THE DIVERSITY AND BIOTECHNOLOGICAL APPLICATION OF MARINE MICROBES PRODUCING OMEGA-3 FATTY ACIDS

BY

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Copyright statement

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Abstract

Omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) play a role in the modulation and prevention of human diseases, in particular cardiovascular diseases. The omega-3 family is found mainly in fish, of which wild stocks are becoming limited. Therefore production of omega-3 PUFAs by marine microbes may provide an alternative source of such componds. The diversity of marine microbes was studied using 16S/18S rRNA gene sequencing of different marine biota with 1500 bacterial strains and 50 microalgae were isolated. The diversity of culturalbe microorganisms inhabiting Mid-Atlantic Ridge (MAR) non-vent sediments was examined for the first time in this area with findings of high diversily of Gram-positive strains, good production of squalene by an unusual strain *Bacillus* sp. MAR089 and the highest yield of EPA ever recovered from strain *Shewanella* sp. MAR441. North Sea sponge associated *Vibrio* sp. strain NSP560 produced considerable levels of EPA, whereas no PUFAs producers were found from tropic Caribbean marine sponge associated bacteria. *Photobacterium* sp. strain MA665, isolated from the coast of North Sea, was described for the first time of this genus and could be cultured easily under atmospheric conditions with appreciable levels of EPA with up to 25 % of total fatty acids (TFA) (or 10.6 mg g^{-1} in dried cell). Strain MAR441 was identified as a new species, designated as *Shewanella dovemarina* sp. nov. (Type strain MAR441^T). The level of EPA production of strain MAR441 has been optimized by varying fermentation conditions, and 15-25 % EPA of TFA (or 17-30 mg g^{-1} in dried cell) could be achieved with 40 % improvement. In order to understand the PUFAs biosynthesis pathways and better predict the maximum EPA production, EPA gene clusters (*pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE*) were cloned and sequenced from the following three species *Shewanella*, *Vibrio* and *Photobacterium*. Great potential was found in marine algae *Phaeodactylum tricornutum* strain M7 with lipid content of 10 % in dry wt biomass and 22-30 % EPA of TFA when it was cultured outdoors under local weather conditions in UK. Under anaerobic conditions, strain MAR441 contained less amount of EPA and produced electricity of ~100 mW m^{-2}. Enhanced electricity production using artificial consortia of estuarine bacteria grown as biofilms was observed with power generation of ~200 mW m^{-2}. In conclusion, bacteria taxonomic resolution based on complete cell fatty acid composition is possible and marine microbes with considerable production of EPA could be potential candidates for industrial production of PUFAs.

Memorandum

Except where acknowledgement is given this thesis is the unaided work of the author. The material presented has never been submitted to Newcastle University or to any other educational establishment for purpose of obtaining a higher degree.

March 2011 Jinwei Zhang

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Abbreviations

AA	Arachidonic acid
ACL	Average chain length
ACP	Acyl carrier protein
ARA	Arachidonic acid
AT	ACP transfer reaction
AD	Acyltransferase domains
ATP	Adenosine-5'-triphosphate
ALA	α-linolenic acid
BCFA	Branched chain fatty acids
BLAST	Basic local alignment search tool
CAD	coronary artery disease
CAGR	Compound annual growth rate
CDS	Coding sequence
CFB	*Cytophaga–Flavobacterium–Bacteriodes*
CL	Chain length factor domain
CV	Cyclic voltammetry
DGGE	Denaturing gradient gel electrophoresis
DGLA	Dihomo-γ-linolenic acid
DH/I	Dehydratase/isomerase reactions
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DPG	Diphosphoglyceride
EFA	Essential fatty acids
EIS	Electrochemical impedance spectroscopy
EPA	Eicosapentaenoic acid
ER	Enoyl reduction reactions
EA	Erucic acid
FAM	Fumaric acid medium
FAME	Fatty acid methyl ester
FAO	The Food and Agriculture Organization
FAS	Fatty acid synthase
FCM	Ferric citrate medium
FFA	Free fatty acids
GC	Gas chromatography
GL	Glycolipids
GLA	Gamma-linolenic acid
GPI	Glycosylphosphatidylinisoto
HD	3-hydroxydecanoyl-ACP dehydratases
HDL	High-density lipoprotein
IR	Internal resistance
KR	3-ketoacyl-ACP reductase
KR	Ketoacyl reduction reaction
KS	3-ketoacyl synthase

LA	Linoleic acid
LC-FA	Long chain fatty acids
LCPUFA	Long chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
LNA	α-Linolenic acid
LP	Lipid particle
LPE	Lysophosphatidylethanolamine
MA	Mead acid
MAR	Mid-Atlantic Ridge
MB	Marine 2216E broth
MCA	Multiple correspondence analysis
MEGA	Molecular evolutionary genetics analysis
MFC	Microbial fuel cells
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
MVSP	Multi-Variate tatistical Package
NAC	North Atlantic Current
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NCBI	The National Center for Biotechnology Information
NEFA	Non-esterified fatty acid
NL	Neutrallipids
NTG	N-methyl-N′-nitro-N-nitrosoguanidine
ORF	Open reading frames
PA	Palmitic acid
PCA	Principal component analysis
PCB	Polychlorinated biphenols
PCO	Principal coordinates analyses
PE	Phosphatidyl ethanolamine
PG	Phosphatidyl glycerol
PKS	Polyketide synthase
PL	Phospholipids
PPTase	Phosphopantetheinyl transferase
PUFA	Polyunsaturated fatty acids
RFLP	Restriction fragment length polymorphism
SA	Stearidonic acid
SAIW	Sub-Arctic Intermediate Water
SCFA	Saturated straight chain fatty acids
SE	Steryl esters
SEM	Scanning electron microscopy
SFA	Saturated fatty acid
SOC	Single cell oils
TEM	Transmission electron microscopic
TFA	Total fatty acids
TLC	Thin-layer chromatography
TLE	Total lipid extract
TTC	Triphenyltetrazolium chloride

UPGAM	Unweighted pair group average method
ZB	Zobell's broth
OA	Oleic acid
SDA	Stearidonic acid
HPA	Heneicosapentaenoic acid
TAG	Triacylglycerol
VLC-FA	Vary long chain fatty acid

Chapter 1. Introduction

1.1 Overview

This section presents a brief overview of an important topic that is addressed in this thesis, more detailed introductions to each topic are presented later in this chapter.

The Inuit, or Eskimo, people of Alaska and Greenland suffer low levels of cardiovascular disease, particularly heart disease, although they often appear to be relatively fat. The overall incidence of cancer in Eskimos is also lower than the Western population (Allport, 2006). Their dietary items are mainly fat and meat of seal, walrus, whale, caribou and fish, which are generally eaten uncooked (Ho et al., 1972). Most of the food has a high cholesterol content and was discovered to be rich in particularly beneficial omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) (Ho et al., 1972; Bersamin et al., 2007).

The flood of scientific information available on EPA and DHA has significantly buoyed the credibility of PUFAs as essential dietary components of many animals. These fatty acids serve as precursors for many hormone and hormone-like regulatory molecules (Lauritzen et al., 2001; Sauer et al., 2001), which could benefit cardiac-health people (Din et al., 2004), or those with a high risk of cardiovascular disease (Angerer and von Schacky, 2000; Kris-Etherton et al., 2003b; Yamagishi et al., 2008; Chang et al., 2009; Lavie et al., 2009), and Alzheimer's disease (Connor and Connor, 2007; Yang et al., 2010). Heightened consumer awareness of the value of omega-3s has increased the growth in demand for marine and algae oil omega-3 products. For example, new analysis from Frost & Sullivan on the European omega-3 market found that annual sales of omega-3 oil were above $580 million in 2008 estimated to reach $840 million in 2013, with an annual grow rate of 10% (http://www.food.frost.com, February 08th, 2011).

The number of aquaculture industries throughout the world is increasing rapidly. The Food and Agriculture Organization (FAO) for instance, has reported a sustained annual growth rate for over 20 years of around 10% increase in volume per annum, and this sustained increase is also causing equally high demands for the supply of fish oil and

fish meal for fish feed diet in aquaculture (FAO/WHO Expert committee, 1994). The proportion of underexploited or moderately exploited marine fishery resources has declined linearly from 40 percent in the mid-1970s to 20 percent in 2007. It is for the first time in decades that real prices of fish have increased (FAO, 2009). The development of alternative lipid sources such as plant oils has been identified as an urgent requirement for industry in order to replace marine fish meal and fish oil (Sargent et al., 2002a). However, plant lipids may not be ideal as an alternative resource for 'marine lipids and fatty acids' as they are unable to produce long-chain PUFAs (LC-PUFAs), such as EPA and DHA, and generally contain omega-6 fatty acids, such as γ-linolenic acid (GLA, 18:3ω6). Therefore, the development of further alternative sources of omega-3 is needed.

All living organisms: micro-organisms, higher plants and animals contain lipids. They occur in all cell types and contribute to cellular structure, provide stored fuel and participate in many biological processes, ranging from transcript of vital metabolic pathway to physiological responses. A major role of lipids and fatty acids is to provide organisms with metabolic energy. However, fatty acids play other roles and the precise fatty acid constituents of the total lipids in a diet may be important in this respect. Animals require the essential fatty acids linoleic acid (LA, 18:2ω6) and α-linolenic acids (ALA, 18:3ω3) in their diet to act as precursors for the ω-3 and ω-6 pathways in order to create long chain C_{20} and C_{22} PUFAs (Gill and Valivety, 1997; Napier, 2002). Plants, on the other hand, synthesize fatty acids to produce LA and ALA, without subsequent synthesis of long carbon chains (Napier, 2002). In general PUFAs are synthesized naturally by an array of desaturase and elongase enzymes present throughout the animal and plant kingdoms with much diversity in microorganisms such as algae, fungi and bacteria (Gill and Valivety, 1997; Napier, 2002). Algae, fungi, bacteria, insects and invertebrates are considered the primary producers of PUFAs, with plants and animals lacking some requisite enzymes making them unable to produce fatty acids over C_{18} (Ratledge, 1994). For instance, certain fatty acids are essential for reproduction in animals, and since these can not be synthesized by the animal itself they must therefore be obtained from the diet. Some fatty acids such as arachidonic acid (AA, 20:4ω6), EPA and DHA are required in brood stock diets for the production of high quality eggs and larvae (Sargent et al., 1999).

The fatty acid composition in particular lipid classes is frequently distinctive and can vary markedly between species and tissues (Sargent et al., 2002b; Zhukova and Titlyanov, 2003). It may be dependent on the nature of the diet of the animal concerned. The normal growth and development of several marine fish larvae depend on the supplementation of omega-3 PUFAs in the diet, particularly EPA and DHA, for which marine fish oils are so highly prized and on which their health-benefit claims are based (Rodríguez *et al.*, 1998). This is a little-appreciated fact that EPA and DHA are not synthesized by the fish, but are produced by marine microorganisms on which the fish either feed or have residing within their intestines (Tocher et al., 2006). It has been assumed that fatty acids in marine food webs come only from microalgae, such as diatoms (Pohnert, 2005). However, it has been recently reported that there are bacteria in the marine environment capable of the biosynthesis of LC-PUFAs, including EPA and DHA (Nichols and McMeekin, 2002). Such bacteria have been found in association Arctic invertebrates (Jostensen and Landfald, 1997) and in polychaetes and other invertebrates (Yazawa et al., 1992; Hirota et al., 2005). Therefore, bacterial PUFA producers may have an important role in the marine food web ecosystems. And finding alternative lipids and fatty acid sources to replace marine fish meal and fish oil is one of the main ways to ensure 'sustainable aquaculture'.

1.2 Structural diversity and physiological functions of fatty acids and lipids

1.2.1 Fatty acid nomenclature

In chemistry or biochemistry, fatty acids are hydrocarbon chains with a carboxyl group at one end, known as the delta (Δ) end, and a methyl group at the opposite end (methyl, n). The general structure of a fatty acid is shown as Figure 1.1. Each carbon atom in a fatty acid chain is either saturated (all four bonds are linked to a separate carbon, hydrogen, or oxygen), or unsaturated (2 adjacent carbons share two bonds) (IUPAC, 1993).

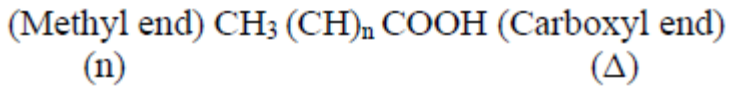

Figure 1.1 The general structure of a fatty acid.

Most naturally occurring fatty acids are unbranched and have a chain of an even number of carbon atoms (4-28) (Figure 1.2 and Table 1.1). They may have various degrees of unsaturation (0-6 double bonds). According to the international nomenclature, unbranched fatty acids can be described in short by y : x, where y represents the number of carbon atoms and x the number of double bonds. Carbon atoms are counted from the carboxyl terminus. The positions of the double bonds are represented by delta (Δ) and a number (IUPAC, 1993). For example, oleic acid (Δ9, 18:1) denotes a fatty acid with 18 carbon atoms and with one double bound at position 9 as counted from the carboxyl terminus (Ackman, 1989; Hagen and Auel, 2001).

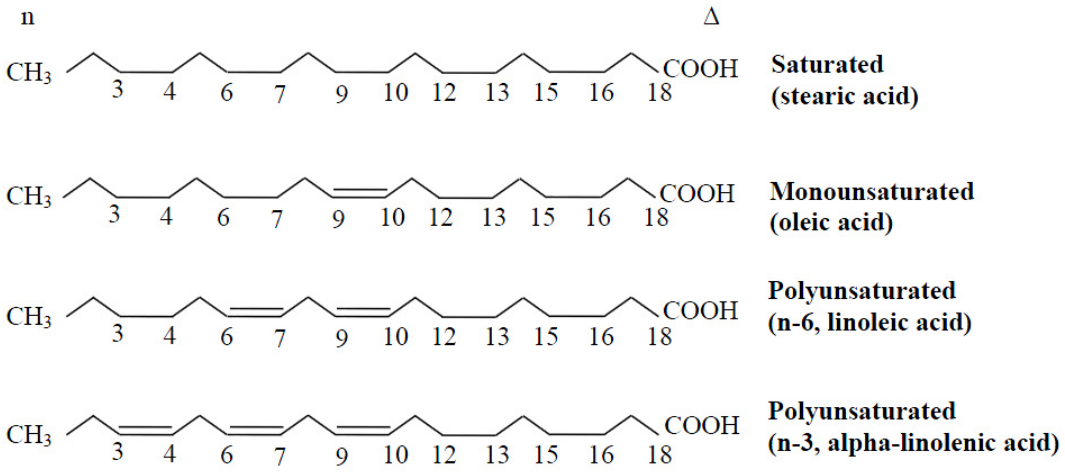

Figure 1.2 Schematic representations to show a saturated (stearic), monounsaturated (oleic), n-6 polyunsaturated (linoleic), and n-3 polyunsaturated (α-linolenic) fatty acid. Adapted from reference (IUPAC, 1993).

Table 1.1 Some naturally occurring fatty acids[a]

Common name	Systematic name*	Short name
Saturated fatty acids		
Lauric acid	Dodecanoic acid	12:0
Myristic acid	Tetradecanoic acid	14:0
Palmitic acid	Hexadecanoic acid	16:0
Stearic acid	Octadecanoic acid	18:0
Monounsaturated fatty acids		
Palmitoleic acid	Δ9-Hexadecenoic acid	Δ9 16:1
Oleic acid	Δ9-Octadecenoic acid	Δ9 18:1
ω-6 Polyunsaturated fatty acids		
Linoleic acid (LA)	Δ12-Octadecadienoic acid	ω-6 18:2
γ-Linolenic acid (GLA)	Δ6, Δ9, Δ12-Octadecatrienoic acid	ω-6 18:3
Arachidonic acid (ARA)	Δ5, Δ8, Δ11, Δ14-Eicosatetraenoic acid	ω-6 20:4
ω-3 Polyunsaturated fatty acids		
α-Linolenic acid (LNA)	Δ9, Δ12, Δ15-Octadecatrienoic acid	ω-3 18:3
Eicosapentaenoic acid (EPA)	Δ5, Δ8, Δ11, Δ14, Δ17-Eicosapentaenoic acid	ω-3 20:5
Docosahexaenoic acid (DHA)	Δ4, Δ7, Δ10, Δ13, Δ16, Δ19-Docosahexaenoic acid	ω-3 22:6

[a]Adapted from reference (IUPAC, 1993); *All double bonds are in *cis*-configuration.

The configuration of double bonds, generally assumed as *cis* (Z) in natural compounds, must be indicated in other cases. The positions of a methyl branch or another group is indicated by the number of the carbon atom on which the chain is substituted, such as 10-methylhexadecanoic acid could be simply as 10-Me-16:0; 2-hydroxydocosanoic acid (2-OH-22:0) and 6-bromo-5,9-heptacosadienoic acid (6-bromo-5,9-27:2) (Berge and Barnathan, 2005).

Long-chain PUFAs have more than one double carbon bond and 18 or more carbon atoms. They are classified according to the position of the first double bond as counted from the methyl end. An omega-3 PUFA has its first double bound at position 3 as counted from the methyl end. As a synonym of ω, the symbol n is often used to classify PUFAs (Figure 1.3). Throughout this thesis, an abbreviated nomenclature is used to designate the structures of fatty acids. For example, docosahexaenoic acid (DHA), the common term for all-*cis*-4,7,10,13,16,19-docosahexaenoic acid, can be designated as Δ4,7,10,13,16,19-22:6 or simply as n-22:6ω3, indicating a fatty acid with 22 carbon atoms and 6 double bonds, the first double bond being found on the third carbon atom from the methyl end group. Trivial names associated with omega-3 series include linolenic (18:3), moroctic and stearidonic (18:4), timnodonic (20:5), clupanodonic (22:5) and cervonic (22:6), though not all of these remain in use.

In biological systems, fatty acids are mostly encountered as components of lipids. Lipids are organic compounds that are insoluble in water and soluble in organic solvents. Chemically, lipids vary to such a great extent that no structural definition is available (Gurr and Harwood, 1991).

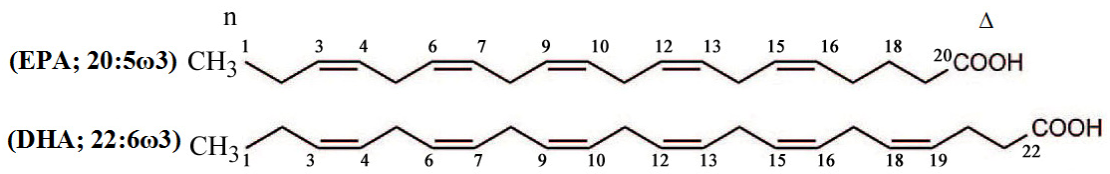

Figure 1.3 Schematic representation of eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) to show the positions of the unsaturated (double) bonds.

1.2.2 Introduction to lipids

There are two general types of lipids: the simple and the complex lipids. The simple lipids, for example cholesterol and other steroids, do not have the ester linkages and cannot be hydrolysed into smaller molecules. The complex lipids, such as the triacylglycerols, fats, oils, and waxes that contain an ester linkages that can be hydrolysed to yield smaller molecules (McMurry, 1988). Glycerophospholipids or phosphoglycerides are glycerol-based phospholipids, which core with fatty acids, are the most familiar lipids. The acylglycerols have a glycerol backbone linked to one, two or three fatty acids via ester bonding, yielding mono-, di- and triacylglycerols, respectively (Fahy et al., 2009). Phosphoglycerols consist of a glycerol backbone to which two fatty acids and a phosphate group could be esterified. Furthermore, phosphoglycerols are very diverse as the phosphate group, which can be attached to a great variety of groups of membrane phospholipids (Gurr and Harwood, 1991).

Fats are lipid materials that stay solid at room temperature, whereas oils are liquid. Natural oils and fats are composed mainly of triacylglycerols (Figure 1.4). Therefore, they can be used to denote triacylglycerols. However, other components, such as monoglycerides and diacylglycerols, phospholipids, waxes, steroids and carotenoids are also included in small amounts in natural fats and oils. (Stauffer, 1996).

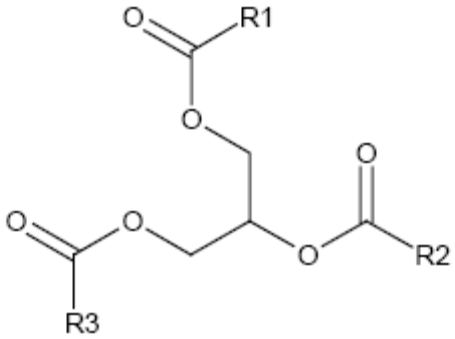

Figure 1.4 Schematic representation of triacylglycerol with in the middle the glycerol backbone and on the outside the fatty acyl groups (R1-CO, R2-CO and R3-CO).

1.2.3 Biological functions of lipids

Lipids are a group of molecules, including fats, waxes, sterols, phospholipids, monoglycerides, diglycerides, triacylglycerols and others, which naturally occur in all organisms. These lipid moleculaes play important roles as storage lipids, structural components of cell membranes and in signaling reactions, which are indispensable for

cell growth and survival. Triacylglycerols are major storage lipids that accumulate in the cells. They have a high free-energy content and tend to form aggregates in water, allowing for compact unhydrated intracellular packing (Stryer, 1998). During the period of food plenty, triacylglycerols can be stored as carbon sources or storage lipids, whereas in times of starvation or strong exercise this storage can be used. Mammals store triacylglycerols mainly in adipose tissue and can excrete them via milk as an energy source for newborn individuals. In plants, triacylglycerols can be stored in the seeds as energy reserves for the germination process (Kattner and Hagen, 1995).

The acylglycerols play no or a little part in membrane structure. The lipids that contribute to the structure and function of biological membranes are called structural lipids. Widely distributed structural lipids include phospholipids, glycolipids (lipids containing a sugar constituent), sphingolipids and steroids. Structural lipids contain a (long) hydrophobic and a (shorter) hydrophilic part. They can form sheet-like double layers where the hydrophobic and hydrophilic parts are oriented to the inside of the layer and to the external water phase, respectively. In addition to lipid bilayers, biological membranes contain about 50% proteins by weight. The formation of lipid bilayers is an energetically preferred process when the glycerophospholipids are in an aqueous environment (Stryer, 1998). Together, structural lipids and membrane proteins form the boundaries of all living cells and intracellular organelles (Gurr and Harwood, 1991).

1.2.4 Physiological and medical effects of omega-3, -6 and -9 fatty acids

Omega-3 fatty acids have three important physiological functions. First, they serve as major components of biological membranes and are important in membrane structure and function (Gawrisch *et al.*, 2003). DHA is found with high concentrations in lipids of the retina, brain and sperm (Nettleton, 1993). Second, they can alter gene expression, by down-regulating and up-regulating some enzymes (Kitajka et al., 2004; O'Shea et al., 2010). Third, EPA has an important role in regulating eicosanoid production from arachidonic acid (AA, 20:4ω6) by competition for the metabolizing enzymes (Calder, 2005). The eicosanoids derived from EPA and AA show different properties, and it is important to keep them in balance appropriately. Mostly, EPA is considered to have a functional role, operating through its metabolites; whereas DHA has a structural role (Kidd, 2007).

The importance of omega-3 PUFA for human health is mostly in its physiological effects. It is known that blood pressure is reduced by DHA, with EPA having a lesser effect, that plasma total and low-density lipoprotein (LDL) cholesterol levels and serum triacylglycerol levels are reduced, and that thrombosis risk is decreased (Theobald *et al.*, 2004). The major impact of omega-3 PUFA on coronary artery disease (CAD) risk reduction appears to be the result of its antiarrhythmic effect, by reducing the risk of ventricular fibrillation and subsequent sudden cardiac death (Reiffel and McDonald, 2006). Omega-3 PUFAs are incorporated into myocardial cell membranes (Harris et al., 2004), in which they serve as a potent inhibitor of voltage-gated Na^+/H^+ channels in cardiac cardiomyocytes, as a result of preventing calcium overload by maintaining L-type calcium channels during periods of ischemic stress (Leaf et al., 2003; Harris, 2010). This effect increases the ventricular refractory period and the electrical threshold required to induce an action potential-depolarization, making the heart less vulnerable to ventricular arrhythmias as has been shown in canine models (Kris-Etherton et al., 2003a). Epidemiologic studies showed that the relative risk for sudden cardiac death is significantly reduced with increasing levels of blood and red blood cell fatty acid levels (Burr et al., 1989; Siscovick et al., 1995; Albert et al., 1998).

Omega-3 fatty acids have the first double bond between the 3rd and 4th carbon atoms from the methyl end, which makes their molecular structure the most flexible in all fat. When the cell membrane is constructed with omega-3 fatty acids, normal cell functions and division occur easily. In contrast, omega-6 fatty acids have the first double bond between the 6th and 7th carbon molecule, which makes their shape is twisty in a cell membrane, as result of flexible of the membrane. This allows for the proper transport of materials though out/in the cell, and for normal cell division. In healthy situations, omega-3 fatty acids compete with omega-6 fatty acids for the same materials, keeping the inflammatory response from continuing when it is not needed. Omega-6 fatty acids are released when a cell is injured (Nettleton, 1995).

The omega-6 PUFA arachidonic acid (AA, 20:4ω6) is the precursor for eicosanoids like thromboxanes, prostaglandins and leukotrienes (Das, 2006). Eicosanoids are molecules that are active in regulation of critical biological functions by altering cell activities. The eicosanoids occur and are biologically active in virtually every mammalian tissue (Stanley and Miller, 1998). Furthermore, PUFAs are essential structural components of phospholipids in cell membranes, where they affect membrane characteristics and

functions such as fluidity, electrolyte transport and hormonal and immunological activities.

The omega-9 fatty acids have the first double bond between the 9^{th} and 10^{th} carbon atoms from the methyl end. Some omega-9 fatty acids are common components of animal fat and vegetable oil. Two omega-9 fatty acids important in industry are: oleic acid (OA, 18:1ω9), a main component of olive oil and other monounsaturated fats; Erucic acid (EA, 22:1ω9), found in rapeseed, wallflower seed, and mustard seed. Rapeseed with high EA content is grown for commercial use in paintings and coatings as a drying oil. Unlike omega-3 and omega-6 fatty acids, omega-9 fatty acids are not classed as essential fatty acids (EFA). This is because they can be synthesized by the human body from unsaturated fat, and are not participating in producing eicosanoids due to the lack of an omega-6 double bond (Phinney *et al.*, 1990).

1.2.5 Omega-3 fatty acids and their metabolism in plants and animals

In the cytoplasm of plant cell, desaturation converts oleate in the form of a phosphatidylcholine to linoleate with the action of a Δ12 desaturase, and converts linoleate as its monogalactosyldiacylglycerol derivative to linolenate by a Δ15 desaturase. The additional double bonds have the *cis* configuration and are in a methylene-interrupted relation to other double bonds. The 1,4-diene unit is characteristic of the common PUFAs and is to be distinguished from the 1,3 (conjugated) systems found in carotenoids and some less-common fatty acids, and the 1,5 system found in polyisoprenoids (Napier, 2007).

In animal systems, a Δ6 desaturase is common but less so in the plant systems. However, it is active in the biosynthesis of γ-linolenic acid (GLA, 18:3ω6) from linoleate and of stearidonic acid (SA, 18:4ω3) from α-linolenate (Napier, 2007). The C_{20} and C_{22} polyenes characterize animal systems, in particular fish lipids, and very rare in plant systems. However, the leaves, stem, and whole plant of purslane (*Portulaca oleracea*) has been reported to be a vegetable source of omega-3 acids, with low levels of the n-20:5ω3, n-22:5ω3 and n-22:6ω3 acids (Omara-alwala *et al.*, 1991).

Plants contain relatively low levels of monounsaturated or saturated very long chain FA with 20 or more carbons (Voelker and Kinney, 2001). Oilseed crops rather than borage and primose seed have been chose for producing GLA and SDA due to the good

cultivate and high yielding. The expression of a Δ6-desaturase gene is required to produce GLA or SDA in oilseed crops (Napier, 2007). Although the production of important LC-PUFA such as ARA, EPA and DHA in plant systems is a challenge, the proof of concept has been provided in transgenic plants. The model plant species *Arabidopsis thaliana* was sequentially transformed with constitutively expressed genes including a Δ9 elongase from the algae *Isochrysis galbana*, a Δ8 desaturase from the protist *Euglena gracilis* and a fungal Δ5 desaturase from *Mortierella alpina*. The accumulation of 7 % ARA and 3 % EPA in total lipids of leaf tissues (Qi et al., 2004). However, much remains to be done before an economically viable system can be developed.

In mammals, saturated FA and monounsaturated FA of the omega-9 and omega-7 series can be synthesized *de novo* from acetyl CoA. However, the omega-6 and omega-3 PUFA cannot be synthesized by mammalian cells due to the absence of the Δ12 and Δ15 desaturase enzymes. These enzymes required for the insertion of a double bond at the 12^{th} and 15^{th} carbons from the carboxyl terminus of 18 carbon chain fatty acids respectively (Innis, 2003; Innis, 2005). Linoleic acid (LA, 18:2ω6) and α-linolenic acid (ALA, 18:3ω3) are the precursors to LC-FA, more highly unsaturated omega-6 and omega-3 fatty acids, respectively; these fatty acids, containing 20 or more carbons, are sometimes referred to as long chain polyunsaturated FA (Innis, 2005). Because the omega-3 and omega-6 PUFA are needed, but cannot be synthesized (Wiese et al., 1958; Benolken et al., 1973; Wheeler et al., 1975; Lamptey and Walker, 1976), they must be obtained from the diet and are termed essential dietary nutrients (Warwick *et al.*, 2007). Once LA and ALA have been ingested they suffer a number of fates, one of which is metabolism by chain-extension and desaturation between existing double bonds and the carboxyl group to produce important polyunsaturated C_{20} and C_{22} acids including ARA, EPA and DHA. It is important to recognize that omega-6 fatty acids cannot be converted to omega-3 fatty acids, and vice versa (Laposata, 1995).

Nutritional attitudes to PUFA have changed over the years. First, they were considered as a single group, distinct from saturated and monounsaturated acids, then they were subdivided into omega-6 and omega-3 PUFA and the ratio of these two was considered as a useful dietary index. For examples, there is some consensus that the PUFA intake should be at least 3 % and preferably 8-23 % of the total lipid intake (Gill and Valivety, 1997). The British Nutrition Foundation recommended a omega-6 to omega-3 PUFA

ratio between 5:1 and 3:1 (British Nutrition Foundation, 1992). Today this concept is considered to be flawed, and separate recommendations are increasingly being given for ALA and for EPA/DHA. ALA is almost entirely of plant origin, while EPA and DHA mostly come from animal sources, particularly fish, and partially from marine biomass, such as microorganisms.

1.2.6 Dietary sources of omega-3 fatty acids

1. *α*-linolenic acid (ALA, 18:3ω3)

Among the so-called commodity vegetable oils, only three contain ALA in significant amounts. These are: soybean oil, with 8 % ALA and an annual production of 35.9 million tones in 2010; rapeseed/canola oil, with 10 % ALA and an annual production of 20.4 million tones in 2010; and linseed oil, with 50 % ALA and an annual production of 0.6 million tones in 2010. Linseed oil is used mainly for industrial purposes and only small amounts are used for human dietary purposes, either as the rapeseen itself or as cold-pressed oil. As a consequence, soybean and rapeseen oils are the major dietary sources of ALA (Gunstone, 2011). In 2004-2005, the agriculture industry produced 3.5 million tones of ALA, mainly from soybean oil (1.9 million tones) and rapeseen/canola oil (1.4 million tones), but the true amount available for dietary intake is less than this because of its high-temperature deodorization to acids with *trans* unsaturated FA (Gunstone, 2005). Also, varieties of both soybean and rapeseed oils with reduced levels of ALA are becoming more widely available (Ghazali et al., 2003; Su et al., 2003). These are attractive to the food industry because they can be used for frying purposes without the need for prior brush hydrogenation to lower the level of ALA. Thus, additional consumption of other sources of ALA, in particular LC-PUFAs, such as EPA or DHA, is necessary as compensations regarding to promote better health care.

2. Stearidonic acid

Stearidonic acid (SDA, 18:4ω3) is the omega-3 equivalent of *γ*-linolenic acid (GLA; 18:3ω6) and is available as a dietary supplement. Only a few seed oils contain SDA but *Echium plantagineum* is cultivated as a source of this acid. With over 60 % of the acids containing three or four double bonds the oil is highly unsaturated. Another convenient source of SDA is the more readily available blackcurrant seed oil, even though it contains only 2.5-3 % of this acid. Stearidonic acid is the first metabolite in the conversion of ALA to EPA and DHA, and arguments for the inclusion of GLA in

dietary supplements can also be applied to SDA (Clough, 2001b; Clough, 2001a). Soybeans with Δ6 desaturase (from borage oil) and Δ15 desaturase were reported to produce an oil containing around 30 % of both ALA and SDA (Eckert et al., 2006).

3. EPA and DHA from fish oils

Long-chain PUFA in the human diet are mainly obtained by consuming fatty fish, or supplements of fish oil enriched in EPA and/or DHA, or by eating food to which fish oil has been added. Table 1.2 contains similar information for a range of commercial fish oils. The sum of EPA, docosapentaenoic acid (DPA, 22:5ω3) and DHA ranging from 11 % to 33 %, with DPA present only as a minor component. The major LC-PUFA is sometimes EPA, sometimes DHA, and sometimes both are found at similar levels (Hjaltason and Haraldsson, 2006a; Hjaltason and Haraldsson, 2006b). DPA is present in fish oils at only low levels, but levels are somewhat higher (up to 5 %) in the fats of marine mammals such as seals. Many fish oils contain small amounts of heneicosapentaenoic acid (HPA, 21:5ω3), which may result from the α-oxidation of DPA (Spurvey et al., 2001; Durnford and Shahidi, 2002).

Table 1.2 Levels of omega-3 fatty acids in commercially available fish oils[a]

Fish	Concentration (% of total fat)			
	EPA (n-20:5ω3)	DPA (n-22:5ω3)	DHA (n-22:6ω3)	Total
Anchovy	22	2	9	33
Jack mackerel	13	2	15	30
Menhaden	14	2	8	24
Sardine/pilchard	16	2	9	27
Capelin	8	-	6	14
Herring	6	1	6	13
Mackerel	7	1	8	16
Norway pout	9	1	14	24
Sand eel	11	1	11	23
Sprat	6	1	9	16
Tuna	6	2	22	30
Cod liver oil	8	1	11	20
Minke whale	4	2	5	11
Harp seal	8	3	10	21
Greenland shark	3	2	6	11
Farmed salmon	7	3	11	21
Blue whiting	9	1	10	20

[a]Adapted from reference (Hjaltason and Haraldsson, 2006a).

4. DHA from microbial oils

Oils containing DHA are now commercially available from microbial sources (Ratledge, 2004b; Ratledge and Hopkins, 2006). Three DHA-rich oils are shown in Table 1.3. As yet there is no single cell source of EPA. The oils are used mainly in infant formulas, or the dried biomass is used to feed animals to produce elevated levels

of DHA in eggs or meat or to feed fish larvae and shrimp. A production level of 650 tones of single cell oils has been reported in 2003 (Ratledge, 2004a).

Table 1.3 Major microbial sources of DHA[a]

Product name	DHA (n-22:6ω3) (%)	Source
DHASCOTM	40-45	*Crypthecodinium cohnii*
DHASCO-STM	25	*Schizochytirum* sp.
DHActiveTM	46	*Ulkenia* sp. (probably)

[a]Adapted from reference (Ratledge and Hopkins, 2006).

5. LC-PUFA from animal fats

Red meat was reported to be the second-best source of LC-PUFA after fish. The levels of these acids in meat are dependent on the diet of the animal (Table 1.4). Pasture-fed animals living on forage rich in ALA produce higher levels of long-chain omega-3 fatty acids (especially DPA) than animals fed on grain or seed meals rich in omega-6 fatty acids. These factors influence the intake of LC-PUFA by Australians according to their dietary habits. Australian intakes are 280 and 140 mg/day of omega-3 LC-PUFA for high and average meat eaters, respectively (Mann, 2005).

Table 1.4 Meat, total fat and LC-PUFA intakes of Australian subjects in four different dietary groups[a]

Dietary component	Intake per dietary group			
	High meat	Moderate meat	Ovolactovegetarian	Vegan
White meat (g/day)	110	54	0	0
Red meat (g/day)	270	109	0	0
Fish (g/day)	28	26	1	1
Total fat (% of energy intake)	38	33	33	28
Fatty acids (mg/day):				
n-20:4ω6	240	100	30	0
n-20:5ω3	70	40	0	0
n-22:5ω3	90	30	0	0
n-22:6ω3	120	70	10	0
Total omega-3 LC-PUFA (mg/day)	280	140	10	0

[a]Adapted from reference (Mann, 2005).

The pasture-fed animals had lower omega-6: omega-3 ratios and contained useful quantities of omega-3 LC-PUFA, though this was mainly in the form of DPA (Nuernberg et al., 2006). By feeding hens diets enriched omega-3 fatty acids through the inclusion of fish oil or of algal biomass, it is possible to produce eggs with enhanced levels of DHA up to about 3 %. These provide a satisfactory dietary means of enhancing circulating blood levels of DHA for mothers and infants during pregnancy

(Smuts *et al.*, 2003). The enrichment of eggs with omega-3 PUFA through dietary fish oil requires vitamin E supplementation to inhibit oxidation (Grune *et al.*, 2001). Tissue levels of ALA, EPA and DHA can be increased by adding each of these acids to the diet of the birds, though the respond to enhanced DHA intake is weaker than for either ALA or EPA (Rymer and Givens, 2005). However, there is little evidence that ALA or EPA is metabolized to other LC-PUFA in these birds.

6. Long-chain PUFA from plant lipids

The need for omega-3 PUFA and the problems of getting adequate supplies of these from fish or from algal resources has been reviewed by Heinz who, along with other research groups, is striving to develop plant sources of these fatty acids (Heinz, 2006). This has already been achieved in several ways involving gene transfer from other organisms. Many difficulties remain to be overcome, not least, for some countries, the fact that these results have only been achieved by genetic engineering (Abbadi et al., 2004; Napier et al., 2004; Qi et al., 2004; Robert et al., 2005; Wu et al., 2005).

1.3 Marine sources of PUFAs

1.3.1 EPA and DHA from fish

Fish are one of the major food sources for mankind, and are being used as the main animal protein diet in many countries. Moreover, the consumption of fish has a range of health benefits for humans including the reduction of coronary heart disease due to its high content of PUFAs. Indeed, oils from fish are characterised by a large range of FA from 12–26 carbon atoms and 0–6 double bonds. Table 1.5 contains information on widely consumed oily fish that represent valuable sources of EPA and DHA. According to the five species studied, the bulk of the fatty acid chains is contributed by saturated FA (29–41 %), monounsaturated FA (41–56 %) and polyunsaturated FA (14–23 %). Among them, the highest proportions of fatty acids are occur in palmitic acid (PA, 16:0) (20-28 %), palmitoleic acid (PA, 16:1ω7) (11-26 %), oleic acid (OA, 18:1ω9) (16-37 %), EPA (3-8 %) and DHA (4-11 %) (Pirestani et al., 2010). Among saturated FA, including $C_{12:0}$ up to $C_{24:0}$ components, some branched chains, such as iso-16:0 and iso-17:0, are present. Among the monounsaturated FA, various amounts of n-16:1ω7, n-20:1ω9 and n-22:1ω11 are also found (Sargent and Tacon, 1999). More than 50 different FA were described in marine fish oil, but eight species frequently represent more than 80 % of the total amount consumed. In fish tissues, the composition of FA

(mainly of triacylglycerols and to a lesser extend of phospholipids), is determined by diet composition and lipid metabolism (Sargent, 1995; Peng et al., 2003). Fish have the ability to synthesize *de novo* saturated and monounsaturated FAs and also to selectively absorb and metabolize dietary FA including LC-PUFA (Bell et al., 1997; Peng et al., 2003), in order to obtain an optimal fatty acid composition (Ackman, 1980). This optimal composition seems to be a characteristic for each species and even each strain (Viga and Grahl-Nielsen, 1990; Pickova et al., 1999). Moreover, the PUFA conversion capacity in fish varies among species and even races (Sargent, 1995). Thus, freshwater fish are generally able to elongate and desaturate α-linolenic acid (ALA, 18:3ω3) to EPA and DHA, whereas marine fish, which lack or have a very low activity of Δ5-desaturase, cannot and require LC-PUFA such as EPA and DHA in the diet (Peng *et al.*, 2003). Long-term absence of essential fatty acid (EFA), such as EPA and/or DHA from the diet leads to deficiency symptoms that, in fish, most often include reduced growth and increased mortality (Glencross, 2009; Tocher, 2010).

Table 1.5 Levels of fat, EPA and DHA in selected fish tissue [a]

Fish	Concentration (g/100g tissue)		
	Fat	EPA (n-20:5ω3)	DHA (n-22:6ω3)
Herring	17.8	2.04	0.68
Sprat	16.6	1.33	1.90
Tuna	15.5	1.08	2.29
Salmon	13.6	0.71	2.15
Sardines in oil	13.9	1.20	1.24

[a]Adapted from reference (Trautwein, 2001).

In addition to food accessibility and lipid metabolism, some environmental parameters also greatly influence the PUFA content (Ould El Kebir *et al.*, 2003). The higher the amount of PUFA components may response to the colder the water. Poikilothermic animals are capable of adjusting the physicochemical characteristics of their membranes according to the prevailing temperatures. This inherent property of cells, known as homeoviscous adaptation of membrane fluidity (Fodor et al., 1995). In fish, the constituent of unsaturated FA increases during its adaptation to reduced temperatures, corresponding with the polar head group and the molecular species composition of membrane phospholipids being reorganized (Lahdes *et al.*, 2000). Evidence suggests that the distribution of fatty acid composition is very individual from species to species and depends on many factors, such as temperature, season, fishing ground, fish species, age, gender or nutritional habits (Bandarra et al., 1997; Tanakol et al., 1999; Trautwein, 2001; Khériji et al., 2003).

Familiar fish species used in the production of fish oil include among others, anchovies, capelin, Atlantic cod, Atlantic herring, Atlantic mackerel, Atlantic menhaden, salmonids, sardines, shark (liver), and tunas (Arts et al., 2001). Of the world's fish oil production, 90 % is produced from fatty fish where lipids are localized mainly under the skin, around the intestines or in the white muscle. In such fishes, the oil content varies but it can reach 21 % (herring) and 18 % (sardines) of the total fat. Such oils are still the least-expensive natural source of preformed LC-PUFA, and several industries (e.g., Croda Leek Ltd, UK; Ocean Nutrition, Halifax, N.S., Canada and Pronova Biocare, Sandefjord, Norway) now specialize in their production and purification through cold pressing, further concentration by winterization (i.e., chilling), and other technologies (Narciso *et al.*, 1999).

There are, however, potential problems associated with fish oils as a source of PUFA such as: taste, odor, stability problems as well as the presence of coextracted contaminants, such a methyl mercury, dioxins, and polychlorinated biphenols (PCB). This has led to some advices to reduce fresh fish intake, especially for pregnant women (Bersamin et al., 2007; Domingo et al., 2007). Some of these problems could be at least partially solved for example by microencapsulation (Marquez-Ruiz *et al.*, 2002) and deodorisation (Hilbert *et al.*, 1998). Nevertheless, the main problem of fish oils is their sustainability due to the worldwide decline of fish stocks (Garcia and Rosenberg, 2010). A better use of raw material as well of by-catch and by-products from fisheries may be one solution, another is to look for other sources, such as marine microbial species.

1.3.2 Marine algae

Fatty acids in marine algae have attracted considerable attention among researchers because they can produce significant amounts of PUFA (Li et al., 2002). The data available on lipids from macroalgae have been reviewed (Dalsgaard *et al.*, 2003). Fatty acid from 11 species of macroalgae from the Brittany coast were studied (Fleurence *et al.*, 1994). Fatty acid compositions of 22 species of marine macrophytes have been reported. These strains were collected from the coast of the Bohai Sea belonging to the three aforementioned algal classes, with typical fatty acid patterns of red, brown and green algae from other regions (Li et al., 2002). In general, red algae from the Bohai Sea contained high levels of C_{20} PUFA, primarily EPA (up to 37.5 % of TFA) and arachidonic acid (AA, 20:4ω6) (up to 29.4 %). The main difference in the FA compositions between red and brown algae was that the latter were richer in C_{18} PUFA,

especially in stearidonic acid (STA, 18:4ω3) (up to 20.1 %). Seven of the ten brown algal species studied also contained EPA as a major component, accounting for 8.4–24.2 %. Green algae studied had the highest level of C_{18} PUFA, mainly α-linoleic (ALA, 18:3ω3) (20.5–27.2 % of total lipids) and STA, and the lowest level of C_{20} PUFA (Li et al., 2002). Red Californian algae contained AA (5.3–23.4 %) and EPA (27.8–45.4 %). Brown algae contained STA (3.6–18.6 %) and EPA (3.1–15.5 %). Two of the three green algae studied contained hexadecatetraenoic acid (16:4ω3) (13.6–16.2 %) and STA (12.1–22.1 %). Both these studies show that red, brown and green algae have different FA profiles that do not depend on the geographical location of the algae and that have a chemotaxonomic significance for seaweeds (Khotimchenko et al., 2002; Li et al., 2002). A comparative study of FA composition of Arctic and Antarctic macroalgae considered their use as indicators of phylogenetic and trophic relationships (Graeve *et al.*, 2002). Several eicosanoids, metabolites of AA, such as hydroxytetraenoic acids, associated with prostaglandins, were identified in a Japanese red alga *Gracilaria asiatica* (Sajiki and Kakimi, 1998). A recent comparative study on fatty acid composition of 12 microalgae as aquaculture food sources (Patil *et al.*, 2007). For instance, *Crypthecodinum cohnii* (dinoflagellates) is rich in DHA (De Swaaf et al., 2003), while another lipid made by the *Mortierella elongata* (phycomycetes) is with high content of AA (Sakuradani et al., 2004), both of the species were good candidates for producing oils. Currently, two American companies OmegaTech and Martek Biosciences have developed special extraction procedures to produce DHA-rich oils from zoo- or phytoplankton or algae (Barclay et al., 1994). The algae oil containing about 40% of EPA and DHA, can be used to enrich food and infant formulas. Furthermore, products based on dried algae preparations, which can be used as animal feed. An example for such a product is the dried algae produced from *Schizochytrium* (OmegaTech & NutraSweet, Kelco, USA).

Thus, EPA producing microalgae may be used as an alternative to fish oil. However, the cultivation of microalgae requires strictly controlled growth conditions in terms of nutrients, light quantity and quality, carbon dioxide levels, which can result in considerable expense (Seto et al., 1984). Much effort is being devoted to developing a commercially feasible technology to produce EPA directly form microalgae based on photoautotrophic growth (Sa´nchez Miro´n et al., 2002; Molina Grima et al., 2003). Unfortunately, photoautotrophic growth is often limited by light caused by mutual shading of cells (Chen, 1996). Consequently, the EPA yield and productivity of

photosynthetic systems are low (Barclay et al., 1994). In contrast, most fungi or bacteria are not fastidious, and can often be grown on the waste products of other agricultural or industrial processes. Therefore, microbial heterotrophic growth process, such as bacteria, is desirable for enhancing EPA production.

1.3.3 Marine fungi

About 800 species of obligate marine fungi have been reported (Hyde *et al.*, 2000). They grow and sporulate exclusively in sea water, and their spores are able to germinate in sea water. In construct, facultative marine fungi are from fresh water or a terrestrial milieu, they may also grow and possibly also sporulate in the marine environment after their physiological adaptations (Kohlmeyer and Kohlmeyer, 1979). Most of them belong to ascomycetes, the anamorphs and a few basidiomycetes. Among the straminipilan fungi, those belonging to *Labyrinthulomycetes*, including the thraustochytrids, aplanochtrids, and labyrinthulids are obligately marine and those belonging to the oomycetes are also fairly widespread in the marine environment (Raghukumar, 2002a). Marine fungi are most common in decomposing wood and plant detritus in coastal waters (Kohlmeyer and Kohlmeyer, 1979; Hyde et al., 2000; Raghukumar et al., 2004), are also common in calcareous animal shells (Raghukumar et al., 1992), algae (Raghukumar, 2006) and corals (Golubic *et al.*, 2005). They have been isolated from deep-sea sediments (Damare and Raghukumar, 2008) and detected in anoxic marine sediments (Stoeck *et al.*, 2003).

Fungi play a key role in the food industry in the production of metabolites and might also supply essential nutrients to detritivores (Philips, 1984). For example, *Penicillium roqueforti* and *Penicillium camemberti* whole cells are good examples of regularly consumed fungi as active parts of Roquefort, Camembert and Gorgonzola cheeses (Carvalho et al., 2010). To obtain more suitable sources of PUFAs, *Mucor* fungi was the first species tried for PUFA production with γ-linolenic acid (GLA, 18:3ω6) as the target metabolites (Suzuki et al., 1981; Ratledge, 1992). Since then, various $C_{18\text{-}22}$ PUFAs have been studied with the aim of effective production. For example, arachidonic acid (AA, 20:4ω6), dihomo-γ-linolenic acid (DGLA, 20:3ω6), and mead acid (MA, 20:3ω9) are now commercially produced by using *Mortierella* fungi (Shimizu and Yamada, 1990; Yamada et al., 1992; Certik et al., 1998; Certik and Shimizu, 1999). Thraustochytrids fall in the order *Labyrinthulida* (Raghukumar, 2002b; Leander et al., 2004), and are considered to be part of the *Stramenopiles* (Patterson,

1999). To date, more than six genera and 38 species have been identified molecularly under the *Thraustochytridae*, which are widely distributed in the marine environments (Honda et al., 1999; Harel et al., 2008). Some *Thraustochytrids* species have a high PUFA content (Lewis et al., 1999; Fan et al., 2001). DHA is now commercially manufactured from thraustochytrids and a large amount of literature and patents describe the current status of this technology (Lewis et al., 1999; Ward and Singh, 2005).

1.3.4 Marine yeast

Yeast lipids have been studied since 1878. Fat production by a beer yeast, and the first report of extracellular lipid production by yeasts has been described (Stodola et al., 1967). Yeasts have received the most attention as source of single cell oil (Hunter and Rose, 1971). *Endomyces vernalis* has been used for producing fat from carbohydrates in Germany during World War I (Prescott and Dunn, 1940). Yeasts capable of excreting lipids has been described (Lodder et al., 1958). In 1963, 65 yeast strains affiliated to 22 species were isolated from the phyllosphere (Ruinen, 1963).

In yeast, triacylglycerol (TAG) is stored in a unique organelle called lipid particle (LP), lipid droplet or adiposome . In these particles, a phospholipid monolayer is surrounding the core of non-polar lipids (Wach, 1996). Yeast LP have a size of 300 to 500 nm in diameter, contain TAG and steryl esters (SE) at equal amounts in the core and small amounts of phospholipids and proteins in the surface monolayer membrane. SE form several ordered shells beneath the surface phospholipid monolayer of LP, whereas TAG are more or less randomly packed in the center of the LP (Czabany et al., 2008). For many decades, TAG has been deemed only as a cellular storage molecule, but recent studies focusing on the relevance of TAG catabolism for cell regulation showed that this non-polar storage lipid is more than just an inert depot (Igal and Coleman, 1996; Rajakumari et al., 2008). In the yeast, long chain fatty acids (LC-FAs) and vary long chain fatty acids (VLC-FAs) are constituents of sphingolipids, but also of inositol glycerophospholipids and the phosphatidylinositol moiety of glycosylphosphatidylinisotol (GPI) anchors (Rajakumaria et al., 2010). All these molecules are important components of lipid rafts and other detergent-insoluble lipid microdomains (Eisenkolb et al., 2002).

In general, yeast cells can grow very quickly, the highest dry cell weight concentration during batch fermentation can reach 14.8 g l^{-1} (Maharajh et al., 2008), and the

fermentation period is very short (Chi et al., 2003). Compared with algal cultivation, it is very easy to manage the large-scale yeast cell production in the fermentor. It is also very easy to collect and concentrate the yeast cells from liquid culture because of their flocculation and large cell size than bacterial cells (Gao et al., 2007). Yeast has the advantage over molds because their single cell mode of growth is easier than mycelial growth of molds and they tend to convert substrate to lipid more efficiently (Bail et al., 1984). Generally, no wild type yeast has been reported with LC-PUFA content. However, genetically modified yeast, such as *Yarrowia lipolytica* with EPA-rich oil, may be an alternative source of fish oil (MacKenzie et al., 2010). Unfortunately, little is known about marine yeasts that have high-lipid content and can be used as aquacultural feed.

1.3.5 Marine bacteria

Marine microbes are fundamental regulators of biogeochemical cycles, playing important roles in cycling and the degradation of organic matter (Dalsgaard et al., 2003; Falkowski et al., 2008). Archaea, bacteria, and protists transform C-, N-, P- and S-containing compounds in ways that affect their availability for biological production, while acquiring resources for metabolism and growth (Strom, 2008). These microbes are play important roles in marine food webs as primary food sources of protein and/or fat and components of the commensal microbial communities of marine animals (Nichols, 2003). Marine sediments are predominant with heterotrophic bacteria, which are colonizers of settling particulate matter followed by phytoplankton bloom (Nadjek et al., 2002). Bacteria incorporate fatty acid mainly in membrance phospholipid (PL) of the cells. Bacterial fatty acids ranging from C_{10} to C_{20} are commonly saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA), whereas PUFA are quite rare. Bacteria can adapt to various environments such as high pressure, cold temperatures and toxic substances by modulating their membrane in a state of fluidity, and therefore maintain the function of membrane proteins involved in respiration and nutrient transport (Aguilar et al., 1998; Russell and Nichols, 1999; Chintalapati et al., 2004). These modifications within the cytoplasmatic membrane may include the changes in the fatty acid composition and interaction between proteins and lipids (Russell and Nichols, 1999). Fatty acids, mainly phospholipid fatty acids are of importance as biomarkers of changes of physiological status of microorganisms caused by external factors (Kaur et al., 2005). Bacterial fatty acids biomarkers are typically odd-numbered, branched trans-

unsaturated and cyclopropyl FA, such as n-15:0, n-17:0, iso- and anteiso-branched SFA and MUFA, 10-Me-16:0 (Dalsgaard et al., 2003).

Oliver and Colwell first reported the finding of PUFAs in marine bacterial isolates (Oliver and Colwell, 1973). The EPA producing marine bacterium *Flexibacter polymorphus* was reported (Johns and Perry, 1977). However, only a narrow group of bacterial genera are capable of producing PUFA. Two bacterial phyla: the *γ-Proteobacteria* (e.g., genera *Shewanella*, *Moritella*, *Colwellia*, *Alteromonas*, *Vibrio*, and *Photobacterium*) and the *Bacteroidetes* (e.g. *Flexibacter* and *Psychroserpens*), are now recognized as major players in producing PUFAs in the marine environment (DeLong et al., 1993; Gauthier et al., 1995; Rossello-Mora et al., 1995; Bowman et al., 1997b; Bowman et al., 1998b).

Studies have noted the occurrence of similar PUFA producing isolates from deep-sea fish and polar invertebrates, of which the fatty acid compositions of isolates (mainly *Colwellia* and *Shewanella* species) were studied (Jøstensen and Landfald, 1997; Yano et al., 1997). A comparatively high association of EPA-producing isolates from the intestines of mackerel species (2.3-2.9% of isolates) has been demonstrated in comparison to other temperate fish species studied (0-1.5%) (Yazawa et al., 1988b). The association of PUFA-producing isolates may not be with any particular marine environment per se, but instead reflect the association of specific bacterial populations (*Colwellia*, *Shewanella* and *Flavobacterium-Cytophaga-Bacteroides* species) with certain environmental niches. Furthermore, the majority of these PUFA producing species occupy the genus *Shewanella* (Russell and Nichols, 1999). High proportions of EPA in *Shewanella* spp. are predominantly associated with cold adaptation mechanisms and the highest production levels occur in cold temperatures (Russell and Nichols, 1999).

1.3.5.1 The genus Shewanella

The genus *Shewanella* comprises a group of Gram-negative, facultatively anaerobic, motile straight or curved rod-shaped bacteria that are nonfermentative in general, although a few species have been reported capable of fermenting glucose (MacDonell and Colwell, 1985; Bowman et al., 1997b; Ivanova et al., 2001). The type species of this genus is *Shewanella putrefaciens*, a bacterium used to be identified as *Pseudomonas putrefaciens* (Owen et al., 1978; MacDonell and Colwell, 1985). The genus includes

psychrophilic and mesophilic species are one of the main players of producing PUFAs, widely distributed in marine environments. At the time of writing, more than 45 *Shewanella* species have been isolated, and half of which have been described with various levels of EPA production (Table 1.6). *Shewanella benthica, S.* abyssi, *S. kaireitica*, *S. violacea*, *S. peizotolerans* and *S. psychrophila* obtained from the deep ocean were found with 2-14 % EPA production of total fatty acids (TFA) (Deming et al., 1984; Delong and Yayanos, 1986; Delong et al., 1997; Nogi et al., 1998b; Miyazaki et al., 2006; Xiao et al., 2007). *Shewanella marinintestina*, *S. schlegeliana*, *S. sairae*, *S. pealeana*, *S. benthica*, *S. baltica*, *S. pneumatohori* and *S. waksmanii* were isolated from the intestine of various marine animals capable of producing mainly 15-37 % EPA of TFA (Yazawa et al., 1992; Leonardo et al., 1999; Satomi et al., 2003; Hirota et al., 2005; Amiri-Jami et al., 2006). *Shewanella hanedai*, *S. frigidimarina* and *S. halifaxensis* isolated from Antarctic marine environments were found with 7-22% EPA (Bowman et al., 1997a; Bowman et al., 1997b; Zhao et al., 2006). *Shewanella affinis* and *S. japonica* were isolated from sea bay or sea water containing 2-8 % EPA of TFA (Ivanova et al., 2001; Ivanova et al., 2004c). Mesophiles, such as *S. olleyana* was obtained form temperate humic-rich river estuary capable of producing 24 % EPA (Skerratt et al., 2002).

Table 1.6 *Shewanella* strains responsible for EPA production

Genus/species	Type strain	EPA (TFA/%)	Source	Reference
S. abyssi	c941	9.1	Deep-sea sediments	(Miyazaki et al., 2006)
S. kaireitica	c931	1.9	Deep-sea sediments	(Miyazaki et al., 2006)
S. marinintestina	JCM 11558	17.5	Squid body	(Satomi et al., 2003)
S. schlegeliana	JCM 11561	18.6	Black porgy intestine	(Satomi et al., 2003)
S. sairae	JCM 11563	15.2	Saury intestine	(Satomi et al., 2003)
S. pealeana	ATCC 700345	11	Squid *Loligo pealei*	(Leonardo et al., 1999)
S. gelidimarina	ACAM 456	16	Congelation ice, Prydz Bay, Antarctica	(Bowman et al., 1997b)
S. benthica	ATCC 43992	16	Intestine, holothurianT	(Bowman et al., 1997)
S. hanedai	ATCC 33224	22.2	Marine sediment, Arctic Ocean	(Bowman et al., 1997)
S. frigidimarina	ACAM 591	6.8	Congelation ice, Prydz Bay, Antarctica	(Bowman et al., 1997)
S. baltica	MAC1	3.5	Mackerel	(Amiri-Jami et al., 2006)
S. violacea	DSS12	14	Deep-sea Ryukyu Trench	(Nogi et al., 1998b)
S. affinis	KMM 3587	2.1	Sea Bay	(Ivanova et al., 2004c)
S. halifaxensis	HAW-EB4	7	Atlantic Ocean	(Zhao et al., 2006)
S. japonica	KMM 3299	8.3	Sea water	(Ivanova et al., 2001)
S. olleyana	ACEM 9	23.6	Humic-rich river estuary	(Skerratt et al., 2002)
S. peizotolerans	WP3	13.4	Pacific deep-sea sediment	(Xiao et al., 2007)
S. psychrophila	WP2	7.1	Pacific deep-sea sediment	(Xiao et al., 2007)
S. pneumatohori	SCRC-2738	36.6	The intestines of Pacific Mackerel	(Yazawa et al., 1992; Hirota et al., 2005)
S. waksmanii	KMM 3823	6.7	Sipuncula (*Phascolosoma japonicum*)	(Ivanova et al., 2003a)

Deep-sea *Shewanella* species was taxonomically divided into two major subgenus branches, one group characterised as high-pressure cold-adapted species produce substantial amounts of EPA and the other group recognized as mesophilic pressure-sensitive species do not produce EPA or produce only scant amounts (Kato and Nogi, 2001). High proportions of EPA in *Shewanella* spp. are mainly associated with cold adaptation mechanisms and the highest production levels occur in the suboptimal region of temperature growth kinetic range (Nichols et al., 1997; Russell and Nichols, 1999). The psychrophilic and piezophilic *Shewanella* strains, including *S. violacea*, *S. benthica and S. piezotolerans*, produce EPA, and thus the production of such LC-PUFAs is a property shared by many deep-sea bacteria to maintain cell-membrane fluidity under conditions of extreme cold and high hydrostatic pressure (Fang et al., 2003; Wang et al., 2008; Wang et al., 2009). A physiological basis for the production of PUFA as a selective adaptation to temperature and/or high pressure environments is well accepted (Nichols *et al.*, 1995). However, *Shewanella olleyana*, *S. japonica* and *S. pacifica* were described to produce significant levels of PUFAs, such as EPA, at relatively high incubation temperatures (25-30 ºC) (Ivanova et al., 2001; Skerratt et al., 2002; Ivanova et al., 2004b).

1.3.5.2 The genus Colwellia

Species of the genus *Colwellia* are defined as facultatively anaerobic and psychrophilic bacteria and the type species of this genus is *C. psychroerythrus*, which used to be classified as *Vibrio psychroerythrus* (Deming et al., 1988). The chemotaxonomic markers of the genus *Colwellia* became clear with the publication of four new species to the genus (*Colwellia demingiae*, *C. psychrotropica*, *C. rossensis* and *C. hornerae*), all of which were isolated from Antarctic sea ice and were able to produce DHA (Bowman et al., 1998b). Fatty acids with n-even chain-length, such as n-14:0 and n-14:1ω7*cis* were dominated in the membrane lipids, correlated with DHA production (Russell and Nichols, 1999). Many *Colwellia* strains were originally assigned as genus *Vibrio*, however the analysis of the fatty acid compositoins could help distinguish these two genera by forming an atypical chemotaxonomic grouping. Thus, the majority of strains previously identified as *Vibrio* spp. that contain DHA and possess the *Colwellia* chemotaxonomic profile may be reassigned to the newly described species of *Colwellia* (Wilkinson, 1988).

Two significant groups are left unaccounted for by this chemotaxonomic identification procedure. First, strains that contain the *Colwellia* fatty acid markers but have a much higher percentage of DHA than the other identified species (Delong and Yayanos, 1986), and second, strains with similar to *Colwellia* fattt acid fingerprint but contain EPA but without DHA (Hamamoto et al., 1995). Further, `*Colwellia*' strains that produce EPA do not contain n-14:1ω7, unlike other species that do produce DHA (Delong and Yayanos, 1986; Hamamoto et al., 1994; Hamamoto et al., 1995; Yano et al., 1997). Levels of LC-PUFAs by *Colwellia* species are shown in Table 1.7. However, novel *Colwellia* species isolated recently were found with no production of EPA and DHA in the membrane layer, whereas high levels of unsaturated fatty acids (n-16:1) are produced, such as *C. piezophila* (Nogi et al., 2004), *C. aestuarii* (Jung et al., 2006), *C. polaris* (Zhang et al., 2008), *C. asteriadis* (Choi et al., 2010) and *C. c*hukchi*ensis* (Yu et al., 2010). These observations suggest that the production of LC-PUFAs should not be a requirement for classification as a psychrophilic and/or piezophilic bacterium, although it is a common property of psychropiezophiles by producing unsaturated fatty acids.

Table 1.7 *Colwellia* strains responsible for long chain PUFA production

Genus/species	Type strain	DHA (TFA/%)	EPA (TFA/%)	Source	Reference
C. demingie	ACAM 459	2.2	-	Antarctic	(Bowman et al., 1998b)
C. hornerae	ACAM 607	2.1	-	Antarctic	(Bowman et al., 1998)
C. psychroerythraea	ACAM 550	8.0	1.5	Antarctic	(Bowman et al., 1998)
C. Psychrotropica	ACAM 179	0.7	0.1	Antarctic	(Bowman et al., 1998)
C. rossensis	ACAM 608	6.0	trace	Antarctic	(Bowman et al., 1998)

(–), Not detectable.

1.3.5.3 The genus Photobacterium

The genus *Photobacterium* was one of the earliest known bacterial taxa, with the report of species *Ph. Phosphoreum* (Beijerinck, 1889). The genus *Photobacterium* comprises a group of Gram-negative, facultatively anaerobic, plump, straight and rod-shaped bacteria that lack an enclosing sheath in their flagelus, and require sodium ions for growth (Seo et al., 2005). The type species of this genus is *Ph. phosphoreum*. This genus affiliates to the *Gammaproteobacteria* and is particularly with high similarity to genus *Vibrio*, on the basis of 16S rRNA gene sequences comparison (Nogi et al., 1998c). Species of the genus *Photobacterium* and other bioluminescent bacteria, such as *Vibrio*, belong to the family *Vibrionaceae*, are widespread in marine environments (Kimura et al., 2000; Shieh et al., 2003). *Photobacterium phosphoreum*, *Ph. leiognathi* and *Ph. profundum* were respectively isolated from sea water, the intestinal contents of

marine animals and deep-sea sediment (Baumann and Baumann, 1984; Nogi et al., 1998c). *Photobacterium iliopiscarium*, *Ph. phosphoreum*, *Ph. profundum* and several other species of the genus *Photobacterium* are psychrophilic, with optimum growth temperature is lower than 20 °C. *Photobacterium profundum* with two type strains DSJ4 and SS9, were the first to be re ported as psychropiezophilic species isolated from the Ryukyu Trench at a depth of 5110 m (Nogi et al., 1998c). *Photobacterium frigidiphilum* was reported to be slightly piezophilic requiring 10 MPa as its optimal pressure for growth (Seo et al., 2005). About 15 *Photobacterium* species have been isolated, but *Ph. profundum* and *Ph. frigidiphilum* are the only two species within this genus known to display piezophily and the only two known to be able to produce the LC-PUFA EPA (Table 1.8). Vary limited species of *Photobacterium* produces LC-PUFA. *Photobacterium profundum* strain SS9 has been well studied regarding to the molecular mechanisms of pressure regulation (Bartlett, 1999) and subsequently genome sequencing and expression analysis (Vezzi et al., 2005).

Table 1.8 *Photobacterium* type strains responsible for EPA production

Genus/species	Type strain	EPA (TFA/%)	Source	Reference
Ph. frigidiphilum	SL13	6	Cold-seep area	(Seo et al., 2005)
Ph. profundum	DSJ4	13	Deep-sea sediment	(Nogi et al., 1998c)
Ph. profundum	SS9	7	Deep-sea sediment	(Allen et al., 1999)

1.3.5.4 The genus Moritella

Moritella marina is the type species of the genus *Moritella* (Urakawa et al., 2000), which was reclassified from *Vibrio marinus* (Colwell and Morita, 1964). Species in this genus were characterised as psychrophilic and/or piezophilic. To date, seven species have been identified in the genus *Moritella*. They were mainly isolated from marine environments, such as fish farms, seawater, marine sediments and the abyssal ocean (Nogi et al., 1998a; Nogi and Kato, 1999; Benediktsdottir et al., 2000; Urakawa et al., 2000; Xu et al., 2003). *Moritella marina* was classified as non-piezophiles, isolated from the North Pacific Ocean (Urakawa et al., 2000). *Moritella japonica* and *M. yayanosii* were identified as piezophiles, isolated from the Japan Trench and Mariana Trench respectively (Nogi et al., 1998a; Nogi and Kato, 1999). The optimal pressure for the growth of *M. yayanosii* strain DB21MT-5 is 80 MPa, and unable to grow at pressures of less than 50 Mpa, but grows well at pressures as high as 100 MPa. *Moritella viscosa*, pathogenic for Atlantic salmon parr, was identified as psychrotolerant species, isolated from the lesions or the internal organs of fish

(Benediktsdottir et al., 2000). *Moritella profunda* and *M. abyssi* were classified as psychropiezophiles, isolated from the deep sea of the eastern tropical Atlantic (Xu et al., 2003). *Moritella dasanensis* was identified a psychrophile, isolated from the coast of Svalbard, Norway (Kim et al., 2008).

The change in cellular fatty acid composition of psychropiezophilic strains in response to pressure changes was observed by presenting higher amount of PUFAs at higher growth pressures. About 70 % of the membrane lipids are unsaturated fatty acids in *M. yayanosii*, indicating its adaptation to very high pressures (Nogi and Kato 1999; Fang et al. 2000). Six species of *Moritella* were reported with variable levels of DHA production (Table 1.9).

