	*Alteromonas macleodii*	2	
R-28018	*Alteromonas macleodii*	2	
R-28039	*Alteromonas macleodii*	2	
R-28047	*Alteromonas macleodii*	2	
R-28053	*Alteromonas macleodii*	2	LMG 24081
R-28032	*Alteromonas macleodii*	2	
R-28033	*Alteromonas macleodii*	5	
R-28069	*Alteromonas macleodii*	5	LMG 24082
R-26190	*Alteromonas macleodii*	5	
R-26143	*Alteromonas macleodii*	5	
R-28696	*Alteromonas macleodii*	4	
R-28740	*Alteromonas macleodii*	4	LMG 24083
R-28741	*Alteromonas macleodii*	4	
LMG 2843[T]	*Alteromonas macleodii*		
LMG 22057[T]	*Alteromonas marina*		
R-28017	*Alteromonas macleodii*	3	LMG 24080
R-28702	*Alteromonas macleodii*	3	
R-28695	*Alteromonas macleodii*	3	
R-26142	*Alteromonas macleodii*	3	
R-25489	*Alteromonas macleodii*	3	
LMG 22958[T]	*Alteromonas hispanica*		

Figure 3.1: Dendrogram representing the BOX-PCR profiles of the isolates and reference strains of the genus *Alteromonas*. Also, BOX-PCR cluster numbers and LMG strain numbers are given.

The DNA G + C content of representatives of each BOX-PCR cluster was determined. DNA was enzymatically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Water Breeze HPLC system and Xbridge Shield RP18 column thermostabilised at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G + C content of representatives of BOX-PCR clusters 1, 2, 3, 4 and 5 ranges from 44.6 mol% to 45.4 mol% which correlates with the mol% G + C reported for the genus *Alteromonas* (Gauthier *et al.*, 1995).

Almost-complete 16S rRNA gene sequences (1339 - 1501 bp) were subsequently obtained for 2 representatives per BOX-PCR cluster using the universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). The EMBL accession numbers of LMG 24078[T], LMG 24079, LMG 24080, LMG 24081, LMG 24082 and LMG 24083 are AM885866, AM887686, AM885868, AM885867, AM885870 and AM887685, respectively. The FASTA program was used to find the most similar sequences in public databases. The sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997). A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using the BioNumerics 4.61 software (Fig. 3.2).

The similarity in 16S rRNA gene sequences among isolates of the same BOX-PCR cluster was always $\geq$ 99.1 % (data not shown). These results confirm that isolates with highly similar rep-PCR profiles are closely related (Rademaker & De Bruijn, 1997, Versalovic *et al.*, 1994). Representatives of BOX-PCR clusters 2, 3, 4 and 5 exhibited relatively high 16S rRNA gene sequence similarities, ranging from 97.6 % to 99.2 %, and were 97.6 % to 99.6 % similar to *Alteromonas macleodii* LMG 2843[T] and *Alteromonas marina* LMG 22057[T], their nearest phylogenetic neighbours (Fig. 3.2). BOX-PCR cluster 1 isolates exhibited less than 97 % 16S rRNA gene sequence similarity to the isolates of BOX-PCR clusters 2, 3, 4 and 5, suggesting that BOX-PCR cluster 1 isolates represent a distinct species (Stackebrandt & Goebel, 1994). The nearest phylogenetic neighbour of BOX-PCR cluster 1 isolates was *A. hispanica* with 98.4 % 16S rRNA gene sequence similarity.

1%

- *Colwellia psychrerythraea* ATCC 27364[T] (AF001375)
- *Colwellia maris* CIP 106458[T] (AB002630)
- *Colwellia rossensis* ACAM 608[T] (U14581)
- *Colwellia psychrotropica* ACAM 179[T] (U85846)
- *Glaciecola pallidula* LMG 21427[T] (U85854)
- *Glaciecola punicea* LMG 21426[T] (U85853)
- *Glaciecola polaris* LMG 21857[T] (AJ293820)
- *Glaciecola mesophila* LMG 22017[T] (AJ488501)
- *Glaciecola agarilytica* LMG 23762T QC oorspr. (DQ784575)
- *Alteromonas hispanica* LMG 22958[T] (AY926460)
- *BOX-PCR cluster 1* LMG 24079 (AM887686)
- *BOX-PCR cluster 1* LMG 24078[T] (AM885866)
- *Alteromonas addita* LMG 22532[T] (AY682202)
- *Alteromonas stellaepolaris* LMG 21861 (AJ295715)
- *Alteromonas tagae* BCRC 17571[T] (DQ836765)
- *Alteromonas litorea* JCM 12188[T] (AY428573)
- *Alteromonas marina* LMG 22057[T] (AF529060)
- *Alteromonas macleodii* LMG 2843[T] (X82145)
- *BOX-PCR cluster 5* LMG 24082 (AM885870)
- *Alteromonas simiduii* BCRC 17572[T] (DQ836766)
- *BOX-PCR cluster 4* LMG 24083 (AM887685)
- *BOX-PCR cluster 3* LMG 24080 (AM885868)
- *BOX-PCR cluster 2* LMG 24081 (AM885867)
- *Aeromonas sobria* LMG 3783[T] (X74683)

Figure 3.2: Neighbour-joining dendrogram representing the 16S rRNA gene sequence similarities of the isolates and their phylogenetic neighbours. Bootstrap values (1000 replicates) > 70 % are given.

DNA-DNA hybridisations experiments were carried out to elucidate the taxonomic position of the isolates. Two representative isolates per BOX-PCR cluster were selected on the basis of their BOX-PCR fingerprints to include isolates with the most diverse BOX-PCR profiles (Fig. 3.1). Their genomic relatedness towards *A. macleodii* LMG 2843[T], *A. hispanica* LMG 22958[T], *A. addita* LMG 22532[T], *A. stellipolaris* LMG 21861[T], *A. litorea* LMG 23846[T] and *A. marina* LMG 22057[T] was also determined. DNA-DNA hybridisation experiments were carried

out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 39°C and reciprocal reactions were performed for every pair of strains.

The DNA-DNA hybridisation values among isolates of the same BOX-PCR cluster were very high, ranging from 80 $\pm$ 1 % (BOX-PCR cluster 5) to 98 $\pm$ 2 % (BOX-PCR cluster 4). This again confirms that the isolates within one BOX-PCR cluster are closely related and belong to the same species (Wayne *et al.*, 1987).

DNA-DNA hybridisation values among representatives of BOX-PCR clusters 2, 3, 4 and 5 were moderate to high (an average value of 66 $\pm$ 1 % was obtained). BOX-PCR clusters 2 and 5 isolates revealed high (> 90 %) DNA-DNA hybridisation values towards *A. macleodii* LMG 2843[T]; BOX-PCR clusters 3 and 4 isolates revealed DNA-DNA hybridisation values of 65 $\pm$ 2 % towards *A. macleodii* LMG 2843[T]. DNA-DNA hybridisation values towards *A. marina* and all other *Alteromonas* species examined were low (ranging from 34 $\pm$ 2 % to 43 $\pm$ 1 %). In addition, DNA-DNA hybridisation values of BOX-PCR cluster 1 isolates towards BOX-PCR clusters 2, 3, 4 and 5 isolates were low (on average 19 $\pm$ 2 %), as were the DNA-DNA hybridisation values between BOX-PCR cluster 1 isolates and *A. hispanica* LMG 22958[T] (37 $\pm$ 2 %), their nearest phylogenetic neighbour, and all other *Alteromonas* species examined (23 $\pm$ 2 %, or less).

The following morphological, physiological and biochemical tests were performed on one representative isolate per BOX-PCR cluster.

Colony morphology was described after 4 days incubation at 20 °C on MA. Cells were tested for their Gram reaction, catalase and oxidase activity.Growth on Nutrient Agar (NA), Trypticase Soy Agar (TSA), R$_2$A and peptone/yeast extract/glucose agar (PYG) (Tan & Rüger, 1999) was recorded. The optimal NaCl concentration and the optimal growth temperature were determined using R$_2$A supplemented with 1 % to 20 % NaCl and incubated for 2 weeks at 20 °C, and MA incubated at 4 °C to 45 °C for 2 weeks, respectively. The effect of pH on the growth was analysed using Marine Broth growth medium (Difco™ 2216) with a pH ranging from 5.0 to 10.0 (at intervals of 0.5 pH units) and incubated at 20 °C for 7 days. Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA (using DNA agar (Difco™) containing 0.01 % Toluidine Blue (Merck)), starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days of incubation at 20 °C. The isolates were inoculated on Sierra's medium to determine their lipolytic activity and were incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was tested on MA plates using the diffusion disc method: ampicilin (25 µg), cefoxitin (30 µg), gentamycin (10 µg), erythromycin (15 µg) and kanamycin (30 µg). Results were read after 3 days of incubation at 20°C.

Biochemical characteristics from commercial microtest galleries (API ZYM, API 20E and API 20NE) were assessed according to the manufacturer's instructions. API ZYM was read after 4 h of incubation at 20 °C whereas API 20E and API 20NE were read after 24 h of incubation at 20 °C.

The cell morphology of BOX-PCR cluster 1 isolate LMG 24078[T] was determined by transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultra-thin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 3.3).
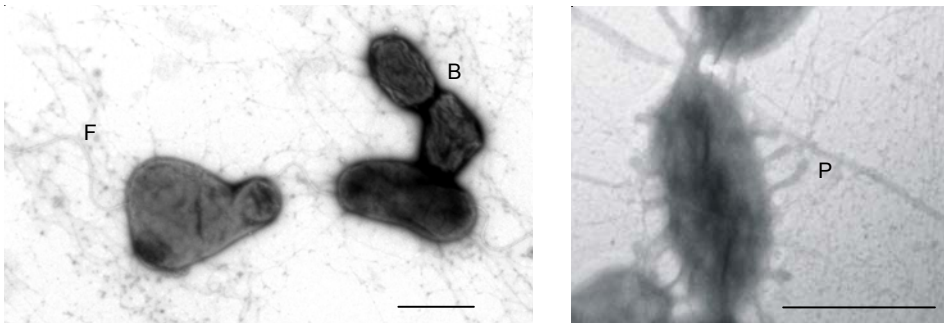
Figure 3.3: Electron micrographs of negatively stained cells of LMG 24078[T], showing flagella (F), buds (B) and prosthecae (P). Bar represents 1 µm.

The results of the phenotypic tests are listed in Table 3.1. Isolates representing BOX-PCR clusters 2, 3, 4 and 5 have the same phenotypic profile as *A. macleodii* LMG 2843[T]. In addition, the phenotypic characteristics clearly discriminated the isolates of BOX-PCR cluster 1 from their nearest phylogenetic neighbour *A. hispanica* and from the other validly named *Alteromonas* species.

1: *A. genovensis* sp. nov. LMG 24078[T]; **2**: *A. hispanica* LMG 22958[T]; **3**: *A. litorea* LMG 23846[T]; **4**: *A. marina* LMG 22057[T]; **5**: *A. macleodii* LMG 2843[T], BOX-PCR cluster 2 LMG 24081, BOX-PCR cluster 3 LMG 24080, BOX-PCR cluster 4 LMG 24083 and BOX-PCR cluster 5 LMG 24082.

+: positive reaction, **-** :negative reaction, **w**: weakly positive reaction, **ND**: no data ; **i**: intermediately susceptible to an antibiotic.

	1	2 *	3 §	4 †	5 ‡
Origin	Marine EAB, Genoa, Italy	Hypersaline water, Spain	Tidal sediment, Korea	Sea water, Korea	Sea water, Hawai, marine EAB
Growth in NaCl at:					
1 %	-	-	+	ND	+
10 %	+	+	+	+	+
15 %	+	-	-	ND	w
Growth at:	+	+	+	+	-
4 °C	-	-	+	+	+
40 °C	-	-	+	-	+
45 °C					
Growth on TSA	-	-	-	w	+
Hydrolysis of					
DNA	-	+	-	ND	+
Starch	w	+	+	+	+
Gelatin	w	+	+	+	+
Tyrosine	-	+	+	+	+
Casein	+	+	+	+	-
Tween 80	w	+	+	+	+
Enzymatic activity:					
α-chymotrypsin	+	-	-	-	-
lipase (C14)	w	w	w	+	+
valine arylamidase	-	w	w	-	+
trypsin	w	w	w	-	-
α-glucosidase	-	-	-	-	+
α-galactosidase	-	-	-	-	+
β-glucosidase	-	-	-	-	+
Susceptible to:					
Ampicilin (10 µg)	-	+	i	-	+
Erytromycin (15 µg)	+	+	+	i	+
Gentamycin (10 µg)	+	ND	+	+	+
Kanamycin (30 µg)	i	+	i	i	+
Cefoxitin (30 µg)	-	-	-	-	+
Mol% G + C	44.5	46.3	46.0	44.5	45.2

Table 3.1: Summary of the phenotypic tests of *Alteromonas genovensis* sp. nov., BOX-PCR clusters 2, 3, 4 and 5 and of *Alteromonas* reference strains.

*: Martinez-Checa *et al.* (2005)

§: Yoon *et al.* (2004)

†: Yoon *et al.* (2003)

‡: Baumann *et al.* (1972, 1984), Gauthier *et al.* (1995), Van Trappen *et al.* (2004), Yi *et al.* (2004) and this study.

In conclusion, although DNA-DNA hybridisation experiments revealed some diversity among BOX-PCR clusters 2, 3, 4 and 5 isolates, the present polyphasic taxonomic study demonstrated that they are all most appropriately classified as *A. macleodii*. Isolates of BOX-PCR cluster 1 constitute a novel species within the genus *Alteromonas* for which the name *Alteromonas genovensis* sp. nov. is proposed.

Description of *Alteromonas genovenis* sp. nov.

Alteromonas genovensis (ge'no.ven.sis. N.L. fem. adj. *genovensis*, pertaining to Genoa (Italy) where the sea water electroactive biofilms originated)

Cells are Gram-negative rods (0.9 μm x 1.8 μm), motile by a single polar flagellum. Buds and prosthecae are formed when the isolates are grown at lower temperatures (15 °C) for more than 3 days.

Prostheca formation is peritrichously and prosthecae are short and straight. These prosthecae are most often found on aggregated cells derived from the bacterial colony. These cell aggregates and, to a lesser extent, isolated bacteria are covered by a layer of fibrillar material. Buds are formed on mother and daughter cell (Fig. 3.3).

Colonies are beige, round-shaped and 2 mm in diameter. The surface is smooth and convex. Growth occurs after 2 days of incubation on MA at 20 °C, no growth occurs on TSA, R_2A or PYG medium.

Moderately halophilic and psychrotolerant. Temperature range for growth is 4 °C - 37 °C; no growth occurs at 40 °C or more. NaCl range for growth is 2 % - 15 %; no growth occurs at 1 % NaCl or at 20 % NaCl. pH range for growth is 6.0 to 8.5 and the optimal pH for growth is 7.0-8.0.

Catalase and oxidase active. Degradation of aesculin and casein but not of tyrosine or DNA. Weak hydrolysis of starch and gelatin; weak lipolysis of Tween 80.

Activity was recorded for the following enzymes: α-chymotrypsin, alkaline phosphatase, leucine arylamidase, cystine arylaminidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, esterase (C4) and esterase lipase (C8) activity. Weak activity of lipase (C14) and trypsin. No activity is detected for β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase or urease. Nitrate is not reduced to nitrite or nitrogen. No indol production, fermentation of glucose or assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid. Resistant to ampicilin (25 μg) and cefoxitin (30 μg) but not to gentamycin (10 μg) or erytromycin (15 μg). Intermediately susceptible to kanamycin (30 μg).

The dominant fatty acids are $C_{10:0}$3-OH (2.1 $\pm$ 0.9 %), $C_{11:0}$3-OH (1.3 $\pm$ 0.6), $C_{12:0}$ (2.6 $\pm$ 0.8 %), $C_{12:0}$3-OH (1.9 $\pm$ 1.4), $C_{14:0}$ (3.7 $\pm$ 0.8 %), $C_{16:0}$ (19.8 $\pm$ 2.4 %), $C_{17:0}$ (2.5 $\pm$ 0.6 %), $C_{17:1\omega8c}$ (5.1 $\pm$ 1.5 %), $C_{18:0}$ (1.1 $\pm$ 0.5), $C_{18:1\omega7c}$ (15.5 $\pm$ 2.0 %), SUMMED FEATURE 2 (which comprises any combination of $C_{12:0}$ aldehyde, an unknown fatty acid of equivalent chain length 10.928, $C_{16:1}$ iso I and $C_{14:0}$3-OH) (4.0 $\pm$ 1.3 %) and SUMMED FEATURE 3 ($C_{16:1\omega7c}$ and/or iso-$C_{15:0}$ 2-OH) (30.5 $\pm$ 2.4 %). The other fatty acids constitute trace fractions only (< 1 %). The DNA G + C content of the type strain is 44.6 mol%.

The 14 isolates were obtained from a marine electroactive biofilm that was grown on a stainless steel cathode (Genoa, Italy). The type strain of *Alteromonas genovensis* sp. nov. is LMG 24078[T] = CCUG 55340[T]; a second isolate with a distinct BOX-PCR fingerprint was deposited in the BCCM/LMG Bacteria Collection as LMG 24079 (Fig. 3.1).

Emended description of *Alteromonas macleodii* Baumann *et al.* 1972 (Approved lists 1980)

The description is as given by Baumann *et al.* (1972, 1984), Gauthier *et al.* (1995), Van Trappen *et al.* (2004) and Yi *et al.* (2004) with the following additions. Hydrolysis of DNA and L-tyrosine. Growth on TSA, but not on NA, R_2A or PYG. The NaCl range for growth is 1 % to 12 %; no growth occurs in the presence of 15 % NaCl. Growth at 45 °C was observed. No H_2S production. Susceptible to the following antibiotics: ampicilin (10 µg), erythromycin (15 µg), gentamycin (10 µg), kanamycin (30 µg) and cefoxitin (30 µg). Activity of lipase (C14), α-glucosidase, α-galactosidase, β-glucosidase, cystine arylamidase and β-galactosidase was recorded which conflicts with results obtained by Yi *et al.* (2004). The reason for this discrepancy is unclear.

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CHAPTER 4: Novel bacterial taxa within the *Roseobacter*-lineage

Preamble

The 91 isolates of FAME cluster IV were studied by BOX-PCR fingerprinting and 6 BOX-PCR clustes comprising isolates with similar DNA profiles could be delineated (Addendum 1, Fig.5.2). Representative isolates were selected for 16S rRNA gene sequence analysis in order to reveal the phylogenetic position of the isolates. The combination of BOX-PCR fingerprinting and 16S rRNA gene sequence analysis did not enable to identify the isolates. Four BOX-PCR clusters and one isolate that had a unique BOX-PCR profile were selected for further experiments.

4.1

Leisingera aquimarina sp. nov., isolated from a marine electroactive biofilm and emended description of *Leisingera methylohalidovorans* Schaefer *et al.* 2002, *Phaeobacter daeponensis* Yoon *et al.* 2007 and *Phaeobacter inhibens* Martens *et al.* 2006

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SUMMARY

Strain LMG 24366[T] was isolated from a marine electroactive biofilm grown on a stainless steel cathode (Genoa, Italy) and was investigated by a polyphasic taxonomic approach. This study demonstrated that LMG 24366[T] constitutes a novel species within the genus *Leisingera* which shared 98.9 % 16S rRNA gene similarity with its nearest phylogenetic neighbour *Leisingera methylohalidivorans*. Strain LMG 24366[T] exhibited growth on betaine (1 mM) as a sole carbon source whereas no growth was observed on L-methionine (10 mM). Both phenotypic and genotypic analyses allowed to differentiate the established *Leisingera* species from this novel species for which the name *Leisingera aquimarina* sp. nov. is proposed. The type strain of *Leisingera aquimarina* sp. nov. is LMG 24366[T] = CCUG 55860[T]; its DNA G + C content is 61.4 mol%.

The genus *Leisingera* was created by Schaefer *et al.* (2002) to accommodate *Leisingera methylohalidovorans*. The only species of this genus was isolated from a tide pool off the coast of California and is able to grow on methyl bromide (MeBr) as a sole energy and carbon source (Schaefer *et al.*, 2002). Although oceans act as a sink for MeBr (Yvon & Butler, 1996), it was the first bacterial species isolated that was able to grow on MeBr. Growth of *L. methylohalidovorans* is also observed on methyl chloride, methyl iodide and methionine. These data indicate a role for *L. methylohalidovorans* in the degradation of methyl halides and thus in the release of bromide and chlorine atoms in the stratosphere which contributes to the catalytic destruction of ozone (Butler, 1999).

The genus *Leisingera* is a member of the *Roseobacter* lineage of the *Alphaproteobacteria*. This lineage is one of the most abundant marine groups (González *et al.*, 2000) as roseobacters can constitute up to 20 % of coastal bacterioplankton communities and are found in almost every marine environment (Buchan *et al.*, 2005).

The present study was part of an analysis of the microbial diversity of a marine electroactive biofilm, grown on a stainless steel cathode (EA-BIOFILMS-508866 (NEST)) exposed to natural marine water at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy (Faimali *et al.*, in press, Vandecandelaere *et al.*, in press). The biofilm was removed from the stainless steel cathode by sonication (Branson™ 3200) (90 s) in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated on Marine Agar (MA) (Difco™ 2216) and incubated aerobically at 20 °C for several days. Pure cultures were obtained and isolates were stored at –20 °C or –80 °C using MicroBank™ vials.

Whole cell fatty acid methyl ester (FAME) analysis (Mergaert *et al.*, 2001) indicated that strain LMG 24366[T] belonged to the *Alphaproteobacteria*. The dominant fatty acids are $C_{10:0}$3-OH (2.0 %), $C_{12:0}$3-OH (2.1 %), $C_{16:0}$ (3.5 %), $C_{16:0}$2-OH (4.2 %), $C_{14:1}$iso E (11.6 %), $C_{18:1\omega7c}$ (71.6 %) and an unknown fatty acid 11.799 (2.7 %). The remaining fatty acids constituted minor fractions only (< 1.0 %).

DNA was extracted according to Pitcher *et al.* (1989) and an almost-complete 16S rRNA gene sequence was obtained (1396 bp) using the universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA 3') (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). The EMBL accession number of the 16S rRNA gene sequence of LMG 24366[T] is AM900415. The FASTA program was used to find the most similar sequences. These sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997). A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using the BioNumerics 4.61 software (Applied Maths, Belgium) (Fig. 4.1). Numerical analysis indicated that the phylogenetic nearest neighbour of strain LMG 24366[T] was *Leisingera methylohalidovorans* LMG 23656[T] with 98.9 % 16S rRNA gene sequence similarity. Members of the genus *Phaeobacter* (*Phaeobacter daeponensis* LMG 24139[T], *P.*

inhibens LMG 22475ᵀ and *P. gallaeciensis* LMG 23163ᵀ) (Martens *et al.*, 2006, Yoon *et al.*, 2007) were also related to strain LMG 24366ᵀ as 16S rRNA gene sequence similarities were ranging from 96.9 % to 97.2 %.

Figure 4.1: Neighbour-joining dendrogram representing the 16S rRNA gene sequence similarities of *Leisingera aquimarina* sp. nov. and its nearest phylogenetic neighbours. Bootstrap values (1000 replicates) above 70 % are given.

Strains LMG 24366[T], *Leisingera methylohalidovorans* LMG 23656[T] *Phaeobacter gallaeciensis* LMG 23163[T] and *P. inhibens* LMG 22475[T] were investigated by repetitive DNA-PCR (rep-PCR) fingerprinting using the BOX-A1R-primer (5' CTA CGG CAA GGC GAC GCT GAC G 3') (Rademaker *et al.*, 2000, Versalovic *et al.*, 1994). Numerical analysis, using the BioNumerics 4.61 software (Applied Maths, St-Martens-Latem, Belgium), showed that strain LMG 24366[T] could be easily distinguished from its nearest phylogenetic neighbours (Fig. 4.2).