Table 1.9 *Moritella* type strains responsible for DHA production

Genus/species	Type strain	DHA (TFA/%)	Source	Reference
M. abyssi	2693	NP	Coast, African	(Xu et al., 2003)
M. japonica	JCM 10249	6	Deep sea	(Nogi et al., 1998a)
M. marina	MP-1	12	Deep sea	(Nogi et al., 1998a)
M. profunda	2674	NP	African coast	(Xu et al., 2003)
M. yayanosil	JCM 10263	11	Ocean floor	(Nogi and Kato, 1999)
M. dasanensis	ArB 0140	NP	Coast, Norway	(Kim et al., 2008)

NP, Specific data not published post-February 2011.

1.3.5.5 The genus Psychromonas

All species in the genus *Psychromonas* are Gram-negative, rod-shaped, cold-temperature aerobic bacteria with an optimum salt concentration of greater than 0 %. Phylogenetically, the genus *Psychromonas* is closely related to the genera *Shewanella* and *Moritella*, and the type species is *Psychromonas Antarctica* (Mountfort et al. 1998). No piezophilic properties shown in this strain which was isolated from a high-salinity pond in Antarctica as an aerotolerant anaerobic bacterium. *Psychromonas kaikoae* is a novel obligatory psychropiezophilic bacterium isolated from the deepest cold-seep environment at a depth of 7,434 m (Nogi et al. 2002). This strain was able to synthesis both EPA and DHA in its cell membrane, with optimal temperature and pressure for the growth at 10 °C and 50 MPa. In contrast, *Psychromonas antarctica* and most of recent identified *Psychromonas* species, such as *P. agarivorans* and *P. boydii* do not produce either EPA or DHA in its membrane layer (Hosoya et al., 2009; Auman et al., 2010). *Psychromonas profunda* is a moderately piezophilic bacterium isolated from deep

Atlantic sediments, similar to the piezo-sensitive strain *Ps. marina*, which also produces small amounts of DHA (Xu et al., 2003). In this genus, only limited species are able to produce low level of PUFAs (Table 1.10).

Table 1.10 *Psychromonas* type strains responsible for long chain PUFA production

Genus/species	Type strain	DHA (TFA/%)	EPA (TFA/%)	Source	Reference
Ps. kaikoae	JT7304	3	2	Deep sea	(Nogi et al., 2002)
Ps. marina	Apr-22	1.6	-	Coast	(Kawasaki et al., 2002)
Ps. profunda	2674	NP	-	Deep sea	(Xu et al., 2003)

NP, Specific data not published post-February 2011; (–), Not detectable.

1.3.5.6 The genus Cyanobacteria

Cyanobacteria, a class of photosynthetic prokaryotes occurring in the phytoplankton, produce C_{18} PUFA esterified to polar lipids, but they do not biosynthesize EPA or DHA (Henderson, 1999). EPA production was obtained by a transgenic marine cyanobacterium carrying a plasmid containing *pfaEABCD* gene cluster involved in EPA biosynthesis from *Shewanella* sp. SCRC-2738, for EPA synthesis with 4-8% EPA of TFA (Table 1.11) (Yu et al., 2000).

Table 1.11 *Cyanobacteria* strains responsible for long chain PUFA production

Genus/species	Strain	EPA (TFA/%)	n-18:3ω3 (TFA/%)	Source	Reference
Synechococcus sp.	NKBG15041c	-	6.4	Coast	(Yu et al., 2000)
Cyanobacterium	Recombinant	4-8	3-7	Recombinant	(Yu et al., 2000)

(–), Not detectable.

1.4 Fatty acid biosynthesis

Essentially, all living cells are capable of *de novo* synthesis of fatty acids and the derived fatty acyl-containing lipids (the exceptions being obligate parasites that rely on the lipid-synthesizing capabilities of their hosts, and also a primitive group of bacteria, known as the *Archaebacteria*, that produce highly branched-chain fatty acids derived from isoprene units and do not biosynthesize straight-chain fatty acids). However, the range of fatty acids that an organism is capable of producing *de novo* (from acetyl subunits) varies enormously. For example, terrestrial plants have limited capabilities for the synthesis of fatty acids with chain length of >18 carbons and none contain LC-PUFA, and therefore with simple fatty acid patterns. In contrast, animal lipids are

generally far more complex and varied, as the fatty acid profile is determined more by the dietary fat intake than by the animal`s innate ability to synthesize fatty acids. Although animal have the ability to synthesize saturated fatty acids, and also possess a whole array of fatty acid desaturases and elongase, they do no possess a complete complement. Specifically, all higher animals lack distal desaturases, such as the Δ15 and Δ12 desaturases, and so animals have a strict dietary requirement for fatty acids - linoleic acid (LA, 18:2ω6), the precursor of the omega-6 fatty acids, and α-linolenic acid (ALA, 18:3ω6), the precursor of the omega-3 fatty acids are dietary requirements for all higher animals (Tran et al., 2003).

Microbial systems are different from those of both animals and terrestrial plants in that species are known that not only are capable of synthesizing PUFA >18 carbons but also possess all the desaturases to synthesize fatty acids of the omega-3 and omega-6 pathways *de novo*. As a consequence, microbial oils possess the dual benefit of having simple fatty acid profiles, like plant oils, while also being potentially able to contain LC-PUFA of the omega-3 pathway (e.g. EPA and/or DHA) like animal oils. Marine fish oils are of great value due to their high content of omega-3 PUFAs, on which their health-benefit claims are based. However, these PUFAs are not synthesized by the fish, but produced by marine microorganisms on which the fish either feed or have residing within their intestines (Tocher et al., 2006), although the fact is still being little appreciated.

The biosynthesis of C_{22} PUFA from C_{18} unsatuarated fatty acids proceeds via a sequence of alternate desaturations and elongations (Figure 1.5). These reactions may be found, in whole or in part, in microorganisms and animals, though in animals the Δ15 and Δ12 desaturases do not occur so that linoleic acid (LA, 18:2ω6) and α-linolenic acid (ALA, 18:3ω3) must be obtained from the diet. In plants, only reactions up to the C_{18} series [including stearidonic acid (STA, 18:4ω3)] occur. Certain marine microorganisms (including a few bacteria and marine microalgae – dinoflagellates and thraustochytrids which are used commercially to produce DHA) have a completely separate biosynthetic mechanism for the synthesis of EPA, DHA and docosapentaenic acid (DPA, 22:5ω3) (Tran et al., 2003).

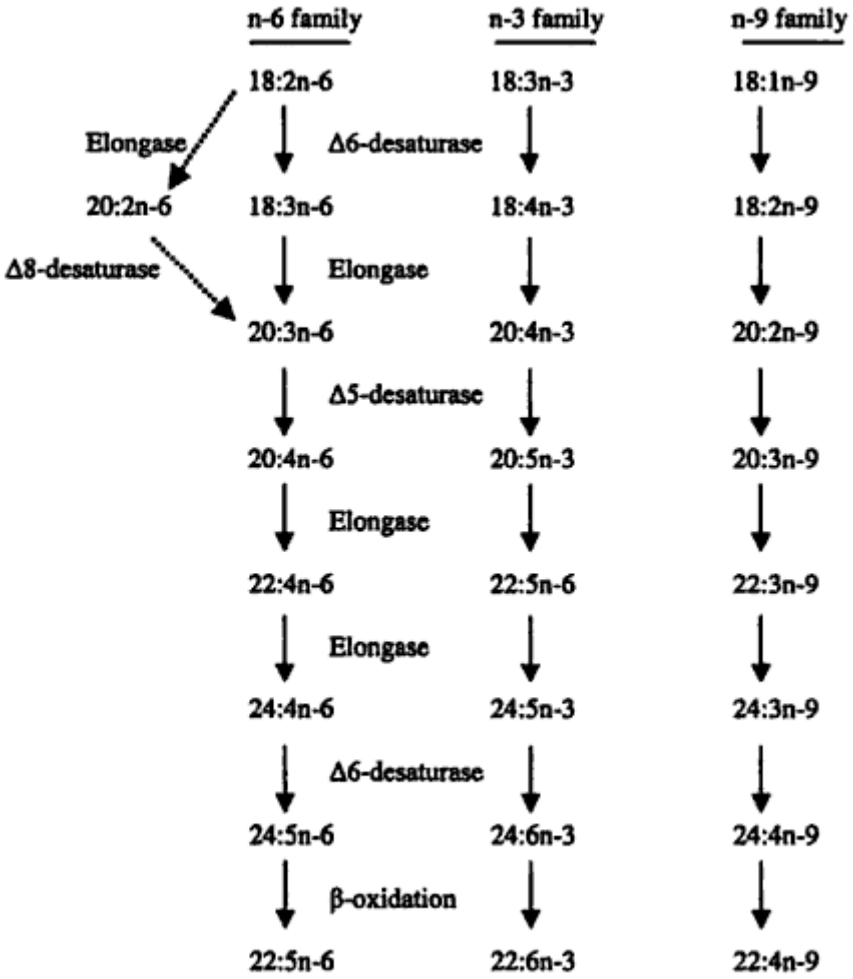

Figure 1.5 The metabolism pathways of biochemical conversion of 18-carbon unsaturated fatty acids by fatty acid desaturases and elongase leading to long-chain polyunsaturated fatty acids (LCPUFA) of the omega-3, omega-6 and omega-9 series. Adapted from (Tran et al., 2003).

1.4.1 Fatty acid synthetase system (FAS)

The enzyme system involved in *de novo* fatty acid synthesis, fatty acid synthase (FAS), is one of the household enzymes of the cell. There are two types of fatty acid synthases. Type I FAS is present in mammals, birds, yeasts, fungi and some special bacteria (Figure 1.6). In this type, multifunctional enzyme complexes harbouring catalytic activities as discrete functional domains are located on one or two polypeptide chains (Schweizer et al., 1984; Schweizer, 1989; Schweizer and Hofmann, 2004). Whereas, in most bacteria as well as in the organelles of prokaryotic descent, for example, mitochondria and chloroplasts, produce fatty acids via the type II FAS in which discrete enzymes encoded by separate genes catalase specific steps of the biosynthetic pathway (Töpfer and Martini, 1994; White et al., 2005a). The dominant cellular fatty acids

produced via the type II FAS typically contain between 14 and 18 carbons, such as via iterative reactions of the elongation and desaturation on the saturated fatty acid palmitic acid (PA, 16:0) (Wallis et al., 2002; Qiu, 2003; Chung et al., 2005), during which palmitate (16:0) can be elongated by steps of 2 carbon atoms (again provided by a malonyl-CoA precursor) to $C_{18:0}$-$C_{24:0}$ (Schweizer, 1989).

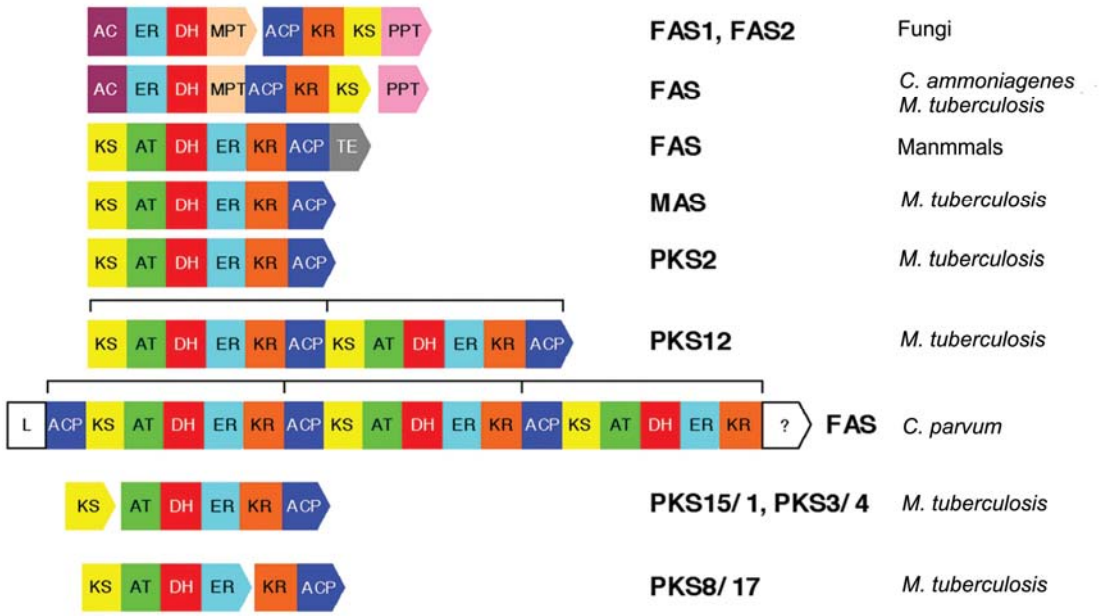

Figure 1.6 Domain organization of known type I FASs and related multienzymes Arrows indicate open reading frames. Their subdivision into functional domains is not shown to scale. With the exception of FAS1 and FAS2, the indicated gene pairs are chromosomally linked in tandem orientation. The indicated PKS combinations encode putative heteromeric multienzymes comprising a complete set of FAS domains. Brackets indicate intramolecular FAS modules. L, acyl-CoA ligase. Adapted from (Cole et al., 1998; Minnikin et al., 2002; Schweizer and Hofmann, 2004).

FAS structural variants may be assigned to three general classes. These FAS enzymes are contrasted by the highly integrated type I FAS multienzymes, which contain the various catalytic activities of the reaction sequence as discrete functional domains, either on a single polypeptide chain or, in some cases, on two different multifunctional proteins of comparable size. Type I FAS multienzymes are characteristically found in the eukaryotic cytoplasm (Lynen, 1980) and, as a remarkable prokaryotic exception, also among the mycolic acid producing subgroup of the *Actinomycetales* (Bloch and Vance, 1977). The type I systems may be further subdivided according to the domain organization of the multifunctional proteins and, concomitantly, according to their subunit stoichiometry. Microbial type I FASs are hexamers with a domain sequence of

AC-ER-DH-MPT/ ACP-KR-KS forming either $\alpha_6\beta_6$ (fungi) or α_6 (bacteria) oligomers (type Ia). In contrast, animal FASs are α_2 dimers with the domain sequence KS-AT-DH-ER-KR-ACP-TE (type Ib) (Schweizer and Hofmann, 2004). Occasionally, more than one set of FAS domains may be fused to a multimodular synthase. For instance, the *pks12* gene of *Mycobacterium tuberculosis* (Cole et al., 1998; Sirakova et al., 2003) has two complete sets of FAS domains, and the FAS gene of the parasitic protist *Cryptosporidium parvum* has three (Zhu et al., 2000) (Figure 1.6). Apart from these structural differences, type I FASs may also be functionally differentiated on the basis of to various parameters, individual FASs may also differ by their specific cellular compartmentation being localized not only in the cytoplasm but also in organelles (Shintani and Ohlrogge, 1994), and microsomal membranes (Gu et al., 1997; Rossler et al., 2003).

1.4.2 Polyketide (anaerobic) pathway

Eckhart and Hofmann (2004) have proposed that FASs may be considered the forebears of most members of the large family of polyketide synthases (PKSs). PKS and FAS systems contain basically the same set of component enzymes. However, in contrast to FASs, the typical PKS pathways are not "iterative" reaction sequences. Instead, they catalyze one or several rounds of FAS-like reaction sequences, each specifically missing one or even more of the canonical FAS reactions. Usually, different enzyme combinations are used in successive PKS cycles ranging from complete to more or less incomplete FAS sequences (Hopwood and Sherman, 1990). Thus, polyketides retain, at distinct positions, the characteristic functional groups of certain FAS intermediates such as keto groups, hydroxyl groups, or double bonds (Schweizer and Hofmann, 2004).

PKS systems have been known for many years in both bacteria and eukaryotic microorganisms as carrying out a wide variety of biosynthesis, with products ranging from relatively simple molecules, such as 6-methylsalicylic acid, to much more complex structures as may be found in the tetracycline and macrolide range of antibiotics (Pfeifer and Khosla, 2001). However, many PKS systems are like FAS in that they use acetyl-CoA as the basic building unit, which is initially converted to malonyl-CoA followed by a condensation of these two molecules. For the biosynthesis of the LC-PUFA, and unlike 'conventional' fatty acid biosynthesis using the FAS system, the product of the condensation reaction between the growing acyl chain and malonyl-ACP in the PKS-catalysed system does not always undergo complete

reduction, but may selectively retain some of the double bonds formed in situ. Some rearrangement of the configuration and position of these double bonds may occur in order to create the final methylene-interrupted *cis*-double-bonded fatty acids. The molecular size of the PKS system (about 500-600 kDa) is, however, much less than that of the FAS (~2000 kDa), which might suggest that, evolutionarily, the PKS may have been derived from genes of the FAS system undergoing some losses as well as additional modifications acid (Hopwood and Sherman, 1990).

Yazawa et al. (1988) observed the production of LC-PUFA (either DHA or EPA) from certain marine bacteria, such as *Vibrio*, *Shewanella* and *Moritella*, resulting in the discovery of this new biosynthesize pathway of fatty acids. Previously, the ability to synthesize significant amounts of LC-PUFA was not associated with prokaryotic systems, with the exception of the cyanobacteria. Furthermore, these prokaryotic LC-PUFA producers were often isolated from environments that were anoxic (without oxygen) and were able to produce LC-PUFA even under these conditions (Bowman et al., 1997b). Although the production of LC-PUFA under anaerobic conditions was apparently at odds with the involvement of oxygen-requiring desaturases, the large cluster of genes that was isolated from one of the LC-PUFA synthesizing bacteria was still assumed to encode conventional fatty acid elongases and desaturases with activities equivalent to those in other systems. These bacterial genes could be isolated and shown to confer the ability to synthesize LC-PUFA when transferred into a host bacterium, such as *Escherichia coli*, not normally able to synthesize such fatty acids (Allen and Bartlett, 2002; Orikasa et al., 2004; Orikasa et al., 2006a; Orikasa et al., 2006b), only a few studies were successful (Valentine and Valentine, 2004; Amiri-Jami and Griffiths, 2010).

Metz et al. (2001) concluded that EPA production shares many features with polyketide synthesis The combined activities of these domains include condensation reactions (KS domains), acyl CoA:ACP transfer reactions (AT), multiple acyl carrier protein domains (ACP), ketoacyl reduction reactions (KR), chain length factor domains (CL) presumably involved in decarboxylation reactions, dehydratase/isomerase reactions (DH/I), and enoyl reduction reactions (ER) (Tanaka et al., 1999; Metz et al., 2001). These domains presumably catalyze the repetitive steps in building the growing acyl chain and molecular oxygen is not involved in any of these steps (Allen and Bartlett, 2002). The presence of repetitive ACP domains is unique to EPA and DHA synthases,

as shown in Figure 1.7, with SS9 pfaA possessing five ACP domains, *Shewanella sp.* six, *Moritella* five, and *Schizochytrium* nine (Cronan and Rock, 1996). The growing acyl chains are presumably bound covalently to these ACP groups as thioesters with AT domains being required for the loading of the starter and extender units. The ability to introduce multiple double bonds into a single acyl chain in the absence of O_2 highlights a major difference with desaturase systems. The ability to produce double bonds anaerobically likely arises from the activities of the DH/I domains present in the microbial synthases, for example, bacterial PfaC homologues and *Schizochytrium* ORF C. Such dehydration/isomerization reactions might be analogous to those catalyzed by FabA (b-hydroxydecanoyl-ACP dehydratase) in bacterial monounsatured fatty acid synthesis, in an anaerobic process (Cronan and Rock, 1996).

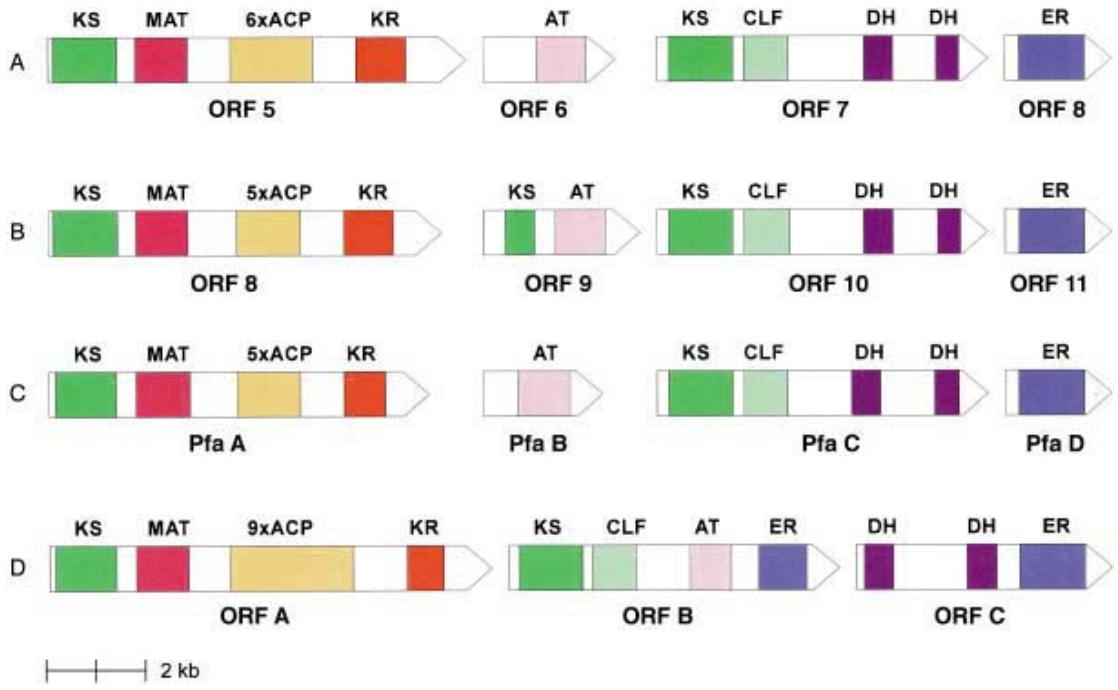

Figure 1.7 Organization of the core regions of PUFA biosynthetic gene clusters. A: *Shewanella* sp. SCRC-2738 (GenBank accession no.: U73935.1); B: *Moritella marinus* strain MP-1 (GenBank accession no.: AB025342.1); C: *Photobacterium profundum* strain SS9 (GenBank accession no.: AF409100, unpublished data); D: *Schizochytrium* (GenBank accession nos.: AF378327, AF378328, AF37832); Adapted from (Kaulmann and Hertweck, 2002).

Therefore, a novel alternative pathway for the biosynthesis of C_{20+} PUFAs has been suggested in a narrow group of predominately marine *Gamma-Proteobacteria* that includes species of the *Shewanella*, *Photobacterium*, *Moritella*, *Colwellia* and *Vibrio*

genera (Yazawa, 1996; Morita et al., 2000; Kaulmann and Hertweck, 2002; Gutierrez et al., 2003). This system is responsible for the specific *de novo* synthesis of the long-chain omega-3 PUFAs EPA and DHA, and the omega-6 PUFA arachidonic acid (AA, 20:4ω6) (Russell and Nichols, 1999). The mechanism of Pfa Synthase for PUFA synthesis proceeds via a novel type I iterative fatty acid synthase/polyketide synthase (FAS/PKS) enzyme complex (Metz et al., 2001).

Metz et al (2001) have proposed plausible schemes of this novel Pfa Synthase for PUFA synthesis system as shown in Figure 1.8. The first elongation step from a putative acetyl-CoA starter molecule and alonyl-CoA as well as a complete cycle of reduction is catalyzed by KS, KR, DH, and ER. The second elongation step occurs by a keto reduction to the β-hydroxyester. The FabA-like bifunctional dehydratase/isomerise would then catalyze dehydration as well as the *trans*-2,3-*cis* rearrangement of the acyl intermediate. In analogy to the requirement of a special β-ketoacyl-ACP synthase in *E. coli* for the elongation of the 3-*cis*-acyl-ACP intermediate, a designated KS might be responsible for chain propagation. After subsequent reduction and dehydration steps, a methylene- interrupted double-bond pattern would require that a *trans*-2,2-*cis* isomerization occurs. A similar reaction is known for the regeneration of 11-*cis*-retinol. For EPA biosynthesis, such set of reactions is then repeated twice, with the last 2,3-isomerization being followed by an elongation in which a full reductive cycle is employed. Similarly, only minor modifications occur during the formation of DHA and AA. However, this proposed pathway needs to be verified and studied by further biochemical analyses. As for fungal iterative type I PKSs, it is completely unresolved how chain length and degree of reduction is controlled at specific steps. The multiple ACPs (5-9 copies) contain a conserved Ser residue that is post-translationally modified with the phosphopantetheinyl moiety of CoA by a phosphopantetheinyl transferase (PPTase) (Rahman et al., 2005), providing a free thiol for tethering the starter and extender units and channeling the intermediates during fatty acid and polyketide biosynthesis (Lambalot et al., 1996; Sanchez et al., 2001).

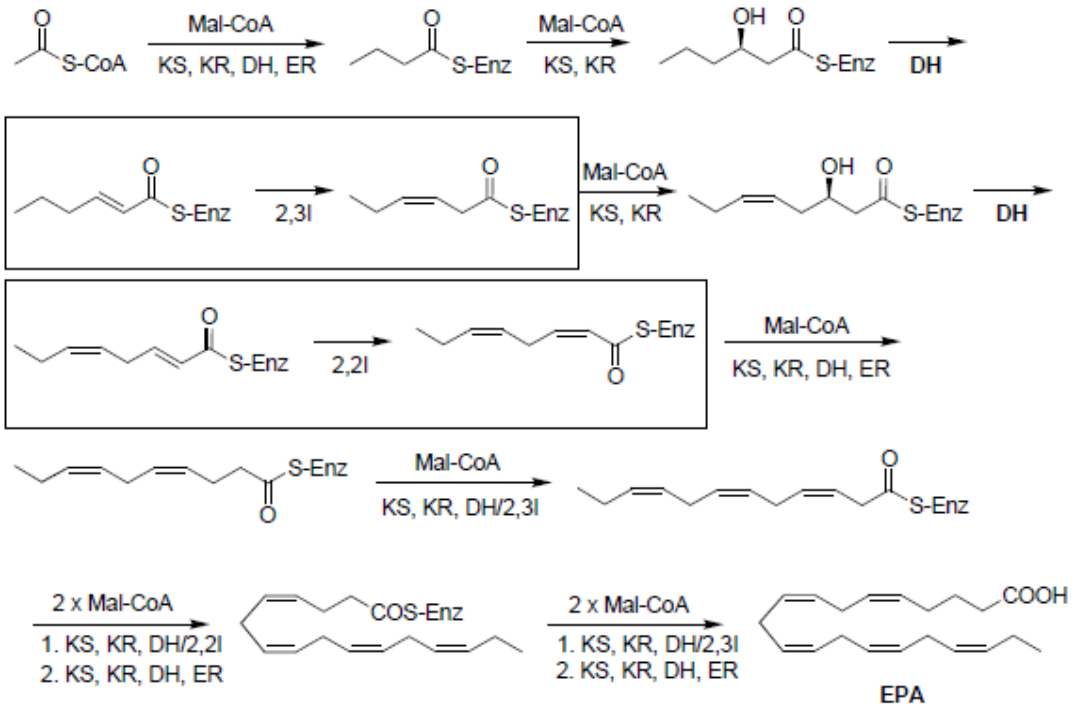

Figure 1.8 Proposed biosynthetic pathway of EPA in *Shewanella* sp. with putative intermediates and key catalytic activities employed**.** Mal-CoA: malonyl-CoA; KS: keto synthase; KR: keto reductase; DH: dehydratase; ER: enoyl reductase; 2,3I: 2,3-isomerase; 2,2I: 2,2-isomerase. Adapted from (Kaulmann and Hertweck, 2002).

1.4.3 PUFAs Polyketide synthase gene

So far EPA and DHA genes have been found only in Gram-negative bacteria, which synthesize an inner and outer membrane structure similar to *E. coli* (Metz et al., 2001; Valentine and Valentine, 2004). The distribution pattern of DHA and EPA in the biosphere studies is that genes for anaerobic production of EPA or DHA are virtually always found in the marine environment (Heidelberg et al., 2002) raising the obvious possibility that these genes have selective advantage in and are tailor-made for this environment. However, as mentioned above, the marine-only rule seems to be broken with the finding of EPA genes in a freshwater isolate called *Shewanella oneidensis*, although this is the only exception to date (Metz et al., 2001). Also, one of the biggest surprises is the presence of the anaerobic pathway in certain marine fungi (Delong and Yayanos, 1986; Jøstensen and Landfald, 1997; Russell and Nichols, 1999).

The classification of omega-3 bacteria has shed light on another interesting aspect regarding the vertical distribution pattern of EPA versus DHA bacteria in seawater. The point is that DHA bacteria were first thought to be found only in the deep sea, compared to EPA strains which are found in both deep and shallow seas (Jøstensen and Landfald,

1997). However, later studies showed that DHA strains are present in high levels in the guts of marine invertebrates living in cold, shallow seas or on sea ice (Gunstone, 1996). There is possible that omega-3 bacteria living the gut might be involved in symbiotic associations with their hosts, as Δ6 desaturase appears to be barely functional in cod under nutritional regulation (Tocher et al., 2006), and therefore essential fatty acids (EFA) are required in ontogeny of marine and freshwater fish (Tocher, 2010). However, further studies are needed to determine the biochemical and molecular basis of EFA requirements and metabolism of fish.

Therefore, in some way, PUFAs genes will help in bacterial taxonomy to redefine old classifications, to create new groupings, and to trace-back evolution (Nichols et al., 1997; Nichols et al., 1999). And, new strains with active and silent copies of the PUFA genes can be tracked by using molecular biology tools, and more might be learned about the world-wide distribution of strains with PUFA-biosynthesis gene clusters and their ecological importance (DeLong and Yayanos, 1985; Nichols et al., 1996b). The genes for PUFA biosynthesis are distinct from previously recognized PKS in both structure and mechanism as well as the novel putative dehydrases/isomerases, and may thus provide new tools for combinatorial biosynthesis of polyketide antibiotics (Leadlay, 1997; Chartrain et al., 2000; Rohlin et al., 2001).

The high degree of sequence similarity between the bacterial (*Shewanella* sp. SCRC-2738, *Moritella marina* strain MP-1 and *Photobacterium profundum* strain SS9) and the eukaryotic microbe *Schizochytrium pfa* genes suggests the possible involvement of horizontal gene transfer in the acquisition of the *pfa* gene clusters in the marine environment, which demonstrated that EPA or DHA polyketide biosynthesis gene clusters from different genera were with high degree of sequence similarity. The result also provided evidence of the common distribution of the novel PUFA synthease pathways among marine microorganisms regardless of their biogeographic variability, which has recently been further testified by the investigation of genetic capacity for production of long-chain fatty acids using a culture-independent approach (Shulse and Allen, 2011). Therefore, possible horizontal transfer of these genes may be an interesting evolutionary question for ecologist to study, as there is no apparent guanine-cytosine (GC) bias among the *pfa A–D* genes nor is there indication of flanking genes possessing functions which could facilitate horizontal transfer.

There are further reasons also for studying bacterial PUFAs. Their occurrence in a selected group of marine psychrophilic, halotolerant bacteria raises questions about their role in membrane structure and stabilization at low temperatures in saline conditions. Their distinctive taxonomic distribution can also be used for classification and identification. Evolutionary relationships may not be confined to bacterial groupings, because the structural similarity of bacterial PUFAs to those that are typical of mammals raises the possibility of lateral gene transfer.

1.5 Bacteria adaptive to low temperature

Psychrophilic and psychrotrophic bacteria are capable of developing over a wide temperature range and they can grow and survive at temperatures close to or below freezing. This ability requires specific adaptative strategies in order to maintain membrane fluidity, the continuance of their metabolic activities, and protein synthesis at low temperature. These strategies are mainly as (i), by altering the size and charge of the polar head groups; (ii), by changing the proportion of short and long chain fatty acids; (iii), by modification the extent of fatty acid desaturation; (iiii), by changing the proportion of *cis* and *trans* fatty acids and changing the composition of carotenoids (Chattopadhyay et al., 1997; Kiran et al., 2004; Zhang and Rock, 2008). However, not all the above strategies are effective. For instance, changes in the polar head groups are less frequent and less effective in modifying the membrane fluidity (Hasegawa et al., 1980; Suutari and Laakso, 1994) and changes through chain length modification is possible only in growing cells (Denich et al., 2003) and therefore may not be the universal method of modulating membrane fluidity. Furthermore, proteins by interacting with lipids contribute to the overall stability of the membrane bilayer, but the interaction itself is dependent on head group acylation, membrane fluidity and membrane thickness, implying that it does not cause the primary effect on fluidity (Takeuchi et al., 1978; Takeuchi et al., 1981; Heipieper and de Bont, 1994; Epand, 1998). Compared to the above strategies, changes in fatty acid desaturation, changes in fatty acid isomerisation and changes in composition of carotenoids appear to be the common modes of modulation of membrane fluidity in cells growing or exposed to low temperatures.

1.5.1 The role of PUFA in the bacterial cold adaptive response

The role of EPA or DHA in bacterial membranes is of great interest and has implications for understanding the biochemical functions of these unique fatty acids in plants, animals and humans. Production of EPA by some bacteria increases as temperature decreases, leading to the hypothesis that these molecules may be important for growth at low temperatures (Delong and Yayanos, 1986; Valentine and Valentine, 2004; Amiri-Jami et al., 2006). Cells must cope with decreases in temperature by modulating the lipid composition of their membrane, which can crystallize or enter nonbilayer phases at low temperatures (Russell and Nichols, 1999). A class of EPA minus mutants of *Shewanella* sp. were isolated, by using a mutational approach involving chemical mutagenesis followed by screening of large numbers of cold-sensitive mutants. Growth of the mutants, such as cs-22 and cs-52 which lack EPA, was found to be dependent on supplying EPA in the medium at 4 °C (Yazawa et al., 1988b). Similar results on requiring EPA for bacterial low temperature growth were also achieved in other *Shewanella* species (Sato et al., 2008; Wang et al., 2009). However, EPA was not required for low-temperature growth in the deep-sea bacterium *Photobacterium profundum* (Allen et al., 1999). Therefore, it is unclear why these bacteria produce omega-3 fatty acids.

A novel EPA recombinant of *E. coli* used as a "reporter" for EPA function demonstrated that EPA is the only significant fluidizing fatty acid available to the cells (Valentine and Valentine, 2004). This confirms earlier studies with yeast, which shows that DHA/EPA are able to fluidize the membranes of a eukaryotic cell (Williams et al., 1973; Walenga and Lands, 1975). About 40 % EPA of the total fatty acids presented in the membrane of the EPA recombinant whereas other potential fluidizing components are largely missing with only n-16:0 which pairs with EPA (Yazawa et al., 1988a). This is the highest level of EPA yet reported in bacteria and also represents the most highly unsaturated membranes seen in *E. coli*. Some n-14:0 is also made (5–9 %) and might contribute some fluidity. It is still unclear that unsaturated fatty acid-requiring mutant of *E. coli* fails to incorporate significant levels of EPA fed exogenously. It may be that EPA generated internally enters the membrane and contributes properties essential for growth of *E. coli* even on strictly respiratory substrates such as proline or succinate (Valentine and Valentine, 2004). The result suggests that EPA-enriched membrane clearly supports proton bioenergetics in *E. coli*. However, other polyunsaturated fatty acids common in marine oils, including n-18:3ω3 and n-20:4ω6, are also effective, for

example, Linoleic acid (LA, 18ω2) shows only traces of activity whereas n-16:1ω7, n-18:1ω9 and n-18:1ω11 are not effective (Watanabe et al., 1994). Preliminary experiments showed that DHA supports growth, but in later studies it was found that this effect is due to preferential uptake of EPA present in trace amounts in the DHA preparation. There is also a possibility that DHA is taken up and then converted to EPA in the cell (Nikaido, 1994). Unsaturated fatty acid auxotrophs of *E. coli* accept fatty acid chains with 1–3 double bonds, but not more. The levels of EPA needed to satisfy the maximum growth requirement are comparable to the values reported for *E. coli*. However, the EPA/DHA recombinant does not behave like a typical *E. coli* cell (Nakano et al., 2000), such as growth occurs around 0.2 M NaCl and restricted to about 12–22 °C (Valentine and Valentine, 2004).

1.6 The importance of PUFAs in marine food web

Many marine organisms lack the capability of synthesizing omega-3 PUFA *de novo* and rely on a dietary supply of EPA, DHA or closely related C_{18} precursors (Kanazawa et al., 1979; Intriago and Jones, 1993). At the pelagic producer–consumer interface, the presence of EPA level in marine food web has been demonstrated of importance in transferring energy and biomass (Muller-Navarra et al., 2000). Microalgae have long been known to be rich in lipids and *de novo* PUFA biosynthesis in marine food webs (Gonzalezbaro and Pollero, 1988) although DHA-rich thraustochytrids have been included as further sources to make an appreciable contribution (Lewis et al., 1999). Marine bacteria with the ability to produce PUFA has been well studied (Russell and Nichols, 1999), and may possible involve in the importance of PUFA in the marine food chain (Delong and Yayanos, 1986).

Energy transferability between trophic levels and the limitation of essential nutrients in marine food webs has gained attention widely. Ecologically, the investigation of prokaryotes is mainly focus on their role in organic matter remineralisation and nutrient cycling. However, only certain bacterial roles in marine food webs have been studied. In particular, bacteria may serve as potential providers of essential nutrients such as B-complex vitamins (Lovley and Phillips, 1994), as a food source in aquaculture-based food chains (Intriago and Jones, 1993). Thus, bacteria server as main food source for sestonivorous, omnivorous and filtering deep-sea animals (Sorokin, 1993) and as

components of the commensal microbial communities of marine animals, were considered to be importance in the marine environment (Nichols, 2003).

To date, only two bacterial phyla: the *Gamma-Proteobacteria* and the *Bacteroidetes*, have been reported as main players for PUFAs production. These PUFAs producing *Gamma-Proteobacteria* are mainly characterised as being psychrophilic, halophilic and predominantly piezophilic or piezotolerant as most of them were isolated from deep-sea ocean or polar zones (Russell and Nichols, 1999; Kato and Nogi, 2001), and few of them are identified as mesophiles isolated from a temperate estuary or shallow seawater (Ivanova et al., 2001; Skerratt et al., 2002; Ivanova et al., 2003b; Frolova et al., 2005). CFB group PUFAs producers are unable to grow at high pressure but they are similarly psychrophilic and halophilic (Kato, 1999). Therefore, these physiological characteristics may contribute to the ecological distribution of bacterial PUFA producers in the marine environment.

The abyssal environment is of great interesting regarding to the *de novo* production of PUFA and transfer to higher trophic levels. High levels of PUFA has been found in the benthic fauna (Ginger et al., 2000), for which the source of dietary is still unclear. Phytodetritus and faecal pellets represent the most common carbon and original PUFA inputs into abyssal environments (Kiriakoulakis et al., 2001), whereas particle-associated populations of flagellates with bacterial communities from surface waters may be considered as PUFA providers (Vanucci et al., 2001). Only limited studies were focused on the ecological diversity of psychrophilic and/or piezophilic bacteria in the benthic ocean (Delong et al., 1997; Yanagibayashi et al., 1999; Eardly et al., 2001). These studies still could not confirm that the existence of piezophiles in abyssal sediment could provide a *de novo* dietary source of PUFA for filtering benthic animals. However, these PUFA-producing piezophiles may act as an inoculum for the intestinal communities of abyssal filter feeders and hence they can benefit from higher nutrient contents (Deming and Colwell, 1982; Delong and Yayanos, 1986; Roberts et al., 2001). Non-detection of PUFA by lipid biomarker study is a common result in surface sediment samples from the Porcupine Abyssal Plain region of the North Atlantic (Eardly et al., 2001), and in abyssal sediments from diverse areas (Kiriakoulakis et al., 2001). There results may suggest that the limited growth and activity of cultivated piezophilic bacteria in abyssal sediment.

The annual sea ice surrounding Antarctica is a habitat where PUFA producers can be isolated. Various microalgae, mostly diatoms present in the bottom of the hard congelation ice (Bunt, 1963; Palmisano and Sullivan, 1983). Up to 50 % of the PUFAs including arachidonic acid (ARA, 20:4ω6), EPA and DHA were produced by microalgae in certain areas (Grossi et al., 1987). Ice-associated bacteria were found mainly psychrophiles containing both free-living *Gamma-Proteobacteria* and epiphytic CFB group (Grossi et al., 1984; Bowman et al., 1997a). Most of these isolates including many new species similar to those isolated from deep sea, were able to produce PUFAs (Kottmeier et al., 1987). However, little is known on the predominant of bacterial PUFA producer within the psychrophilic bacterial population that dominate the Antarctic prokaryotic sea ice community. The PUFA producers with 100 % 16S rDNA identity as *Shewanella frigidimarina* were isolated from the Arctic and Antarctica (Junge et al., 2002). This suggests that bacterial PUFA producer may play an important role in the food web of global polar marine ecosystems.

The intestinal contents of marine fish and invertebrates are commonly reported with isolation of bacterial PUFA producers. About 112 bacterial PUFA producers were found by comparing 7000 bacterial strains from the intestinal contents of temperate fish, zooplankton, shellfish and surrounding seawater/sediment (Nichols, 2003). In total 98 from 258 isolates produced EPA or DHA (38 % average) rising to 50 % of isolates for two bivalve and one amphipod species. In some invertebrates, PUFA-producing bacteria were found to be predominant of the culturable bacteria (Jøstensen and Landfald, 1997). It was estimated that DHA-producing bacteria accounted for 14 % and EPA containing bacteria 30 % of total cell counts in the intestinal contents of seven deep-sea fish. In contrast, no DHA producers but 40 EPA-producing strains were isolated from 112 strains of intestinal bacteria from 10 shallow-sea animals (Yano et al., 1997). It was concluded that PUFA-producing bacteria accounting for a large proportion of the bacterial community actively grow in the intestines of deep-sea fish (Iwanami et al., 1995; Watanabe et al., 1997).

Nichols (2003) suggested that the potential benefit of the association between PUFA bacterial producer and certain animals must be considered in order to establish a hypothetical rationale for their ecological role in the marine food web. The rate of survival and growth of detritovors may be enhanced by feeding microbial enriched detritus, which may contain bacterially derived vitamins or other micronutrients (Lovley

and Phillips, 1994). It has been demonstrated that the transfer of bacterially derived fatty acids, and specifically PUFA, between marine bacteria and higher trophic levels was possible (Ederington et al., 1995; Nichols et al., 1996b). However, it is still unclear, whether such transfer occurs in the intestinal environment of marine organisms harbouring PUFA-producing bacterial populations, has ecological significance (Nichols, 2003).

PUFA-producing bacteria could be used as feedstock for organisms, such as rotifers, by which introduce them into a marine food web for PUFA-rich oils production by aquaculture. By varying the primary bacterial feedstock, specific PUFA enrichment can be achieved, and several studies have now demonstrated the trophic transfer of both EPA and DHA from bacteria to the rotifer *Brachionus plicatilis*, which is used in the cultivation of smallmouthed larval fish (Watanabe et al., 1992; Nichols et al., 1996b; Lewis et al., 1998).

Therefore a better understanding of the biosynthetic pathway and its regulation in bacteria would pave the way for increasing levels of PUFAs, and the relative ease of genetic manipulation in bacteria makes them attractive for studying enzyme regulation and also gives the potential for gene transfer into other organisms to enhance or modify their PUFA-producing capacity.

1.7 Advantages of microbial omega-3 PUFAs

More attention has been given to the dietary importance of omega-3 PUFAs. Currently, fish oils are the primary and cheapest source of DHA and EPA, which may not be the best long-term solution to the increasing demand for these desirable fatty acids. Biomass from microbes grown under very closely controlled conditions could be a possible way to sustainable produce PUFAs (Spolaore et al., 2006). Their production can permit very close regulatory scrutiny from a range of regulatory bodies. Therefore, PUFAs from microbes can be certified as vegetarian or even vegan, as well as being potentially kosher and halal. DHA produced by Martek Biosciences are currently certified kosher by the Jewish Orthodox Union and halal by the Islamic Food and Nutritional Council of America. These oils can be incorporated into a wide range of food products without any risk of making the products unsuitable for any consumer group as a result of dietary restrictions. Moreover, the cost of biological production of DHA by using *Schizochytrium* sp. by Marteck biosciences has been demonstrated lower

than those of certain fish oils. These may be contributed by the advantages of microbial fatty acids: simple fatty acid profile; not affected by climatic, geographical or environmental factors; quality and supply can be guaranteed; vary rich in fatty acid of interest; Kosher, halal and vegetarian source; and, superior sensory and stability profile (Abril et al., 2003; Raghukumar, 2008; Fedorova-Dahms et al., 2010). Although, the microbial biomass production capacity might be limited and microbial PUFAs may potentially adverse public perception (Hammond et al., 2002).

1.8 Thesis aims and objectives

In this thesis, a number of hypotheses have been developed: 1, marine bacteria isolated from cold marine environments, such as Mid-Atlantic Ridge deep-sea sediments, North England coastal water, North-sea sponges and algae, may be able to produce PUFAs; 2, strains isolated from tropical marine environments such as Caribbean marine water and sponges may produce less or not produce PUFAs; 3, bacterial cells FA composition/patterns may be modified responding to the changes of temperature; 4, PUFA biosynthesis, speciation and the interaction of PUFA with other fatty acid types in the adaptive responses of bacteria to changing environmental conditions could be manipulated; 5, the phytoplanktonic diatoms isolated from the North Sea may highly produce EPA/DHA under local weather conditions; and 6, microbial PUFAs may play important role in marine food web.

The aims of the thesis were to: 1, study culturable microbial communities and their fatty acid composition from different marine environments; 2, biotechnological production of PUFAs under various fermentation conditions; 3, molecular analysis of bacterial PUFA biosynthesis pathway; and 4, bio-electrochemically study power generation by bacteria under anaerotic conditions.

The thesis had 9 objectives: 1, bacterial diversity and their fatty acid composition of Mid-Atlantic Ridge non-vent sediments; 2, fatty acid composition marine bacteria associated with North-sea and Caribbean marine sponge; 3, fatty acid composition marine algae associated with North-sea macro algae and microalgae; 4, taxonomic identification of novel PUFA producing bacteria; 5, study the bacterial cold-adaption mechanism by observing the modification of cell fatty acid compositions responding to temperatures; 6, optimization of EPA production under various fermentation conditions

using novel bacterial strains; 7, molecular identification of bacterial PUFA biosynthase genes in order to understand microbial EPA/DHA biosynthesis via the PKS-like pathway; 8, EPA production by marine microalgae indoors/outdoors in Northern England; and 9, electricity production by marine bacteria and estuary bacteria.

Chapter 2 presents a full study on the fatty acid production by microbial communities from Mid-Atlantic Ridge non-vent sediments; Chapter 3 reveals marine sponge-associated bacteria community from North Sea and Caribbean as sources for omega-3 fatty acid; Chapter 4 the characterization of marine macro/micro algae and their associated bacteria community from North Sea as sources for omega-3 fatty acid study; Chapter 5 presents a study on *Shewanella dovemarina* sp. nov., a psychrotrophic bacterium producing high level of polyunsaturated fatty acid and electricity, isolated from deep-sea sediments; Chapter 6 describes the optimization of eicosapentaenoic acid production by deep-sea strain *Shewanella* sp. MAR441; Chapter 7 elaborates the biosyntheses of polyunsaturated fatty acids by polyketide synthases in *Gammaproteobacteria*; Chapter 8 provides the outdoor production of eicosapentaenoic acid by marine micro algae in the UK; Chapter 9 an approach for the enhanced electricity production using reconstituted artificial consortia of estuarine bacteria grown as biofilms; and finially, Chapter 10 integrates the results of the studies described in the thesis and presents the conclusion.

References and Appendix for the work are attached at end of the thesis.

Chapter 2. Fatty acid production by microbial communities from non-vent Mid-Atlantic Ridge sediments

2.1 Abstract

Little is known about the diversity or ecology of microorganisms inhabiting Mid-Atlantic Ridge (MAR) sediments found away from hydrothermal vent systems. We isolated 312 strains of psychrotrophic bacteria from non-vent habitats, at depths between 2,400 m and 2,750 m. Gram-positive bacteria were found to be the most abundant, followed by *Gamma-Proteobacteria* and *Alpha-Proteobacteria* based on 16S rRNA gene analysis. Twenty seven strains representing different genera within the *Alpha* and *Gamma* subgroups of the *Proteobacteria*, *Firmicutes* and CFB group were analyzed for temperature-induced changes in their whole cell fatty acid (FA) compositions. Principal coordinates analysis resulted in hierarchical grouping of the bacterial community which matched the relationship revealed by molecular analysis. FA composition altered strongly with temperature. *Gram-positive* strains, mainly the *Firmicutes*, employed anteiso-15:0 and iso-15:0 FA, whereas *Alpha-*, *Gamma-Proteobactria* and CFB strains synthesized unsaturated fatty acids in response to a temperature drop. One strain, *Shewanella* sp. strain MAR445, which was phylogenetically unusual, and was found to produce 21 % eicosapentaenoic acid (EPA) in total fatty acid (TFA). Furthermore, we observed the production of squalene in a marine *Gram-positive Bacillus* strain MAR019.

2.2 Introduction

Psychrophiles and psychrotrophs are important in global ecology as a large proportion of our planet is cold (below 5 °C). Psychrophilic microorganisms are able to grow at 0 °C or lower with an optimal growth temperature at about 15 °C and a maximal growth temperature at about 20 °C, whereas, psychrotrophic microorganisms may have a maximum temperature for growth above 20 °C (Morita, 1975; Gow and Mills, 1984). There is a growing interest in studying the psychrotrophic bacteria based on their possible biotechnological applications (Aguilar, 1996). One such application is to produce polyunsaturated fatty acids (PUFAs), especially dietetic important omega-3 PUFAs, such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA,

22:6ω3). They were well documented for providing beneficial effects on human being and prevention of human diseases (Connor and Connor, 2007). EPA and DHA are natural substances mainly extracted from fish oils. However, potential problems associated with fish oils as a source of PUFA such as: taste, odor, stability problems as well as the presence of coextracted contaminants (Bersamin et al., 2007; Domingo et al., 2007). Nevertheless, the crucial problem of those oils is their sustainability due to the worldwide decline of fish stocks (Garcia and Rosenberg, 2010). Therefore natural production of omega-3 by marine microbes is a possible way to find alternative sources of nutrients. Psychrophiles and psychrotrophs may constitute an economic alimentary source recently analysed for aquaculture industries (Nichols and Russell, 1996; Yazawa, 1996). The studies have demonstrated the trophic transfer of both EPA and DHA from bacteria to the rotifer *Brachionus plicatilis*, which is used in the cultivation of smallmouthed larval fish (Watanabe et al., 1992; Nichols et al., 1996b; Lewis et al., 1998).

Gamma-Proteobacteria and the *Bacteroidetes* are the two main bacterial phyla containing PUFAs producers in the marine environment (Nichols and McMeekin, 2002). Ecologically, the most commonly reported are the *Gram-negative Alpha-*, *Beta-* and *Gamma-Proteobacteria* and the *Cytophaga–Flavobacterium–Bacteriodes* (CFB) phylum. *Coryneforms*, *Arthrobacter* sp. and *Micrococcus* sp. are the most frequently found Gram-positive bacteria (D'Amico et al., 2006), however, there are limited reports on the diversity and ecophysiology, and secondary metabolite production by Gram-positive psychrotrophic bacteria from deep sea environments. So far, bacterially derived PUFAs were mainly from Gram-negative strains (Valentine and Valentine, 2004). However, partially due to the difficulty of collecting samples, such comparative information on deep sea bacteriology is rare.

Psychrophiles and psychrotrophs may adjust their enzymes and cellular membranes in order to be metabolically active in cold environments. They can decrease their membrane fluidity by altering the lipid composition to reduce the melting point of its constituent phospholipids, and have been suggested as a homeoviscous adaptive response (Kato and Nogi, 2001), which may be achieved by increasing the degree of monounsaturated fatty acids (MUFAs) and PUFAs (Melchiodr, 1982; Russell, 1990; Skerratt et al., 2002). The finding is supported by *Shewanella* mutants deficient in EPA production, showing cold and/or pressure sensitive (Sato et al., 2008; Wang et al.,

2009). However, EPA defective mutants in *Photobacterium profundum* SS9 show no appreciable growth deficit at elevated pressure or reduced temperature (Allen et al., 1999). Thus, the function of PUFAs in the microbial membrane remains enigmatic. Therefore, more different bacterial species including no-PUFAs producing bacteria should be included for the cold-adaption study.

Mid-Atlantic Ridge (MAR) is a unique ecosystem in the deep sea, with large expanses of hard substrate, complex topography and oceanography, hydrothermal activity and an absence of a terrigenous input of sediment and organic matter. Microbial communites associated with hydrothermal vent sites on the MAR have been studied in hydrothermal plumes, bacterial mats, as endo- and ectosymbionts, and in suspension (Polz and Cavanaugh, 1995; Chin et al., 1998; Reysenbach et al., 2000; Lopez-Garcia et al., 2003a; Nercessian et al., 2005). However, relatively little attention has been given to the ecology and diversity of the free-living microbial communities that occupy the "non-vent" sites. Northern sections of the MAR (>41 ºN) are believed to be devoid of hydrothermal activity (German et al., 1994; Søilanda et al., 2008). Three sites from this region were chosen and their bacterial diversity and the fatty acid content of isolated strains were examined, in order to obtain cold adapted bacteria capable of PUFA production. In this chapter, the diversity and fatty acid metabolism of cultured psychrotrophic bacteria from MAR "non-vent" sites, were studied for the first time in this area.

2.3 Materials and Methods

2.3.1 Sample collection

Sediment samples were collected between 48° and 54°N using a megacore from three stations on the Mid-Atlantic Ridge (MAR) north and south east of the Charlie-Gibb Fracture Zone (CGFZ) on board the R.R.S James Cook from 13th July to 18th August 2007 (Table 2.1). These stations are located at depths between 2400 m and 2750 m, oceanographically separated by the Arctic Sub-polar Front and physically by the CGFZ and the ridge axis. They are remote from any islands and seamounts, with no known hydrothermal activity. Cores with intact surface sediments were chosen for sampling. One core was selected from each station and sectioned at 0 to 5 mm (surface) and 5 to 10 mm (subsurface) depth horizons. The sediment was then frozen and stored in glass vials at -80 ºC.

Table 2.1 Locations of MAR non-vent sites sediment sample collection

Station	Date	Lat	Long	Depth Water (m)	Sediment Depth (cm)	Number of isolates
Southeast	2007/7/22	49°05.40'N	27°50.22'W	2734	0-5	21
Southeast	2007/7/22	49°05.40'N	27°50.22'W	2734	5-10	133
Northwest	2007/8/4	54°01.00'N	36°13.3'W	2566	0-5	19
Northwest	2007/8/4	54°01.00'N	36°13.3'W	2566	5-10	47
Northeast	2007/8/9	54°00.65'N	34°10.42'W	2500	0-5	17
Northeast	2007/8/9	54°00.65'N	34°10.42'W	2500	5-10	75

2.3.2 Isolation of psychrophilic and psychrotrophic bacteria

The sediments (1 g wet sediment) were subsequently diluted with 4 ml autoclaved seawater passed through a 0.2 um-pore-size filter. After vigorous shaking for 30 s, the sediments were allowed to settle for 5 min before 50 µl was inoculated onto different marine agar plates and spread with an alcohol sterilized glass rod. Processed samples were inoculated onto 9 different isolation marine agar media (MA1 to MA9), which consisted of the following: MA1, Difco™ Marine Broth 2216E; MR2 (modified Zobell's agar), 10 g agar, 10 g peptone, 10 glucose, 1 g yeast extract and 1 g meat extract in 1000 ml natural seawater; MA3, 10 g agar, 10 g glycerol, 1 g yeast extract and 1 g peptone in 1000 ml natural seawater; MA4, 10 g agar, 10 g triacylglyceride, 1 g yeast extract and 1 g peptone in 1000 ml natural seawater; MA5, 10 g agar, 10 g tween 80, 1 g yeast extract and 1 g peptone in 1000 ml natural seawater; MA6, 10 g agar, 10 g casein, 1 g yeast extract and 1 g peptone in 1000 ml natural seawater; MA7, 10 g agar, 10 g soluble starch, 1 g yeast extract and 1 g peptone in 1000 ml natural seawater; MA8, 10 g agar, 1 g soluble starch, 0.5 g yeast extract, 0.5 g peptone and 0.2 g glycerophosphate (disodium pentahydrate) in 1000 ml natural seawater; MA9, 10 g agar in 1000 ml natural seawater. Plates were incubated in the dark at 4 and 15 ºC for about 45 days, for isolating anaerobic bacteria, the plates were placed in an anaerobic container system (GasPak™ EZ, BD, Maryland) containing anaerobic GasPaks (Oxoid), and 312 cold-adapted (psychrotrophic) strains were isolated consequently, 52 of which were isolated under anaerobic conditions. Base on the morphological differences, such as colony color, edge-shape, etc, 36 morphologically distinct bacterial strains were found. All these strains were cultivated at both 4 °C and 37 °C in marine broth medium,

strains grew well at 4 °C but did not grow at 37 °C, were further cultured at 4, 15 and 25 °C to get enough cells for lipid extraction.

2.3.3 MFC construction and operation

The glass dual-chamber MFC was constructed from two 250 ml bottles (Corning Inc.) with H2315 carbon cloth (4×5 cm) (Freudenberg FCCT KG, Germany) electrodes. A proton exchange membrane (inner diameter: 1.3 cm, NafionR 117, Dupont Co., Wilmington, USA) was installed between two chambers as described in Chapter 9.

2.3.4 Strain growth

For biomass production, strains were inoculated into 10 ml of Zobell's broth (ZB) (ZoBell, 1946), and incubated at 15 °C until turbidity was apparent. The 10 ml cultures were then used to inoculate 90 ml volumes of marine 2216E broth contained in 500 ml conical flasks pre-rinsed in chloroform. Flasks were incubated at 4, 15 and 25 °C respectively with agitation provided by a magnetic stirrer or orbital shaker (180 rpm) for 24-48 h until sufficient mass of estimated late-log phase cells were present for harvest. The bacterial growth curved was measured as methods mentioned in Chapter 6.

Cell mass from broth cultures was collected by centrifugation at 4500 g for 20 min. Cell pellets were resuspended in 200 ml M9 solution (22mM KH_2PO_4, 22mM Na_2HPO_4, 85 mM NaCl, 1mM $MgSO_4$) and recentrifuged followed by rinsing with 0.1 % ammonium acetate and frozen. The washed cell pellets were suspended in 2.0 ml saline and lyophilised in preweighed containers prior to lipid extraction.

2.3.5 Lipid extraction, transesterification, gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

Samples were harvested by centrifugation (4500 g, 4 °C) and frozen at -20 °C followed by -80 ^{o}C, before freeze-drying. The dry weights of pellets were determined. Fatty-acyl methyl esters were prepared by using the method sulfuric-acid-catalysed trans-esterification (Komagata and Suzuki, 1987; Christie, 1989). After the transmethylation, fatty acid methyl esters were extracted with n-hexane, concentrated under a stream of oxygen-free dry nitrogen at 37 °C. Analyses of the FAME preparations were performed with a Hewlett-Packard model 7890A gas chromatograph (Varian CP-3800, Varian, Inc. 2700 Mitchell Drive Walnut Creek, CA 94598-1675/USA) equipped with type DB225

capillary column (BPX70, 10 m x 0.1 mm, 0.2 μm; J & W Scientific, Folsom, Ca, USA) with programmed temperature of 170 °C–220 °C, a linear increase at 5 °C min^{-1}, injection and detection temperature maintained at 250 and 260 °C, respectively, and helium as the carrier gas. GC/MS analysis was carried out with Agilent 5975 GC/MS (Agilent Technologies Co., Ltd., Palo Alto , USA) equipped with HP-5ms Capillary GC-MS Column (Agilent, 19091S-433, 30 m x 0.25 mm, 0.25 μm), temperature programme 120 °C for 1 min, increased at 8 °C min^{-1} to 260 °C, which was maintained for 10 min with He as the carrier gas. MS operating conditions were as follows: electron multiplier, 2,000 V; transfer line, 250 °C; electron impact energy, 70 eV; scan threshold, 50; 1.3 scans s21 with a mass range of 50 to 500 atomic mass units; and solvent delay, 2.35 min. Compounds were identified by comparison of their retention times with those of known standards, and sample mass spectra data were compared to the mass spectra data of 275, 000 compounds in the Wiley 275 spectra library.

2.3.6 Preparation of genomic DNA and 16S rRNA gene analysis

Genomic DNA was extracted from the strains using the PureLink™ Genomic DNA Mini Kit (Invitrogen Ltd, Paisley, U.K) and used as templates for PCR amplification of the 16S rRNA gene fragments according to the methods described previously (Rainey et al., 1996). The Primers used were Eubac27F (5'-AGAGTTTGATCCTGGCTCAG-3') and Eubac1492R (5'-GGTTACCTTGTTACGACTT-3') (DeLong, 1992). The PCR products were sequenced by Eurofins MWG Operon after purification with PureLink™ PCR Purification Kit (Invitrogen Ltd, Paisley, U.K) following the manufacturer's protocol.

2.3.7 Phylogenetic analysis and diversity estimates

The 16S rRNA gene sequences determined were checked for similarities to DNA sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) and RDPII (http://rdp.cme.msu.edu) database using the Basic Local Alignment Search Tool (BLAST). The alignment and phylogenetic analysis of sequences were achieved with the neighbour-joining method by DNAMAN software package (Version 5.1), cluster and molecular evolutionary genetics Analysis (MEGA) Version 4.0.

2.3.8 Nucleotide sequence accession numbers

The nucleotide sequences of 16S rRNA gene have been deposited in EMBL under the accession numbers: FR744769-FR744821 (MAR003-MARG45).

2.3.9 Multivariate analyses

Relative abundances of major FAs after whole cell hydrolysis (Tables 2.3–2.6) were used for statistical analysis; compounds representing <1 % of total FAs were excluded. The fatty acid patterns similarities were numerically analyzed with a classification using an unweighted pair group average method (UPGAM) clustering method. Principal coordinates analyses (PCO) and box and whisker plots was used to analyze the distribution of fatty acid patterns in relation to the bacterial species. The classification and ordination analyses were carried out using the statistical software Multi-Variate statistical Package (MVSP) version 3.2 (Kovach Computing Service, Pentraeth, UK) (Kovach, 1999). Then, an eigenanalysis was performed on the matrix, resulting in different CA axes. A scatter plot was performed on the first and the second CA axis, representing the highest eigenvalues. The number of axes to extract was identified by Kaiser's rule (Kaiser, 1974).

2.4 Results

2.4.1 Location of sediment sampling sites

Deep-sea sediments were collected, between 49ºN and 54ºN (Table 2.1). Previous investigations have found high-temperature venting between 12°N and 41°N (Chin et al., 1998), while no hydrothermal vents have been located from the northern sections of the MAR (>41 ºN). The MAR-ECO expedition in June and July 2004, undertook 39 deep CTD stations at uneven spacing along the MAR between 41ºN and 61ºN and detected no buoyant hydrothermal plumes. Meanwhile lots of valuable data have been well provided from this research for our selected sections, e.g. the North Atlantic Current (NAC) crosses the MAR in a minimum of two and a maximum of four branches between 45ºN and 52ºN; stations south of 56º30`N are dominated by Sub-Arctic Intermediate Water (SAIW); and photographic evidence demonstrated the deposition of phytodetritus at depths between 2000 to 4000 m at the temperate latitudes of the

Northeast Atlantic (Billett et al., 1983; Lampitt, 1985). Analysis of the material collected by a Multiple Corer showed that it was typical of the spring bloom in the overlaying waters, which also suggested the material sank rapidly at a rate of 100-150 m day^{-1} (Billett et al., 1983). The phenomenon was supported in other regions of the world which had collected sinking organic matter in sediment trapss (Deuser and Ross, 1980). The transport of organic matter in the form of faecal pellets is important because they contain smaller particles that would not sink unless they became incorporated into something heavier (Dunbar and Berger, 1981) and are sites for bacterial growth (Azam et al., 1994). The rapid nature by which these faecal pellets sink, means the organic matter arriving at the sea floor is less degraded than aggregations of slow sinking phytodetritus (Pfannkuche, 1993).

2.4.2 Strains isolation

From a total of 6 deep-sea sediment samples, 312 bacteria were isolated at low temperatures under aerobic condition, using 9 different isolation marine agar media (MA1 to MA9). Of these strains, 49.3 % was from southeast station (49°05.40'N, 27°50.22'W), 21.2 % was from northwest and 29.5 % from northeast (54°01.00'N, 36°13.3'W). Base on the morphological differences, such as colony color, edge-shape, etc, 36 morphologically distinct bacterial strains were found. Psychrophilic strains were seldom isolated as most of the strains could still grow from 20 °C to 30 °C, although 108 strains, most of which isolated from subsurface, grew fast at 4 °C and with optimal growth temperature at 15 °C (e.g. Figure 6.1 in Chapter 6), whereas at lest 152 strains have some growth at 4 °C with optimal growth temperature at around 25 °C, therefore all the strains isolated could be assigned as psychrotrophs. Gram-positive bacteria accounted for 68 % of the strains based on the gram staining technique and further confirmed by molecular identification. Gram-positive strains were for the most part brightly pigmented, psychrotrophic, and some of them possessed an oxidative metabolism. About 82 % of the isolates were found to be presented in most surface sediment samples (0-5 mm depth) and relative less from the subsurface sediments (5-10 mm depth), for example, about 43 % of isolates from subsurface in Southeast station, only 15 % from subsurface sediments in Northeast and about 24 % from subsurface sediments in Northwest (Table 2.1).

2.4.3 Phylogenetic groups and identification of the benthic bacteria

From a total of 6 deep-sea sediment samples, 312 bacteria were isolated, of which, 105 were chosen for phylogenetic analysis based on colony colour and morphology difference. They fell into nine major lineages of the domain Bacteria (Hugenholtz et al., 1998). Their ratios in each bacterial community were shown in Figure 2.1. Gram-positive bacteria with low G+C content (*Firmicutes*) constituted the most abundant division of the bacterial populations, followed by *Gamma-Proteobacteria* in these deep-sea sediments. Only a few strains fall into Gram-positive with high G+C content (*Mcicrococcaceae*), *Alpha-Proteobacteria* and C*ytopahga-Flexibacteria-Bacteroides* (CFB) group. NCBI nucleotide BLAST searches using the partial 16S rRNA gene sequences of these 105 strains revealed that 73 (69.5 %) of the isolates were Gram positive and shared a phylogenetic affiliation with members of the *Bacillaceae*, *Planococcaceae*, *Nocardiaceae* and *Micrococcaceae*. These results were further confirmed by gram staining and microscope pictures. Sixty-seven strains belonged to the phylogenetic group of as "low G+C Gram positive" representatives of the family *Bacillaceae* including 11 taxonomic units and showed *B. pseudofirmus* (19.7 %), *B. safensis* (15.2 %), *Jeotgalibacillus marinus* (13.6 %), *B. pumilus* (12.1 %), *B. sonorensis* (10.6 %) and *B. decolorationis* (7.6 %) as major isolates (8-20 %) and contained less *B. licheniformis*, *B. tequilensis*, *B. horneckiae*, *B. hwajinpoensis*, *B. circulans, B. simplex* and *Thalassobacillus devorans* (2-5 %), by exhibiting similarity values with representative type strains of this family ranging from 93.4 to 99.9 % (Table 2.2).

Strain MAR899 was most closely affiliated with 16S rRNA gene reference sequences of the family *Planococcaceae* by exhibiting the highest similarity value of 98.8 % with type strain *Paenisporosarcina quisquiliarum* SK 55^T. Two families *Micrococcaceae* and *Nocardiaceae* were felled into high G+C Gram positive bacteria division, containing 4 different species: MAR443 showing the highest similarity value of 97.2 % with *Micrococcus terreus* V3M1^T; MAR806 showing most closely affiliated (99.6 %) with *Micrococcus yunnanensis* YIM 65004^T; MAR387 and MAR801 exhibiting 99.6 % similar to *Kocuria marina* KMM 3905^T and *Kocuria rhizophila* DSM 11926 respectively; and anaerobic isolates MARG10 and MARG12^T exhibiting the highest similarity (99.6 %) with *Rhodococcus erythropolis* NBRC 100887.