LMG 23163[T]   *Phaeobacter gallaeciensis*

LMG 23656[T]   *Leisingera methylohalidivorans*

LMG 22475[T]   *Phaeobacter inhibens*

LMG 24366[T]   *Leisingera aquimarina* sp. nov.
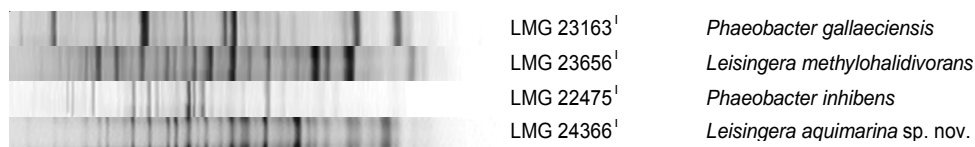
Figure 4.2: BOX-PCR fingerprints of *Leisingera aquimarina* sp. nov. and its nearest phylogenetic neighbours.

The DNA G + C content of strain LMG 24366[T] was subsequently determined. DNA was enzymatically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Water Breeze HPLC system and Xbridge Shield RP18 column thermostabilised at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G + C content of LMG 24366[T] was 61.4 mol%, which correlates with the DNA G + C content of the genus *Leisingera* as described by Schaefer *et al.* (2002).

DNA-DNA hybridisations experiments were performed to elucidate the taxonomic position of LMG 24366[T] and were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 45 °C and reciprocal reactions were performed for every pair of strains. The average DNA-DNA hybridisation value between LMG 24366[T] and its nearest phylogenetic neighbour, *L. methylohalidivorans* LMG 23656[T], was 56 ± 2 %; the average DNA-DNA hybridisation value between LMG 24366[T] and *Phaeobacter gallaeciensis* LMG 23163[T] was very low (2 ± 0 %). These data indicated that LMG 24366[T] constitutes a novel species within the genus *Leisingera* (Wayne *et al.*, 1987).

The following morphological, physiological and biochemical characteristics were evaluated for strain LMG 24366[T]. Colony morphology was described after 4 days of incubation at 20 °C

on MA. Cells were tested for their Gram reaction and the presence of catalase and oxidase activity.

Growth was examined on Nutrient Agar (NA), Trypticase Soy Agar (TSA), R_2A and peptone/yeast extract/glucose agar (PYG) (Tan & Rüger, 1999). Growth on L-methionine (10 mM) and betaine (1 mM) was tested as described by Martens *et al.* (2006). The optimal salinity and the optimal growth temperature were determined using respectively R_2A supplemented with 1 % to 20 % NaCl, incubated for 2 weeks at 20 °C and MA incubated at 4 °C to 45 °C for 2 weeks. The effect of pH on the growth was analysed using Marine Broth growth medium (Difco™ 2216) with a pH ranging from 5.0 to 10.0 (at intervals of 0.5 pH units) and incubated at 20 °C for 7 days.

Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA (using Difco™ DNA agar containing 0.01 % Toluidine Blue (Merck)), starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days of incubation at 20 °C. Strain LMG 24366[T] was inoculated on Sierra's medium to determine their lipolytic activity and was incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was examined on MA plates using the diffusion disc method: cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg), tetracyclin (30 µg), streptomycin (25 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg). Results were read after 5 days of incubation at 20 °C.

Biochemical characteristics from the commercial microtest galleries API ZYM and API 20NE were studied according to the manufacturer's instructions. API ZYM was read after 4 h of incubation at 20 °C whereas API 20NE was read after 48 h of incubation at 20 °C. The results of the phenotypic features are summarized in Table 4.1.

1: *Leisingera aquimarina* sp. nov. LMG 24366[T]; **2**: *Leisingera methylohalidovorans* LMG 23656[T] (data from Schaefer *et al.*, 2002; Martens *et al.*, 2006); **3**: *Phaeobacter inhibens* LMG 22475[T] (data from Martens *et al.*, 2006); **4**: *Phaeobacter gallaeciensis* LMG 23163[T] (data from Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006); **5**: *Phaeobacter daeponensis* LMG 24139[T] (data from Yoon *et al.*, 2007).

+: Characteristic is present; **-**: Characteristic is absent; **w**: Weak activity was recorded; **ND**: No data available; **i**: intermediately susceptible to an antibiotic
***: Data from this study

	1*	2	3	4	5
Origin	Marine EAB, Italy	Tidal pool, USA	Tidal flat, Germany	Scallop, Spain	Tidal flat, Korea
Colony colour	Dark beige-pink	Unpigmented	Dark brown	Brown	Yellowish white
Growth at:					
4°C	+	+	+	-	+
40°C	-	-	-	-	+
Growth in NaCl at:					
1 %	w	-	+	+	+
2 %	+	+	+	+	+
7 %	w	-	+	+	+
10 %	-	-	-	+	-
Growth on:					
NA	-	-*	-*	ND	w*
TSA	-	w*	w*	ND	w*
Growth on:					
Betaine (1 mM)	+	+	+	-	+*
Methionine (10 mM)	-	+	+	+	+*
Degradation of:					
L-tyrosine	-	-*	+	-*	+
Starch	-	+	-	-	-
Gelatin	w	-*	-	-	-
Reduction of NO_3 to NO_2	-	-	-	-	+
Susceptible to:					
Erythromycin (15 µg)	+	+*	i*	+	i*
Tetracyclin (30 µg)	+	+*	-*	ND	-
Gentamicin (30 µg)	-	i*	-*	+	+
Streptomycin (25 µg)	+	+*	+	+	+
Vancomycin (30 µg)	-	-*	-*	ND	+*
Enzymatic activity *:					
Alkaline phosphatase	w	-	-	ND	+
Esterase (C 4)	-	-	-	ND	+
Esterase lipase (C 8)	w	-	-	ND	+
Valine arylamidase	-	w	-	ND	-
Acid phosphatase	-	-	w	ND	+
Naphthol-AS-BI-phosphohydrolase	w	w	-	ND	-
α-glucosidase	-	-	w	ND	-
DNA G + C content (mol%)	61.4	60.5	55.7	58	64.9

Table 4.1: Summary of the phenotypic features of *Leisingera aquimarina* sp. nov., *Leisingera methylohalidivorans* and *Phaeobacter* species.

The cell morphology of strain LMG 24366^T was determined by transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultra-thin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 4.3).
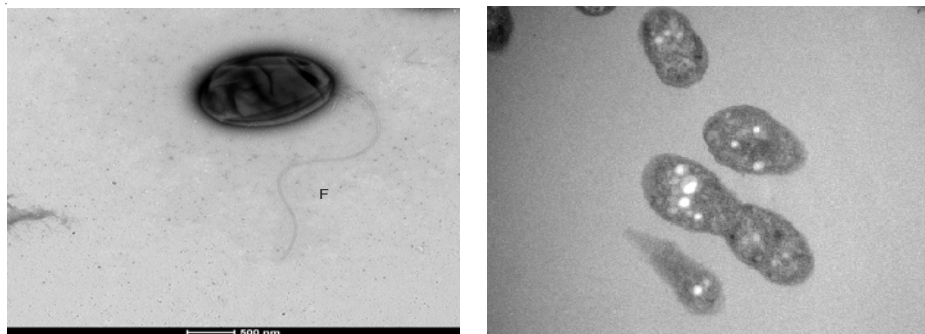
Figure 4.3: Electron micrographs of LMG 24366^T, showing the polar flagellum (left panel) and poly-β-hydroxybutyrate inclusion (P) bodies (right panel).

In conclusion, strain LMG 24366^T is easily discriminated from *Leisingera methylohalidovorans* and *Phaeobacter* species. On the basis of phylogenetic, genomic and phenotypic data, we conclude that LMG 24366^T constitutes a novel species within the genus *Leisingera* for which the name *Leisingera aquimarina* sp. nov. is proposed.

Description of *Leisingera aquimarina* sp. nov.

(A.qui.ma.ri'na. L. fem. N. *aqua* water; L. adj. *marinus* from the sea; N. L. adj. *aquimarina* from the seawater)

Cells are ovoid (1 x 1.4 µm), Gram-negative and motile by a single polar flagellum. Poly-β-hydroxybutyrate inclusion bodies are present (Fig. 4.3). Colonies are dark beige-pink, round and 1-2 mm in diameter after 3 days incubation on MA.

Growth occurs after 2 days of incubation at 20 °C on MA but not on R$_2$A, NA, TSA and PYG. Temperature range for growth is 4 °C-37 °C; no growth at 40 °C or higher. NaCl range for growth is 1 % - 7 %. pH range for growth is 5.5 - 9.0 and the optimal pH for growth is 6.5 - 8. Growth is observed on betaine (1 mM) as a sole carbon source; no growth on L-methionine (10 mM).

Catalase and oxidase active. Weak degradation of gelatine; no degradation of L-tyrosine, DNA, starch, casein, chitin, aesculin or Tween 80.

Leucine arylamidase activity is detected; weak activity is detected for alkaline phosphatase and naphthol-AS-BI-posphohydrolase. No activity is observed for the following enzymes: esterase (C4), esterase lipase (C8), valine arylamidase, acid phosphatase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosamidase, α-mannosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, arginine hydrolase, urease or α-fucosidase.

Nitrate is not reduced to nitrite or to nitrogen. No production of indole or fermentation of glucose. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid.

Susceptible to cefoxitin (30 µg), erythromycin (15 µg), tetracyclin (30 µg) and streptomycin (25 µg) but resistant to vancomycin (30 µg), trimethoprim (1.25 µg), clindamycin (2 µg) and gentamicin (30 µg).

The dominant fatty acids are $C_{10:0}$3-OH (2.0 %), $C_{12:0}$3-OH (2.1 %), $C_{16:0}$ (3.5 %), $C_{16:0}$2-OH (4.2 %), $C_{14:1}$iso E (11.6 %), $C_{18:1\omega7c}$ (71.6 %) and an unknown fatty acid 11.799 (2.7 %); the remaining fatty acids constituted trace fractions only (< 1.0 %).

DNA G + G content is 61.4 mol%. The type strain is LMG 24366^T and was isolated from a marine electroactive biofilm (Genoa, Italy).

Emended description of *Leisingera methylohalidivorans* Schaefer *et al.* 2002

The description is as given by Schaefer *et al.* (2002) and Martens *et al.* (2006) with the following additions. No degradation of L-tyrosine, casein or DNA. No hydrolysis of aesculin or gelatin. No indole production or fermentation of glucose. Grows weakly on TSA; no growth on NA, R_2A or PYG. Leucine arylamidase is present; weak activity is detected for valine arylamidase and naphthol-AS-BI-phosphohydrolase. No activity is observed for the following enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase and urease. No assimilation of D-mannose, D-mannitol, D-maltose, potassium gluconate, capric acid, adipic acid or phenylacetic acid. Susceptible to cefoxitin (30 µg), erythromycin (15 µg), streptomycin (25 µg) and tetracyclin (30 µg); intermediately susceptible to gentamicin (10 µg). Resistant to vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg).

Emended description of *Phaeobacter inhibens* Martens *et al.* 2006

The description is as given by Martens *et al.* (2006) with the following additions. Weak growth on TSA; no growth on NA, PYG or R_2A. No hydrolysis of DNA or aesculin. No fermentation of glucose. Leucine arylamidase is present; weak activity is detected for acid phosphatase and α-glucosidase. No activity is observed for the following enzymes: esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phospohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosamine, α-mannosidase, α-fucosidase, arginine dihydrolase and urease. Susceptible to cefoxitin (30 µg); intermediately susceptible to erythromycin (15 µg) and streptomycin (25 µg). Resistant to tetracyclin (30 µg), gentamicin (10 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg).

Emended description of *Phaeobacter daeponensis* Yoon *et al.* 2007

The description is as given by Yoon *et al.* (2007) with the following additions. Weak growth on TSA and NA; no growth on PYG or R_2A. No hydrolysis of DNA or fermentation of glucose. Urease activity is not detected. Susceptible to cefoxitin (30 µg) and vancomycin (30 µg); intermediately susceptible to erythromycin (15 µg). Resistant to trimethoprim (1.25 µg) and clindamycin (2 µg).

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4.2

Ruegeria scottomollicae sp. nov., isolated from a marine electroactive biofilm

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SUMMARY

Seventy isolates were obtained from a marine electroactive biofilm that was generated on a cathodically polarized stainless steel electrode (Genoa, Italy). The genetic diversity was investigated by BOX-PCR fingerprinting and two clusters of isolates with similar BOX-PCR profiles were delineated. Whole cell fatty acid methyl ester analysis and 16S rRNA gene sequence analysis showed that the isolates belong to the *Roseobacter* lineage of the *Alphaproteobacteria*. DNA-DNA hybridisation experiments and a biochemical analysis demonstrated that 4 isolates belonged to *Ruegeria mobilis*. On the other hand, the 66 isolates of the second BOX-PCR cluster constitute a novel species within the genus *Ruegeria* for which the name *Ruegeria scottomollicae* sp. nov. is proposed. Its DNA G + C content is 61.0 $\pm$ 0.4 %. The type strain is LMG 24367^T = CCUG 55858^T.

Uchino *et al.* (1998) created the genus *Ruegeria* to accommodate the generically misclassified species *Agrobacterium atlanticum*, *Agrobacterium gelatinovorum* and *Roseobacter algicola* with *Ruegeria atlantica* as type species. This genus harbours marine aerobic Gram-negative, catalase and oxidase positive rods. Arahal *et al.* (2005) subsequently reclassified *Ruegeria gelatinovorans* as *Thalassobius gelatinovorus* and Martens *et al.* (2006) restricted the genus *Ruegeria* to its type species by transferring *Ruegeria algicola* to the genus *Marinovum*. More recently, Yi *et al.* (2007) transferred the two species of the genus *Silicibacter* (*Silicibacter lacuscaerulensis* (Petursdottir & Kristjansson, 1997) and *Silicibacter pomeroyi* (González *et al.*, 2003) into the genus *Ruegeria*; *Ruegeria mobilis* and *Ruegeria pelagia* were also described (Lee *et al.*, 2007, Muramatsu *et al.*, 2007). At the time of writing, the genus *Ruegeria* thus comprises 5 validly named species: *R. atlantica*, *R. pomeroyi*, *R. lacuscaerulensis*, *R. pelagia* and *R. mobilis*, although the allocation of *R. pomeroyi* and *R. lacuscaerulensis* is controversial (Muramatsu *et al.*, 2007).
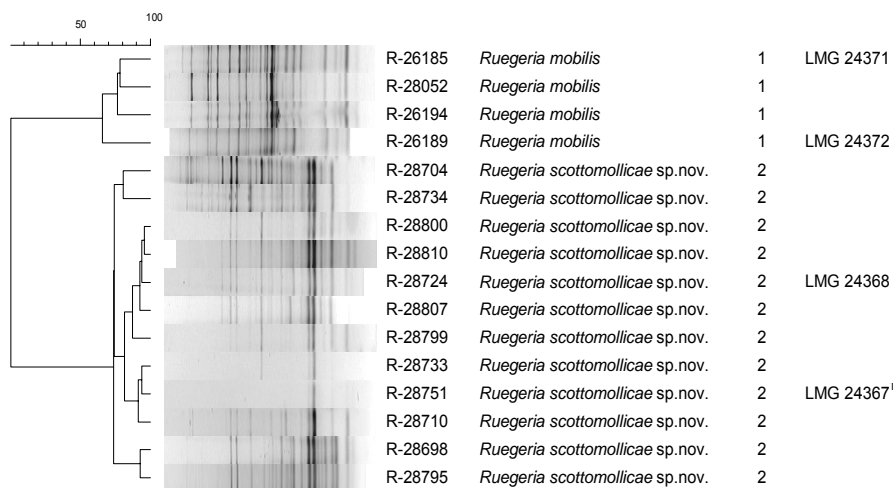
The present taxonomic study was performed in the context of the analysis of the microbial population present in a marine electroactive biofilm (EA-BIOFILMS-508866 (NEST)), generated on a stainless steel cathode that was exposed to natural marine water at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy (Faimali *et al.*, in press, Vandecandelaere *et al.*, in press). The biofilm was removed from the stainless steel cathode by sonication (Branson^{TM} 3200) (90 s) in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated on Marine Agar (MA) (Difco^{TM} 2216) and incubated aerobically at 20 °C for several days. Pure cultures were obtained and the isolates were stored at –20 °C or –80 °C using MicroBank^{TM} vials.

DNA was extracted from the 70 isolates according to Pitcher *et al.* (1989) and the genetic diversity was investigated by repetitive DNA-PCR (rep-PCR) fingerprinting using the BOX-A1R-primer (5' CTA CGG CAA GGC GAC GCT GAC G 3') (Rademaker *et al.*, 2000, Versalovic *et al.*, 1994). The obtained DNA profiles were numerically analysed using the BioNumerics 4.61 software (Applied Maths, St-Martens-Latem, Belgium). Two clusters of isolates with similar BOX-PCR profiles were delineated: BOX-PCR cluster 1 comprising 4 isolates sharing at least 65.8 % profile similarity and BOX-PCR cluster 2 representing 66 isolates sharing at least 76 % profile similarity. Fig. 4.4 illustrates BOX-PCR fingerprints and a numerical analysis of the profiles of BOX-PCR cluster 1 isolates and of a selection of BOX-PCR cluster 2 isolates. In both BOX-PCR clusters, but especially in BOX-PCR cluster 2, some isolates exhibited almost identical DNA profiles, indicating that they likely originated from the same strain. In contrast, both clusters also comprised isolates with clearly distinguishable profiles suggesting the presence of multiple strains.

Pearson correlation (Opt:0.37%) [0.0%-100.0%]

BOX-PCR　　　　　　**BOX-PCR**

R-26185	*Ruegeria mobilis*	1	LMG 24371
R-28052	*Ruegeria mobilis*	1	
R-26194	*Ruegeria mobilis*	1	
R-26189	*Ruegeria mobilis*	1	LMG 24372
R-28704	*Ruegeria scottomollicae* sp.nov.	2	
R-28734	*Ruegeria scottomollicae* sp.nov.	2	
R-28800	*Ruegeria scottomollicae* sp.nov.	2	
R-28810	*Ruegeria scottomollicae* sp.nov.	2	
R-28724	*Ruegeria scottomollicae* sp.nov.	2	LMG 24368
R-28807	*Ruegeria scottomollicae* sp.nov.	2	
R-28799	*Ruegeria scottomollicae* sp.nov.	2	
R-28733	*Ruegeria scottomollicae* sp.nov.	2	
R-28751	*Ruegeria scottomollicae* sp.nov.	2	LMG 24367[T]
R-28710	*Ruegeria scottomollicae* sp.nov.	2	
R-28698	*Ruegeria scottomollicae* sp.nov.	2	
R-28795	*Ruegeria scottomollicae* sp.nov.	2	

Figure: 4.4: Dendrogram representing the BOX-PCR profiles of a selection of isolates. The BOX-PCR cluster number and the LMG reference strain number are given.

All isolates were examined by whole cell fatty acid methyl ester (FAME) analysis (Mergaert *et al.*, 2001) and were tentatively identified as *Alphaproteobacteria*. The dominant fatty acids of the 70 isolates were $C_{10:0}$3-OH (4.9 $\pm$ 1.0 %), 11-methyl-$C_{18:1\omega7c}$ (3.4 $\pm$ 1.4 %), $C_{16:0}$ (1.8 $\pm$ 0.5 %), $C_{16:0}$2-OH (6.0 $\pm$ 0.8 %), $C_{18:1}$2-OH (3.9 $\pm$ 0.8 %), $C_{18:1\omega7c}$ (73.8 $\pm$ 2.5 %) and an unknown fatty acid 11.799 (4.1 $\pm$ 0.8 %). Largely the same types and proportions of fatty acids were present in both BOX-PCR clusters, except C_{12}3-OH (1.4 $\pm$ 0.9 %) and $C_{18:0}$ (1.3 $\pm$ 0.6 %) which were only detected in BOX-PCR cluster 1 isolates.

Isolates from both BOX-PCR clusters were selected for 16S rRNA gene sequencing on the basis of their BOX-PCR profiles as we looked for the ones with the most different DNA profiles (Fig. 4.4). Almost-complete 16S rRNA gene sequences (1343 - 1403 bp) were obtained for representatives of BOX-PCR cluster 1 (LMG 24371 and LMG 24372) and of BOX-PCR cluster 2 (LMG 24367[T] and LMG 24368) using the universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA 3') (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). A partial 16S rRNA gene sequence (430 bp) was obtained for a BOX-PCR cluster 1 isolate (R-28052) and partial 16S rRNA gene sequences ranging from 331 to 414 bp were obtained for 6 isolates of BOX-PCR cluster 2 (R-28698, R-28710, R-28733, R-28795, R-28799 and R-28807) by using the universal primer pD (5' GTA TTA CCG CGG CTG CTG 3') as described by Coenye *et al.* (1999) (data not shown). The EMBL accession numbers of LMG 24367[T], LMG 24368, LMG 24371 and LMG 24372 are AM905330, AM905331, AM905332 and AM905333, respectively.

The FASTA program was used to find the most similar sequences for LMG 24371, LMG 24372, LMG 24367[T] and LMG 24368 in public databases. These 16S rRNA gene sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using the BioNumerics 4.61 software (Fig. 4.5). The tree topology was confirmed by maximum parsimony and maximum likelihood analyses (data not shown).
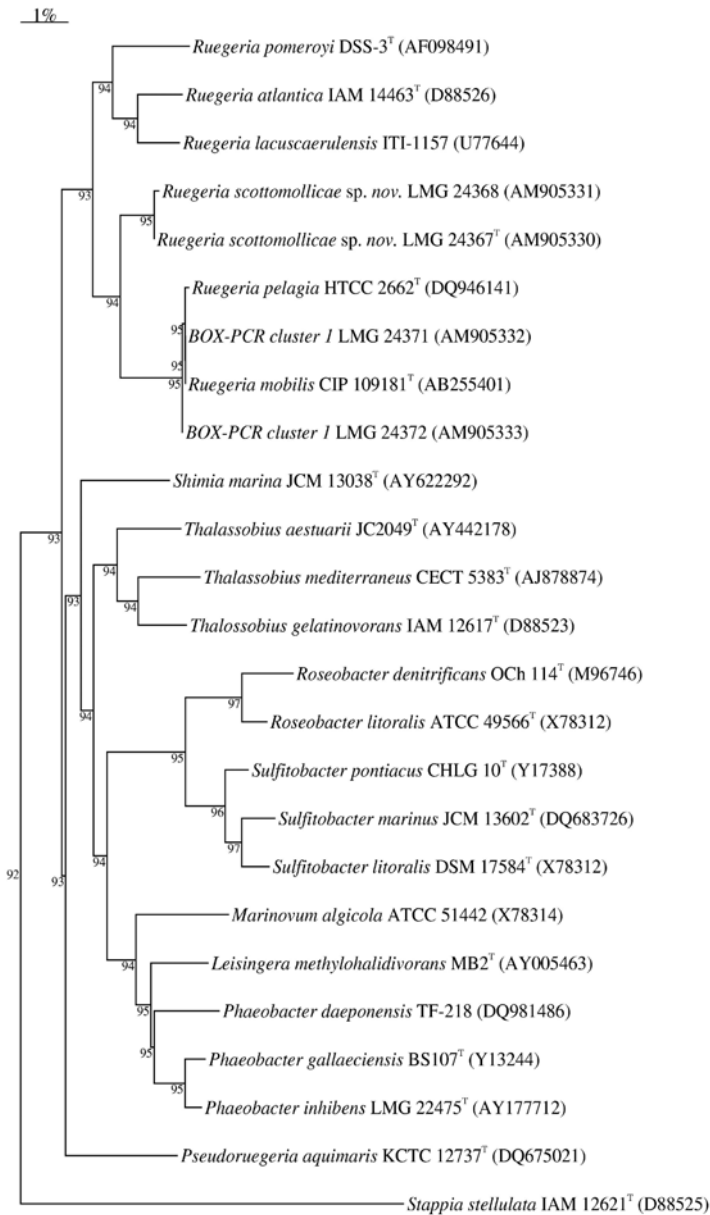
Figure 4.5: Neighbour-joining dendrogram representing the 16S rRNA gene sequence similarities of *Ruegeria scottomollicae* sp. nov., BOX-PCR cluster 1 isolates and their nearest phylogenetic neighbours. Bootstrap values (1000 replicates) > 70 % are shown.

The 16S rRNA gene sequence similarities between the 3 representatives of BOX-PCR cluster 1 (LMG 24371, LMG 24372 and R-28052) were high, ranging from 99.2 to 99.9 %. These data suggested that BOX-PCR cluster 1 isolates belong to the same species (Stackebrandt & Ebers, 2006) and confirmed that isolates with highly similar BOX-PCR profiles are closely related (Rademaker & De Bruijn, 1997). The nearest phylogenetic neighbours of BOX-PCR cluster 1 isolates were *Ruegeria pelagia* HTCC 2662^T and *R. mobilis* CIP 109181^T with 99.9 - 100 % 16S rRNA gene sequence similarity. In contrast, the phylogenetic relationship to the type strains of the other established *Ruegeria* species (*Ruegeria lacuscaerulensis* LMG 23162^T, *R. pomeroyi* LMG 23168^T and *R. atlantica* LMG 23161^T) was more distant as 16S rRNA gene sequence similarities of 96.1 to 96.6 % were detected (Fig. 4.5).

The 8 selected representatives of BOX-PCR cluster 2 (LMG 24367^T, LMG 24368, R-28698, R-28710, R-28733, R-28795, R-28799 and R-28807) were also closely related as 16S rRNA gene sequence similarities ranging from 99.7 to 100 % were detected, strongly suggested that BOX-PCR cluster 2 isolates belong to the same species (Stackebrandt & Ebers, 2006). The nearest phylogenetic neighbours of BOX-PCR cluster 2 isolates were *Ruegeria mobilis* CIP 109181^T (97.9 – 98 .1 % 16S rRNA gene sequence similarity), *R. pelagia* HTCC 2662^T (97.8 – 97.9 % sequence similarity) and BOX-PCR cluster 1 isolates (97.8 - 98.1 % sequence similarity). The 16S rRNA gene sequence similarities between representatives of BOX-PCR cluster 2 and the other established *Ruegeria* species (*R. pomeroyi* LMG 23168^T, *R. lacuscaerulensis* LMG 23162^T and *R. atlantica* LMG 23161^T) were low (95.8 – 96.6 %) (Fig. 4.5).

DNA-DNA hybridisations experiments were performed with BOX-PCR cluster 1 strains (LMG 24371 and LMG 24372) and BOX-PCR cluster 2 strains (LMG 24367^T and LMG 24368) to elucidate their taxonomic position and were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 45 °C and reciprocal reactions were performed for every pair of isolates. DNA-DNA hybridisation values among representatives of the same BOX-PCR cluster were high (76 $\pm$ 5 % and 95 $\pm$ 5 % for BOX-PCR cluster 1 and BOX-PCR cluster 2, respectively). The average DNA-DNA hybridisation value between a representative of BOX-PCR cluster 1 and its phylogenetic nearest neighbour *Ruegeria mobilis* CIP 109181^T was high (87 $\pm$ 3 %), demonstrating that BOX-PCR cluster 1 isolates belong to *R. mobilis* (Wayne *et al.*, 1987). On the other hand, the DNA-DNA hybridisation values between BOX-PCR cluster 2 strain LMG 24367^T and *Ruegeria* reference strains (*Ruegeria atlantica* LMG 23161^T, *R. lacuscaerulensis* LMG 23162^T, *R. pomeroyi* LMG 23168^T and *R. mobilis* CIP 109181^T) were low (ranging from 8 $\pm$ 6 % to 30 $\pm$ 2 %), indicating that BOX-PCR cluster 2 isolates constitute a novel species within the genus *Ruegeria*.

The DNA mol G + C content of LMG 24371 and LMG 24372 (BOX-PCR cluster 1) and of LMG 24367[T] and LMG 24368 (BOX-PCR cluster 2) was determined. DNA was enzymatically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Water Breeze HPLC system and Xbridge Shield RP18 column thermostabilised at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G + C content of strains LMG 24371 and LMG 24372 (BOX-PCR cluster 1) and LMG 24367[T] and LMG 24368 (BOX-PCR cluster 2) was 59.0 $\pm$ 0.5 mol% and 61.0 $\pm$ 0.4 mol%, respectively, which is in the range of the DNA mol G + C content of the established species of the genus *Ruegeria* (González *et al.*, 2003, Lee *et al.*, 2007, Muramatsu *et al.*, 2007, Petursdottir & Kristjansson, 1997, Uchino *et al.*, 1998).

The following morphological, physiological and biochemical characteristics were analysed for strains LMG 24371 and LMG 24372 (BOX-PCR cluster 1) and LMG 24367[T] and LMG 24368 (BOX-PCR cluster 2). Colony morphology was described after 4 days of incubation at 20 °C on MA. Cells were tested for their Gram reaction, catalase and oxidase activity. Growth was verified on Nutrient Agar (NA), Trypticase Soy Agar (TSA), R_2A and peptone/yeast extract/ glucose agar (PYG) (Tan & Rüger, 1999). The optimal salinity and the optimal growth temperature were determined using R_2A supplemented with 1 % to 20 % NaCl and incubated for 2 weeks at 20 °C, and MA incubated at 4 °C to 45 °C for 2 weeks, respectively. The effect of pH on the growth was analysed using Marine Broth growth medium (Difco™ 2216) with of a pH ranging from 5.0 to 10.0 (at intervals of 0.5 pH units) and incubated at 20 °C for 7 days. Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA (using Difco™ DNA agar, containing 0.01 % Toluidine Blue (Merck)), starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days of incubation at 20 °C. The isolates were inoculated on Sierra's medium to determine their lipolytic activity and were incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was tested on MA plates using the diffusion disc method: cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg), streptomycin (25 µg), tetracyclin (30 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg). Results were read after 5 days of incubation at 20 °C.

Biochemical characteristics from the commercial microtest galleries API ZYM and API 20NE were assessed according to the manufacturer's instructions. API ZYM was read after 4 h of incubation at 20 °C whereas API 20NE was read after 48 h of incubation at 20 °C. The results of the phenotypic analysis are summarized in Table 4.2.

1: *Ruegeria scottomollicae* sp. nov. (LMG 24367^T, LMG 24368); **2**: BOX-PCR cluster 1 (LMG 24371, LMG 24372); **3**: *R. atlantica* (data from Rüger & Höfle, 1992; Uchino *et al.*, 1998; Martens *et al.*, 2006; Muramatsu *et al.*, 2007; Yi *et al.*, 2007) **4**: *R. lacuscaerulensis* (LMG 23162^T) (data from Petursdottir & Kristjansson, 1997; Yi *et al.*, 2007); **5**: *R. pomeroyi* (LMG 23168^T) (Gonzalez *et al.*, 2003; Yi *et al.*, 2007); **6**: *R. mobilis* (Muramatsu *et al.*, 2007) **7**: *R. pelagia* KCCM 42378^T (Lee *et al.*, 2007).

*: Data from this study; **+**: Characteristic is present; **-**: Characteristic is absent; **w**: Weak activity was recorded; **ND**: No data available; **i**: Intermediately susceptible to an antibiotic

	1*	2	3	4	5	6	7
Origin	Marine EAB, Genoa, Italy	Marine EAB, Genoa, Italy	Marine sediments, NW Atlantic ocean	Geothermal lake Iceland	Coastal seawater, USA	Marine slime, Japan	Western Sargasso Sea
Growth at:							
4 °C	+	w	-	-	-	-	-
40 °C	w	w	-	+	+	-	+
45 °C	-	w	-	+	-	-	-
Growth in NaCl at							
1 %	+	+	-	+	+	+	+
2 %	+	+	-	+	+	+	+
7 %	+	+	+	+	-	+	+
10 %	+	+	-	-	-	+	+
15 %	+	-	-	-	-	-	-
Reduction of NO$_3$ to NO$_2$	-	-	+	+	-	-	-
Degradation of:							
DNA	-	+	-	ND	ND	-	ND
Tween 80	+	+	-	+	+	-	+
Starch	-	-	-	-	-	-	ND
Aesculin	+	+	+	+	+	+	+
Gelatin	-	-	-	-	+	ND	-
Susceptible to:							
Erythromycin (15 µg)	i	i	i*	i*	+*	i*	+
Tetracyclin (30 µg)	-	-	-*	+	i*	-*	+
Gentamicin (30 µg)	i	-	-*	+*	+*	-*	-
Streptomycin (25 µg)	+	+	+*	-	+*	+*	+
Vancomycin (30 µg)	-	-	+*	-*	-*	-*	-
Enzymatic activity:							
Esterase (C4)	w	-	-	w	-	-*	+
Esterase lipase (C8)	w	-	-	w	w	-*	+
Valine arylamidase	-	w	w	w	-	-*	+
Acid phosphatase	+	+	w	+	-	w*	+
Naphthol-AS-BI-phosphohydrolase	+	+	-	+	-	w*	+
α-glucosidase	w	+	w	+	-	-*	+
N-acetyl-β-glucosaminidase	+	+	-	-	-	-*	+
β-galactosidase	-	-	-	w	-	-*	-
DNA G + C content (mol%)	61	59.5	55-58	66	68	58.5-58.7	58.4

Table 4.2: Summary of the phenotypic features of representatives of *Ruegeria scottomollicae* sp. nov., BOX-PCR cluster 1 strains and established *Ruegeria* species (*R. lacuscaerulensis*, *R. pomeroyi*, *R. atlantica*, *R. pelagia* and *R. mobilis*).

The cell morphology of strain LMG 24371 (BOX-PCR cluster 1) and LMG 24367^T (BOX-PCR cluster 2) was determined by transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultra-thin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 4.6). Cells of LMG 24371 are rod-shaped (0.8 x 1.4 µm) with a single polar flagellum. Poly-β-hydroxybutyrate inclusion bodies were observed for only a minority of cells.
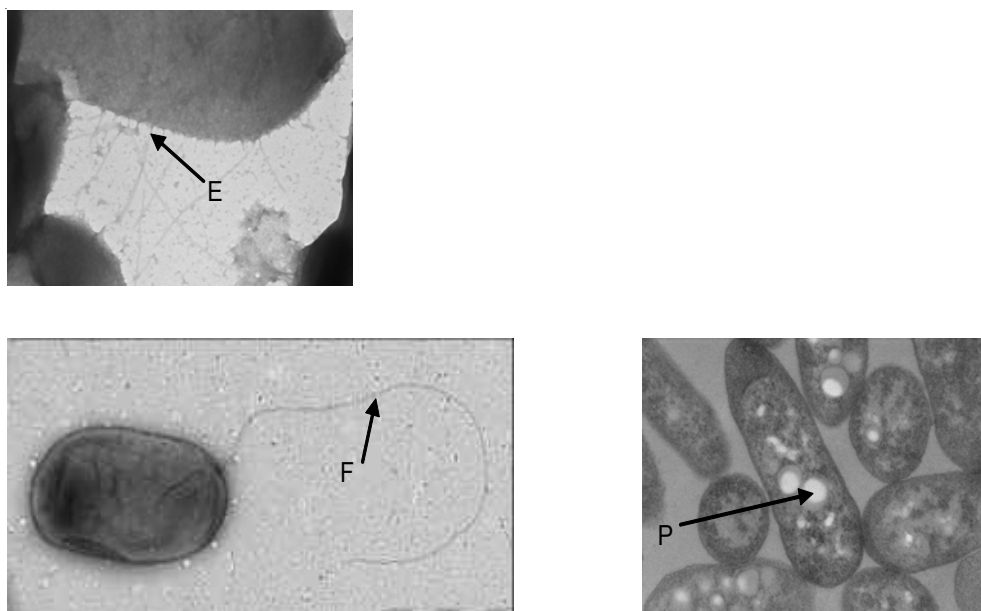
Figure 4.6: Electron micrographs of LMG 24367^T, showing a polar flagellum (F), poly-β-hydroxybutyrate inclusion bodies (P) and fibrillar extensions (E).

In conclusion, BOX-PCR cluster 2 isolates can easily be discriminated from the established *Ruegeria* species on the basis of their phenotypic features. We conclude that BOX-PCR cluster 2 isolates constitute a novel species within the genus *Ruegeria* for which the name *Ruegeria scottomollicae* sp. nov. is proposed, while BOX-PCR cluster 1 isolates were identified as *Ruegeria mobilis*.

Description of *Ruegeria scottomollicae* sp. nov.

(N.L. gen. n. scottomollicae, of Scotto and Mollica, in honour of Dr. Victoria Scotto-Mollica and Dr. Alfonso Mollica who were pioneers in the field of microbial induced corrosion of steels and the generation of electroactive sea water biofilms)

Cells are Gram-negative ovoid rods (1 x 1.3 µm) and are motile by 2 polar flagella. Long slender fibrillar extensions on the cell surface and poly-β-hydroxybutyrate inclusion bodies are observed (Fig. 4.6).

Round, beige colonies with a diameter of 2 mm are formed after 4 days of incubation on MA at 20 °C.

Growth occurs after 2 days at 20°C on MA; after 5 days on TSA. Weak growth on R_2A and NA. No growth on PYG. Temperature range for growth is 4 – 40 °C; growth at 40 °C is weak. No growth at 45 °C or higher. NaCl range for growth is 1 % - 15 %; no growth at 16 % or more. The pH range for growth is 5 - 9 and the optimal pH for growth is 6.5 - 8.5.

Exhibits catalase and oxidase activity. Degradation of tyrosine, Tween 80 and aesculin but not of DNA, starch, chitin, casein or gelatin.

Activity is observed for leucine arylamidase, alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosamidase. Weak activity is detected for the following enzymes: esterase (C4), esterase lipase (C8), α-galactosidase and α-glucosidase. No activity is observed for valine arylamidase, β-glucuronidase, β-glucosidase, α-mannosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, arginine hydrolase, urease, β-galactosidase or α-fucosidase. Nitrate is not reduced to nitrite or to nitrogen.

Indole is not produced and glucose is not fermented. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid.

Susceptible to streptomycin (25 µg) and cefoxitin (30 µg). Intermediately susceptible to erytromycin (15 µg) and gentamicin (30 µg). Resistant to tetracylin (30 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg).

Major fatty acids are $C_{10:0}3$-OH (4.9 $\pm$ 1.0 %), 11-methyl $C_{18:1\omega7c}$ (3.4 $\pm$ 1.3 %), $C_{16:0}$ (1.8 $\pm$ 0.4), $C_{16:0}2$-OH (6.1 $\pm$ 0.7 %), $C_{18:1}2$-OH (4.0 $\pm$ 0.8 %), $C_{18:1\omega7c}$ (73.7 $\pm$ 2.5 %) and unknown fatty acid 11.799 (4.1 $\pm$ 0.7 %). All other fatty acids constitute trace fractions only (< 1 %).

DNA G + C content is 61.0 $\pm$ 0.4 mol%. All 66 isolates were obtained from a marine electroactive biofilm (Genoa, Italy) and the type strain is LMG 24367^T. A second isolate with a different BOX-PCR profile was deposited in the BCCM/LMG collection; LMG 24368 (Fig. 4.4).

Emended description of *Ruegeria mobilis* Muramatsu *et al.* 2007

The description is as given by Muramatsu *et al.* (2007) with the following additions. Susceptible to streptomycin (25 µg) and cefoxitin (30 µg); intermediately susceptible to erythromycin (15 µg). Resistant to trimethoprim (1.25 µg), tetracyclin (30 µg), vancomycin (30 µg), gentamicin (10 µg) and clindamycin (2 µg).

Alkaline phosphatase activity and leucine arylamidase activity is observed. Weak activity of acid phosphatase and naphthol-AS-BI-phosphohydrolase. No activity is observed for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, , β-glucosidase, α-mannosidase or α-fucosidase.

Variable results were obtained for the hydrolysis of DNA and Tween 80 and for the activity of α-glucosidase and N-acetyl-β-glucosaminidase.

Emended description of *Ruegeria lacuscaerulensis* (Petursdottir and Kristjansson 1997) Yi *et al.* 2007

The description is as given by Petursdottir & Kristjansson (1997) and Yi *et al.* (2007) with the following additions. Susceptible to cefoxitin (30 µg); intermediately susceptible to erythromycin (15 µg). Resistant to vancomycin (30 µg), trimethoprim (1.25 µg), clindamycin (2 µg) and gentamicin (10 µg).

Emended description of *Ruegeria atlantica* (Rüger and Höfle 1992) Uchino *et al.* 1999

The description is as given by Rüger & Höfle (1992), Uchino *et al.* (1998), Martens *et al.* (2006), Muramatsu *et al.* (2007) and Yi *et al.* (2007) with the following additions. Susceptible to cefoxitin (30 µg), vancomycin (30 µg) and streptomycin (25 µg); intermediately susceptible to erythromycin (15 µg). Resistant to trimethoprim (1.25 µg), tetracyclin (30 µg), gentamicin (10 µg) and clindamycin (2 µg).

Emended description of *Ruegeria pomeroyi* (González *et al.* 2003) Yi *et al.* 2007

The description is as given by González *et al.* (2003) and Yi *et al.* (2007) with the following additions. Susceptible to cefoxitin (30 µg), erythromycin (15 µg), gentamicin (10 µg) and streptomycin (25 µg); intermediately susceptible to tetracyclin (30 µg). Resistant to trimethoprim (1.25 µg), vancomycin (30 µg) and clindamycin (2 µg).

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4.3

Phaeobacter caerulea sp. nov., a blue-coloured colony forming bacterium isolated from a marine electroactive biofilm

Ilse Vandecandelaere, Eveline Segaert, Alfonso Mollica, Marco Faimali, Peter Vandamme

SUMMARY

Three isolates obtained from a marine electroactive biofilm that was grown on a cathodically polarized electrode were investigated by a polyphasic taxonomic approach. Whole cell fatty acid methyl ester analysis and 16S rRNA gene sequencing identified the isolates as members of the genus *Phaeobacter* of the *Roseobacter* lineage of the class *Alphaproteobacteria*. Both genotypic and phenotypic analyses demonstrated that the three isolates constitute a novel species within the genus *Phaeobacter* for which the name *Phaeobacter caerulea* is proposed. The type strain is LMG 24369^T = CCUG 55859^T with a DNA G + C content of 63.6 mol%.

Martens *et al.* (2006) created the genus *Phaeobacter* to harbour the species *Phaeobacter inhibens* and the misclassified '*Roseobacter gallaeciensis*'. *Phaeobacter inhibens* was isolated from the German Wadden Sea and produces an antibiotic, tropodithietic acid, which exhibits inhibitory effects against various marine bacteria and algae (Brinkhoff *et al.*, 2004, Martens *et al.*, 2007). *Phaeobacter gallaeciensis*, the type species of the genus *Phaeobacter*, was obtained from the larval cultures and the collectors of the scallop *Pecten maximus* (Ruiz-Ponte *et al.*, 1998) whereas *Phaeobacter daeponensis*, was isolated from a tidal flat at Daepo Beach (Yellow Sea, Korea) (Yoon *et al.*, 2007).

The present study was performed during the analysis of the microbial diversity of a marine electroactive biofilm (EA-BIOFILMS-508866 (NEST)), grown on a cathodically polarized stainless steel cathode exposed to natural seawater at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy (Faimali *et al.*, in press, Vandecandelaere *et al.*, in press). The biofilm was removed from the stainless steel cathode by sonication (Branson™ 3200) (90 s) in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated on Marine Agar (MA) (Difco™ 2216) and incubated aerobically at 20 °C for several days. Pure cultures were obtained and the isolates were stored at – 80 °C using MicroBank™ vials.

Whole cell fatty acid methyl ester (FAME) analysis (Mergaert *et al.*, 2001) tentatively identified the three isolates as *Paracoccus denitrificans* (mean identification score of 0.688 $\pm$ 0.034) by comparison of the obtained FAME profiles to a commercial (MIS) database (MIDI, Newark). The major fatty acids were $C_{10:0}$3-OH (2.8 $\pm$ 0.4 %), 11-methyl-$C_{18:1\omega7c}$ (1.3 $\pm$ 0.4 %), $C_{12:0}$3-OH (2.4 $\pm$ 0.2 %), $C_{16:0}$ (4.2 $\pm$ 0.5 %), $C_{16:0}$2-OH (2.4 $\pm$ 0.4 %), $C_{18:0}$ (1.0 $\pm$ 0.1 %), $C_{18:1\omega7c}$ (81.5 $\pm$ 0.8 %) and an unknown fatty acid with an equivalent chain length value of 11.799 (2.9 $\pm$ 0.4 %); the remaining fatty acids constituted only minor fractions (< 1 %).

DNA was extracted according to Pitcher *et al.* (1989). The three isolates were investigated by repetitive DNA-PCR (rep-PCR) fingerprinting using the BOX-A1R-primer (5' CTA CGG CAA GGC GAC GCT GAC G 3') (Rademaker *et al.*, 2000, Versalovic *et al.*, 1994) in order to investigate their genetic diversity. The DNA profiles demonstrate that the isolates represent three genetically distinct strains (Fig. 4.7).

LMG 24369[T]
R-26156
LMG 24370
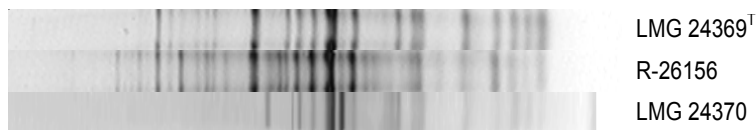
Figure 4.7: The BOX-PCR profiles of the three isolates.

An almost-complete 16S rRNA gene sequences was obtained for strain LMG 24369[T] (1422 bp) using the universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC AGC CG CA 3') (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). A partial 16S rRNA gene sequence (522 bp) was obtained for strain LMG 24370 by using the universal primers pA and pD (5' GTA TTA CCG CGG CTG CTG 3') as described by Coenye *et al.* (1999) (data not shown). The EMBL accession numbers of the 16S rRNA gene sequences of LMG 24369[T] and LMG 24370 are AM943630 and AM943631, respectively. The FASTA software was used to find the most similar sequences in public databases. These sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997). A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using the BioNumerics 4.61 software (Applied Maths, Belgium) (Fig. 4.8). The tree topology was confirmed by maximum likelihood and maximum parsimony analyses (data not shown).

Numerical analysis demonstrated that strains LMG 24369[T] and LMG 24370 shared 99.1 % 16S rRNA gene sequence similarity whereas only 96.1 to 97.6 % 16S rRNA gene similarity was observed between strain LMG 24369[T] and the nearest phylogenetic neighbours (*P. daeponensis*, *P. gallaeciensis* and *P. inhibens*), suggesting that the 3 isolates may represent a novel species within the genus *Phaeobacter* (Stackebrandt & Ebers, 2006) (Fig. 4.8).