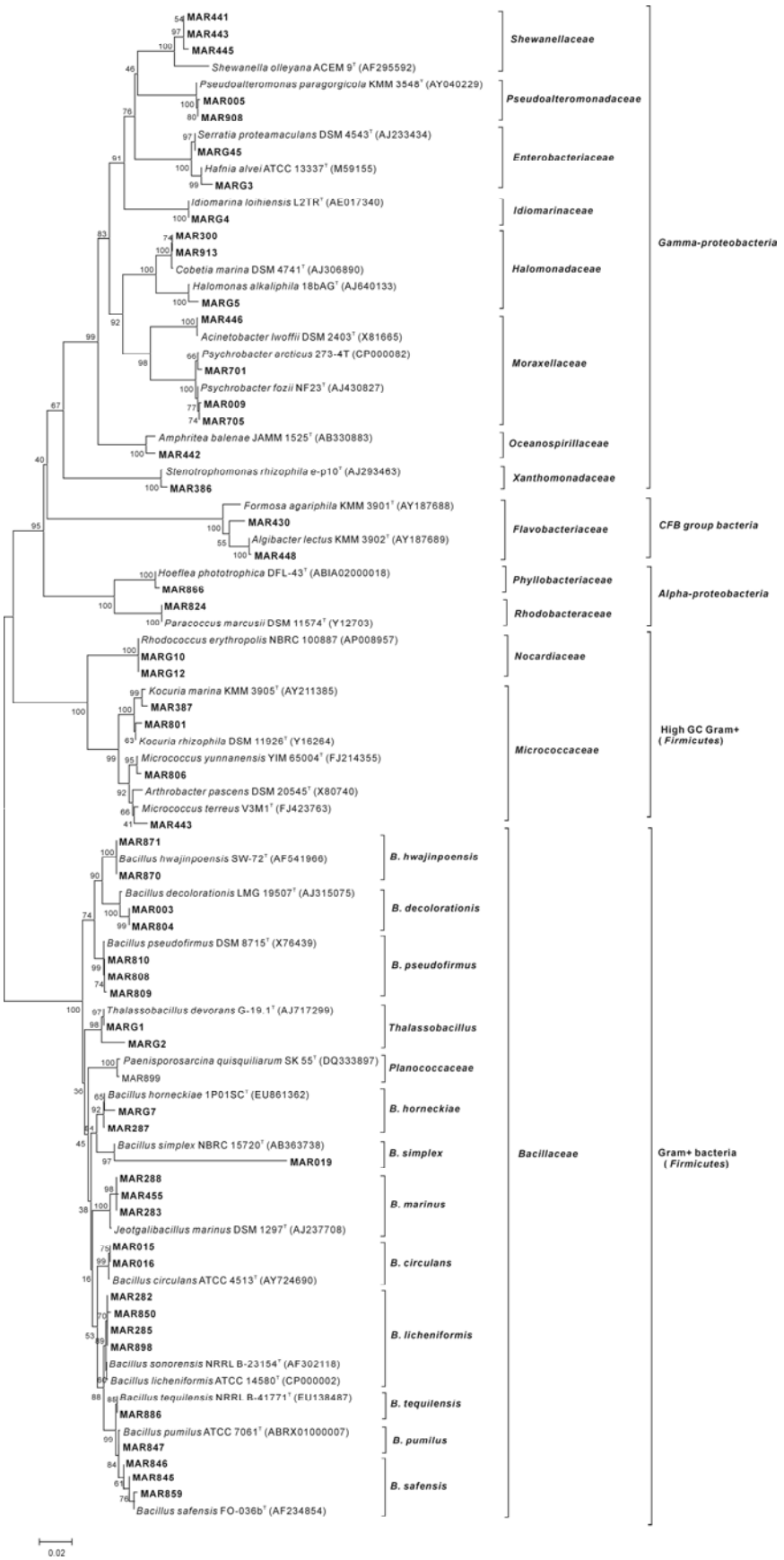

Figure 2.1 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of 53 representative strains observed in this study (indicated in bold) and their nearest type strains.

Table 2.2 Bacterial strains isolated from Mid-Atlantic Ridge non-vent sediments[a]

Phylogenetic group (family)	Representative isolates	No. of strains in OTU	Nearest type strain in Bank (accession number)	Similarity (%)	Sediment Depth (cm)	Source of nearest type strain
Firmicutes						
Bacillaceae	**MAR886**	1	*Bacillus tequilensis* NRRL B-41771^T (EU138487)	99.951	5-10	Culture Collection
	MAR282; MAR285; MAR289; MAR802; MAR834; MAR835; MAR840	7	*Bacillus sonorensis* NRRL B-23154^T (AF302118)	96.275	5-10	Soil in the Sonoran Desert, Arizona
	MAR898; MAR850	2	*Bacillus licheniformis* ATCC 14580^T (CP000002)	99.176	0-5	Persian petroleum reservoir
	MAR003; MAR001; MAR002; **MAR804**; MAR805	5	*Bacillus decolorationis* LMG 19507^T (AJ315075)	97.915	5-10	Mural paintings
	MAR807; MAR836; MAR837; MAR839; MAR841; **MAR845**; **MAR846 MAR859**; MAR860; MAR861	10	*Bacillus safensis* FO-036b^T (AF234854)	99.568	5-10	Sspacecraft and assembly-facility surfaces
	MAR847; MAR849; MAR851; MAR853; MAR854; MAR855; MAR856; MAR857	8	*Bacillus pumilus* ATCC 7061^T (ABRX01000007)	99.646	5-10	
	MAR808; MAR820; MAR821; MAR825; MAR826; MAR827; MAR828; MAR829	8	*Bacillus pseudofirmus* DSM 8715^T (X76439)	99.240	5-10	pig skins/bovine bones
	MAR809; **MAR810**; MAR814; MAR816; MAR818;	5	*Bacillus pseudofirmus* DSM 8715^T (X76439)	99.101	5-10	pig skins/bovine bones
	MAR283 ; MAR288 ; MAR302; MAR303; MAR305; MAR306	6	*Jeotgalibacillus marinus* DSM 1297^T (AJ237708)	96.931	5-10	deep sea sediments
	MAR454; **MAR455**; MAR456	3	*Jeotgalibacillus marinus* DSM 1297^T (AJ237708)	97.992	5-10	deep sea sediments
	MAR287, MARG7	2	*Bacillus horneckiae* 1P01SC^T (EU861362)	98.327	5-10	spacecraft-assembly clean room
	MAR870; MAR871	2	*Bacillus hwajinpoensis* SW-72^T (AF541966)	99.723	0-5	sea water
	MAR015; MAR016; MAR017	3	*Bacillus circulans* ATCC 4513^T (AY724690)	99.053	5-10	Soil
	MAR019; MAR020	2	*Bacillus simplex* NBRC 15720^T (AB363738)	93.376	0-5	Mural paintings
	MARG1, MARG2	2	*Thalassobacillus devorans* G-19.1^T (AJ717299)	98.538	5-10	phenol enrichment
Planococcaceae	**MAR899**	1	*Paenisporosarcina quisquiliarum* SK 55^T (DQ333897)	98.812	0-5	landfill soil
high GC Gram+						
Micrococcaceae	MAR443	1	*Micrococcus terreus* V3M1^T (FJ423763)	97.156	0-5	forest soil
	MAR806	1	*Micrococcus yunnanensis* YIM 65004^T (FJ214355)	99.638	0-5	Polyspora axillaris roots
	MAR387	1	*Kocuria marina* KMM 3905^T (AY211385)	99.583	0-5	marine sediment
	MAR801	1	*Kocuria rhizophila* DSM 11926^T (Y16264)	99.582	5-10	rhizoplane of the narrow-leaved cattail
Nocardiaceae	MARG10, MARG12	2	*Rhodococcus erythropolis* NBRC 100887 (AP008957)	99.643	5-10	Pacific Ocean
Alpha-proteobacteria						
Phyllobacteriaceae	MAR866	1	*Hoeflea phototrophica* DFL-43^T (ABIA02000018)	99.852	5-10	cultures of marine dinoflagellates
Rhodobacteraceae	MAR824	1	*Paracoccus marcusii* DSM 11574^T (Y12703)	99.926	0-5	agar plate
CFB group bacteria						
Flavobacteriaceae	MAR430	1	*Formosa agariphila* KMM 3901^T (AY187688)	98.253	5-10	Sea water
	MAR448	1	*Algibacter lectus* KMM 3902^T (AY187689)	99.790	5-10	sea urchin in Troitsa Bay
Gamma-proteobacteria						
Xanthomonadales; Xanthomonadaceae	MAR386	1	*Stenotrophomonas rhizophila* e-p10^T (AJ293463)	99.237	0-5	various patient sites
Oceanospirillaceae	**MAR442**	1	*Amphritea balenae* JAMM 1525^T (AB330883)	98.084	0-5	sediment adjacent to sperm whale
Halomonadaceae	**MARG5**	1	*Halomonas alkaliphila* 18bAG^T (AJ640133)	98.897	0-5	salt pool
	MAR300	1	*Cobetia marina* DSM 4741^T (AJ306890)	96.637	0-5	Marine water
	MAR913	1	*Cobetia marina* DSM 4741^T (AJ306890)	97.697	5-10	Marine water
Shewanellaceae	MAR441, MAR444 ; **MAR445**	3	*Shewanella olleyana* ACEM 9^T (AF295592)	97.874	0-5	temperate estuary
Enterobacteriaceae	**MARG3**, MARG8, MARG11, MARG13	4	*Hafnia alvei* ATCC 13337^T (M59155)	90.571	5-10	
	MARG45	1	*Serratia proteamaculans* DSM 4543^T (AJ233434)	99.794	5-10	Soil
Idiomarinaceae	MARG4	1	*Idiomarina loihiensis* L2TR^T (AE017340)	99.787	5-10	Hydrothermal vent
Pseudoalteromonadaceae	**MAR005**; MAR025; MAR026; MAR027; MAR029;	5	*Pseudoalteromonas paragorgicola* KMM 3548^T (AY040229)	98.643	5-10	A sponge
	MAR880; **MAR908**	2	*Pseudoalteromonas paragorgicola* KMM 3548^T (AY040229)	99.788	0-5	
Moraxellaceae	MAR446	1	*Acinetobacter lwoffii* DSM 2403^T (X81665)	98.955	0-5	
	MAR009; MAR010; MAR028; **MAR705**	4	*Psychrobacter fozii* NF23^T (AJ430827)	99.864	0-5	Antarctic coastal marine environments
	MAR701; MAR703	2	*Psychrobacter arcticus* 273-4^T (CP000082)	99.128	0-5	A terrestrial environment

[a]Strains chosen for phylogenetic analysis were indicated in bold text.

The remaining bacteria cultured, 32 strains (30.5 % of the 105 isolates) were divided into three groups: *Gamma-Proteobacteria* (26.7 %), *Alpha-Proteobacteria* (4 %) and CFB group bacteria (4 %). Of these Gram-negative sequences identified, 2 (6.3 %), MAR886 and MAR824 were phylogenetically affiliated with *Alpha-Proteobacteria* phylum and the order *Rhizobiales*, which are most closely related to 2 separate family level groupings: *Phyllobacte* and *Rhodobacteraceae*. MAR886 was exhibited 99.8 % the highest similarity with *Hoeflea phototrophica* DFL-43^T and MAR824 was 99.9 % similar to *Paracoccus marcusii* DSM 11574^T. Another two isolates (6.3 %), MAR430 showing most closely affiliated (98.2 %) with *Formosa agariphila* KMM 3901^T and MAR448 exhibiting 99.7 % similarity with *Algibacter lectus* KMM 3902^T, were phylogenetically affiliated with the same family *Flavobacteriaceae* of C*ytopahga-Flexibacteria-Bacteroides* (CFB) group. Then the most dominant Gram negative group was *Gamma-Proteobacteria* with 14 different species (87.5 %). These isolates formed highly diverse clades (8 families): *Xanthomonadaceae* with 1 isolate MAR386, showing 99.2 % the highest similarity with *Stenotrophomonas rhizophila* e-p10^T; *Oceanospirillaceae* with 1 isolate, MAR442*,* both exhibiting 98.1 % the most closely affiliated with *Amphritea balenae* JAMM 1525^T; *Halomonadaceae* with 3 isolates, MAR300 and MAR913 showing the highest similarity (96.6 % and 97.7 %) to *Cobetia marina* DSM 4741^T, and anaerobic isolate MARG5 showing 98.9 % to *Halomonas alkaliphila* 18bAG^T*; Shewanellaceae* with 3 isolates, MAR441, MAR443 and MAR445, showing 97.9 % to *Shewanella olleyana* ACEM 9^T; *Enterobacteriaceae* with 5 strains isolated under anaerobic conditions, MARG3, MARG8, MARG11 and MARG13 exhibiting the closest similarity (90.6 %) to *Hafnia alvei* ATCC 13337^T, and MARG45 showing 99.8 % to *Serratia proteamaculans* DSM 4543^T; *Idiomarinaceae* with 1 anaerobic isolate MARG4, 99.8 % to *Idiomarina loihiensis* L2TR^T; *Pseudoalteromonadaceae* with 7 isolates, MAR005, MAR025, MAR026, MAR027, MAR029, MAR880 and MAR908 showing 98.6-99.7 % similarities to *Pseudoalteromonas paragorgicola* KMM 3548^T; and *Moraxellaceae* with 9 isolates, MAR446 showing 98.9 % homology to *Acinetobacter lwoffii* DSM 2403^T, MAR009, MAR010, MAR028 and MAR705 exhibiting 99.8 % similarity to *Psychrobacter fozii* NF23^T, MAR701 and MAR703 exhibiting 99.1 % homology to *Psychrobacter arcticus* 273-4^T.

Among the above isolates, 72 strains (68.6 %) showed 98.0-99.9 % the highest similarities with their nearest type strains, whereas 33 strains (31.4 %) exhibited <98 %

homology. These strain may be considered as separated species in that 16S rRNA gene sequence showing <98 % sequence similarity according to previous studies (Fry et al., 1991; Dighe et al., 2004; Janda and Abbott, 2007).

2.4.4 Fatty acid composition of MAR benthic isolates

2.4.4.1 Alpha-Proteobacteria

The *Alpha-Proteobacteria Hoeflea* sp. strain MAR886 and *Paracoccus* sp. strain MAR824 showed the highest sequence similarities to *Hoeflea phototrophica* DFL-43^T (ABIA02000018*)* (99.8 %) and *Paracoccus marcusii* DSM 11574^T (Y12703) (99.9 %) respectively on the basis of the levels of 16S rRNA gene sequence similarity. They both had a very simple pattern, with the n-18:1ω7c component accounting for >70 % of the FAs (Table 2.3). Among the remaining FAs, for strain MAR886, only n-16:0, n-18:0 and n-18:1ω9c was present in significant abundance (3-9 %), whereas only traces (<1.4 %) of n-14:0, n-16:1ω7 and n-16:1ω9 acids were detected (Table 2.3); for strain MAR824, contained less proportion of n-10:0, n-16:0, n-16:1ω7 and n-18:0 acids (4-8 %). These patterns strongly resembles those of other *Hoeflea* species (Biebl et al., 2006) and closely related Alphaproteobacteria-like members of the *Paracoccus* sp. (Harker et al., 1998; Freese et al., 2008). Cells of *Hoeflea* sp. MAR886 showed no remarkable shift in FA composition and average chain length with changing growth temperature. The relative amounts of monounsaturated FAs (MUFAs) and saturated straight chain fatty acids (SCFAs) remained almost constant at ca. 88-90 % and 7-10 %, respectively (Table 2.3 and Figure 2.3). Traces of branched chain FAs (BCFAs), such as i-15:0, ai-15:0 and i-17:0 were detected only in cells grown at 4 °C and 15 °C.

2.4.4.2 Cytophaga–Flavobacterium–Bacteroides (CFB) bacteria

CFB group bacteria *Formosa* sp. strain MAR430 exhibited 98.3 % homology to *Formosa agariphila* KMM 3901^T (AY187688) based on 16S rRNA gene phylogenetic studies. The cellular fatty acid composition of strain MAR430 was found with various branched-chain fatty acids, such as i-13:0, i-15:0 and ai-15:0 (Table 2.3). The branched-chain fatty acids formed at stable level with a fraction of 15 % when growing at 4 and 15 °C, while only 9 % at 25 °C. Among the remaining FAs, n-15:0, n-16:0, n-16:1ω9, n-17:1ω6, n-18:1ω7c and n-18:1ω7c were present in significant abundance (8-23 %). The pattern of the predominant fatty acids are comparable with those from *Formosa*

agariphila KMM 3901^T and *Formosa algae* (Ivanova et al., 2004a; Freese et al., 2008), mainly iso-15:0, n-15:0, i-15:1, ai-15:0 and i-17:0. When compared with another strain of CFB genus, *Aquiflexum balticum* strain BA160^T, the number of fatty acid compounds detected in strain MAR430 is similar and the composition differs considerably (Brettar et al., 2004). The major difference is the lower abundance of iso-15:0 and ai-15:0 in strain MAR430 (strain BA160^T, 22.6 and 18.5 %). However, strain BA160^T with more BCFAs, such as i-16:1, iso- and ai-17:0. Cells of strain MAR430 showed remarkable shift in FA composition with changing growth temperature from 25 to 4 ºC, the increasing amounts of MUFAs was at the expense of saturated analogues and the average chain length remained constant (Figure 2.2).

2.4.4.3 Gamma-Proteobacteria

The isolates affiliated with the *Gamma-Proteobacteria* showed remarkable variations in FA compositions. The strain affiliated with the genus *Psychrobacter* had patterns dominated by n-18:1ω9 acid (30–70 %), a FA found only in comparatively small amounts (<15 %) in the other gammaproteobacterial isolates (Table 2.4). In fact, the dominance of the n-18:1ω9 acid appears to be typical of *Psychrobacter* species (Bowman et al., 1996; Romanenko et al., 2004; Shivaji et al., 2004; Yoon et al., 2005b; Freese et al., 2008). 16S rRNA gene phylogenetic studies confirmed that *Psychrobacter* sp. strain MAR701 was most closely related to *Psychrobacter arcticus* 273-4^T (CP000082) (99.1 %). Strain MAR701 contained significantly lower amounts (5–10 %) of n-14:1ω7, n-16:0, n-17:1ω8 and n-18:0 acids. The temperature modulation of the FA patterns in strain MAR701 did not follow a unidirectional trend. An increase in growth temperature from 4 ºC to 25 ºC led to a decrease in MUFAs (83–30 %) in favour of SCFAs (5–25 %), also with the compensation of BCFAs (12–44 %). Cells grown at 25 ºC showed almost the same pattern as cells grown at 10 ºC. The average chain length remained more or less constant between 4 ºC and 15 ºC, but with 1 carbon less at 25 ºC due to a great level decreasing of long chain fatty acids, MUFAs (Figure 2.2).

Table 2.3 Major FAs after whole cell hydrolysis (% of total FAs) in bacterial strains affiliating with the *Proteobacteria* and *CFB Group* bacteria

	Hoeflea phototrophica			*Paracoccus marcusii*			*Formosa agariphila*			*Stenotrophomonas rhizophila*			*Amphritea balenae*			*Pseudoalteromonas paragorgicola*		
	MAR866			MAR824			MAR430			MAR386			MAR442			MAR005		
	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC
n-10:0	-	-	-	4.4	4.5	7.8	-	-	-	-	-	-	-	-	-	-	-	-
n-12:0	-	-	-	-	-	-	1.9	1.5	5.1	2.7	2.5	3.4	0.5	2	3.7	1.7	1.8	1.6
i-13:0	-	-	-	-	-	-	9.5	8.7	6.1	19.7	18.8	20.5	-	-	-	-	-	-
n-13:0	-	-	-	-	-	-	1.3	1.1	2.1	0.8	0.4	1.2	-	-	-	0.1	0.1	0.5
n-14:0	0.5	0.4	0.4	0.6	1.1	1.6	2.2	2.9	5.5	3.3	4.1	7.4	0.7	2	2.1	-	-	-
n-14:1ω7c	-	-	-	-	-	-	-	-	-	-	-	-	2.8	1.2	1.5	0.8	0.4	0.2
i-15:0	0.1	0.2	-	-	-	-	1.2	2.7	1.7	11.4	12	13.5	-	-	-	0.4	0.3	0.5
ai-15:0	0.2	0.1	-	-	-	-	3.9	3.4	1.1	0.5	0.3	0.4	-	-	-	-	-	-
n-15:1ω6	-	-	-	-	-	-	1.4	2.5	2.2	4.6	4	3.5	-	-	-	2.5	2	1.7
n-15:0	0.1	0.2	0.1	0.2	0.5	0.3	8.0	11.6	13.9	11.8	13.1	14.5	-	-	-	0.4	2.6	3.5
n-16:1ω9	1.4	0.7	0.5	-	-	-	23.7	18.7	14.8	26.5	21.2	12.9	49.2	43	38.7	3.7	1.2	0.5
n-16:1ω7	1.2	2.2	2.1	4.5	6.6	5.5	-	-	-	-	-	-	-	-	-	56	41.4	25
n-16:1ω5	-	-	-	-	-	-	0.6	2.1	1.7	-	-	-	-	-	-	3.1	1.3	0.3
n-16:0	3.5	4.0	4.2	2.1	4.2	5.3	10.2	12.5	15.4	10.4	11.6	13.3	16.3	29	34.8	7.8	26.6	28.5
ai-17:1ω7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.7	3.3	1.6
i-17:0	0.2	0.1	0.3	-	-	-	-	-	-	6.1	6.5	6.3	-	-	-	1.2	1.9	1.3
n-17:1ω8	-	-	-	-	-	-	-	-	-	2.2	5.5	3.1	-	-	-	5.2	5.8	6.3
n-17:1ω6	-	-	-	-	-	-	13.7	9.2	8.5	-	-	-	0.9	0.8	0.5	1	0.5	0.2
n-17:0	-	-	-	-	-	-	7.2	9.5	11.5	-	-	-	-	-	-	2.5	3.3	3.7
n-18:1ω9c	9.1	7.1	5.5	-	-	-	1.0	1.4	1.1	-	-	-	29.6	22	18.7	0.5	0.2	0.2
n-18:1ω7c	80.2	79.4	80.8	82.9	77	71.0	11.7	8.5	5.4	-	-	-	-	-	-	7.4	6.7	24
n-18:0	3.5	5.6	6.1	5.3	6.6	8.5	1.9	2.9	3.4	-	-	-	-	-	-	0.3	0.6	0.4
n-19:0	-	-	-	-	-	-	0.5	0.8	0.5	-	-	-	-	-	-	-	-	-
Σ TFA	100	100	100	100	100	100	100	102	100	100	100	100	100	100	100	100	100	100
Σ SCFA	7.6	10.2	10.8	13	17	24	31.9	41.7	55.3	29	31.7	39.8	17.5	33	40.6	13	35	38.2
Σ BCFA	0.5	0.4	0.3	-	-	-	14.6	14.8	8.9	37.7	37.6	40.7	-	-	-	7.3	5.5	3.4
Σ MUFA	91.9	89.4	88.9	87	83	77	52.1	42.4	33.7	33.3	30.7	19.5	82.5	67	59.4	80	59.5	58.4
ACL[a]	17.8	17.8	17.8	18	17	17	15.9	15.8	15.6	15	15.1	14.8	16.5	16	16.2	16	16.2	16.5

[a] Average chain length (ACL) calculated after (White et al., 2005a); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; and (–), not detectable.

Pseudoalteromonas sp. strain MAR005 was most related to *Pseudoalteromonas paragorgicola* KMM 3548^T (AY040229) based on 16S rRNA gene identification with 98.6 % similarity. FA patterns of strain MAR005 were dominated by the n-18:1ω7, n-17:1ω8, ai-17:1ω7, n-16:1ω7 and n-16:0 acids and showed minor (1–4 %) contributions from n-12:0, n-14:0, n-15:0, n-15:1ω6, n-16:1ω5, n-16:1ω9, i-17:0, and n-17:0 acids (Table 2.4). Similar patterns have been found in other *Pseudoalteromonas* strains (Romanenko et al., 2003b; Al Khudary et al., 2008). Strain MAR005 also showed a relatively remarkable response to changing growth temperature. With increasing temperature from 4 ºC to15 ºC, the proportion of MUFAs decreased (80–60 %), also BCFAs decreased slightly (7.3–5.5 %) whereas SCFAs increased (13–35 %). However the differences on MUFAs and SCFAS between 15 ºC and 25 ºC were very slightly (Figure 2.2).

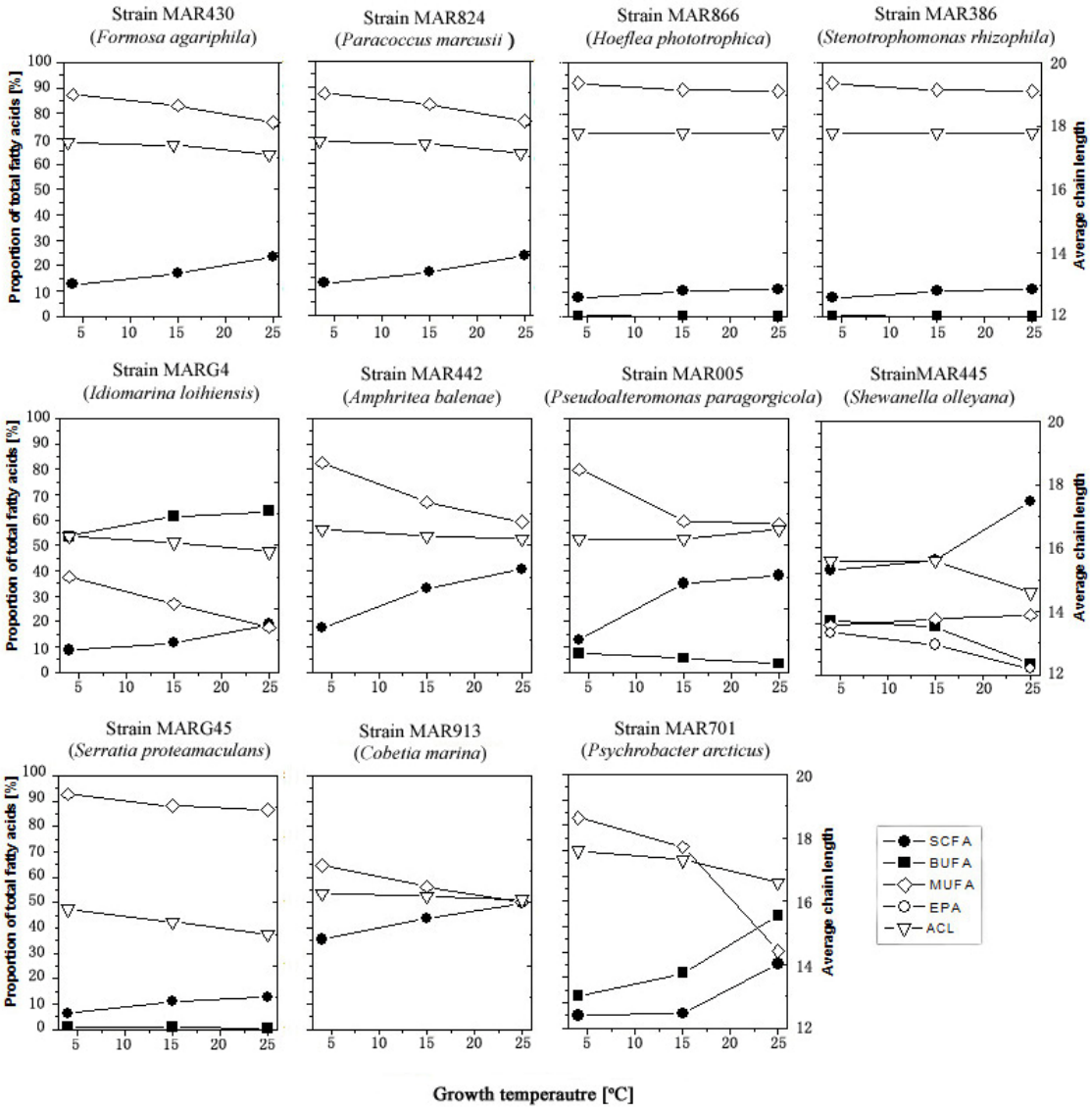

Figure 2.2 Change in average chain length (inverted triangles) and relative proportion of whole cell FAs in *Alpha*-, *Gamma-Proteobacteria* and CFB group strains grown at 4, 15 and 25 ^{o}C. Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open diamonds), eicosapentaenoic acid (EPA, open circles) and nearest type strains were indicated below the isolates. Values are means of three samples based on Table 2.3 and Table 2.4.

Shewanella sp. strain MAR445 exhibited 97.8 % the highest similarity on 16S rRNA gene sequence level with *Shewanella olleyana* ACEM 9^{T} (AF295592). FA patterns of strain MAR445 was dominated by n-13:0, i-15:0, n-16:0, n-16:1ω9 and n-20:5ω3 acids (10-40 %) as usual for this genus, with minor contributions (1-7 %) of n-12:0, i-13:0, n-14:0 and n-15:0 acids (Table 2.4). The *Shewanella* strain differed from the other Gammaproteobacteria by the presence of i-13:0 and i-15:0 branched FAs. Strain MAR445 contained the unusual polyunsaturated eicosapentaenoic acid (EPA), n-20:5ω3 (up to 18 %), which was supposed to be fell in the group 1 *Shewanella* species

which were subdivided based on the temperature range for growth and lipid pattern (Kato and Nogi, 2001). Group 1 species were characterised as high pressure and/or cold-adapted species that produce substantial amounts of EPA (11–16 %), whereas group 2 species comprised mesophilic, pressure-sensitive species that produce no or only low levels of EPA (3–5 %). *Shewanella* sp. strain MAR445 grew well at the range between 4 to 25 °C with optimal growth temperature at 15 °C, exhibiting sigmoidal growth even at 0 °C. The effect of growth temperature on the percentage composition of individual fatty acids in MAR445 grown between 4 ºC and 25 ºC is shown in Table 2.3. Growth within the optimal region (4 ºC and 15 ºC) resulted in the highest percentage of i-13:0, i-15:0 and EPA, while n-16:1ω9 and the sum of MUFAs were at their lowest level. At growth temperatures above the optimal region, the percentage of n-13:0 and n-16:0 and SCFAs were maximal, while the percentage of n-14:0 decreased with increasing growth temperature. Overall the increase the percentage of PUFAs and BCFAs were with the expense of SCFAs and MUFAs. The average chain length decreased apparently at 25 °C due to the decreasing production of PUFAs (Figure 2.2). These findings are agreeable to the previously research on EPA which is a very distinctive feature of psychrophilic and piezophilic bacteria (Nichols et al., 1997; Yang et al., 2007).

Stenotrophomonas sp. strain MAR386 showed the highest 16S rRNA gene sequence similarity (99.2 %) to *Stenotrophomonas rhizophila* e-p10^{T} (AJ293463), and with i-13:0, i-15:0, n-15:0, n-16:1ω9 and n-16:0 as major FAs (10-20 %) and contained less n-12:0, n-14:0, n-15:1ω6, i-17:0 and n-17:1ω6 acids (2-5 %). Only minor proportions of n-13:0 and ai-15:0 acids (0.5-1.5 %) were found (Table 2.3). Strain MAR386 showed a relatively weak response to changing growth temperature, and the average chain length remained constant. With increasing temperature, the proportion of MUFAs decreased slightly with the rise of SCFAs and BCFAs whereas BCFAs remained stable at 4 ºC and 15 ºC (Figure 2.2).

Amphritea sp. strain MAR442 was most closely related to *Amphritea balenae* JAMM 1525^{T} (AB330883) (98.1 %) based on 16S rRNA gene phylogenetic studies. Strain MAR442 showed only n-16:1ω9, n-16:0 and n-18:1ω9 as major FAs (16-50 %) and only minor proportions of n-12:0, n-14:0, n-14:1ω7 and n-17:1ω6 acids (0.5-5 %) (Table 2.3). The FA profiles of the strain MAR442 indicated similarity to the type strain of *A. atlantica* M41^{T} which was also isolated from Mid-Atlantic Ridge at Logatchev

hydrothermal vent field (Gartner et al., 2008; Miyazaki et al., 2008). Strain MAR442 showed a relatively strong response to changing growth temperature. With increasing temperature from 4 ºC to 25 ºC, the proportion of MUFAs decreased from 82 % - 59 % with the increasing of SCFAs, and the average chain length remained constant (Figure 2.2).

Table 2.4 Major FAs after whole cell hydrolysis (% of total FAs) in bacterial strains affiliating with the *Proteobacteria*

	Shewanella olleyana			*Idiomarina loihiensis*			*Serratia proteamaculans*			*Cobetia marina*			*Psychrobacter arcticus*		
	MAR445			MARG4			MARG45			MAR913			MAR701		
	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC
n-10:0	-	-	-	0.6	0.5	0.9	-	-	-	0.7	0.8	1.3	1	0.3	1.7
n-12:0	2.1	1.9	2.3	0.9	1.1	3.2	0.8	1	1.4	1.2	1.7	1.9	0.2	1	3
i-13:0	7.2	7.3	1.2	1.5	1	1.1	-	-	-	-	-	-	0.1	0.4	1.6
n-13:0	20.9	23.0	47.5	-	-	-	-	-	-	-	-	-	-	-	-
i-14:0	0.7	0.7	0.1	-	-	-	-	-	-	-	-	-	-	-	-
n-14:0	4.1	3.9	2.1	-	-	-	4.2	8.5	9.1	2.6	3.4	4.6	-	-	-
n-14:1ω7c	-	-	-	-	-	-	-	-	-	-	-	-	0.2	6.8	4.2
i-15:0	12.3	10.3	1.8	24.5	35.3	39.2	1.1	0.8	0.5	-	-	-	-	-	-
ai-15:0	0.2	0.1	0.3	0.6	0.4	0.5	-	-	-	-	-	-	-	-	-
n-15:1ω6	0.1	0.0	0.0	2.5	2.3	1.4	-	-	-	2.5	2.3	1.9	-	-	-
n-15:0	2.3	2.6	0.3	0.5	1.8	2.5	0.4	0.5	0.9	3.5	4.8	5.8	0.1	-	6.2
n-16:1ω9	13.7	14.8	18.1	10.5	7.2	4.5	29.5	27.8	22	-	-	-	-	-	-
n-16:1ω7	1.1	0.3	0.85	-	-	-	-	-	-	33.1	31.6	27.1	-	-	-
n-16:1ω5	-	-	-	6.2	6.3	8.1	16.3	26.1	33.1	-	-	-	-	-	-
n-16:0	11.0	12.1	14.7	15.8	14.3	9.1	-	-	-	18.2	21.7	23.6	2.3	0.8	11
i-17:1ω7	-	-	-	-	-	-	-	-	-	-	-	-	12	21	38.9
ai-17:1ω7	-	-	-	11.2	10.5	13.5	-	-	-	-	-	-	-	-	-
i-17:0	-	-	-	1.6	0.9	0.8	21.5	15.2	11.9	-	-	-	0.1	1.1	3.7
n-17:1ω8	0.3	1.5	0.8	1.6	1.5	1.8	-	-	-	14.9	11.7	12.1	9.3	5.3	-
n-17:1ω6	-	-	-	-	-	-	0.2	0.5	0.5	-	-	-	-	-	-
n-17:0	0.6	0.7	0.5	8.4	5.4	2.5	-	-	-	8.5	9	9.0	-	-	-
n-18:1ω9c	0.5	0.6	0.2	11.9	8.3	6.1	23.7	19.6	18.5	-	-	-	73	59	26.2
n-18:1ω7c	4.9	5.1	4.7	0.6	1.8	4.2	0.6	0.6	0.9	14	10.7	9.3	-	-	-
n-18:0	0.4	1.3	1.0	1.1	1.4	0.6	-	-	-	0.8	2.3	3.4	1.3	3.7	3.5
n-18:2ω6t	0.1	-	-	-	-	-	-	-	-	-	-		-	-	-
n-18:3n3	0.1	0.3	0.9	-	-	-	-	-	-	-	-	-	-	-	-
n-18:4n3	0.3	0.6	0.0	-	-	-	1.7	1.4	1.2	-	-	-	-	-	-
n-20:4n3	0.6	0.7	0.1	-	-	-	-	-	-	-	-	-	-	-	-
n-20:5ω3	16.6	11.9	2.4	-	-	-	-	-	-	-	-	-	-	-	-
n-22:5ω3	-	0.4	0.3	-	-	-	-	-	-	-	-	-	-	-	-
Σ TFA	100	100	100	100	100	100	100	102	100	100	100	100	100	100	100
Σ SCFA	41.4	45.3	68.3	8.8	11.5	18.9	6.2	11.1	12.8	35.5	43.7	49.6	4.9	5.8	25.4
Σ BCFA	21.5	18.8	4.3	53.6	61.5	63.4	1.1	0.8	0.5	-	-	-	13	22	44.2
Σ MUFA	19.4	22	23.7	37.6	27	17.7	92.7	88.1	86.7	64.5	56.3	50.4	83	71	30.4
Σ PUFA	18.2	13.9	3.6	-	-	-	-	-	-	-	-	-	-	-	-
EPA	16.6	11.9	2.4	-	-	-	-	-	-	-	-	-	-	-	-
ACL[a]	15.6	15.6	14.6	16.3	16.1	15.8	15.8	15.4	15	16.3	16.2	16.1	18	17	16.6

[a] Average chain length (ACL) calculated after (White et al., 2005a). Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

The nucleotide sequence of the 16S rRNA gene in *Idiomarina* sp. strain MARG4 shared 99.8 % similarity over 1408 nt with that of *Idiomarina loihiensis* L2TR^T

(AE017340), its nearest cultivated neighbour in this respect. The fatty acid profile of strain MARG4 displayed the same dominance by iso-branched fatty acids that characterizes other members of the genus *Idiomarina* (Donachie et al., 2003). A high contribution of isobranched fatty acids has previously only been seen for the *Xanthomonas*-branch *Proteobacteria* (Finkmann et al., 2000), and has been observed for genera closely related to the genus *Idiomarina* (Brettar et al., 2003). Indeed, anteiso-branched fatty acids comprised <1 % of the total fatty acid pool. The dominant fatty acids for cells of strain MARG4 grown anaerobically at 4, 15 and 25 ºC were i-15:0 (31-35 %), i-17:1 (11-17 %), i-17:0 (10-15 %), n-18:1ω7 (6-10 %), n-18:1ω9 (2-6 %), n-16:1ω7 (5-9 %) and n-16:0 (4-8 %) (Table 2.4). The composition of the fatty acids of strain MARG4 was analysed after cultivation at lower (4 ºC) and higher (25 ºC) temperatures in comparison to the standard cultivation at 15 ºC. At 4 ºC, the proportion of SCFAs decreased while the proportion of MUFAs increased, with n-16:1ω7, 18:1ω7 and n-18:1ω9 showing increasing of their contribution. At 25 ºC, the proportion of BCFAs increased and the proportion of MUFAs decreased. With the decrease in the growth temperature from 25 to 4 ºC, the proportion of MUFAs increased from 17 to 37 % and the average chain length showed slightly decreased at the same time (Figure 2.2).

Halomonas sp. strain MAR913 exhibited 97.7 % the highest similarity with *Cobetia marina* DSM 4741^T (AJ306890) based on 16S rRNA gene phylogenetic studies, and showed n-16:1ω7, n-16:0, n-17:1ω8 and n-18:1ω7 as major FAs (10-30 %) and contained less n-14:0, n-15:1ω6, n-15:0, n-17:0 and n-18:0 acids (1-9 %), and not branched FAs were detected (Table 2.4). FA profiles of strain MAR913 was not resembled that of type strain of *H. desiderata* DSM 9502^T, of which the n-18:1ω7c component accounting for 70 % in the TFAs, and with n-10:0, n-12:0, n-14:0 and n-16:0 as major FAs (Kim et al., 2007a). Strain MAR913 showed a relatively response to changing growth temperature. With increasing temperature, the decreasing proportion of MUFAs (64-50 %) was mirrored by increasing of SCFAs increased (35-49 %), and the average chain length showed slightly decreased (Figure 2.2).

The nucleotide sequence of the 16S rRNA gene in *Serratia* sp. strain MARG45 shared 99.8 % similarity with that of *Serratia proteamaculans* DSM 4543^T (AJ233434). And the fatty acid profile of strain MARG45 consisted of n-16:1ω7, n-16:0, n-17:1ω8 and n-18:1ω7 as major FAs (10-30 %) and contained less n-14:0 and i-15:0 acids (1-9 %) when anaerobically cultured at temperatures ranging from 4 to 25 ºC (Table 2.4). The

major cellular fatty acids profile of strain MARG45 was compared with those of other members of the same *Serratia* genus (Bhadra et al., 2005). The composition of the fatty acids of strain MARG45 was analysed after cultivation at lower (4 ºC) and higher (25 ºC) temperatures in comparison to the standard cultivation at 15 ºC. At 4 ºC, the proportion of SCFAs decreased while the proportion of MUFAs increased, with n-16:1ω7 and n-18:1ω9 showing increasing of their contribution. At 25 ºC, the proportion of BCFAs increased and the proportion of MUFAs decreased. With the decrease in the growth temperature from 25 to 4 ºC, the proportion of MUFAs increased from 86 to 92 %. The average chain length showed slightly with decreaing temperature (Figure 2.2).

2.4.4.4 Temperature modulation of the whole cell FA patterns of low-GC Firmicutes

The patterns for the strains affiliated with the *Firmicutes* generally appeared more complex than those of the Gram-negative isolates. A striking feature of strain MAR846 similar to *Bacillus safensis*, strain MAR886 homology to *Bacillus tequilensis,* strain MAR285 similar to *Bacillus sonorensis,* strain MAR015 homology to *Bacillus circulan*, strain MAR287 similar to *Bacillus horneckiae*, strain G2 homology to *Thalassobacillus devorans*, strain MAR019 homology to *Bacillus simplex*, strain MAR283 similar to *Jeotgalibacillus marinus*, strain MAR808 homology to *Bacillus pseudofirmus*, strain MAR870 homology to *Bacillus hwajinpoensis,* strain MAR804 similar to *Bacillus decolorationis.*

Bacillus safensis strain MAR846 showed i-15:0, ai-15:0, n-16:0 and i-17:0 as major FAs (11-30 %) and contained less i-14:0, n-14:0, i-16:0 and ai-17:0 acids (3-7 %), and only minor proportions of LCFAs, such as n-18:1ω9 and n-18:0 acids (0.8-2 %). Not n-18:1ω9 was detected at 25 ºC (Table 2.5). With decreasing temperature from 25 ºC to 4 ºC, the proportion of MUFAs and BCFAs increased slightly with the expense of SCFAs, while the average chain length remained constant (Figure 2.3).

The major FAs of *Bacillus tequilensis* strain MAR886 were the branched i-15:0 (25–30 %), ai-15:0 (40-42 %), ai-17:1ω7 (~8 %) and i-17:0 acids (~10 %) as well as the monounsaturated chain n-18:1ω9 acid (3-6 %). Several other FAs were present in relatively small amount (<3 %) such as, i-14:0, i-16:0, i-17:1ω7, ai-17:0 acids. BCFAs account for 93-98 % of the TFAs (Table 2.5). Strain MAR886 showed a relatively week response to changing growth temperature and the average chain length remained constant at all temperatures (Figure 2.3).

Bacillus sonorensis strain MAR285 showed i-14:0, i-15:0, ai-15:0, i-16:0, i-17:0, ai-17:0 and n-18:1ω9 as major FAs (9-25 %) and contained minor proportions of n-14:0, n-17:1ω6 and n-18:0 acids (0.5-3 %) (Table 2.5). With decreasing temperature from 25 ºC to 4 ºC, the proportion of BCFAs and MUFAs increased slightly with the expense of SCFAs, whereas the average chain length remained more or less constant (Figure 2.3).

Bacillus circulan strain MAR015 showed i-15:0, ai-15:0 and n-18:1ω9 as major FAs (9-25 %) and contained less proportions of n-16:0, i-17:0, ai-17:0 and n-18:0 acids (1-6 %) (Table 2.5). With decreasing temperature from 25 ºC to 4 ºC, the proportion of MUFAs increased slightly with the expense of SCFAs, while BCFAs stayed similar between 4 ºC and 25 ºC, and less at 15 ºC, and the average chain length showed apparently decreased at 25 ºC (Figure 2.3).

Bacillus horneckiae strain MAR287 showed only i-15:0 (16-25 %) and ai-15:0 (42-60 %) as major FAs and contained minor proportions of i-14:0, n-14:0, n-15:0, i-16:0, n-16:1ω9, n-16:1ω7, i-17:0, ai-17:0, n-17:1ω6 and i-18:0 acids (1-8 %) (Table 2.5). With decreasing temperature from 25 ºC to 4 ºC, the proportion of BCFAs increased slightly with the expense of SCFAs. The proportion of MUFAs was higher at 15 ºC than that at 4 ºC and 25 ºC, while the average chain length remained constant (Figure 2.3).

Strain MARG2 falls within the radiation of the cluster comprising members of the genus *Thalassobacillus* based on 16S rRNA gene sequence analysis. The predominant fatty acids of this strain anaerobically cultured under temperature ranging from 4 to 25 ºC were ai-15:0 (24-57 %), i-16:0 (7-23 %), i-15:0 (5-11 %), ai-17:0 (7-9 %), ai-17:1ω7 (2-14 %), n-18:1ω9 (5-7 %) and iso-14:0 (4-6 %) (Table 2.5). Strain MARG2 and the phylogenetically related genera contained similar fatty acid profiles, having ai-15:0 as a major fatty acid (Garcia et al., 2005), but with production of LCFAs of n-18:0 and n-18:1ω9. With decreasing temperature from 25 ºC to 4 ºC, the proportion of MUFAs increased slightly with the expense of SCFAs, whereas the proportion of BCFAs was remained stable. The average chain length was slightly increased with increasing temperature due to higher lever of i-16:0 and ai-17:1ω7 at 25 ºC (Figure 2.3).

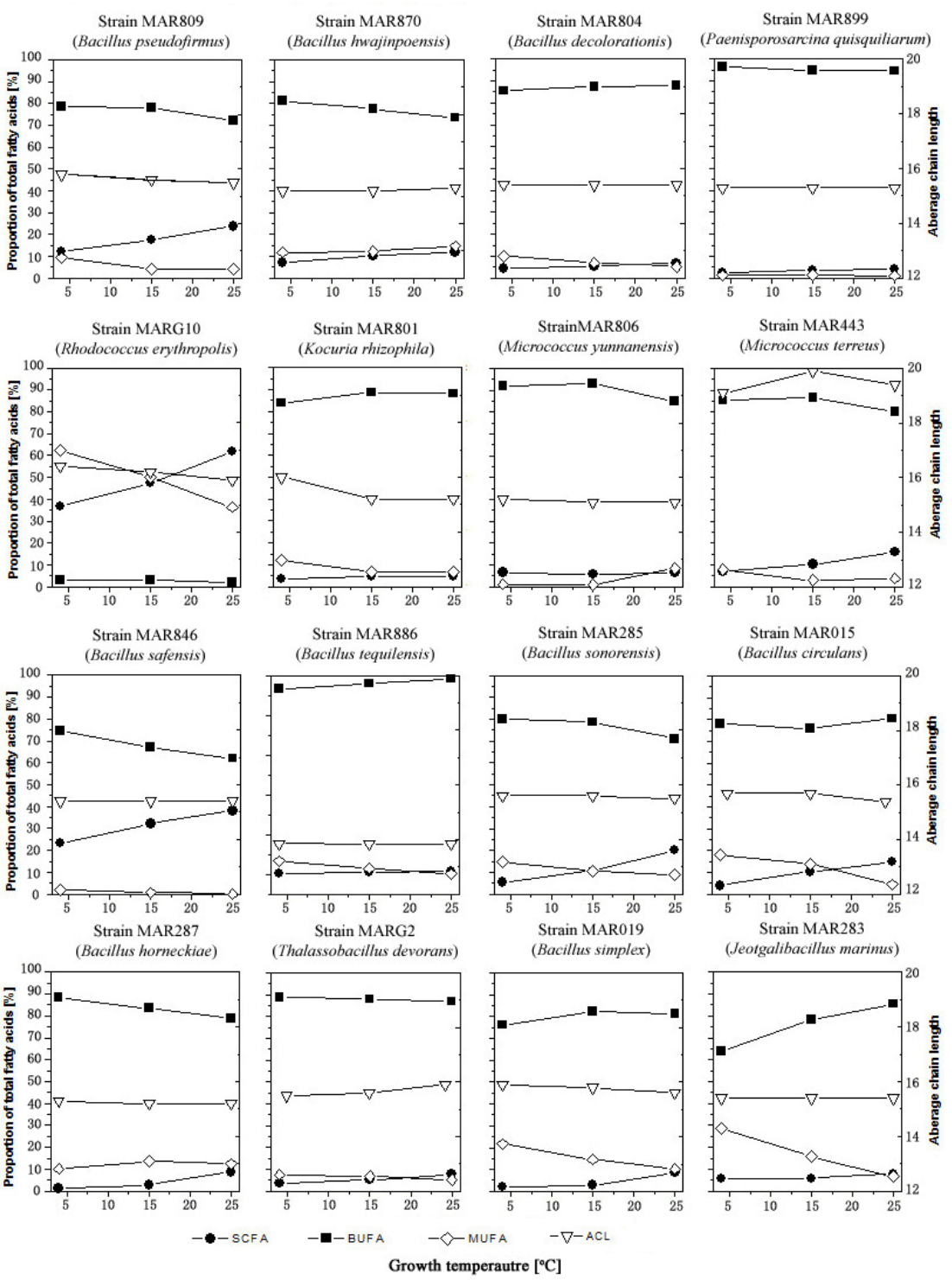

Figure 2.3 Change in average chain length (inverted triangles) and relative proportion of whole cell FAs in *Gram-positive* strains grown at 4, 15 and 25 °C. Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open diamonds) and nearest type strains were indicated below the isolates. Values are means of three samples based on Tables 2.5 and 2.6.

Table 2.5 Major FAs after whole cell hydrolysis (% of total FAs) in bacterial strains affiliating with the *Firmicutes*

	Bacillus safensis			*Bacillus tequilensis*			*Bacillus sonorensis*			*Bacillus circulans*			*Bacillus horneckiae*			*Thalassobacillus devorans*			*Bacillus simplex*			*Jeotgalibacillus marinus*		
	MAR846			MAR886			MAR285			MAR015			MAR287			MARG2			MAR019			MAR283		
	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC
i-14:0	6.5	4	2.1	2.6	1.8	1.1	15.6	12	7.9	0.7	0.3	0.4	2.6	2.5	2.1	6.1	4.8	5.3	2.3	3.0	2.5	26.9	17.7	10.2
n-14:0	3.1	5.5	7.6	0	0.2	0.5	0.3	0.9	3.6	0.3	0.5	1.8	0.4	0.5	2.5	-	-	-	2.1	2.9	8.5	2	2.6	5.5
i-15:0	18.5	19.1	22.2	25.4	28.5	29.5	8.7	10.1	17.0	27.2	25	32.9	60.7	52.3	41.5	5.5	9.2	11.4	4.4	6.7	8.9	19.6	26	32.7
ai-15:0	28.5	25.3	20.6	39.2	41.3	42.0	26	25.2	24.8	42.8	41	35.5	15.9	17.6	25.8	57.4	48.1	23.9	35.0	29.5	22.1	2.5	2.1	1.5
n-15:0	-	-	-	-	-	-	0.8	-	-	-	1.8	2.2	0.8	1.3	3.4	-	-	-	-	-	-	1.2	1.4	1.3
i-16:1ω8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	18.7	22.9	26.0	-	-	-
i-16:0	5.5	6.5	7.2	1.9	1.5	1.1	3.6	13.5	5.0	1.2	1.5	2.1	3	2.9	3.5	7.7	12.5	23.7	7.8	9.5	9.0	6.9	19	23
n-16:1ω9	-	-	-	-	-	-	-	-	-	-	-	-	3	3.7	3.2	-	-	-	-	-	-	8.2	5.4	3.5
n-16:1ω7	-	-	-	-	-	-	-	-	-	-	-	-	2.8	6.7	7.8	-	-	-	2.1	2.5	3.2	13	6.9	2
n-16:0	19.5	26.1	29.5	0.5	0.9	1.1	3.8	9	15.9	0.6	1.9	5.7	0.2	1	2.8	3.2	4.3	6.7	-	-	-	2.4	1.8	1.1
i-17:1ω7	-	-	-	2.9	3	3.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ai-17:1ω7	-	-	-	11.6	9.8	10.9	-	-	-	-	-	-	-	-	-	2.6	5.1	14.8	-	-	-	2.7	6.5	8.5
i-17:0	11.4	6	2.3	7.8	8.8	8.7	9.2	7.9	7.5	2.9	3.3	4.1	2	1.9	2.5	-	-	-	1.2	2.0	0.5	0.8	0.4	0.6
ai-17:0	4.2	6.2	7.6	1.5	1	1.1	16.8	10	8.9	3.2	4.7	5.6	1.1	4.2	2.9	9.6	8.3	7.8	6.8	8.9	12.2	4.6	6.5	9
n-17:1ω6	-	-	-	-	-	-	1.5	0.5	-	0.8	-	-	4.4	3.3	1.4	-	-	-	-	-	-	-	-	-
i-18:0	-	-	-	0.2	0.2	0.5	-	-	-	-	-	-	3.1	2.1	0.6	-	-	-	-	-	-	1.7	0.3	0
n-18:1ω9	2	0.7	0.0	6.6	3	0.2	13.2	10.1	8.8	17.3	14	4.5	-	-	-	7.6	6.8	5.3	12.6	5.6	0.6	7.5	3.4	1.1
n-18:0	0.8	0.6	0.9	-	-	-	0.5	0.8	0.6	3	6.1	5.2	-	-	-	0.3	0.9	1.1	-	-	-	-	-	-
n-30: 6ω2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.0	7.5	6.5	-	-	-
Σ TFA	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Σ SCFA	23.4	32.2	38	0.5	1.1	1.6	5.4	10.7	20.1	3.9	10	14.9	1.2	2.8	8.7	3.5	5.2	7.8	2.1	2.9	8.5	5.6	5.8	7.9
Σ BCFA	74.6	67.1	62	93.1	95.9	98.2	79.9	78.7	71.1	78	76	80.6	88.6	83.5	78.9	88.9	88	86.9	76.2	82.5	81.2	64	78.2	85.5
Σ MUFA	2	0.7	0.0	6.6	3	0.2	14.7	10.6	8.8	18.1	14	4.5	10.2	13.7	12.4	7.6	6.8	5.3	21.7	14.6	10.3	28.7	15.7	6.6
anteiso/iso	0.8	0.9	0.8	1.3	1.2	1.2	1.2	0.8	0.9	1.4	1.5	1.0	0.2	0.3	0.5	3.6	2.3	1.2	0.8	0.6	0.4	0.2	0.2	0.3
ACL[a]	15.4	15.4	15.4	15.5	15.3	15.3	15.6	15.6	15.5	15.7	16	15.4	15.3	15.2	15.2	15.5	15.6	15.9	15.9	15.8	15.6	15.4	15.4	15.4

[a] Average chain length (ACL) calculated after (White et al., 2005a). Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; Squalene, C30 H50 (n-30:6ω2); and (–), not detectable.

Bacillus simplex strain MAR019 showed ai-15:0, i-16:1ω8, i-16:0, ai-17:0 and n-18:1ω9 as major FAs (10-35 %) and contained less i-14:0, n-14:0, i-15:0, i-16:0, n-16:1ω7, i-17:0 acids and n-30:6ω2 (Squalene) (2-9 %). Higher amount of LC-PUFA, n-18:1ω9 was produced at 4 ºC (Table 2.5). The production of Squalene was contant at all temperatures with 6-7 % in the TFAs. With decreasing temperature from 25 ºC to 4 ºC, the proportion of MUFAs increased slightly with the expense of SCFAs and BCFAs. The proportion of BCFAs was stable at 15 ºC and 25 ºC, and the average chain length showed slightly decreased with increasing temperature (Figure 2.3).

Jeotgalibacillus marinus strain MAR283 showed i-14:0, i-15:0, i-16:0, ai-17:0 and n-18:1ω9 as major FAs (7.5-32 %) and contained minor proportions of n-14:0, ai-15:0, n-15:0, n-16:0 and n-18:0 acids (1-5 %) (Table 2.5). With decreasing temperature from 25 ºC to 4 ºC, the proportion of MUFAs increased slightly with the expense of SCFAs and BCFAs, while the average chain length remained constant (Figure 2.3).

Bacillus pseudofirmus strain MAR808 showed i-15:0, ai-15:0, i-16:0 and ai-17:0 acids as major FAs (10-30 %) and contained less i-16:1ω8 and n-18:1ω9 acids (2-8 %) (Table 2.6). With increasing temperature from 4 ºC to 25 ºC, the proportion of SCFAs increased slightly with the expense of BCFAs and MUFAs, while the average chain length showed slightly decreased (Figure 2.3).

Bacillus hwajinpoensis strain MAR870 exhibited only i-14:0, i-15:0, ai-15:0 and n-16:1ω9 acids as major FAs (10-38 %) and contained less i-16:0, n-16:1ω7, n-16:0, and n-18:1ω9 acids (2-8 %) (Table 2.6). With decreasing temperature from 25 ºC to 4 ºC, the proportion of BCFAs increased slightly with the expense of SCFAs and MUFAs, while the average chain length remained more or less constant (Figure 2.3).

Bacillus decolorationis strain MAR804 showed only ai-15:0 as major FA (62-65 %) and contained less i-15:0, i-16:0, ai-17:0 and n-18:1ω9 acids (5-11 %), and only minor proportions of i-14:0, n-14:0, n-16:1ω7, n-16:0 and ai-17:1ω7 acids (1-4 %) (Table 2.6). With decreasing temperature from 25 ºC to 4 ºC, the proportion of MUFAs increased slightly with the expense of SCFAs and BCFAs. The proportion of BCFAs was stable at 15 ºC and 25º C, while the average chain length remained constant (Figure 2.3).

Paenisporosarcina quisquiliarum strain MAR899 showed i-14:0 (11-12 %), ai-15:0 (35-40 %), i-16:1ω8 (23-25 %) and ai-17:0 acids (6-13 %) as major FAs and contained less proportions of i-15:0, i-16:0 and i-17:0 acids (3-5 %). BCFAs were the main composition of the TFAs (92-95 %) (Table 2.6). With decreasing temperature from 25 ºC to 4 ºC, the proportion of BCFAs increased slightly with the expense of SCFAs, whereas the proportion of MUFAs was higher at 15 ºC than that at 4 ºC and 25 ºC while the average chain length remained constant (Figure 2.3).

Bacillus decolorationis strain MAR287*, Paenisporosarcina quisquiliarum* strain MAR899, *Kocuria rhizophila* strain MAR801*, Micrococcus yunnanensis* strain MAR806 and *Micrococcus terreus* strain MAR443 was the dominance of saturated and monounsaturated branched chain iso and anteiso FAs with carbon numbers ranging from 14 to 18, a pattern typical for *Bacillus* and related genera (Haque and Russell, 2004; Lopez et al., 2006; Kim et al., 2007b).

2.4.4.5 Temperature modulation of the whole cell FA patterns of high-GC firmicutes

Strain MARG10 falls within the radiation of the cluster comprising members of the genus *Rhodococcus* based on 16S rRNA gene sequence analysis, the predominant fatty acids of *Rhodococcus* sp. strain MARG10 anaerobically cultured under temperature ranging from 4 to 25 ºC were n-16:0 (23-39 %), n-18:1ω9 (17-28 %), n-16:0 (11-21 %), n-16:1ω5 (11-18 %) and n-16:1ω7 (6-13 %) (Table 2.6). The fatty acid profile of strain MARG10 displayed the same dominance by MUFAs that characterizes other members of the genus *Rhodococcus* (Gutierrez et al., 2003). The composition of the fatty acids of strain MARG10 was analysed after cultivation at lower (4 ºC) and higher (25 ºC) temperatures in comparison to the standard cultivation at 15 ºC. At 4 ºC, the proportion of SCFAs decreased while the proportion of MUFAs increased, with n-16:1ω5, n-16:1ω7 and n-18:1ω9 showing increasing of their contribution. With the increase in the growth temperature from 4 to 25ºC, the increase of SCFAs was mirrored by the decreasing of MUFAs, while the average chain length showed slightly increased (Figure 2.3).

Kocuria sp. strain MAR801 showed 99.6 % 16S rRNA gene sequence similarity to *Kocuria rhizophila* DSM 11926^T (Y16264) based on 16S rRNA gene phylogenetic

studies, and with only ai-15:0 as major FAs (57-67 %) and contained less i-15:0, n-16:1ω9 and ai-17:1ω7 acids (5-15 %). Only minor proportions of i-14:0, n-14:0, i-16:0, i-17:0 and n-18:1ω7 acids (1-5 %) were found (Table 2.6). The fatty acid profile showed high levels of similarity to those of reference strains were of the branched-chain saturated isoanteiso type and showed ai-15:0 as the predominating component (Kovacs et al., 1999). Strain MAR801 showed a relatively weak response to changing growth temperature. The proportion of MUFAs increased slightly at 4 ºC with the expense of SCFAs and BCFAs whereas SCFAs, BCFAs and MUFAs, as well as the average chain length remained constant at 15 ºC and 25 ºC (Figure 2.3).

Micrococcus sp. strain MAR806 exhibited 99.6 % 16S rRNA gene sequence similarity to *Micrococcus yunnanensis* YIM 65004^T (FJ214355) and showed only ai-15:0 (60-67 %) and ai-17:1ω7 (11-12 %) as major FAs and contained less proportions of i-14:0, n-14:0, i-15:0, i-16:0, n-16:0 and i-17:0 acids (2-6 %). BCFAs were the main composition of the TFAs (85-93 %) (Table 2.6). The FAs composition remained stable between 4 ºC and 21 ºC, and some n-16:1ω9 (8.5 %) and minor proportion of n-15:0 were found at 25 ºC. The average chain length remained more or less constant at all temperatures (Figure 2.3).

Arthrobacter sp. strain MAR443 was most related to *Micrococcus terreus* V3M1^T (FJ423763) based on 16S rRNA gene identification with 97.2 % similarity and showed i-C15:0, ai-15:0, i-16:0, i-17:0 and n-16:0 as major FAs (10-43 %) and contained less n-14:0 and n-16:1ω9 acids (1-4 %). Only minor proportions of n-15:0, i-17:1ω7 and n-17:1ω8 acids were found (Table 2.6). The fatty acid profile showed fair levels of similarity to those of reference strains, such as *Arthrobacter* sp. strain Sphe3 and *Arthrobacter halodurans* strain JSM 078085T (Chen et al., 2009; Kallimanis et al., 2009). Strain MAR443 showed a relatively weak response to changing growth temperature, and the proportion of MUFAs was relative higher at 4 ºC and stable at 15 ºC and 25 ºC, while SCFAs and BCFAs were higher at 25 ºC, and the average chain length showed higher at 15 ºC (Figure 2.3).

Table 2.6 Major FAs after whole cell hydrolysis (% of total FAs) in bacterial strains affiliating with the *Firmicutes*

	Bacillus pseudofirmus			*Bacillus hwajinpoensis*			*Bacillus decolorationis*			*Paenisporosarcina quisquiliarum*			*Rhodococcus erythropolis*			*Kocuria rhizophila*			*Micrococcus yunnanensis*			*Micrococcus* terreus		
	MAR809			MAR870			MAR804			MAR899			MARG10			MAR801			MAR806			MAR443		
	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC	4ºC	15ºC	25ºC
i-14:0	1.7	1.9	1.1	14.3	11	7.2	2.1	2.9	3	12.1	11.5	12.3	-	-	-	2.3	2.0	1.2	2.8	2.5	1.4	-	-	-
n-14:0	1.2	1.1	1.2	0.4	1.2	1.6	1	1.1	1.2	0.5	1	1.5	11.3	16.6	21.5	3.2	1.7	1.9	3.9	3.5	2.9	1.1	1.4	5.2
i-15:0	19.1	24.4	26.5	21.9	30	38.3	5.7	4.4	2.7	5	4.5	4.1	-	-	-	7.4	8.5	14.5	6.3	5.7	17.0	15.3	14.3	19
ai-15:0	24.3	27.6	30.3	38.5	32	21.3	65	63.9	61.8	40.4	37.6	35.1	0.4	0.7	0.8	57.5	68.2	66.7	64.2	67.7	59.5	28.7	36.2	43
n-15:0	0.4	0.6	0.5	-	-	-	0.2	0.6	1.1	0.6	0.9	1.1	0.3	0.6	0.5	0.2	1.2	0.3	-	-	0.4	0.7	0.6	1.2
i-16:1ω8	8.3	4.8	1.1	-	-	-	0.4	-	-	25.3	25.5	23.0	-	-	-	-	-	-	1.2	1.1	-	-	-	-
i-16:1ω6	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
i-16:0	13.6	13.9	8.7	3.2	1.8	2.5	5.3	4.3	4.5	4.4	3.8	3.3	-	-	-	3.4	1.8	1.4	2.7	2.4	1.7	19.6	17.7	9.2
n-16:1ω9	0.5	0.5	0.9	7.5	8.9	11.7	0.3	0.5	0.2	-	-	-	-	-	-	6.8	6.6	6.9	-	-	8.5	6.9	2.5	3.8
n-16:1ω7	0.7	0.6	0.7	2.1	2	2.4	2.2	1.6	1.2	1.2	1.5	1.1	13.5	9.6	6.4	-	-	-	-	-	-	-	-	-
n-16:1ω5	-	-	-	-	-	-	0.9	0.6	0.4	-	-	-	18.3	13.9	11.5	1	-	-	-	-	-	-	-	-
n-16:0	9.6	14.1	18.5	5.4	6.9	8.0	1.8	2.2	2.1	1.2	1.7	1.6	23.1	29.6	39.5	0.1	1.8	2.6	2.7	2.4	3.2	5.1	8.3	9.6
i-17:1ω7	0.8	-	-	-	-	-	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.1	2.5	1.2
ai-17:1ω7	0.3	0.3	0.2	1.1	1.5	1.6	2.2	3.5	3.8	-	-	-	-	-	-	10.4	4.8	2.5	12.4	11.3	3.1	-	-	-
i-17:0	0.2	0.8	0.6	1.3	1.6	1.5	0.7	0.6	0.5	3.2	3.5	3.1	-	-	-	3.1	3.4	2.0	3.8	3.4	2.3	19.6	16	7.9
ai-17:0	10.2	4.6	3.7	0.8	0.9	1.1	3.4	7.6	11.5	6.1	8.6	13.8	0.5	1.8	0.9	-	-	-	-	-	-	-	-	-
n-17:1ω8	0.8	0.7	1.2	-	-	-	0.5	0.2	-	-	-	-	1.9	1.2	0.9	-	-	-	-	-	-	0.9	0.5	0.1
n-17:1ω6	0.3	0.4	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n-18:1ω9	7.0	1.9	0.8	2.2	1.4	0.5	6.2	4.1	3.5	-	-	-	28.6	25.4	17.6	4.6	-	-	-	-	-	-	-	-
n-18:0	1.0	1.8	3.5	1.3	2.1	2.3	1.4	1.6	2.5	-	-	-	2.1	0.6	0.4	-	-	-	-	-	-	-	-	-
Σ TFA	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Σ SCFA	12.2	17.6	23.7	7.1	10	11.9	4.4	5.5	6.9	2.3	3.6	4.2	36.8	47.4	61.9	3.5	4.7	4.8	6.6	5.9	6.5	6.9	10.3	16
Σ BCFA	78.5	78.2	72.2	81.1	78	73.5	86	87.5	87.8	96.5	95	94.7	3	3.1	2.1	84	88.7	88.3	92	93	85	85.3	86.7	80
Σ MUFA	9.3	4.1	4.1	11.8	12	14.6	10	7	5.3	1.2	1.5	1.1	62.3	50.1	36.4	12	6.6	6.9	1.2	1.1	8.5	7.8	3	3.9
anteiso/iso	0.8	0.7	0.9	1.0	0.7	0.5	3.7	3.4	3.0	0.7	0.7	0.6	-	-	-	4.2	4.6	3.6	4.9	5.6	2.8	0.5	0.7	1.2
ACL[a]	15.8	15.6	15.5	15.2	15	15.3	15.4	15.3	15.4	15.3	15.3	15.3	16.4	16.2	15.9	16	15.2	15.2	15	15	15.1	19.1	19.9	19

[a] Average chain length (ACL) calculated after (White et al., 2005a). Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; and TFA, total fatty acids; and (–), not detectable.

2.4.5 Electrochemical properties of the isolated deep-sea bacteria

Among these 52 bacteria isolated under anaerobic condictions, 4 strains were found with the ability of electricity production, such as Strains MARG5, MARG6 and MARG9 were homology to *Halomonas* were found with the highest power density of 75-90 mW/m^2 when growing in a fresh anaerobic corresponding growth medium with 10 mM peptone as the electron donor in the above mentioned MFC units, and MARG45 with high similar to *Serratia* was found with the ability of reducing Fe(III) and higher electricity production of 210 mW/m^2. Strain MAR445 identified as *Shewanella* species, although it was isolated under aerobic condition, could grew very well under anaerobic condition with ability of reducing Fe(III) and power generation of 115-150 mW/m^2 (See Chapter 5, Figure 5.3).

2.5 Discussion

2.5.1 Deep sea sediments and the density of the bacterial isolates

The deep sea is a vast habitat that covers approximately 62 % of the world's surface and 79 % of the volume occupied by living organisms which live below a depth of 1000 m (Childress, 1995). Food availability in the deep sea, declines with depth and distance from the continental shelf edge (Warrant and Locket, 2004). The benthic environment of the deep sea is nearly entirely heterotrophic with the majority of organisms' dependent of food sources descending from higher up in the water column (Danovaro et al., 2008). The supply of food to the deep sea depends on primary production in the photic zone, except for hydrothermal vent areas (Thingstad and Rassoulzadegan, 1999).