1%
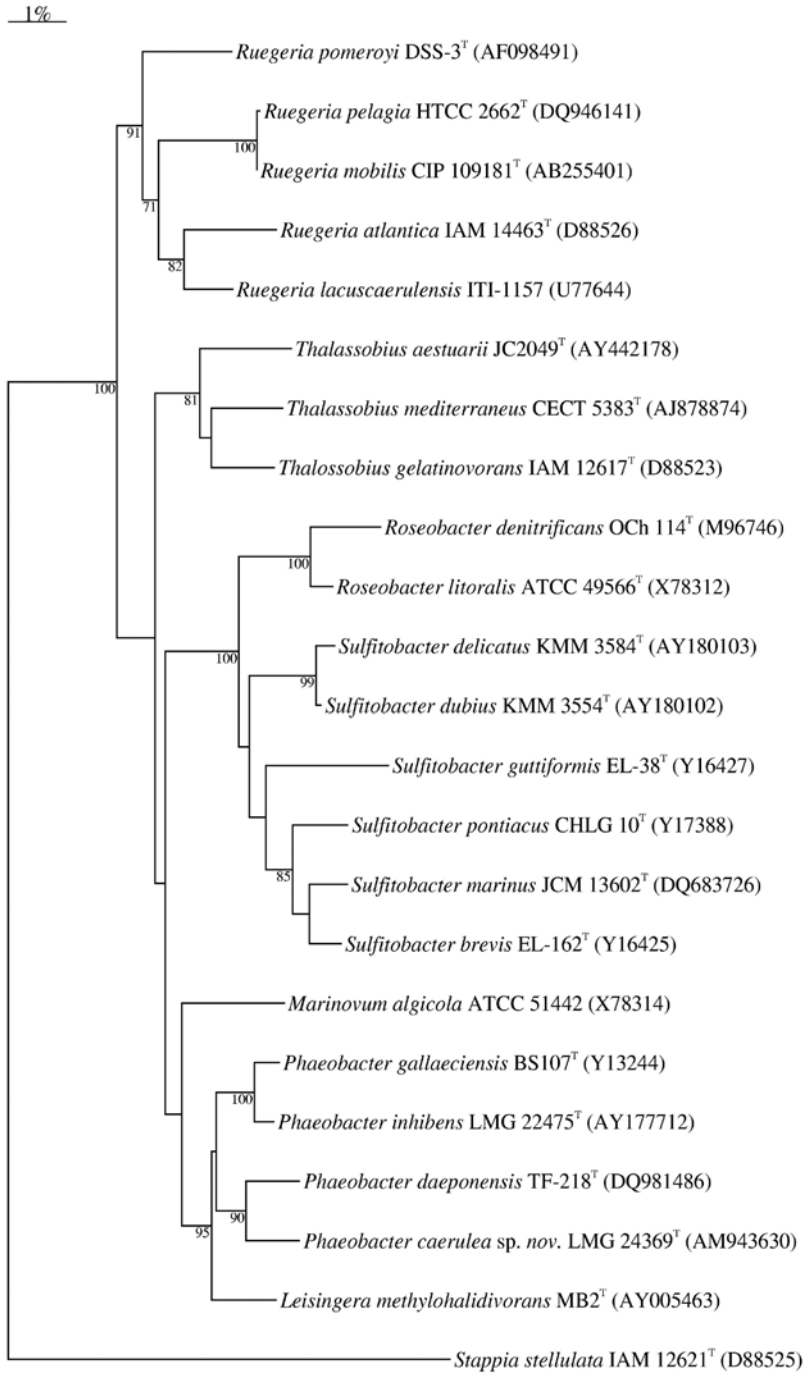
Figure 4.8: Neighbour-joining dendrogram representing the 16S rRNA gene sequence similarities of *Phaeobacter caerulea* sp. nov. and its nearest phylogenetic neighbours. Bootstrap values (1000 replicates) > 70 % are shown.

DNA was enzymatically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Water Breeze HPLC system and Xbridge Shield RP18 column thermostabilised at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G + C content of strain LMG 24369[T] was 63.6 mol%.

DNA-DNA hybridisations experiments were performed to further elucidate the taxonomic position of the strains and were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 45 °C and reciprocal reactions were performed for every pair of strains. Strains LMG 24369[T] and LMG 24370 were chosen on the basis of their distinct BOX-PCR profiles as we looked for the most divergent strains. The DNA-DNA hybridisation values between LMG 24369[T] and LMG 24370 were high (98 $\pm$ 2 %), indicating that the 3 isolates represent a single species. The DNA-DNA hybridisation values between strain LMG 24369[T] and the type strains of its closest phylogenetic neighbours *Phaeobacter inhibens* LMG 22475[T], *Phaeobacter daeponensis* LMG 24139[T], *Phaeobacter gallaeciensis* LMG 23163[T], *Ruegeria atlantica* LMG 23161[T], *Ruegeria lacuscaerulensis* LMG 23162[T], *Ruegeria pomeroyi* LMG 23168[T] and *Leisingera methylohalidovorans* LMG 23656[T] were relatively low, ranging from 18 $\pm$ 4 % to 55 $\pm$ 1 %, indicating that the three isolates represent a novel species.

The following morphological, physiological and biochemical characteristics were evaluated for strains LMG 24369[T] and LMG 24370. Colony morphology was described after 4 days of incubation at 20 °C on MA. Cells were tested for their Gram reaction and the presence of catalase and oxidase activity.

Growth was examined on Nutrient Agar (NA), Trypticase Soy Agar (TSA), R_2A and peptone/yeast extract/glucose agar (PYG) (Tan & Rüger, 1999). The optimal salinity and the optimal growth temperature were determined using respectively R_2A supplemented with 1 % to 20 % NaCl, incubated for 2 weeks at 20 °C and MA incubated at 4 °C to 45 °C for 2 weeks. The effect of pH on the growth was analysed using Marine Broth growth medium (Difco™ 2216) with a pH ranging from 5.0 to 10.0 (at intervals of 0.5 pH units) and incubated at 20°C for 7 days.

Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA (using DNA agar from Difco™ containing 0.01 % Toluidine Blue (Merck)), starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days of incubation at 20 °C. The isolates were inoculated onto Sierra's medium to determine their lipolytic activity and were incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was examined on MA plates using the diffusion disc method: cefoxitin (30 μg), gentamicin (10 μg), erythromycin (15 μg), tetracyclin

(30 µg), streptomycin (25 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg). Results were read after 5 days of incubation at 20 °C.

Biochemical characteristics in the commercial microtest galleries API ZYM and API 20NE were performed according to the manufacturer's instructions. API ZYM was read after 4 h of incubation at 20 °C whereas API 20NE was read after 48 h of incubation at 20 °C.

The cell morphology of strain LMG 24369^T was determined by transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultra-thin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 4.9).
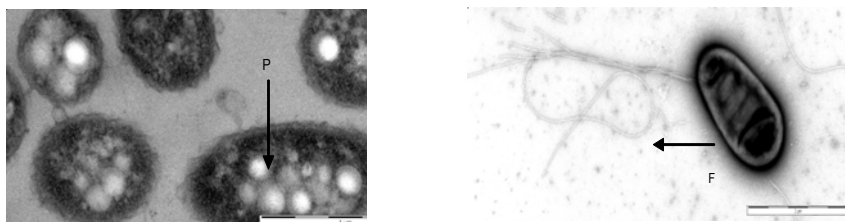
Figure 4.9: Electron micrographs of LMG 24369^T, showing a polar flagellum (F) and poly-β-hydroxybutyrate inclusion bodies (P).

The results of the phenotypic analysis are summarized in Table 4.3. Strains LMG 24369^T and LMG 24370 were easily discriminated from the established *Phaeobacter* species. On the basis of phylogenetic, genomic and phenotypic data, we conclude that the three strains constitute a novel species within the genus *Phaeobacter* for which the name *Phaeobacter caerulea* sp. nov. is proposed.

1: *Phaeobacter caerulea* sp. nov. LMG 24369[T] and LMG 24370; **2**: *Phaeobacter inhibens* LMG 22475[T] (data from Martens *et al.*, 2006); **3**: *Phaeobacter gallaeciensis* LMG 23163[T] (data from Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006); **4**: *Phaeobacter daeponensis* LMG 24139[T] (data from Yoon *et al.*, 2007).

+: Characteristic is present; **-**: Characteristic is absent; **w**: Weak activity is recorded; **i**: Intermediately susceptible to an antibiotic; **ND**: No data available
*: Data from this study

	1*	2	3	4
Origin	Marine EAB, Genoa, Italy	Tidal mud flat, Wadden Sea, Germany	Scallop *Pecten maximus*, Spain	Tidal flat, Yellow Sea, Korea
Colony colour	Blue	Dark brown	Brown	Yellowish white
Growth at:				
4°C	+	+	-	+
40°C	+	-	-	+
5°C	+	-	-	-
Growth in NaCl at:				
1 %	w	+	+	+
2 %	+	+	+	+
7 %	-	+	+	+
10 %	-	-	+	-
Growth on:				
R$_2$A	w	ND	ND	ND
NA	-	-*	ND	w*
TSA	-	w*	ND	w*
Reduction of NO$_3$ to NO$_2$	+	-	-	+
Degradation of:				
L-tyrosine	+	+	-*	+
Starch	-	-	-	-
Tween 80	+	+	-	-
Susceptible to:				
Erythromycin (15 µg)	i	i*	+	i*
Tetracyclin (30 µg)	-	-*	ND	-
Gentamicin (30 µg)	+	-*	+	+
Streptomycin (25 µg)	+	+	+	+
Vancomycin (30 µg)	-	-*	ND	+*
Enzymatic activity *:				
Alkaline phosphatase	w	-	ND	+
Esterase (C 4)	-	-	ND	+
Esterase lipase (C 8)	-	-	ND	+
Valine arylamidase	-	-	ND	-
Acid phosphatase	w	w	ND	+
Naphthol-AS-BI-phosphohydrolase	+	-	ND	-
α-glucosidase	-	w	ND	-
DNA G + C content (mol%)	63.6	55.7	58	64.9

Table 4.3: Summary of the phenotypic characteristics of *Phaeobacter caerulea* sp. nov. and *Phaeobacter* species.

Description of *Phaeobacter caerulea* sp.nov.

Phaeobacter caerulea (cae.ru'le.a. L. fem. adj. *caerulea* dark blue coloured, referring to the colony colour of the isolates)

Cells are Gram-negative rods (0.9 x 1.8 µm) with bundles of polar flagellae. Poly-β-hydroxybutyrate inclusion bodies are observed (Fig. 4.9).

Colonies are round shaped, 2 mm in diameter and blue after 3 days of incubation on MA at 20 °C. The colony surface shows a concentric pattern of dark and bright blue circles. The colony colour becomes darker as the incubation time extends.

Growth occurs on MA after 2 days at 20 °C. Weak growth on R_2A after 5 days of incubation at 20 °C but no growth on NA, PYG and TSA. Temperature range for growth is 4 °C to 45 °C and NaCl range for growth is 2 – 5 %. The pH range for growth is 6.0 - 9.0 and the optimal pH for growth is 6.5 - 8.0.

Catalase and oxidase positive. Degradation of L-tyrosine, DNA, Tween 80 and aesculin but no degradation of gelatin, starch, casein or chitin.

Activity was recorded for leucine arylamidase and naphthol-AS-BI-phosphohydrolase and weak activities were detected for alkaline phosphatase and acid phosphatase. No activity was observed for the presence of esterase (C4), esterase lipase (C8), valine arylamidase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosamidase, α-mannosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, arginine hydrolase, urease and α-fucosidase.

Nitrate is reduced to nitrite. Indole is not produced and glucose is not fermented. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid. Susceptible to gentamicin (30 µg), streptomycin (25 µg) and cefoxitin (30 µg) and intermediate susceptible to erythromycin (15 µg). Resistant to tetracyclin (30 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg).

The major fatty acids are $C_{10:0}$3-OH (2.8 $\pm$ 0.4 %), 11-methyl-$C_{18:1\omega7c}$ (1.3 $\pm$ 0.4 %), $C_{12:0}$3-OH (2.4 $\pm$ 0.2 %), $C_{16:0}$ (4.2 $\pm$ 0.5 %), $C_{16:0}$2-OH (2.4 $\pm$ 0.4 %), $C_{18:0}$ (1.0 $\pm$ 0.1 %), $C_{18:1\omega7c}$ (81.5 $\pm$ 0.8 %) and an unknown fatty acid with an equivalent chain length value of 11.799 (2.9 $\pm$ 0.4 %); the remaining fatty acids constituted only minor fractions (< 1 %).

All three strains were isolated from a marine electroactive biofilm (Genoa, Italy). The type strain is LMG 24369[T] with a DNA G + C content of 63.6 mol %.

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4.4

Nautella italica gen. nov., sp. nov., isolated from a marine electroactive biofilm

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SUMMARY

Five isolates obtained from a marine electroactive biofilm grown on a stainless steel cathode were investigated by a polyphasic taxonomic approach.

Whole cell fatty acid methyl ester analysis and 16S rRNA gene sequence analysis showed that the isolates belonged to the *Roseobacter* lineage of the *Alphaproteobacteria*.

Both phenotypic and genotypic analyses demonstrated that the 5 isolates formed a single species, which did not represent an established member of the *Roseobacter* lineage. Therefore, we propose to classify the 5 isolates into a novel species of a new genus for which the name *Nautella italica* gen. nov., sp. nov. is proposed. The type strain of *Nautella ica* gen. nov., sp. nov. is LMG 24365^T = CCUG 55857^T; its DNA G + C content is 61 mol%.

The *Roseobacter* lineage of the *Alphaproteobacteria* is one of the most dominant marine bacterial groups representing up to 20 % of coastal and up to 15 % of mixed-layer bacterioplankton populations (González & Moran, 1997, González *et al.*, 2000, Selje *et al.*, 2004, Suzuki *et al.*, 2001). Members of the *Roseobacter* lineage are found in almost every marine environment i.e. from coastal to open ocean seawater and from sea ice to sea floor sediments (e.g. Brinkmeyer *et al.* (2003), Gonzàlez & Moran (1997), Inagaki *et al.* (2003), Selje *et al.* (2004)). They exhibit a diverse range of metabolic and physiological characteristics including aerobic anoxygenic phototrophy (Shiba, 1991), sulphur transformations (Moran *et al.*, 2003), carbon monoxide oxidation (King, 2003, Moran *et al.*, 2004) and aromatic compound degradation (Buchan *et al.*, 2000, González *et al.*, 1996). Several studies indicated an important role of roseobacters in marine biofilm formation (Dang & Lovell, 2000) where they use the production of antibiotics and secondary metabolites to outcompete other bacteria (Martens *et al.*, 2007).

The present study was part of an analysis of the microbial diversity of a marine electroactive biofilm, grown on a stainless steel cathode exposed to natural seawater at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy (Faimali *et al.*, in press, Vandecandelaere *et al.*, in press). The biofilm was removed from the stainless steel cathode by sonication (Branson™ 3200) (90 s) in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated onto Marine Agar (MA) (Difco™ 2216) and incubated aerobically at 20 °C for several days. Pure cultures were stored at – 80 °C using MicroBank™ vials.

All isolates were investigated by whole cell fatty acid methyl ester (FAME) analysis as described by Mergaert *et al.* (2001). Comparison of the FAME profiles of the 5 isolates of the present study to a commercial (MIS) database tentatively identified them as *Paracoccus* sp. with low to moderate identification scores (0.192 to 0.361). The dominant fatty acids were $C_{10:0}$3-OH (3.2 $\pm$ 0.4 %), 11-methyl-$C_{18:1\omega7c}$ (4.5 $\pm$ 2.5 %), $C_{12:0}$3-OH (2.4 $\pm$ 0.3 %), $C_{16:0}$ (1.8 $\pm$ 0.3 %), $C_{16:0}$2-OH (5.5 $\pm$ 0.7 %), $C_{18:0}$ (1.4 $\pm$ 0.3 %), $C_{18:0}$12-OH (2.6 $\pm$ 0.6 %), $C_{18:1\omega7c}$ (74.5 $\pm$ 3.6 %) and an unknown fatty acid with an equivalent chain length value of 11.799 (3.6 $\pm$ 0.4 %); the remaining fatty acids constitute minor fractions only (< 1.0 %).

DNA was extracted according to Pitcher *et al.* (1989). Almost-complete 16S rRNA gene sequences (1425-1432 bp) were obtained for the isolates LMG 24364, LMG 24365^T, R-28717, R-28753 and R-25532 using the universal primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA 3') (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). The EMBL accession numbers of the 16S rRNA gene sequences of LMG 24365^T, LMG 24364, R-25532, R-28717 and R-28753 are AM904562, AM904563, AM944520, AM944521 and AM944522, respectively. The FASTA software was used to find the most similar sequences in public databases. The sequences were aligned

using CLUSTAL_X (Thompson *et al.*, 1997). A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using the BioNumerics 4.61 software (Fig. 4.10). The tree topology was confirmed by maximum parsimony and maximum likelihood analyses (data not shown). This numerical analysis showed that the isolates LMG 24365[T], LMG 24364, R-28753, R-28717 and R-25532 had 100 % 16S rRNA gene sequence similarity. The established species of the genus *Phaeobacter* (*Phaeobacter inhibens*, *Phaeobacter daeponensis* and *Phaeobacter gallaeciensis*) showed to be their nearest phylogenetic neighbours, although with relatively low 16S rRNA gene sequence similarities (96.6 % to 97.2 %).

1%

Pseudoruegeria aquimaris KCTC 12737[T] (DQ675021)

Ruegeria pomeroyi DSM 15171[T] (AF098491)

Ruegeria pelagia HTCC 2663 (DQ916141)

Ruegeria mobilis NBRC 101030[T] (AB255401)

Ruegeria atlantica ATCC 700000[T] (D88526)

Ruegeria lacuscaerulensis DSM 11314 (U77644)

Thalassobacter stenotrophicus CECT 5294[T] (AJ631302)

Nereida ignava 2SM4[T] (AJ748748)

Sulfitobacter pontiacus LMG 19752[T] (Y17388)

Roseobacter denitrificans LMG 19751[T] (M96746)

Roseobacter litoralis LMG 19750[T] (X78312)

Thalassobius aestuarii DSM 15283[T] (AY442178)

Thalassobius mediterraneus XSM19[T] (AJ878874)

Thalossobius gelatinovorans LMG 8174[T] (D88523)

Nautella italica gen. nov., sp. nov. LMG 24365[T] (AM904562)

Nautella italica gen. nov., sp. nov. LMG 24364 (AM904563)

Nautella italica gen. nov., sp. nov. R-28753 (AM944522)

Nautella italica gen. nov., sp. nov. R-25532 (AM944520)

Nautella italica gen. nov., sp. nov. R-28717 (AM944521)

Ruegeria algicola ATCC 51442 (X78314)

Leisingera methylohalidivorans DSM 14336[T] (AY005463)

Phaeobacter daeponensis TF-218 (DQ981486)

Phaeobacter gallaeciensis LMG 19757[T] (Y13244)

Phaeobacter inhibens LMG 22475[T] (AY177712)

Shimia marina JCM 13038[T] (AY962292)

Stappia stellulata LMG 8178[T] (D88525)

Figure 4.10: Neighbour-joining dendrogram representing the 16S rRNA gene sequence similarities of *Nautella italica* gen. nov., sp. nov. and its nearest phylogenetic neighbours. Bootstrap values (1000 replicates) > 70 % are shown.

DNA-DNA hybridisation experiments were performed with isolates LMG 24365^T, LMG 24364 and representatives of their phylogenetic nearest neighbours using photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), and a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 45 °C and reciprocal reactions were performed for every pair of strains. The DNA-DNA hybridisation values between LMG 24365^T and LMG 24364 were high (88 $\pm$ 5 %). DNA-DNA hybridisation values between LMG 24365^T and the type strains of *P. gallaeciensis*, *P. daeponensis* and *P. inhibens* were low, ranging from 7 $\pm$ 2 % to 17 $\pm$ 0 %. These data demonstrate that the 5 isolates constitute a novel species, which is best allocated into a novel genus within the *Roseobacter* lineage of *Alphaproteobacteria* (Fig. 4.10).

The genetic diversity of the 5 isolates was examined by repetitive DNA-PCR (rep-PCR) fingerprinting using the BOX-A1R-primer (5' CTA CGG CAA GGC GAC GCT GAC G 3') (Rademaker & De Bruijn, 1997, Versalovic *et al.*, 1994). Differences between BOX-PCR fingerprints of all 5 isolates were observed, indicating the presence of genetically distinct strains (Fig. 4.11).

LMG 24365^T
R-28717
R-28753
R-25532
LMG 24364
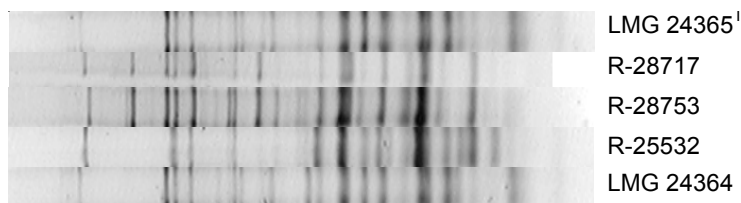
Figure 4.11: The BOX-PCR fingerprints of the five strains of *Nautella italica* gen. nov., sp. nov.

DNA was subsequently enzymatically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Water Breeze HPLC system and Xbridge Shield RP18 column thermostabilised at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G + C content of LMG 24365^T and LMG 24364 was 61.0 $\pm$ 0.8 %.

The following morphological, physiological and biochemical characteristics were determined for strains LMG 24365^T and LMG 24364. Colony morphology was described after 4 days of incubation at 20 °C on MA. Cells were tested for their Gram reaction, catalase and oxidase activity. Growth was verified on Nutrient Agar (NA), Trypticase Soy Agar (TSA), R$_2$A and peptone/yeast extract/glucose agar (PYG) (Tan & Rüger, 1999). The optimal salinity and the optimal growth temperature were determined using R$_2$A supplemented with 1 % to 20 % NaCl and incubated for 2 weeks at 20 °C, and MA incubated at 4 °C to 45 °C for 2 weeks,

respectively. The effect of pH on the growth was analysed using Marine Broth growth medium (Difco™ 2216) with of a pH ranging from 5.0 to 10.0 (at intervals of 0.5 pH units) and incubated at 20 °C for 7 days.

Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA (using DNA agar (Difco™), containing 0.01 % Toluidine Blue (Merck)), starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days of incubation at 20 °C. The isolates were inoculated onto Sierra's medium to determine their lipolytic activity and were incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was tested on MA plates using the diffusion disc method: cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg), streptomycin (25 µg), tetracyclin (30 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg). Results were read after 5 days of incubation at 20 °C.

Biochemical characteristics in commercial microtest galleries (API ZYM and API 20NE) were assessed according to the manufacturer's instructions. API ZYM was read after 4 h of incubation at 20 °C whereas API 20NE was read after 48 h of incubation at 20 °C.

The cell morphology of strain LMG 24365[T] was determined by transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultra-thin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 4.12).

Figure 4.12: Electron micrographs of LMG 24365[T], showing a polar flagellum (F) and poly-β-hydroxybutyrate inclusion bodies (P).

The results of the phenotypic tests are summarized in Table 4.4. In conclusion, we demonstrated that the five isolates represent genetically different strains constituting a novel species within a novel genus for which we propose the name *Nautella italica* gen. nov., sp. nov.

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1: *Nautella italica* gen. nov., sp. nov. LMG 24365[T] and LMG 24364; **2**: *Phaeobacter inhibens* LMG 22475[T] (data from Martens *et al.*, 2006 and this study); **3**: *Phaeobacter gallaeciensis* LMG 23163[T] (data from Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006 and this study); **4**: *Phaeobacter daeponensis* LMG 24139[T] (data from Yoon *et al.*, 2007 and this study).

+: Characteristic is present; **-**: Characteristic is absent; **w**: Weak activity was recorded; **ND**: No data available; *: Data from this study

	1*	2	3	4
Origin	Marine EAB, Genova, Italy	Tidal mud flat, Wadden Sea, Germany	Scallop '*Pecten maximus*', Spain	Tidal flat, Yellow Sea, Korea
Colony colour	Beige	Dark brown	Brown	Yellowish-white
Growth at				
4°C	w	+	-	+
40°C	+	-	-	+
45°C	+	-	-	-
Growth in NaCl at				
1%	+	+	+	+
2%	+	+	+	+
7%	w	+	+	+
10%	-	-	+	-
Growth on				
NA	-	-*	ND	w*
TSA	-	w*	ND	w*
Degradation of				
L-tyrosine	-	+*	-*	+
DNA	-	-*	-	-*
Tween 80	+	+	-	-
Aesculin	+	-*	-	-
Gelatin	-	-	-	-
Reduction of NO_3 to NO_2	-	-	-	+
Susceptible to				
Erythromycin (15 µg)	+	i*	+	i*
Tetracyclin (30 µg)	i	i*	ND	-
Gentamycin (30 µg)	-	-*	+	+
Streptomycin (25 µg)	+	+	+	+
Vancomycin (30 µg)	-	-	ND	+
Enzymatic activity				
Alkaline phosphatase	+	-*	ND	+
Esterase (C4)	-	-*	ND	+
Esterase lipase (C8)	w	-*	ND	+
Valine arylamidase	w	-*	ND	-
Acid phosphatase	+	w*	ND	+
Naphthol-AS-Bl-phosphohydrolase	+	-*	ND	-
α-galactosidase	w	-*	ND	-
β-glucuronidase	v	-*	ND	-
α-glucosidase	+	w*	ND	-
β-glucosidase	w	-*	ND	-
DNA G + C content (mol%)	61.0 ± 0.8	55.7	58	64.9

Table 4.4: Summary of the phenotypic characteristics of *Nautella italica* gen. nov., sp. nov. and of the established species of the genus *Phaeobacter*.

Description of *Nautella* gen. nov.

Nautella (Gr. n. nautes, seaman; L. dim. suf. –ella; N. L. fem. n. *Nautella* referring to the marine habitat of this novel bacterial genus)

Gram-negative, motile rods which are moderately halophilic and strictly aerobic. Catalase and oxidase tests are positive. Temperature range for growth is 4 °C to 45 °C. The type species is *Nautella italica* gen. nov., sp. nov.

Pending on the isolation and characterization of additional species, the description of *Nautella* gen. nov. is as given below for the type species *Nautella italica* gen. nov., sp. nov.

Description of *Nautella italica* gen. nov., sp. nov.

Nautella italica (i.ta'li.ca. L. fem. adj. *italica* from Italy, where this species was first isolated)

Cells are Gram-negative rods (0.9 x 1.8 μm) that are motile by a single polar flagellum. Poly-β-hydroxybutyrate inclusion bodies are observed (Fig. 4.12).

The colonies are beige, round-shaped and 1 to 2 mm in diameter after 3 days of incubation on MA at 20 °C. The colony surface is smooth and convex with entire margins.

Growth occurs after 2 days at 20 °C on MA, but not on TSA, R_2A, NA or PYG. Temperature range for growth is 4 °C to 45 °C (only weak growth at 4 °C). Moderately halophilic (1 to 5 % NaCl); only weak growth in the presence of 7 % NaCl and no growth in the absence of NaCl. The pH range for growth is 5.5 - 9 and the optimal pH for growth is 6.5 - 8.0.

Catalase and oxidase tests are positive. Degradation of Tween 80 and aesculin but not of L-tyrosine, DNA, starch, chitin, gelatin or casein.

Activity was recorded for leucine arylamidase, alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Weak enzymatic activity was observed for esterase lipase (C8), valine arylamidase, α-galactosidase and β-glucosidase. Variable results were obtained for the presence of the β-glucuronidase enzyme. No activity was detected for esterase (C4), N-acetyl-β-glucosamidase, α-mannosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, arginine hydrolase, urease and α-fucosidase.

Nitrate is not reduced to nitrite. Indol is not produced and glucose is not fermented. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid.

Susceptible to cefoxitin (30 µg), erythromycin (15 µg) and streptomycin (25 µg). Intermediately susceptible to tetracyclin (30 µg). Resistant to gentamicin (30 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg).

The dominant fatty acids are $C_{10:0}$3-OH (3.2 ± 0.4 %), 11-methyl-$C_{18:1\omega7c}$ (4.5 ± 2.5 %), $C_{12:0}$3-OH (2.4 ± 0.3 %), $C_{16:0}$ (1.8 ± 0.3 %), $C_{16:0}$2-OH (5.5 ± 0.7 %), $C_{18:0}$ (1.4 ± 0.3 %), $C_{18:0}$12-OH (2.6 ± 0.6 %), $C_{18:1\omega7c}$ (74.5 ± 3.6 %) and an unknown fatty acid with an equivalent chain length value of 11.799 (3.6 ± 0.4 %); the remaining fatty acids constitute only minor fractions (< 1.0 %).

The DNA G + G content is 61.0 ± 0.8 %. All 5 strains were obtained from a marine electroactive biofilm (Genoa, Italy) and the type strain is LMG 24365[T] = CCUG 55857[T].

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PART IV

GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSIONS

CHAPTER 5: GENERAL DISCUSSION AND PERSPECTIVES

At present, the world energy consumption is immense and is predicted to increase even more. Currently, the main energy sources are fossil fuels but their combustion contributes to the global climate change due to the discharge of large amounts of carbon dioxide into the atmosphere. Therefore, the search and development of green and renewable energy sources is of utmost importance. One of the possible solutions is energy production by microbial fuel cells (MFCs) in which bacteria, forming electroactive biofilms (EABs) on the electrode surface, produce electricity.

5.1 THE MICROBIAL DIVERSITY OF A MARINE ELECTROACTIVE BIOFILM

Several studies already investigated the microbial populations of benthic MFCs (Chapter 1) but the bacterial population of an aerobic marine stainless steel EAB was never characterized before. The EAB was generated by placing a cathodically polarized stainless steel electrode (-200 mV Ag/AgCl) in natural aerated seawater in the harbour of Genoa (Italy) (Faimali *et al.*, unpublished). Biofilm formation was observed (Addendum 2) and the electrical current increased and stabilized at a maximal value of 0.5 A / m² in less than 10 days (Addendum 2). The cathodes were disconnected from the electrical circuits after 14 days and the formed EABs were analysed by culture dependent and culture independent analyses as the application of both approaches is the best way to reveal the diversity of a bacterial population (Chapter 1.3).

The EAB was removed from the cathode by sonication and multiple dilutions (10^0 to 10^{-6}) of the cell suspensions were inoculated onto three different growth media (Trypticase Soy Agar, R_2A and Marine Agar) at 20°C. Three hundred and fifty six isolates, representing different morphotypes on the initial isolation plates, were picked up (Chapter 2). Thus, the sampled diversity among the isolates was not completely proportional to the observed diversity on the primary isolation plates as we aimed to obtain isolates that represent as many different morphotypes as possible.

The aim of this study was to identify the general microbial population of the marine electroactive biofilms. Therefore, we applied three general isolation media. The use of these three media resulted in the isolation of phylogenetically different bacteria. So, the three media were appropriate to fulfil the aim of the study. In addition, several isolates demonstrated to have electrochemical properties (Chapter 2). However, if we want to isolate and enrich electrochemically active bacteria, other media can be used. For instance, attempts can be made to mimic as much as possible the conditions in which the biofilm was grown. Therefore, we can dip the cathode in a general isolation medium to which the natural seawater containing the common bacterial population and subsequently, we can put a potential of -200 mV Ag/

AgCl on the cathode. Probably, this would results in the enrichment and isolation of electrochemically active bacteria.

The cultivable fraction of the marine EAB included members of the classes *Alpha-* (the genera *Phaeobacter*, *Ruegeria* and *Leisingera*) and *Gammaproteobacteria* (the genera *Alteromonas*, *Pseudoalteromonas*, *Acinetobacter*, *Marinobacter*, *Idiomarina* and *Halomonas*), the phyla *Firmicutes* (the genera *Bacillus*, *Staphylococcus* and *Exiguobacterium*) and *Actinobacteria* (the genera *Arthrobacter* and *Frigoribacterium*) and the family *Flavobacteriaceae* (the genera *Maribacter* and *Winogradskyella*) (Chapter 2, Addendum 1). A substantial fraction (40 %) of the isolates were Gram-positive bacteria. Although Gram-negative bacteria constitute the major bacterial fraction in seawater (Jensen & Fenical, 1995), Gram-positive bacteria are also indigenously present in marine environments such as sediments (Gontang *et al.*, 2007), surface microlayers (Agogué *et al.*, 2005), coastal sediments (Köpke *et al.*, 2005) and near-shore tropical regions (Jensen & Fenical, 1995). Interestingly, Gram-positive bacteria are more abundant in polluted coastal ecosystems (Agogué *et al.*, 2005) such as harbour water.

The phylogenetic diversity of the EAB investigated was high, which contrasted with the microbial population of EABs reported in literature. For instance, members of the class *Deltaproteobacteria* (and more especially members of the family *Geobacteraceae*) dominated the microbial biofilm populations of anodes buried in marine sediments (Bond *et al.*, 2002, Holmes *et al.*, 2004, Tender *et al.*, 2002). As members of the family *Geobacteraceae* are anaerobic bacteria that oxidize organic matter in the sediment and transfer the obtained electrons to the anode, it is logical that they were not detected in the cathodic, aerobic biofilm of the present study. At present, only one study analysed the microbial populations of cathodes exposed to aerobic seawater (although on carbon electrodes instead of stainless steel electrodes) and reported the dominance of *Alpha-* and *Gammaproteobacteria* in the bacterial community (Holmes *et al.*, 2004). In the present study, the latter taxa also represent the largest fractions among the isolates.

Denaturating gradient gel electrophoresis of the PCR amplified V_3 regions of the bulk 16S rRNA genes demonstrated that the dominant bacterial populations of the EAB and of the surrounding seawater were highly similar, demonstrating that the bacterial EAB population and the seawater comprised common marine organisms in similar ratios (Chapter 2, Fig. 2.3). The identification results of the culture dependent analyses indeed confirmed that the biofilm primarily consisted of common marine bacteria. All *Alphaproteobacteria* (n=91 isolates; 26 %) were identified as members of the *Roseobacter* lineage which is one of the most abundant marine bacterial groups (Buchan *et al.*, 2005, Wagner-Dobler & Biebl, 2006) and which is found in almost every marine environment (Buchan *et al.*, 2005, González & Moran, 1997, González *et al.*, 2000, Selje *et al.*, 2004, Suzuki *et al.*, 2001). Quite a large fraction of

our isolates (n = 115; 32 %) were identified as members of the class *Gammaproteobacteria*, which was also not surprising as members of this class represent a large phylogenetic group that is globally distributed and easily cultivated (Agogué *et al.*, 2005). The gammaproteobacterial isolates in the present study were mainly identified as *Pseudoalteromonas* and *Alteromonas* species, which are common marine organisms (Baumann *et al.*, 1972, Eilers *et al.*, 2000, Romanenko *et al.*, 2003, Van Trappen *et al.*, 2004a). Another large fraction of our isolates (n = 50; 14 %) was identified as members of different genera of the phylum *Actinobacteria* (Addendum 1). Several studies reported the presence of members of the phylum *Actinobacteria* in a variety of marine environments such as sea surface microlayers (Agogué *et al.*, 2005), the water column (Venter *et al.*, 2004) and marine sediments (Gontang *et al.*, 2007). In addition, *Actinobacteria* were also already detected in marine environments by using culture independent techniques (Ward & Bora, 2006). Similarly, the presence of members of the phylum *Firmicutes* among our isolates (n = 76; 21 %) was not unusual as they have been detected before in marine sediments (Gontang *et al.*, 2007), the water column (Siefert *et al.*, 2000, Venter *et al.*, 2004) and coastal subsurface sediments (Köpke *et al.*, 2005). Finally, the detection of members of the family *Flavobacteriaceae* among our isolates (n = 10; 3 %) was not surprising as representatives of the *Flavobacteriaceae* too are common marine organisms (Bernardet *et al.*, 2002, Van Trappen *et al.*, 2003, Van Trappen *et al.*, 2004b, Vela *et al.*, 2007, Yi *et al.*, 2005). All the identification results mentioned above were obtained by FAME and subsequent 16S rRNA gene sequence analyses of representative isolates (96 %). However, fourteen isolates (4 %) displayed unique FAME profiles and thus grouped separately at the 70 % profile similarity level. These isolates were only tentatively identified by FAME analysis and also represented different phylogenetic groups (Chapter 2, Addendum 1).

Previous studies reported that the microbial populations of different MFCs were phylogenetically less diverse than those of the initial inoculum or the control electrodes. Bacterial communities on current harvesting electrodes are therefore considered enriched with specific bacteria (Jong *et al.*, 2006, Kim *et al.*, 2004, Kim *et al.*, 2006, Lee *et al.*, 2003). In contrast, the marine EAB population of the present study was diverse and not enriched when compared to the surrounding seawater. This may be explained by the growth time of the present biofilm (14 days) which is definitely lower compared to the ones of the EABs described previously which range from a few months to several years (Kim *et al.*, 2004, Moon *et al.*, 2006). It is unclear if, and if so, when the community structure of the present EAB becomes stable. Although the electrical current is stable after 10 days (Addendum 2), confocal laser scanning microscopy demonstrated that the biofilm structure is still developing. Typical microcolonies are already formed but some parts of the biofilm are only constituted of thin cell layers (Addendum 2) (Faimali *et al.*, unpublished).

Future research of the present marine EAB should address several outstanding issues. The study of its population dynamics should reveal whether prolonged exposure of the cathode to seawater will result in the enrichment of specific bacterial groups which may or may not contribute to electroactivity. If extended incubation time results in an enrichment of specific bacterial groups while electrical current production remains stable, it will be easier to study and to determine which members of the community are responsible for electrical current production and which mechanisms are used for electricity production. On the other hand, if the EAB population remains as phylogenetically diverse as observed in the present study, this may indicate that the electrochemical features of this EAB involves the action of many bacteria. Some recent reports seem to confirm the latter hypothesis as it was observed that the diversity among electrochemical active bacterial populations was larger than initially expected (Logan & Regan, 2006). In addition, the inoculation of MFCs with a variety of samples such as wastewater, sediments and sludge resulted in electrical current production indicating that electrochemically active bacteria are widely distributed (Logan & Regan, 2006). As FAME cluster IV bacteria represented the most abundant taxon among the isolates, representatives were examined by cyclic voltammetry including *Nautella italica* gen. nov., sp. nov. LMG 24364, *Ruegeria scottomollicae* sp. nov. R-28704, *Phaeobacter caerulea* sp. nov. R-26156, *Ruegeria pelagia* R-26162 and two unidentified members of the *Alphaproteobacteria* R-26165 and R-28809 (Addendum 1). These bacteria all belong to the *Roseobacter* lineage of which members exhibit a whole range of metabolic features that may play a role in electrochemical activity e.g. carbon monoxide and sulphur transformations (Buchan *et al.*, 2005). Also, *Halomonas aquamarina* R-28817 (*Gammaproteobacteria*) was examined (Addendum 1). All seven isolates efficiently catalysed the reduction of oxygen as revealed by cyclic voltammetry on glassy carbon electrodes (Parot *et al.*, in press). In fact, these results indicated that electrochemically activity is present among different species of different genera. So, it is not unlikely that several members of different bacterial groups may contribute to the electrochemical properties of the present biofilm. In order to confirm this hypothesis, intensive electrochemical screening of the obtained isolates should be performed in the future. In addition, the mechanisms of electricity production by this EAB are not yet revealed. Preliminary studies of Faimali *et al.* (in press) and Parot *et al.* (in press) suggested a role of enzymes in electroactivity as determined by the use of specific enzyme inhibitors and cyclic voltammetric studies, respectively. They hypothesized that the biofilm bacteria release redox proteins that are entrapped in the extracellular matrix and these proteins may play a role in electron transfer. Further studies such as proteomic analysis of the EAB will help to elucidate the mechanisms beyond electrochemical activity.

Although many aspects about the present EAB are yet to be resolved, this type of EAB is a good candidate for electricity production. Relatively easy inoculation and set-up provide a major advantage compared to benthic MFCs. In addition, if further tests demonstrate that members of the *Roseobacter* lineage are indeed electrochemically active, one can imagine

the electrical current production by the metabolically versatile roseobacters in polluted marine environments (Chapter 1.2.5).

In conclusion, the present study demonstrated that the dominant bacterial fractions of the 14 days old marine EAB does not differ from those of the surrounding seawater. Yet already at this 'age' a clear and maximal electrochemical effect is observed. Preliminary experiments by Parot *et al.* (in press) demonstrated that a range of bacteria might contribute to the electrochemical characteristics of this EAB.

5.2 EVALUATION OF THE METHODS USED TO STUDY THE BACTERIAL POPULATION

The bacterial diversity of the marine EAB was studied by a polyphasic taxonomic approach. All 356 isolates were investigated by whole cell fatty acid methyl ester (FAME) analysis while 16S rRNA gene sequence analysis was performed on representative isolates of the FAME clusters. Two FAME clusters (I and IV) were investigated in more detail by BOX-PCR fingerprinting, DNA-DNA hybridisation experiments and phenotypic tests. In addition, a clone library was constructed and denaturing gradient gel electrophoresis of PCR amplified V_3 regions of bulk 16S rRNA genes was performed in order to study the bacterial diversity in a culture independent way.

During this study, FAME analysis and BOX-PCR analysis were the key methods to investigate the bacterial population of the marine EAB and are discussed below. The polyphasic identification strategy entailed the detection and description of several novel marine bacteria.

5.2.1 Whole cell fatty acid methyl ester analysis as a primary tool to analyse the diversity of a large number of isolates

All 356 isolates were first examined for their FAME composition. FAME analysis is a rapid and relatively inexpensive standardized screening tool that allows to group isolates in clusters of strains with similar FAME profiles. Typically, strains within such a FAME cluster belong to closely related species. In addition, the comparison of FAME profiles to a commercial database (MIDI, Newark) tentatively identifies the isolates examined (Vandamme *et al.*, 1996).

During the present study, similar FAME profiles were grouped on the basis of at least 70 % profile similarity (relying on the Bray-Curtis coefficient). The Bray-Curtis coefficient offers two main advantages for the analysis of FAME profiles of a large set of isolates. First, the

exclusion or inclusion of characters (i.e. a given fatty acid), which are not present in either sample, does not affect the resemblance between two samples ("Independence of joint absence"). In addition, the inclusion of an additional sample (i.e. an isolate) in the analysis does not affect the resemblance between existing samples ("Localisation") (Clarke *et al.*, 2006). Numerical analysis delineated twenty FAME clusters, comprising 2 to 91 isolates, whereas fourteen isolates grouped separately at the 70 % profile similarity level and thus formed separate branches. Comparison of the obtained FAME profiles to the TSBA50 (5.00) database (MIDI, Newark) yielded an approximate identification and the isolates were tentatively identified as members of different phylogenetic groups (Chapter 5.1). The identification scores were variable within the FAME clusters and the standard deviation values were relatively high (Addendum 1, Chapter 2).

At least one isolate per FAME cluster was selected for 16S rRNA gene sequence analysis in order to obtain a more accurate identification. The results of the 16S rRNA gene sequence and FAME analyses correlated well at the genus level for 8 FAME clusters (28 % of all isolates) whereas the identification results of the remaining FAME clusters only correlated at higher taxonomic levels. In general, when low FAME identification scores (< 0.400) were obtained, genus level identification by FAME was not reliable. In contrast, when FAME identification scores were higher, genus level identification of FAME analysis and 16S rRNA gene sequence analysis were congruent (e.g. FAME cluster VII Addendum 1). As discussed in Chapter 2, several reasons may account for the discrepancy between FAME and 16S rRNA gene sequence analyses identification results.

The FAME clusters were delineated on the basis of at least 70 % profile similarity but the FAME profile similarity within the FAME clusters ranged from 71.6 % (FAME cluster I) to 98.5 % (FAME cluster XX) (Addendum 1). Typically, the higher the profile similarities within a FAME cluster, the more phylogenetically coherent the clusters were. For instance, isolates of FAME cluster XX, exhibiting 98.5 % profile similarity, belonged to a single species (*Halomonas aquamarina*). In addition, the profile similarity within one species of a FAME cluster was high. For instance, the strains of *Nautella italica* gen. nov., sp. nov. exhibited high profile similarities (> 90 %). In contrast, when the FAME profile similarities within a FAME cluster were low, the isolates were phylogenetically diverse. For instance, the isolates of FAME cluster I (71.6 % profile similarity) and FAME cluster IV (76.3 %) belonged to different genera within the class of *Gamma-* and *Alphaproteobacteria*, respectively.

The identification scores, obtained by comparison of the profiles to those of a commercial database, were very diverse (Chapter 2, Addendum 1). If the identification scores were uniformly high within a FAME cluster, the isolates were phylogenetically closely related, in other words, the FAME cluster was phylogenetically coherent. For instance, the identification

scores of the isolates of FAME cluster IX were high (0.747 $\pm$ 0.157) and most of those isolates (26 of 28) were identified as belonging to the genus *Bacillus*. In contrast, the opposite is not always correct. Low identification scores do not necessarily imply low phylogenetic coherence within a FAME cluster. For instance, low identification scores were obtained for the isolates of FAME cluster III (0.204 $\pm$ 0.093) but the isolates demonstrated to belong to the same genus (Addendum 1). In addition, the FAME identification scores varied strongly within some FAME clusters and relatively high standard deviation values were obtained (Table 2.1). For instance, the identification scores ranged from 0.031 to 0.796 within FAME cluster X (n = 30). For such clusters, it appeared not possible to predict the identity or the phylogenetic coherence of its members. For instance, the identification scores within FAME cluster II (n = 5) varied from 0.140 to 0.733 and the isolates demonstrated to be phylogenetically coherent as they were all identified as *Acinetobacter* species. On the other hand, the identification scores within FAME cluster X (n = 30) ranged from 0.031 to 0.796 but the isolates were phylogenetically diverse as they belonged to different genera of the phylum *Actinobacteria*.

In conclusion, FAME analysis was used to group the 356 isolates into smaller groups. Depending on the heterogeneity of the FAME clusters (i.e. the profile similarity within a FAME cluster), the clusters comprised members of multiple species which were closely related or which represented multiple genera. Homogenous FAME clusters demonstrated to be phylogenetically more coherent. In general, FAME analysis did not reveal the exact taxonomic diversity within the delineated FAME clusters. Therefore, other methods with a higher level of taxonomic resolution had to be applied.

5.2.2 BOX-PCR fingerprinting as a tool to study the taxonomic diversity within a FAME cluster

Rep-PCR fingerprinting, using the BOX-A1R primer, is a DNA-based typing method that reveals the genetic diversity within a group of isolates. The genomic diversity of FAME clusters I and IV bacteria was investigated by BOX-PCR fingerprinting. Isolates of these FAME clusters were predominant accounting for 45 % of all isolates and also represented the most abundant morphotypes on the initial isolation plates. Eleven BOX-PCR clusters with at least 60 % profile similarity and comprising 2 to 14 isolates were delineated among FAME cluster I isolates whereas ten isolates had unique BOX-PCR fingerprints (Fig. 5.1). Some isolates of FAME cluster I generated BOX-PCR profiles with only a few bands (e.g. BOX-PCR cluster 3) whereas other isolates generated more complex DNA profiles (e.g. BOX-PCR cluster 1) (Fig. 5.1, Chapter 3). Therefore, at least one isolate per BOX-PCR cluster (except BOX-PCR clusters 9 and 11) was selected for (GTG)$_5$-PCR fingerprinting in order to reveal if the

(GTG)$_5$-PCR primer was more suitable for the analysis of FAME cluster I isolates. Unfortunately, no or very weak DNA profiles were obtained using the (GTG)$_5$-PCR primer (data not shown), demonstrating that the (GTG)$_5$-PCR primer is not suitable.

Six BOX-PCR clusters, with at least 60 % profile similarity, were delineated among FAME IV isolates. The BOX-PCR clusters comprised 2 to 66 isolates and 7 isolates had unique BOX-PCR fingerprints (Fig. 5.2).