The depth profile indicated that the bacterial community structure in the MAR "non-vent" sites shifted with increasing depth in the sediment, and the microbial diversity in shallow (0-5 mm) sediments was greater than that in deep (5-10 mm) sediments. Among the 312 isolates from the six deep sea sediments, 82 % were found isolated from surface sediments. It may be that the locations of surface (core-top) sediment samples contain planktonic foraminiferal faunas (Morey et al., 2005). The finding is agreeable to other benthic life studies in deep-sea sediments, such as a marginal decrease in the number of bacteria from surface to 30 cm depth of the

Central Indian Ocean Basin in the Indian pioneer area (Raghukumar et al., 2001), the highest numbers of stained foraminifera are invariably found at the sediment surface, whereas numbers decrease exponentially deeper in the sediment in the southern Adriatic Sea (De Stigter et al., 1998). This may indicate that the productivily of the benthic sediments is generally differred by several orders of magnitude.

The southeast station was found to have higher microbial density than those from another two, Northeast and Northwest, both of which are north of the Charlie Gibbs Fracture Zone (CGFZ). It may be that Southeast (Lat 49°05.40'N) with more organic matters transported by North Atlantic Current (NAC) which crosses the MAR in a minimum of two and a maximum of four branches between 45ºN and 52ºN, and therefore the core sediment might be affected by the currents across the ocean floor which carrying the surface sediments along in the water flow; whereas the sedimentary organic carbon contents of Northeast (Lat 54°01.00'N) and Northwest (Lat 54°00.65'N) remained about constant at less levels (Billett et al., 1983; Lampitt, 1985).

2.5.2 Phylogenetic diversity of MAR bacterial isolates

It was interesting was that the bacteria belonging to the *Protobacteria* group, Gram-positive and CFB group formed a site-specific microbial ecosystem of MAR non-vent sites based on phylogenetic analysis of 16S rRNA gene sequences, which were different from other microbial diversity studies. For example, the genus *Pseudomonas* which seemed highly prevalent in other oceans (Hengstmann et al., 1999; Bano and Hollibaugh, 2002), and many *Colwellia* strains have been isolated from the deep sea as common in the deepest oceanic regions, containing psychrophilic and barophilic species (Bowman et al., 1997a; Delong et al., 1997). However, these two species were not present in these MAR regions or seldom isolated by using the above mentioned culture techniques in this study. Whereas, our study revealed that in deep-sea sediments from the MAR, members belonging to genus *Bacillus* were dominant not only in *Gram positive*, but also in the whole bacteria communities.

The identification results assigned 69.5 % of the isolates to the Gram-positive species in the MAR sediments. Gram-positive bacteria have been cultivated from seawater and marine invertebrates (Ivanova et al., 1999; Han et al., 2003; Yi et al., 2004; Montalvo et al., 2005), but marine sediments (Takizawa et al., 1993; Jensen and Fenical, 1995; Moran et al., 1995; Mincer et al., 2002; Jensen et al., 2005; Gontang et al., 2007), including deep-sea sediments (Weyland, 1969; Li et al., 1999a; Ruger et al., 2000), are the primary oceanic habitat from which they have been recovered (Austin, 1988).

In this study, isolates related to low-G+C Gram-positive formed the largest cluster in terms of diversity and abundance, with 12 phylogenetically distinct organisms predominant in subsurface sediments. Psychrotrophic *Bacillus* species consisted mainly of *B. pumilus* (27.3 %), *B. pseudofirmus* (19.7 %) and *B. licheniformis* (12.1 %), with spore-forming ability according the previous reports (Bonde, 1981). This would be the first record of *Bacilli* and their associated high diversity level from MAR deep-sea sediments, which resembled that of previously reports in other marine environments, such as deep sea (Gontang et al., 2007; Ettoumi et al., 2009).

While it is probable that some marine-derived Gram-positive bacteria are terrigenous microorganisms, washed or blown into the marine environment, species occurring exclusively in the sea have been described (Helmke and Weyland, 1984; Han et al., 2003; Yi et al., 2004). Among the *Bacilli* strains isolated in this study, only species of *B. pumilus*, *B. subtilis*, *B. lichenifirmis*, *B. marinus* were reported to have been isolated from marine environments (Ivanova et al., 1999). *Bacillus baekryungensis* is a provisional species isolated from the Yellow Sea (Yoon et al., 2004). Strain GSP77 related to *B. pseudofirmus* was a facultatively alkaline isolated from the deep sea (Caton et al., 2004). *Bacillus decolorationis* and *B. simplex*, were used to be isolated from wall paintings (Heyrman et al., 2003), and *B. pseudofirmus* and *B. longiquaesitum* were used to be isolated from soil samples (Das-Bradoo et al., 2004; Kojima et al., 2006). *Bacillus horneckiae* was isolated from spacecraft (Vaishampayan et al., 2010). *Thalassobacillus devorans* was reported to have been isolated from hypersaline habitats (Garcia et al., 2005). Therefore, *Bacillus decolorationis*, *B. simplex*, *B. longiquaesitum*, *B. horneckia* and *Thalassobacillus devorans* were the first reported to have been isolated from marine environment in this study. Strain MAR019 homology to *Bacillus simplex* species was found with

high production of squalene. This is another report on squalene production by a marine Gram-positive bacterium after a Gram-negative strain *Rubritalea squalenifaciens* HOact23^T, which was isolated from the marine sponge *Halichondria okadai* and was reported with the production of such natural organic compound (Kasai et al., 2007).

Isolates related to high G+C Gram-positives formed a small cluster in terms of diversity, with 2 phylogenetically distinct organisms: *Arthrobacter-Micrococcus* lineage and genera of the *Nocardiaceae*.

The *Cytophaga–Flavobacterium–Bacteroides (CFB)* group of bacteria is considered to be of special relevance for aquatic environments, and is an especially dominant component of the microbial assemblage in anaerobic marine sediments (Coleman et al., 1993; Brettar et al., 2004). However, only few CFB species were isolated in this study.

Indigenous marine bacteria occurring in most marine sediments belong mainly to different subclasses of *Proteobacteria* and are actively involved in geobiochemical cycles (Teske et al., 2000; Lopez-Garcia et al., 2003b), which may highlight the sediment structure and characteristics, organic matter loading and punctual changes, as well as the extent of grazing and viral infection in particular marine sediments (Bowman and McCuaig, 2003; Weinbauer et al., 2006).

Most microbial diversity studies showed the ubiquitous presence of marine *Alpha-Proteobacteria* in the water column of all oceans and in marine snow (Fuhrman et al., 1993; Mullins et al., 1995; Rath et al., 1998), and are especially abundant in coastal seawater (Teske et al., 2000). Our study found that *Alpha-Proteobacteria* only occanted for 4 % of total bacteria isolated in the MAR sediments. These bacteria in the sediments could be derived from those coastal populations. An important ecological role of several members of the marine subclass is to degrade and decompose organic sulfur compounds produced mostly by eukaryotic phytoplankton and salt marsh plants (Pakulski and Kiene, 1992; Gonzalez et al., 1999).

High prevalence of *Gamma-Proteobacteria* has been reported from various marine environments, including different geographic areas and ecosystems. Members of the group have been found in clone libraries, denaturing gradient gel electrophoresis bands, and isolates retrieved from the Pacific Ocean (Crump et al., 1999; Beja et al., 2000; Suzuki et al., 2001; Connon and Giovannoni, 2002; Cho and Giovannoni, 2004), Atlantic Ocean (Britschgi and Giovannoni, 1991; Rappe´ et al., 1997; Gonzalez et al., 2000; Kelly and Chistoserdov, 2001), North Sea (Eilers et al., 2001; Zubkov et al., 2001), Arctic Ocean (Bano and Hollibaugh, 2002), Antarctic Sea (Bowman et al., 2000), Mediterranean Sea (Schafer et al., 2001), Gulf of Elat (Weidner et al., 1996), Tokyo Bay (Urakawa et al., 2000), Suruga Bay (Li et al., 1999a) and Tyrrhenian Sea (Ettoumi et al., 2010). Only few of these isolates originated from shallow or deep marine sediments (Li et al., 1999a; Li et al., 1999b; Bowman et al., 2000; Urakawa et al., 2000). Most cultured members have been obtained from temperate environments (Eilers et al., 2001; Connon and Giovannoni, 2002). Our study found that *Gamma-Proteobacteria* was accounted for 26.7 % of total bacteria isolated in the MAR sediments. Isolates related to *Gamma-Proteobacteria* formed the second largest cluster in terms of diversity and high abundance, with 11 phylogenetically distinct organisms distinguished by 8 main subclusters. MAR441 and MAR445 were most closely related to *Shewanella* species found with high production of EPA and can grow anaerobically by dissimilatory Fe(III) reduction, as well as generating electricity by utilizing peptone or glucose.

Though predominantly aerobic, some of the deep sea bacteria develop anaerobically as reported previously (Lovley and Phillips, 1994; Teske et al., 2002; Wery et al., 2002; Prokofeva et al., 2005; Byrne et al., 2009; Bruck et al., 2010). In this study, of the 52 anaerobic isolates, 46 were isolated from core sediment samples. All these 52 strains were phylogenetically divided into 7 distinct organisms: *Thalassobacillus devorans*, *Hafnia alvei*, *Idiomarina loihiensis*, *Halomonas alkaliphila*, *Bacillus horneckiae, Rhodococcus erythropolis* and *Serratia proteamaculans*. Strains MARG5, MARG6 and MARG9 were homologous to *Halomonas*, and MARG45 with, high similarity to *Serratia proteamaculans*, were found with the ability of reducing Fe(III) and electricity production. Strain MARG10 and MARG12 were homology to *Rhodococcus erythropolis*, which is involved in desulfurisation (Denome et al., 1994; Matsui et al., 2001). *Idiomarina loihiensis* related strains

were also isolated from the sediments, which might be related to biomineralization processes as it was reported with mineral precipitation (González-Muñoz et al., 2008).

2.5.3 Comparison of temperature-induced changes among representatives of different phyla

Whole-cell FA patterns of 27 isolates grown at different temperatures were analyzed. The dataset indicates that there is no unequivocal response in FA pattern to changing temperature. Significant differences were found among single organisms, not only between those belonging to different bacterial phyla, but sometimes even between representatives of a single genus. Such intrageneric variability appears to be quite common. Similar results were obtained with *Arthrobacter* (White et al., 2000), *Bacillus* (Haque and Russell, 2004), and *Sphingomonas* spp. (Mannisto and Puhakka, 2001).

For many isolates, changes in composition with temperature corresponded with literature data, such as monounsaturated FAs decreasing with increasing temperature, whereas saturated fatty acids increased (Hazel and Williams, 1990), for example in the *Stenotrophomonas* sp. MAR386, *Paracoccus* sp. MAR824, *Amphritea* sp. MAR442, *Idiomarina* sp. MARG4, *Halomonas* sp. MAR913, *Psychrobacter* sp. MAR701, *Rhodococcus* sp. MARG10 and in a few of the *Bacillus* strains (e.g. MAR886, MAR285, MAR015, MAR019 and MAR283). In some cases, however, the results were inconsistent, for example for *Bacillus* sp. MAR287 and MAR870 and *Micrococcus* sp. MAR806. For these strains the expected changes were observed over one temperature range, whereas over another opposite effects occurred. Currently we do not know whether these organisms employ other adaptation mechanisms besides FA side chain modification.

Equivocal results were obtained for BCFAs. These are generally attributed to maintaining membrane fluidity at lower temperature (Haque and Russell, 2004). This is supported by a number of isolates affiliated with the *Firmicutes* or *Gammaproteobacteria*. However, in the strains *Idiomarina* sp. MARG4, *Psychrobacter* sp. MAR701, *Bacillus* sp. (MAR886, MAR283 and MAR804), BCFAs often decreased with decreasing temperature. In these strains BCFAs often mirrored straight chain FAs. Some of the isolates showed almost no or only very little temperature-driven variation

(*Hoeflea* sp. MAR866, *Thalassobacillus* sp. MARG2, *Sporosarcina* sp. MAR899 and *Bacillus* sp. MAR870). This may indicate that they adjust membrane viscosity using other mechanisms (e.g. hopanoid or protein content or phospholipid head groups). Alternatively, it is possible that, in the temperature range over which the organisms were grown, an adjustment in membrane viscosity was not necessary. Therefore, it is apparently more advantageous for the organisms not to need to alter the FA chains and can be seen as an example of being well adapted to their habitat.

Likewise, for many other isolates changes did not appear gradually over the whole temperature range. For example, in strain *Stenotrophomona*s sp. MAR386 almost no differences in FA pattern were found between cells grown at 4 or 15 ºC, whereas for cells grown at 25 ºC the relative proportion of MUFAs decreased from about 30 % to more than 19 %. Whereas, as another example, in strain *Pseudoalteromonas* sp. MAR005 almost no differences in FA pattern were found between cells grown at 15 or 25 ºC, whereas for cells grown at 4 ºC the relative proportion of MUFAs increased from about 59 % to more than 80 %.

The bacterial isolates originating in MAR sediments, where the biogeography patterns have not been characterised, will experience temperature as low as 4 ºC. The alteration in FA side chains is the most effective and energy saving method (Hazel and Williams, 1990) for adapting membrane viscosity to temperature change. We assume that it is also the quickest method in that we found some *Gram-negatives*, such as *Stenotrophomonas* sp. MAR386, *Idiomarina* sp. MARG4 and *Psychrobacter* sp. MAR701; CFB group, e.g. *Formosa* sp. MAR430; and most of *Gram-positives* including genus *Bacillus* in this study with branched-chain fatty acids as major constituents, especially ai-15:0, i-15:0, ai-17:0, i-17:0 and i-16:0 acids. This branched-chain fatty acid family occurring in bacteria is very significant (Kaneda, 1977; Lechevalier, 1977), membranes with this family are manipulated mainly by 12-methyltetradecanoic acid (ai-15:0) and 13-methyltetradecanoic acid (i-15:0) and subsequently their membrane fluidity is modified (Kaneda, 1991). For example, fatty acids of strains *Idiomarina* sp. MARG4 and *Shewanellas* sp. MAR445 were partly controlled by i-15:0, while most of the genus *Bacillus* (e.g strains MAR846, MAR886, MAR285, MAR015, MAR287, MARG2, MAR019 and MAR283) were mainly manipulated by ai-15:0 and i-15:0 acids.

The branched-chain fatty acid family is not nearly as common as that of the straight-chain fatty acid family. The fluidity of membranes composed of straight chain fatty acids is adjusted to the proper level by the inclusion of MUFAs and/or PUFAs, such as in *Shewanellas* sp. MAR445, with increasing temperature from 4 ºC to 25 ºC, the proportion of MUFAs (mainly n-16:1ω9 and n-18:1ω7c) and PUFAs (mainly n-20:5ω3) decreased, with the increasing of SCFAs (mainly n-13:0 and n-16:0), whereas in strains *Paracoccus* sp. MAR824, *Amphritea* sp. MAR442 and *Cobetia* sp. MAR913, the variation of MUFAs was only mirrored by SCFAs.

2.5.4 Phylogenetic resolution of FA patterns

In many cases, the patterns of fatty-acid variables have been analyzed by many classical multivariate data analyses methods, such as principal coordinates analysis (PCO) (Freese et al., 2008), correspondence analysis (CA) (Malmgren et al., 1978), principal component analysis (PCA) (Viga and Grahl-Nielsen, 1990; Patil et al., 2007), Multiple correspondence analysis (MCA). In this study, the FA patterns at 15 ºC were subjected to PCO (Figure 2.4), which allowed separation of isolates representing different phyla: *Firmicutes* groups mainly *Bacillus*, *Paenisporosarcina*, *Kocuria*, *Micrococcus* and *Rhodococcus*; *Gamma-Proteobacteria* including *Stenotrophomonas*, *Amphritea*, *Halomonas*, *Hafnia*, *Cobetia*, *Shewanella*, *Obesumbacterium*, *Serratia*, *Idiomarina*, *Pseudoalteromonas* and *Psychrobacter*; *Alpha-Proteobacteria* comprising *Hoeflea* and *Paracoccus*; (Figure 2.4). The ordination pattern clearly separated the 27 genus, and segregated all *Firmicutes* genus expect *Rhodococcus* in Group (I); most of *Gamma-Proteobacteria* and *Rhodococcus* in Group (II); and *Alpha-Proteobacteria* with two *Gamma-Proteobacteria* (*Hoeflea* and *Pseudoalteromonas*) in Group (III). The result was agreeable to the data clustered by using hierarchical UPGMA analysis (Figure 2.5), which was divided into three groups as mentioned above. This indicates that a taxonomic resolution on the basis of complete FA patterns is possible if isolates belonging to different phyla were not sometimes dominated by the same FAs. However, different isolates belonging to diffient phyla were sometimes dominated by the same major fatty acids and therefore resulting in unsuccessfully separation. For example, *Rhodococcus* sp. MARG10 affiliated with the *Firmicutes* phylum and *Formosa* sp. MAR430 belonged to CFB group based on phylogenetic analysis of 16S rRNA gene sequences, but they were clustered into *Gamma-Proteobacteria* based on fatty acid composition. As it was indicated that the diversity of FA patterns among representatives of a single sub-phylum greater than the differences between representatives of different

phyla might be possible in that the patterns of isolates belonging to different phyla were sometimes dominated by the same FAs (Freese et al., 2008). In this case, the problem could be solved by distinguishing the presence of the fatty acids in lower abundance. Examples for such 'marker FAs' for the individual isolates exhibited the same major FAs, are n-16:1ω7 for the *Rhodococcus* strain, and n-19:0 for the *Formosa* strain.

Generally, FAs patterns obtained at 4, 15 and 25 ºC showed different variation after PCO. Some of them clustered closely at all these three temperatures, such as *Hoeflea* sp. MAR866, *Amphritea* sp. MAR442, *Idiomarina* sp. MARG4, *Bacillus* sp. MAR846, *Bacillus* sp. MAR886, *Bacillus* sp.MAR809, *Bacillus* sp. MAR804, *Rhodococcus* sp. MARG10 and *Micrococcus* sp. MAR806 (nos. 1, 5, 8, 12, 13, 20, 22, 24 and 26); a few of them clustered closely at both 15 and 25 ºC, such as *Formosa* sp. MAR430, *Stenotrophomonas* sp. MAR386, *Pseudoalteromonas* sp. MAR005, *Bacillus* sp. MAR285 and *Micrococcus* sp. MAR443 (nos. 3, 4, 6, 14 and 27), and some only clusted closely at 4 and 25 ºC, such as *Paracoccus* sp. MAR824, *Amphritea* sp. MAR442, *Halomonas* sp. MAR913, *Bacillus* sp. MAR015, *Thalassobacillus* sp. MARG2, *Bacillus* sp. MAR019, *Bacillus* sp. MAR870, *Paenisporosarcina* sp. MAR899 and *Kocuria* sp. MAR801 (nos. 2, 5, 10, 15, 17, 18, 21, 23 and 25), while patterns in the PCO plot varied significantly on strains *Shewanella* sp. MAR445, *Serratia* sp. MARG45, *Halomonas* sp. MAR913, *Bacillus* sp. MAR287 and *Bacillus* sp. MAR283 (nos. 7, 9, 10, 16 and 19), they gave separate clusters at all three temperatures. FA patterns among representatives of different phylum at different temperature clustered closely or overlapped, such as *Serratia* sp. MARG45 at 4 ºC and *Psychrobacter* sp. MAR701 at 15 ºC (nos. 9 and 11), *Kocuria* sp. MAR801 at 15 ºC and *Bacillus* sp. MAR283 at 25 ºC (nos. 19 and 25). Therefore taxonomic identification on the basis of complete FA patterns need to be very careful on isolates belonging to the same phyla in that more or less of temperature change might result in some change of branched, saturated or monounsaturated FAs.

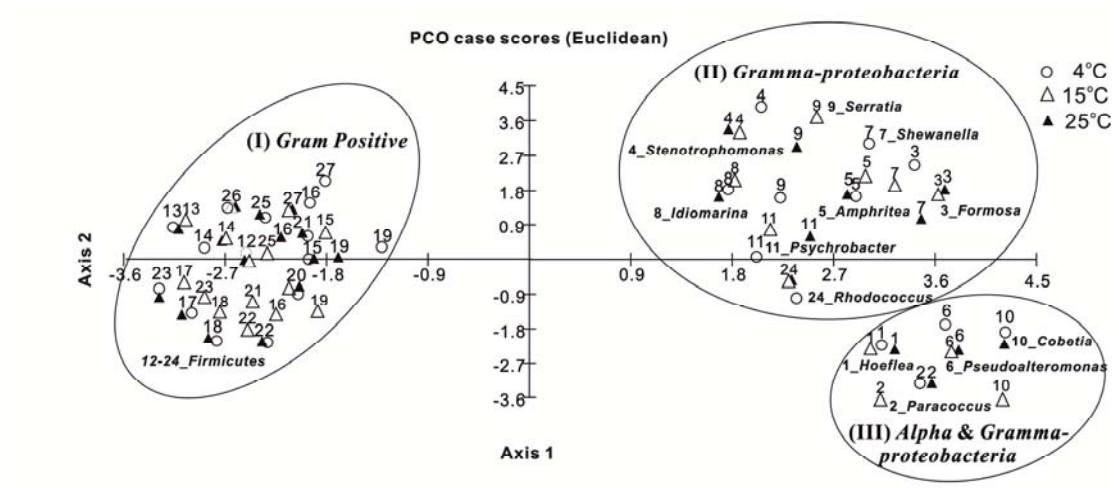

Figure 2.4 Principal coordinates analyses (PCO) of major FA abundance data from Tables 2.3-2.6 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 2.5)

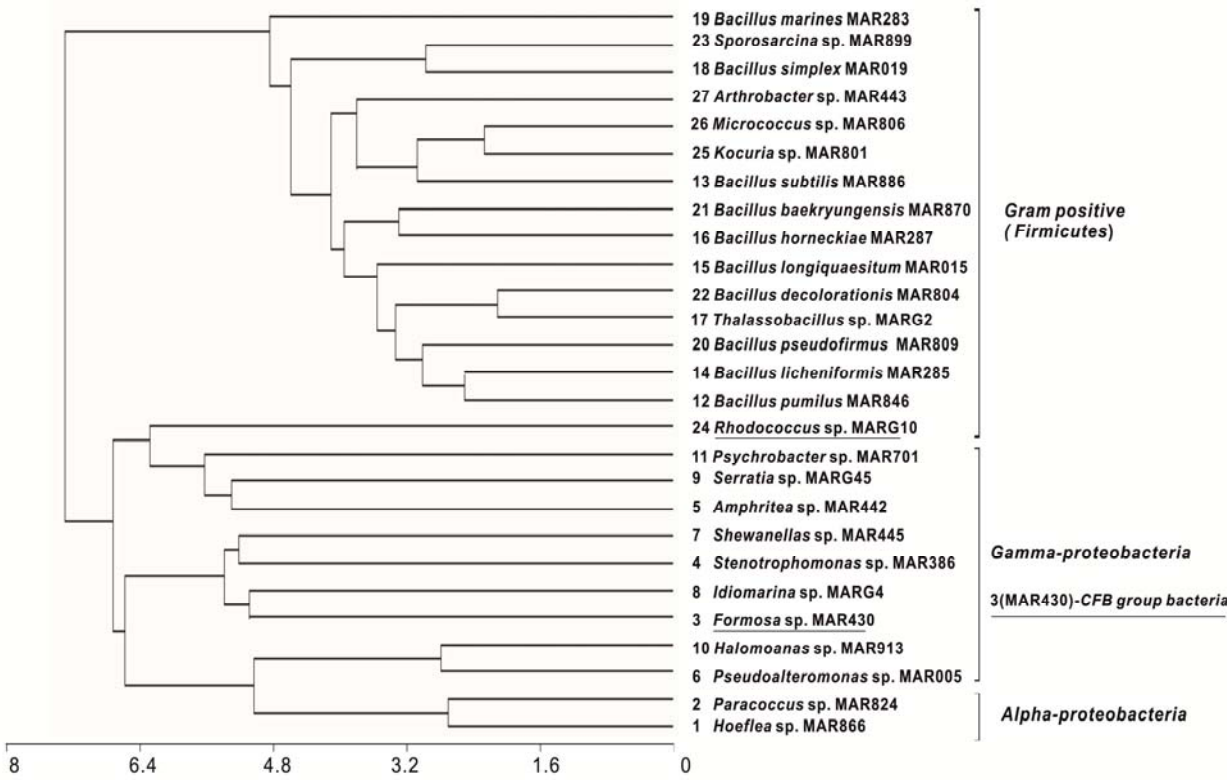

Figure 2.5 Similarity analysis based on whole cell fatty acid composition at 15 ^{o}C from 27 bacterial species. The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 2.4 and nearest type strains were indicated beside the isolates.

Both the distribution and the percentage contribution of each fatty acid to the total fatty acid across taxa at species level of *Gamma-Proteobacteria*, *Alpha-Proteobacteria* and

Gram-positive bacteria were plotted respectively shown in Figures 2.6, 2.7 and 2.8. The trends and relationships of fatty acid composition of each group under different temperautres or each species within *Gamma-Proteobacteria*, *Alpha-Proteobacteria* and *Gram-positive bacteria* respectively could be presented using this method. Within *Gamma-Proteobacteria*, wide distribution of fatty acid composition was observed, ranging from short-chain FAs (e.g. i-12:0, n-13:0, i-15:0 and n-15:0); middle-chain FAs (e.g. n-16:1ω9, n-16:1ω7, n-16:0, i-17:0, n-17:1ω8 and n-17:0 and n-18:1ω9c), to long-chain FAs (e.g. n-20:5ω3), in which EPA was found. The fatty acids of *Alpha-Proteobacteria* were dominated by middle-chain FAs, such as n-18:1ω9c and n-18:1ω7c. Similarly, both *Alpha-* and *Gamma-Proteobactria* strains synthesized unsaturated fatty acids in response to a temperature drop (Figures 2.6 and 2.7). Most of the *Gram*$^{+}$ species contained i-15:0, ai-15:0, i-16:0, ai-17:1ω7, i-17:0, ai-17:0 and n-18:1ω9c as their major fatty acids, and mainly employed mainly i-15:0 and ai-15:0 acids in response to a temperature drop (Figure 2.8).

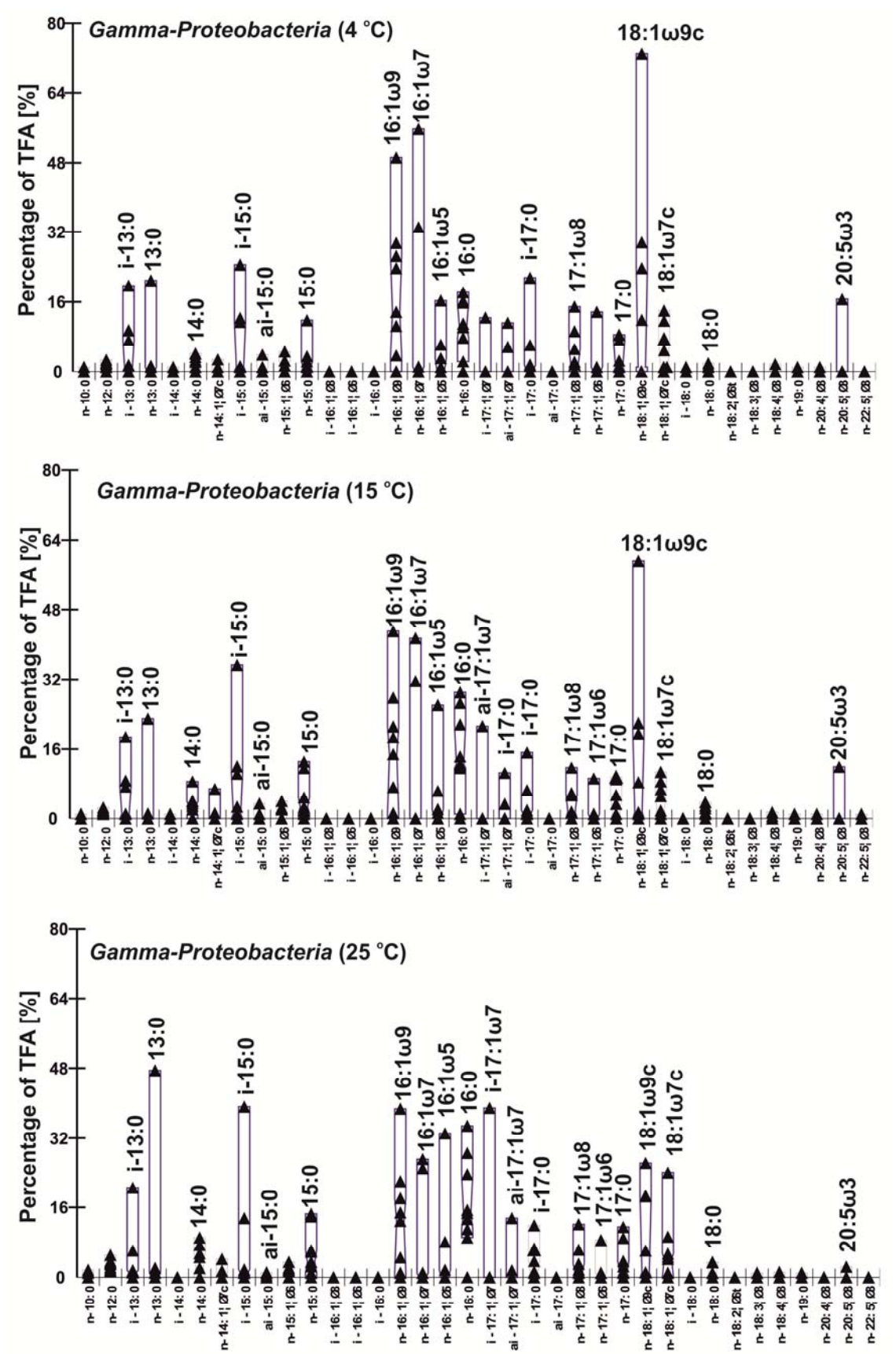

Figure 2.6 Box plots representing the distribution and variability in the percentage contribution of each fatty acid to the total fatty acids within *Gamma-Proteobacteria* at 4, 15 and 25 ^{o}C. The triangles represent the presence of each fatty acid (Axis X) from different species analyzed, with percentage of TFA indicated by Axis Y.

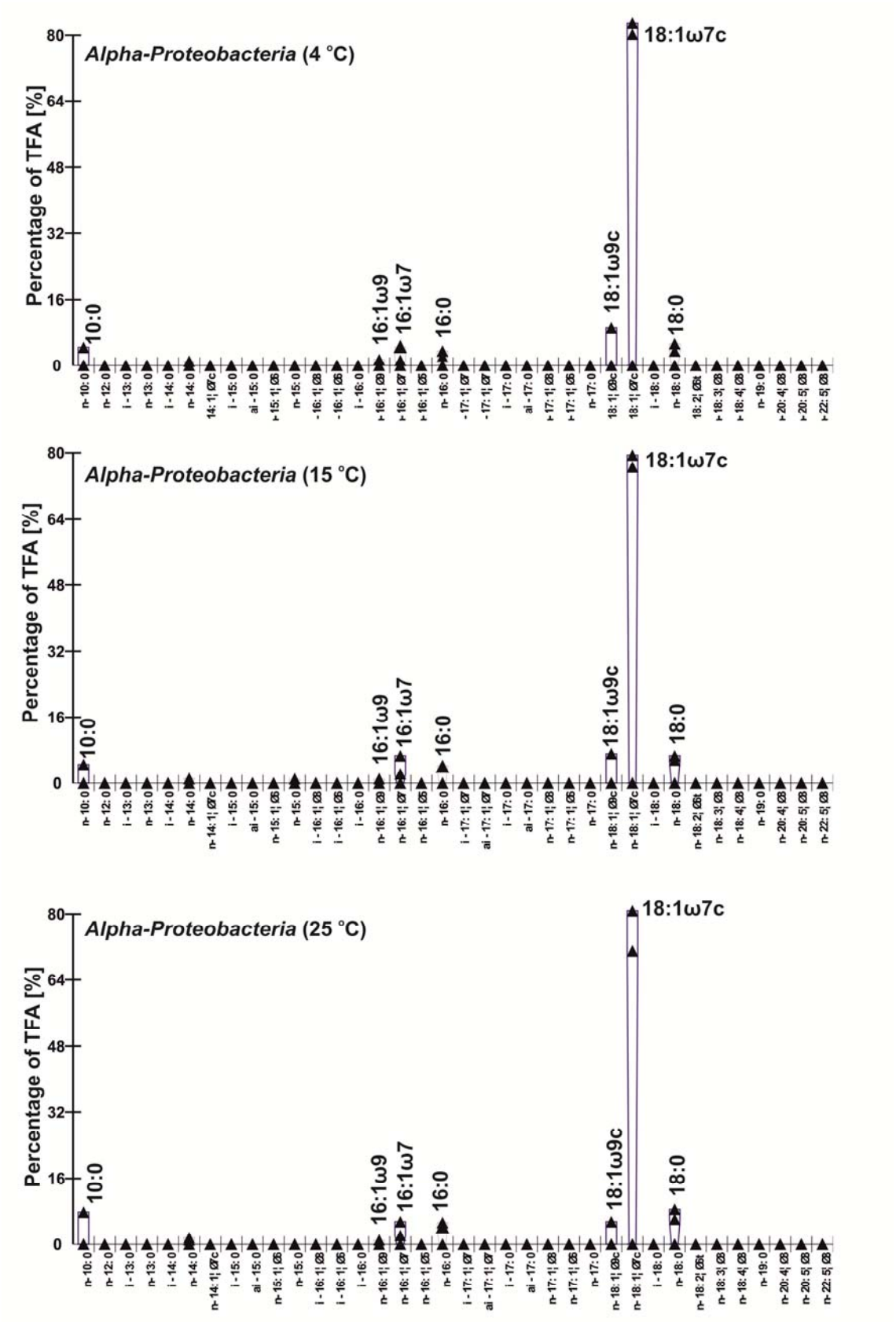

Figure 2.7 Box plots representing the distribution and variability in the percentage contribution of each fatty acid to the total fatty acids within *Alpha-Proteobacteria* at 4, 15 and 25 ^{o}C. The triangles represent the presence of each fatty acid (Axis X) from different species analyzed, with percentage of TFA indicated by Axis Y.

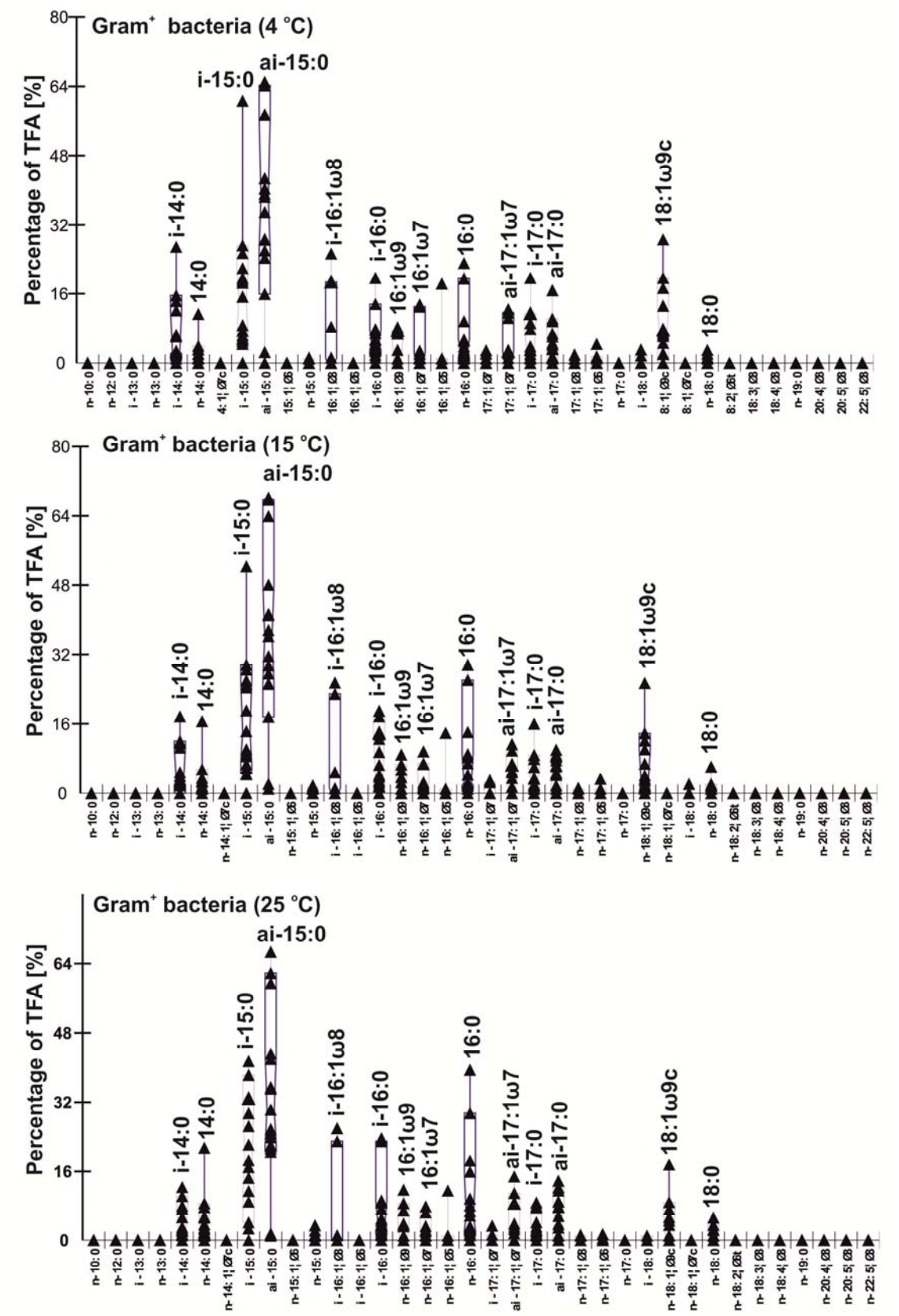

Figure 2.8 Box plots representing the distribution and variability in the percentage contribution of each fatty acid to the total fatty acids within *Gram*$^+$ (*Firmicutes*) at 4, 15 and 25 ^{o}C. The triangles represent the presence of each fatty acid (Axis X) from different species analyzed, with percentage of TFA indicated by Axis Y.

2.6 Conclusions

In conclusion, the comparison between the bacterial community and the distribution and the percentage contribution of their fatty acid composition is helpful in studying bacterial taxonomy and recognizing their important role in certain marine food web, such as in MAR "non-vent" sites sediments.

Although marine microorganisms have only recently become a target for natural product drug discovery and energy production (Bernan et al., 1997; Reimers et al., 2001; Bond et al., 2002; Blunt et al., 2006), it has become increasingly clear that Gram-positive strains are a rich source of new structures that possess promising antimicrobial and anticancer activities (Bernan et al., 2004; Blunt et al., 2006; Kwon et al., 2006) and that a better understanding of microbial diversity will provide important insight into how to devise intelligent strategies for discovering natural product (Bull, 2004), and environmental power sources (Tender et al., 2002; Lovley, 2006). The present research firstly reports the microbial biodiversity of MAR "non-vent" sediments with findings of Gram-negative EPA producers, and Gram-positive squalene producers and bacterial electricity producers. The work helps to establish a fundamental understanding of the diversity of culturable deep-sea bacteria in the MAR sediments and provides a diverse, marine environment-derived assemblage of cultured bacteria whose chemical and biosynthetic diversity can be investigated.

Furthermore, FA compostion of certain isolates could be well defined under certain conditions, e.g. temperature, pressure or grown medium. However, some strains strongly modify their FA patterns when their culture temperature changes and several strains did not show any modifications. FA patterns of microbial communities could represent their taxonomic structure in certain environmental nitch. However, it should be done with great care if strains were identified on the basis of fatty acid compositions. The FA patterns of MAR non-vent microbial communities in reponse to changing temperature showed that, Gram positive strain, mainly *Firmicutes* affiliated with branched-chain fatty acid family, employed anteiso-15:0 and iso-15:0 acids to adjust their membrane viscosity, whereas other strains, such as *Alpha*, *Gamma-Proteobactria* and CFB group belong to straight-chain fatty acid family which required unsaturated fatty acids for growth and membrane ciscosity manipulation.

Chapter 3. Marine sponge-associated bacteria as sources for omega-3 fatty acids

3.1 Abstract

Sponge associated bacteria are of great interest in that highly diverse microbes and novel biologically active chemical compounds (e.g. glycosphingolipids) could be found. However, sponge associated bacteria as a source for omega-3 fatty acids has received little attention. In this study, we investigated biogeographic variability of bacterial communities and fatty acid compositions between temperate and tropical sponges by isolating bacteria from the temperate North-sea sponge *Halichondria panicea* and the tropical Caribbean sponge *Agelas clathrodes*. Phylogenetic analysis of the sponge associated bacterial communities based on 16S rRNA gene sequences indicated an abundance of *Gamma-Proteobacteria* (90 %) in *Halichondria panicea*, whereas Gram-positive bacteria mainly occured in *Agelas clathrodes*. Fatty acid analysis indicated that *Vibrio* and *Shewanella*, isolated from *Halichondria panicea*, were able to produce eicosapentaenoic acid (EPA) (2-10 % of total fatty acids), and no strains capable of producing EPA were isolated from *Agelas clathrodes*. Principal coordinates analysis (PCO) on the marine sponge associated bacteria by whole cell fatty acid compositions indicated that taxonomic resolution on the basis of complete FA patterns is possible.

3.2 Introduction

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), are essential to human health and nutrition, providing important visual, mental, and cardiovascular benefits throughout life (Heird and Lapillonne, 2005; Brouwer et al., 2006; Muskiet and Kemperman, 2006). Microbial omega-3 PUFAs have raised attention from vegetarians as an alternative source for EPA or DHA, instead of fish oil, which is limited (Garcia and Rosenberg, 2010). Furthermore, bacteria could be used as a primary PUFA-rich feedstock in artificial food chains in the aquaculture industry, thereby protecting natural fish stocks (Watanabe et al., 1992; Nichols et al., 1996b). Several EPA-producing bacteria have been isolated, such as psychro- or piezophiles from polar regions and the deep sea (Delong and Yayanos, 1986; Bowman et al., 1997b; Russell and Nichols, 1999; Gentile et al., 2003),

from a temperate estuary (Skerratt et al., 2002), from shallow seawater samples (Ivanova et al., 2001; Ivanova et al., 2003a; Frolova et al., 2005), and from an intertidal flat (Freese et al., 2008; Freese et al., 2009). However, little research has been undertaken on bacteria from marine sponges producing omega-3 PUFAs.

Marine invertebrates, especially sponges, have a two layer structure of outer and inner endosomal membrane, and sequester food by filtering seawater, making sponges an ideal habitat for microorganisms. Sponges harbour dense and diverse microbial consortia, which may contribute up to 40 % of sponge tissue volume and exceed the numbers of microorganisms in seawater by two to three orders of magnitude (Wilkinson, 1978). Sponge microbes span all three domains of life, of which at least 18 bacterial and archaeal phyla have been described from sponge hosts (Taylor et al., 2007). Various unprecedented chemical structures of fatty acids, and lipid-containing fatty acids, have recently been discovered from sponges (Berge and Barnathan, 2005). For example, marine glycosphingolipids, chiefly isolated from sponges, such as *Agelas clathrodes* (Ding et al., 2006), show interesting biological activities such as immunomodulation and antitumoral activity (Fattorusso and Mangoni, 1997). The fatty acyl chains linked to these classes of compounds are common but several new and original structures have been reported recently (Costantino et al., 1999; Costantino et al., 2003). A novel series of α-methoxylated FA have been reported from Caribbean sponges from the genera *Amphimedon*, *Callyspongia* and *Spheciospongia* (Carballeira and Colon, 1999; Carballeira and Alicea, 2002). Biosynthesis genes encoding polyketide synthases (PKSs) or nonribosomal peptide synthetases (NRPSs) were presented in *Halichondria panicea* associated *Actinobacteria*, indicating the existence of natural products (Schneemann et al., 2010). Therefore, it would be interesting to find sponge associated bacteria which produce bioactive compounds.

In the present investigation an attempt has been made to examine the variability of bacterial communities and fatty acid patterns between temperate and tropical sponges by isolating bacteria from North-sea sponge *Halichondria panicea* and Caribbean sponge *Agelas clathrodes*. The taxonomic analysis of sponge associated bacterial community structure based on complete FA patterns was also conducted.

3.3 Materials and Methods

3.3.1 Sample collection

The sponge sample *Halichondria panicea* was collected by the author at low tide in the coastal area off the Dove Marine Laboratory, Cullercoats, North Sea (54°54'25"N, 1°21'35"W) by scraping the rocks. The sponge sample *Agelas clathrodes* (the Orange Elephant Ear Sponge) inhabiting the depths of 22 to 55 m, was collected from the coast of Grenada near St. George's University Caribbean by snorkelling from the reef. Collected sponge samples (100 g) were transferred in zip-lock bags on ice to UK. Portions of these specimens were thoroughly washed three times with autoclaved seawater passed through a 0.2 μm-pore-size filter to remove loosely attached bacteria.

3.3.2 Isolation of sponge associated bacteria

For the isolation of the sponge associated bacteria, one 1 cm^3 of sponge tissue was excised from the middle of the whole sponge using a pair of sterile scissors. The excised portion was thoroughly washed three times with filtered sea water to remove any bacteria within current canals and then the tissue was homogenaised using a sterile mortar and pestle. The resultant homogenate was serially diluted with filtered sea water and pre-incubated at 15 °C for 1 h. The aliquot was plated on marine agar plates (Difco) and incubated at 4 and 15 °C in dark aerobic conditions until visible colonies appeared. Colonies with different morphologies on this medium were selected and purified through the third generation. Gram staining was used for initial bacterium classification; after staining the bacteria were stored in glycerol broth 15 % v/v (-80 °C). Altogether 53 marine sponge associated bacterial isolates were obtained, all of which were further analysed for fatty acid production.

3.3.3 Strain growth

For biomass production, strains were inoculated into 10 ml of Marine Broth 2216E (Difco), and incubated at 15 °C until turbidity was apparent. The 10 ml cultures were then used to inoculate 90 ml volumes of marine 2216 broth contained in 500 ml conical flasks pre-rinsed in chloroform. Flasks were incubated at 15 °C respectively with agitation provided by a magnetic stirrer or orbital shaker (180 rpm) for 24-48 h until sufficient mass of estimated late-log phase cells were present for harvest.

Cell mass from broth cultures was collected by centrifugation at 4500 g for 20 min. Cell pellets were resuspended in 200 ml M9 solution (22 mM KH_2PO_4, 22 mM Na_2HPO_4, 85 mM NaCl, 1mM $MgSO_4$) and recentrifuged followed by rinsing with 0.1 % ammonium acetate and frozen. The washed cell pellets were suspended in 2.0 ml saline and lyophilised in preweighed containers prior to lipid extraction.

3.3.4 Fatty acids analysis as methods mentioned in Chapter 2

3.3.5 16S rRNA gene identification as methods mentioned in Chapter 2

3.3.6 Nucleotide sequence accession numbers

The nucleotide sequences of 16S rRNA gene have been deposited in EMBL under the accession numbers: FR750928-FR750956 (NSP480-NSP683) and FR750957-FR750980 (CMS161-CMS382).

3.3.7 Multivariate analyses as methods memtioned in Chapter 2

Relative abundances of major FAs after whole cell hydrolysis (Tables 3.3–3.7) were used for statistical analysis as memtioned in Chapter 2.

3.4 Results

3.4.1 Phylogenetic identification of the North-sea sponge (Halichondria panicea) associated bacteria

From the North-sea marine sponge (*Halichondria panicea*), a total of 29 morphologically distinct heterotrophic associated bacterial colonies were isolated and identified on the basis of 16S rRNA gene sequence analysis (Table 3.1). Phylogenetic analysis of these 29 strains revealed that *Gamma-Proteobacteria* was the most abundant division of the bacterial component (86.2 %), followed by Gram positives (10.3 %) and high CFB group bacteria (3.5 %). *Gamma-Proteobacteria* was phylogenetically affiliated with 6 members of *Pseudoalteromonadaceae*, *Vibrionaceae*, *Alteromonadaceae*, *Shewanellaceae*, *Halomonadaceae* and *Gilvimarinus*, including 17 taxonomic units of *Pseudoalteromonas carrageenovora*, *Ps. marina*, *Ps. prydzensis*, *Ps.*

arctica, *Ps.undina*, *Ps. tetraodonis*, *Ps. issachenkonii*, *Ps. prydzensis*, *Ps. aliena*, *Vibrio tasmaniensis*, *V. splendidus*, *Alteromonas stellipolaris*, *Glaciecola mesophila*, *Shewanella pealeana*, *Sh. piezotolerans*, *Gilvimarinus chinensis* and *Cobetia marina*. Gram positive contained 3 taxonomic units of Arthrobacter parietis, *Bacillus stratosphericus* and *Paenisporosarcina quisquiliarum*. And one strain identified as *Cellulophaga baltica* belonged to CFB group bacteria (Figure 3.1).

Table 3.1 List of North Sea sponge (*Halichondria panicea*) associated bacterial strains

Phylogenetic group (family)	Representative isolates	No. of strains in OTU	Nearest type in the GenBank (accession number)	Sequence identity (%)[b]	Source of nearest type strain
Gamma-Proteobacteria					
Pseudoalteromonadaceae	NSP482	1	*Pseudoalteromonas carrageenovora* ATCC 12662^T (X82136)	99.112	**ATCC collection**
	NSP507; NSP508; NSP513; NSP517; NSP562	5	*Pseudoalteromonas marina* Mano4^T (AY563031)	99.5-99.9	**Yellow Sea**
	NSP511; NSP558	2	*Pseudoalteromonas prydzensis* MB8-11^T (U85855)	98.3-99.9	**Antarctic sea ice**
	NSP519	1	*Pseudoalteromonas arctica* A 37-1-2^T (DQ787199)	99.378	**Spitzbergen**
	NSP530	1	*Pseudoalteromonas undina* NCIMB 2128^T (X82140)	99.614	**ATCC collection**
	NSP541; NSP549	2	*Pseudoalteromonas tetraodonis* IAM 14160^T (AF214730)	99.899	**Red alga**
	NSP542	1	*Pseudoalteromonas issachenkonii* KMM 3549^T (AF316144)	100.000	**Brown algae**
	NSP681	1	*Pseudoalteromonas aliena* KMM 3562^T (AY387858)	99.856	**Sea-water**
Vibrionaceae	NSP501; NSP560	2	*Vibrio tasmaniensis* LMG 21574^T (AJ514912)	99.4-99.5	**Atlantic salmon**
	NSP559	1	*Vibrio splendidus* ATCC 33125^T (X74724)	99.326	**ATCC collection**
Alteromonadaceae	NSP481	1	*Alteromonas stellipolaris* LMG 21861^T (AJ295715)	99.635	**Antarctic sea**
	NSP683	1	*Glaciecola mesophila* KMM 241^T (AJ488501)	99.509	**Halocynthia aurantium**
Shewanellaceae	NSP523	1	*Shewanella pealeana* ATCC 700345^T (CP000851)	99.653	**Atlantic squid**
	NSP561	1	*Shewanella piezotolerans* WP3^T (CP000472)	98.695	**West Pacific deep-sea sediment**
Gilvimarinus	NSP500	1	*Gilvimarinus chinensis* QM42^T (DQ822530)	94.975	**Coastal seawater**
Halomonadaceae	NSP484; NSP488	2	*Cobetia marina* DSM 4741^T (AJ306890)	99.381	**ATCC collection**
	NSP520	1	*Cobetia marina* DSM 4741^T (AJ306890)	99.098	**ATCC collection**
High GC Gram+					
Micrococcaceae	NSP487	1	*Arthrobacter parietis* LMG 22281^T (AJ639830)	99.781	**Mural paintings**
Firmicutes					
Bacillaceae	NSP483	1	*Bacillus stratosphericus* 41KF2a^T (AJ831841)	99.498	**Air samples**
Planococcaceae	NSP480	1	*Paenisporosarcina quisquiliarum* SK 55^T (DQ333897)	98.529	**Landfill soil**
CFB group bacteria					
Flavobacteriaceae	NSP516	1	*Cellulophaga baltica* NN015840^T (AJ005972)	99.151	**Seawater samples**

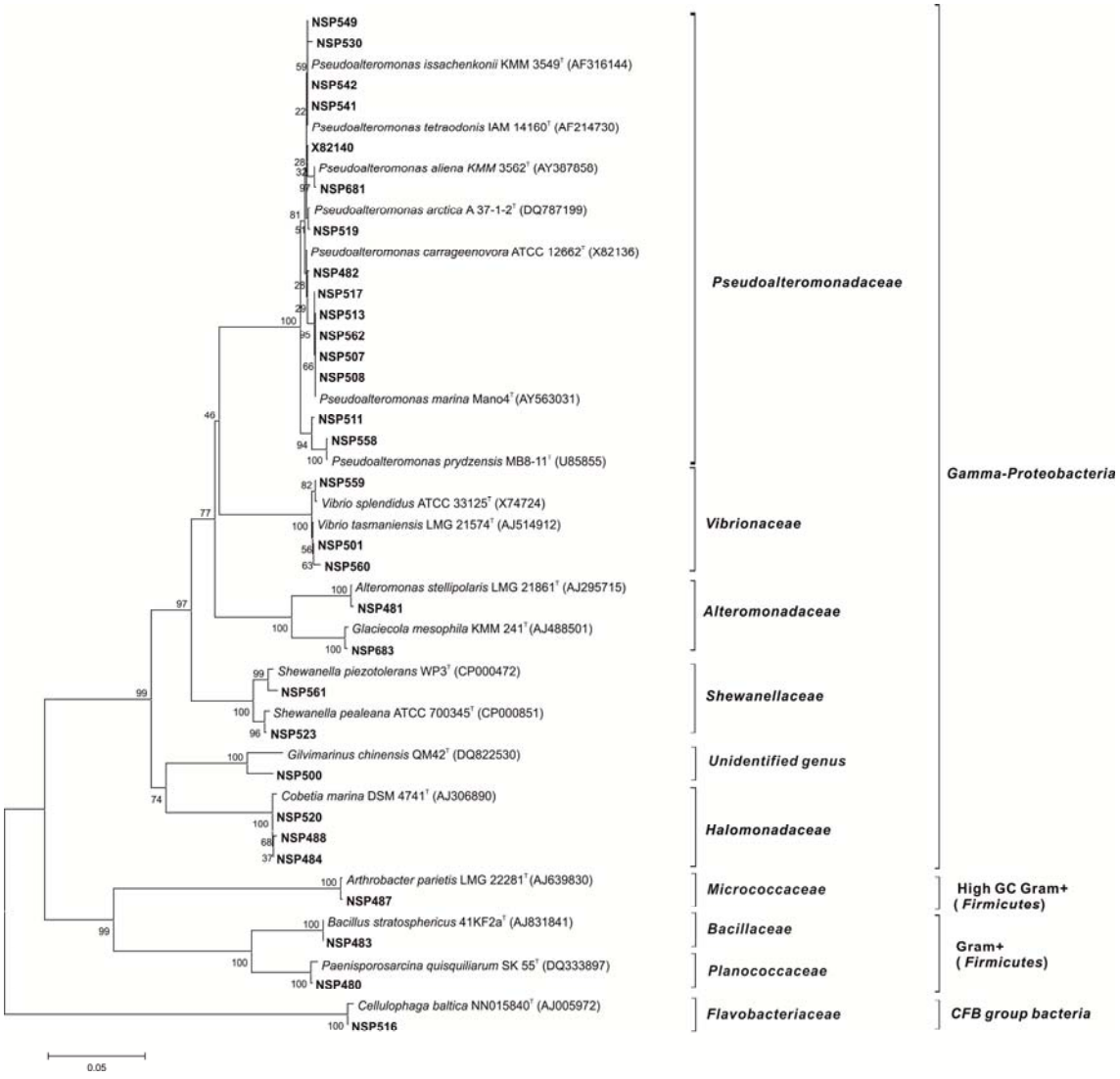

Figure 3.1 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of 29 bacteria associated with North-sea sponge (*Halichondria panicea*) in this study and their nearest type strains. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.05 nucleotides substitution per site.

3.4.2 Phylogenetic groups and identification of bacterial associated with the Caribbean sponge (Agelas clathrodes)

Twenty-four bacterial strains were isolated and identified on the base of 16S rRNA gene sequence analysis (Table 3.2). Phylogenetic analysis of these 24 strains revealed that Gram positive was the most abundant division of the bacterial populations (75 %), followed by *Gamma-Proteobacteria* (16.6 %), *Alpha-Proteobacteria* (4.2 %) and CFB group bacteria (4.2 %). Gram-positive strains were phylogenetically affiliated with 7

members of the *Bacillaceae, Paenibacillaceae, Planococcaceae, Aerococcaceae, Cellulomonadaceae, Nocardiaceae* and *Micrococcaceae*. *Gamma-Proteobacteria* contained 3 members of *Moraxellaceae*, *Granulosicoccaceae* and *Rhodobacteraceae,* including 3 taxonomic units of *Psychrobacter nivimaris* and *Granulosicoccus coccoides*. One strain has homology to *Labrenzia alba*, affiliated with *Alpha-Proteobacteria*, and one strain, identified as *Algibacter lectus*, belonged to CFB group bacteria (Figure 3.2).

Table 3.2 List of Caribbean sponge (*Agelas clathrodes*) associated bacterial strains

Phylogenetic group (family)	Representative isolates	No. of strains	Nearest type in the GenBank (accession number)	Similarity (%)	Source of nearest type strain
Firmicutes					
Bacillaceae	CMS179	1	*Bacillus licheniformis* ATCC 14580^T (CP000002)	98.962	Soil
	CMS176	1	*Brevibacterium frigoritolerans* DSM 8801^T (AM747813)	98.689	N/A
	CMS178; CMS187	2	*Bacillus hwajinpoensis* SW-72^T (AF541966)	99.5-99.9	Yellow Sea
	CMS188	1	*Halobacillus salinus* HSL-3^T (AF500003)	98.653	East Sea
	CMS190; CMS193; CMS195; CMS206	4	*Oceanobacillus profundus* CL-MP28^T (DQ386635)	99.7-99.9	Deep-sea sediment core
Paenibacillaceae	CMS172	1	*Paenibacillus pinihumi* S23^T (GQ423057)	98.440	Rhizosphere of Pinus densiflora
Planococcaceae	CMS223	1	*Sporosarcina psychrophila* IAM 12468^T (D16277)	99.863	N/A
Aerococcaceae	CMS378; CMS380	2	*Aerococcus viridans* ATCC 11563^T (M58797)	99.6-99.9	Spanish Type Culture Collection
High GC Gram+					
Cellulomonadaceae	CMS382	1	*Demequina aurantiaca* YM12-102^T (AB522641)	98.139	Algae
Nocardiaceae	CMS164	1	*Rhodococcus maanshanensis* M712^T (AF416566)	*99.357*	Soil
	CMS166; CMS199	2	*Rhodococcus erythropolis* NBRC 100887 (AP008957)	99.8-99.9	Pacific Ocean
Micrococcaceae	CMS197	1	*Micrococcus luteus* NCTC 2665^T (CP001628)	99.582	N/A
Gamma-Proteobacteria					
Moraxellaceae	CMS161; CMS165; CMS185	3	*Psychrobacter nivimaris* 88/2-7^T (AJ313425)	99.5-99.8	South Atlantic
Granulosicoccaceae	CMS377	1	*Granulosicoccus coccoides* Z 271^T (FJ535355)	99.135	Leaves of seagrass
Alpha-Proteobacteria					
Rhodobacteraceae	CMS163	1	*Labrenzia alba* CECT 5094^T (AJ878875)	99.355	Mediterranean oysters
CFB group bacteria					
Flavobacteriaceae	CMS186	1	*Algibacter lectus* KMM 3902^T (AY187689)	98.693	Green algae

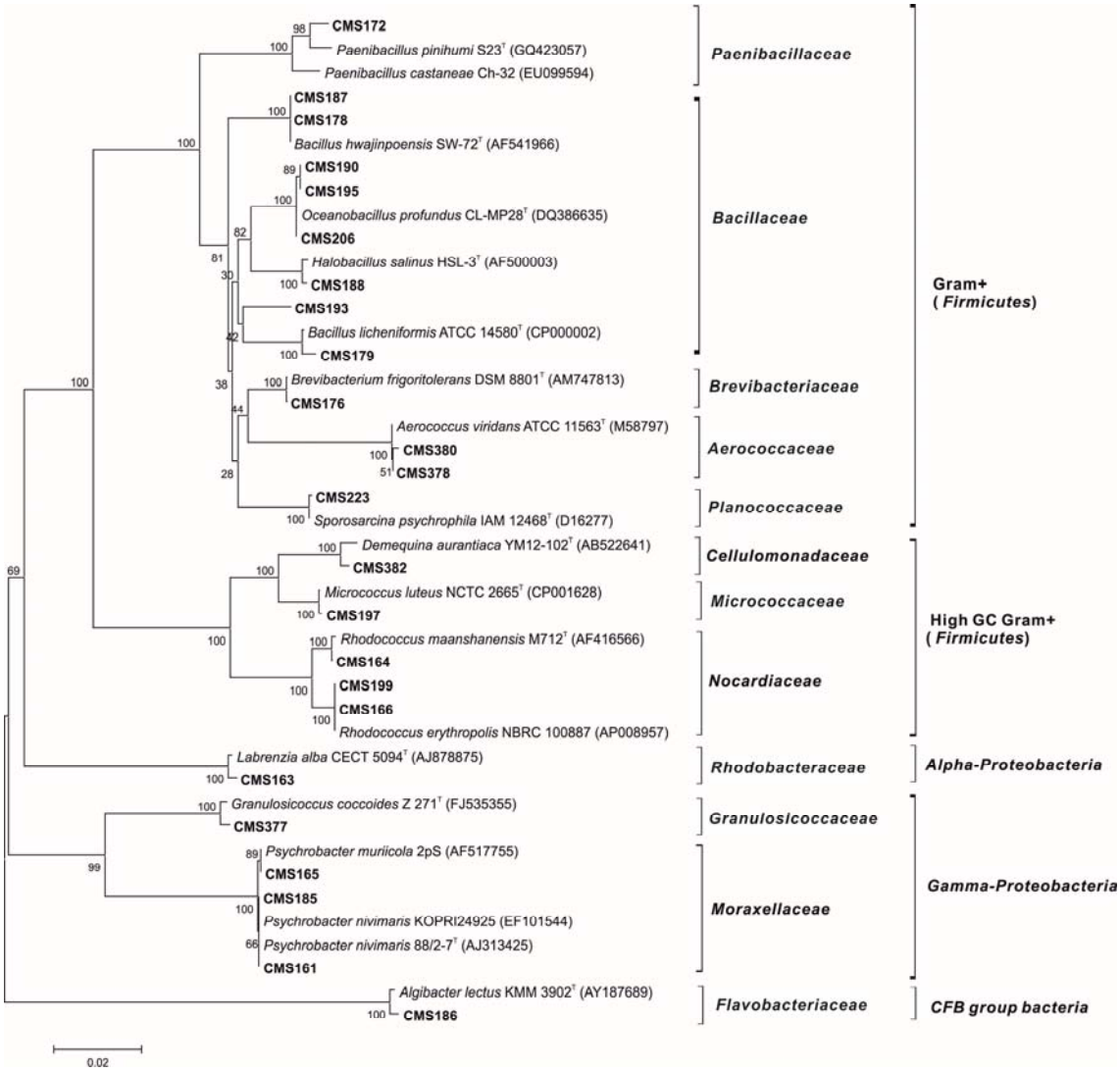

Figure 3.2 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of 24 bacteria associated with Caribbean marine sponge (*Agelas clathrodes*) in this study and their nearest type strains. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.02 nucleotides substitution per site.

3.4.3 Fatty acid composition of sponge associated bacteria

3.4.3.1 Fatty acid composition of Gamma-Proteobacteria

Strains NSP482, NSP511, NSP513, NSP519, NSP530, NSP541, NSP542, NSP558 and NSP681 exhibited the highest homology (98-99 % 16S rRNA gene identity) to type strains *Pseudoalteromonas carrageenovora, Ps. prydzensis*, *Ps. marina*, *Ps. arctica*, *Ps. undina*, *Ps. tetraodonis*, *Ps. issachenkonii, Ps. prydzensis* and *Ps. aliena* respectively. FA patterns of these *Pseudoalteromonadaceae* strains were dominated by n-16:0, n-16:1ω7 and n-18:1ω7c (8-45 %), with minor contributions (1-7 %) of n-12:0, 3-OH-12:0, n-14:0, n-15:1ω8, n-17:0 and n-17:1ω8 acids. Low levels of saturated iso-branched fatty acids and 3-OH fatty acids were also detected, but these were at levels too low to be reliable for differentiation of the groups. The lipid profiles were very similar to those found for other *Pseudoalteromonas* species and *Alteromonas macleodii* (Ivanova et al., 2002; Nam et al., 2007; Al Khudary et al., 2008). No BCFA and PUFA were detected of the species, and the ACL was variable (15.5-16) (Table 3.3).

Strains NSP484 and NSP520 exhibited the highest homology (99.1-99.4 % 16S rRNA gene identity) to the type strain *Cobetia marina*. FA patterns of these *Halomonadaceae* strains were dominated by 3-OH-12:0, n-16:0, n-16:1ω7, cyclo-17:0 and n-18:1ω7c acids (9-26 %), with minor contributions (1-7 %) of n-10:0, n-12:0, i-15:0, n-18:0 and cyclo-19:0 acids (1-7 %), and ACL of 14.9-15.6 (Table 3.3). The FA profiles were comparable with those from type strains (Yumoto et al., 2004).

Strains NSP560 and NSP559 showed the highest sequence similarities (99.3-99.9 % 16S rRNA gene identity) to type strains *Vibrio tasmaniensis* and *V. splendidus* respectively. FA patterns of these *Vibrionaceae* strains were dominated by n-14:0, n-16:0, n-16:1ω7 and n-18:1ω7c (8-40 %), with minor contributions (1-6 %) of n-12:0, 3-OH-12:0, i-15:0, n-16:1ω11 and i-16:1ω6 acids. The major cellular fatty acids profile of these strains was compared with those of other members of the same *Vibrio* genus (Thompson et al., 2003). MUFAs were the most dominance, and PUFAs, such as EPA, was only in trace level (1-4 %) and ACL was between 15.2-15.8 (Table 3.4).

Table 3.3 Major FAs after whole cell hydrolysis (% of TFAs) in the bacteria associated North-sea sponge (*Halichondria panicea*)

Fatty acids	*Pseudoalteromonas* sp.									*Cobetia* sp.	
	NSP482	NSP511	NSP513	NSP519	NSP530	NSP541	NSP542	NSP558	NSP681	NSP484	NSP520
n-10:0	-	-	-	1.1	-	-	-	-	-	2.3	3.1
n-12:0	0.3	1.2	0.9	2.7	2.2	1.7	1.8	1.1	2.1	4	5.5
3-OH-12:0	0.6	0.5	6.1	6.7	6.2	3.5	0.4	1.6	0.1	12.6	20.3
n-13:0	0.5	0.5	0.1	0.3	0.2	0.1	0.2	0.3	0.5	-	-
i-14:0	-	-	-	-	-	-	-	-	-	-	-
n-14:0	1.7	2.2	1.1	1.2	1.1	1.9	1.7	1.5	1.2	1.5	1.7
n-14:1ω7c	0.6	1.1	-	-	-	-	1.3	-	0.5	-	-
i-15:0	-	-	-	-	-	-	-	-	-	2.5	1.8
n-15:1ω6	0.6	0.7	0.8	0.2	0.5	0.6	-	0.4	-	-	-
n-15:1ω8	3.7	6.3	8.6	1.6	2.1	6.4	5.1	3.6	6.7	-	-
n-15:0	8.6	12.5	17.1	11.9	16.4	7.5	8.5	7.2	15.5		
n-16:1ω7	39.5	40.5	24.7	41.1	42.5	37.4	46.5	40.2	41.5	15.1	18.5
n-16:1ω5	0.4	0.1	0.5	0.3	0.1	0.3	0.1	-	-	-	-
n-16:0	19.8	15.4	22.5	15.8	10.8	16.7	15.3	23.7	15.7	23.4	22.3
ai-17:1ω7	0.8	-	0.2	-	-	-	0.5	0.4	0.1	-	-
n-17:1ω8	9.2	6.2	4.7	6.9	6.5	13.1	8.5	8.8	4.2	-	-
n-17:0	6.7	4.6	5.2	1.2	4.1	4.4	2.5	3.1	0.7	13.6	9.6
n-18:1ω9c	-	0.7	0.8	0.5	0.3	0.2	0.2	0.3	0.5	-	-
n-18:1ω7c	4.5	5.5	5.1	6.6	5.8	4.1	6.3	6.5	8.5	19.7	11.5
n-18:0	0.9	0.3	0.2	0.3	0.3	0.5	0.4	0.2	0.4	0.5	1.7
cyclo-19:0	-	-	-	-	-	-	-	-	-	3.6	2.5
Others	1.6	1.7	1.4	1.6	0.9	1.6	0.7	1.1	1.8	1.2	1.5
Σ TFA	100	100	100	100	100	100	100	100	100	100	100
Σ SCFA	39.1	37.2	53.2	41.2	41.3	36.3	30.8	38.7	36.2	61.5	66.7
Σ BCFA	-	-	-	-	-	-	-	-	-	2.5	1.8
Σ MUFA	59.3	61.1	45.4	57.2	57.8	62.1	68.5	60.2	62	34.8	30
ACL[a]	15.8	15.6	15.4	15.4	15.4	15.6	15.9	15.8	15.6	15.6	14.9

[a]Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; TFA, total fatty acids; and (–), not detectable.