Every BOX-PCR clusters harbours a collection of isolates with similar DNA profiles. Nevertheless, differences can be observed between DNA profiles of some isolates within such a BOX-PCR cluster (e.g. BOX-PCR cluster 1 of FAME cluster I), suggesting the presence of multiple strains within one BOX-PCR cluster (Fig. 5.1, Chapter 3).

At least one representative isolate per BOX-PCR cluster was selected for 16S rRNA gene sequencing. Often, the 16S rRNA gene sequencing results did not allow the identification of the isolates but determined the nearest phylogenetic neighbours of the isolates due to the limited taxonomic resolution of comparative 16S rRNA gene sequence analysis. These closest relatives were also analysed by BOX-PCR fingerprinting but only a low profile similarity was observed between the isolates and their closest phylogenetic neighbours and thus no identification was obtained by BOX-PCR fingerprinting (Chapter 3). In order to elucidate if the isolates constituted novel bacterial taxa, DNA-DNA hybridisation experiments were performed between representative isolates of the BOX-PCR clusters and the type strains of their closest phylogenetic relatives. Two representative isolates per BOX-PCR cluster were selected for DNA-DNA hybridisations on basis of their BOX-PCR profiles as we looked for the most divergent strains. DNA-DNA hybridisation values (> 70 %) demonstrated that isolates within one BOX-PCR cluster belonged to the same species, confirming that isolates with highly similar BOX-PCR profiles belong to the same species (Rademaker & De Bruijn, 1997). Five BOX-PCR clusters of FAME cluster I were investigated by DNA-DNA hybridisation experiments of which four were identified as *Alteromonas macleodii* and one constituted a novel species, *Alteromonas genovensis* sp. nov. (Chapter 3, Fig. 5.1). In addition, the isolates of four BOX-PCR clusters and one isolate with an unique BOX-PCR fingerprint of FAME cluster IV were studied by DNA-DNA hybridisation of which one was identified as *Ruegeria mobilis* and four constituted novel bacterial taxa i.e. *Leisingera aquimarina* sp. nov., *Ruegeria scottomollicae* sp. nov., *Phaeobacter caerulea* sp. nov. and *Nautella italica* gen. nov., sp. nov. (Chapter 4).

In conclusion, the use of BOX-PCR profiling to investigate a collection of isolates enabled to reveal the genetic diversity within a FAME cluster and allowed, in combination with 16S rRNA gene sequence analysis, the selection of representative isolates for DNA-DNA hybridisation experiments. However, due to the genetic diversity within a bacterial species, it was often impossible to recognize species only on the basis of their BOX-PCR profiles.

Indeed, several BOX-PCR clusters represented established species. For instance, four BOX-PCR clusters of FAME I were identified as *Alteromonas macleodii* although a low profile similarity was observed towards the type strain of *A. macleodii* (LMG 2843^T) (Chapter 3, Fig. 5.1).

In general, the strategy of sequentially applying techniques with different taxonomic resolution demonstrated to be very efficient to investigate and identify a large number of isolates. This way, six novel bacterial taxa were detected. The isolates of these novel groups were subsequently examined using a diverse range of phenotypic tests as required for the description of novel bacterial species (Kämpfer *et al.*, 2003) (Chapter 3, Chapter 4).

5.2.3 Comparison of culture dependent versus culture independent analyses

The bacterial diversity of a marine electroactive biofilm was investigated by culture dependent (e.g. FAME, BOX-PCR fingerprinting, 16S rRNA gene sequence analysis) and culture independent (clone library and DGGE) techniques. The aim of the culture independent analyses performed during this study was to verify if we succeeded to cultivate the dominant bacterial population of the marine electroactive biofilm (by constructing a clone library) and to compare the bacterial composition of the biofilm and the surrounding seawater (by DGGE) (Chapter 2).

Analyzing the clone library demonstrated that largely the same bacterial groups were obtained by culture dependent and culture independent methods (Chapter 2). Nevertheless, some differences between both approaches were observed, for instance, no actinobacterial sequences were detected in the clone library. Also, although largely the same bacterial groups were detected, the diversity among the clones was smaller than among the isolates. For instance, all *Firmicutes*-like sequences in the clone library were identified as *Bacillus*-like sequences whereas the *Firmicutes* among the isolates were identified as *Bacillus* sp., *Staphylococcus* sp. and *Exiguobacterium* sp. Several reasons may account for these differences. First, bacteria that were not detected in the library may constitute only minor fractions of the biofilm population. In addition, the cultivation conditions used may have favoured the growth of these bacteria, leading to an overestimation of the presence of these groups in the bacterial population. Furthermore, the techniques used may be less suitable for the construction of a clone library with DNA from these bacteria.

In general, although the combination of culture dependent and culture independent analyses is absolutely required to investigate the bacterial population of a given sample, some caution is necessary when interpreting the results.

Pearson correlation (Opt:1.29%) [0.0%-100.0%]
BOX-PCR BOX-PCR

R-number	Identification	Cluster	LMG
R-28711	Pseudoalteromonas sp.	9	
R-28784	Pseudoalteromonas sp.	9	
R-28712	Pseudoalteromonas sp. *	UP	
R-25611	Pseudoalteromonas sp. *	10	
R-26141	Pseudoalteromonas sp. *	10	
R-25608	Vibrio sp. *	UP	
R-28033	Alteromonas macleodii	5	
R-28069	Alteromonas macleodii	5	LMG 24082
R-26190	Alteromonas macleodii	5	
R-26143	Alteromonas macleodii	5	
R-26196	Alteromonas macleodii	5	
R-25514	Pseudoalteromonas sp.	6	
R-25577	Pseudoalteromonas sp.	6	
R-28060	Alteromonas macleodii	2	
R-28062	Alteromonas macleodii	2	
R-28061	Alteromonas macleodii	2	
R-28048	Alteromonas macleodii	2	
R-28018	Alteromonas macleodii	2	
R-28039	Alteromonas macleodii	2	
R-28047	Alteromonas macleodii	2	
R-28053	Alteromonas macleodii	2	LMG 24081
R-28032	Alteromonas macleodii	2	
R-28696	Alteromonas macleodii	4	
R-28740	Alteromonas macleodii	4	LMG 24083
R-28741	Alteromonas macleodii	4	
LMG 2843T	Alteromonas macleodii		
R-25605	Pseudoalteromonas sp. *	UP	
R-25515	Pseudoalteromonas sp. *	UP	
LMG 22958T	Alteromonas hispanica		
LMG 21861T	Alteromonas stellipolaris		
LMG 22532T	Alteromonas addita		
R-28697	Alteromonas genovensis sp.nov.	1	
R-28713	Alteromonas genovensis sp.nov.	1	
R-28744	Alteromonas genovensis sp.nov.	1	LMG 24079
R-28813	Alteromonas genovensis sp.nov.	1	
R-28731	Alteromonas genovensis sp.nov.	1	
R-25500	Alteromonas genovensis sp.nov.	1	
R-28754	Alteromonas genovensis sp.nov.	1	
R-28776	Alteromonas genovensis sp.nov.	1	
R-28716	Alteromonas genovensis sp.nov.	1	
R-28714	Alteromonas genovensis sp.nov.	1	
R-28729	Alteromonas genovensis sp.nov.	1	LMG 24078T
R-28820	Alteromonas genovensis sp.nov.	1	
R-28767	Alteromonas genovensis sp.nov.	1	
R-25502	Alteromonas genovensis sp.nov.	1	
R-28757	Pseudoalteromonas sp. *	UP	
R-28699	Pseudoalteromonas sp. *	UP	
R-25508	Pseudoalteromonas sp. *	UP	
LMG 23846T	Alteromonas litorea		
R-26168	gammaproteobacteria*	11	
R-26169	gammaproteobacteria*	11	
R-25579	Aeromonas sp. *	UP	
R-28017	Alteromonas macleodii	3	LMG 24080
R-28702	Alteromonas macleodii	3	
R-26142	Alteromonas macleodii	3	
R-28695	Alteromonas macleodii	3	
R-25489	Alteromonas macleodii	3	
R-25603	Pseudoalteromonas sp.	7	
R-28068	Pseudoalteromonas sp.	7	
R-25609	Pseudoalteromonas sp.	7	
R-28050	Pseudoalteromonas sp.	7	
R-28051	Pseudoalteromonas sp.	7	
R-25492	Paucimonas sp. *	UP	
R-28723	Pseudoalteromonas sp.	8	
R-28742	Pseudoalteromonas sp.	8	
R-28727	Pseudoalteromonas sp.	8	
R-26197	Pseudoalteromonas sp.	8	
LMG 22057T	Alteromonas marina		
R-28760	Pseudoalteromonas sp. *	UP	

Figure 5.1: BOX-PCR profiles and dendrogram based on numerical analysis of the profiles by means of UPGMA and relying on the Pearson's correlation coefficient. The R-number, the identification results, the BOX-PCR cluster number and the LMG number, if available, are given.
*: Only a FAME analysis based identification was obtained
UP: Unique profile

Pearson correlation (Opt 0.24%) [0.0% - 100.0%]
BOX-PCR   **BOX-PCR**

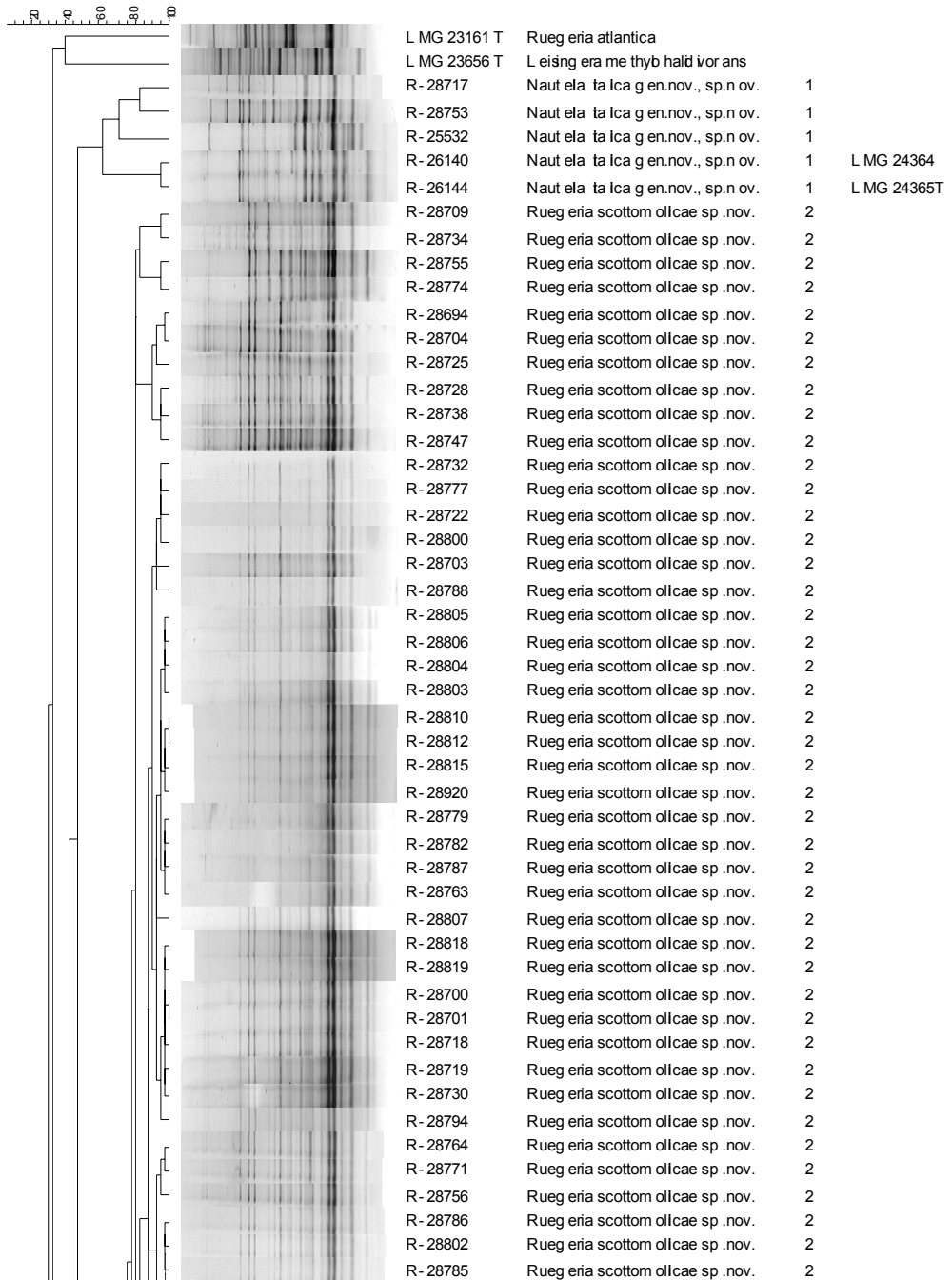|		
LMG 23161 T	Ruegeria atlantica	
LMG 23656 T	Leisingera methybalidivorans	
R-28717	Nautela talca g en.nov., sp.nov.	1
R-28753	Nautela talca g en.nov., sp.nov.	1
R-25532	Nautela talca g en.nov., sp.nov.	1
R-26140	Nautela talca g en.nov., sp.nov.	1	LMG 24364
R-26144	Nautela talca g en.nov., sp.nov.	1	LMG 24365T
R-28709	Ruegeria scottomollicae sp.nov.	2
R-28734	Ruegeria scottomollicae sp.nov.	2
R-28755	Ruegeria scottomollicae sp.nov.	2
R-28774	Ruegeria scottomollicae sp.nov.	2
R-28694	Ruegeria scottomollicae sp.nov.	2
R-28704	Ruegeria scottomollicae sp.nov.	2
R-28725	Ruegeria scottomollicae sp.nov.	2
R-28728	Ruegeria scottomollicae sp.nov.	2
R-28738	Ruegeria scottomollicae sp.nov.	2
R-28747	Ruegeria scottomollicae sp.nov.	2
R-28732	Ruegeria scottomollicae sp.nov.	2
R-28777	Ruegeria scottomollicae sp.nov.	2
R-28722	Ruegeria scottomollicae sp.nov.	2
R-28800	Ruegeria scottomollicae sp.nov.	2
R-28703	Ruegeria scottomollicae sp.nov.	2
R-28788	Ruegeria scottomollicae sp.nov.	2
R-28805	Ruegeria scottomollicae sp.nov.	2
R-28806	Ruegeria scottomollicae sp.nov.	2
R-28804	Ruegeria scottomollicae sp.nov.	2
R-28803	Ruegeria scottomollicae sp.nov.	2
R-28810	Ruegeria scottomollicae sp.nov.	2
R-28812	Ruegeria scottomollicae sp.nov.	2
R-28815	Ruegeria scottomollicae sp.nov.	2
R-28920	Ruegeria scottomollicae sp.nov.	2
R-28779	Ruegeria scottomollicae sp.nov.	2
R-28782	Ruegeria scottomollicae sp.nov.	2
R-28787	Ruegeria scottomollicae sp.nov.	2
R-28763	Ruegeria scottomollicae sp.nov.	2
R-28807	Ruegeria scottomollicae sp.nov.	2
R-28818	Ruegeria scottomollicae sp.nov.	2
R-28819	Ruegeria scottomollicae sp.nov.	2
R-28700	Ruegeria scottomollicae sp.nov.	2
R-28701	Ruegeria scottomollicae sp.nov.	2
R-28718	Ruegeria scottomollicae sp.nov.	2
R-28719	Ruegeria scottomollicae sp.nov.	2
R-28730	Ruegeria scottomollicae sp.nov.	2
R-28794	Ruegeria scottomollicae sp.nov.	2
R-28764	Ruegeria scottomollicae sp.nov.	2
R-28771	Ruegeria scottomollicae sp.nov.	2
R-28756	Ruegeria scottomollicae sp.nov.	2
R-28786	Ruegeria scottomollicae sp.nov.	2
R-28802	Ruegeria scottomollicae sp.nov.	2
R-28785	Ruegeria scottomollicae sp.nov.	2

R-number	Identification	BOX-PCR cluster	LMG number
R-28 720	Ruegeria scottomolicae sp. nov.	2	
R-28 724	Ruegeria scottomolicae sp. nov.	2	LMG 243 68
R-28 801	Ruegeria scottomolicae sp. nov.	2	
R-28 707	Ruegeria scottomolicae sp. nov.	2	
R-28 737	Ruegeria scottomolicae sp. nov.	2	
R-28 761	Ruegeria scottomolicae sp. nov.	2	
R-28 762	Ruegeria scottomolicae sp. nov.	2	
R-28 781	Ruegeria scottomolicae sp. nov.	2	
R-28 811	Ruegeria scottomolicae sp. nov.	2	
R-28 759	Ruegeria scottomolicae sp. nov.	2	
R-28 758	Ruegeria scottomolicae sp. nov.	2	
R-28 769	Ruegeria scottomolicae sp. nov.	2	
R-28 795	Ruegeria scottomolicae sp. nov.	2	
R-28 698	Ruegeria scottomolicae sp. nov.	2	
R-28 792	Ruegeria scottomolicae sp. nov.	2	
R-26 164	Ruegeria scottomolicae sp. nov.	2	
R-28 726	Ruegeria scottomolicae sp. nov.	2	
R-28 710	Ruegeria scottomolicae sp. nov.	2	
R-28 751	Ruegeria scottomolicae sp. nov.	2	LMG 243 67T
R-28 706	Ruegeria scottomolicae sp. nov.	2	
R-28 733	Ruegeria scottomolicae sp. nov.	2	
R-28 791	Ruegeria scottomolicae sp. nov.	2	
R-28 799	Ruegeria scottomolicae sp. nov.	2	
LMG 2316 3T	Phaeobacter gallaeciensis		
R-26 151	alphaproteobacterium *	UP	
LMG 2316 2T	Ruegeria lacuscaerulensis		
R-26 147	alphaproteobacterium *	UP	
LMG 2247 5T	Phaeobacter inhibens		
R-26 159	Leisingera aquimarina sp. nov.	UP	LMG 243 66T
LMG 2316 8T	Ruegeria pomeroyi		
R-26 160	Phaeobacter sp.	UP	
R-26 146	Phaeobacter caerulea sp. nov.	4	LMG 243 69T
R-26 156	Phaeobacter caerulea sp. nov.	4	
R-26 149	Phaeobacter caerulea sp. nov.	4	LMG 243 70
R-26 157	no match *	6	
R-26 165	alphaproteobacterium *	6	
R-26 145	Ruegeria pelagia	UP	
R-26 163	alphaproteobacterium *	UP	
R-26 188	alphaproteobacterium *	UP	
R-26 185	Ruegeria mobilis	1	LMG 243 71
R-28 052	Ruegeria mobilis	1	
R-26 194	Ruegeria mobilis	1	
R-26 189	Ruegeria mobilis	1	LMG 243 72
R-26 162	Ruegeria sp.	5	
R-26 166	Ruegeria sp.	5	
R-26 155	Ruegeria sp.	5	
R-28 809	alphaproteobacterium *	UP	

Figure 5.2: BOX-PCR profiles and dendrogram based on numerical analysis of the profiles by means of UPGMA and relying on the Pearson's correlation coefficient. The R-number, the identification results, the BOX-PCR cluster number and the LMG number if available are given.
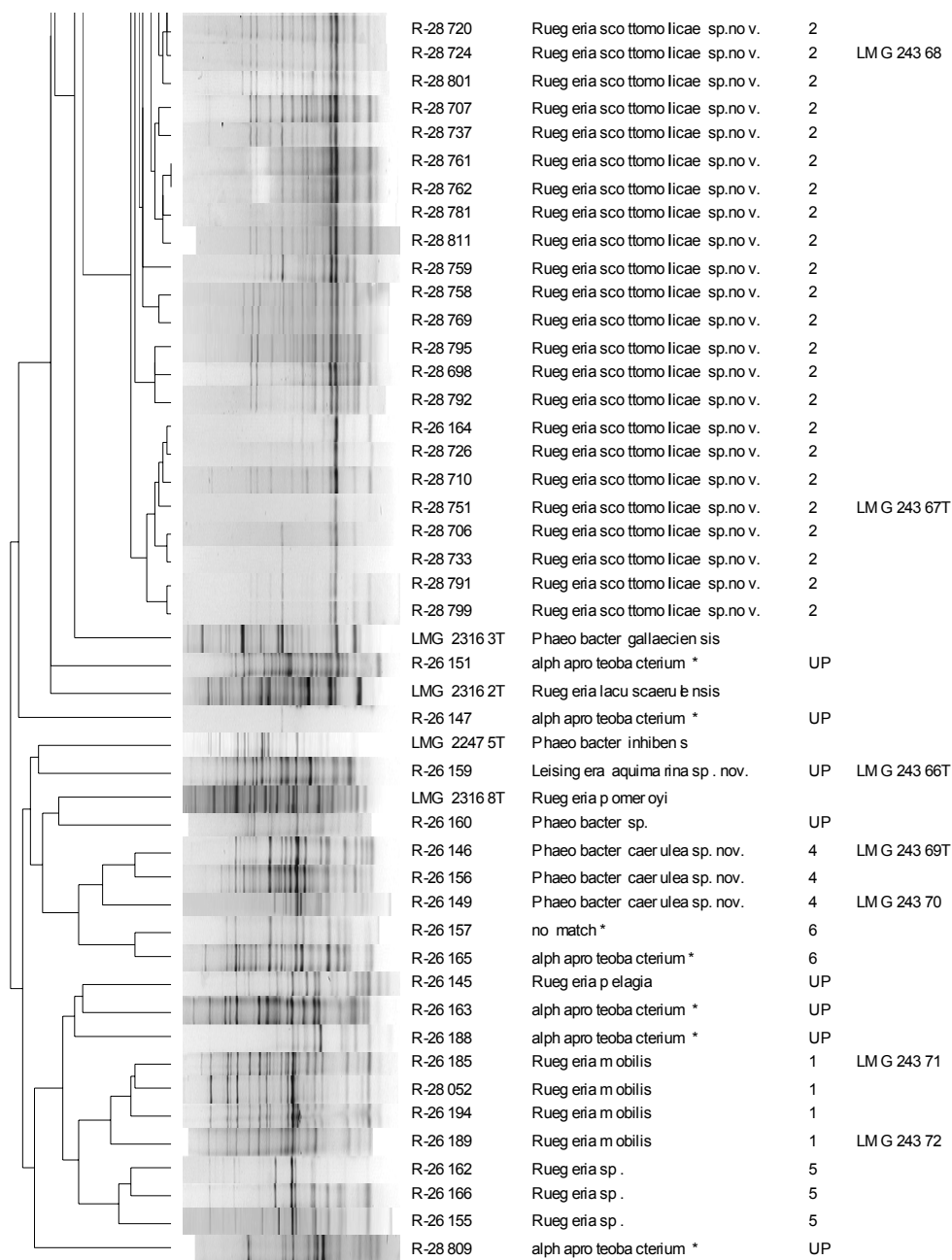*: Only a FAME analysis based identification was obtained
UP: Unique profiles

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CHAPTER 6: CONCLUSION & SAMENVATTING

6.1 CONCLUSION

At present, the energy demand of the world population is immense and is predicted to increase even more. The "Energy information administration/ international energy outlook 2006" forecasts that the world energy consumption will increase by 71 percent from 2003 to 2030. Currently, fossil fuels are the major industrial energy sources, but the extensive burning of these fuels jeopardizes the climate on Earth. In addition, a depletion of fossil fuels is expected during the next decades. Therefore, the development of new and green energy sources is extremely important. One of the possibilities is energy production by microbial fuel cells in which bacteria generate current. Bacteria transfer electrons to or from the electrode and are present as an electroactive biofilm (EAB) community on the surface of the electrode. The present study was part of a European project that was entitled "Electrochemical control of biofilm-forming micro-organisms: screening, identification and design of new knowledge-based technologies (EA-BIOFILM-508866 (NEST))". A cathodically polarized stainless steel electrode (-200 mV Ag/AgCl) was placed in natural aerated seawater at the harbour of Genoa, Italy. Biofilm formation was observed on the cathodes and current increased and stabilized at a maximal value of 0.5 A / m^2 in less than 10 days. A polyphasic taxonomic approach was used to investigate the microbial population of this marine EAB through culture dependent and culture independent analyses.

Three hundred and fifty six isolates were obtained from three different bacterial growth media and were grouped on the basis of their FAME profiles. Twenty FAME clusters, with at least 70 % profile similarity and comprising 2 to 91 isolates, were delineated. At least one representative per FAME cluster was selected for 16S rRNA gene sequence analysis in order to obtain a more accurate identification. The isolates demonstrated to belong to a range of bacterial groups as members of the class *Alpha*- (the genera *Ruegeria*, *Phaeobacter* and *Leisingera*) and the *Gammaproteobacteria* (the genera *Alteromonas*, *Pseudoalteromas*, *Acinetobacter*, *Marinobacter*, *Idiomarina* and *Halomonas*), the phylum *Firmicutes* (the genera *Bacillus*, *Staphylococcus* and *Exiguobacterium*), the phylum *Actinobacteria* (the genera *Arthrobacter* and *Frigoribacterium*) and the family *Flavobacteriaceae* (the genera *Maribacter* and *Winogradskyella*) were detected. This high diversity contrasts with previous studies, which reported the enrichment of specific bacterial groups. This may be explained by the minimal growth time (14 days) of the EAB investigated compared to previously described EABs which had been developing for several months to years. In addition, denaturing gradient gel electrophoresis of PCR amplified V_3 regions of bulk 16S rRNA genes demonstrated that the microbial populations of the marine EAB and of the surrounding seawater were highly similar, indicating that the EAB is mainly constituted of typical marine bacteria, which was confirmed by the identification results of the culture dependent analyses.

The isolates of two FAME clusters (I and IV) were most abundant (45 % of all isolates) and comprised the predominant morphotypes on the initial isolation plates. The isolates of these two FAME clusters were further investigated by BOX-PCR fingerprinting, DNA-DNA hybridisation experiments and phenotypic tests, which resulted in the description of several novel bacterial taxa. One novel species was delineated within the class *Gammaproteobacteria* (FAME cluster I) i.e. *Alteromonas genovensis* sp. nov. Detailed analyses of FAME cluster IV resulted in the description of three novel species within the *Roseobacter*-lineage of the class *Alphaproteobacteria* namely *Leisingera aquimarina* sp. nov., *Ruegeria scottomollicae* sp. nov. and *Phaeobacter caerulea* sp. nov. and in the delineation of one novel genus within the *Roseobacter*-lineage i.e. *Nautella italica* gen. nov., sp. nov.

FAME analysis and BOX-PCR fingerprinting were the key methods in order to investigate the microbial diversity of this marine EAB. FAME analysis was very useful to group isolates in clusters comprising phylogenetically closely related bacterial taxa but did not reveal the exact taxonomic diversity of the isolates within the FAME clusters. BOX-PCR fingerprinting was used to determine the genetic diversity within two selected FAME clusters (I and IV) and showed to be very useful in order to select strains for further experiments. However, due to its high taxonomic resolution level and the genetic diversity within bacterial species, it is often impossible to delineate species on the basis of their BOX-PCR profiles and strains from different BOX-PCR clusters may represent the same species.

Seven isolates (six members of different genera of the *Roseobacter*-lineage and one gammaproteobacterium) were analysed by cyclic voltammetry in order to investigate their potential electrochemical activity. All isolates efficiently catalysed the reduction of oxygen, indicating that electrochemical activity is present among different bacterial taxa.

In conclusion, the microbial population of a marine, 14 days old EAB was phylogenetically highly diverse and comprised typical marine bacteria. Largely the same phylogenetic groups of bacteria were detected with culture dependent and culture independent techniques, indicating that the dominant fractions of the bacterial population of the EAB were isolated by cultivation. The application of a diverse set of culture dependent and culture independent techniques was required to reveal the bacterial EAB population and to generate pure culture for further experiments.

6.2 SAMENVATTING

Tegenwoordig is de vraag naar energie immens. Er wordt zelfs voorspeld dat de energievraag nog zal stijgen. Momenteel zijn fossiele brandstoffen de belangrijkste energiebronnen, maar de enorme uitstoot van koolstofdioxide door de verbranding van deze brandstoffen is schadelijk en erg belastend voor het milieu. Daarnaast wordt er een uitputting van fossiele brandstoffen verwacht in de volgende decades. De zoektocht naar en de ontwikkeling van nieuwe en groene energiebronnen is van cruciaal belang om aan de alsmaar groeiende energievraag te voldoen. Eén van de mogelijkheden zijn microbiële brandstofcellen waarin bacteriën elektrische stroom produceren. Bacteriën katalyseren het elektronentransport naar of van de elektrode wat resulteert in stroomproductie. Deze bacteriën vormen hierbij elektroactieve biofilms (EAB) op het oppervlak van de elektrode.

Voorgestelde studie kaderde in een Europees project getiteld "Electrochemical control of biofilm-forming micro-organisms: screening, identification and design of new knowledge-based technologies (EA-BIOFILM-508866 (NEST))". Roestvrij staal coupons werden kathodisch gepolariseerd (-200 mV Ag/AgCl) in zeewater in de haven van Genua (Italië), wat resulteerde in stroomproductie. De stroomproductie was maximaal na 10 dagen (0.5 A/m²) en bleef nadien constant. De vorming van een biofilm werd waargenomen op het oppervlak van de kathode. De microbiële gemeenschap in deze mariene EAB werd onderzocht aan de hand van een reeks cultuurafhankelijke en cultuuronafhankelijke technieken, steunend op een polyfasische taxonomische aanpak.

Driehonderd zesenvijftig isolaten werden opgepikt van drie verschillende groeimedia en werden allemaal geanalyseerd door middel van cellulaire vetzuuranalyse (FAME). De isolaten werden gegroepeerd op basis van hun FAME profielen en zo werden er twintig groepen, met ten minste 70 % profiel gelijkenis, afgebakend. De FAME groepen bevatten 2 tot 91 isolaten en ten minste 1 isolaat per FAME cluster werd geselecteerd voor 16S rRNA gensequentieanalyse om een nauwkeuriger identificatie te bekomen. De isolaten bleken te behoren tot verschillende fylogenetische groepen namelijk vertegenwoordigers van de klasse *Alpha-* (de genera *Ruegeria*, *Phaeobacter* en *Leisingera*) en *Gammaproteobacteria* (de genera *Alteromonas*, *Pseudoalteromonas*, *Acinetobacter*, *Marinobacter*, *Idiomarina* en *Halomonas*), het fylum *Actinobacteria* (de genera *Arthrobacter* en *Frigoribacterium*), de familie *Flavobacteriaceae* (de genera *Maribacter* and *Winogradskyella*) en het fylum *Firmicutes* (de genera *Bacillus*, *Staphylococcus* en *Exiguobacterium*) waren aanwezig. Deze hoge bacteriële diversiteit in de mariene EAB is in tegenspraak met eerdere studies van de microbiële gemeenschappen van EABs. Deze studies toonden aan dat er aanrijking van specifieke bacteriële groepen plaatsvond. Deze tegenstelling kan verklaard worden door de korte

incubatietijd (14 dagen) van de EAB van voorgestelde studie terwijl bij vorige studies de incubatietijd van de EABs varieerde van enkele maanden tot 5 jaar.

De isolaten van twee FAME groepen (I en IV) waren dominant (45 % van alle isolaten) en vertegenwoordigden ook de dominante morphotypes op de initiële isolatieplaten. De isolaten van deze twee FAME groepen werden in detail onderzocht aan de hand van BOX-PCR fingerprinting, DNA-DNA hybridisatie experimenten en fenotypische testen. Dit leidde tot de beschrijving van verschillende nieuwe bacteriële soorten. Eén nieuwe bacteriële soort kon beschreven worden binnen een bestaand genus van de klasse *Gammaproteobacteria* (FAME cluster I) namelijk *Alteromonas genovensis* sp. nov.. Gedetailleerd onderzoek van de isolaten van FAME cluster IV resulteerde in de beschrijving van 3 nieuwe bacteriële soorten binnen bestaande genera van de *Roseobacter*-groep van de klasse *Alphaproteobacteria* namelijk *Ruegeria scottomollicae* sp. nov., *Phaeobacter caerulea* sp. nov., *Leisingera aquimarina* sp. nov. en in de beschrijving van een nieuwe genus in de *Roseobacter*-groep namelijk *Nautella italica* gen. nov., sp. nov.

Cellulaire vetzuuranalyse (FAME) en BOX-PCR fingerprinting waren de sleutelmethodes om de bacteriële diversiteit van de mariene EAB te bestuderen. FAME analyse bleek erg nuttig om een grote set isolaten te groeperen in meer werkbare clusters van fylogenetisch nauw verwante stammen. Daarentegen was het onmogelijk om met FAME analyse de exacte taxonomische diversiteit van isolaten binnen een FAME groep na te gaan. BOX-PCR fingerprinting werd gebruikt om de genetische diversiteit binnen geselecteerde FAME groepen te analyseren en was uitermate geschikt om stammen te selecteren voor verdere experimenten. Niettegenstaande, de hoge taxonomische resolutie van deze techniek en de aanwezigheid van genetische verschillende stammen binnen een bacteriële soort maken het meestal onmogelijk om soorten enkel op basis van hun BOX-PCR profielen te identificeren.

Algemeen, de microbiële gemeenschap van de onderzochte mariene EAB was fylogenetisch erg divers en bestond uit typische mariene bacteriën. Grotendeels dezelfde fylogenetische groepen van bacteriën werden geïdentificeerd met cultuurafhankelijke en – onafhankelijke technieken, wat aantoont dat we erin geslaagd zijn om de dominante bacteriële gemeenschap op te kweken. Dus het toepassen van een reeks methodes met een verschillende taxonomische resolutie bleek erg nuttig te zijn om de diversiteit van deze mariene EAB gemeenschap te bestuderen.

ADDENDA

Addendum 1: Lists of the 356 isolates obtained from the marine EAB

[1]: Reference number of the isolate (deposited in the Research collection of LM-UGent)
[2]: Reference number of the isolate when deposited in the BCCM/LMG public culture collection
[3]: Identification results as obtained by FAME analysis
[4]: 16S rRNA gene based identification: the best match and the percent similarity
[5]: The accession number of the 16S rRNA gene sequence of the isolate as deposited in the EMBL database
[6]: Isolates analysed by DNA-DNA hybridisation experiments
[7]: The number of isolates within a FAME cluster
[8]: The profile similarity within a FAME cluster

R-nr[1] *Cluster I* n = 68[7] 71.6 %[8]	LMG-nr[2]	FAME genus level ID[3]	FAME species level ID[3]	Score[3]	16S rRNA gene sequence analysis[4]	Acc-nr[5]	Analysed by DDH[6]
BOX-cluster1							
25500		*Pseudoalteromonas*	*nigrifaciens*	0.410			
25502		*Vibrio*	*hollisae*	0.327			
28697		*Vibrio*	*hollisae*	0.349			
28713		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.520			
28714		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.300			
28716		*Vibrio*	*hollisae*	0.280			
28729[T]	24078[T]	*Aeromonas*	*ichthiosmia / A. hydrophila*	0.484	*Alteromonas macleodii* LMG 2843[T] (97.9%)	AM885866	*Alteromonas genovensis* sp.nov.
28731		*Aeromonas*	*ichthiosmia / A. hydrophila*	0.414			
28744	24079	*Aeromonas*	*ichtiosmia / A. hydrophila*	0.066	*Alteromonas hispanica* LMG 22958[T] (98.9%)	AM887686	*Alteromonas genovensis* sp.nov.
28754		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.365			
28767		*Vibrio*	*proteolyticus*	0.283			
28776		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.468			
28813		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.472			
28820		*Vibrio*	*fluvialis / furnissii*	0.564			
BOX-cluster 2							
28018		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.371	*Alteromonas macleodii* LMG 2843[T] (99.8 %)		
28032		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.381	*Alteromonas macleodii* LMG 2843[T] (99.1%)		*Alteromonas macleodii*
28039		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.379	*Alteromonas marina* LMG 22057[T] (98.4%)		
28047		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.453			
28048		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.476			
28053	24081	*Pseudoalteromonas*	*tetraodonis*	0.570	*Alteromonas marina* LMG 22057[T] (99.5%)	AM885867	*Alteromonas macleodii*
28060		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.384	*Alteromonas marina* LMG 22057[T] (99.3%)		
28061		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.371	*Alteromonas macleodii* LMG 2843[T] (98%)		
28062		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.420			
BOX-cluster 3							
28017	24080	*Vibrio*	*hollisae*	0.221	*Alteromonas macleodii* LMG 2843[T] (99%)	AM885868	*Alteromonas macleodii*
25489		*Pseudoalteromonas*	*tetraodonis*	0.646			
26142		*Aeromonas*	*jandaei*	0.409			
28695		*Pseudoalteromonas*	*nigrifaciens*	0.438			*Alteromonas macleodii*
28702		*Pseudoalteromonas*	*tetraodonis*	0.450			
BOX-cluster 4							
28696	24083	*Aeromonas*	*Ichtiosmia / A. hydrophila*	0.230	*Alteromonas macleodii* LMG 2843[T] (99.1%)	AM887685	*Alteromonas macleodii*
28740		*Aeromonas*	*Ichtiosmia / A. hydrophila*	0.191			*Alteromonas macleodii*
28741		*Enterobacter*	*hormaechei*	0.253			
BOX-cluster 5							
26143		*Vibrio*	*hollisae*	0.492	*Alteromonas marina* LMG 22057[T] (99.5%)		*Alteromonas macleodii*
26190		*Aeromonas*	*jandaei*	0.141			
28033	24082	*Pseudoalteromonas*	*nigrifaciens*	0.391	*Alteromonas macleodii* LMG 2843 (100%)	AM885870	*Alteromonas macleodii*
28069		*Pseudoalteromonas*	*tetraodonis*	0.302			*Alteromonas macleodii*
26196		*Aeromonas*	*ichtiosmia / A. hydrophila*	0.018			

ID	Genus	species	value	Closest relative	Accession
BOX-cluster 6					
25514	*Paucimonas*	*lemoignei*	0.305		
25577	*Vibrio*	*hollisae*	0.401		
BOX-cluster 7					
25603	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.390	*Pseudoalteromonas spongiae* JCM12884[T] (100%)	AM944021
25609	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.331	*Pseudoalteromonas ruthenica* LMG 19699[T] (98.6%)	AM944022
28050	*Colwellia*	*psychrerythraea*	0.073	*Pseudoalteromonas ruthenica* LMG 19699[T] (99.0%)	
28051	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.164		
28068	*Pseudoalteromonas*	*tetraodonis*	0.168	*Pseudoalteromonas ruthenica* LMG 19699[T] (97%)	
BOX-cluster 8					
28723	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.288		
28727	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.295		
28742	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.377		
26197	*Pseudoalteromonas*	*tetraodonis*	0.719	*Pseudoalteromonas* sp. FR1302 (98.9%)	AM944023
BOX-cluster 9					
28711	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.298		
28784	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.640	*Pseudoalteromonas* sp. SM9913 (97.2%)	
BOX-cluster 10					
25611	*Pseudoalteromonas*	*tetraodonis*	0.653		
26141	*Pseudoalteromonas*	*tetraodonis*	0.133		
BOX-cluster 11					
26168	*Aeromonas*	*veronii GC subgr A*	0.680		
26169	*Vibrio*	*fluvialis / furnissii*	0.664		
Unique profiles					
25508	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.191		
25515	*Pseudoalteromonas*	*tetraodonis*	0.665		
25579	*Aeromonas*	*ichtiosmia / A. hydrophila*	0.817		
25608	*Vibrio*	*hollisae*	0.033		
25492	*Paucimonas*	*lemoignei*	0.176		
28699	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.641		
28712	*Pseudoalteromonas*	*tetraodonis*	0.322		
28757	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.369	*Alteromonas hispanica* LMG 22958[T] (98%)	
28760	*Pseudoalteromonas*	*tetraodonis*	0.788		
25605	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.198		
No profiles					
28027	*Aeromonas*	*jandaei*	0.256		
26158	*Aeromonas*	*ichtiosmia / A. hydrophila*	0.534		
25527	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.882		
28036	*Pseudoalteromonas*	*haloplanktis haloplanktis*	0.178		
28775	*Vibrio*	*hollisae*	0.358		

Cluster / Strain	Genus	Species	Value	Closest relative	Proposed name	Accession
Cluster II						
n = 5						
75.1 %						
25565	*Acinetobacter*	*calcoaceticus*	0.462			
25580	*Acinetobacter*	*calcoaceticus*	0.337			
25578	*Acinetobacter*	*calcoaceticus*	0.140			
25504	*Acinetobacter*	*calcoaceticus*	0.532			
26193	*Acinetobacter*	*lwoffii GC subgroup B*	0.733	*Acinetobacter johsonii* DSM 6963^T (99%)		AM944024
Cluster III						
n = 4						
83.5 %						
25575	*Bacillus*	*circulans*	0.255			
25590	*Bacillus*	*circulans*	0.307			
25620	*Bacillus*	*alcolophilus*	0.111			
25632	*Bacillus*	*circulans*	0.142	*Bacillus drentensis* LMG 21831^T (99%)		AM944025
Cluster IV						
n= 91						
76.3 %						
BOX-cluster 1						
28717	*Gluconobacter*	*asaii/cerinus/oxydans*	0.192	*Phaeobacter gallaeciensis* LMG 23163^T (97.2 %)		AM944521
28532	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.351	*Phaeobacter gallaeciensis* LMG 23163^T (97.2 %)		AM944520
24364	*Paracoccus*	*denitrificans*	0.429	*Phaeobacter gallaeciensis* LMG 23163^T (97.2 %)	*Nautella italica* gen. nov., sp.nov.	AM904563
24365^T	*Gluconobacter*	*asaii/cerinus/oxydans*	0.361	*Phaeobacter gallaeciensis* LMG 23163^T (97.1 %)	*Nautella italica* gen. nov., sp.nov.	AM904562
28753	*Gluconobacter*	*asaii/cerinus/oxydans*	0.202	*Phaeobacter gallaeciensis* LMG 23163^T (97.2 %)		AM944522
BOX-cluster 2						
26164	*Paracoccus*	*denitrificans*	0.602			
28694	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.284			
28698	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.270			
28700	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.258			
28701	*Gluconobacter*	*asaii /oxydans*	0.168			
28703	*Gluconobacter*	*asaii/ cerinus /oxydans*	0.292			
28704	*Gluconobacter*	*asaii/ cerinus /oxydans*	0.158	*Ruegeria atlantica* LMG 23161^T (99.9 %)		
28706	*Curtobacterium*	*pusilum*	0.208			
28707	*Paracoccus*	*denitrificans*	0.410			
28709	*Gluconobacter*	*asaii/cerinus/oxydans*	0.285			
28710	*Gluconobacter*	*asaii/cerinus/oxydans*	0.263			
28718	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.214			
28719	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.264			
28720	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.274			
28722	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.258			
24368	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.312	*Ruegeria atlantica* LMG 23161^T (99.7%)	*Ruegeria scottomollicae* sp.nov.	AM905331
28725	*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.315	*Ruegeria atlantica* LMG 23161^T (99.8%)		

Strain	Genus	Species	Value	Identification
28726	*Paracoccus*	*denitrificans*	0.354	
28728	*Gluconobacter*	*asaii/cerinus/oxydans*	0.268	
28730	*Gluconobacter*	*asaii/cerinus/oxydans*	0.354	
28732	*Gluconobacter*	*asaii/cerinus/oxydans*	0.318	*Ruegeria atlantica* LMG 23161[T] (99.4%)
28733	*Gluconobacter*	*asaii/cerinus/oxydans*	0.294	*Ruegeria atlantica* LMG 23161[T] (99.4%)
28734	*Gluconobacter*	*asaii/cerinus/oxydans*	0.314	
28737	*Gluconobacter*	*asaii/cerinus/oxydans*	0.256	*Ruegeria atlantica* LMG 23161[T] (99.6 %)
28738	*Gluconobacter*	*asaii/cerinus/oxydans*	0.156	
28747	*Gluconobacter*	*asaii/cerinus/oxydans*	0.302	
28751[T]	*Gluconobacter*	*asaii/cerinus/oxydans*	0.286	24367[T] AM905330 *Ruegeria scottomollicae* sp.nov. *Ruegeria mobilis* CIP109181[T] (97.2 %)
28755	*Gluconobacter*	*asaii/cerinus/oxydans*	0.309	
28756	*Gluconobacter*	*asaii/cerinus/oxydans*	0.319	*Ruegeria atlantica* LMG 23161[T] (99.7%)
28758	*Gluconobacter*	*asaii/cerinus/oxydans*	0.294	
28759	*Gluconobacter*	*asaii/cerinus/oxydans*	0.230	
28761	*Gluconobacter*	*asaii/cerinus/oxydans*	0.271	
28762	*Gluconobacter*	*asaii/cerinus/oxydans*	0.277	
28763	*Gluconobacter*	*asaii/cerinus/oxydans*	0.309	
28764	*Gluconobacter*	*asaii/cerinus/oxydans*	0.305	
28769	*Gluconobacter*	*asaii/cerinus/oxydans*	0.283	
28771	*Gluconobacter*	*asaii/cerinus/oxydans*	0.348	
28774	*Gluconobacter*	*asaii/cerinus/oxydans*	0.289	
28777	*Gluconobacter*	*asaii/cerinus/oxydans*	0.310	
28779	*Gluconobacter*	*asaii/cerinus/oxydans*	0.356	
28781	*Gluconobacter*	*asaii/cerinus/oxydans*	0.302	
28782	*Gluconobacter*	*asaii/cerinus/oxydans*	0.337	
28785	*Gluconobacter*	*asaii/cerinus/oxydans*	0.277	
28786	*Gluconobacter*	*asaii/cerinus/oxydans*	0.275	
28787	*Gluconobacter*	*asaii/cerinus/oxydans*	0.311	
28788	*Gluconobacter*	*asaii/cerinus/oxydans*	0.274	
28791	*Gluconobacter*	*asaii/cerinus/oxydans*	0.251	
28792	*Paracoccus*	*denitrificans*	0.473	
28794	*Gluconobacter*	*asaii/cerinus/oxydans*	0.303	
28795	*Gluconobacter*	*asaii/cerinus/oxydans*	0.270	
28799	*Gluconobacter*	*asaii/cerinus/oxydans*	0.271	
28800	*Gluconobacter*	*asaii/cerinus/oxydans*	0.269	*Ruegeria atlantica* LMG 23161[T] (99.4%)
28801	*Gluconobacter*	*asaii/cerinus/oxydans*	0.311	
28802	*Gluconobacter*	*asaii/cerinus/oxydans*	0.265	
28803	*Gluconobacter*	*asaii/cerinus/oxydans*	0.270	
28804	*Gluconobacter*	*asaii/cerinus/oxydans*	0.171	
28805	*Gluconobacter*	*asaii/cerinus/oxydans*	0.281	
28806	*Gluconobacter*	*asaii/cerinus/oxydans*	0.288	
28807	*Gluconobacter*	*asaii/cerinus/oxydans*	0.336	