Strain NSP481 and NSP683 exhibited the highest homology (99.5-99.6 % 16S rRNA gene identity) to type strains *Alteromonas stellipolaris* and *Glaciecola mesophila.* Both species showed very similar fatty acid profiles, of which the major constituents included n-16:1ω7, n-16:0, n-17:1ω8, n-18:1ω9c and/or n-18:1ω7c (7-45 %). Hydroxylated fatty acids and alcohol derivatives of the fatty acids n-16:0 and n-16:1ω7c were also present as minor components or at trace levels. The fatty acid profiles of these two strains clearly resembled those determined for other *Alteromonas* and *Glaciecola* species (Van Trappen et al., 2004; Yong et al., 2007). The ACLs of these two species were the highest (16.0 and 16.1) among the North-sea isolates due to the high content of MUFAs (Table 3.4).

Table 3.4 Major FAs after whole cell hydrolysis (% of TFAs) in the bacteria associated North-sea sponge (*Halichondria panicea*)

Fatty acids	*Shewanella*		*Vibrio*		*Alteromonas*	*Glaciecola*	*Gilvimarinus*	*Arthrobacter*	*Bacillus*	*Paenispo-rosarcina*	*Cellulophaga*
	NSP523	NSP561	NSP560	NSP559	NSP481	NSP683	NSP500	NSP487	NSP483	NSP480	NSP516
n-10:0	-	-	3.1	3.5	1.5	-	3.4	-	-	-	-
n-12:0	3.7	4.6	-	-	3.1	2.7	8.2	-	-	-	-
3-OH-12:0	1.2	-	7.5	9.2	1.1	-	3.2	-	-	-	-
i-13:0	15.1	9.5	-	-	-	-	-	0.8	-	1.6	-
n-13:0	1.8	1.2	-	-	-	-	-	0.5	-	-	-
i-14:0	1.2	-	-	-	-	-	-	-	0.5	4.8	-
n-14:0	5.5	3.5	11	9.5	2.2	1.5	2	2.3	-	-	0.7
n-14:1ω7c	-	-	-	-	-	-	-	-	-	-	-
i-15:0	16.8	10.5	3.5	1.2	-	-	4.2	29.5	14.7	37.8	14.4
ai-15:0	-	-	-	-	-	-	-	46.7	24.6	18.2	1.7
i-15:1ω10c	-	-	-	-	-	-	-	-	-	-	7.5
n-15:1ω6	-	-	-	0.5	-	-	-	-	-	-	2.1
n-15:1ω8	-	-	-	-	0.8	0.7	-	-	-	-	-
n-15:0	2.7	2.8	-	-	3.1	1.6	0.8	3.7	-	1	10.2
i-16:0	-	-	-	-	2.2	-	-	2.6	4.2	6.2	7.5
i-16:1ω6	-	-	1.2	2.7	-	-	-	-	-	1.5	0.5
3-OH-16:0	-	-	-	-	-	-	-	-	-	-	1.5
3-OH-i-16:0	-	-	-	-	-	-	-	-	-	-	14.5
n-16:1ω11	-	-	2.5	1.4	-	-	-	-	10.5	2.3	-
n-16:1ω7	19.6	25.6	36.7	30.5	30.5	44.4	22.3	2.7	2.3	10.5	15.6
n-16:0	17.5	12.8	20.2	29.5	21.5	22.2	21.4	2.5	5.7	1.8	3.2
i-17:0	1.2	-	-	-	-	1.2	-	1.2	28.8	1.5	0.3
ai-17:0	-	-	-	-	-	-	-	5.8	3.9	3.6	-
i-17:1ω9c	-	-	-	-	-	-	-	-	-	2.5	7.1
ai-17:1ω9c	-	-	-	-	-	-	-	-	-	5.3	0.8
3-OH-i-17:0	-	-	-	-	-	-	-	-	-	-	11.5
n-17:1ω8	3.1	5.6	-	-	12.5	7.7	-	-	-	-	-
n-17:0	1.2	2.3	-	-	-	2.2	1.1	0.5		0.6	-
n-18:1ω9c	2.5	2.1	-	-	-	14.1	-	-	-	-	-
n-18:1ω7c	-	9.5	6.7	8.9	18.3		21.2	-	2.2	-	-
n-18:0	0.3	1.6	0.8	0.5	2.1	0.5	11.2	-	1.5	-	-
n-20:5ω3	5.6	7.5	5.8	1.2	-	-	-	-	-	-	-
Others	1	0.9	1	1.4	1.1	1.2	1	1.2	1.1	0.8	0.9
Σ TFA	100	100	100	100	100	100	100	100	100	100	100
Σ SCFA	33.9	28.8	42.6	52.2	34.6	30.7	51.3	9.5	7.2	3.4	14.1
Σ BCFA	34.3	20	3.5	1.2	2.2	1.2	4.2	86.6	76.7	73.7	35.4
Σ MUFA	25.2	42.8	44.6	42.6	62.1	66.9	43.5	2.7	4.5	19.8	33.6
Σ PUFA	5.6	7.5	5.8	1.2	-	-	-	-	-	-	-
ACL[a]	15.2	15.8	15.5	15.2	16	16.1	15.8	15	15.8	15.3	15.7

[a]Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

Table 3.5 Major FAs after whole cell hydrolysis (% of TFAs) in the bacteria associated Caribbean marine sponge (*Agelas clathrodes*)

Fatty acids	*Algibacter*	*Labrenzia*	*Granulo-sicoccus*	*Psychro-bacter*	*Rhodococcus*		*Demequina*	*Aerococcus*
	CMS186	CMS163	CMS377	CMS161	CMS166	CMS164	CMS382	CMS378
3-OH-10:0	-	-	-	1.8	-	-	-	-
3-OH-12:0	-	-	-	2.8	-	-	-	-
n-12:0	0.6	-	1	2.6	1.5	1.1	-	1.1
n-13:0	-	-	2.5	-	1.5	2.1	-	-
i-14:0	0.4	-		-	-	-	1.2	-
n-14:0	1.1	1.1	0.7	-	8.2	6.9	1.6	4.5
3-OH-14:0		3.2		-	-	-		-
i-15:0	16.7	-	-	-	-	-	3.5	-
ai-15:0	6.8	-	-	-	-	-	44.2	-
i-15:1ω10c	14.2	-	-	3.8	-	-	1.5	-
ai-15:1	-	-	-	-	-	-	11.3	-
n-15:1ω6	12.7	-	-	-	-	-		-
2-OH-i-15:0	-	-	0.8	3.3	11.7	15.5		-
n-15:0	25.3	-	-	-	8.4	6.7	4.3	3.2
i-16:0	2.1	-	-	-	-	-	5.7	
3-OH-16:0	-	1.6	-	-	-	-	-	-
i-16:1ω6	0.8	-	-	1.5	-	-	-	
n-16:1ω9c	-	-	-	1.3	1.4	4.5	-	13.7
n-16:1ω7	2.3	0.7	29.7	6.5	-	-	-	5.8
n-16:0	0.8	2.2	16.7	2.4	26.7	25.5	11.4	19.5
i-17:0	-	-	-	-	-	-	0.8	-
ai-17:0	-	-	-	-	-	-	12.5	-
i-17:1ω9c	1.2	-	-	-	-	-	-	-
ai-17:1ω9c	0.6	-	-	-	-	-	-	-
3-OH-i-17:0	9.6		1.6	-	-	-	-	-
n-17:1ω6	2.1	-	-	-	-	-	-	-
n-17:1ω8		-	-	11.2	4.5	1.7	-	-
n-17:0	0.7	-	0.6		0.6	-	0.7	-
n-18:1ω9c	0.6	3.1	3.2	57.8	6.6	6.5	-	16.6
n-18:1ω7c	-	68.7	40.5	-	-	-	-	3.5
10-OH-18:0	-	-	-	-	-	-	-	0.5
n-18:0	0.2	5.9	1.6	1.6	3.8		-	3.2
10-Met-18:0	-	-	-	-	12.4	20.3	-	-
11-Met-18:6t	-	3.4	-	-	-	-	-	-
n-19:1ω9c	-	-	-	2.5	-	-	-	-
n-19:0 cyc 9,10	-	-	-	-	7.2	2.8	-	15
n-20:1ω7c	-	8.6	-	-	-	-	-	12.5
n-20:0	-	-	-	-	4.7	5.4	-	-
Others	1.2	1.5	1.1	0.9	0.8	1	1.3	0.9
Σ TFA	100	100	100	100	100	100	100	100
Σ SCFA	28.7	14	23.1	11.2	75	70.8	18	47
Σ BCFA	35.6	-	2.4	3.3	11.7	15.5	67.9	-
Σ MUFA	34.5	84.5	73.4	84.6	12.5	12.7	12.8	52.1
ACL[a]	15.2	17.6	16.6	16.8	16.3	16.2	15.2	17.1

[a]Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; TFA, total fatty acids; and (–), not detectable.

Strains NSP523 and NSP561 exhibited the highest homology (98.7-99.6 % 16S rRNA gene identity) to type strains *Shewanella pealeana* and *Sh. piezotolerans* respectively. The predominant cellular fatty acids of these *Shewanellaceae* strains were i-13:0, i-15:0, n-16:1ω7 and n-16:0 acids (10-20 %), with more or less n-12:0, n-14:0, n-17:1ω8 and n-

20:5ω3 acids (Table 3.4). The proportion of EPA varied inversely with species of these strains as reported previously (Leonardo et al., 1999; Xiao et al., 2007).

Strain NSP500 exhibited the highest homology (95.0 % 16S rRNA gene identity) to the type strain *Gilvimarinus chinensis*. The main cellular fatty acids of species *Gilvimarinus* were n-12:0, n-16:1ω7, n-16:0, n-18:1ω7c and n-18:0 acids (8-22 %), which are similar to those of *Gilvimarinus* species (Du et al., 2009), mainly composed of SCFAs and MUFAs, with ACL of 15.8 (Table 3.4).

Strain CMS377 showed the highest similarity (99.1 % 16S rRNA gene identity) with *Granulosicoccus coccoides*. The main cellular fatty acids are n-16:1ω7, n-16:0 and n-18:1ω7c (17-40 %) (Table 3.5), and with ACL of 16.6, as was described on this genus (Kurilenko et al., 2010).

Strain CMS161 homology to *Psychrobacter nivimaris* (99.8 % 16S rRNA gene identity), with predominating fatty acid component of n-18:1ω9c (57.8 %), and less of i-15:1ω10c, 2-OH-i-15:0, n-16:1ω7 and n-17:1ω8 (4-11 %). This fatty acid profile was similar to those of other *Psychrobacter* species shown previously, although there were differences in the proportions of some fatty acids that might have been caused by different cultivation conditions (Yoon et al., 2005a; Yoon et al., 2005b). The fatty acids were predominated by MUFAs (85 %), with ACL of 16.8 (Table 3.5).

3.4.3.2 Alpha-proteobacteria

Strains CMS163 showed the highest 16S rRNA gene sequence similarities (99.4 %) to the type strain *Labrenzia alba*, with fatty acid n-18:1ω7c as the main component (68 %), and minor amount of others, namely 3-OH-14:0, n-16:0, n-18:1ω9c, n-18:0, 11-methyl-18:6t and n-20:1ω7c acids. Fatty acids dominated by n-18:1ω7c is consistent for virtually all members of the *Alpha-proteobacteria*, a fact usually missed in the majority of species descriptions relating to members of this major evolutionary group (Biebl et al., 2007). The fatty acids were dominated by MUFAs (85 %), with ACL of 17.6 (Table 3.5).

3.4.3.3 Cytophaga–Flavobacterium–Bacteroides (CFB) bacteria

Strain NSP516 and CMS186 showed the highest homology (98.7-99.5 % 16S rRNA gene identity) to *Cellulophaga baltica* and *Algibacter lectus*. The predominant cellular fatty acids of strain NSP516 were branched-chain saturated and unsaturated fatty acids and straight-chain saturated and monounsaturated fatty acids, namely i-15:0, i-15:1ω10c, n-15:0, i-16:0, 3-OH-i-16:0, n-16:1ω7c, i-17:1ω7c and 3-OH-i-17:0 acids (7-16 %) (Table 3.5). The results support the affiliation of the sea water isolates to the family *Flavobacteriaceae*, all members of which are characterised by the predominance of fatty acids n-15:0, i-15:0 and 3-OH-i-17:0, whereas The fatty acids i-15:1ω10c, i-15:0, ai-15:0, n-16:1ω7c, i-17:1ω7c and 3-OH-i-17:0 appear to be the most useful for the discrimination of *Cellulophaga* species from other members of the *Flavobacteriaceae* (Bowman, 2000). The novel isolates contained a high proportion of BCFAs (35 %) and MUFAs (35 %), with ACL of 15.2 and 15.7, which is a characteristic feature of the family.

3.4.3.4 Gram Positive

Strain NSP487 showed the highest homology (99.1 % 16S rRNA gene identity) to *Arthrobacter parietis*. The predominant fatty acids were mainly composed of i-15:0 and ai-15:0 acids (30-46 %), with less of n-14:0, n-15:0, i-16:0, n-16:1ω7, n-16:0, i-17:0 and ai-17:0 (1-5 %), as described on this genus (Heyrman et al., 2005). The fatty acids were predominated by BCFAs (87 %), with ACL of 15.

Strain NSP483 showed the highest homology (99.5 % 16S rRNA gene identity) to *Bacillus stratosphericus*, with i-15:0, ai-15:0, n-16:1ω11c, n-16:0 and i-17:0 acids as the predominant fatty acids, which supported the strain as a member of the genus *Bacillus*, as reported on similar genus (Shivaji et al., 2006). The fatty acids were mainly BCFAs (77 %), with ACL of 15.8.

Strain NSP480 showed the highest homology (99.5 % 16S rRNA gene identity) to *Paenisporosarcina quisquiliarum*, contained the fatty acids ai-15:0, i-15:0, n-16:1ω7c alcohol and ai-17:1ω9c as major components (5-38 %), similar to that reported previously (Krishnamurthi et al., 2009), although some minor fatty acids were present at different levels. This could be attributed to the different cultivation conditions and methods used for the analyses. The fatty acids were mainly BCFAs (74 %) and MUFAs (20 %), with ACL of 15.3.

Strain CMS166 and CMS164 homology (99.4-99.8 % 16S rRNA gene identity) to *Rhodococcus erythropolis* and *Rh. maanshanensis* respectively. The cellular fatty acid profile containing straight-chain saturated, unsaturated and 10-methyl-branched fatty acids, and had n-16:0 as the major fatty acid (27 %) and relatively high proportions of n-14:0, 2-OH-i-15:0, n-15:0, n-18:1ω9c, 10-Me-18:0, n-19:0 and n-20:0 (5-20 %). Branched saturated and hydroxy fatty acids were not detected. This is also similar to the composition of other species of *Rhodococcus* (Yoon et al., 2000), with predominant of SCFAs (70-75 %) and considerable of BUFAs and MUFAs, with ACL of 16.2-16.3.

Strain CMS382 exhibited the highest similarity (98.1 % 16S rRNA gene identity) to *Demequina aurantiaca*, contained straight-chain saturated, iso-methyl-branched, and anteiso-methyl-branched saturated fatty acids with ante ai-15:0 (4 4 %), ai-15:1 (11 %) and n-16:0 (11 %) representing the major fatty acids, and less of i-15:0, i-16:0 and i-17:0 acids (3-6 %). The fatty acid profiles were similar to other members of the genus *Demequina* (Yi et al., 2007), mainly with BCFAs (68 %) and considerable of SUFAs and MUFAs, with ACL of 15.2.

Strain CMS378 showed the highest similarity (99.6 % 16S rRNA gene identity) to *Aerococcus viridans*. The predominant fatty acids of the *Aerococci* were as follows: n-16:1ω9c, n-16:0, n-18:1ω9c, cyco-19:0 and n-20:1ω7 (12-20 %), with smaller amounts of n-14:0, n-15:0, n-16:1ω7, n-18:1ω7c and n-18:0 acids (3-6 %). The FA patterns were agreeable to those from similar genus described previously (Bosley et al., 1990), only constituted by SCFAs and MUFAs, with ACL of 17.1.

Strain CMS223 was identified as *Sporosarcina psychrophila*. The major fatty acids of this isolate were ai-15:0 (65 %), i-15:1ω10c and i-17:1ω9c, being similar to those present in recognized species of the genus *Sporosarcina* (Kwon et al., 2007), with mainly BCFAs (76 %) and ACL of 15.1 (Table 3.6).

Strain CMS172 exhibited the highest similarity (98.4 % 16S rRNA gene identity) with *Paenibacillus pinihumi*. The major cellular fatty acids were i-15:0 (14 %), ai-15:0 (50 %) and i-16:0 (15 %) with less of i-14:0, n-14:0, n-16:1ω7, n-16:0 and i-17:0 acids (2-7 %). The fatty acid content of strain CMS172 is similar to that of closely related type

strains of *Paenibacillus* (Kim et al., 2009), predominated by BCFAs (89 %) with ACL of 15.2 (Table 3.6).

Strain CMS176, CMS178, CMS179, CMS188, CMS190 and CMS197 showed the highest similarity (98.6-99.7 % 16S rRNA gene identity) with *Brevibacterium frigoritolerans*, *Bacillus hwajinpoensis*, *Bacillus licheniformis*, *Halobacillus salinus*, *Oceanobacillus profundus* and *Micrococcus luteus* respectively. The fatty acid profile was dominated by branched fatty acids, mainly by ai-15:0 (36-65 %) and less of i-14:0, i-15:0, i-16:0, n-16:0 and ai-17:0. The relative proportions of the predominant fatty acids shared somewhat similar to their type strains of the genus (Yoon et al., 2003; Yoon et al., 2004; Kim et al., 2007b; Kati et al., 2010), mainly by BCFAs (80-97 %), with more of less of SCFAs and MUFAs, and ACL of 15.2-15.5 (Table 3.6).

Table 3.6 Major FAs after whole cell hydrolysis (% of total FAs) in Caribbean marine sponge associated bacteria

Fatty acids	*Micrococcus*	*Sporosarcina*	*Paenibacillus*	*Oceano-bacillus*	*Halobacillus*	*B. hwajinpoensis*	*Brevibacterium*	*B.licheniformis*
	CMS197	CMS223	CMS172	CMS190	CMS188	CMS178	CMS176	CMS179
n-10:0	-	-	0.9	-	-	-	-	-
n-13:0	-	0.9	-	-	-	-	-	-
i-14:0	3.7	3.5	3.5	5.8	8.9	8.7	1.2	12.1
n-14:0	3.8	1.2	2.1	1.1	-	0.5	-	0.7
i-15:0	6.5	6.8	13.6	5.9	16.7	4.4	11.5	12.5
ai-15:0	60.5	65.3	49.5	57.8	35.8	50.2	52.5	28.7
2-OH-15:0	-	-	-	0.6	-	-	-	-
n-15:0	-	0.6	-	0.5	1.6	2.2	2.3	1.8
i-16:0	3.5	-	15.2	7.2	17	17.9	3.2	13.5
3-OH-i-16:0	1.3	-	-	-	-	-	-	-
n-16:1ω11c	-	1.9	0.3	0.3	-	-	-	-
n-16:1ω7	-	2.5	-	0.5	5.5	3	-	-
n-16:0	3.7	1.3	6.5	9.7	1.7	-	-	7.8
i-17:0	3.5	0.2	2.8	0.8	3.5	0.7	1.1	8.5
ai-17:0	0.9		4.5	8.8	8.6	10.5	27.5	13.2
i-17:1ω9c	11.5	13.8	-	-	-	0.7	-	-
n-17:0	-		0.4	-	-	-	-	-
n-18:1ω9c	-	0.5	-	-	-	-	-	-
n-18:0	-	0.2	-	-	-	-	-	-
Others	1.1	1.3	0.7	1	0.7	1.2	0.7	1.2
Σ TFA	100	100	100	100	100	100	100	100
Σ SCFA	7.5	4.2	9.9	11.9	3.3	2.7	2.3	10.3
Σ BCFA	79.9	75.8	89.1	86.3	90.5	92.4	97	88.5
Σ MUFA	11.5	18.7	0.3	0.8	5.5	3.7	-	-
ACL [a]	15.2	15.1	15.2	15.2	15.3	15.2	15.5	15.3

[a] Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; TFA, total fatty acids; and (–), not detectable.

3.4.3.5 Phylogenetic resolution of FA patterns

In this study, all the bacterial cell were obtained from 15 ºC marine broth cultures for FAs patterns analysis, and were subjected to principal coordinates analysis (PCO), which allowed separation of isolates representing different phyla. For examples, in the case of FAs from North-sea sponge-associated bacteria, different groups, such as FAs of *Gamma-Proteobacteria* (I), FAs of CFB group bacteria (II) and FAs of *Firmicutes* (III) (Figure 3.3). The result of the ordination pattern was agreeable to the data clustered by using hierarchical UPGMA analysis, which clearly separated the 20 isolated bacteria associated with *Halichondria panicea* (Figure 3.4), and grouped them into the three phyla as above. Similarly, the analysis of FAs from bacteria associated with Caribbean sponge (*Agelas clathrodes*) showing that four FA groups were divided: *Gamma-Proteobacteria* (I), CFB group bacteria (II), *Firmicutes* (III) and *Alpha-Proteobacteria* (IV) (Figure 3.5). All 16 bacterial strains associated with *Agelas clathrodes* were hierarchically separated into four phyla analyzed by UPGMA (Figure 3.6).

PCO analysis on FA composition of all 38 strains from *Agelas clathrodes* and *Halichondria panacea* associated bacteria exhibited the separation by 4 groups: *Gamma-Proteobacteria* (I), *CFB Group bacteria* (II), *Firmicutes* (III) and *Alpha-Proteobacteria* (IV) (Figure 3.7), although, the Bray Curtis distance of each bacterial FA changed due to larger data set analysis. However, all these FA compositions were hierarchically separated into four phyla analyzed by UPGMA (Figure 3.8), as presented above (Figures 3.4 and 3.6). Both the distribution and the percentage contribution of each fatty acid to the total fatty acid across taxa at species level were plotted shown in Figure 3.9. The trends and relationships of fatty acid composition of each species within *Gamma-Proteobacteria*, *CFB Group*, *Gram*$^+$ (*Firmicutes*) and *Alpha-Proteobacteria* respectively could be presented using this method. Within *Gamma-Proteobacteria* (I), wide distribution of fatty acid composition was observed, ranging from short-chain FAs (e.g. n-12:0 12:0 3-OH and n-14:0); middle-chain FAs (e.g. n-16:1ω9, n-16:1ω7, n-17:1ω8, n-17:0, n-18:1ω9c and n-18:1ω7c), to long-chain FAs (n-20:5ω3), in which EPA was found only in this group. For *CFB Group bacteria* (II), the predominant fatty acids are middle-chain FAs (e.g. i-15:0, i-15:1ω10c, n-15:1ω6, n-15:0, i-16:0 3-OH, n-16:1ω7, i-17:1ω9c and i-17:0 3-OH), in which most of the branched-chain FAs were analyzed. Similar to *CFB Group bacteria*, *Gram*$^+$ bacteria (*Firmicutes*) (III) mainly contained branched-chain FAs (e.g. i-14:0, i-15:0, ai-15:0, i-16:0, n-16:1ω11, n-16:1ω7,

n-16:0, i-17:0, ai-17:0 and i-17:1ω9c), and dominated by i-15:0 and ai-15:0. The fatty acids of *Alpha-Proteobacteria* (IV) were dominated by middle-chain FAs, such as n-16:1ω7, n-16:0, n-18:1ω7c, and contained less of long-chain FA (n-20:1ω7). Thus, n-16:1ω7 and n-16:0 of *Gamma-Proteobacteria*, i-15:0 and n-15:0 of *CFB Group*, i-15:0 and ai-15:0 of *Gram*$^{+}$ (*Firmicutes*), and n-18:1ω7c of *Alpha-Proteobacteria*, may be chose as fatty acid markers in each group respectively.

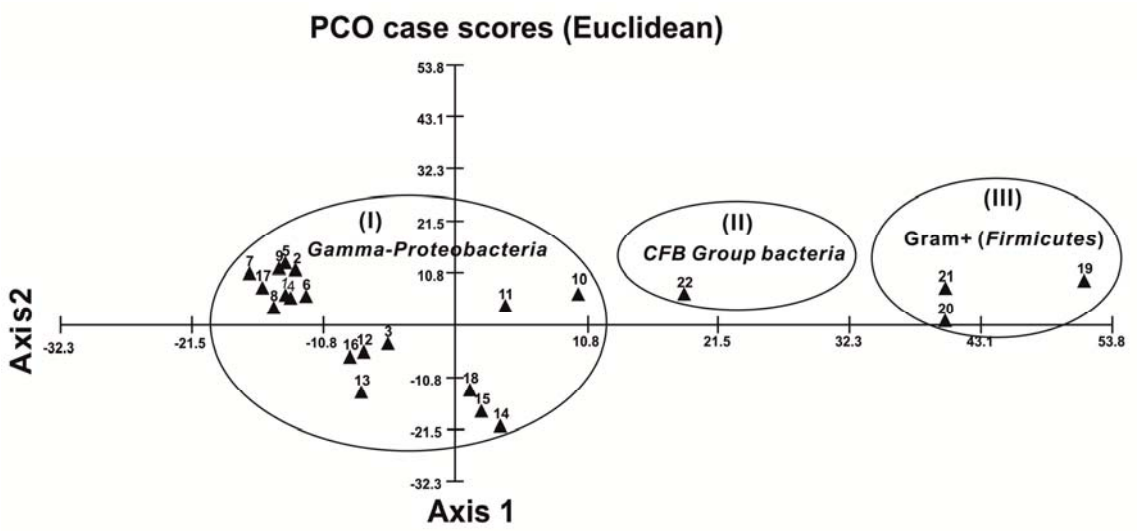

Figure 3.3 Principal coordinates analyses (PCO) of major FA abundance data from bacteria associated with North-sea sponge (*Halichondria panicea*) from Tables 3.3-3.4 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 3.4).

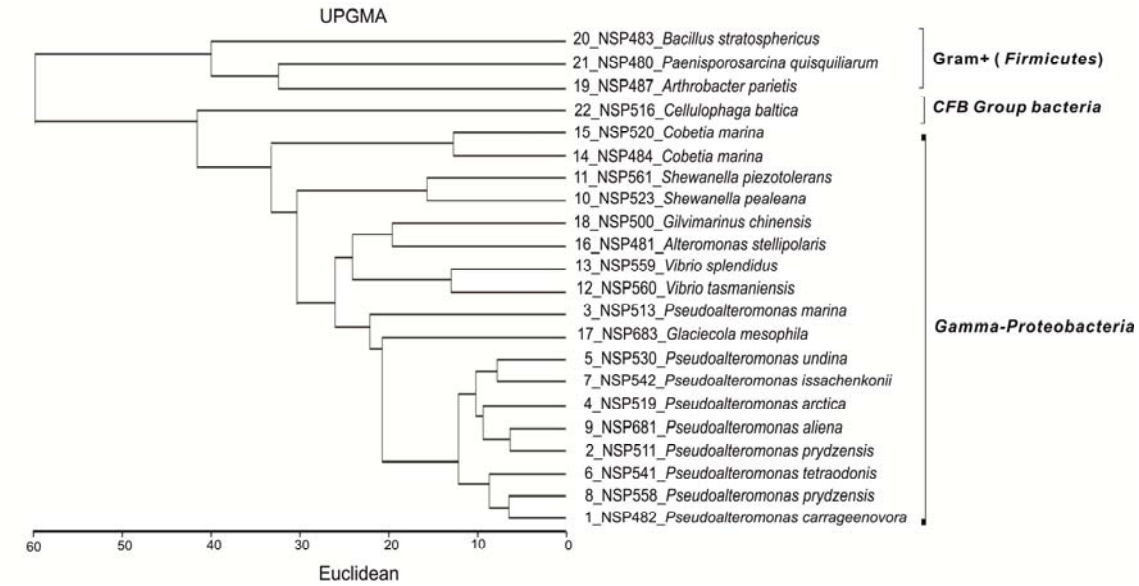

Figure 3.4 Similarity analysis based on whole cell fatty acid composition from bacterial species associated with North-sea sponge (*Halichondria panicea*). The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering

method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 3.3 and nearest type strains were indicated beside the isolates.

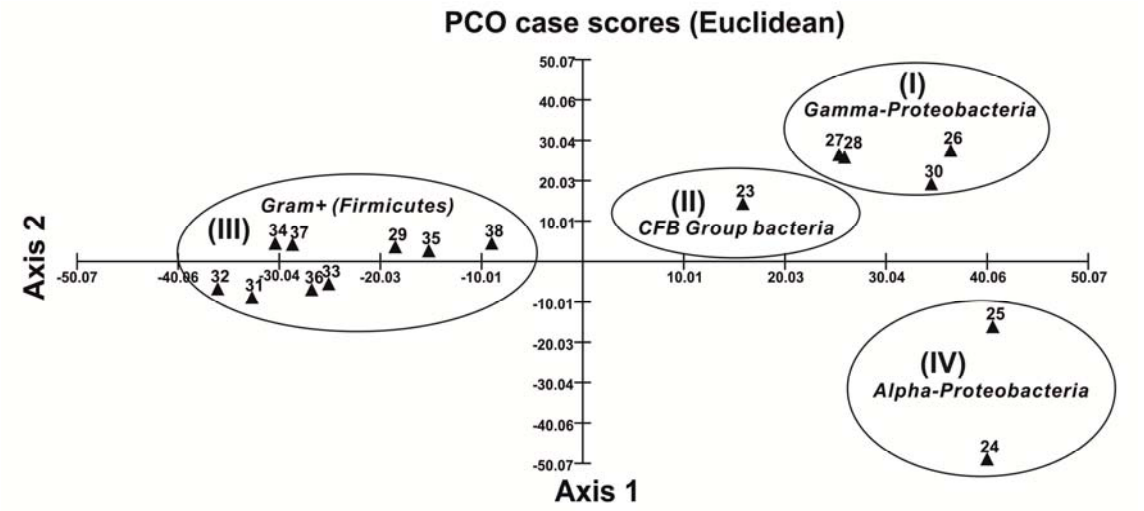

Figure 3.5 Principal coordinates analyses (PCO) of major species FA abundance data from bacteria associated with Caribbean marine sponge (*Agelas clathrodes*) from Tables 3.5-3.6 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 3.6)

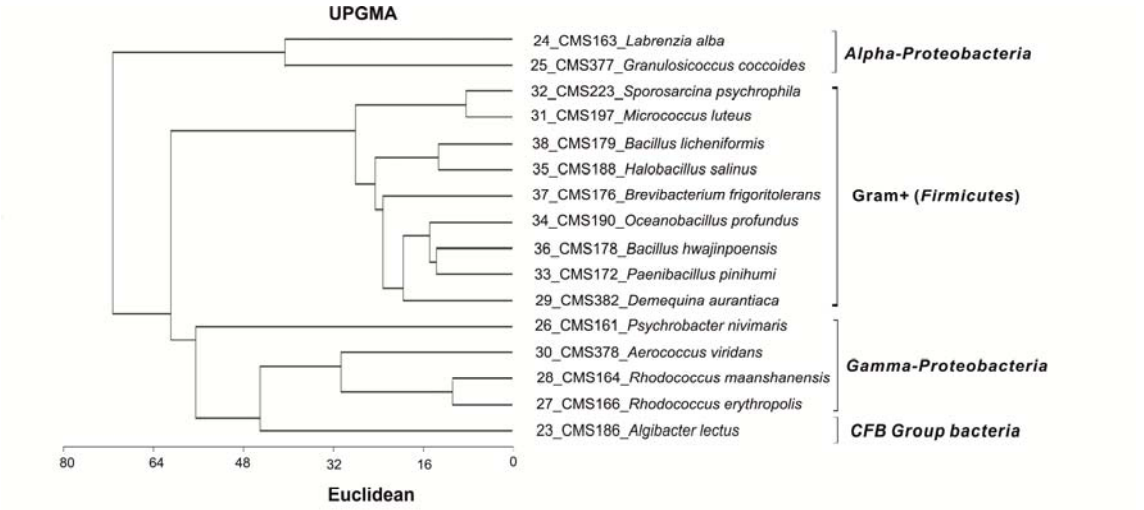

Figure 3.6 Similarity analysis based on whole cell fatty acid composition from bacterial species associated with Caribbean marine sponge (*Agelas clathrodes*). The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 3.5 and nearest type strains were indicated beside the isolates.

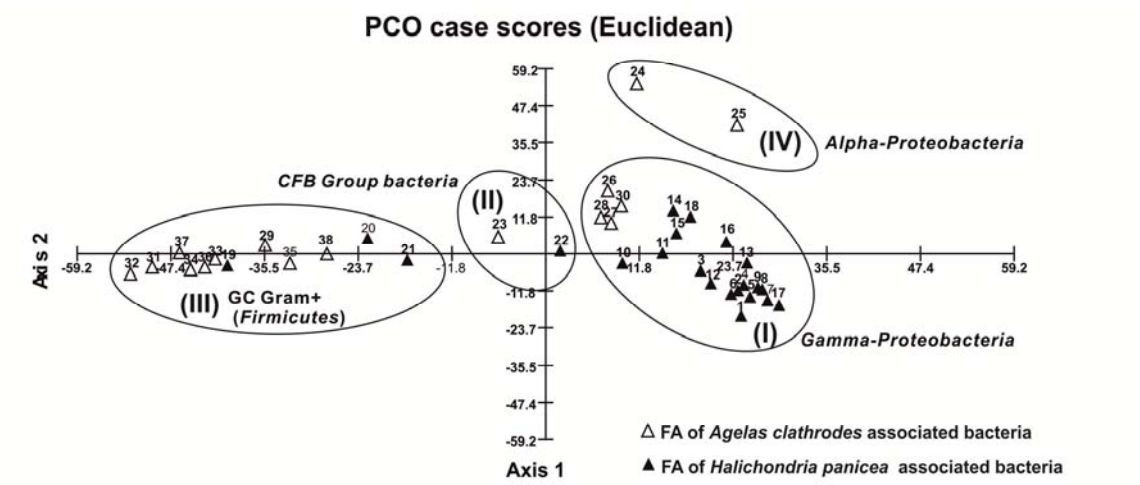

Figure 3.7 Principal coordinates analyses (PCO) of major species FA abundance data from bacteria associated with North-sea sponge (*Halichondria panicea*) and Caribbean marine sponge (*Agelas clathrodes*) from Tables 3.3-3.6 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 3.8)

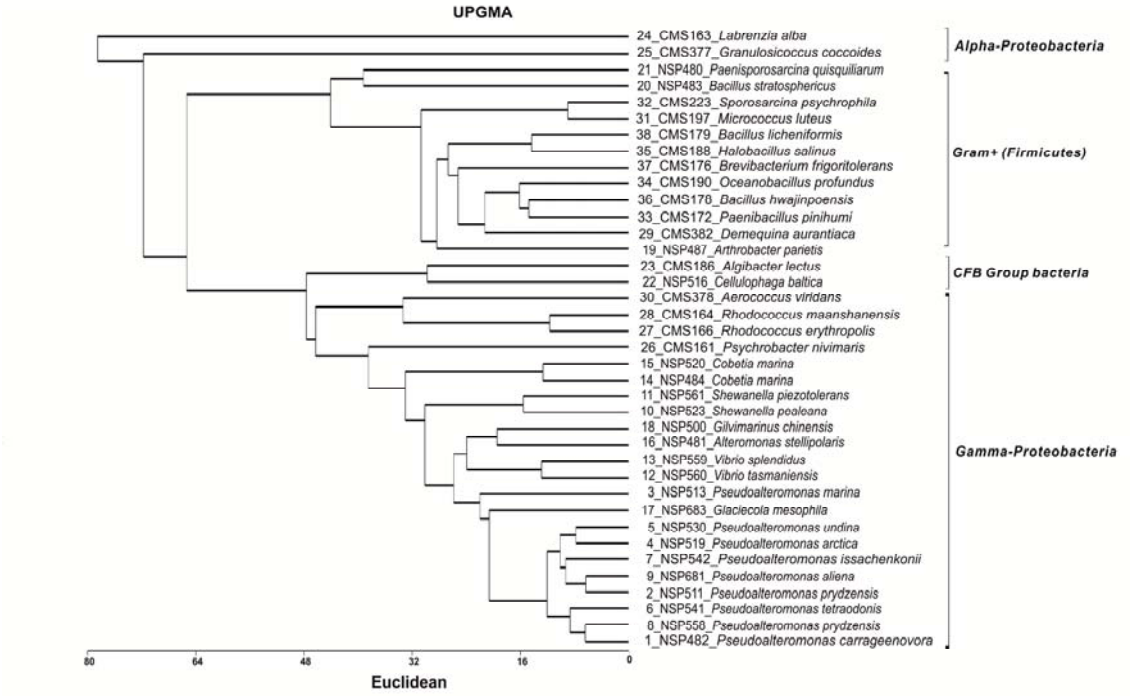

Figure 3.8 Similarity analysis based on whole cell fatty acid composition from bacterial species associated with North-sea sponge (*Halichondria panicea*) and Caribbean marine sponge (*Agelas clathrodes*). The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 3.7 and nearest type strains were indicated beside the isolates.

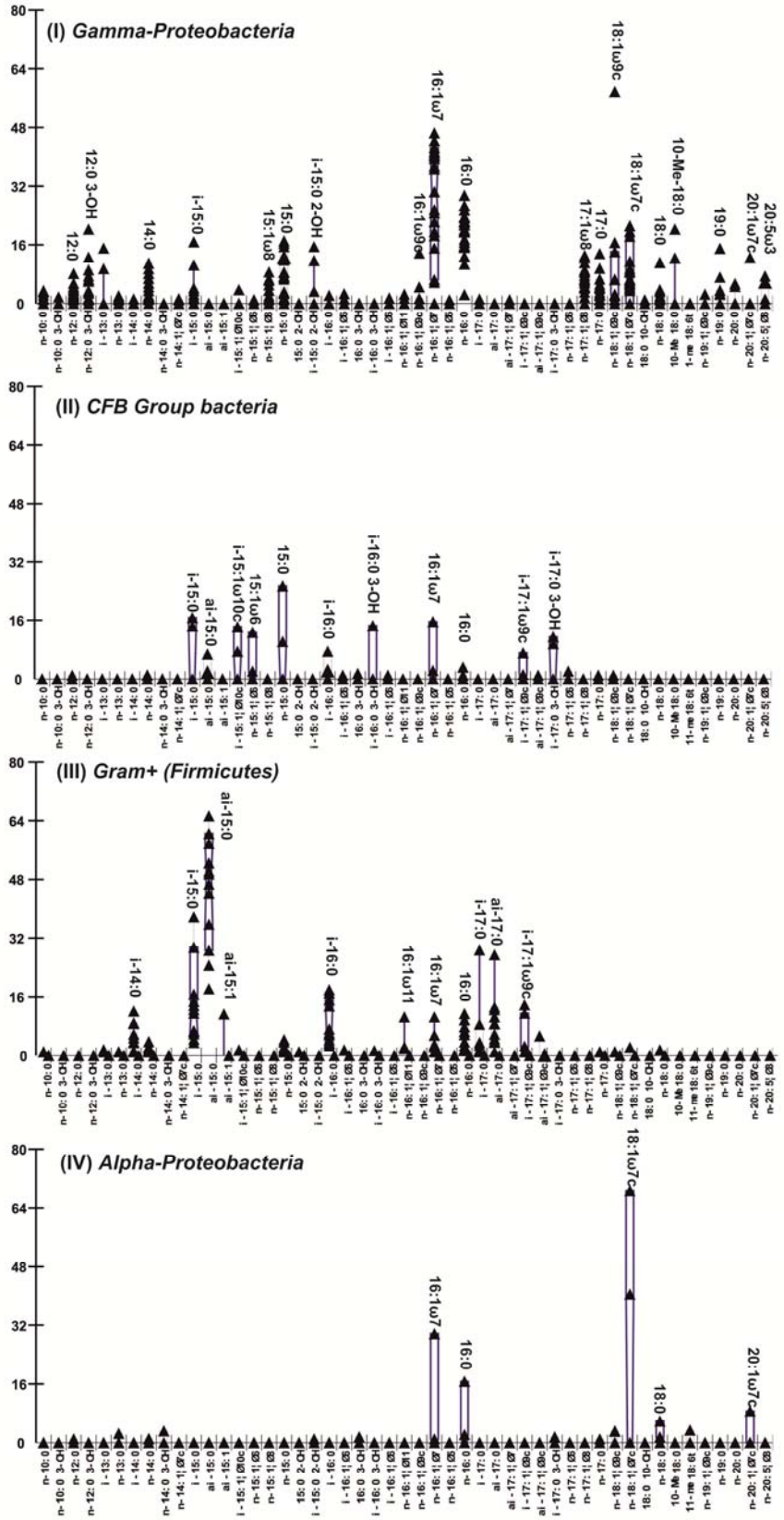

Figure 3.9 Box plots representing the distribution and variability in the percentage contribution of each fatty acid to the total fatty acids within *Gamma-Proteobacteria*, *CFB Group*, *Gram*$^{+}$ *(Firmicutes)* and *Alpha-Proteobacteria*. Groupings are as indicated in Figures 3.7 and 3.8. The triangles represent the presence of each fatty acid (Axis X) from different species analyzed, with percentage of TFA indicated by Axis Y.

3.5 Discussion

3.5.1 Phylogenetic diversity of sponge associated bacterial isolates

Sponges (phylum *Porifera*) are excellent models for the study of marine host-associated bacteria. For understanding the community and diversity of sponge-associated microorganisms, electron microscopic techniques were used to obtain morphological data (Manz et al., 2000; Usher et al., 2001); standard isolation and cultivation methods were employed to identified some sponge bacteria (Burja et al., 1999); due to the sponge-associated microorganisms are not easily cultivated and isolated (Lopez et al., 1999), culture-independent technique, such as 16S rRNA gene-Denaturing gradient gel electrophoresis (DGGE) fingerprinting has been used to monitor changes in a sponge-associated microbial community over time (Friedrich et al., 1999; Taylor et al., 2004) and transplantation (Thoms et al., 2003).

Phylogenetic diversity of North-sea marine sponge (*Halichondria panicea*) associated bacteria showed the abundant presence of *Gamma-Proteobacteria*, in accordance with the early culture-based studies of marine microbiology, approximately 95 % of bacterial isolates were found to be *Gram negative* (ZoBell, 1946). However, high proportion of gram-positive bacteria found in Caribbean marine sponge (*Agelas clathrodes*) as it has recently been underestimated (Jensen and W., 1994). Biochemical characterization of culturable sponge-associated microorganisms from *Ceratoporella nicholsoni* revealed that at least 78 % of these bacteria were members of the *Gamma-Proteobacteria* genera *Vibrio* and *Aeromonas* (Santavy et al., 1990). However, evidence for uniformity in sponge microbial communities between different oceans and host species were also presented by choosing sponges *Aplysina aerophoba* and *Theonella swinhoei* for construction of the bacterial 16S rRNA gene library (Hentschel et al., 2002). Therefore, the structure of microbial communities from the same sponge species or two different sponge species may be varies according to different analysis techniques employed.

Deep-sea microorganism, such as *Shewanella piezotolerans*, used to be identified as psychropiezophiles model strain (Xiao et al., 2007; Wang et al., 2008). Interestingly, similar species, such as strain NSP561 was isolated from North-sea sponge, and with the ability of EPA production under the atmospheric conditions. Furthermore, most of the nearest type strains of these sponge-associated bacteria were most marine origin, such as from sea water or marine algae.

3.5.2 Phylogenetic resolution of FA patterns

Fatty acids are one of the most important building blocks of cellular materials. In bacterial cells, fatty acids occur mainly in the cell membranes as the acyl constituents of phospholipid (Goldfine, 1984). It has become practical to use gas chromatography of whole cell fatty acid methyl esters to identify a wide range of organisms (Henis et al., 1966; Larsson et al., 1978; Athalye et al., 1985; Freese et al., 2008). In this study, the fatty acid composition of North-sea and tropical Caribbean sponge associated bacteria was demonstrated by employing multivariate data analyses methods, such as principal coordinates analysis (PCO), which has been used for analyzing the patterns of fatty-acid variables (Freese et al., 2008). The studies indicated that a taxonomic resolution on the basis of complete FA patterns is possible, as the cultivation conditions for all the bacteria cultures were consistent. When isolates belonging to different phyla were not sometimes dominated by the same FAs, or different isolates belonging to different phyla were sometimes dominated by the same major fatty acids, and unsuccessfully separation may be happened (Freese et al., 2008), as fatty acid compositions will be variable according to their culture conditions (e.g. temperature and medium) (Yumoto et al., 2004). For example, *Granulosicoccus* sp. CMS377 affiliated with the *Firmicutes* phylum based on phylogenetic analysis of 16S rRNA gene sequences, but it was clustered into *Alpha-proteobacteria* based on fatty acid composition. Whereas, *Psychrobacter* sp. CMS161 and *Aerococcus* sp. CMS378 belonged to the *Firmicutes* phylum were similar to *Gamma-Proteobacteria* based on fatty acid composition.

Most of *Gram Positives* including genus *Bacillus* in this study with branched-chain fatty acids as major constituents, especially ai-15:0, i-15:0, ai-17:0, i-17:0 and i-16:0. This branched-chain fatty acid family occurring in bacteria is very significant (Kaneda, 1977; Lechevalier, 1977), membranes with this family is controlled mainly by 12-methyltetradecanoic acid (anteiso-15:0) and 13-methyltetradecanoic acid (iso-15:0) and subsequently their membrane fluidity is modified (Kaneda, 1991). For example, fatty acids of strains *Vibrio* sp. NSP559 and *Shewanellas* sp. NSP561 were partly controlled by i-15:0, while most of the genus *Bacillus* (e.g. strains NSP483, CMS197, CMS188, CMS178 and CMS172) and *CFB Group* bacteria (e.g. strains NSP487, NSP516 and CMS186) were mainly manipulated by ai-15:0 and i-15:0. However, no branched-chain fatty acids were detected from *Alpha-proteobacteria* (strain CMS163) and all the *Pseudoalteromonas* strains. Most of these *Alpha-*, *Gamma-Proteobactri*a and *CFB*

Group bacteria, contained considerable amount of MUFAs or PUFAs, which may be required for their growth and membrane viscosity manipulation. The change of the degree of PUFAs may result in the modification of membrane fluidity by altering the lipid composition of the membrane (Melchiodr, 1982; Russell, 1990).

3.6 Conclusions

In conclusion, the function and physiology of sponge-associated microbes are increasingly important research topics, reflecting our current paucity of knowledge about many of the microbial associates of sponges. In this study, bacterial symbionts in sponges were isolated as pure cultures and phylogenetically identified on the basis of 16S rRNA gene sequences and complete FA patterns, both of which successfully separated different bacterial genus/species hierarchically for bacterial community structure study. Furthermore, North-sea sponge associated bacteria was found with dominant of *Gamma-Proteobacteria*, containing EPA producing bacteria, whereas Caribbean sponge associated bacteria was mainly Gram positives, with no PUFAs producing bacteria was found. The variance might be due to geographic temperatures and difference of phytoplankton hosts, as well as the metabolism difference between *Gram Negatives* and *Gram Positives*. It will be interesting to examine how the sponge associated bacteria cooperate with their host during their metabolism for PUFAs production and/or by investigating their PUFA biosynthesis pathway, which so far remains a black box, should also be aided by our ongoing experiments including genomic analysis. Models of sponge-associated bacterial structure and a better understanding of their fatty acid compositions will help us better predict the biogeographic variability of bacterial communities among different sponges from different latitudes, due to which certain marine food web/chain characterizations may be speculated.

Chapter 4. Marine algae and algae associated bacteria from the North Sea as sources of omega-3 fatty acids

4.1 Abstract

Macro/micro algae provide valuable nutrients due to the high content of omega-3 fatty acids, such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), therefore bacteria associated with their surface might also be a good source for obtaining omega-3 fatty acids. We collected different macro algal samples and cultured micro algae isolated from North Sea. These algal samples were identified as brown algae (*Ascophyllum nodosum*, *Egregia menziesii* and *Fucus serratus*), green algae (*Codium fragile*), red algae (*Palmaria palmata*), green micro algae (*Tetraselmis* sp.) and diatoms (*Phaeodactylum tricomutum* and *Bacillariophyta*) based on 18S rRNA gene sequence analysis. Fatty acids analysis showed that these algal samples are most with high content of EPA (10-43 % of total fatty acids), and only some macro algae are with trace level of DHA. Bacterial communities associated with micro algae and macro algae were phylogenetically identified respectively based on 16S rRNA gene sequences, indicating that the abundance of *Gamma-Proteobacteria* (90 %). Macro algae associated bacteria responsible for EPA or DHA production were mainly members of genera *Vibrio* and *Colwellia*, whereas genera *Vibrio*, *Photobacterium* and *Shewanella* were presented in the micro algal culture as bacterial EPA producers. Principal coordinates analysis (PCO) on the algal and bacterial whole cell fatty acid compositions, indicating that taxonomic resolution on the basis of complete FA patterns is possible.

4.2 Introduction

Polyunsaturated fatty acids (PUFAs), in particular omega-3 fatty acids such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), have received much attention for their remarkably wide range of physiological (Fontani et al., 2005) and clinical effects (Siddiqui et al., 2004; MacLean et al., 2006). For instance, DHA is essential for the proper visual and neurological development of infants because of its roles as structural lipid component (Nettleton, 1993), while EPA plays a role in cardiovascular health, can reduce inflammation and may have a role in mental disorders (Kelly, 1991). Currently, the main sources of dietary EPA and DHA are marine fish.

However, the fish population of some commercially targeted species is declining and extracted products from fish oil may have an undesirable fishy odour and is expensive (Arts et al., 2001; Garcia and Rosenberg, 2010). All these factors have led to an interest in alternative sources of EPA and DHA. These sources include fungi, marine algae, diatoms, and some bacteria.

Seaweeds are of ecological importance in their role as one of the primary producers in the marine food chain. They provide a good source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins and minerals (Faulkner, 1984; Faulkner, 1986). 60 % of these metabolites are reported to be terpenes, 20 % are fatty acids and 10 % are mixed biosynthesis (Van Alstyne and Paul, 1988). Extracts from edible/common marine algae, such as *Undaria pinnatifida*, *Laminaria* and *Sargassum* species were found to have potential antitumor promoting properties (Ohigashi et al., 1992). However, there may be problems with heavy metal contamination in seaweeds due to the natural environment or industrial discharges (Giusti, 2001; Caliceti et al., 2002). Thus, new generation technologies in the area of seaweed cultivation and utilization may be developed by using *in vitro* cell culture technology (Reddy et al., 2008). Nevertheless, seaweed associated microbes may be an alternative source for obtaining similar bioactive compounds.

Marine microalgae have several advantages over conventional (energy) crops for converting carbon dioxide into biofuel. They are the primary producers of omega-3 PUFAs and fish usually obtain EPA via bioaccumulation in the food chain. Much effort has been devoted to developing a commercially feasible technology to produce EPA directly form microalgae based on photoautotrophic growth (Sa´nchez Miro´n et al., 2002; Molina Grima et al., 2003). Unfortunately, photoautotrophic growth is often limited by insufficiency of light caused by mutual shading of cells (Chen, 1996). Consequently, the EPA yield and productivity of photosynthetic systems are low (Barclay et al., 1994). Therefore, microbial heterotrophic growth process, such as bacteria, is desirable for enhancing EPA production.

Microbes adapt to their habitat, react to external pressure and, in turn, interact with biogeochemical cycles of carbon and nutrients, especially in aquatic environments (Azam and Worden, 2004). Some of the metabolites produced by marine bacteria can be used for drug development, especially those bacteria associated with living surfaces are

rich sources of bioactive metabolites (Fenical, 1993; Grossart et al., 2004). For example, bacteria living in complex associations with animals are often proposed to be the real producers of 'invertebrate' metabolites (Proksch et al., 2002). Marine epibiotic bacteria growing on the surface of seaweeds and other invertebrates live in a highly competitive environment where space and access to nutrients are limited. Bioactive compound production in these bacteria could be attributed to the competition among them for space and nutrition (Burgess et al., 1999). Studies have been focused on the antibacterial activity of the seaweed associated bacteria (Lemos et al., 1986; Steinberg and de Nys, 2002; Bull, 2004; Kanagasabhapathy et al., 2006; Rungprom et al., 2008), algal toxins production from microalgae associated bacteria (Crocia et al., 2006), or toxicity modulation between bacteria and diatoms (Bates et al., 2004). However, the comparison on fatty acid compositions between algae and their associated bacteria has not been investigated.

In the present investigation, we collected algal samples (including seaweeds, micro algae and diatoms) from the coast of North Sea and cultured micro algae indoors and outdoors in the laboratory. Phylogenetic taxonomy study was conducted on these marine planktons and their associated bacteria, on the basis of 16S rRNA or 18S rRNA gene sequences. The analysis of algal and bacterial community structure based on complete FA patterns was also conducted.

4.3 Materials and Methods

4.3.1 Sample collection

The macro algae (seaweed) *Ascophyllum nodosum* (SW1), *Palmaria palmata* (SW2), *Fucus serratus* (SW3), *Egregia menziesii* (SW4), *Codium fragile* (SW5) and micro algae (green micro algae and diatoms) were collected at low tide in the coastal area off the Dove Marine Laboratory, Cullercoats, North Sea (54°54'25"N, 1°21'35"W). Collected seaweed samples (100 g) were transferred in zip-lock bags on ice. Portions of these specimens were thoroughly washed three times with autoclaved seawater passed through a 0.2 μm-pore-size filter to remove loosely attached bacteria. Bacterial samples were taken from the surface seaweeds with a sterile cotton swab. Once the sample had been taken, the macro-algal surface was rinsed again with AFSW, dried with sterile paper, and stored in a sterile flask.

Collected sea water contained micro algae/diatoms were cultured in sea water supplemented with F/2 solution (Sigma). The algae were subjected to purification by serial dilution followed by plating. The microscopic observations of the isolated algae revealed its colonial existence. The individual colonies were isolated and inoculated into F/2 medium and incubated at 15 ± 1 ºC under natural light-night period in the UK, for biomass accumulation. The purity of the culture was ensured by repeated plating and by regular observation under microscope. The stock culture was maintained on F/2 medium. Bacterial colonies were isolated during purification of the micro algae culture on the agar plates.

4.3.2 Isolation of macro/micro algae associated bacteria

A sterile swab was used to rub the seaweed surface and inoculating the removed bacteria on marine agar plates (Difco). The inoculated plates were incubated for 3 days at 15 °C until colonies were observed. Bacterial colonies were isolated during purification of the micro algae culture on the agar plates. Colonies with different morphologies on this medium were selected and purified through the third generation. Gram stain was used for initial bacterium classification; after staining the bacteria were stored in glycerol broth 15 % v/v (-80°C). Altogether 74 isolates were obtained from the surfaces of a range of marine seaweed and micro algae. All obtained isolates were further scored for fatty acids production.

4.3.3 Strain growth as methods mentioned in Chapter 3

4.3.4 Fatty acid analysis as methods mentioned in Chapter 2

4.3.5 Preparation of genomic DNA and 18S/16S rRNA gene analysis

Algal genomic DNA was extracted from the strains using the PureLink™ Genomic Plant DNA Purification Kit (Invitrogen Ltd, Paisley, U.K) and used as templates for PCR amplification of the 18S rRNA gene fragments based on the published methods (Goff and Coleman, 1988). Gene fragments sequencing and phylogenetic analysis were conducted as methods mentioned in Chapter 2. The nucleotide sequences of 18S/16S rRNA gene have been deposited in EMBL under the accession numbers: FR744744, FR744750 and FR744763 (micro algae); FR744764- FR744768 (macro algae);

FR744822- FR744859 (macro algae associated bacteria) and FR744860- FR744896 (micro algae associated bacteria).

4.3.6 Multivariate analyses as methods mentioned in Chapter 2

Relative abundances of major FAs after whole cell hydrolysis (Tables 4.3–4.7) were used for statistical analysis as methods mentioned in Chapter 2.

4.4 Results

4.4.1 Phylogenetic groups and identification of the macro/micro algal strains

Based on 18S rRNA gene sequence analysis, 7 algal strains were divided into five different groups: brown algae (*Ascophyllum nodosum*, *Egregia menziesii* and *Fucus serratus*), green algae (*Codium fragile*), red algae (*Palmaria palmata*), green micro algae (*Tetrselmis*) and diatoms (*Phaeodactylum tricomutum* and *Bacillariophyta*) (Figure 4.1), which was consistent with the studies by morphology or microscopy. Phylogenetically, macro algae *Palmaria palmate* SW2 and *Codium platylobium* SW5 were closer to those microalgal species, such as *Tetraselmis* sp. M1.

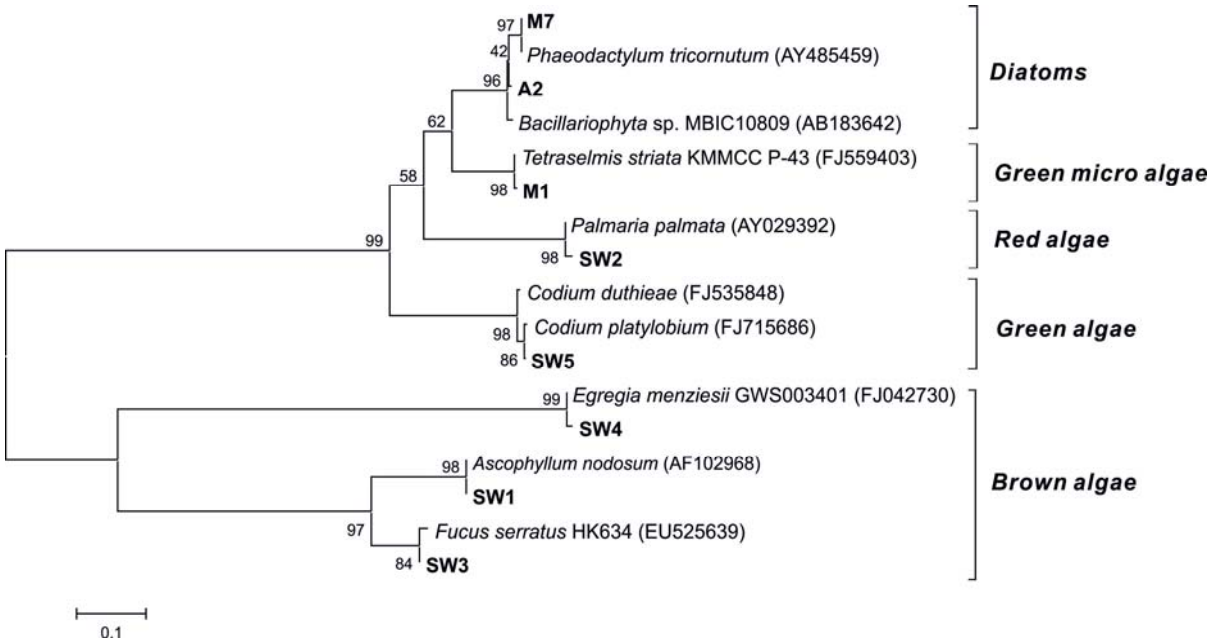

Figure 4.1 Neighbour-joining distance tree based on the nearly complete and aligned 18S rRNA gene sequences of 5 macro algae and 3 micro algae in this study and their nearest strains in the database. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.1 nucleotides substitution per site.

4.4.2 Phylogenetic groups and identification of the macro algae associated bacteria

From the five species of marine macro algae collected, a total of 37 morphologically distinct heterotrophic associated bacterial colonies were isolated and identified on the base of 16S rRNA gene sequence analysis. Of these strains, most of the epibiotic bacteria were non-pigmented, 24 % (9 strains) were isolated from *Ascophyllum nodosum* (SW1), 27 % (10) from *Palmaria palmata* (SW2), 19 % (7) from *Fucus serratus* (SW3), 14 % (5) from *Egregia menziesii* (SW4) and 16 % (6) from *Codium fragile* (SW5) (Table 4.1).

NCBI nucleotide BLAST searches using the partial 16S rRNA gene sequences of these 37 strains revealed that *Gamma-Proteobacteria* was the most abundant division of the bacterial populations (83.5 %), followed by CFB group bacteria (13.5 %) and high GC Gram positive (3 %). *Gamma-Proteobacteria* was phylogenetic affiliation with 6 members of the *Vibrionaceae*, *Pseudoalteromonadaceae*, *Oceanospirillaceae*, *Halomonadaceae*, *Alteromonadaceae* and *Colwelliaceae*, including 17 taxonomic units of *Vibrio lentus*, *V. splendidus*, *V. tasmaniensis*, *V. splendidus*, *V. kanaloae*, *Aliivibrio fischeri*, *Pseudoalteromonas elyakovii*, *Ps. nigrifaciens*, *Marinomonas balearica*, *M. ushuaiensis*, *M. arenicola*, *Cobetia marina*, *C. crustatorum*, *Amphritea balenae*, *Glaciecola psychrophila*, *G. mesophila* and *Colwellia psychrerythraea*. CFB group bacteria contained 3 taxonomic units of *Polaribacter butkevichii*, *Flavobacterium fluvii* and *Cellulophaga fucicola*. And only one strain identified as *Gordonia terrae* belonged to Gram positive (Figure 4.2).

Table 4.1 List of bacterial strains associated with macro algae

Phylogenetic group (family)	Representative isolates	No. of strains	Nearest type in the GenBank (accession nomber)	Similarity (%)	Source of nearest type strain
Gamma-Proteobacteria					
Vibrionaceae	SW1-5	1	*Vibrio lentus* 4OM4^T (AJ278881)	99.5	Mediterranean oysters
	SW2-5	1	*Vibrio lentus* 4OM4^T (AJ278881)	99.4	Mediterranean oysters
	SW3-2	1	*Vibrio splendidus* ATCC 33125^T (X74724)	99.4	ATCC Collection
	SW3-5	1	*Vibrio tasmaniensis* LMG 21574^T (AJ514912)	99.5	Atlantic salmon
	SW2-3C1	1	*Vibrio splendidus* ATCC 33125^T (X74724)	99.5	ATCC Collection
	SW2-3C2	1	*Vibrio splendidus* ATCC 33125^T (X74724)	99.6	ATCC Collection
	SW3-8	1	*Vibrio splendidus* ATCC 33125^T (X74724)	99.5	ATCC Collection
	SW5-2	1	*Vibrio kanaloae* LMG 20539^T (AJ316193)	99.3	LMG Collection
	SW5-1	1	*Aliivibrio fischeri* ATCC 7744^T (X74702)	99.8	ATCC Collection
Pseudoalteromonadaceae	SW1-9	1	*Pseudoalteromonas elyakovii* KMM 162^T (AF082562)	99.9	Laminaria japonica
	SW2-3D; SW4-8	2	*Pseudoalteromonas nigrifaciens* NCIMB 8614^T (X82146)	99.9	ATCC Collection
	SW4-2	1	*Pseudoalteromonas elyakovii* KMM 162^T (AF082562)	99.7	Laminaria japonica
	SW4-3	1	*Pseudoalteromonas espejiana* NCIMB 2127^T (X82143)	99.4	ATCC Collection
	SW4-4	1	*Pseudoalteromonas espejiana* NCIMB 2127^T (X82143)	98.1	ATCC Collection
	SW5-6	1	*Pseudoalteromonas espejiana* NCIMB 2127^T (X82143)	99.2	ATCC Collection
Oceanospirillaceae	SW1-3	1	*Marinomonas balearica* IVIA-Po-185^T (EU188441)	98.7	Seagrass
	SW1-7	1	*Marinomonas ushuaiensis* U1^T (AJ627909)	97.3	Coastal sea water
	SW3-1	1	*Marinomonas arenicola* KMM 3893^T (AB467281)	99.5	Marine sediment
	SW3-3	1	*Marinomonas arenicola* KMM 3893^T (AB467281)	99.3	Marine sediment
	SW3-7; SW5-4	2	*Marinomonas arenicola* KMM 3893^T (AB467281)	99.7	Marine sediment
	SW2-3B	1	*Amphritea balenae* JAMM 1525^T (AB330883)	98.4	Marine sediment
Halomonadaceae	SW1-1	1	*Cobetia marina* DSM 4741^T (AJ306890)	99.4	Culture collections
	SW1-8	1	*Cobetia marina* DSM 4741^T (AJ306890)	99.4	Culture collections
	SW3-9	1	*Cobetia crustatorum* JO1^T (EU909460)	99.8	Fermented seafood
	SW4-5	1	*Cobetia crustatorum* JO1^T (EU909460)	99.4	Fermented seafood
Alteromonadaceae	SW1-13	1	*Glaciecola psychrophila* 170^T (DQ007436)	97.3	Arctic
	SW5-3	1	*Glaciecola mesophila* KMM 241^T (AJ488501)	99.5	Marine water
Colwelliaceae	SW2-3A	1	*Colwellia psychrerythraea* 34H (CP000083)	97.7	Arctic marine sediments
	SW2-3E	1	*Colwellia psychrerythraea* 34H (CP000083)	98.9	Arctic marine sediments
CFB group bacteria					
Flavobacteriaceae	SW2-4	1	*Polaribacter butkevichii* KMM 3938^T (AY189722)	99.0	N/A
	SW2-7	1	*Polaribacter butkevichii* KMM 3938^T (AY189722)	98.1	N/A
	SW1-18; SW2-1	2	*Flavobacterium fluvii* H7^T (EU109724)	96.8	Stream sediment
	SW5-7	1	*Cellulophaga fucicola* NN015860^T (AJ005973)	99.6	Fucus serratus
High GC Gram+					
Gordoniaceae	SW1-11	1	*Gordonia terrae* DSM 43249^T (X79286)	96.6	N/A

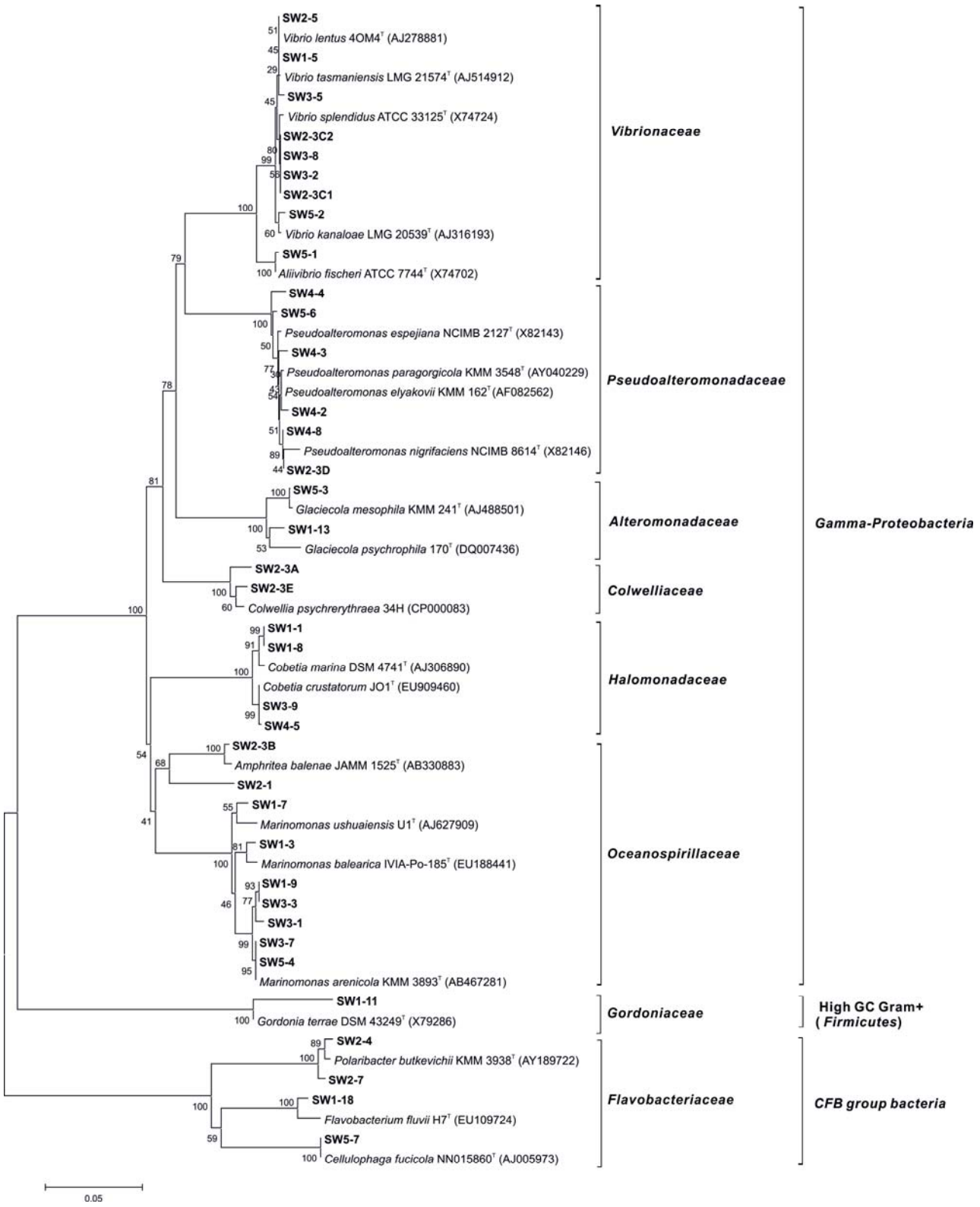

Figure 4.2 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of 37 macro algae associated bacteria in this study and their nearest type strains. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.05 nucleotides substitution per site.