Strain	LMG	Genus	Species	Value	Closest relative (similarity)	Proposed name	Accession
28810		*Gluconobacter*	*asaii/ cerinus/ oxydans*	0.215			
28811		*Gluconobacter*	*asaii/cerinus/oxydans*	0.247			
28812		*Gluconobacter*	*asaii/cerinus/oxydans*	0.275			
28815		*Gluconobacter*	*asaii/cerinus/oxydans*	0.306			
28818		*Gluconobacter*	*asaii/cerinus/oxydans*	0.284			
28819		*Gluconobacter*	*asaii/cerinus/oxydans*	0.215			
28920		*Gluconobacter*	*asaii/cerinus/oxydans*	0.251			
BOX-cluster 3							
26185	**24371**	*Gluconobacter*	*asaii/cerinus/oxydans*	0.229	*Ruegeria mobilis* CIP 109181^T (100 %)	*Ruegeria mobilis*	AM905332
26052		*Gluconobacter*	*asaii/cerinus/oxydans*	0.251	*Ruegeria mobilis* CIP 109181^T (99.9 %)	*Ruegeria mobilis*	AM905333
26189	**24372**	*Paracoccus*	*denitrificans*	0.297			
26194		*Paracoccus*	*denitrificans*	0.587			
BOX-cluster 4							
26146^T	**24369^T**	*Paracoccus*	*denitrificans*	0.714	*Phaeobacter daeponensis* LMG 24139^T (98.1 %)	*Phaeobacter caerulea* sp. nov.	AM943630
26149	**24370**	*Paracoccus*	*denitrificans*	0.713	*Phaeobacter daeponensis* LMG 24139^T (98.1 %)	*Phaeobacter caerulea* sp. nov.	AM943631
26156		*Paracoccus*	*denitrificans*	0.637			
BOX-cluster 5							
26155		*Paracoccus*	*denitrificans*	0.649			
26162		*Paracoccus*	*denitrificans*	0.602	*Ruegeria pelagia* HTCC 2662^T (99.1 %)		AM944026
26166		*Paracoccus*	*denitrificans*	0.667			
BOX-cluster 6							
26157		*NO MATCH*					
26165		*Brevundimonas*	*vesicularis*	0.141			
Unique							
26159	**24366^T**	*Gluconobacter*	*asaii/cerinus/oxydans*	0.117	*Leisingera methylohalidivorans* LMG 23656^T (98.9%)	*Leisingera aquimarina* sp. nov.	AM900415
26145		*Gluconobacter*	*aspaii/cerinus/oxydans*	0.049	*Ruegeria pelagia* HTCC 2662^T (99.2 %)		AM944027
26147		*Paracoccus*	*denitrificans*	0.711			
26151		*Gluconobacter*	*asaii/cerinus/oxydans*	0.175	*Phaeobacter gallaeciensis* LMG 23163^T (97.3 %)		AM944028
26160		*Gluconobacter*	*asaii/cerinus/oxydans*	0.379			
26163		*Brevundimonas*	*diminuta*	0.492			
26188		*Paracoccus*	*denitrificans*	0.334			
28809		*Sphingomonas*	*paucimobilis*	0.336			
Cluster V							
n= 5							
75 %							
25591		*Kocuria*	*erythromyxa*	0.621			
25592		*Kocuria*	*erythromyxa*	0.692			
25598		*Kocuria*	*erythromyxa*	0.750	*Arthrobacter oxydans* ATCC 14358^T (99.7%)		AM944029
25582		*Micrococcus*	*luteus* GC subgroup D	0.648			
26174		*Brevibacillus*	*centrosporus*	0.228			

Cluster VI
n= 29
79.9 %

Strain	Genus	Species	Value	Reference
25499	Staphylococcus	xylosus	0.550	
25535	Staphylococcus	xylosus	0.568	
25510	Staphylococcus	xylosus	0.625	
25493	Staphylococcus	xylosus	0.632	
25657	Staphylococcus	cohnii	0.648	*Staphylococcus cohnii* CCUG 7322[T] (99%) AM944030
25658	Staphylococcus	xylosus	0.639	
25655	Staphylococcus	xylosus	0.603	
25531	Staphylococcus	xylosus	0.641	
25649	Staphylococcus	xylosus	0.650	
25536	Staphylococcus	xylosus	0.699	
25550	Staphylococcus	saprophyticus	0.900	
25647	Staphylococcus	saprophyticus	0.838	
25633	Staphylococcus	saprophyticus	0.870	
25638	Staphylococcus	saprophyticus	0.713	
25585	Staphylococcus	saprophyticus	0.844	
25607	Staphylococcus	saprophyticus	0.812	
25566	Staphylococcus	saprolyticus	0.830	
25634	Staphylococcus	saprophyticus	0.891	*Staphylococcus saprophyticus* LMG 13350[T] (98.2%)
25650	Staphylococcus	saprophyticus	0.843	
25538	Staphylococcus	saprophyticus	0.669	
25517	Paenibacillus	validus	0.489	
25627	Paenibacillus	validus	0.495	
25546	Paenibacillus	validus	0.369	
25548	Paenibacillus	validus	0.534	
25623	Paenibacillus	validus	0.489	
25564	Paenibacillus	validus	0.438	
26177	Staphylococcus	cohnii	0.833	
25639	Staphylococcus	xlosus	0.599	
25528	Bacillus	subtilis	0.591	

Cluster VII
n= 3
83.4 %

Strain	Genus	Species	Value	Reference
25596	Bacillus	GC subgroup 22	0.840	*Bacillus firmus* LMG 7125[T] (99.6%) AM944031
25600	Bacillus	GC subgroup 32	0.841	
25534	Bacillus	GC subgroup 22	0.488	

Cluster VIII
n= 5
78.6 %

ID	Genus	Species	Value		Identification
25542	Staphylococcus	warneri	0.789		
26148	Staphylococcus	epidermidis GC subgroup C	0.682		
25543	Staphylococcus	aureus GC subgroup C	0.716	AM944032	Bacillus pumilus LMG 7132^T (99%)
26191	Staphylococcus	epidermidis GC subgroup C	0.389		
26192	Staphylococcus	aureus GC subgroup C	0.589		

Cluster IX
n= 28
77.9 %

ID	Genus	Species	Value		Identification
25494	Bacillus	pumilus GC subgroup B	0.881		
25526	Bacillus	pumilus GC subgroup B	0.881		
25641	Bacillus	pumilus GC subgroup B	0.867		
25621	Bacillus	pumilus GC subgroup B	0.885		
25628	Bacillus	pumilus GC subgroup B	0.906		
25645	Bacillus	pumilus GC subgroup B	0.907		
25524	Bacillus	pumilus GC subgroup B	0.892		
25518	Bacillus	pumilus GC subgroup B	0.833		
25616	Bacillus	pumilus GC subgroup B	0.823		
25618	Bacillus	pumilus GC subgroup B	0.837		
25622	Bacillus	pumilus GC subgroup B	0.818		
25640	Bacillus	pumilus GC subgroup B	0.818		
25615	Bacillus	pumilus GC subgroup B	0.762		
25617	Bacillus	pumilus GC subgroup B	0.795	AM944033	Bacillus pumilus LMG 7132^T (99.8%)
25624	Bacillus	pumilus GC subgroup B	0.781		
25496	Bacillus	pumilus GC subgroup B	0.785		
25643	Bacillus	pumilus GC subgroup B	0.762		
25509	Bacillus	pumilus GC subgroup B	0.852		
25521	Kurthia	sibirica	0.599		
25613	Bacillus	pumilus GC subgroup B	0.768		
28055	Bacillus	megaterium GC subgroup A	0.609		
28739	Bacillus	megaterium GC subgroup A	0.572		
28766	Bacillus	megaterium GC subgroup A	0.416		
26164	Bacillus	pumilus GC subgroup B	0.711		
25539	Kurthia	sibrica	0.440		
28778	Bacillus	laevolacticus	0.305		
25495	Bacillus	megaterium GC subgroup A	0.706		
25497	Bacillus	megaterium GC subgroup A	0.703		

Cluster X
n= 30
71.9 %

Strain	Genus	Species	Value	Accession	Closest relative
25554	_Clavibacter_	_michiganensis_	0.563		
25560	_Nesterenkonia_	_halobia_	0.477		
25549	_Curtobacterium_	_flaccumfaciens betae/oortii_	0.530		
26176	_Clavibacter_	_sepedonicum_	0.669		
26172	_Clavibacter_	_sepedonicum_	0.679		
26186	_Microbacterium_	_hominis_	0.736		
25625	_Microbacterium_	_barkeri_	0.778		
25552	_Curtobacterium_	_flaccumfaciens_	0.926		
26178	_Microbacterium_	_saperdae/imperiale_	0.722		
26179	_Microbacterium_	_testaceum_	0.832		
25568	_Bacillus_	_coagulans_	0.407		
26183	_Bacillus_	_coagulans_	0.125		
25589	_Microbacterium_	_barkeri_	0.291		
25563	_Rothia_	_dentocariosa_	0.487		
26153	_Nesterenkonia_	_halobia_	0.442		
25547	_Microbacterium_	_saperdae_	0.444		
25540	_Microbacterium_	_saperdae_	0.483		
25635	_Kocuria_	_kristinae_	0.709		
25642	_Paenibacillus_	_polymyxa_	0.509		
25601	_Micrococcus_	_luteus GC subgroup B_	0.680		
26187	_Microbacterium_	_arabinogalactonolyticum_	0.362		
25604	_Kocuria_	_kristinae_	0.974		
28042	_Kocuria_	_kristinae_	0.796		
25545	_Paenibacillus_	_alginolyticus_	0.547		
25561	_Rothia_	_dentocariosa_	0.071		
25562	_Rothia_	_dentocariosa_	0.171		
26184	_Microbacterium_	_flavescens_	0.143		
25593	_Paenibacillus_	_validus_	0.031	AM944034	_Frigoribacterium faeni_ KMM 3907[T] (99%)
25533	_Microbacterium_	_flavescens_	0.163		
25586	_Microbacterium_	_flavescens_	0.502		

Cluster XI

n= 15

75.3 %

Strain	Genus	Species	Value	16S rRNA match	Accession
25523	Micrococcus	luteus GC subgroup C	0.766		
25636	Micrococcus	luteus GC subgroup C	0.874		
25597	Micrococcus	luteus GC subgroup C	0.820		
25599	Micrococcus	luteus GC subgroup C	0.812		
26181	Arthrobacter	aurescens	0.740		
25606	Micrococcus	luteus GC subgroup C	0.800		
25619	Micrococcus	luteus GC subgroup C	0.685		
25656	Arthrobacter	agilis	0.813		
25644	Arthrobacter	aurescens	0.885		
25626	Arthrobacter	agilis	0.663		
26170	Arthrobacter	agilis	0.800	*Arthrobacter agilis* LMG 17244[T] (99.5%)	AM944035
26171	Arthrobacter	agilis	0.800		
28708	Brevibacillus	choshinensis	0.283		
25637	Brevibacillus	choshinensis	0.172		
25595	Paenibacillus	pabuli	0.545		

Cluster XII

n= 4

74.5 %

Strain	Genus	Species	Value	16S rRNA match	Accession
26161	Zobellia	uliginosa	0.345		
26167	Zobellia	uliginosa	0.333		
28796	Zobelia	uliginosa	0.353	*Maribacter dokdoensis* DSM 17201[T] (99.8%)	AM944036
28790	Zobellia	uliginosa	0.570		

Cluster XIII

n= 6

76.6 %

Strain	Genus	Species	16S rRNA match	Accession
26150	NO MATCH		*Winogradskyella poriferorum* JCM 12885[T] (99.5%)	AM944037
26154	NO MATCH		*Winogradskyella poriferorum* JCM 12885[T] (99.6%)	AM944038
25511	NO MATCH			
26173	NO MATCH			
28026	NO MATCH			
25651	NO MATCH			

Cluster XIV
n= 23
79.2 %

ID	Genus	Species	Value	Match	Accession
28024	NO MATCH				
28059	Marinobacter	hydrocarbonoclasticus	0.245		
28046	Marinobacter	hydrocarbonoclasticus	0.082		
28043	Marinobacter	hydrocarbonoclasticus	0.406		
28044	Marinobacter	hydrocarbonoclasticus	0.534		
28034	Marinobacter	hydrocarbonoclasticus	0.435		
28066	Marinobacter	hydrocarbonoclasticus	0.426		
28029	Marinobacter	hydrocarbonoclasticus	0.414		
28028	Marinobacter	hydrocarbonoclasticus	0.343		
28037	Marinobacter	hydrocarbonoclasticus	0.360		
28022	Marinobacter	hydrocarbonoclasticus	0.515		
28045	Marinobacter	hydrocarbonoclasticus	0.402		
28038	Marinobacter	hydrocarbonoclasticus	0.482		
28057	NO MATCH				
28056	Marinobacter	hydrocarbonoclasticus	0.571	_Marinobacter hydrocarbonoclasticus_ DSM 8798^T (100%)	AM944039
28058	Marinobacter	hydrocarbonoclasticus	0.280		
28067	Marinobacter	hydrocarbonoclasticus	0.233		
28064	Marinobacter	hydrocarbonoclasticus	0.287		
28025	Marinobacter	hydrocarbonoclasticus	0.355		
28030	Marinobacter	hydrocarbonoclasticus	0.401		
26139	Marinobacter	hydrocarbonoclasticus	0.113		
28063	Marinobacter	hydrocarbonoclasticus	0.056		
28065	Marinobacter	hydrocarbonoclasticus	0.121		

Cluster XV
n= 7
75.6 %

ID	Genus	Species	Value	Match	Accession
25567	NO MATCH				
25569	NO MATCH				
25570	NO MATCH			_Exiguobacterium aurantiacum_ CCUG 44910^T (98.9%)	AM944040
25571	NO MATCH				
25587	NO MATCH			_Exiguobacterium acetylicum_ CCUG 32630^T (98.9%)	AM944041
25588	NO MATCH			_Exiguobacterium aurantiacum_ CCUG 44910^T (98.9%)	AM944042
25573	Staphylococcus	gallinarum GC subgroup A	0.079		

Cluster XVI
n= 6
78.5 %

Strain	Genus	Species	Value	Closest match	Accession
28040	Lysobacter	enzymogenes	0.223	*Idiomarina loihiensis* DSM 15497[T] (99.8%)	AM944044
28715	Stenotrophomonas	maltophilia	0.177		
28031	Lysobacter	enzymogenes	0.069		
28041	Lysobacter	enzymogenes	0.057		
28035	Lysobacter	enzymogenes	0.350		
28054	Stenotrophomonas	maltophilia	0.241		

Cluster XVII
n= 4
84.5 %

Strain	Genus	Species	Value	Closest match	Accession
28768	Vibrio	hollisae	0.025	*Marinobacter koreensis* DSM 17924[T] (99.7 %)	AM944524
28770	Vibrio	hollisae	0.063	*Marinobacter koreensis* DSM 17924[T] (99.7 %)	AM944523
25530	Vibrio	hollisae	0.134	*Marinobacter koreensis* DSM 17924[T] (99.7 %)	
28023	NO MATCH				

Cluster XVIII
n= 3
72.2 %

Strain	Genus	Species	Value	Closest match	Accession
25576	Vibrio	hollisae	0.173		
25501	Vibrio	hollisae	0.086		
26152	Aeromonas	hydrophila	0.160	*Alteromonas macleodii* LMG 2843[T] (98.8 %)	AM944044

Cluster XIX
n= 4
77.6 %

Strain	Genus	Species	Value	Closest match	Accession
28019	Pseudoalteromonas	nigrifaciens	0.245		
28020	Pseudoalteromonas	tetraodonis/nigrifaciens	0.360	*Pseudoalteromonas* sp. FR 1302 (98.9%)	AM944045
28021	Pseudoalteromonas	nigrifaciens	0.436		
28049	Pseudoalteromonas	nigrifaciens	0.486		

Cluster XX
n= 2
98.5 %

Strain	Genus	Species	Value	Closest match	Accession
28816	Halomonas	aquamarina	0.354	*Halomonas aquamarina* LMG 2853[T] (99.4%)	AM944046
28817	Halomonas	aquamarina	0.294		

n= 14

25648	Stenotrophomonas	maltophilia	0.153
26180	Cellulomonas	flavigena	0.333
25506	Stenotrophomonas	maltophilia	0.010
25520	Stenotrophomonas	maltophilia	0.059
26182	Brevibacillus	centrosporus	0.125
26175	Bacillus	cereus GC subgroup A	0.604
25602	Bacillus	spaericus GC subgroup A	0.424
25646	Flavobacterium	ferrugineum	0.133
25507	Nocardiodes	albus GC subgroup A	0.039
28070	NO MATCH		
25614	Rhodococcus	fascians GC subgroup A	0.617
25652	NO MATCH		
25544	Pseudomonas	fluorescens biotype F	0.578
25537	NO MATCH		

Addendum 2: Confocal laser scanning micrographs of the 14 days old EAB present on the surface of a cathodically polarized stainless steel electrode. LIVE/DEAD staining was performed and the photographs are taken at a magnification of 200fold (a, b).

The evolution of the current ($\mu A/cm^2$) is given as a logarithmic function of the growth time (c).
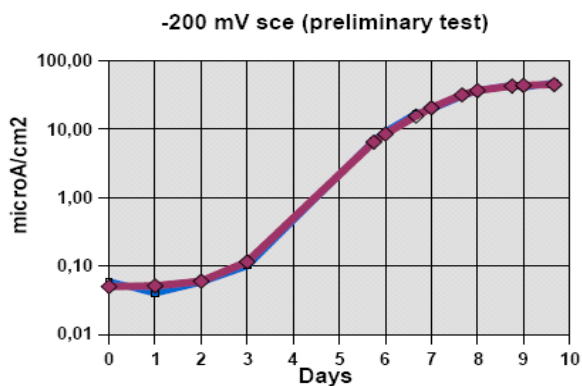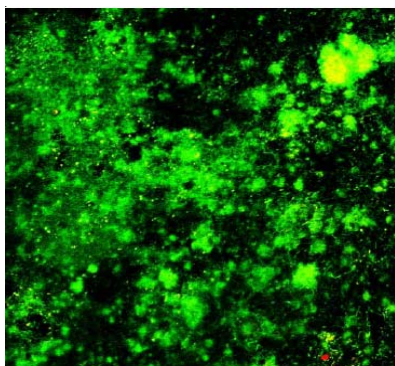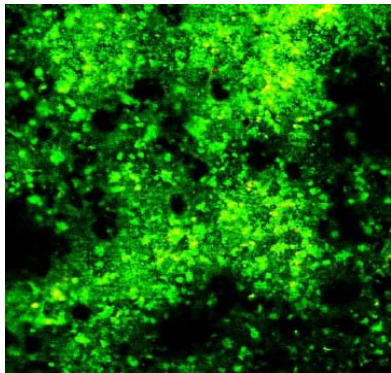
(c)

CURRICULUM VITAE

Curriculum vitae

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Personalia

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Born in Roeselare, Belgium, on January 3 1981

Educational background

1987-1993:   Primary School, Vrije Basisschool Houthulst

1993-1999:   St.-Aloysiuscollege, Diksmuide

1999-2004:   Ghent University

1999-2003:   Licentiate Biotechnology (graduaded cum laude)
Dissertation: "Diversity of heterotrophic bacteria from Antarctic lakes"
(graduaded magna cum laude)

2003-2004:   Master of Medical Molecular biotechnology
Dissertation: "Further characterization of the interaction between human TNF
and murine TNF muteins" (graduaded magna cum laude)

Scientific activities

2005   Monika Drabarek (master thesis)
"Identification and characterization of bacteria in an electroactive biofilm"

2006   Monika Kijanska (master thesis)
"Identification of *Roseobacter* clade affiliated isolates from a marine biofilm
by DNA-DNA hybridisation experiments"

2007   Till Dhaene (master-after-master thesis)

"Identification of bacteria in electroactive biofilms"

2007   Gwen Duytschaever (master thesis)

"*Samenstelling van de bacteriële huidflora van Salamandra salamandra terrestris*"

2008   Murad Ehliblu (master thesis)

"Genomic fingerprinting of *Staphylococcus* sp., isolated from a marine and an activated sludge electroactive biofilm"

Scientific outputs

Van Trappen, S., <u>Vandecandelaere, I.</u>, Mergaert, J., Swings, J. (2004). *Flavobacterium degerlachei* sp. nov., *Flavobacterium frigoris* sp. nov., and *Flavobacterium micromati* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. *International Journal of Systematic and Evolutionary Microbiology* 54, 85-92.

Van Trappen, S., <u>Vandecandelaere, I.</u>, Mergaert, J., Swings, J. (2004). *Gillisae limnae* gen. nov., sp. nov., a new member of the *Flavobacteriaceae* isolated from a microbial mat in Lake Fryxell, Antarctica. *International Journal of Systematic and Evolutionary Microbiology* 54, 445-448.

Van Trappen, S., <u>Vandecandelaere, I.</u>, Mergaert, J., Swings, J. (2004). *Algoriphagus antarcticus* sp. nov., a novel psychrophile from microbial mats in Antarctic lakes. *International Journal of Systematic and Evolutionary Microbiology* 54, 1969-1973.

Van Trappen, S., <u>Vandecandelaere, I.</u>, Mergaert, J., Swings, J. (2004). *Flavobacterium fryxellicola* sp. nov., and *Flavobacterium psychrolimnae* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. *International Journal of Systematic and Evolutionary Microbiology* 55, 769-772.

Parot, S., <u>Vandecandelaere, I.</u>, Délia, M.L., Vandamme, P., Bergel, A. (in press). Catalysis of electrochemical reduction of oxygen by bacteria isolated from electrochemically marine biofilm. *Electrochimica Acta*.

<u>Vandecandelaere, I.</u>, Nercessian, O., Segaert, E., Achouak, W., Mollica, A., Faimali, M., De Vos, P., Vandamme, P. (accepted). *Alteromonas genovensis* sp. nov., isolated from a

marine electroactive biofilm and emended description of *Alteromonas macleodii* Baumann *et al.* 1972 (Approved lists 1980). *International Journal of Systematic and Evolutionary Microbiology*.

Vandecandelaere, I., Nercessian, O., Segaert, E., Achouak, W., Faimali, M., Vandamme, P. (accepted). *Ruegeria scottomollicae* sp. nov., isolated from a marine electroactive biofilm. *International Journal of Systematic and Evolutionary Microbiology*.

Vandecandelaere, I., Segaert, E., Mollica, A., Faimali, M., Vandamme, P. (accepted). *Leisingera aquamarina* sp. nov., isolated from a marine electroactive biofilm and emended description of *Leisingera methylohalidivorans* Schaefer *et al.* 2002, *Phaeobacter daeponensis* Yoon *et al.* 2007 and *Phaeobacter inhibens* Martens *et al.* 2006. *International Journal of Systematic and Evolutionary Microbiology*.

Vandecandelaere, I., Nercessian, O., Segaert, E., Achouak, W., Mollica, A., Faimali, M., Vandamme, P. (submitted). Bacterial diversity of a marine electroactive biofilm. for publication, *Environmental Microbiology*.

Vandecandelaere, I., Segaert, E., Vandamme, P. (submitted). *Phaeobacter caerulea* sp. nov., isolated from a marine electroactive biofilm. *International Journal of Systematic and Evolutionary Microbiology*.

Vandecandelaere, I., Nercessian, O., Segaert, E., Achouak, W., Mollica, A., Faimali, M., Vandamme, P. (submitted). *Nautella italica* gen. nov., sp. nov., isolated from a marine electroactive biofilm. *International Journal of Systematic and Evolutionary Microbiology*.

Vandecandelaere, I., Strathman, M., Wingender, J., Segaert, E., Flemming, H.C., Vandamme, P. (in preparation). Bacterial diversity of an electroactive biofilm generated in the anoxic zone of a wastewater treatment plant. *Environmental Microbiology*.
Poster presentations

Vandecandelaere, I., Nercessian, O., Segaert, E., Conrod, S., Achouak, W., De Vos, P., Vandamme, P. Polyphasic characterization of a marine biofilm grown on a stainless steel surface. 11th International Symposium on Microbial Ecology, August 20-25 2006, Vienna, Austria.