4.4.3 Phylogenetic groups and identification of the microalgae associated bacteria

From the three species of marine micro algal sea water cultures, 37 bacterial strains were isolated and identified on the base of 16S rRNA gene sequence analysis. Of these strains, 62 % (23 strains) were isolated from *Tetraselmis* sp. (strain M1), 22 % (8) from *Amphora Montana* (strain A2) and 16 % (6) from *Phaeodactylum tricornutum* (strain M7) (Table 4.2).

NCBI nucleotide BLAST searches using the partial 16S rRNA gene sequences of these 37 strains revealed that *Gamma-Proteobacteria* was the most abundant division of the bacterial populations (91 %), followed by Gram positive (4 %), *Alpha-Proteobacteria* (2 %) and CFB group bacteria (2 %). *Gamma-Proteobacteria* was phylogenetic affiliation with 8 members of the *Pseudoalteromonadaceae*, *Vibrionaceae*, *Shewanellaceae*, *Pseudomonadaceae, Oceanospirillaceae*, *Alteromonadaceae*, *Halomonadaceae* and *Rhodobacteraceae,* including 22 taxonomic units of *Pseudoalteromonas elyakovii, P. undina, P. elyakovii, P. tetraodonis, P. atlantica, Vibrio gigantis, V. fortis, Photobacterium profundum, Ph. frigidiphilum, Shewanella algae, S. pacifica, S. gaetbuli, S. japonica, S. abyssi, S. colwelliana, Flavimonas oryzihabitans, Pseudomonas poae, Amphritea atlantica, A. japonica, A. balenae, Cobetia marina and Sulfitobacter pontiacus*. Gram positive group contained 2 taxonomic units of *Arthrobacter agilis* and *Bacillus simplex*. One strain identified as *Sulfitobacter pontiacus* affiliated with *Alpha-Proteobacteria* and one strain identified as *Lacinutrix mariniflava* belonged to CFB group bacteria (Figure 4.3).

Table 4.2 List of bacterial strains associated with marine microalgae

Phylogenetic group (family)	Representative isolates	No. of strains	Nearest type strain in the GenBank (accession number)	Similarity (%)	Source of nearest type strain
Gamma-Proteobacteria					
Pseudoalteromonadaceae	A2B1	1	*Pseudoalteromonas elyakovii* KMM 162^T (AF082562)	99.9	*Laminaria japonica*
	A2B10	1	*Pseudoalteromonas elyakovii* KMM 162^T (AF082562)	99.6	*Laminaria japonica*
	A2B8	1	*Pseudoalteromonas undina* NCIMB 2128^T (X82140)	99.6	Sea water
	MA63	1	*Pseudoalteromonas tetraodonis* IAM 14160^T (AF214730)	99.8	Sea water
	MA64	1	*Pseudoalteromonas atlantica* IAM 12927^T (X82134)	99.7	Deep sea
Vibrionaceae	A2B2	1	*Vibrio gigantis* CAIM 25^T (EF094888)	99.5	Biocorrosion phenomena
	A2B5	1	*Vibrio fortis* LMG 21557^T (AJ514916)	99.8	Aquatic animals
	MA66	1	*Photobacterium profundum* DSJ4^T (D21226)	97.7	Coastal water
	MA665	1	*Photobacterium frigidiphilum* SL13^T (AY538749)	99.2	Deep-sea sediments
Shewanellaceae	MA667	1	*Shewanella algae* ATCC 51192^T (AF005249)	99.8	Gut microflora of abalone
	MA321; MA325; MA357	3	*Shewanella pacifica* KMM 3597^T (AF500075)	98.8-99.3	Sea water
	MA322	1	*Shewanella gaetbuli* TF-27^T (AY190533)	97.1	Tidal flat
	MA323	1	*Shewanella japonica* KMM 3299^T (AF145921)	99.4	Sea water
	MA327	1	*Shewanella abyssi* c941^T (AB201475)	99.9	Deep-sea sediment
	MA342	1	*Shewanella colwelliana* ATCC 39565^T (AY653177)	99.8	ATCC
Pseudomonadaceae	A2B3	1	*Flavimonas oryzihabitans* IAM 1568^T (D84004)	98.3	Rice Paddy
	A2B7	1	Pseudomonas poae DSM 14936^T (AJ492829)	98.9	Phyllosphere of grasses
Oceanospirillaceae	MA68; MA326	2	*Amphritea atlantica* M41^T (AM156910)	99.1-99.8	Deep sea hydrothermal vent
	MA324 ; MA328	2	*Amphritea japonica* JAMM 1866^T (AB330881)	99.2-99.9	Deep-sea sediment
Alteromonadaceae	MA58; MA320; MA458; MA462; MA463; MA466; MA467; MA468; MA471	9	*Amphritea balenae* JAMM 1525^T (AB330883)	98.3-99.5	Maine sediments
Halomonadaceae	MA669	1	*Cobetia marina* DSM 4741^T (AJ306890)	99.2	Sea water
Alpha-Proteobacteria					
Rhodobacteraceae	MA69	1	*Sulfitobacter pontiacus* DSM 10014^T (Y13155)	99.7	Marine
High GC Gram+					
Micrococcaceae	MA319	1	*Arthrobacter agilis* DSM 20550^T (X80748)	99.2	Filtration substrate
Firmicutes					
Bacillaceae	A2B4	1	*Bacillus simplex* NBRC 15720^T (AB363738)	97.1	N/A
CFB group bacteria					
Flavobacteriaceae	MA343	1	*Lacinutrix mariniflava* AKS432^T (DQ167239)	99.6	Marine algae

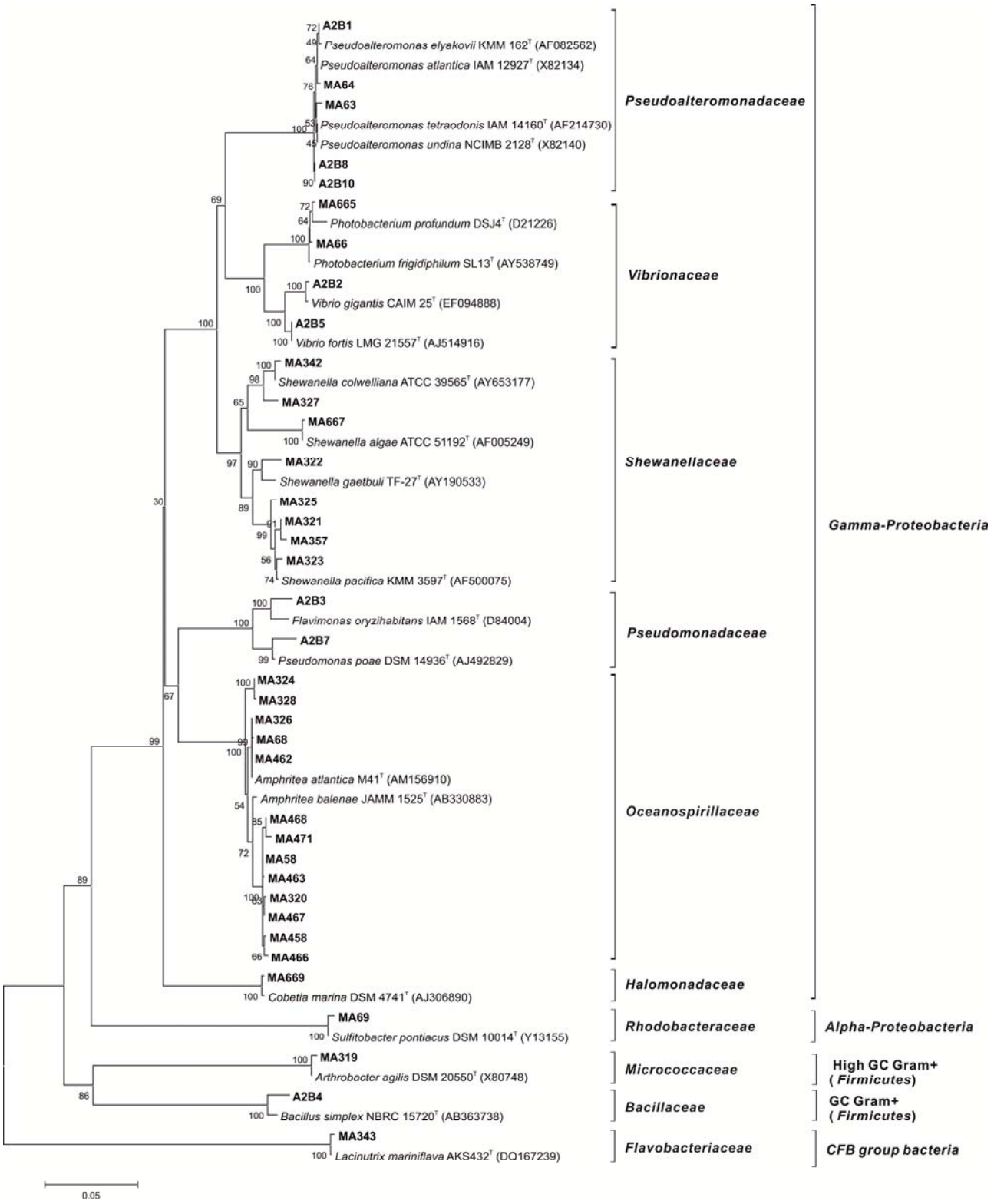

Figure 4.3 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of 37 micro algae associated bacteria in this study and their nearest type strains. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.05 nucleotides substitution per site.

4.4.4 Fatty acid composition of seaweeds and their associated bacteria

4.4.4.1 Fatty acid composition of seaweeds and micro algal cultures

Seaweed samples SW1, SW2, SW3, SW4 and SW5 were identified as *Ascophyllum nodosum*, *Palmaria palmate*, *Fucus serratus, Egregia menziesii* and *Codium fragile* respectively based on morphology study and partial 18S rRNA gene sequences analysis. The fatty acid profiles of these seaweed isolates were mainly n-14:0, n-16:0, n-18:1ω9c, n-18:2ω6t and n-20:5ω3 acids (10-43 % in TFAs), with minor contributions of n-13:0, n-18:3ω3, n-18:4ω3 and n-22:6ω3 acids (Table 4.3).

Table 4.3 Major FAs after whole cell hydrolysis (% of TFAs) in algal strains

Fatty acids	*Ascophyllum nodosum*	*Palmaria*	*Fucus serratus*	*Egregia menziesii*	*Codium fragil*	*Tetraselmis* sp.	*Amphora Montana*	*Phaeodactylum tricornutum*
	SW1	SW2	SW3	SW4	SW5	M1	A2	M7
n-13:0	4.7	5.3	1.7	5.7	4.6	1	-	-
n-14:0	12.5	8.2	13.1	3.3	0.7	2.7	1.4	7.8
n-14:1ω7c	1.3	1.4	0.4	-	-	-	-	-
n-15:0	0.9	0.3	0.4	0.3	-	-	-	-
n-16:1ω9	1	1.8	0.9	3.2	1.4	-	-	-
n-16:1ω7	2.7	3.2	2.3	0.4	1.7	1.4	2.2	16.4
n-16:1ω5	0.7	0.7	0.7	-	-	-	-	-
n-16:2ω4	-	-	-	-	-	4.9	18.4	4.4
n-16:3ω4	-	-	-	-	-	1.7	2.2	12.9
n-16:4	-	-	-	-	-	3.2	2.4	-
n-16:0	17.7	25.4	16.6	29.5	28.7	17.2	19.3	15.5
n-17:1ω8	1.1	0.8	0.7	0.4	8.5	-	-	-
n-17:0	-	-	-	-	2.2	-	-	-
n-18:1ω9c	0.2	5.1	20.9	12.3	11.4	16.3	15.3	2.1
n-18:1ω7c	-	0.2	-	0.3	-	10.9	0.9	1.1
n-18:0	9.9	0.9	-	0.8	-	0.4	7.5	1.3
n-18:2ω6t	5.5	0.5	12.2	1.2	7.3	7.7	2.5	3.5
n-18:3ω6	0.5	0.4	0.8	0.2	1.5	-	-	-
n-18:3ω3	6.5	0.6	5.5	-	12.9	20.9	2.6	1.3
n-18:4ω3	6.5	-	3.9	-	11.1	3.2	0.6	0.6
n-20:1ω9	0.3	-	0.2	0.2	0.2	1.6	2.1	1.2
n-20:2ω2	0.8	1	0.2	-	-	-	0.5	0.7
n-20:3ω6	2.3	0.3	0.2	23.2	0.5	-	-	-
n-20:3ω3	11.7	-	11	-	1.3	-	-	-
n-20:4ω6	1.7	-	-	-	0.4	0.8	0.5	0.6
n-20:4ω3	0.3	-	-	-	-	0.6	1.8	2.2
n-20:5ω3	10.2	43.2	7.8	18.7	3.1	6.5	18.7	28.4
n-24:1n9	0.3	-	0.1	-	0.5	-	-	-
n-22:2	-	-	-	0.2	2	-	-	-
n-22:5ω3	-	-	-	-	-	-	1.1	-
n-22:6ω3	0.7	0.7	0.4	0.1	-	-	-	-
Σ TFA	100	100	100	100	100	100	100.0	100
Σ SCFA	45.7	40.1	31.8	39.6	36.2	20.3	28.2	24.6
Σ MUFA	7.6	13.2	26.2	16.8	23.7	30.2	20.5	20.8
Σ PUFA	46.7	46.7	42	43.6	40.1	49.5	51.3	54.6
ACL[a]	17.325	17.63	17.356	17.762	17.219	17.51	17.57	17.37

[a] Average chain length (ACL) calculated after (White et al., 2005a); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (20:5ω3); DHA, docosahexaenoic acid (22:6ω3); and (–) not detectable.

These five species of seaweed were all with high level of PUFAs (40-46 %). SW2 and SW4 were with high content of EPA (43 and 19 % respectively), while DHA was seldom detected from SW5. The average chain lengths of the TFAs of these seaweeds were similar (17.2-17.6). Marine micro algal cultures M1, A2 and M7 were identified as *Tetraselmis* sp., *Amphora Montana* and *Phaeodactylum tricornutum* respectively based on 18S rRNA gene sequences analysis. The fatty acid profiles of these micro algal cultures were mainly n-16:1ω7, n-16:2ω4, n-16:3ω4, n-16:0, n-18:1ω9c, n-18:1ω7c, n-18:2ω6t, n-18:3ω3 and n-20:5ω3 acids. These three species of algae were also with high level of PUFAs (50-54 %), especially strain M7 with high level of EPA in TFAs (28 %) and remarkable lipid content of 104.40 mg g^{-1}.

4.4.4.2 Fatty acid composition of Gamma-Proteobacteria

Strains MA665 and MA66 also belong to *Vibrionaceae*, they showed the highest sequence similarities (97.3-99.7 %) to type strains *Photobacterium frigidiphilum* and *Ph. profundum* respectively. The dominant fatty acid components detected in these two strains extracts were n-16:1ω9, n-16:0, n-18:1ω9c and n-20:5ω3 acids (6-40 %), and less of n-12:0, n-14:0, n-15:0, n-16:1ω7 and n-18:0 acids, as reported of this genus isolated from deep sea (Nogi et al., 1998b; Seo et al., 2005). Higher lever of EPA could be detected from *Photobacterium* species than that from *Vibiro*, and the ACL was also longer of 16.2 (Table 4.4).

Strains MA667, MA322, MA667, MA323, MA327 and MA342 exhibited the highest homology (97.1-99.9 % 16S rRNA gene identity) to type strains *Shewanella algae, S. gaetbuli, S. pacifica, S. japonica, S. abyssi* and *S. colwelliana* respectively. The predominant cellular fatty acids of these *Shewanellaceae* strains were i-13:0, i-15:0, n-15:0, n-16:1ω7, n-16:0 and n-17:1ω8 acids, with more or less of n-12:0, n-18:1ω9c and n-20:5ω3 acids (Table 4.4). The proportion of EPA varied inversely with species of these strains as reported previously (Yang et al., 2007).

Strains SW1-13 and SW5-3 exhibited the highest homology (97.3-99.6 % 16S rRNA gene identities) to type strains *Glaciecola psychrophila* and *G. mesophila* respectively. The predominant cellular fatty acids of these *Alteromonadaceae* strains were n-12:0, n-

16:0, n-16:1ω7, n-17:1ω8 and n-18:1ω7 (5-48 %) (Table 4.4), which are similar to those of *Alteromonas* and *Glaciecola* strains (Romanenko et al., 2003a; Zhang et al., 2006a).

Table 4.4 Major FAs after whole cell hydrolysis (% of TFAs) in algae associated **bacteria** affiliating with the *Gamma-Proteobacteria*

	Photobacterium sp.		*Shewanella* sp.						*Glaciecola* sp.		
Fatty acids	MA665	MA66	MA667	MA322	MA357	MA323	MA327	MA342	SW1-13	SW5-3	SW4-2
n-10:0	-	-	-	-	-	-	-	-	1.2	-	1.1
n-12:0	2.1[b]	4.7	1.9	3.5	1.7	3.8	2.6	1.2	3.5	2.5	2.5
3-OH-12:0	-	-	-	-	-	-	-	-	6.6	-	6.5
i-13:0	-	-	3.1	9.4	9.9	6.9	23.5	7.2	-	-	-
n-13:0	0.3	2.1	-	1.5	0.8	0.5	1.5	3.5	-	-	0.3
i-14:0	0.1	-	0.8	2.7	1.2	0.9	-	0.6	-	-	-
n-14:0	2.8	1.5	2	5.1	3.1	4.5	3.2	2.1	7.9	1.3	0.8
n-14:1ω5c	1	-	-	-	-	-	-	-	-	-	-
i-15:0	0.5	-	20.5	20.8	34.9	16.2	13.6	14.5	-	-	-
ai-15:0	-	-	0.5	1.3	2.6	2.3	-	0.3	-	-	-
ai-15:1	-	-	-	-	3.5	0.2	-	1.2	-	-	-
n-15:1ω6	1	-	-	-	0.2	0.3	-	2.5	-	-	0.7
n-15:1ω8	-	-	-	-	0.1	0.1	-	1.1	2.1	0.9	0.2
n-15:0	1.3	2.3	3.7	4.5	3.6	1.1	11.6	14.1	2.3	1.8	0.8
i-16:0	0.2	1.2	-	-	0.2	0.2	-	0.5	-	-	-
n-16:1ω9	40.1	35.7	1.3	-	-	-	-	-	-	-	-
n-16:1ω7	1.3	1.4	15.2	22.3	17.1	25.2	19.7	20.2	39.6	47.5	38.9
n-16:0	24.3	26.1	21.6	18.4	-	15.6	11.2	8.5	18.5	21.6	13.9
i-17:0	0.1	1.1	1.7	-	1.9	0.9	-	-	-	-	-
n-17:1ω8	0.1	0.8	12.1	6.2	7.4	0.2	2.5	13.2	3.2	7.3	4.2
n-17:1ω6	0.3	-	-	-	0.3	1.5	-	0.7	-	-	-
n-17:0	0.1		2.3	-	0.8	0.4	1.5	5.3	1.2	1.7	1.3
n-18:1ω9c	13.2	12.2	4.7	-	1.2	1.6	1.3	0.7	8.5	13.2	0.5
n-18:1ω7c	0.4	-	4.2	2.9	2.1	9.5	-	0.5	-	-	27.4
n-18:0	2.4	3.5	3.1	1.1	0.6	0.5	-	0.6	3.1	0.7	0.5
n-20:5ω3	7.5	6.5	-	-	5.2	7.1	6.3	0.7	-	-	-
Others	0.9	0.9	1.3	0.3	1.6	0.5	1.5	0.8	2.3	1.5	0.4
Σ TFA	100	100	100	100	100	100	100	100	100	100	100
Σ SCFA	33.3	40.2	34.6	34.1	10.6	26.4	31.6	35.3	42	27.8	26.9
Σ BCFA	0.9	2.3	26.6	34.2	50.7	27.4	37.1	23.1	-	-	-
Σ MUFA	57.4	50.1	37.5	31.4	31.9	38.6	23.5	40.1	53.4	68.9	71.9
Σ PUFA	7.5	6.5	-	-	5.2	7.1	6.3	0.7	-	-	-
ACL[a]	16.28	16.15	15.72	15.21	15.21	15.78	14.91	15.34	15.23	16	16.1

[a] Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (20:5ω3); and (–) not detectable.

Strains SW4-2, SW4-4, SW4-8, MA63 and A2B8 exhibited the highest homology (98-99 % 16S rRNA gene identity) to type strains *Pseudoalteromonas elyakovii, P. espejiana*, *P. nigrifaciens*, *P. tetraodonis* and *P. undina* respectively. FA patterns of these *Pseudoalteromonadaceae* strains were dominated by n-16:0, n-16:1ω7 and n-18:1ω7c (8-45 %), with minor contributions (1-7 %) of n-12:0, 3-OH-12:0, n-14:0, n-15:1ω8, n-17:0 and n-17:1ω8 acids (Table 4.5). The pattern of the predominant fatty acids are comparable with those from *P. elyakovii*, *P. haloplanktis*, *P. tetraodonis*, *P.*

agarivorans and *P. atlantica* (Al Khudary et al., 2008). No BCFA and PUFA were detected of the species, the ACL was variable (15.5-16).

Strain SW2-3A and SW2-3E exhibited the highest homology (97.3-98.9 % 16S rRNA gene identity) to type strains *Colwellia psychrerythraea.* The predominating fatty acid components detected in these *Colwelliaceae* strains extracts were n-14:0, n-14:1ω7c, n-15:0, n-16:1ω9, n-16:1ω7, n-16:0 and n-22:6ω3 acids (Table 4.5). Both strains contained DHA with trace levels of EPA as the type strains isolated from Antarctic sea water samples (Bowman et al., 1998a).

Strains SW1-3, SW1-7, SW3-1, SW2-3B, MA68 and MA324 showed the highest sequence similarities (97.3-99.7 % 16S rRNA gene identity) to type strains *Marinomonas balearica*, *M. ushuaiensis*, *M. arenicola, Amphritea balenae*, *A. atlantica* and *A. japonica*. The fatty acid profiles of these *Oceanospirillaceae* strains were characterised by mainly 3-OH-10:0, n-16:0, n-6:1ω7c and n-8:1ω7c acids (Table 4.5 and Table 4.6), which were comparable with those from type strains isolated from seagrass or sediment (Miyazaki et al., 2008; Romanenko et al., 2009; Lucas-Elio et al., 2010).

Strains A2B3 and A2B7 exhibited the highest homology (98.2-99.0 % 16S rRNA gene identity) to type strains *Flavimonas oryzihabitans* and *Pseudomonas poae* respectively. The major cellular fatty acids of these *Pseudomonadaceae* strains contained large amounts of even-numbered straight-chain, such as n-16:1ω9, n-16:0 and n-18:1ω9c acids (17-40 %), small amounts of even-numbered straight-chain, n-12:0 and n-18:0 acids, and the hydroxy acids 3-OH-10:0, 2-OH-12:0 and 3-OH-12:0 were also detected (Table 4.5), as reported on this genus (Verhille et al., 1999).

Strains SW1-1 and SW3-9 exhibited the highest homology (99.4-99.8 % 16S rRNA gene identity) to type strains *Cobetia marina* and *C. crustatorum* respectively. FA patterns of these *Halomonadaceae* strains were dominated by 3-OH-12:0, n-16:0, n-16:1ω7, n-17:0 cyclo and n-18:1ω7c acids (9-26 %), with minor contributions of n-10:0, n-12:0, i-15:0, n-18:0 and cyclo-19:0 acids (1-7 %) (Table 4.6). The FA profiles were comparable with those from type strains (Choi et al., 2010).

Table 4.5 Major FAs after whole cell hydrolysis (% of TFAs) in algae associated bacteria affiliating with the *Gamma-Proteobacteria*

	Colwellia sp.		*Pseudoalteromonas* sp.					*Marinomonas* sp.			*Pseudomonas* sp.	
Fatty acids	SW2-3A	SW2-3E	SW4-4	SW4-8	A2B1	MA63	A2B8	SW1-3	SW1-7	SW3-1	A2B3	A2B7
n-10:0	-	-	-	-	1.2	-	1.1	6.5	9.7	8.8	2.1	2.5
n-12:0	-	-	3.1	2.9	2.3	1.6	2.8	9.7	4.6	10.8	5.4	4.5
2-OH-12:0	-	-	-	-	-	-	-	-	-	-	2.7	2.6
3-OH-12:0	-	-	4.1	-	5.9	3.7	2.4	-	-	3.4	4.5	5.8
n-13:0	-	-	-	0.2	0.3	0.3	0.1	-	-	-	-	-
n-14:0	4.8	6.7	2.7	1.3	0.7	1.7	0.5	1.3	3.7	1.1	-	0.2
n-14:1ω7c	7.5	10.1	3.3	-	-	-	-	-	-	-	-	-
n-15:1ω6			0.7	0.3	0.5	0.4	0.5	-	-	-	-	-
n-15:1ω8	0.2	1.7	6.7	3.1	0.3	6.1	2.1	-	-	-	-	-
n-15:0	8.5	5.3	3.5	-	0.9	7.2	1.3	-	0.8	-	-	-
n-16:1ω9	7.3	7.5	-	-	-	-	-	-	-	-	39.5	37.8
n-16:1ω7	37.5	30.3	45.1	39.7	35.1	36.9	40.5	19.6	23.5	19.3	-	-
n-16:1ω5	-	-	-	-	-	0.1	0.2	-	-	-	-	-
n-16:0	23.5	27.1	14.2	28.5	12.3	17.5	25.6	17.5	15.8	16.5	27.1	25.5
ai-17:1ω7	-	-	-	-	3.8	13.2	4.6	-	-	-	-	-
n-17:1ω8	0.7	0.8	3.2	6.3	-	-	-	-	-	-	-	-
n-17:0	1.1	1.5	2.1	7.2	1.2	4.9	0.6	2.8	-	-	-	0.7
n-18:1ω9c	0.6	0.3	0.3	0.3	0.6	0.3	0.7	-	-	-	17.2	19.5
n-18:1ω7c	0.5	1.6	9.4	8.5	32.6	4.3	14.7	37.7	39.5	35.5	-	-
n-18:0	1.5	2.2	0.4	0.8	0.7	0.5	1.1	4.1	2.3	4.1	0.6	0.5
n-20:5ω3	0.2	0.1	-	-	-	-	-	-	-	-	-	-
n-22:6ω3	5.1	4.2	-	-	-	-	-	-	-	-	-	-
Others	1	0.6	1.2	0.9	1.6	1.3	1.2	0.8	0.9	0.5	0.9	0.4
Σ TFA	100	100	100	100	100	100	100	100	100.8	100	100	100
Σ SCFA	39.4	42.8	26.6	40.9	24.6	30.2	34.2	41.9	36.1	44.7	42.4	42.3
Σ MUFA	54.3	52.3	65.4	58.2	72.9	61.3	63.3	57.3	63	54.8	56.7	57.3
Σ PUFA	5.3	4.3	-	-	-	-	-	-	-	-	-	-
ACL[a]	15.89	15.86	15.55	16	16.03	15.68	15.86	15.93	15.97	15.59	15.58	15.67

[a] Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); DHA, docosahexaenoic acid (n-22:6ω3); and (–) not detectable.

Strains SW1-5, SW3-2, SW3-5, SW5-2, SW5-1, A2B2 and A2B5 showed the highest sequence similarities (99.1-99.8 %) to type strains *Vibrio lentus*, *V. splendidus*, *V. tasmaniensis*, *V. kanaloae, Aliivibrio fischeri, V. gigantis* and *V. fortis* respectively. FA patterns of these *Vibrionaceae* strains were dominated by n-14:0, n-16:0, n-16:1ω7 and n-18:1ω7c (8-40 %), with minor contributions (1-6 %) of n-12:0, 3-OH-12:0, i-15:0, n-16:1ω11 and i-16:1ω6 acids. The major cellular fatty acid profile of these strains was compared with those of other members of the same *Vibrio* genus (Lambert et al., 1983; Faury et al., 2004), MUFAs were the most dominance, and PUFAs, such as EPA, was only in trace level (1-4 %) and ACL was between 15.2-15.8 (Table 4.6).

Table 4.6 Major FAs after whole cell hydrolysis (% of TFAs) in algae associated bacterial strains isolated from North Sea

	Vibrio sp.							*Amphritea* sp.				*Cobetia* sp.		
Fatty acids	SW2-3C	SW3-2	SW5-2	SW3-5	SW1-5	A2B2	A2B5	MA68	MA324	MA58	SW2-3B	SW1-1	SW3-9	MA669
n-10:0	4.9	3.6	4.3	2.9	2.4	4.8	4.2	2	2.4	0.5	0.8	2.2	4.1	2.3
n-12:0	-	-	-	-	-	-	-	2.2	0.2	0.2	-	3.7	7.5	3.8
3-OH-12:0	3.4	8.8	2.2	6.8	5.5	2.1	0.3	2.5	3.1	4.2	4.1	12.1	26.7	12.7
n-14:0	-	9.3	5.1	10.5	9.2	4.7	5.8	-	-	-	-	-	1.5	0.7
n-14:1ω7c	4.7	1.2	-	-	-	2.1	3.4	-	-	-	-	-	-	-
i-15:0	-	0.8	3.2	3.6	2.8	-	2.9	-	-	-	-	2.2	1.4	2.6
i-16:1ω6	2.2	3.5	6.8	0.8	0.9	2.2	9.5	-	-	-	-	-	-	-
n-16:1ω11	8.4	1.6	2.2	2.8	3.2	-	0.8	-	-	-	-	-	-	-
n-16:1ω7	41.1	31.6	39.7	34.7	39.7	40.5	37.6	40.5	38.5	44.2	45.5	14.9	18.7	14.1
n-16:0	15.3	28.7	24.7	26.9	25.5	24.5	19.5	27.7	17.6	15.5	14.5	25.7	22.5	24.7
n-17:0	-	-	-	-	-	-	-	-	-	-	-	14.8	8.9	15.8
n-18:1ω7c	14	8.1	9.8	7.9	8.5	13.7	11.9	23.2	36.6	33.5	33.2	18.5	5.5	19.2
n-18:0	2.8	0.3	0.8	0.5	-	-	1.3	0.7	0.8	0.9	1.1	0.3	1.2	0.5
n-19:0	-	-	-	-	-	-	-	-	-	-	-	3.5	0.5	2.7
n-20:5ω3	2.7	1.1	-	0.8	1.7	3.5	1.5	-	-	-	-	-	-	-
Others	0.5	1.4	1.2	1.8	0.6	1.9	1.3	1.2	0.8	1	0.8	2.1	1.5	0.9
Σ TFA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Σ SCFA	23	41.9	34.9	40.8	37.1	34	30.8	35.1	24.1	21.3	20.5	62.3	72.9	63.2
Σ BCFA	-	0.8	3.2	3.6	2.8	-	2.9	-	-	-	-	2.2	1.4	2.6
Σ MUFA	70.4	46	58.5	46.2	52.3	58.5	63.2	63.7	75.1	77.7	78.7	33.4	24.2	33.3
Σ PUFA	2.7	1.1		0.8	1.7	3.5	1.5	-	-	-	-	-	-	-
ACL [a]	15.84	15.2	15.4	15.22	15.57	15.6	15.64	16	16.34	16.32	16.35	15.51	14.34	15.65

[a] Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–) not detectable.

4.4.4.3 Alpha-Proteobacteria

Strain MA69 exhibited the highest homology (99.6 % 16S rRNA gene identity) to *Sulfitobacter pontiacus*. The major cellular fatty acids of strain MA69 are unsaturated fatty acids such as n-18:1ω7c (70 %) and 11-methyl 18:1ω7c (8.3 %) (Table 4.7). The fatty acid composition was similar to that observed in other members of the genus *Sulfitobacter*. The presence of n-18:1ω7c as the dominant fatty acid is representative of several major phyletic groups within the *Alphaproteobacteria* (Labrenz et al., 2000).

4.4.4.4 Cytophaga–Flavobacterium–Bacteroides (CFB) bacteria

CFB bacteria strains SW2-4 and SW2-7 exhibited 98.1-99.0 % 16S rRNA gene sequence homology to *Polaribacter butkevichii* KMM 3938^T (AY189722) based on 16S rRNA gene phylogenetic studies, while strains SW1-18 and SW2-1 were the most related to *Flavobacterium fluvii* (96.8 %), and strain SW5-7 exhibited 99.6 % the highest similarity with *Cellulophaga fucicola*. The cellular fatty acid composition of these *Flavobacteriaceae* strains showed more branched chain fatty acids, mainly with i-13:0, i-14:0, n-15:0, i-15:0, i-15:1, n-15:1ω6, 2-OH-15:0, 3-OH-15:0 and i-16:1ω6 acids (Table 4.7), similar to the reports on related type strains previously (Bowman, 2000; Nedashkovskaya et al., 2005; Lee et al., 2010).

Strain MA343 showed 99.5 % similarity (16S rRNA gene identity) to *Lacinutrix mariniflava*. The main cellular fatty acids of strain MA343 were i-15:0, ai-15:0, 3-OH-i-15:0, n-16:1ω7c, 3-OH-i-16:0 and 3-OH-i-17:0 acids (8-13 %). Despite the existence of differences in culture conditions and analytical methods, fatty acid composition and the proportion of most components were similar to that observed in other members of the genus *Lacinutrix* (Nedashkovskaya et al., 2008).

4.4.4.5 Gram Positive

Strain SW1-11 showed the highest 16S rRNA gene sequence similarity (96.6 % 16S rRNA gene identity) to *Gordonia terrae*, and with 2-OH-i-15:0, n-16:0, n-18:1ω9c and 10-OH-18:0 as major FAs (16-28 %) and contained less n-14:0, n-15:0, n-15:1ω6, n-16:1ω9, n-17:0 and n-18:0 acids (1-2.7 %) (Table 4.7), which possessed similar whole-cell fatty acid profiles and the dominant fatty acids with those from the type strains (Yoon et al., 2000).

Strain MA319 showed the highest homology (99.2 % 16S rRNA gene identity) to *Arthrobacter agilis*. The predominant fatty acids were ai-15:0, i-16:0, ai-17:0 and n-17:0 acids (7-49 %), with less of n-15:0, n-14:0, n-16:1ω7, n-16:0, n-18:0 and n-20:1 acids (0.5-4 %) (Table 4.7), as described on this genus (Reddy et al., 2000).

Table 4.7 Major FAs after whole cell hydrolysis (% of total FAs) in algae associated bacteria affiliating with the *Alpha-Proteobacteria*, *Firmicutes* and CFB group bacteria

Fatty acids	*Polaribacter*		*Flavobacterium*		*Cellulophaga*	*Gordonia*	*Lacinutrix*	*Bacillus*	*Arthrobacteri*	*Sulfitobacter*
	SW2-4	SW2-7	SW1-18	SW2-1	SW5-7	SW1-11	MA343	A2B4	MA319	MA69
3-OH-10:0	-	-	-	-	-	-	-	-	-	8.1
3-OH-12:0	-	-	-	-	-	-	-	-	-	2.4
i-13:0	10.1[b]	16.9	2.1	0.5	-	-	-	-	-	-
n-13:0	0.6	0.7	-	-	-	-	-	-	-	-
i-14:0	3.1	8.5	0.3	0.1	-	-	0.2	0.7	-	-
n-14:0	1.2	0.8	0.5	0.6	0.8	2.7	0.2	-	2.3	-
i-15:0	8.1	6	10.2	24.4	22.7	-	13.5	19.2	-	-
ai-15:0	6.4	2.1	17.8	15	4.7	-	17.3	30.5	48.5	-
i-15:1ω10c	7.5	8.7	3.7	5.5	9.9	-	10.9	-	-	-
ai-15:1	-	-	-	-	-	-	8.5	-	-	-
n-15:1ω6	15.7	10.2	8.7	6.9	1.5	1.1	1.5	-	-	-
2-OH-15:0	9.2	17.5	2.4	1.6	-	-	2.6	-	-	-
3-OH-15:0	8.5	5	0.8	2.1	2.1	-	-	-	-	-
2-OH-i-15:0	-	-	-	-	-	16.2	-	-	-	-
3-OH-i-15:0	3.4	2.1	0.8	1.3	7.9	-	13.2	-	-	-
n-15:0	8.8	7.8	9.4	6.7	10.6	1.3	1.5	-	3.7	-
i-16:0	0.7	0.5	0.7	0.5	1.4	-	3.1	4.3	16.5	-
3-OH-16:0	1.2	-	5.6	4.7	7.5	-	-	-	-	-
3-OH-i-16:0	4.2	5.4	12.1	0.7	5.5	-	7.2	-	-	-
i-16:1ω6	6.8	3.7	16.3	19.5	1.9	-	-	-	-	-
n-16:1ω11c	-	-	-	-	-	-	-	8.9	-	-
n-16:1ω9c	-	-	-	-	-	1.5	-	2.1	-	-
n-16:1ω7	-	-	-	-	16.7	-	7.8	-	0.6	-
n-16:0	0.7	0.6	3.8	4.5	5.2	28.2	-	3.5	2.5	8.8
i-17:0	-	-	-	-	-	-	-	25.2	-	-
ai-17:0	-	-	-	-	-	-	-	3.3	7.3	-
i-17:1ω9c	-	-	-	-	-	-	1.5	-	-	-
ai-17:1ω9c	-	-	-	-	-	-	1.8	-	-	-
3-OH-i-17:0	-	-	-	-	-	-	8.1	-	-	-
n-17:1ω6	2.3	1.8	3.9	4.2	0.8	-	-	-	-	-
n-17:0	-	-	-	-	-	1.5	-	-	12.9	-
n-18:1ω9c	-	-	-	-	-	22.5	-	-	-	-
n-18:1ω7c	-	-	-	-	-		-	1.2	-	69.5
10-OH-18:0	-	-	-	-	-	21.4	-		-	
n-18:0	0.4	0.2	-	-	-	2.5	-	0.2	3.5	1.4
11-Me-18:1ω7c		-	-	-	-	-	-	-	-	8.3
n-20:1	-		-	-	-		-	-	0.5	-
Others	1.1	1.5	0.9	1.2	0.8	1.1	1.1	0.9	1.7	1.5
Σ TFA	100	100	100	100	100	100	100	100	100	100
Σ SCFA	30.6	32.6	22.5	20.2	26.2	57.6	4.3	3.7	24.9	20.7
Σ BCFA	36	41.5	44	42.5	42.2	16.2	62.6	83.2	72.3	-
Σ MUFA	32.3	24.4	32.6	36.1	30.8	25.1	23.5	12.2	1.1	77.8
ACL[a]	14.77	14.47	15.28	15.19	15.27	16.53	15.24	15.66	15.45	16.76

[a] Average chain length (ACL) calculated after (White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; TFA, total fatty acids; and (–) not detectable.

Strain A2B4 showed the highest similarity (97.2 % 16S rRNA gene identity) to *Bacillus simplex*. The major fatty acids were mainly i-15:0, ai-15:0, n-16:1ω11c and i-17:0 acids

(9-30 %), with less of i-16:0, n-16:1ω9 cis, and ai-17:0 (2-4 %) (Table 4.7), similar to the sutdy on this sepcies reported (Sikorski et al., 2008).

4.4.4.6 Phylogenetic resolution of FA patterns

In this study, all the bacterial cell were obtained from 15 ºC marine broth cultures for FAs patterns analysis, and were subjected to principal coordinates analysis (PCO), which allowed separation of isolates representing different phyla. For examples, in the case of macro algae and their associated bacteria, different groups, such as FAs of *Gamma-Proteobacteria,* FAs of *Firmicutes*, FAs of CFB group bacteria and FAs of macro algae (Figure 4.4). The result of ordination pattern was agreeable to the data clustered by using hierarchical UPGMA analysis, which clearly separated the 30 genus including 5 algal species (Figure 4.5), and divided them into four phyla as mentioned above. The result was further confirmed by the analysis of FAs from micro algae and their associated bacteria (Figure 4.6), in which six groups has been divided: *Gamma-Proteobacteria*, *Gram positive (Firmicutes), Alpha-Proteobacteria*, CFB group bacteria, micro algae and *Photobacterium* (Figure 4.7), all 22 species including the micro algae and their associated bacteria were hierarchically separated into six phyla, from which FAs pattern of *Photobacterium* was fall into *Gamma-Proteobacteria* and showed close evolutionary distance to micro algae and diatoms (Figure 4.8).

PCO analysis on FA composition of all 56 strains including marine macroalgae and microalgae and their associated bacteria exhibited a separation by 7 groups: *Gamma-Proteobacteria* (I), *CFB Group bacteria* (II), *Gram*$^+$ (*Firmicutes*) (III), *Alpha-Proteobacteria* (IV), algae (V), *Photobacterium* (VI) and high GC *Gram*$^+$ *bacteria* (VII) (Figure 4.9). All these FA compositions were hierarchically separated into four phyla analyzed by UPGMA (Figure 4.10), as presented above (Figures 4.6 and 4.8). Both the distribution and the percentage contribution of each fatty acid to the total fatty acid across taxa at species level were plotted shown in Figures 4.11 and 4.12. The trends and relationships of fatty acid composition of different groups or each species within *Gamma-Proteobacteria*, *CFB Group*, *Gram*$^+$ (*Firmicutes*), *Alpha-Proteobacteria*, algae, *Photobacterium* and high GC *Gram*$^+$ *bacteria* respectively were found to be similar using this method. Within *Gamma-Proteobacteria* (I), wide distribution of fatty acid composition was observed, ranging from short-chain FAs (e.g. n-12:0, 12:0 3-OH, n-14:0 and n-15:0); middle-chain FAs (e.g. n-16:1ω9, n-16:1ω7, n-16:0, n-17:1ω8, n-17:0, n-18:1ω9c and n-18:1ω7c), to long-chain FAs (e.g. n-20:5ω3

and n-22:6ω3), in which EPA and DHA were found. For *CFB Group bacteria* (II), the fatty acids were mainly short-chain (e.g. i-13:0 and i-14:0) and middle-chain FAs (e.g. i-15:0, i-15:1ω10c, n-15:1ω6, n-15:0, i-16:0 3-OH, n-16:1ω7, i-17:1ω9c and i-17:0 3-OH), predominant by branched-chain FAs. Similar to *CFB Group bacteria*, *Gram*$^+$ bacteria (*Firmicutes*) (III) mainly contained branched-chain FAs (e.g. i-15:0, ai-15:0, i-16:0, n-16:1ω11 and i-17:0), and dominated by i-15:0, ai-15:0 and i-17:0. The fatty acids of *Alpha-Proteobacteria* (IV) were dominated by middle-chain FAs, such as ai-15:0, i-16:0 and n-17:0. In marine macroalgae and microalgae (V), highly diverse of middle-chain and long-chian FAs were found with much more unsaturated, for example, n-16:1ω7, n-16:2ω4, n-16:3ω4, n-16:0, n-17:1ω8, n-18:1ω9c, n-18:1ω7c, n-18:2ω6t, n-18:3ω3, n-18:4ω3, n-20:3ω6 and n-20:5ω3. Similar to algal FA composition, but simply presented in *Photobacterium* species (VI) with mainly middle-chain FAs (e.g. n-16:1ω9, n-16:0 and n-18:1ω9c) and long-chain FAs (e.g. n-20:5ω3). In high GC *Gram*$^+$ *bacteria* (VII), i-15:0 2-OH, n-16:0, n-18:1ω9c and n-18:0 10-OH acids were mainly found. Comparatively, n-16:1ω7, n-16:0 and n-18:1ω7 acids may serve as FA markers for *Gamma-Proteobacteria*, while n-16:0, n-18:1ω9 and n-20:5ω3 acids for algae.

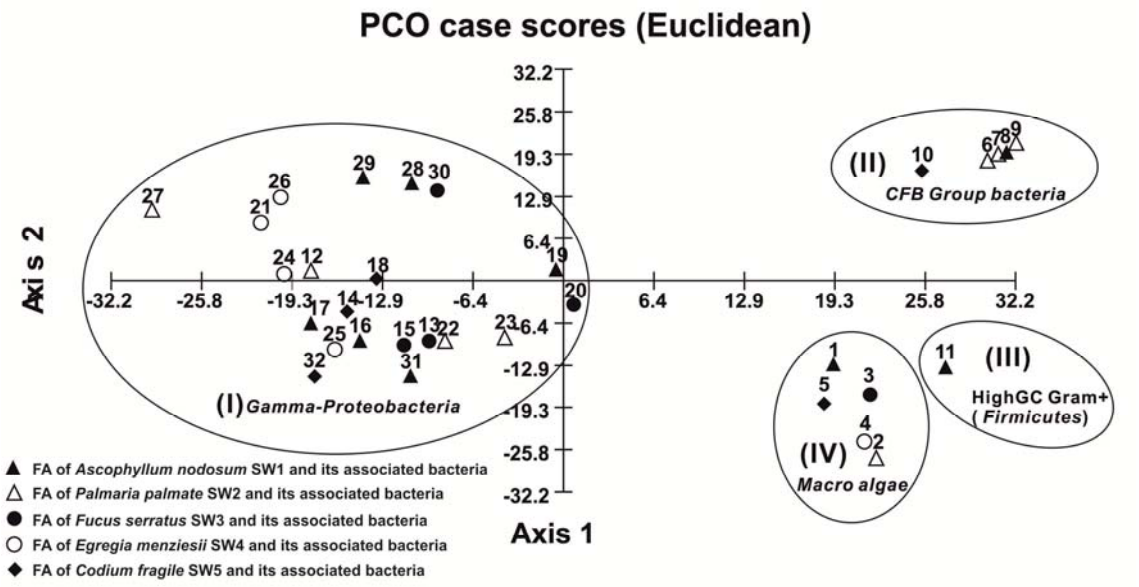

Figure 4.4 Principal coordinates analyses (PCO) of macro algae and their associated bacterial major FA abundance data from Tables 4.3-4.7 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 4.5)

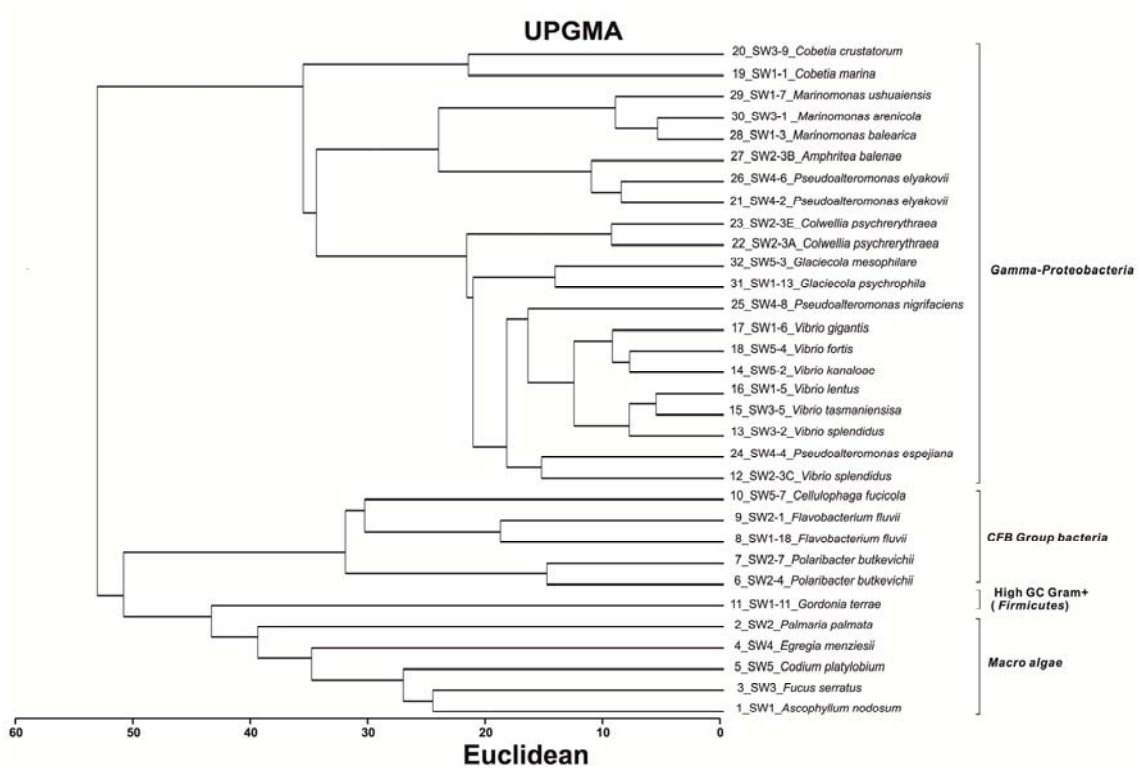

Figure 4.5 Similarity analysis based on whole cell fatty acid composition from macro algae and their associated bacterial species. The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 4 and nearest type strains were indicated beside the isolates.

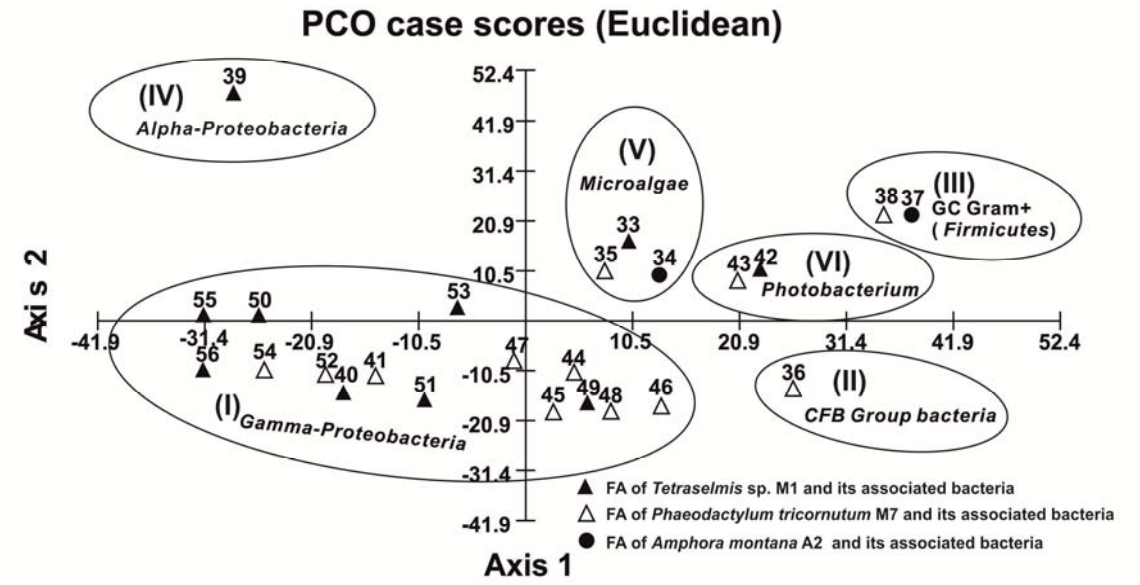

Figure 4.6 Principal coordinates analyses (PCO) of microalgae and their associated bacterial major FA abundance data from Tables 4.3-4.7 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 4.7)

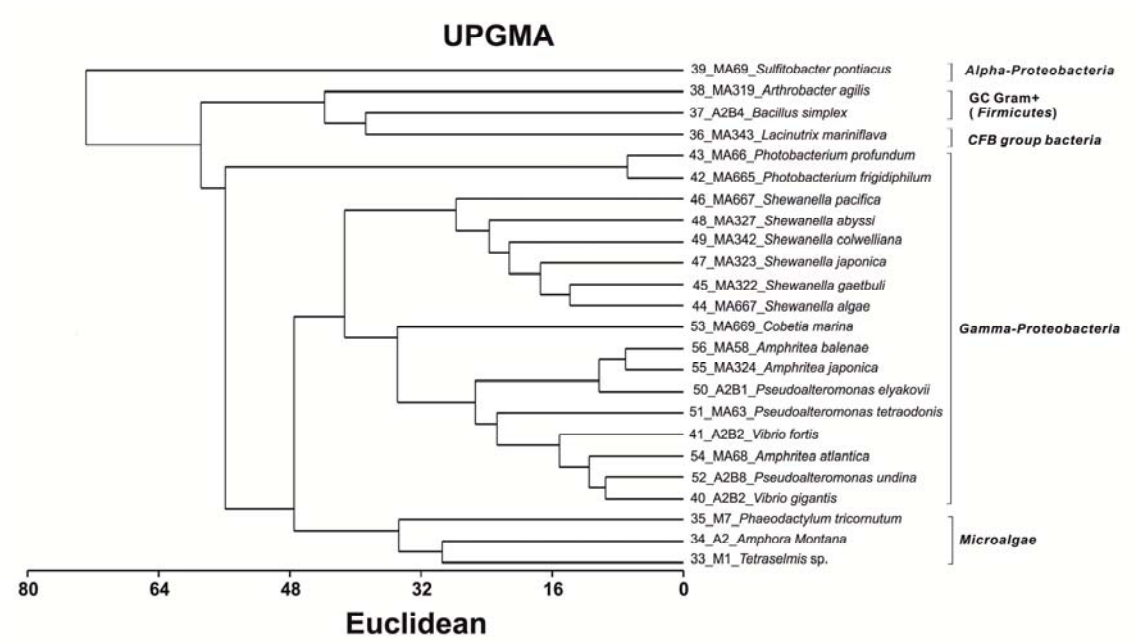

Figure 4.7 Similarity analysis based on whole cell fatty acid composition from microalgae and their associated bacterial major species. The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 4.6 and nearest type strains were indicated beside the isolates.

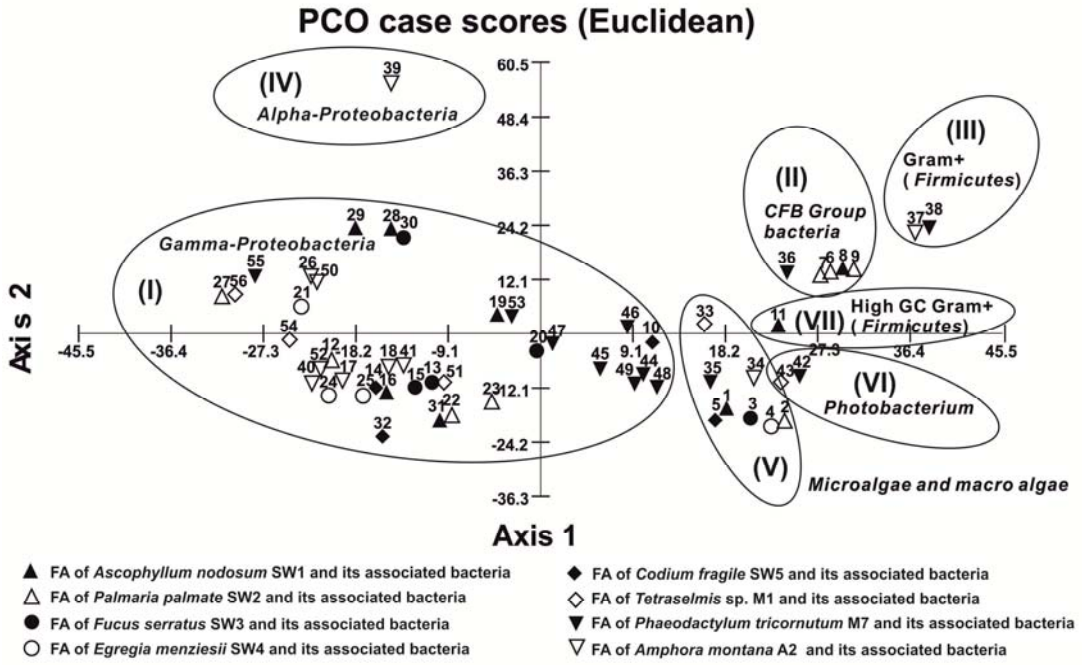

Figure 4.8 Principal coordinates analyses (PCO) of macroalgae, microalgae and their associated bacterial major FA abundance data from Tables 4.3-4.7 measuring Bray Curtis distance. Numbers indicate bacterial strains (see Figure 4.9)

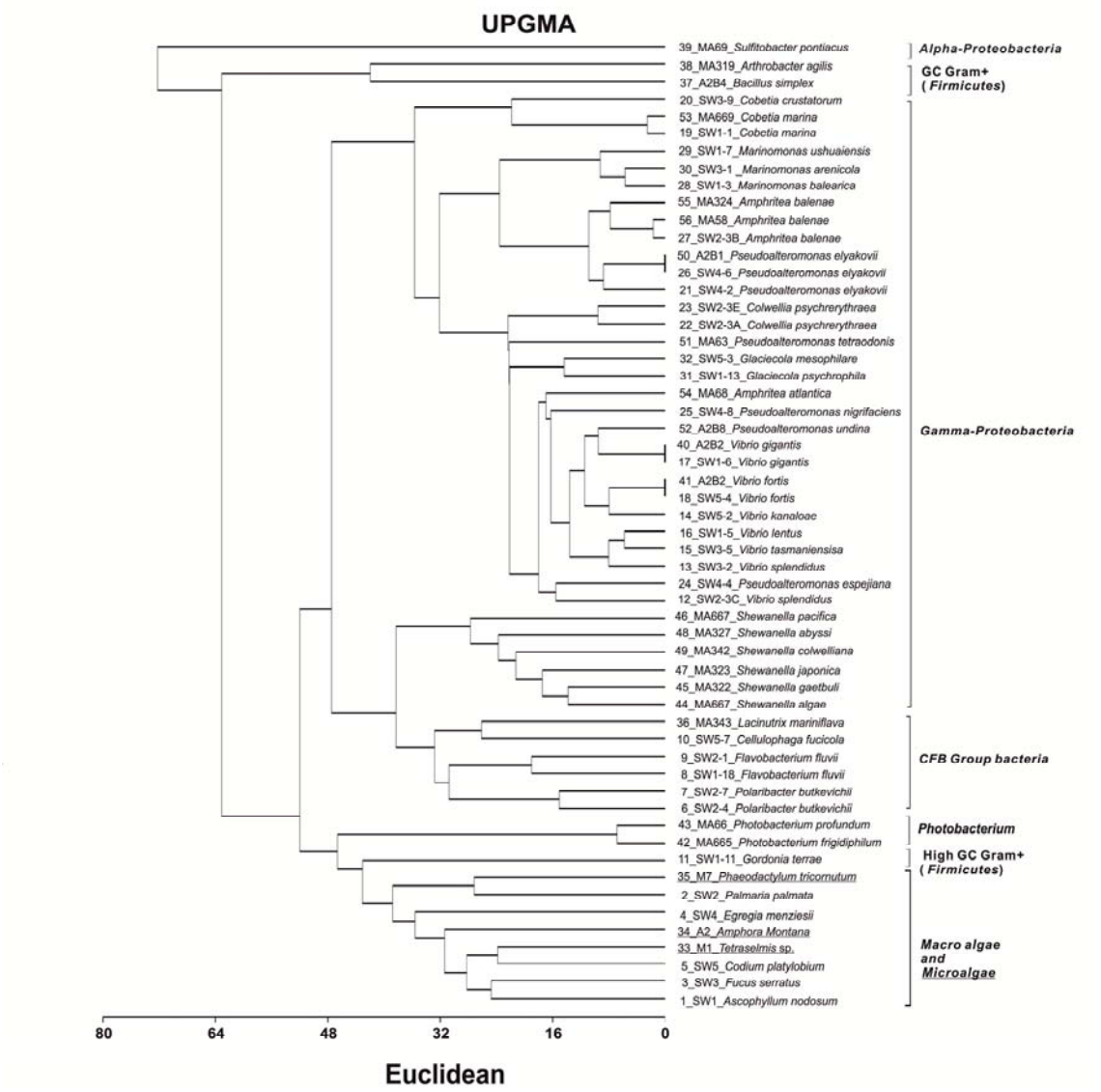

Figure 4.9 Similarity analysis based on whole cell fatty acid composition from macroalgae, microalgae and their associated bacterial major species. The phylogenetic tree was constructed by unweighted pair group average method (UPGAM) clustering method using the programs of MVSP package. Numbers indicated in the figure are the same as in Figure 4.8 and nearest type strains were indicated beside the isolates.

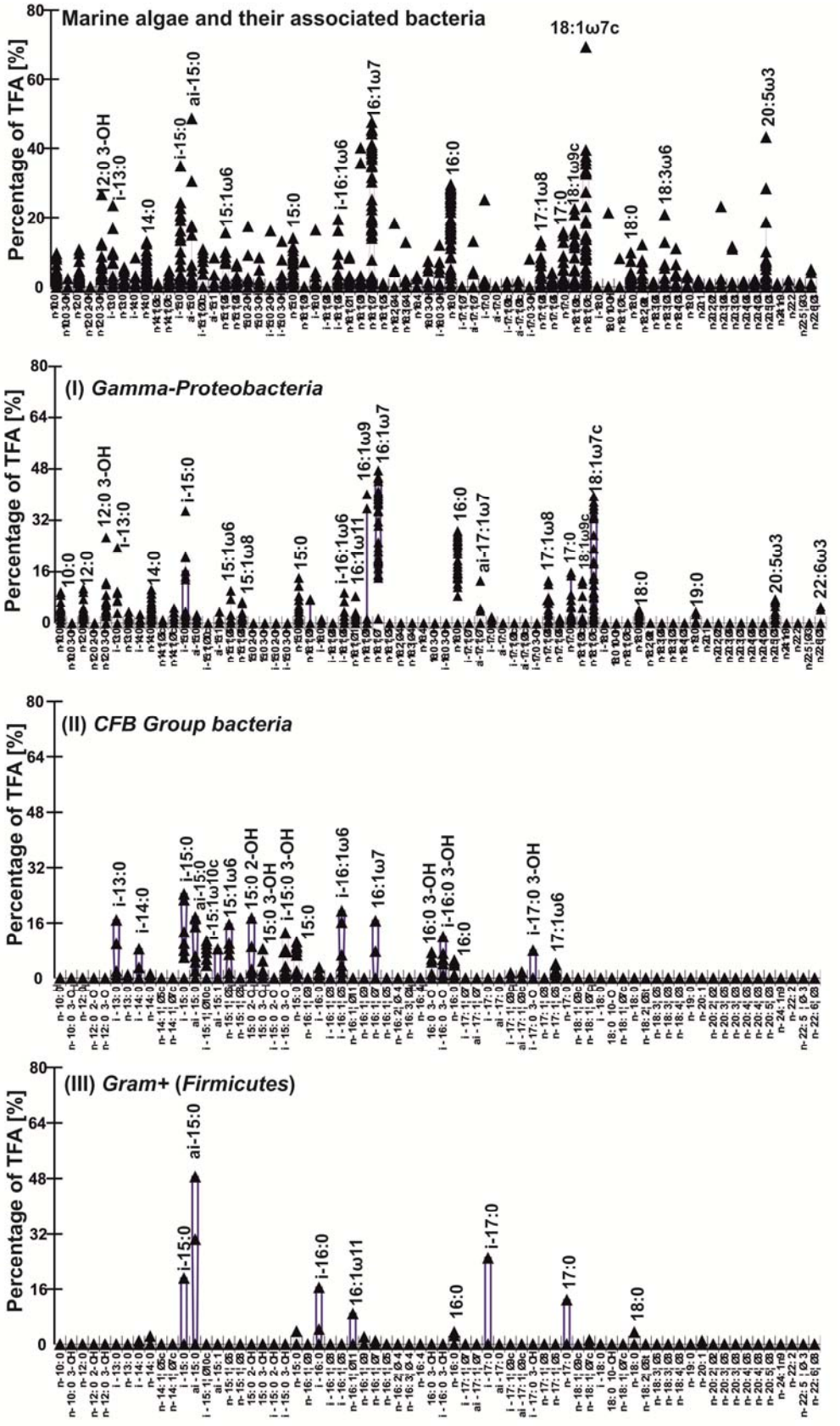

Figure 4.10 Box plots representing the distribution and variability in the percentage contribution of each fatty acid to the total fatty acids within *Gamma-Proteobacteria*, *CFB Group*, *Gram*$^{+}$ *(Firmicutes)* and *Alpha-Proteobacteria*. Groupings are as indicated in Figures 4.8 and 4.9. The triangles represent the presence of each fatty acid (Axis X) from different species analyzed, with percentage of TFA indicated by Axis Y.

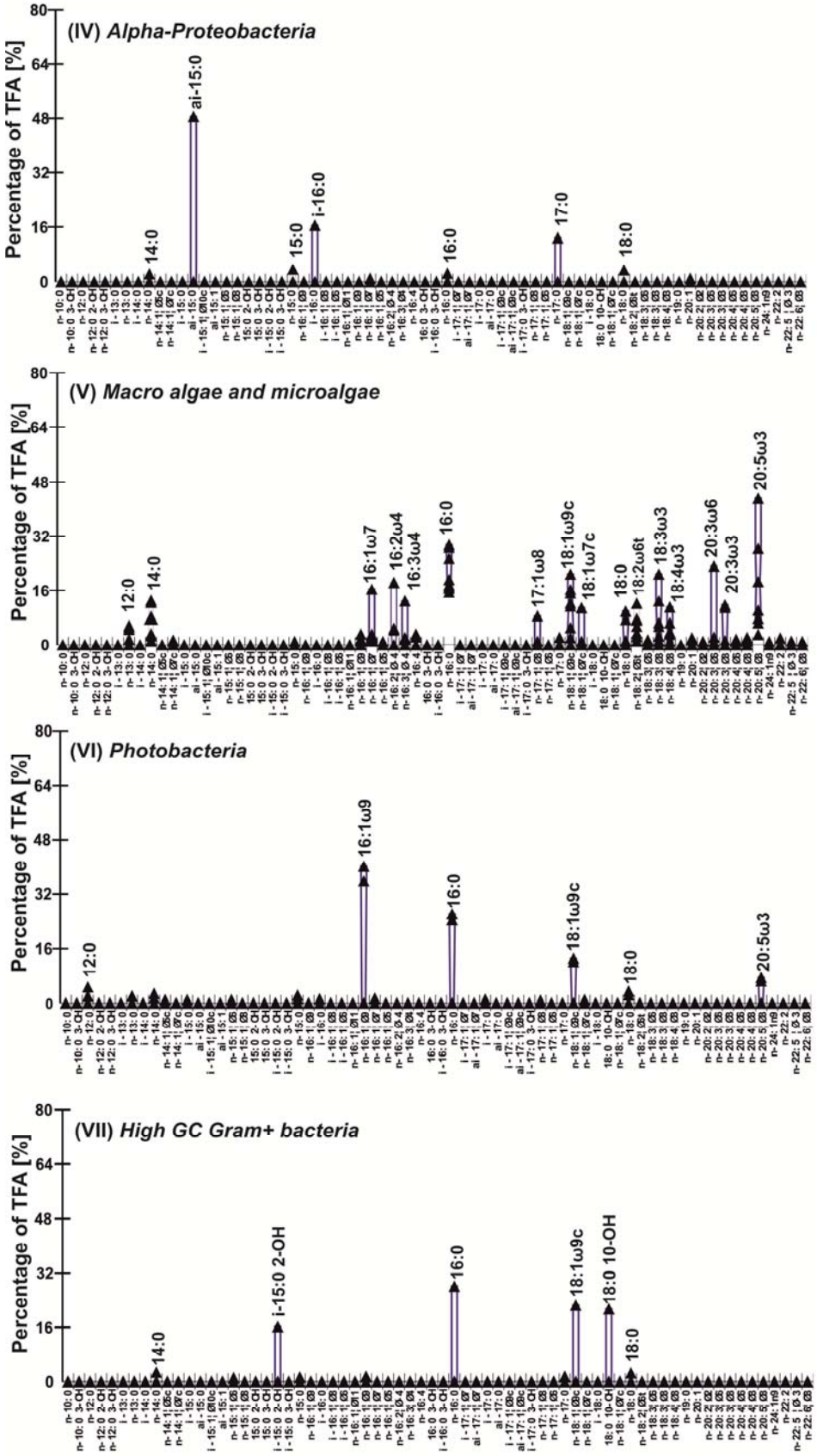

Figure 4.11 Box plots representing the distribution and variability in the percentage contribution of each fatty acid to the total fatty acids *Gamma-Proteobacteria*, *CFB Group*, *Gram*$^{+}$ *(Firmicutes)* and *Alpha-Proteobacteria*. Groupings are as indicated in Figures 4.8 and 4.9. The triangles represent the presence of each fatty acid (Axis X) from different species analyzed, with percentage of TFA indicated by Axis Y.

4.5 Discussion

4.5.1 Phylogenetic diversity of seaweed associated bacterial isolates

Both studies on phylogenetic diversity of macro and micro associated showed that the abundant presence of *Gamma-Proteobacteria* with fewer CFB group bacteria and Gram positive, which is consistent with the previous bacterioplankton studies have shown that the majority of marine bacteria are Gram negative. However, Gram positive bacteria as main population were also reported from other algal samples (Wahbeh and Mahasneh, 1984; Jensen and Fenical, 1995; Kanagasabhapathy et al., 2006). The variation in the distribution pattern of the bacterial genera may be related partly to inhibiting substances such as flavones, phenolic acids and tannins, excretion of metaboloytes which can act as nutrients for bacteria, and partly to differences in the quantity and quality of soluble organic exudates released by the plants (Wahbeh and Mahasneh, 1984), or could be variable according to the external climatic conditions (Martin and Bianchi, 1980).

Deep-sea microorganisms, such as *Photobacterium profundum* and *Ph. Frigidiphilum*, were used to be identified as psychropiezophiles model strains (Nogi et al., 1998b; Seo et al., 2005). Interestingly, similar species, such as strains MA665 and MA66 were isolated from the marine micro algal cultures, and with the ability of EPA production under atmospheric conditions. Meanwhile, *Colwellia psychrerythraea* was the only species in the genus *Colwellia* reported capable of producing DHA, isolated from Antarctic sea ice (Bowman et al., 1998a). Similar strains like SW2-3A and SW2-3E were isolated from seaweed surface and with the ability to produce DHA.

4.5.2 Algae–bacteria interactions

Marine biofouling communities are complex, highly dynamic ecosystems consisting of a diverse range of organisms. The development of such communities begins with bacterial attachment followed by the colonization of higher organisms such as invertebrate larvae and algal spores. Studies have examined bacterial attachment to algae (Droop and Elson, 1966; Rausch de Traubenberg and Soyer-Gobillard, 1990; Bowman et al., 1998a; Alavi et al., 2001; Biegala et al., 2002; Grossart et al., 2004), to diatoms (Rosowski, 1992; Bates et al., 2004), and to seaweeds (Shiba and Taga, 1980; Wahbeh and Mahasneh, 1984; Lemos et al., 1986; Nedashkovskaya et al., 2004; Rungprom et al., 2008).

The interactions between algae and bacteria in aquatic environments are numerous, possibly by the following reasons: (a) competition for available organic substrates or inorganic nutrient; (b) provision of extra-cellular material by algae which are of benefit to bacteria or vice versa; and (c) production of toxic metabolites which retard the growth of bacteria and vice versa (Jolley and Jones, 1977; Cole, 1982; Grossi et al., 1984; Grossart et al., 2004). Interestingly, bacteria may also mediate allelochemical interactions between different algal taxa (Keating, 1978).

In all the seaweeds and micro algae studied the most abundant fatty acids was n-16:0 and n-20:5ω3 (EPA), and the essential fatty acids such as n-18:2ω6 (linoleic acid), n-18:3ω3 (linolenic acid) and the eicosanoid precursors n-20:4ω6 (arachidonic acid) were presented at various low levels as reported previously (Sánchez-Machado et al., 2004). The fatty acids of seaweeds generally have linear chains, an even number of carbon atoms, and one or more double bonds (Shameel, 1990), and particularly, they can be a source of EPA (Khotimchenko, 1995). The average chain lengths of algal fatty acids were among 17.2-17.8, longer than any bacteria isolated in this study (14.5-16.8) as they contained higher levers of longer chain fatty acids, such as EPA.

Interestingly, genus *Vibrio* and *Colwellia* responsible for EPA or DHA production were among the macro algae associated bacteria, while genus *Vibrio*, *Photobacterium* and *Shewanella* were presented in the micro algal culture as EPA producers. These results are evidence that the presence of EPA/DHA in the genus *Colwellia*, *Vibrio*, *Photobacterium* and *Shewanella* might be related to the specific environment they attached associated with as all the algal samples were found with high content of EPA. Whereas DHA could only be detected both from the macro algae, such as *Palmaria palmata* (SW2) and its associated bacteria (*Colwellia*), and bacterial EPA producers (*Vibrio*, *Photobacterium* and *Shewanella*) are general exist as all the algal samples were found with high content of EPA.

Production of EPA/DHA is an important physiological and descriptive component that allows differentiation between algal/bacterial species, and may also have an important ecological role acting as a nutrient source for certain marine biota requiring essential fatty acids yet unable to synthesize omega-3 fatty acids *de novo*.

4.5.3 Phylogenetic resolution of FA patterns

Multivariate data analyses methods, such as principal coordinates analysis (PCO) has been used for analyzing the patterns of fatty-acid variables (Freese et al., 2008). In this study, two cases, fatty acid compositions of macro algae and their associated bacteria and FAs pattern of micro algae and their associated bacteria, have been demonstrated. The studies indicated that a taxonomic resolution on the basis of complete FA patterns is possible, as the cultivation conditions for all the bacteria cultures were consistent. However, when isolates belonging to different phyla were not sometimes dominated by the same FAs, or different isolates belonging to different phyla were sometimes dominated by the same major fatty acids, and unsuccessfully separation may be happened (Freese et al., 2008), as fatty acid composition will be variable according to their culture conditions (e.g. temperature and medium) (Yumoto et al., 2004). Therefore, the culture conditions should be carefully controlled, when taxonomy study was conducted based on fatty acid compositions.

4.6 Conclusions

In conclusion, marine macro/micro algae have a high content of omega-3 PUFAs, providing highly valuable supplement to human diets. Marine algae associated bacteria is good source for omega-3 PUFAs study. The proportion of EPA bacterial producers varied inversely with their host, indicating that environment remains the primary controlling factor in PUFA synthesis. Taxonomic resolution on the basis of complete FA patterns could be a possible way for study bacterial community structure.

Chapter 5. *Shewanella dovemarina* sp. nov., a psychrotrophic bacterium from deep-sea sediments producing high yields of eicosapentaenoic acid and electricity

5.1 Abstract

Two strains of bacteria, MAR441^T and MAR445 were isolated from Mid-Atlantic Ridge (MAR) sediments recovered from a depth of 2,734 m, and found to be members of the genus *Shewanella*. The strains were rod shaped, facultatively anaerobic, pigmented, non-motile, and capable of anaerobic growth either by fermentation of carbohydrates or by anaerobic respiration. Both strains can utilize a variety of electron acceptors, including nitrate and ferric compounds. The strains can deliver a stable power output of ~150-200 mW/m^2 when grown anaerobically in a two-chambered microbial fuel cell (MFC) using peptone as substance in the anode. The major fatty acids were typical of the genus *Shewanella*; however, PUFAs mostly composed of eicosapentaenoic acid (EPA) were produced at high levels (9.5--21.4 % of total fatty acids). Sequence analysis indicated that strain MAR441^T was most closely related to *Shewanella olleyana* (sequence similarity 97.9 %). Phenotypic characteristics confirmed that the isolate constituted a novel species of the genus *Shewanella*, which is designated *Shewanella dovemarina* sp. nov. (Type strain MAR441^T).

5.2 Introduction

Shewanella was described as a new genus in 1985 (MacDonell and Colwell, 1985), although historically it was initially classified as *Alteromonas* or *Pseudomonas* (Derby and Hammer, 1931; Long and Hammer, 1941). The genus *Shewanella* comprises a group of Gram-negative, facultatively anaerobic, motile straight or curved rod-shaped bacteria that are nonfermentative in general, although a few species have been reported capable of fermenting glucose (Bowman *et al.*, 1997b; Ivanova *et al.*, 2001). At the time of writing, at least 54 members of the genus *Shewanella* have been reported. An interesting feature of species of the genus *Shewanella* is the ability to produce polyunsaturated fatty acids (PUFAs) particularly eicosapentaenoic acid (EPA). The genus includes psychrophilic and mesophilic species and are widely distributed in

marine environments. Only a narrow group of predominately marine *Gamma-Proteobacteria* has the ability to produce PUFAs.

The species, *Shewanella benthica, S. abyssi*, *S. kaireitica*, *S. violacea*, *S. peizotolerans* and *S. psychrophila* isolated from the deep ocean could produce EPA at the level of 2-14 % of total fatty acids (TFA) (Deming et al., 1984; Delong and Yayanos, 1986; Delong et al., 1997; Nogi et al., 1998b; Miyazaki et al., 2006; Xiao et al., 2007). *Shewanella marinintestina*, *S. schlegeliana*, *S. sairae*, *S. pealeana*, *S. benthica*, *S. baltica*, *S. pneumatohori* and *S. waksmanii* were isolated from the intestinal tract of various marine animals, and could produce EPA at 15-37 % EPA of TFA (Yazawa et al., 1992; Leonardo et al., 1999; Satomi et al., 2003; Hirota et al., 2005; Amiri-Jami et al., 2006). *Shewanella hanedai*, *S. frigidimarina* and *S. halifaxensis* isolated from Antarctic marine environments were found with EPA at 7-22 % of TFA (Bowman *et al.*, 1997a; Bowman *et al.*, 1997b; Zhao *et al.*, 2006). *Shewanella affinis* and *S. japonica* were isolated from marine habitats and produced EPA at 2-8 % of TFA (Ivanova et al., 2001; Ivanova et al., 2004c). The mesophile *S. olleyana*, was isolated form a temperate estuary and capable of producing EPA at 24 % of TFA (Skerratt et al., 2002). Although the proportion of EPA as a percentage of TFA is reported, the high yield of total lipid with considerable EPA content would be much more desirable.

Deep-sea *Shewanella* species are taxonomically divided into two major subgenra, one characterised as high-pressure, cold-adapted species which produce substantial amounts of EPA and the other recognized as mesophilic pressure-sensitive species which do not produce EPA or produce only small amounts (Kato and Nogi, 2001). A high proportion of EPA in *Shewanella* isolates are mainly associated with cold adaptation (Nichols et al., 1997; Russell and Nichols, 1999). A physiological basis for the production of PUFA as a selective adaptation to reduced temperature and/or high pressure is well accepted (Nichols *et al.*, 1995). However, *Shewanella olleyana*, *S. japonica* and *S. pacifica* could produce significant levels of EPA at relatively high temperatures of 25–30 ºC (Ivanova et al., 2001; Skerratt et al., 2002; Ivanova et al., 2004b).

Members of the genus *Shewanella* were among the first microbes shown to grow by dissilimatory metal and radionuclide reduction. They may therefore influence the aqueous geochemical process occurring in the sea through biogeochemical reactions (Hau and Gralnick, 2007; Lassak et al., 2010). This genus has been extensively studied

in biogeochemical cycling and environmental clean-up (Moser and Nealson, 1996; Marshall et al., 2006b), and as also as exoelectrogens (Biffinger et al., 2007; Bretschger et al., 2007). Appendages of *S. oneidensis* were shown to be responsible for electricity conduction (Reguera et al., 2005; El-Naggar et al., 2010), whereas *S. putrefaciens* was reported to transfer electrons directly to an electrode (Kim et al., 2002).

In this study, two novel EPA producing psychrotrophs of the genus *Shewanella* were characterised. The bacteria were isolated from Mid-Atlantic Ridge (MAR) "non-vent" sediments at a depth of 2,734 m, and are capable of producing high levels of EPA at relatively low incubation temperatures and generating electricity in peptone-fed two-chamber microbial fuel cells (MFCs). This work was part of a taxonomic survey of microbial populations of the non-vent MAR deep sea sediments, to which relatively little attention has been given on the biological ecology study (Chapter 2).

5.3 Materials and methods

5.3.1 Sample collection and strain isolation as methods mentioned in Chapter 2

5.3.2 Morphological and biochemical identification

The two strains were routinely cultured on marine broth 2216E (Difco) before inoculation of biochemical and growth test media. Biochemical tests were performed with API-NE and API 20E test strips (bioMérieux, UK). For carbon and energy source tests, most of the test compounds were used at a concentration of 0.2 % (w/v), with the exception of carbohydrates, which were tested at a concentration of 0.5 % (w/v). Filled sea water was used for preparing medium, which was solidified with 1.4 % agar. Comparison of media with a control which lacked an added carbon source was used to assess carbon substrate use. Semi-solid agar containing triphenyltetrazolium chloride (TTC) was used to test motility, and compared with a positive control type strain *Shewanella japonica* KMM 3299^T. Other phenotypic tests used were conducted as described by Bowman et al. (1997). Anaerobic growth tests used media formulated by Coates et al. (1998) except that the basal medium was used filled sea water instead, and incubated in a 2.5 l anaerobic container system (GasPak™ EZ, BD, Maryland) containing anaerobic GasPaks (Oxoid) and included media controls prepared without an electron acceptor.

5.3.3 Strain growth

The temperature-growth response (4-30 °C) of strain MAR441 was conducted for growth in marine 2216E broth 2216E (MB) (Difco). Growth was observed and samples collected every day for five days, centrifuged and washed with sterile solution. For biomass production, strains were inoculated into 10 ml of Zobell's broth (ZB) (ZoBell, 1946), and incubated at 15°C until turbidity was apparent. The 10 ml cultures were then used to inoculate 90 ml volumes of marine 2216E broth in 500 ml conical flasks. Flasks were incubated at 15 °C with agitation provided by a magnetic stirrer or orbital shaker (180 rpm) for 24-48 h until sufficient mass of late-log phase cells were present for harvest.

5.3.4 16S rRNA gene analysis as methods mentioned in Chapter 2

The GenBank accession numbers for the 16S rRNA gene sequences of MAR441^T and MAR445 are FR744784 and FR744787.

5.3.5 Scanning electron microscopy

The cells were processed for scanning electron microscopy (SEM) (Cambridge Stereoscan 240). The samples were fixed in 2 % glutaraldehyde in 0.2 M sorensons phosphate buffer (pH 6.8) for 12 hrs, then rinsed in sorensons phosphate buffer twice for 15 mins and dried in alcohol series up to 100 %. The electrode samples were then CO_2-critical point dried by a Samdri 780 Critical Point Dryer, mounted on an aluminium stub with Achesons Silver ElectroDag and coated with 15nm gold/palladium (40/60) using a polaron SEM coating unit and then observed in SEM.

5.3.6 Transmission electron microscopy

Liquid grown cells at stationary phase were collected, washed and suspended in potassium phosphate buffer (pH 7.0), then fixed in 2 % glutaraldehyde in 0.2 M sorensons phosphate buffer (pH 6.8) for 12 hrs. The morphologies of living and nonliving stained cells were determined by light microscopy and transmission electron microscopy, respectively. For negative staining, one drop of a culture was placed on a copper grid coated with Pioloform and carbon and stained with 1 % potassium phosphotungstic acid adjusted to pH 7.0 with potassium hydroxide. The negatively-

stained cells were observed with a Philips CM100 transmission electron microscope (Biomedical EM Unit, Newcastle University) operating at 60 kV. Photographs were made on Kodak electron imaging film with type 4463 photographic emulsion.

5.3.7 Fatty acid analysis as methods mentioned in Chapter 2

5.3.8 MFC construction and measurement as methods mentioned in Chapter 9

5.4 Results and discussion

5.4.1 Morphological identification

The strains demonstrated cellular and colonial morphologies and phenotypic profiles typical of *Shewanella* species. Cells were rod-shaped, Gram-negative, 1.5–4.5 μm in length, 0.4–0.76 μm in diameter, without flagella (Figure 5.1). Colonies on marine agar plates were tan-pigmented, butyrous in consistency, smooth, and circular and convex in shape with an entire edge. Colonies of 2-4 mm in diameter were formed following 2 days incubation at 15 °C. The agar beneath colonies on marine 2216E agar became softened, but not liquefied, however became transparent and increasingly mucoid with prolonged incubation. Flooding the agar surface with Lugols's iodine solution revealed hydrolysis zones around the growth, suggesting that the strains have an agarolytic ability. No hydrolisis zone was formed in triacylglycerol agar plates, indicating that the strains are unable to produce lipase. MAR441^T and MAR445 cells mobility were observed negative when grown on plates of semi-solid motility test media containing 0.5 % triphenyltetrazolium chloride (TTC), whereas *Shewanella japonica* KMM 3299^T showed fuzzy growth (indicated by pink color) away from the line of inoculation, which denoted motility.

The strains were psychrotolerant and stenohaline. Growth was observed between 4 and 30 °C with best growth at 15-20 °C. Growth on agar media at 4 °C was slower (2-4 d) and less prolific than that at 10-25 °C (1 d). No growth was observed for either strain above 30 °C. Weak growth was observed for MAR441^T and MAR445 at 30 °C in marine broth. Strains required Na^+ for growth and grew between 0.05 and 0.7 M NaCl

(0.3-7 %), with best growth at 0.05-0.5 M NaCl (0.3-3 %). NaCl concentrations below or above the optimal range slightly inhibited PUFA synthesis. No growth detected at 8 % NaCl. However, the two strains grew very well in the medium supplemented with only K^+ or Fe^{3+}, but poor in the medium supplemented with only Zn^{2+} or Ca^{2+}.

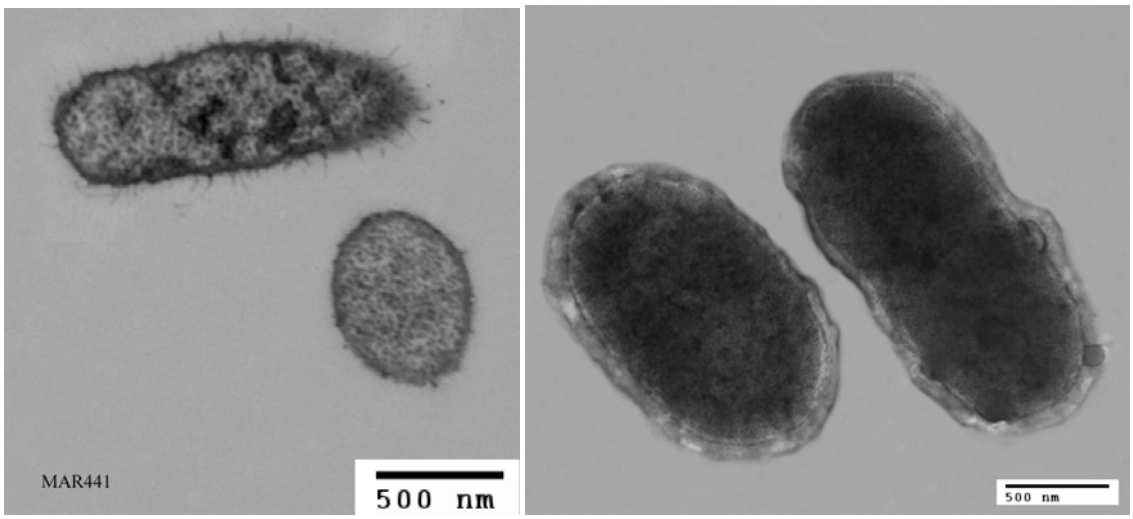

Figure 5.1 Scanning electron microscopy (Left, Bar 500 nm) and transmission electron microscopy (Right, Bar 500 nm) of a negatively-stained cell of *Shewanella* sp. MAR441

5.4.2 Biochemical identification

Oxidase- and catalase-positive. Reduces nitrate to nitrite. Arginine dihydrolase and lysine decarboxylase are not observed. Haemolytic, produces amylase, esterase (Tween 20, 40, 80), proteinase (gelatinase), and agarase. Chitin is not hydrolysed. H_2S is formed from thiosulfate anaerobically. Indole is not formed from L-tryptophan. Voges–Proskauer test is negative. D-Glucose, glycerol, starch, cellulose, agarose, L-leucine, L-serine, L-alanine, L-proline, Tween 80, Tween 60 and Tween 40 are utilized as sole source of carbon for EPA production (see Chapter 6). Does not utilize D-galactose, D-fructose, N-acetylglucosamine, succinate, D-mannose, lactose, propionic acid, fumarate or L-tyrosine, triacylglycerol, cellulose, chitin, dextran, casein, elastin, DNA or uric acid. The following substrates (according to Biolog) are utilized: dextrin, starch, cellulose, agarose, Tween 40, 60 and 80, L-arabinose, D-cellobiose, D-Glucose, maltose, c-hydroxybutyric acid, a-ketobutyric acid, aketoglutaric acid, a-ketovaleric acid, D-saccharic acid, succinic acid, L-alanine, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, L-leucine, DL-carnitine, c-aminobutyric acid,

urocanic acid, putrescine, pyruvate, 2-aminoethanol, 2,3-butanediol, glycerol, DL-a-glycerol phosphate, a-D-glucose 1-phosphate and Dglucose 6-phosphate.

5.4.3 Molecular identification

Identification by bacterial 16S rRNA gene sequence analysis showed that strains MAR441^T and MAR445 exhibited 16S rRNA gene sequence similarity of 92.6-97.9 % and 92.5-97.8 % respectively to the type strains of the other 54 *Shewanella* species. Among these, four type strains showed 16S rRNA gene sequence similarity of more than 97 % to strains MAR441^T and MAR445. Strains MAR441^T and MAR445 were most closely related (97.9 % and 98.0 % respectively) to *Shewanella pacifica* KMM 3597^T (AF500075), which were isolated from Sea of Japan, Pacific Ocean with production of EPA (Ivanova et al., 2004b); 97.9 % to *Shewanella olleyana* strain ACEM 9^T (NR_025123) isolated from a temperate estuary with high lever of EPA production (Skerratt *et al.*, 2002); 97.9 % to *Shewanella japonica* KMM 3299^T (NR_025012) (Ivanova *et al.*, 2001) and 97.5 % to *Shewanella donghaensis* strain LT17^T (AY326275) isolated from deep-sea sediments with high production of PUFAs (Yang et al., 2007), and 95.5 % to other type strains *Shewanella arctica* 40-3T (AJ877256), *Shewanella baltica* OS185 (AJ000216) (Ziemke *et al.*, 1998), *Shewanella massilia* (AJ006084) (Dos Santos *et al.*, 1998), *Shewanella gaetbuli* isolate UL19 (AM180742) (Marshall et al., 2006a). However, strain MAR441^T was phylogenetically affiliated with type strain *Shewanella olleyana* strain ACEM 9 (NR_025123) in the same clade on the phylogenetic tree, although they only shared 16S rRNA gene sequence similarity of 97.9 %. Therefore strain MAR441^T and MAR445 can be considered as a separate species in that 16S rRNA gene sequence showing <98 % sequence similarity according to previous studies (Fry et al., 1991; Dighe et al., 2004; Janda and Abbott, 2007). Thus we placed these strains in the genus *Shewanella*, as *Shewanella* sp. strains MAR441^T and MAR445. The similarity between MAR441^T and MAR445 is 99.8 % (Figure 5.2).

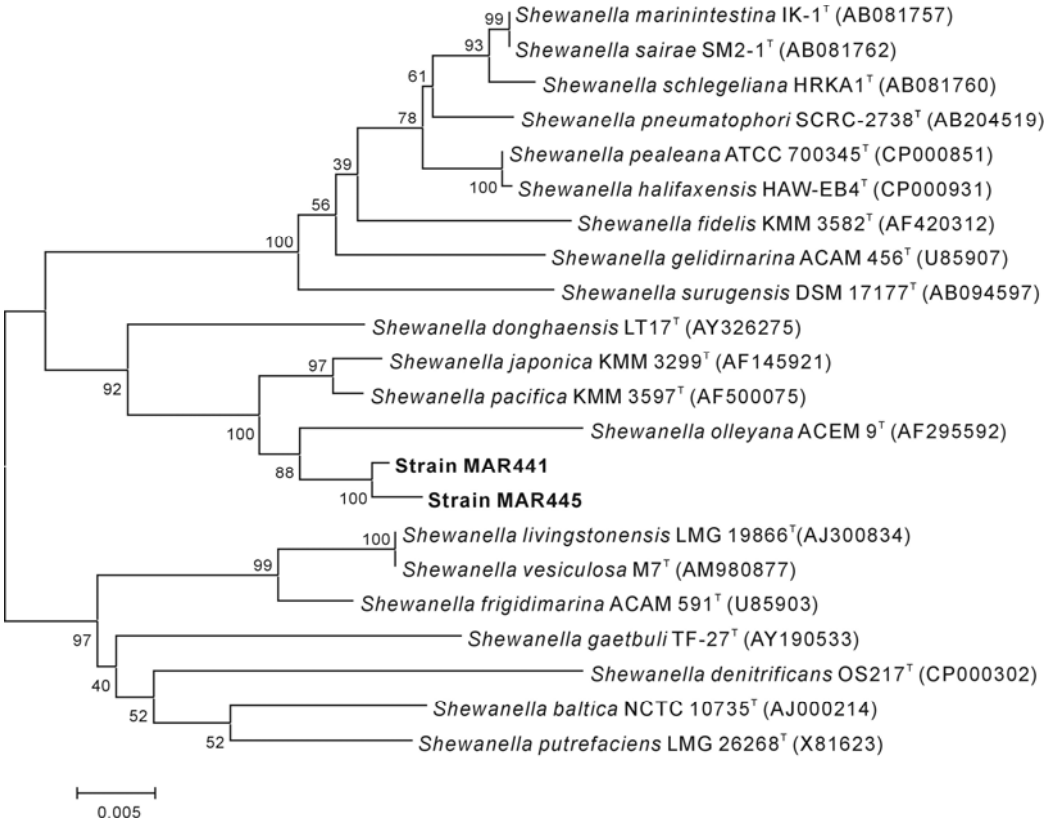

Figure 5.2 Phylogenetic tree based on 16S rRNA gene sequences of strains MAR441^T, MAR445 and various most related *Shewanella* type species was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.005 nucleotide substitution per site.

5.4.4 Electricity production

The strains MAR445 and MAR441^T were facultatively anaerobic. Respiratory anaerobic growth was supported on a variety of electron acceptors when sodium lactate or sodium acetate was used as the electron donor. Electron acceptors used included Fe(III) (50 mM ferric citrate or 10 mM amorphous ferric oxides), 10 mM sodium nitrate, 25 mM sodium fumarate and sodium nitrite (5 mM) and sodium sulfite (10 mM) also supported the growth.

Both strains can grow well anaerobically in peptone-fed two-chamber microbial fuel cells (MFCs) when they were cultivated in a fresh anaerobic corresponding growth medium with 10 mM peptone as the electron donor. Generally, the microbial fuel cells exhibited a lag phase (about 2 days) before voltage started to increase. Figure 5.3 shows

the voltage output produced by one of the microbial fuel cells inoculated with MAR441^T and MAR445 respectively, delivering a stable power output of ~150-200 mW/m^2 for 6 days, and then decreased gradually, probably due to peptone depletion. The control microbial fuel cell remained sterile and did not show voltage increase (Figure 5.3).

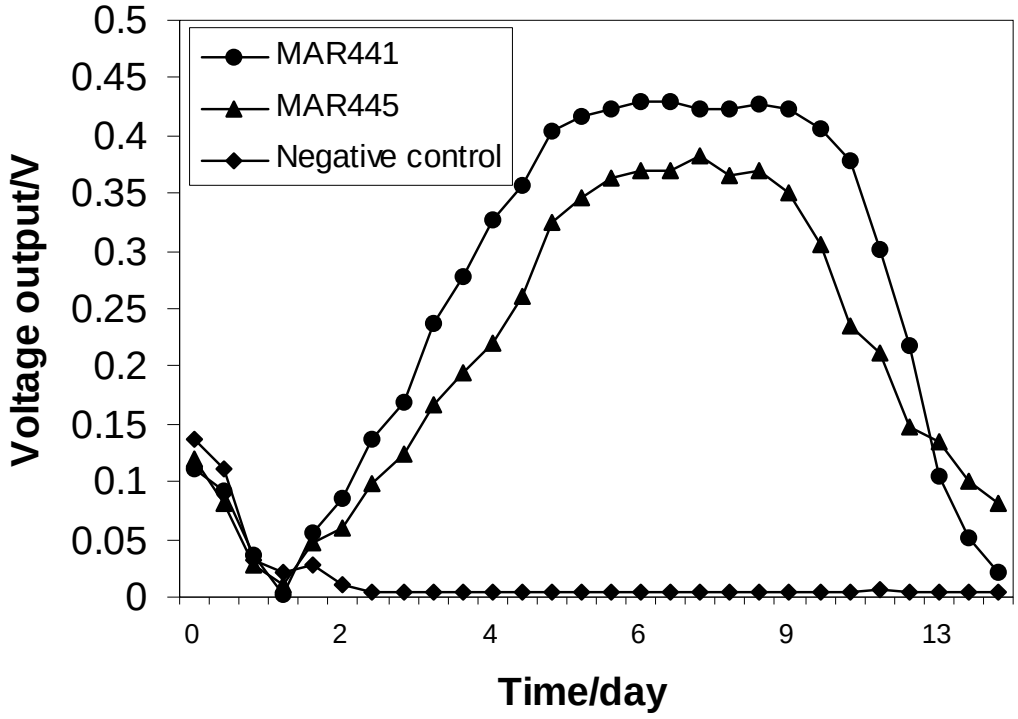

Figure 5.3 Voltage output produced by MFCs containing MAR441^T culture (filled circles) and MAR445 culture (filled triangles). One set-up with no inoculum was also operated in parallel as a control (filled diamonds). The experiments were carried out in duplicate and values are means of two samples.

5.4.5 Fatty acid compositions

The fatty acid composition of MAR441^T exhibited changes in response to growth temperature. Growth at temperatures within or below the optimal region resulted in an increased percentage of PUFAs (27.8 % at 4 °C versus 2 % at 25 °C), and a decreased proportion of short-chain saturated components (Table 5.1). Both the percentage and the quantitative level of EPA decreased markedly at growth temperatures above the optimal region (21.4 % at 4 °C versus 0.6 % at 25 °C), indicating that PUFA may play a critical role in the modulation of membrane fluidity and the homeostatic adaptation of cellular membrane viscosity (Russell and Nichols, 1999). As growth temperature increased, MAR441^T also demonstrated a novel adaptational response, involving an increase in the

percentage of n-13:0 and i-15:0 with corresponding decrease of n-16:1ω7 and n-18:1ω7, which might be concerned the role of fatty acid primers selection within this bacterium as an adaptational response.

Table 5.1 Temperature dependence of the fatty acid composition of strain MAR441^T and MAR445 grown in marine broth 2216E medium

	MAR441^T				MAR445			
Fatty acids	4 °C	15 °C	15 °C[b]	25 °C	4 °C	15 °C	15 °C[b]	25 °C
n-12:0	1.3	2.2	2.2	0.3	2.1	1.9	1.8	2.3
n-13:0	13.5	23.6	4.2	32.1	20.9	23	6.9	47.5
n-14:0	3.9	2.9	3.3	2.7	4.1	3.9	4.2	2.1
n-15:0	3.5	4.5	1.5	1.9	2.3	2.6	0.8	0.3
n-16:0	5.3	14.5	20.7	12.4	11	12.1	22.5	14.7
n-17:0	0.3	0.8	0.5	5.7	0.6	0.7	0.5	0.5
n-18:0	0.5	1.3	0.2	1	0.4	1.3	0.3	1
Σ SCFA	**28.3**	**49.7**	**32.6**	**56**	**41.4**	**45.5**	**37**	**68.4**
i-13:0	11.6	4.9	6.9	11.2	7.2	7.3	7.8	1.2
i-14:0	0.7	0.4	0.1	0.2	0.7	0.7	0.4	0.1
ai-15:0	1.2	0.4	0.2	0.8	0.2	0.1	0.3	0.3
i-15:0	7.2	10.2	1.2	14.2	12.3	10.3	3.3	1.8
i-17:0	0.5	0.2	0.2	0.2	-	-	-	-
Σ BCFA	**21.2**	**16.2**	**8.6**	**26.6**	**20.4**	**18.4**	**11.8**	**3.4**
n-15:1ω6	0.1	0.9	0.2	0.6	0.1	-	-	-
n-16:1ω9	-	-	-	-	13.7	14.8	14.5	18.1
n-16:1ω7	16.9	11.2	30.5	9.3	1.1	0.3	16.4	0.85
n-17:1ω8	0.2	2.8	9.3	4	0.3	1.5	4	0.8
n-18:1ω7c	5.2	4.2	16.3	1.1	4.9	5.1	12.1	4.7
Σ MUFA	**23.3**	**19.7**	**56.3**	**15.5**	**20.1**	**21.7**	**47**	**24.45**
n-18:2ω6t	2.8	1.5	0.1	0.3	0.1	-	0.3	-
n-18:3ω3	0.3	0.6	-	0.2	0.1	0.3	-	0.9
n-20:5ω3	21.4	9.5	1.4	0.6	16.6	11.9	2.7	2.4
n-22:5ω3	0.8	0.3	-	0.1	-	0.4	-	0.3
Σ PUFA	**27.8**	**14.4**	**1.5**	**2**	**16.8**	**12.6**	**3**	**3.6**
Others	3.9	3	1	1.2	1.3	1.8	1.2	0.1
Total	100	100	100	100	100	100	100	100
ACL	16.3	15.5	15.8	14.7	15.6	15.6	15.6	14.6
EPA (mg g^{-1})	24.7	9.7	1.4	0.2	17.0	12.1	2.6	2.4
TFA (mg g^{-1})	115.2	102.5	99.7	96.5	102.2	101.5	98.5	100.5
Cells(g l^{-1})[a]	2.25	2.6	1.76	0.6	2.3	2.5	1.65	0.9

[a]Cellular dry weight; [b]Cells were obtained from anaerobic culture; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

The values of average chain length (ACL) (from 16.29-14.67) and quantitative level of EPA decreased with increasing growth temperature (24–0.2 mg g^{-1} cells dry weight) at all growth temperatures from 4-25 °C. When strain MAR441^T was cultured

anaerobically in marine broth at 15 °C, the MUFAs was greatly increased to 56 % in TFAs with accumulating n-16:1ω7 and n-18:1ω7c, by decreasing SCFAs, BCFAs and PUFAs, and only with 1.4 % EPA presented. Fatty acid compositions responding to different temperatures (4-25 °C) in MAR445 were similar to those of MAR441^T, except considerable amount of n-16:1ω7 was detected in MAR445. This fatty acid profile of both strains was similar to those of related *Shewanella* species.

5.5 Discussion

Morphologically, on marine agar plate, strain MAR441^T shared most of the similarity with Estuary *Shewanella* species (Skerratt *et al.*, 2002), but with more temperature sensitive and showed weak grow above 30 °C and can not produce lipase. Biochemically, strain MAR441^T can utilize maltose. These are the key differences from the two phylogenetically close type strains: KMM 3597^T and ACEM 9^T (Skerratt et al., 2002; Ivanova et al., 2004b). Generally, it was believed that flagella-based motility is typical of genus *Shewanella* (Table 5.1), however, this is not universal in that strain MAR441^T showed no presence of flagella, but only fimbriae on the cell surface. These fimbriae were helpful for biofilm formation on a solid surface according to a non-fimbriae mutant by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) mutation experiment (Chapter 6, Figure 6.5).

The presence of EPA is an important physiological and descriptive component that allows differentiation between *Shewanella* species (Skerratt *et al.*, 2002). Strain MAR441^T is one of the highest EPA bacterial producers by proportions and/or quantitative comparing to other high EPA-producing *Shewanella* species isolated from polar, deep sea and estuarine environments (Bowman et al., 1997b; Nichols et al., 1997; Kato and Nogi, 2001; Skerratt et al., 2002). The proportion of EPA varied inversely with temperature by regulating some other fatty acids, e.g. n-13:0 and i-15:0, for strain MAR441^T (Table 5.1), indicating that temperature remains the primary controlling factor in PUFA synthesis. Production of EPA by some bacteria increases as temperature decreases, leading to the hypothesis that these molecules may be important for growth at low temperatures (Delong and Yayanos, 1986; Valentine and Valentine, 2004; Amiri-Jami et al., 2006). Cells must cope with decreases in temperature by modulating the composition of their lipid membrane, which can crystallize or enter nonbilayer phases at low temperatures (Russell and Nichols, 1999). High content of unsaturated fatty acids

was observed from the MAR441^T anaerobic culture, probably due to the activation of oxygen-independent (anaerobic) pathway catalysed by a fatty acid synthetase (Yano *et al.*, 1998). A shortage of oxygen easily occurs in deep-sea environments where reduced sulfur compounds or other metals are supplied constantly as final electron acceptor for microbes in their respiratory pathway (Woulds *et al.*, 2007).

Strains of *Shewanella*, especially those metal reducing bacteria, were also found with active of power generation since the first direct proof of electrical current generation in an MFC by *S. putrefaciens* IR-1 (von Canstein *et al.*, 2008), by donating electrons to the anode directly through outer membrane cytochromes or through the reduction of redox mediators (quinones and quinolines) secreted by the bacteria (Lovley, 2006). The power density produced by strain MAR441 was competitive comparing to that from strain *S. oneidensis* MR-1 (Watson and Logan, 2010).

Strains MAR441^T and MAR445 can be distinguished easily from *Shewanella olleyana* by their capacity to utilize D-glucose and maltose. They can grow at 4 °C or in the presence of 6 % NaCl, unlike *S. japonica* which does not require Na^+ ions for growth, and unlike *S. pacifica* can not utilize succinate. A lack of motility and the inability to produce lipase also clearly differentiated strains MAR441^T and MAR445 from other *Shewanella* species (Table 5.2). Therefore, on the basis of phenotypic, chemotaxonomic and phylogenetic data, strains MAR441^T and MAR445 represent a distinct species within the genus *Shewanella*, for which the name *Shewanella dovemarina* sp. nov. is proposed.

Description of *Shewanella dovemarina* sp. nov.

Shewanella dovemarina (dove.ma`ri.na. N.L. fem. adj. *dovemarina* from Dove Marine Laboratory, Newcastle University, where these strains were isolated).

Growth Cells are rod-shaped, Gram-negative, 1.5-4.5 μm in length, 0.4-0.8 μm in diameter and have no flagella. Temperature range for growth is 0-30 °C. Optimum temperature for growth was 15 °C. Requires Na^+ ions for growth (minimum 0.05 M, optimal 0.1-0.5 M, maximum 1.2 M). Oxidase- and catalase positive. Facultatively anaerobic chemoheterotroph. Can grow anaerobically by respiration using ferric citrate, fumarate, amorphic ferric oxide, nitrate, thiosulfate, trimethylamine *N*-oxide (TMAO) and anthraquinone-2,6-disulfonate (AQDS) as electron acceptors and lactate as an

electron donor. Carbohydates are fermented with production of electricity. Nitrate is reduced to nitrite in the presence of oxygen. Ornithine decarboxylase, arginine dihydrolase and lysine decarboxylase are not observed. Hydrolyses dextrin, starch, cellulose, agarose, Tween 40, 60 and 80, L-arabinose, D-cellobiose, D-Glucose, maltose, c-hydroxybutyric acid, α-ketobutyric acid, aketoglutaric acid, α-ketovaleric acid, D-saccharic acid, succinic acid, L-alanine, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, L-leucine, DL-carnitine, c-aminobutyric acid, urocanic acid, putrescine, pyruvate, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate and Dglucose 6-phosphate, but not D-galactose, D-fructose, N-acetylglucosamine, succinate, D-mannose, lactose, propionic acid, fumarate or L-tyrosine, triacylglycerol, cellulose, chitin, dextran, casein, elastin, DNA or uric acid. Agarolytic activity is positive. Production of H_2S from L-cysteine is negative (from API 20E test strip results) but H_2S was formed from thiosulfate anaerobically. Indole is not formed from L-tryptophan. Voges-Proskauer test is negative. Forms tan-pigmented, butyrous in consistency, smooth, and circular and convex in shape with an entire edge colonies 2-4 mm in diameter following 2 d incubation at 15 °C. Major fatty acids are n-13:0, iso-13:0, iso-15:0, n-16:0, n-16:1ω7, n-18:1ω7 and n-20:5ω3 (Table 5.2). Based on 16S rDNA nucleotide sequence analysis, the species belongs to the family *Alteromonadaceae*, order `*Alteromonadales*' and class *Gammaproteobacteria*'. The type strain is MAR441^T isolated from Mid-Atlantic Ridge (MAR) “non-vent” sediments at a depth of 2,734 m.

Table 5.2 Characteristics that differentiate *Shewanella dovemarina* from the most phylogenetically related species.

Phenotypic characteristic	*Shewanella dovemarina* MAR441^T	*Shewanella dovemarina* MAR445	*Shewanella pacifica* KMM 3597^T	*Shewanella olleyana* ACEM 9^T	*Shewanella japonica* KMM 3299^T	*Shewanella donghaensis* LT17^T	*Shewanella baltica* NCTC 10735^T	*Shewanella gaetbuli* TF-27^T	*Shewanella frigidimarina* ACAM 591^T	*Shewanella livingstonensis* LMG 19866^T
Requires Na^+ ions for growth	+	+	+	+	-	+	+	+	-	+
Growth in NaCl at:										
0 %	-	-	-	-	+	-	+	-	+	+
3 %	+	+	+	+	+	+	+	+	+	+
6 %	+	+	+	+	-	-	+	-	+	-
Growth at 4 °C	+	+	+	+	-	+	+	-	+	+
Growth at 30 °C	-	+	+	+	+	-	-	+	-	-
Growth at 35 °C	-	-	-	-	+	-	-	+	-	-
Ornithine decarboxylase	-	-	-	-	ND		+		ND	+
NO3 ⟶ NO2 (+O2)	+	+	+	ND	+	+	+	-	+	+
Production of										
DNase	-	-	ND	-	+	ND	+	ND	+	ND
Amylase	+	+	+	+	+	-	-	+	-	-
Lipase	-	-	+	+	+	+	ND	+	ND	+
Gelatinase	+	+	+	-	+	+	+	+	ND	+
Chitinase	-	-	-	-	-	-	ND	ND	-	-
Utilization of:										
D-Glucose	+	+	+	-	+	-	+	-	+	+
N-Acetylglucosamine	-	-	-	-	+	-	+	-	+	-
Maltose	+	+	ND	-	+	-	+	-	-	-
Sucrose	-	-	ND	+	-	-	+	-	+	-
D-Gluconate	+	+	ND	+	-	-	+	-	-	-
DL-Lactate	-	-	-	-	-	-	ND	-	+	+
Succinate	-	-	+	-	-	-	ND	-	+	ND
Citrate	-	-	-	-	-	-	+	-	+	-
EPA synthesis	+	+	+	+	+	+	-	-	+	-
Electricity production	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Motile	-	-	+	+	+	+	+	+	+	+
Mol % GC	ND	ND	40	44	43-44	38.8	46	42	45	41

+, Test is positive; -, test is negative; d, test results vary amongst strains of the species ; ND, data not available. Data for reference species were taken from Bowman *et al*. (1997), Ziemke *et al*. (1998), Ivanova *et al*. (2001), Bozal *et al*. (2002), Skerratt *et al*. (2002), Ivanova *et al*. (2004), Yang *et al*. (2004) and Yang *et al*. (2007).

Chapter 6. Optimization of eicosapentaenoic acid production by deep-sea strain *Shewanella* sp. MAR441

6.1 Abstract

Bacteria capable of producing omega-3 fatty acids have been widely reported. However, biotechnological production of bacterial omega-3 is rarely studied. We isolated a strain from Mid-Atlantic Ridge (MAR) deep-sea sediments, *Shewanella* sp. strain MAR441, which was phylogenetically unusual, and could produce the highest recorded yields of eicosapentaenoic acid (EPA), to date. The strain required Na^+ for growth and EPA synthesis, cells harvested at late exponential or early stationary phase with higher content of EPA. Fatty acid composition was influenced by provision of potential acyl chain precursor as sole carbon sources. Both the highest amounts and percentage of EPA occurred from growth on L-proline (15 mg g^{-1} and 15.6 %). The combination medium of L-proline and $(NH_4)_2SO_4$ greatly improved the amount and percentage of EPA (20 mg g^{-1} and 18 %). In the cerulenin-treated cells, decreases in levels of middle-chain fatty acids and remarkable increases in levels of EPA were observed. Monounsaturated fatty acid components and higher content of EPA were concentrated in phosphatidylglycerol (PG), while the proportion of branched-chain fatty acids was elevated in phosphatidylethanolamine (PE). EPA was also a large component (18.7 %) of a non-esterified fatty acid (NEFA) fraction within the total lipid extract of the bacterium.

6.2 Introduction

Omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) play a role in the modulation and prevention of human diseases, in particular heart, cardiovascular and Alzheimer's disease (Kris-Etherton et al., 2003b). The omega-3 family is found mainly in fish. There are, however, potential problems associated with fish oils as a source of PUFA such as: taste, odor, stability as well as coextracted contaminants (Arts et al., 2001). Nevertheless, the crucial problem of those oils is their sustainability due to the worldwide decline of fish stocks (Garcia and Rosenberg, 2010). Therefore, natural production of omega-3 by marine microbes is a potential alternative source of nutrients. EPA producing microalgae such as *Chlorella* may be used as an alternative to fish oil,

but the cultivation of microalgae require strictly controlled growth conditions in terms of nutrients, light quantity and quality, oxygenation and carbon dioxide levels, which can result in considerable expense (Seto et al., 1984). In contrast, most bacteria are not fastidious, and can often be grown on the waste products of other agricultural or industrial processes.

So far, bacterially derived PUFAs were mainly from Gram-negative strains (Valentine and Valentine, 2004), which contain limited genera *Shewanella*, *Moritella*, *Colwellia*, *Alteromonas*, *Photobacterium*, *Flexibacter* and *Psychroserpens* (Nichols and McMeekin, 2002). These EPA/DHA derived bacteria include psychrophiles or piezophiles were isolated from polar regions and the deep sea (Delong and Yayanos, 1986; Bowman et al., 1997b; Nogi et al., 1998a; Nichols et al., 1999; Kato and Nogi, 2001; Gentile et al., 2003; Wang et al., 2009), as well as mesophiles isolated from a temperate estuary (Skerratt et al., 2002) and from shallow seawater samples (Ivanova et al., 2001; Ivanova et al., 2003a; Frolova et al., 2005). However, it is unclear why these bacteria produce omega-3 fatty acids. Production of EPA by some bacteria increases as temperature decreases, leading to the hypothesis that these molecules may be important for growth at low temperatures (Delong and Yayanos, 1986; Valentine and Valentine, 2004; Amiri-Jami et al., 2006). Cells must cope with decreases in temperature by modulating the lipid composition of their membrane, which can crystallize or enter nonbilayer phases at low temperatures (Russell and Nichols, 1999). EPA was not required for low-temperature growth in the deep-sea bacterium *Photobacterium profundum* (Allen et al., 1999), but it may be required for low temperature growth in *Shewanella* (Valentine and Valentine, 2004; Sato et al., 2008; Wang et al., 2009).

It has additionally been suggested that bacterial EPA may play an antioxidative role (Nishida et al., 2006). Whereas few studies available on the physiology of bacterial PUFA production under varying culture conditions (Akimoto et al., 1990; Suzuki et al., 1991; Suzuki et al., 1992; Henderson et al., 1993; Hamamoto et al., 1994; Nichols et al., 1994; Bowman et al., 1997b; Nichols et al., 1997; Gentile et al., 2003), which highlighted that PUFA biosynthesis, speciation and the interaction of PUFA with other fatty acid types in the adaptive responses of bacteria to changing environmental conditions could be manipulated. Thus, continued research utilizing a variety of bacterial strains is warranted to more fundamentally understand PUFA biosynthesis, speciation and adaptive responses.

In this study we describe the physiological characterization of a psychrotrophilic strain *Shewanella* sp. MAR441 isolated from the Mid-Atlantic Ridge (MAR) "non-vent" site at the depth of 2,500 m. MAR441 was a phylogenetically unusual *Shewanella* species based on conventional phenotypic and phylogenetic analyses (another study). Production of PUFA was scored under cultivation with different carbon sources, artificial sea water with different concentration of Na^+, different time course of the culture and at different temperatures, as well as on the cerulenin-treating cells. The acyl chain speciation of the major phospholipid classes and non-esterified fatty acid (NEFA) fraction in MAR441 are also described.

6.3 Materials and methods

6.3.1 Bacterial strain and cultivation as methods mentioned in Chapters 2 and 5

Strain MAR441 was isolated from Mid-Atlantic Ridge (MAR) "non-vent" site at the depth of 2,500 m, and was found to be a member of the genus *Shewanella* based on phenotypic characterization, biochemical studies and 16S rRNA gene sequence analysis with most closely related to *Shewanella olleyana* (sequence similarity 97.9 %) (See Chapter 5). Strain was routinely cultured in marine 2216E broth (MB) (Difco) before inoculation of following growth conditions/media.

6.3.2 Cultivation conditions based on temperature

The temperature-growth response (4-30 °C) of strain MAR441 was conducted by growing in marine broth (MB). Growth was observed and samples collected every day for five days, centrifuged and washed with sterile solution. The generation time (*g*) was used to evaluate the cell growth in the exponential phase according to previous methods (Zhang and Zeng, 2007). The collected samples were then stored at -20 °C followed by -80 °C, for fatty acids analysis.

6.3.3 Cultivation conditions based on carbon and nitrogen sources

Strain MAR441 was grown on various sole carbon sources (L-alanine, L-leucine, L-proline, L-serine, propionic acid, glucose, glycerol, Tween 80, 60 and 40) and nitrogen sources (Urea and $(NH_4)_2SO_4$) in triplicate or duplicate (see Table 2). Modified media for sole carbon source cultures was (Dobson et al., 1991): 0.5 % (w/v) carbon source

and 0.01 % (w/v) yeast extract in 0.22 mm filtered and sterilized natural seawater. Cultures were incubated at 15 °C, in triplicate 50-ml ZB broths contained within 200-ml flasks pre-rinsed in chloroform with orbital shaker (180 rpm) for 36-48 h until sufficient mass of estimated late-log phase cells were present for harvest.

6.3.4 Cultivation with cerulenin

The 10 ml MAR441 seed cultures were used to inoculate 90 ml volumes of marine broth contained in 500 ml conical flasks pre-rinsed in chloroform, where the antibiotic, cerulenin (MERCK), in 50 % (v/v) ethanol was added at various concentrations (0, 0.5, 1, 2.5, 5 and 7.5 μg ml^{-1}) prior to cultivation. Flasks were incubated at 4 and 15 °C respectively. The growth of cells was monitored turbidometrically at 600 nm.

6.3.5 Induction of mutations with nitrosoguanidine (NTG)

Freshly prepared N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (1 mg ml^{-1} in sterile water) was added to the washed culture to a final concentration of 100 g/ml in Tris maleic acid buffer at pH 6.0. The bacteria were incubated with NTG for 30 min. The NTG-treated cells were grown in nutrient broth to express any mutations that were induced. Bacterial colonies were replica-plated in supplemented minimal agar plates.

NTG obtained from Tokyo Kasei Kogyo Co., Ltd. Japan, was used as chemical mutagen. The chemical mutagenesis was carried out according to the literature (Kotchoni et al. 2003; Liu et al. 2004). Exponentially growing cells of *Shewanella* sp. strain MAR441 were harvested from 3 ml of fermentation broth by centrifuging at 1600 g for 10 min at room temperature. The pellet was washed twice with 0.85 % NaCl solution and then resuspended in 5 ml 0.1 M phosphate buffer, producing a suspension containing ~10^5 cfu l^{-1} (colony forming units l $broth^{-1}$). The cells were then exposed to two NTG concentrations (300 μg l^{-1} and 500 μg l^{-1}) for 12 hr at 4 and15 °C by adding appropriate volumes of NTG stock (720 μg l^{-1} NTG in 0.1 M phosphate buffer, pH 6.5) to the cell suspension in flask. One-millilitre samples of the serially-diluted culture were then spread on agar plates for mutant screening. The agar plates were incubated for 2 days at 4 and 15 °C respectively, and the resulting colonies (more than 1000 colones) were taken off the agar plates by random selection based on morphlogy (e.g. size and colour), 50 colones was further chose for evaluation of their ability to produce PUFAs.

The transformation experiments involving the selected mutants were conducted using the culture methods for the parent strain.

6.3.6 Scanning electron microscopy as methods mentioned in Chapter 5

6.3.7 Lipid extraction, preparation of fatty acid methyl esters and analysis

Samples were harvested by centrifugation (4500 g, 4 °C) and frozen at -20 °C followed by -80 °C, before freeze-drying. Freeze dried biomass was accurately weighed, an internal standard (2-Terthiophene triheneicosanoin, n-21:0, Sigma) was added. Fatty-acyl methyl esters were prepared by using the method sulfuric-acid-catalysed trans-esterification (Komagata and Suzuki, 1987; Christie, 1989). After the transmethylation, fatty acid methyl esters were extracted with n-hexane, concentrated under a stream of oxygen-free dry nitrogen at 37 °C, to give a total lipid extract (TLE). Fractionation of phospholipids from the TLE was accomplished by thin-layer chromatography (TLC). Portions of sample TLEs were applied to silica gel plates (Silica gel 60 F254, Merck) that had been activated at 100 °C for 1h. Plates were developed in $CHCl_3$/MeOH/CH_3COOH/H_2O (85:15:10:3.5, v/v/v/v). Samples were visualised by iodine vapour and identified by comparison with known standards which were identified with rhodamine-6-G, ninhydrin and Dragendorff stains (Kates, 1986). The lipid classes were separated by silica gel (1:30 w/w of lipid) column chromatography by successive elution with chloroform (1:10 m/v of lipid), acetone-methanol (9:1 v/v;1:15 w/v of lipids) and methanol (1:10 w/v of lipid) to get neutral-(NL), glyco-(GL) and phospho-(PL) lipids respectively. All fractions along with total lipids were transmethylated using sodium methoxide (0.5 M) to obtain the fatty acid methyl esters (FAMEs). Analyses of the FAME preparations were performed with a Hewlett-Packard model 7890A GC (Varian CP-3800, Varian, Inc. 2700 Mitchell Drive Walnut Creek, CA 94598-1675/USA) equipped with type DB225 capillary column (BPX70, 10 m x 0.1 mm, 0.2 μm; J & W Scientific, Folsom, Ca, USA) with programmed temperature of 170 °C–220 °C, a linear increase at 5 °C min^{-1}, injection and detection temperature maintained at 250 and 260 °C, respectively, and helium as the carrier gas. GC/MS analysis was carried out with Agilent 5975 GC/MS (Agilent Technologies Co., Ltd., Palo Alto , USA) equipped with HP-5ms Capillary GC-MS Column (Agilent 19091S-433, 30 m x 0.25 mm, 0.25 μm), temperature programme 120 °C for 1 min, increased at 8 °C min^{-1} to 260 °C, which was maintained for 10 min with He as the carrier gas. MS

operating conditions were as follows: electron multiplier, 2,000 V; transfer line, 250 °C; electron impact energy, 70 eV; scan threshold, 50; 1.3 scans s21 with a mass range of 50 to 500 atomic mass units; and solvent delay, 2.35 min. Compounds were identified by comparison of their retention times with those of known standards, and sample mass spectra data were compared to the mass spectra data of 275, 000 compounds in the Wiley 275 spectra library.

6.4 Results

6.4.1 Fatty acid composition of phospholipid classes

Strain MAR441 is psychrotrophic and showed good biomass production at temperatures 10-15 °C. The amount of total lipid was 10.3 % of dry cell mass from 10 °C MB cultures and the content of phospholipids and neutral lipids were about 82 % and 28 % of total lipid, respectively (Table 6.1). As identified by TLC from fractionation of TLEs, phosphatidyl ethanolamine (PE) was the dominant lipid class in phospholipids (50 %) followed by phosphatidyl glycerol (PG) (40 %). About 5 % of diphosphoglyceride (DPG) and 3 % of lysophosphatidylethanolamine (LPE) were also detected with some unidentified phospholipids (2 %). The fatty acid compositions of TLEs and their derived PE, PG and DPG fractions are shown in Table 6.1.

Table 6.1 Lipid composition of *Shewanella* sp. strain MAR441 when grown in marine broth medium at 10 °C[a]

Biomass (g dry cell l^{-1})	2.66
Total lipid (% of dry weight)	12.5
Neutral lipids (% of total lipids)	28
Free fatty acid (FFA) (% of total lipids)	15
Phospholipids (PL) (% of total lipids)	72
Phosphatidylethanolamine (PE) (% of PL)	50
Phosphatidylglycerol (PG)	40
Diphosphatidylglycerol (DPG)	5
Lysophosphatidylethanolamine (LPE)	3
Unidentified phospholipid	2
EPA (% of total lipids)	13.56
EPA (mg g^{-1})	16.95
Lipid g l^{-1}	0.3325
Lipid (mg g^{-1})	125

[a]The values are means of three samples.

Table 6.2 Distribution of major fatty acid in total and different lipid class in *Shewanella* sp. MAR441 strain when grown in marine broth medium at 10 °C

Fatty acids	TFA	FFA	DPG	PE	PG
n-12:0	2.1	2.2	2.5	0.3	0.5
n-13:0	22.92	17.6	28.4	24.8	19.5
n-14:0	4.11	2.85	3.1	3.7	4.2
n-15:0	2.25	1.3	2.8	1.8	2.7
n-16:0	10.99	5.5	9.2	10.5	12.2
n-17:0	0.56	0.1	0.5	0.7	0.5
n-18:0	0.44	0.1	0.5	0.4	0.3
Σ SCFA	**43.37**	**29.65**	**47**	**42.2**	**39.9**
i-13:0	7.23	3.9	9.4	8.9	5.7
i-14:0	0.33	0.67	0.2	0.6	0.3
ai-15:0	0.68	0.35	1.7	0.6	0.46
i-15:0	10.29	7.5	15.7	14.5	7.6
i-17:0	0.2	0.1	0.15	0.4	0.5
Σ BCFA	**18.73**	**12.52**	**27.15**	**25.0**	**14.6**
n-15:1ω6	0.09	0.88	0.2	1.2	1.3
n-16:1ω7	13.65	26.5	12.5	12.5	19.4
n-17:1ω8	0.28	0.3	0.3	0.1	0.5
n-18:1ω9c	0.53	0.42	0.2	0.45	0.7
n-18:1ω7c	4.88	4.24	3.5	4.5	6.1
n-20:1ω9	0.19	0.3	0.1	0.2	0.3
Σ MUFA	**19.62**	**32.64**	**16.8**	**19.0**	**28.3**
n-18:2ω6t	1.16	2.5	0.7	1.2	0.8
n-18:3ω6t	0.09	0.2	0.05	-	0.1
n-18:3ω3	0.11	0.5	0.8	0.2	0.1
n-18:4ω3	0.31	0.4	0.1	0.6	0.3
n-20:2	0.08	0.1	-	0.1	0.1
n-20:3ω6	0.04	0.1	-	-	-
n-20:4ω6	0.2	0.2	0.1	0.2	0.1
n-20:3ω3	0.06	0.2	-	0.1	0.1
n-20:4ω3	0.59	0.8	-	1.1	0.6
n-20:5ω3	**15.01**	**18.7**	**6.5**	**9.5**	**14.2**
n-22:2ω6	0.04	0.2	0.1	0.1	-
n-22:4ω6	0.04	0.1	-	-	-
n-22:5ω3	0.49	0.5	0.2	0.35	0.5
Σ PUFA	**18.22**	**24.5**	**8.55**	**13.45**	**16.9**
Others	0.06	0.69	0.5	0.45	0.34
Total	100	100	100	100	100
ACL[a]	15.62	16.02	14.83	15.27	15.79

[a]ACL, average chain length (calculated after White et al., 2005); Values are means of three samples; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (20:5ω3); and (–), not detectable.

There was a near equal proportion of SCFAs within the PE and PG fractions. However, PE contained a higher proportion of BCFAs components (25 % PE versus 15 % PG) due mainly to a higher percentage of i-13:0 (9 % PE versus 6 % PG) and i-15:0 (15 % PE versus 8 % PG). In contrast, PG contained a greater proportion of MUFAs (28 % versus 19 %) which was due to slightly higher proportions of all monounsaturated acyl species. PUFA were present in both phospholipid classes, although PG contained a higher percentage of total PUFAs (17% versus 14%) and EPA (14 % versus 10 %). Free fatty acids (FFA) or non-esterified fatty acid (NEFA) was recovered from the TLC plates, and found with high content of MUFAs and MUFAs, especially the high content of EPA and n-16:1ω7c (18.7 % and 26.5 %, respectively) and with less SCFAs and BCFAs. Whereas, DPG contained higher SCFAs and BCFAs, especially n-13:0 and i-15:0 (28.4 % and 15.7 %, respectively), and less MUFAs and PUFAs, from which 6.5 % of EPA was also detected. Thus, a mass balance calculation of the percentage of EPA, based on a qualitative assessment of probable phospholipid class distribution did balance (Table 6.2).

6.4.2 Effect of growth temperature

Strain MAR441 can grow well at the range between 4 to 25 °C with optimal growth temperatures at 15 °C but showed poor growth conditions under 30 °C or above under atmospheric pressure in marine broth medium (Figure 6.1). After 72 h at 4 °C, the cells had reached 5.2×10^8 c.f.u. ml^{-1}; and at 81 h, the amounts of dry cells, total lipids, and EPA were 2.25 g, 115.2 mg, and 24.7 mg per litre of culture, respectively. For 10 °C MAR441 cells culture, the biomass achieved 6.3×10^8 c.f.u. ml^{-1} when grown for 72 h, the quantitative yield of dry cells, total lipids, and EPA were 2.66 g, 103.1 mg, and 15.5 mg respectively per litre of culture. While after 50 h at 15 °C, the cells had reached 7.5×10^8 c.f.u. ml^{-1}; and at 68 h, the amounts of dry cells, total lipids, and EPA were 2.6 g, 102.5 mg, and 9.7 mg per litre of culture, respectively. Under warmer temperature 25 °C, the cells had reached 1.2×10^8 c.f.u. ml^{-1} after 40 h, the amounts of dry cells, total lipids, and EPA at 50 h were 1.0 g, 36.5 mg, and 0.2 mg per litre of culture, respectively.

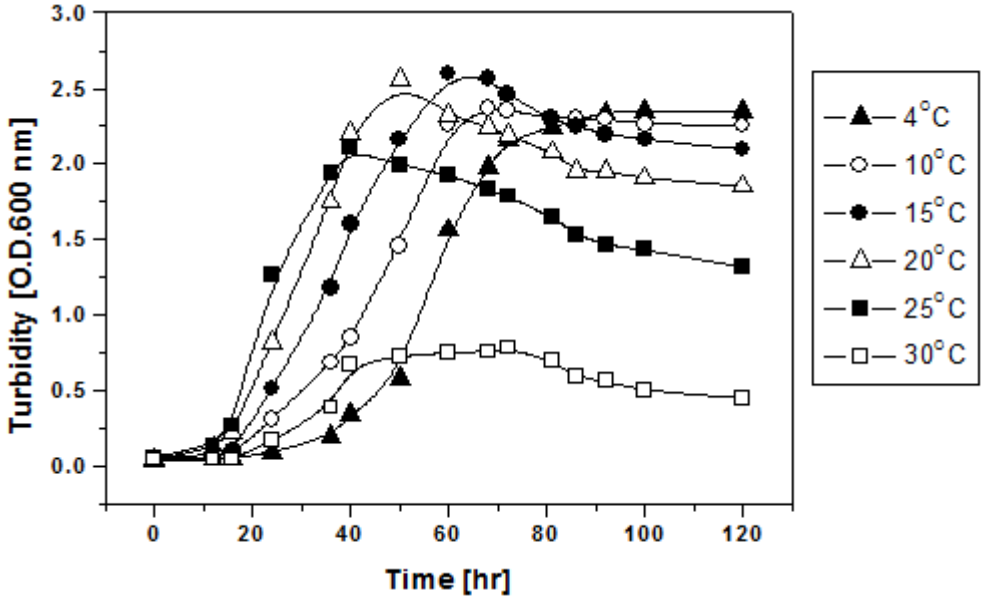

Figure 6.1 Growth curves of strain MAR441 with shaking at various temperatures under atmospheric pressure in marine broth medium. The experiments were carried out in duplicate and values are means of two samples.

The effect of growth temperature on the percentage composition of individual fatty acids in MAR441 grown between 4 – 25 °C is shown in Table 6.3. Growth at temperatures 4 °C to 10 °C below the optimal growth temperature region 15–20 °C, resulted in a higher percentage of EPA and n-16:1ω7c, and generally a lower percentage of n-13:0 and n-16:0 compared to growth within 15 °C or above 20 °C the optimum growth temperature region. Growth within the optimal region resulted in the highest percentage of n-15:0 and n-16:0, while iso-tridecanoic acid (i-13:0) and the sum of monounsaturated fatty acids were at their lowest level. At growth temperatures above the optimal region, the percentage of iso-pentadecanoic acid (i-15:0), n-17:0, n-17:1ω8 and were maximal, while the percentage of n-13:0 increased with increasing growth temperature. With increasing temperature from 4 ºC to 25 ºC, the proportion of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) decreased, with the increasing of straight chain fatty acids (SCFAs), whereas, the branched chain fatty acids (BCFAs) at its lowest point when at 15 °C. The values of average chain length (ACL) (from 16.29-14.67) and quantitative level of EPA decreased with increasing growth temperature (24–0.2 mg g^{-1} cells dry weight) at all growth temperatures from 4-25 °C (Figure 6.2).

Table 6.3 Temperature dependence of the fatty acid composition of *Shewanella* sp. MAR441 grown in marine broth medium

Temperatures	Percentage composition				
Fatty acids	4 °C	10 °C	15 °C	20 °C	25 °C
n-12:0	1.3	2.1	2.2	2.5	0.3
n-13:0	13.5	22.9	23.6	30.4	32.1
n-14:0	3.9	4.1	2.9	2.1	2.7
n-15:0	3.5	2.3	4.5	2.2	1.9
n-16:0	5.3	11.0	14.5	12.2	12.4
n-17:0	0.3	0.6	0.8	2.5	5.7
n-18:0	0.5	0.4	1.3	1.1	1.0
Σ SCFA	**28.3**	**43.4**	**49.7**	**53.0**	**56.0**
i-13:0	11.6	7.2	4.9	7.1	11.2
i-14:0	0.7	0.3	0.4	0.2	0.2
ai-15:0	1.2	0.7	0.4	0.2	0.8
i-15:0	7.2	10.3	10.2	12.7	14.2
i-17:0	0.5	0.2	0.2	1.2	0.2
Σ BCFA	**21.2**	**18.7**	**16.2**	**21.4**	**26.6**
n-15:1ω6	0.1	0.1	0.9	0.3	0.6
n-16:1ω7	16.9	13.7	11.2	10.2	9.3
n-17:1ω8	0.2	0.3	2.8	3.5	4.0
n-18:1ω9c	0.6	0.5	0.4	0.2	0.3
n-18:1ω7c	5.2	4.9	4.2	1.2	1.1
n-20:1ω9	0.4	0.2	0.2	0.3	0.1
Σ MUFA	**23.3**	**19.6**	**19.7**	**15.7**	**15.5**
n-18:2ω6t	2.8	1.2	1.5	0.5	0.3
n-18:3ω6t	0.2	0.1	0.2	-	0.4
n-18:3ω3	0.3	0.1	0.6	0.8	0.2
n-18:4ω3	0.7	0.3	0.3	-	-
n-20:2	0.2	0.1	0.2	0.5	0.2
n-20:3ω6	0.1	0.0	-	-	-
n-20:4ω6	0.2	0.2	0.1	-	-
n-20:3ω3	0.1	0.1	0.4	0.3	0.1
n-20:4ω3	0.7	0.6	0.7	-	-
n-20:5ω3	**21.4**	**15.0**	**9.5**	**5.6**	**0.6**
n-22:2ω6	0.1	0.0	0.3	0.8	0.1
n-22:4ω6	0.2	0.0	0.2	0.6	0.1
n-22:5ω3	0.8	0.5	0.3	0.4	0.1
Σ PUFA	**27.8**	**18.2**	**14.4**	**9.5**	**2.0**
Others	0.4	0.1	0.1	0.4	0.0
Total	100.0	100.0	100.0	100.0	100.0
ACL	16.29	15.62	15.52	15.04	14.67
EPA (mg g^{-1})	24.7	15.48	9.7	5.6	0.6
TFA (mg g^{-1})	115.2	103.1	102.5	100.7	96.5
Cells(g l^{-1}) [a]	2.25	2.66	2.6	2.4	0.6

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (20:5ω3); and (–), not detectable.

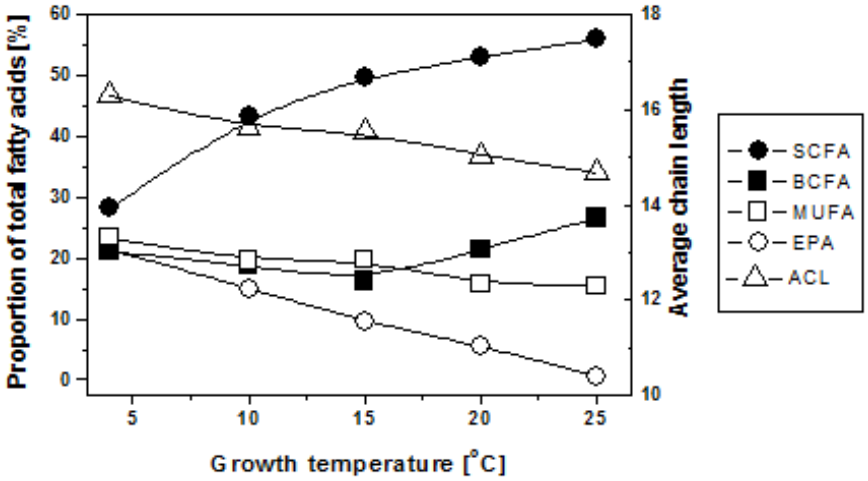

Figure 6.2 Change in average chain length (ACL, triangles) and relative proportion of whole cell FAs in Shewanella sp. MAR441 grown at 4, 10, 15, 20 and 25 °C in marine broth medium. Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open boxes); eicosapentaenoic acid (EPA, open circles). The experiments were carried out in triplicate and values are means of three samples based on Table 6.3.

6.4.3 Time course of cell growth and FAs production

Fatty acids are intracellular products in *Shewanella* sp. MAR441 shows a typical time course of EPA production by *Shewanella* sp. MAR441 suspension culture in MB medium at 15 °C (Figure 6.3, Figure 6.4 and Table 6.4). The increase in lipid and EPA content parallels that of cell growth (Figure 6.3).

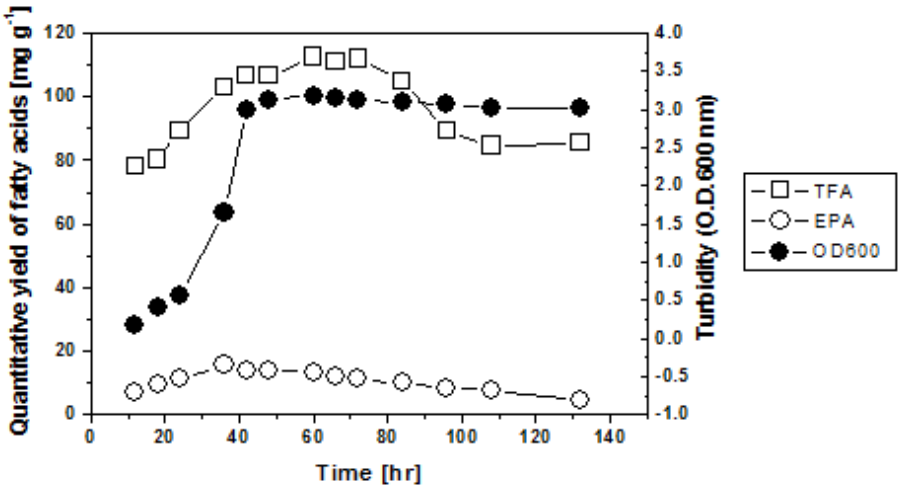

Figure 6.3 Time period of cell growth (filled circles) and quantitative yield of whole cell TFAs and EPA in *Shewanella* sp. MAR441 grown in marine broth medium at 15 °C during the time period. Total fatty acids (TFAs, open boxes); eicosapentaenoic acid (EPA, open circles) and optical density 600 nm (O.D. 600 nm, filled circles). The experiments were carried out in triplicate and values are means of three samples based on Table 6.4.

After 36 hrs of cultivation, the cells entered the late exponential or early stationary phase and the content of TFAs, PUFAs and EPA reached their maximum of 103.1 mg g^{-1}, 17.7 mg $g^{-1}$and 15.48 mg g^{-1} respectively, when EPA in its highest content of 15 % in TFAs. In this experiment, 5 % culture inoculum was used, and the maximum TFAs concentration reached about 113 mg g^{-1} after 60 hrs culture, with ca. 16.8 mg g^{-1} of PUFA and 13.4 mg g^{-1} of EPA, when the percentage of EPA dropped to 12 % in TFAs. The TFAs and EPA maintained a stable quantitative yield of 90-12 mg g^{-1} and 11-15 mg g^{-1} for 60 hours from 24 hr to 84 hr time period culture, during which the percentage composition of SCFAs, BCFAs, MUFAs, PUFAs and EPA was kept at a relative stable levers of 41-46 %, 19-22 %, 18-20 %, 13-17 % and 10-15 % respectively, and the average chain length remained more or less constant from 15.21-15.73. However, an increase in cell growth during the exponential phase led to a decrease in SCFAs from a high lever of 60-43 % in favour of BCFAs, MUFAs and PUFAs, due to the great decrease of n-13:0 with a corresponding increase of n-16:0, i-C15:0, n-16:1ω7 and EPA, which contributing the increase of average chain length. Interestingly, the cell death during the later stationary phase and decline phase of cell culture led to a great increase in SCFAs from 50-73 % at the expense of BCFAs, MUFAs and PUFAs, due to a surprising rise of n-13:0 from 29-63 %, which was accompanied by a decrease of n-16:0, i-15:0, n-16:1ω7 and EPA, as well as the average chain length (Figure 6.4).

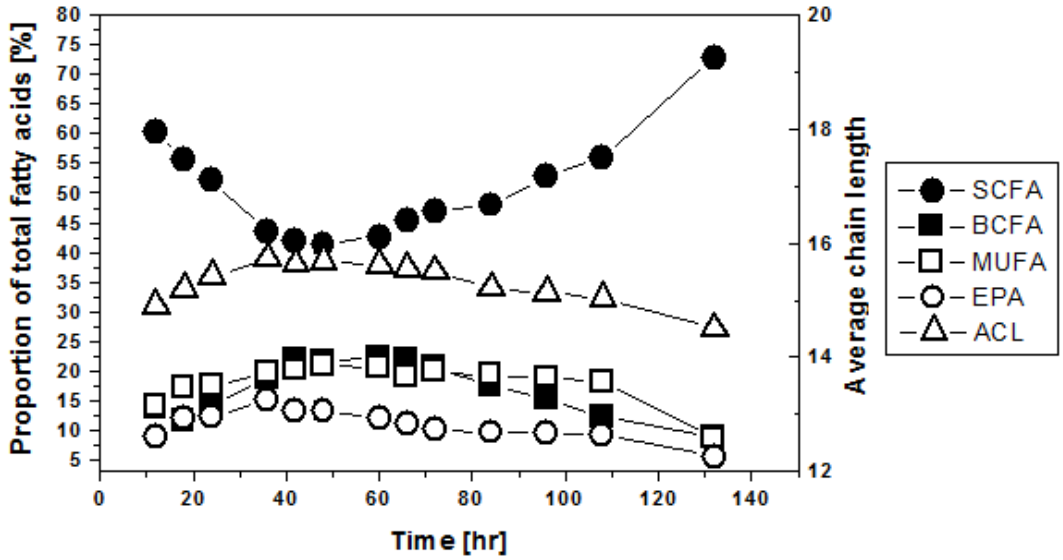

Figure 6.4 Change in average chain length (ACL, triangles) and relative proportion of whole cell FAs in *Shewanella* sp. MAR441 grown in marine broth medium at 15 °C during the time period. Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open boxes); and eicosapentaenoic acid (EPA, open circles). The experiment was carried out in Values are means of three samples based on Table 6.4.

Table 6.4 Fatty acid composition of *Shewanella* sp. MAR441 during the time course of cell growth in marine broth medium at 15 °C

Time period	Percentage composition												
Fatty acids	12hr	18hr	24hr	36hr	42hr	48hr	60hr	66hr	72hr	84hr	96hr	108hr	132hr
n-12:0	2.76	2.16	2.2	2.1	1.8	1.7	2.6	2.7	3.3	2.9	2.7	1	0.2
n-13:0	42.09	37.6	31.92	22.92	20.6	21.1	22.5	24.1	23.5	29.2	33.5	40	63.1
n-14:0	3.79	3.49	5.11	4.11	4.5	4.9	4.6	4.2	4.1	4.1	4.4	3.4	1.7
n-15:0	1.71	1.77	2.12	2.25	2.6	2.1	2.4	2.4	2.7	1.3	0.3	0.5	0.6
n-16:0	7.76	8.5	9.6	10.99	11.2	10.8	9.7	10.9	12.5	12.7	11.7	9.5	5.3
n-17:0	0.33	0.31	0.87	0.56	0.6	0.3	0.5	0.6	0.5	0.3	0.1	0.59	0.6
n-18:0	1.75	1.7	0.42	0.44	0.5	0.45	0.3	0.4	0.3	0.2	0.1	1	1.5
Σ SCFA	**60.19**	**55.53**	**52.24**	**43.37**	**41.8**	**41.35**	**42.6**	**45.3**	**46.9**	**50.7**	**52.8**	**55.99**	**72.9**
i-13:0	6.34	5.12	6.1	7.23	8.23	8.5	9.1	8.2	7.1	6.5	5.1	5.3	2.7
i-14:0	0.72	0.62	0.58	0.68	0.8	0.7	0.6	0.5	0.5	0.55	0.5	0.24	0.2
ai-15:0	0.72	0.72	0.3	0.2	0.5	0.6	0.5	0.7	0.9	0.6	0.4	1.1	0.1
i-15:0	5.35	4.7	6.7	10.29	12.2	11.6	11.7	12.3	11.8	9.8	8.9	4.93	4.9
i-17:0	0.33	0.31	0.43	0.33	0.21	0.22	0.2	0.2	0.2	0.25	0.2	0.65	0.7
Σ BCFA	**13.46**	**11.47**	**14.11**	**18.73**	**21.94**	**21.62**	**22.1**	**21.9**	**20.5**	**17.7**	**15.1**	**12.22**	**8.7**
n-15:1ω6	0.12	0.11	0.07	0.09	0.1	0.2	0.3	0.3	0.2	0.2	0.2	0.1	0.1
n-16:1ω7	10.64	14.1	12.65	13.65	14.2	15	14.4	13.2	13.5	13.3	13.4	14.3	6.1
n-17:1ω8	0.18	0.16	0.23	0.28	0.42	0.4	0.8	0.7	0.4	0.4	0.3	0.1	0.1
n-18:1ω9c	0.38	0.25	0.45	0.53	0.4	0.3	0.5	0.3	0.2	0.2	0.2	0.1	0.3
n-18:1ω7c	2.43	2.11	3.88	4.88	4.7	4.7	4.3	4.1	5.2	4.8	4.5	3.3	1.5
n-20:1ω9	0.32	0.32	0.23	0.19	0.23	0.3	0.2	0.3	0.3	0.25	0.2	0.1	0.4
Σ MUFA	**14.07**	**17.05**	**17.51**	**19.62**	**20.05**	**20.9**	**20.5**	**18.9**	**19.8**	**19.15**	**18.8**	**18**	**8.5**
n-18:2ω6t	0.36	0.38	0.23	0.11	0.21	0.22	0.25	0.3	0.2	0.3	0.5	0.5	0.7
n-18:3ω6t	-	-	0.29	0.09	0.04	0.1	0.1	0.1	0.1	0.1	-	-	0.7
n-18:3ω3	0.37	0.35	0.3	0.11	0.13	0.14	0.15	0.2	0.1	0.1	0.6	0.8	0.4
n-18:4ω3	0.21	0.24	0.28	0.31	0.27	0.3	0.5	0.45	0.3	0.3	0.4	0.2	-
n-20:2	0.3	0.32	0.18	0.08	0.08	0.05	0.1	0.15	0.1	0.2	0.3	0.7	0.7
n-20:3ω6	0.1	-	0.14	0.04	0.05	0.07	0.1	0.12	0.1	0.3	0.5	0.3	0.4
n-20:4ω6	-	0.11	0.23	0.2	0.14	0.15	0.18	0.2	0.25	0.2	-	-	-
n-20:3ω3	0.69	0.71	0.66	0.06	0.08	0.08	0.1	0.11	0.2	0.3	0.4	0.5	0.5
n-20:4ω3	0.23	0.26	0.34	0.59	0.49	0.53	0.63	0.53	0.5	0.4	0.1	0.1	0.1
n-20:5ω3	**8.84**	**12**	**12.3**	**15.01**	**13.2**	**13.2**	**11.9**	**10.9**	**10**	**9.6**	**9.5**	**9.1**	**5.5**
n-22:2ω6	-	-	-	0.04	0.05	0.08	0.1	0.1	0.1	0.1	-	-	-
n-22:4ω6	0.12	0.11	0.08	0.04	0.03	0.04	0.05	0.05	0.1	0.3	0.5	0.3	0.5
n-22:5ω3	0.25	0.23	0.37	0.49	0.35	0.4	0.5	0.6	0.5	0.2	0.3	0.1	0.3
Σ PUFA	**11.47**	**14.71**	**15.4**	**17.17**	**15.12**	**15.36**	**14.7**	**13.81**	**12.6**	**12.4**	**13.1**	**12.6**	**9.7**
Others	0.81	0.74	0.74	1.11	1.09	0.77	0.14	0.09	0.25	0.8	0.21	0.89	0.0
Total	100	100	100	100	100	100	100	100	100	100	100	100	100.0
ACL	14.9	15.2	15.42	15.73	15.63	15.67	15.6	15.55	15.5	15.21	15.1	15.04	14.5
EPA (mg g^{-1})	6.9	9.6	11.1	15.48	14.1	14	13.4	12.1	11.2	10	8.5	7.7	4.7
TFA (mg g^{-1})	78.1	80.2	89.5	103.1	106.8	106.6	113	111	112	105	89.5	84.6	85.4
Cells(g l^{-1}) [a]	0.6	0.8	1.4	1.96	2.42	2.66	2.7	2.68	2.7	2.6	2.5	2.2	2.2

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

6.4.4 Effect of Na^+ on cell growth and PUFA production

This bacterium required Na^+ for growth, growth occurred at Na^+ concentrations ranging from 0.051 M (0.3 %) to 1.197 M (7 %) with an optimum between 0.051 and 0.513 M (3 %), which is consistent with the sea-water environment containing about 19.45 g l^{-1} Na^+; the growth was inhibited at Na^+ concentrations above 1.368 M (8 %). Effects of different concentrations of sodium chloride were supplemented to the regular ZB medium and their involved in biomass formation, fatty acid biosynthesis and desaturation reactions were investigated (Table 6.5 and Figure 6.5).

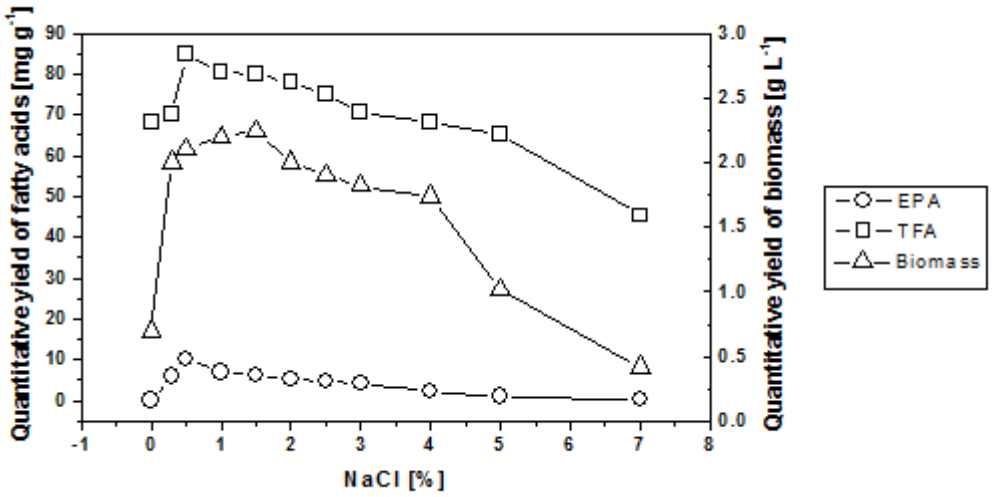

Figure 6.5 Quantitative yield of biomass (triangles), TFA and EPA in *Shewanella* sp. MAR441 grown in ZB medium at 15 °C with various concentration of NaCl. Total fatty acids (TFAs, open boxes); eicosapentaenoic acid (EPA, open circles). The experiments were carried out in triplicate and values are means of three samples based on Table 6.5.

A change in biomass formation was observed between cultures within optimum Na^+ concentrations and cultures without Na^+ or with high Na^+ concentrations. Growth inhibition was noted with NaCl at a concentration less of 0.3 % or with concentration more than 7 %. 1.7-2.2 g l^{-1} biomass and 2-10 mg g^{-1} EPA were achieved when the Na^+ in the ZB medium were controlled between 0.051-0.684 M, while only 0.4-1.0 g l^{-1} biomass and 0-0.18 mg g^{-1} EPA were produced when the medium were supplemented without or with high concentration of Na^+ (>0.85 M). When the ZB medium was prepared using distilled water to make no any metallic ions were added, and very low percentage composition (0.2 %) of EPA was produced by MAR441 cells, and with high lever of SCFAs (64.5 %). As the ZB medium supplemented with Na^+ from low

concentration (0.3 %), which resulting in a great decrease of SCFAs in favour of BCFAs, MUFAs and PUFAs, due to the great decrease of n-13:0 with a corresponding increase of n-14:0, n-16:0, i-13:0, n-16:1ω7, n-18:1ω7 and EPA, which contributing the increase of average chain length from 14.27 to 15.32. The productivity of EPA was significantly increased from 0.4 % to 8.6 % when only 0.3 % Na^+ was supplemented. EPA reached its maximum when the concentration of Na^+ was maintained at 0.5 % with proportion of 15.4 % in TFAs. However, the increasing supplemented of Na^+, the led to a decrease in the percentages of EPA from 15.4 % to 0.4 % when 7 % of Na^+ was added in the medium. Nevertheless, in 0.5-3 % of Na^+ supplemented ZB medium, the percentage composition of SCFAs, BCFAs, MUFAs and PUFAs in MAR441 cells was more or less maintained at stable levers. However, growth on 4-7 % of Na^+ supplemented ZB medium increased the proportion of SCFAs, which accompanied by a decrease in the percentages of BCFAs, MUFAs and PUFAs, and this was mirrored by a corresponding decrease of average chain length (Figure 6.6).

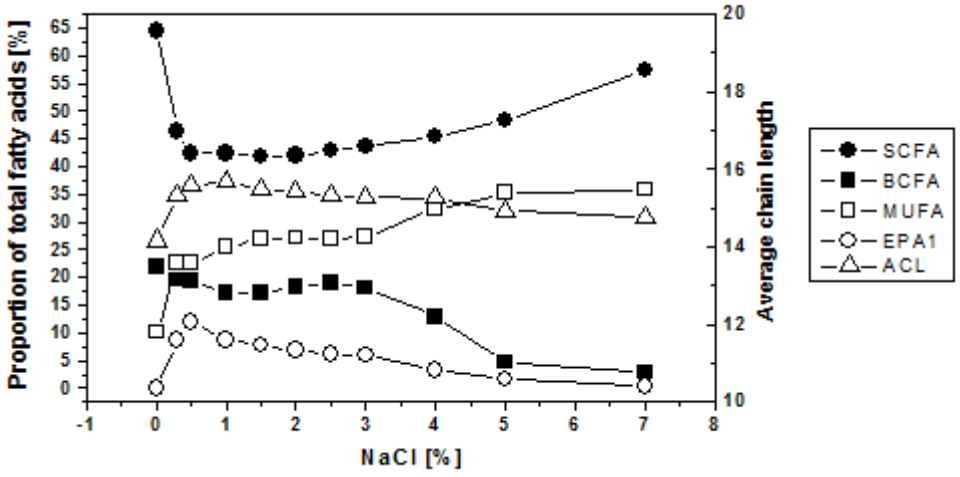

Figure 6.6 Change in average chain length (ACL, triangles) and relative proportion of whole cell FAs in *Shewanella* sp. MAR441 grown in ZB medium at 15 °C with various concentration of NaCl. Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open boxes); eicosapentaenoic acid (EPA, open circles). The experiments were carried out in triplicate and values are means of three samples based on Table 6.5.

Table 6.5 Fatty acid composition of *Shewanella* sp. MAR441 grown on various concentration of NaCl in ZB liquid medium at 15 °C

Fatty acids	**Percentage composition**										
NaCl (%)	**0**	**0.3**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**4.0**	**5.0**	**7.0**
n-12:0	0.7	3.0	1.9	2.8	1.7	2.3	1.8	2.0	2.8	3.5	1.7
n-13:0	56.2	25.2	20.0	18.5	19.1	20.1	22.5	24.1	27.5	32.4	43.9
n-14:0	0.9	4.4	3.9	4.4	2.9	3.3	3.0	2.9	3.0	3.1	0.9
n-15:0	0.8	2.8	2.6	2.1	3.2	4.2	4.9	4.5	2.4	0.8	0.9
n-16:0	4.2	8.4	12.1	12.4	11.3	9.1	8.3	8.1	4.7	2.2	6.7
n-17:0	0.2	0.5	0.7	0.2	1.5	1.5	1.2	0.8	2.8	3.5	1.4
n-18:0	1.5	2.1	1.3	2.0	2.2	1.5	1.2	1.3	2.2	2.8	2.0
Σ SCFA	**64.5**	**46.4**	**42.4**	**42.4**	**41.9**	**42.0**	**42.9**	**43.6**	**45.4**	**48.3**	**57.5**
i-13:0	5.5	6.4	7.3	6.7	6.4	7.4	7.7	7.7	4.4	0.1	0.2
i-14:0	0.6	0.5	0.7	0.3	0.5	0.5	0.3	0.4	0.2	0.2	0.2
ai-15:0	0.2	0.5	0.7	0.4	0.5	0.6	0.5	0.4	0.3	0.2	0.2
i-15:0	14.2	12.0	10.5	9.8	9.7	9.6	10.5	9.4	7.9	4.0	2.0
i-17:0	1.4	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.3
Σ BCFA	**21.8**	**19.5**	**19.3**	**17.3**	**17.2**	**18.2**	**19.1**	**18.1**	**12.9**	**4.7**	**2.9**
n-15:1ω6	1.8	-	-	0.5	0.5	0.6	0.8	0.9	6.9	8.1	9.3
n-16:1ω7	5.3	12.2	15.2	16.2	16.2	15.7	14.8	16.7	18.0	19.2	22.4
n-17:1ω8	0.2	2.5	1.5	2.5	3.9	5.3	4.8	3.0	0.5	0.2	0.3
n-18:1ω9c	0.5	0.2	0.6	0.5	0.5	0.2	0.5	0.4	0.3	0.2	0.2
n-18:1ω7c	1.9	7.6	5.1	5.6	5.5	5.3	5.9	6.1	6.4	7.2	3.3
n-20:1ω9	0.5	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.3	0.3	0.4
Σ MUFA	**10.1**	**22.6**	**22.5**	**25.5**	**26.9**	**27.2**	**26.9**	**27.3**	**32.4**	**35.2**	**35.9**
n-18:2ω6t	-	0.2	0.3	0.8	0.8	0.6	0.7	0.5	1.0	1.5	0.1
n-18:3ω6t	-	0.2	0.3	0.3	0.3	0.3	0.1	0.2	0.2	-	-
n-18:3ω3	1.0	0.3	0.3	0.8	0.9	1.0	0.4	0.6	0.6	1.8	0.1
n-18:4ω3	-	0.5	0.6	0.7	0.7	0.6	1.7	1.3	-	-	-
n-20:2	1.0	0.3	0.4	0.8	0.7	0.5	0.2	0.2	1.2	1.5	1.7
n-20:4ω6	-	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	-	-
n-20:3ω3	0.6	0.1	0.2	0.5	0.7	0.7	0.2	0.4	0.8	1.3	0.4
n-20:4ω3	-	0.5	0.7	0.6	0.6	0.5	0.5	0.7	-	-	-
n-20:5ω3	**0.2**	**8.6**	**11.9**	**8.6**	**7.8**	**6.8**	**6.2**	**5.8**	**3.2**	**1.6**	**0.4**
n-22:2ω6	0.3	0.1	0.1	0.4	0.4	0.4	0.1	0.3	0.8	0.8	0.3
n-22:4ω6	0.3	0.1	0.1	0.5	0.3	0.2	0.2	0.2	0.9	0.6	0.3
n-22:5ω3	0.2	0.3	0.4	0.4	0.5	0.3	0.3	0.3	0.4	0.4	0.3
Σ PUFA	**3.6**	**11.3**	**15.4**	**14.6**	**13.9**	**12.0**	**10.7**	**10.6**	**9.2**	**9.5**	**3.7**
Others	0.0	0.2	0.4	0.2	0.2	0.6	0.4	0.4	0.2	0.1	0.0
Total	100	100	100	100	100	100	100	100	100	100	100
ACL	14.27	15.32	15.58	15.63	15.69	15.42	15.32	15.28	15.26	14.93	14.77
EPA (mg g^{-1})	0.14	6.02	10.2	6.92	6.24	5.3	4.65	4.2	2.18	1.04	0.18
TFA (mg g^{-1})	68.1	70.1	85	80.5	80	78	75	70.5	68.2	65.3	45.3
Cells(g l^{-1}) [a]	0.7	2	2.1	2.2	2.24	2	1.9	1.82	1.74	1.02	0.42

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (20:5ω3); and (–), not detectable.

6.4.5 Effect of sole carbon and nitrogen source on FAs

To elucidate global adaptation mechanisms of membrane fatty acids in strain MAR441 in response to growth at a low temperature on seven different carbon sources, the degree of saturation (i.e. saturated/unsaturated ratio), the degree of polyunsaturation (i.e. PUFA/MUFA ratio) and average chain length (ACL) of fatty acids were determined and compared. The results obtained are summarized in Table 6.6.

The changes in fatty acid composition of MAR441 grown on various sole carbon/nitrogen sources at 15 °C are shown in Table 6.5. Growth on L-leucine, L-alanine, L-serine, L-proline, glucose, glycerol, pyruvate, Tween 80, Tween 60, Tween 40, urea and $(NH_4)_2SO_4$ produced a fatty acid composition similar to that obtained in complex media MB or ZB. Only some differences in percentage composition or the quantitative level of TFA were apparent.

Growth on complex media MB or ZB achieved high production of n-16:1ω7 (14 %) and EPA (13-15 %). However, MB media contributed less production of SCFAs, such as n-13:0 (23 % versus 36 %), which was mirrored by a corresponding increase in BCFAs due to the higher amount of i-13:0 (7 % versus 3 % for ZB) and i-15:0 (10 % versus 3 %), as well as by a higher quantitative yield of EPA (15.5 mg g^{-1} versus 14.2 mg g^{-1}) but with similar value of average chain length (15.57 versus 15.49). PUFA levels produced by MAR441 strain in MB and ZB (0.91 and 1.11 of polyunsaturation degree) were substantially higher compared with other organic substrates, where almost equal quantities of PUFA have been observed (0.1–0.86 of polyunsaturation degree).

Growth on L-alanine and L-serine produced a very similar fatty acid percentage composition as well as average chain length (15.39 versus 15.38), with very low proportion of n-13:0 (24-26 % versus 32-51 % for other carbon sources excluding glycerol). This was accompanied by a rise in the percentages of other fatty acids with different levels, especially on n-16:1ω7 (11-15 %) and EPA (11-13 %). However, grown on L-alanine led to higher quantitative yield of EPA (12.57 mg g^{-1} versus 11.13 mg g^{-1}).

Table 6.6 Fatty acid composition of *Shewanella* sp. MAR441 grown on various sole carbon/nitrogen sources at 15 °C

Medium	**Percentage composition**															
Fatty acids	**ZB1**	**MB**	**ZB**	**NC**	**Ala**	**Leu**	**Pro**	**Ser**	**Pyr**	**Glu**	**Gly**	**T 80**	**T 60**	**T 40**	**Urea**	**NH_4**
n-12:0	2.3	2.1	1.6	1.9	2.9	1.2	2.2	2	2.4	2.4	2.8	0.3	2.7	3.3	1	0.5
n-13:0	25	22.92	36.4	48.4	24.6	45.7	32.2	26.7	30.1	36.3	21	23.2	25.7	23.1	42.7	51.1
n-14:0	3.3	4.11	4.1	2.8	4.2	2.4	3.3	3.6	4.8	3.2	3.5	2.6	3.6	1.7	2.2	0.8
n-15:0	0.7	2.25	1.4	1.1	2.3	-	1.1	2.6	1.4	2	1.1	0.2	0.4	0.1	0	1.2
n-16:0	13.8	10.99	9.2	8.3	10.7	7.2	9.7	11.6	11.8	9.8	11.1	8.1	10.8	7.2	8.2	6.3
n-17:0	0.6	0.56	0.3	1.9	1.5	1.5	2.2	2.5	0.5	1.5	2.9	0.6	1	0.4	1.2	2.1
n-18:0	1.9	0.44	2.4	3.1	1.9	2.5	2.3	4.2	2.1	2.8	1.2	3.1	33.3	15.4	3.2	2
Σ SCFA	**47.6**	**43.37**	**55.4**	**67.5**	**48.1**	**60.5**	**53**	**53.2**	**53.1**	**58**	**43.6**	**38.1**	**77.5**	**51.2**	**58.5**	**64**
i-13:0	2.6	7.23	3.6	6.8	9.8	7.8	4.3	8.3	6.6	6.7	3.4	3	1.9	3.4	7.8	6.7
i-14:0	0.9	0.33	0.4	0.2	0.3	0.5	0.2	0.1	0.4	0.6	0.4	0.6	0.2	0.15	0.2	0.25
ai-15:0	0.2	0.68	0.3	0.1	0.1	0.15	0.2	0.3	0.2	0.5	0.1	0.3	0.3	0.2	0.23	0.3
i-15:0	2.5	10.29	3	1.7	6	6.5	4.4	6	4.3	4	4.6	2.5	0.5	3.7	5.1	4
i-17:0	0.2	0.54	0.4	0.44	0.15	0.24	0.21	0.5	0.38	0.49	0.42	1.2	0.11	0.15	0.36	0.13
Σ BCFA	**6.4**	**19.07**	**7.7**	**9.24**	**16.35**	**15.19**	**9.31**	**15.2**	**11.88**	**12.29**	**8.92**	**7.6**	**3.01**	**7.6**	**13.69**	**11.38**
n-15:1ω6	0.1	0.09	-	0.2	0.26	0.3	0.1	0.5	0.4	0.3	0.1	-	0.5	0.7	0.2	0.1
n-16:1ω7	20.4	13.65	14.1	10.5	14.6	9.1	11.3	11.4	13.6	12.1	28.8	10.6	5.4	24.7	8.9	6.2
n-17:1ω8	0.1	0.28	0.2	2.2	2.3	2.2	1.6	1.2	0.6	1.2	0.7	1.3	2	1.5	2.1	3
n-18:1ω9c	0.3	0.53	0.3	0.1	0.3	0.2	0.1	0.1	0.2	0.3	0.2	35.3	0.4	0.6	0.2	0.5
n-18:1ω7c	4	4.88	2.8	4.4	3.3	2.1	7.1	4.8	6.1	3.6	4.4	1.5	3.8	4.5	6.4	2.5
n-20:1ω9	0.4	0.19	0.1	0.3	0.2	0.6	0.1	0.2	0.4	0.2	0.5	0.1	0.1	-	0.2	0.4
Σ MUFA	**25.3**	**19.62**	**17.5**	**17.7**	**20.96**	**14.5**	**20.3**	**18.2**	**21.3**	**17.7**	**34.7**	**48.8**	**12.2**	**32**	**18**	**12.7**
n-18:2ω6t	0.5	1.16	0.7	0.27	0.15	0.25	0.11	0.13	0.14	0.15	0.2	0.1	0.35	0.21	0.54	0.3
n-18:3ω6t	0.5	0.09	0.7	0.2	0.25	0.38	0.31	0.27	0.3	0.5	0.45	0.35	0.24	-	0.21	-
n-18:3ω3	0.2	0.11	0.1	0.2	0.12	0.28	0.08	0.21	0.15	0.25	0.4	0.15	0.3	0.6	0.53	0.7
n-18:4ω3	0.4	0.31	0.8	0.15	-	0.17	0.04	0.15	0.2	0.1	0.1	0.2	0.5	0.34	0.22	0.4
n-20:2	0.5	0.08	0.5	0.3	0.25	0.33	0.2	0.14	0.15	0.48	0.2	0.1	-	0.4	0.1	-
n-20:3ω3	0.3	0.06	0.1	0.29	0.41	0.26	0.06	0.28	0.08	0.1	0.1	0.2	0.4	0.7	0.5	0.5
n-20:4ω3	0.3	0.59	0.2	0.35	0.26	0.54	0.59	0.49	0.53	0.63	0.53	0.5	0.1	0.3	0.1	0.1
n-20:5ω3	**17.6**	**15.01**	**13.8**	**3.4**	**12.8**	**6.4**	**15.6**	**11**	**11.4**	**9.4**	**10.2**	**3.2**	**4.5**	**5.6**	**6.2**	**8.5**
n-22:4ω6	0.1	0.04	2.2	0.3	0.2	1.1	0.2	0.4	0.35	0.2	0.1	0.6	0.75	0.8	1.3	1.2
n-22:5ω3	0.3	0.49	0.3	0.1	0.15	0.1	0.2	0.33	0.42	0.2	0.5	0.1	0.15	0.25	0.11	0.25
Σ PUFA	**20.7**	**17.94**	**19.4**	**5.56**	**14.59**	**9.81**	**17.39**	**13.4**	**13.72**	**12.01**	**12.78**	**5.5**	**7.29**	**9.2**	**9.81**	**11.9**
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
ACL	15.91	15.57	15.49	14.53	15.39	14.73	15.58	15.38	15.32	15.03	15.62	16.16	16.03	15.79	14.91	14.79
EPA (mg g^{-1})	**20.28**	**15.48**	**14.2**	**1.9**	**12.57**	**5.68**	**15.23**	**11.13**	**11.00**	**9.94**	**10.66**	**7.26**	**4.06**	**5.18**	**5.90**	**8.19**
TFA (mg g^{-1})	115.2	103.1	102.5	55.3	98.2	88.7	97.6	101.2	96.5	105.7	104.5	88.5	90.2	92.5	95.1	96.3
Cells(g l^{-1})[a]	3.1	2.66	2.6	0.75	1.95	2.1	2	1.85	2	2.3	2.2	1.65	1.7	1.5	1.8	1.85

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable. ZB1: ZB supplemented with L-proline and $(NH_4)_2SO_4$; MB, Marine broth; ZB, Zobell broth; NC, Negative control; Ala, Alanine; Leu, Leucine; Pro, Proline; Ser, Serine; Pyr, Pyruvate; Glu, Glucose; Gly, Glycerol; T80, Tween 80; T60, Tween 60; T40, Tween 40; NH_4^+, $(NH_4)_2SO_4$.

Growth on L-leucine resulted in the highest production of n-13:0 (45.7 % versus 21-41 % for other carbon sources) and i-15:0 (6.5 %) at the expense of MUFAs and PUFAs, e.g. n-16:1ω7, n-18:1ω7c and EPA. This was mirrored by a corresponding decrease in the quantitative yield of EPA (5.68 mg g^{-1}) and the value of average chain length (14.73).

Growth on L-proline decreased the proportion of BCFAs, such as i-13:0 (4 % versus 5-9 % for other carbon/nitogen sources excluding glycerol and Tween medium). This was accompanied by a rise in the percentages of n-18:1ω7 (7.1 % versus 2–6 % excluding Tween 60) and EPA (15.6 % versus 4–12 %). Furthermore, the increased proportion of EPA was mirrored by a corresponding increase in the quantitative yield (15.23 mg g^{-1} versus 4-11 mg g^{-1} for other carbon/nitrogen sources), which was competing with MB cultures (15.5 mg g^{-1}), and the average chain length was in higher value (15.58).

The fatty acid composition of glucose cultured cells was similar to that of ZB cultured cells. The only difference was that the former one with higher content of BCFAs (12.3 % versus 7.7 % for ZB) and lower proportion of PUFAs (12 % versus 19 %).

Growth on glycerol led to lower SCFAs and BCFAs due to the lowest proportion of n-13:0 (21 % versus 24-51 % for other carbon/nitrogen sources) and i-13:0 (3.4 % versus 4-9 %, excluding Tween medium), whereas n-16:1ω7 at its highest point (29 % versus 6-15 %) which contributed a high lever of MUFAs (34.7 % versus 12-27 %), in TFAs (saturation degree of 0.92), and percentage of EPA production was in a sound level (10 %) as well as the quantitative yield (10.66 mg g^{-1}).

Growth on Tween 80, 60 and 40 exhibited a marked alteration in fatty acid composition and the quantitative yield of EPA (4-7 mg g^{-1} versus 10-12 mg g^{-1} for other carbon sources) and cells (1.5-1.7 g l^{-1} versus 1.8-2.3 g l^{-1}). The cultivation of strain MAR441 cells with Tween 80 (35 % content of n-18:1ω9c oleic acid) caused the increase of MUFAs up to 48 % in cellular lipids (saturation degree of 0.7) and contemporary inhibited the PUFAs production (polyunsaturation degree of 0.113). Growth on Tween 60 led to 10 % of n-16:0 and 33 % of n-18:0 causing the increase of SCFAs up to 77 % in TFAs (saturation degree of 4.0) and therefore inhibited the BCFAs, MUFAs and PUFAs production. Growth on Tween 40 resulted in high content of n-18:0 (15 %) and n-16:1ω7c (25 %), causing the increase of MUFAs up to 32 % in TFAs, the degree of

saturation and polyunsaturation were 1.2 and 0.29 respectively. However, the values average chain length from these Tween medium were among the highest levers with 15.79-16.16 versus 14.73-15.62 for other carbon sources.

The percentage of EPA from the L-leucin, L-seine and L-glucose cultures was comparable to that obtained when grown in ZB at 15 °C (10.2 % versus 9–11 %). And these higher proportions of EPA were mirrored by a corresponding the highest level in the quantitative yield (1-3.9 mg g^{-1}) with higher level of TFA (4.8-9.9 mg g^{-1}).

However, when the MAR441 grown on the single nitrogen medium by supplemented only with urea or $(NH_4)_2SO_4$, which led to low proportion of MUFAs and PUFAs and in favour of SCFAs, due to a great increase of n-13:0 (42.5 % for urea and 51.1 % for $(NH_4)_2SO_4$) with corresponding decrease of n-16:1ω7, n-18:1ω7 and EPA, and less average chain length (14.79 for urea and 14.91 for $(NH_4)_2SO_4$) in that the deficiency of carbon sources used for developing backbone.

Thus, ZB medium (ZB1) supplemented with L-proline and $(NH_4)_2SO_4$ for growing MAR441 cells led to a higher production of MUFAs and PUFAs with the proportion of 20.7 % and 25.3 % respectively due to the rise of n-16:1ω7 (20.4 %) and EPA (17.6 %), which was mirrored by the quantitative yield of TFA (115.2 mg g^{-1}) and EPA (20.28 mg g^{-1}), as well as the higher value of average chain length (15.91).

6.4.6 Effect of cerulenin treatment on the amount of biomass, lipids and EPA in Shewanella sp. MAR441 cells

Biomass production, lipid and EPA yields obtained from *Shewanella* sp. MAR441 cells grown in the medium containing cerulenin up to 7.5 μg ml^{-1} at 4 °C and 15 °C are summarized in Table 6.7, Figures 6.7 and 6.8. The cell biomass was 3.2 g dry cells l^{-1} when cells were grown in the medium containing cerulenin at 0.5, 1, 2.5, 5 and 7.5 μg ml^{-1}. This value was 20 % increased as that of non-treated cells. The lipid yield (g l^{-1}) was unchanged by the concentration of cerulenin in the range from 0 to 7.5 μg ml^{-1}. However, the biomass was slightly influenced by the concentration of cerulenin to the medium up to 7.5 μg ml^{-1}, indicating some effects of cerulenin to the growth of *Shewanella* sp. MAR441 cells. The highest EPA yield at 81.5 μg ml^{-1} was obtained from cells treated with cerulenin at 0.5 μg ml^{-1} at 4 °C. This yield was 53 % increased than that obtained from non-treated cells. However, at 15 °C, EPA was 93 % increased

when the cells was treated with 1 μg ml^{-1} cerulenin and the percentage of PUFAs was 201 % increased due to n-18:2ω6t and n-18:3ω3 were also increased at high level. All these results clearly show that the optimization of cerulenin treatment enhances EPA or PUFAs production as well as the short chain fatty acids, such as n-13:0 and n-15:0, and most of the middle chain FAs, such as n-16:0, n-18:0, i-15:0, n-15:1ω6, n-16:1ω7, n-17:1ω8 and n-18:1ω7c acids were inhibited in *Shewanella* sp. MAR441 cells when various concentration of cerulenin were presented in the growth medium.

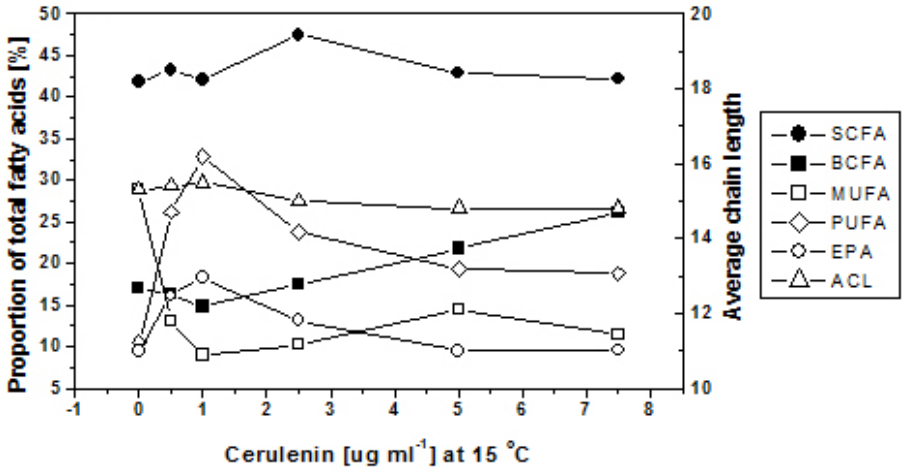

Figure 6.7 Change in average chain length (ACL, triangles) and relative proportion of whole cell FAs in *Shewanella* sp. MAR441 grown in marine broth medium at 15 °C with various concentration of cerulenin. The experiments were carried out in triplicate and values are means of three samples based on Table 6.7.

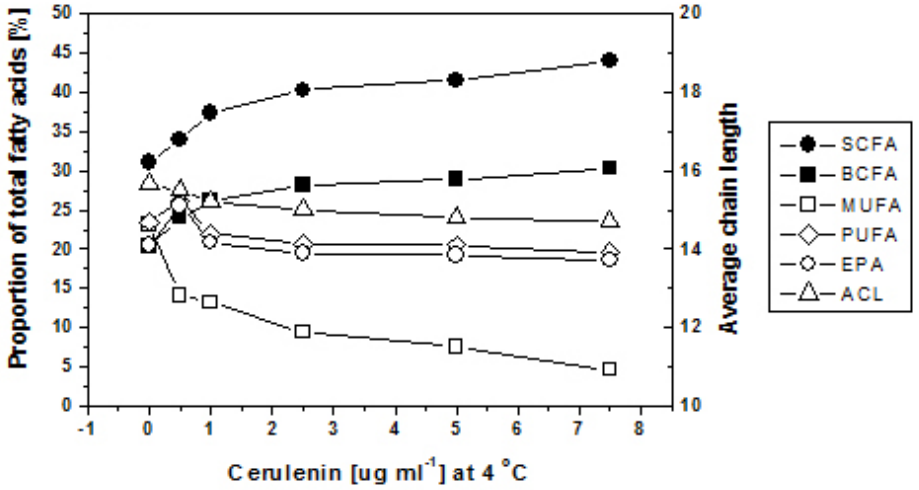

Figure 6.8 Change in average chain length (triangles) and relative proportion of whole cell FAs in strain MAR441 grown in marine broth medium at 4 °C with various concentration of cerulenin. Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open boxes); polyunsaturated fatty acids (MUFAs, open diamonds); eicosapentaenoic acid (EPA, open circles). The experiments were carried out in triplicate and values are means of three samples based on Table 6.7.

Table 6.7 Fatty acid composition of *Shewanella* sp. MAR441 grown on various concentration of cerulenin in marine broth medium at 4 °C and 15 °C

Fatty acids	Cultures with Cerulenin (μg ml^{-1}) at 15 °C						Cultures with Cerulenin (μg ml^{-1}) at 4 °C					
Cerulenin	0	0.5	1	2.5	5	7.5	0	0.5	1	2.5	5	7.5
n-12:0	3.6	5.4	5.7	8.1	7.5	6.6	2.3	2.1	2.5	2.7	3.1	4.5
n-13:0	16.8	17.7	19.4	20.9	17.3	16.3	13.5	14.5	17.1	18.5	19.7	21.5
n-14:0	2.9	4.1	4.2	5.8	4.1	4.5	3.9	3.7	4.1	4.2	4.7	4.8
n-15:0	4.7	10.8	11.6	12.1	13.5	14.3	4.5	7.3	8.5	11.5	12.3	11.1
n-16:0	11.5	5.3	1.2	0.6	0.5	0.5	6.4	5.6	5.1	3.4	1.7	2.1
n-18:0	2.4	0.0	0.0	0.0	0.0	0.0	0.5	0.8	0.1	-	-	-
Σ SCFA	41.9	43.3	42.1	47.5	42.9	42.2	31.1	34.0	37.4	40.3	41.5	44.0
i-13:0	5.9	8.7	8.0	12.0	14.3	15.3	12.7	17.8	20.6	22.9	23.8	25.8
i-15:0	11.2	7.7	6.8	5.5	7.5	10.8	7.7	6.2	5.5	5.3	5.1	4.5
Σ BCFA	17.1	16.4	14.8	17.5	21.8	26.1	20.4	24.0	26.1	28.2	28.9	30.3
n-15:1ω6	0.9	0.2	0.6	0.2	1.7	1.6	0.1	0.6	0.6	0.3	0.2	0.3
n-16:1ω7	18.2	10.9	6.3	8.1	10.8	8.5	16.9	13.4	12.6	9.1	7.2	4.3
n-17:1ω8	3.8	0.1	0.1	0.0	0.4	0.0	0.2	0.1	-	-	-	-
n-18:1ω7c	5.9	1.8	2.0	1.8	1.5	1.3	6.0	-	-	-	-	-
Σ MUFA	28.8	13.0	9.0	10.2	14.4	11.4	23.2	14.1	13.2	9.4	7.5	4.6
n-18:2ω6t	0.5	6.3	9.1	7.6	7.2	7.1	2.8	0.5	0.4	0.4	0.5	0.4
n-18:3ω3	0.6	3.8	5.4	2.9	2.7	2.1	0.3	0.2	0.7	0.7	0.8	0.7
n-20:5ω3	9.5	16.1	18.3	13.1	9.5	9.6	20.5	25.5	20.9	19.5	19.2	18.6
Σ PUFA	10.6	26.2	32.8	23.7	19.4	18.8	23.5	26.2	22.0	20.6	20.5	19.6
Others	1.6	1.1	1.3	1.2	1.5	1.5	1.8	1.7	1.3	1.5	1.7	1.5
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
UFAs/SFAs	0.9	0.9	1.0	0.7	0.8	0.7	1.5	1.2	0.9	0.7	0.7	0.5
ACL	15.3	15.4	15.5	15.0	14.8	14.8	15.65	15.5	15.2	15.0	14.8	14.7
EPA (mg g^{-1})	9.7	16.7	18.7	13.3	9.7	9.8	23.6	30.2	25.5	23.5	22.9	21.9
TFA (mg g^{-1})	102.5	103.5	102.0	101.5	102.1	102.2	115.2	118.6	122.0	120.5	119.1	118.0
EPA(mg l^{-1})	25.2	54.3	59.8	42.6	30.9	30.6	53.1	81.5	75.2	65.8	64.1	59.1
Cells(g l^{-1}) [a]	2.6	3.25	3.2	3.2	3.19	3.12	2.25	2.7	2.95	2.8	2.8	2.7

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

6.4.7 Fatty acid production by NTG-mutant strains (A4 and A13)

Two mutants A4 and A13 were morphologically different from the wild type strain MAR441. Colonies of wild-type strain MAR441 on marine agar plates were 2-4 mm in diameter, tan-pigmented, butyrous in consistency, circular and convex in shape with an

smooth edge, and the central rough area is adherent to or embeds into the agar and is not easy to emulsify. Whereas, colonies of NTG-mutant strains A4 and A13 on marine agar plates were 3-5 mm in diameter, tan-pigmented, opaque, dull, with dentate margin or undulate edge, and the central rough area is attached to the agar loosely, which make it easily be pushed/moved away by pipette tips (Figure 6.9A). The cells of A4 and A13 were found with absence of fimbriae after the NTG mutation under scanning electron microscopy (SEM) (Figure 6.9B).

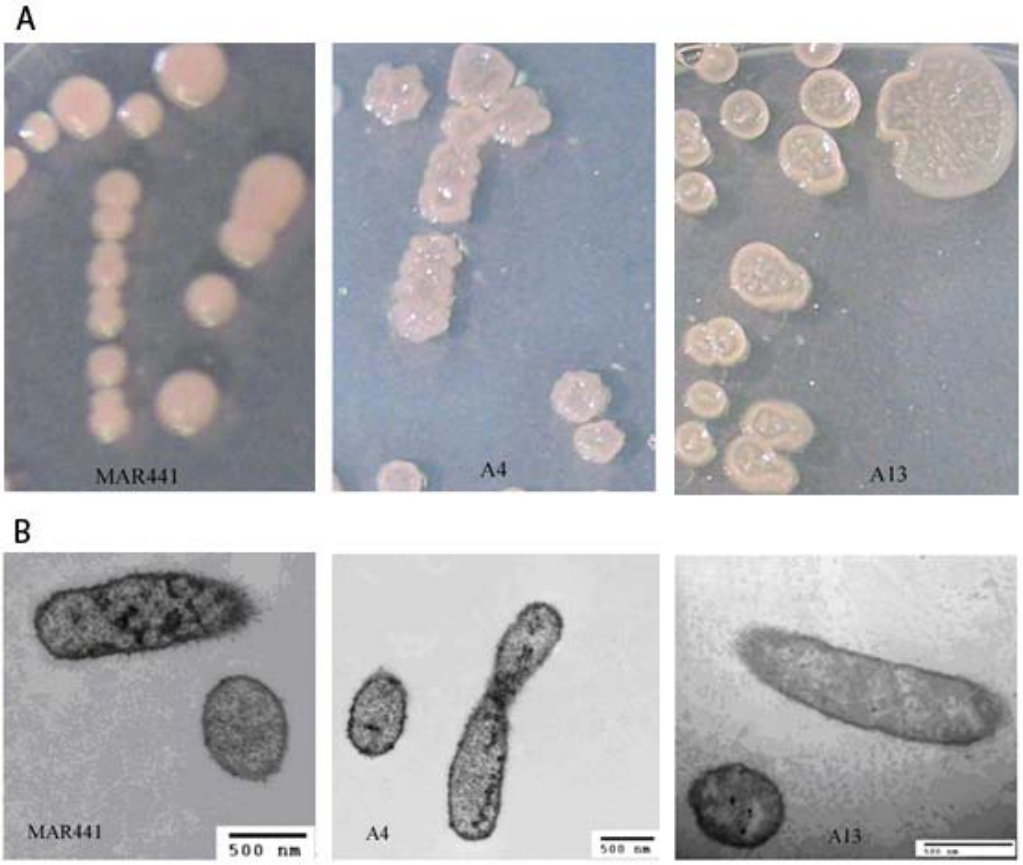

Figure 6.9 Colonies of strain MAR441 and its NTG mutants (A4 and A13) on marine agar plates at 15 ºC for 3 days; (B) scanning electron microscopy (Right, Bar 500 nm) of a negatively-stained cells of strain MAR441 and its NTG mutants (A4 and A13).

The effect of growth temperature on the percentage composition of individual fatty acids in *Shewanella* sp. MAR441 and its NTG-mutants (A4 and A13) grown between 4 °C, 15 °C and 25 °C is shown in Figure 6.10 and Table 6.8. Comparing to the fatty acid compositions of strain MAR441, the mutants were found with lower levers of SCFAs and higher percentage of BCFAs at 15 °C and 25 °C, and lower levers of EPA at 4 °C. However, the percentage of EPA in mutant A13 was higher than that in wild type strain at 15 °C, and decreasing the EPA levels 4 °C and 25 °C. By increasing temperatures

from 4 °C to 25 °C, the values of average chain length (ACL) in these mutants were decreasing. The quantitative level of lipid content of the mutants were similar to that of MAR441 at 4 °C and 15 °C, while increasing levels of EPA were found at 25 °C. Mutant A13 could reach relatively higher lever of EPA of 15.8 mg g^{-1} cells dry weight at 15 °C (Figure 6.10).

Table 6.8 Fatty acid composition of *Shewanella* sp. MAR441 and its NTG mutants (A4 and A13) grown on marine broth medium at 15 °C

Fatty acids	MAR441			A4			A13		
Temperatures	4°C	15°C	25°C	4°C	15°C	25°C	4°C	15°C	25°C
n-12:0	1.3	2.2	0.3	4	4.5	5.5	3.8	3.6	4.6
n-13:0	13.5	23.6	32.1	8.3	7.2	9.5	7.2	8.2	9.7
n-14:0	3.9	2.9	2.7	3.7	4.4	6.1	4.5	4.7	6.5
n-15:0	3.5	4.5	1.9	0.5	0.8	0.7	0.4	1.9	0.5
n-16:0	5.3	14.5	12.4	14.1	17.9	9.3	12.4	12.3	16.1
n-17:0	0.3	0.8	5.7	1	0.8	4.8	0.9	0.8	2.2
n-18:0	0.5	1.3	1.0	3	2.2	0.3	2.1	1.7	2.3
Σ SCFA	28.3	49.7	56.0	34.6	37.8	36.2	31.3	33.2	41.9
i-13:0	11.6	4.9	11.2	16.5	10.1	12.2	14.2	9.1	13.1
i-14:0	0.7	0.4	0.2	1.5	0.5	0.9	-	-	0.3
ai-15:0	1.2	0.4	0.8	-	-	-	0.4	0.7	0.4
i-15:0	7.2	10.2	14.2	6.6	13.3	27.6	11.1	16.6	22.7
i-17:0	0.5	0.2	0.2	0.4	0.3	0.5	1.7	0.5	0.4
Σ BCFA	21.2	16.2	26.6	25	24.2	41.2	27.4	26.9	36.9
n-16:1ω7	16.9	11.2	9.3	18.4	20.2	13.1	15.2	17.9	12.1
n-17:1ω8	0.2	2.8	4.0	3.3	2.6	2.5	2.1	2.1	2.1
n-18:1ω7c	5.2	4.2	1.1	6.3	6.4	3.5	4.2	2.8	1.2
Σ MUFA	22.3	18.3	14.5	28	29.2	19.1	21.5	22.8	15.4
n-18:2ω6t	2.8	1.5	0.3	0.3	0.4	-	0.3	0.5	0.5
n-18:3ω3	0.3	0.6	0.2	0.5	0.3	-	0.3	0.3	-
n-20:3ω3	0.8	0.3	0.1	-	-	-	5.7	-	-
n-20:5ω3	21.4	9.5	0.6	10.7	6.8	2.6	12.8	15.3	4.5
Σ PUFA	25.3	11.9	1.2	11.5	7.5	2.6	19.1	16.1	5
others	2.9	3.9	1.9	0.9	1.3	0.9	0.7	1	0.8
Total	100	100	100	100	100	100	100	100	100
ACL	16.29	15.52	14.67	15.45	15.35	14.82	15.92	15.64	14.94
EPA (mg g^{-1})	24.7	9.7	0.6	10.7	6.8	2.4	12.8	15.8	4.3
TFA (mg g^{-1})	115.2	102.5	96.5	112.7	100.6	91.2	110.4	103.7	95.6
Cells(g l^{-1}) [a]	2.25	2.6	0.6	2.3	2.6	0.62	2.35	2.66	0.6

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length (calculated after White et al., 2005); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

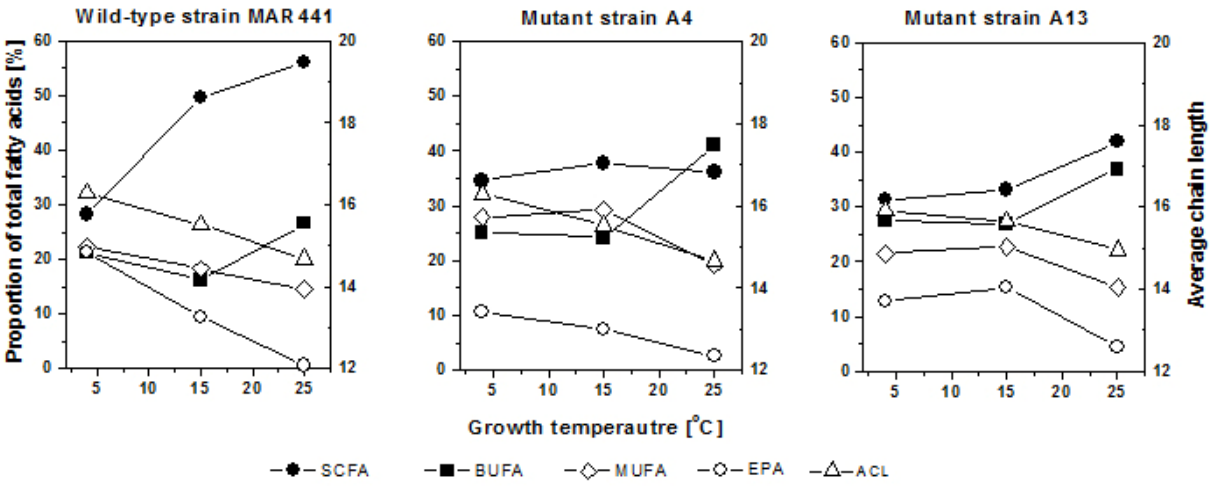

Figure 6.10 Change in average chain length (ACL, triangles) and relative proportion of whole cell FAs in *Shewanella* sp. MAR441 and its mutants (A4 and A13) grown at 4, 15 and 25 °C.Straight chain fatty acids (SCFAs, filled circles), branched chain fatty acids (BCFAs, filled boxes), monounsaturated fatty acids (MUFAs, open boxes) and eicosapentaenoic acid (EPA, open circles). The experiments were carried out in triplicate and values are means of three samples based on Table 6.8.

6.5 Discussion

6.5.1 Environmental adaptation

The fatty acid composition of MAR441 exhibited changes in response to growth temperature and sole carbon/nitrogen source, as have been reported on some *Shewanella* PUFAs producers (Nichols et al., 1997; Gentile et al., 2003), and non-PUFAs producers, such as *Cobetia marina* (Yumoto et al., 2004). Both the percentage and the quantitative level of EPA markedly changed at different growth temperatures, indicating that PUFA may play a critical role in the modulation of membrane (Nichols and Russell, 1996; Nichols et al., 1997). The mechanism why these bacteria produce omega-3 fatty acids is still unclear, although many PUFA synthase genes responsible for EPA/DHA synthesis have been cloned and sequenced (Allen and Bartlett, 2002; Okuyama et al., 2007), and been successfully expressed in *E. coli* (Valentine and Valentine, 2004; Amiri-Jami and Griffiths, 2010). As growth temperature increased, MAR441 also demonstrated a novel adaptational response by increasing the percentage of n-13:0 and i-15:0 acids with corresponding decrease of n-16:1ω7 and n-18:1ω7 acids, which might due to the role of fatty acid precursors selection within this bacterium as an adaptational response (Jostensen and Landfald, 1996). This finding is also supported by

some other strains possessing similar fatty acid compositions, which sharing similar adaptational response, such as *Shewanella gelidimarina* ACAM 456^T exhibited a rise proportion of n-15:0 and decrease of n-16:1ω7 and EPA as increasing the culture temperature (Bowman et al., 1997b), *S. olleyana* ACEM 9^T showed an increase in the percentage of n-16:0 and i-15:0 with corresponding decrease of n-16:1ω7, n-18:1ω7 and EPA when growth temperature increased (Skerratt et al., 2002).

Growth on the sole carbon sources L-proline, Tween 80, Tween 60 or Tween 40 demonstrated that in this bacterium the fatty acid composition can be manipulated by the provision of potential acyl chain precursor (Bowman et al., 1997b). The large increase in the observed percentage of EPA and less of i-13:0 and i-15:0 from L-proline cultures. While an observed increase of i-13:0 and to a lesser extent i-15:0 from L-alanine and L-leucine cultures that there is a preference for 4-5 cycles of chain elongation from the alanine- or leucine-derived primer molecule according to previously researches (Butterworth and Bloch, 1970; Kaneda, 1991). This is similar to *S. gelidimarina* ACAM 456^T, which exhibited a similar change in fatty acid composition when it was grown in L-leucine medium (Bowman et al., 1997b). The degree of acyl chain elongation may therefore be primer-specific. L-alanine and L-proline also increased the level of TFA, and therefore EPA, suggesting an increase in the level of total lipid resulted from growth on these substrates. In contrast, FAs patterns were dominated by the n-18:1ω9 in Tween 80 cultures, while mainly n-18:0 in Tween 60 cultures and with great rise of n-16:1ω7 when grown in Tween 40, which greatly decrease the percentage of PUFAs or polyunsaturation degree. These data are in agreement with the results obtained for EPA-producing *Shewanella* strain GA-22, the Tween 80-growing cells caused the increase of monounsaturated fatty acids up to 78 % in cellular lipids and contemporary inhibited the PUFA production (Gentile et al., 2003). These findings were consequences of the nature of substrates added, because Tween 80 contains oleic (n-18:1) acid, Tween 60 has stearic acids (n-18:0) and Tween 40 is composed of n-16:0. Although Tween 80, 60 and 40 usually served as surfactants, they might also be used as carbon sources.

However, the strain preferred growing in the complex medium, such as marine broth and ZoBell broth, from which the higher level of biomass, lipid or TFA and EPA was achieved, instead of cultivating in single carbon/nitrogen medium. This corroborates previous reports that, production of PUFA at low temperature of cultivation was

enhanced more than two-fold and reached 5 % of total fatty acids in the strain GA-22 cells grown on marine broth (Gentile et al., 2003). In our study, L-proline and $(NH_4)_2SO_4$ were selected as the most suitable carbon and nitrogen sources used in combination for preparing ZB medium, which greatly improved the production of biomass (two-fold increased) and EPA (1.5-fold). The carbon-to-nitrogen ratio (C:N) in the substance may affect the production of biomass and EPA.

MAR441 required Na^+ for growth and EPA synthesis. The Na^+-requiring for MAR441 cells growth is agreeable with other marine strains, such as *S. halifaxensis*, *S. sediminis*, *S. pealeana* and *S. woodyi*, they required Na^+ and preferred low temperatures for growth and thus considered as cold-adapted obligate marine *Shewanella* (Zhao et al., 2005; Zhao et al., 2006). Studies on protein coding sequences (CDS) from two obligate marine *S. halifaxensis* and *S. sediminis*, found that many genes coding Na^+-dependent nutrient transporters were recruited to use the high Na^+ content as an energy source. For example, many unique Na^+-dependent nutrient symporters and Na^+/nutrient symporters for transport of L-glutamine acid, L-proline, dicarboxylate and amino acids (Zhao et al., 2010). Based on genome annotations of all *Shewanella*, L-glutamine acid was predicted to be an essential precursor for biosynthesis of heme, nucleobase (purine, pyrimidine), peptiglycan, aminosugar, and fatty acids in *Shewanella* (Makemson and Hastings, 1979; Padan et al., 2001). Requirement of Na^+ as a motive force for transport of these essential growth substrates are consistent with the nature of strain MAR441 being obligate marine bacteria as strains *S. halifaxensis* and *S. sedimini*.

Cerulenin specifically blocks the activity of β-keto acyl thioester synthetase, which may account for the inhibition of overall fatty acid synthesis (Vance et al., 1972). However, EPA production was greatly improved in *Shewanella* sp. MAR441 by treated with cerulenin, and middle-chian fatty acids were almost deficit. The resulted were suggested that cerulenin inhibited the *de novo* synthesis of middle-chain fatty acids, but not the synthesis of EPA/DHA and short-chain fatty acids (Morita et al., 2000). Therefore, *Shewanella* sp. MAR441 may similar to *Moritella marina* strain MP-1 and *Shewanella marinintestina* strain IK-1, using two fatty acid-biosynthetic systems, e.g. independently synthesize middle-chain fatty acids and LC-PUFAs, by using a common stating material(s) as FA precursor (Amiri-Jami et al., 2006).

The NTG mutagenesis is particularly useful chemical mutagenesis of a variety of Gram-

negative bacteria (Lewenza et al., 2005). However, NTG mutagenesis has not been reported as a means for increasing PUFA production from the environmental bacteria, although *Shewanella putrefaciens* strain 2738 has been treated by NTG to get cold-sensitive EPA-requiring mutants at low temperature (Valentine and Valentine, 2004). Therefore, by treating NTG at higher temperatures, we got less temperature sensitive mutants with improved levers of lipid and EPA content at higher temperatures.

6.5.2 EPA yield

The yield of EPA from strain MAR441 ranged from 2 to 20 mg g^{-1} (30 mg g^{-1} of the cerulenin-treating cultures) (cells dry weight) or 6 to 63 mg l^{-1} while depending on cultural conditions, and showed the highest quantitative yield of 11 % TFA of dry cell weight and 20 mg $l^{-1}day^{-1}$ of EPA production in marine broth at 15 °C for 1.5 days, values which compare favourably with literature reports for other EPA-producing bacteria. *Shewanella putrefaciens*-like strain SCRC-8132 produced 4-15 mg g^{-1} (cells dry weight) of EPA and 2 % TFA of dry cell weight, although it was reported with high percentage of EPA (24-40 %) (Yazawa et al., 1988b; Yazawa, 1996). Under various temperatures by utilizing different carbon sources, *Shewanella gelidimarina* ACAM 456^T could yield 1 to 16 mg g^{-1} (cells dry weight) of EPA (Bowman et al., 1997b), whereas another *Shewanella putrefaciens*-like strain SCRC-2738, is presently identified as *Shewanella pneumatophori* SCRC-2738 (Hirota et al., 2005), produced 4 to 11 mg g^{-1} of EPA (Yazawa et al., 1988a), while enhanced to 17 mg g^{-1} by Akimoto et al. (Akimoto et al., 1990).

Many wild-type strains of autophotrophic microalgae produce similar levels of EPA to the bacteria mentioned above, such as *P. tricornutum* and *Monodus subterraneus*, has been investigated (Yongmanitchai and Ward, 1991b). By employing continuous culture, with the heterotrophic diatom *Nitzschia laevis*, resulted in the highest EPA productivities of 73 mg l^{-1} day^{-1} using a glucose feed (Wen and Chen, 2003). In a high cell density system maximum cell dry weight and EPA yields were 22.1 g l^{-1} and 695 mg l^{-1}, respectively, in a14-day incubation (Wen et al., 2002). While, the productivity of *P. tricornutum* by using batch culture was 25 mg l^{-1} day^{-1} (Yongmanitchai and Ward, 1991a), and improved up to 40 mg l^{-1} day^{-1} by employing photobioreactor (Miron et al., 1999), *N. alba* ATCC 40775 was optimized with high production EPA of 100-300 mg l^{-1} day^{-1} (40-50 g l^{-1} biomass) (Boswell et al., 1992), *Thraustochytrid* strains were reported with EPA productivity of 47 mg L^{-1} day^{-1} in 50 l tubular photobioreactor

(Grima et al., 1994), *Mortierella alpina* 1S-4 and *M. subterraneus* were reported with EPA production of 30 and 50 mg l^{-1} day^{-1} respectively (Shimizu et al., 1988; Grima et al., 1994), and *M. alpine* was with high biomass production of 43 g l^{-1} and EPA productivity of 60 mg l^{-1} day^{-1} (Bajpai and Ward, 1991). However, the majority of these algal species generally require strictly controlled growth conditions in terms of nutrients, light quantity and quality, oxygenation and carbon dioxide levels; these factors can result in considerable expense. In contrast, MAR441 strain with high EPA productivity of 20 mg $l^{-1}day^{-1}$ (2 g l^{-1} of biomass) when grown in marine broth at 15 °C for 1.5 days could be a cheap and reliable source of PUFA, therefore it is of great interest in many industrial and health fields, although biomass yield per unit volume and culture time may affect production economics.

6.5.3 EPA in phospholipid and non-esterified fatty acids

The occurrence of non-esterified fatty acids (NEFA), or free fatty acids (FFA) in MAR441 was identified through TLC and GC, accounting for 15 % of the total lipid and contained 18.7 % EPA. Phospholipids were found as the main content of total lipid (72 % versus 28 % for neutral lipid). Major phospholipid classes exhibited phosphatidyl ethanolamine (PE) and phosphatidyl glycerol (PG) as the main components (50 % versus 40 %), appreciable levels of EPA were detected from both of them (9.5 % versus 14.2 % for PG) and 6.5 % EPA in diphosphoglyceride (DPG) which accounted for 5 % of total lipid. The presence of non-esterified fatty acids within a PUFA-producing *Vibrio* sp. was reported to be 13.3 % of total lipid, and EPA accounted for 13 % of NEFA (Henderson et al., 1993). PE (with 5.5% of EPA) and PG (with 10.6 % of EPA) accounted for 61 % and 19 % of phospholipids respectively in *Aeromonas* sp. 3010, and high content of EPA and palmitoleic acid (19.7 and 50 %, respectively) in the free fatty acid fraction was found (Cho and Mo, 1999). For *Shewanella* strain ACAM 456, PG contained a higher in the percentage of total PUFA (14 % versus 9 % for PG) and EPA (13 % versus 9 %), and NEFA accounted for 9 % of total lipid and contained 22 % EPA (Bowman et al., 1997b). By employing the FAB-MS-MS technique, acyl chains, such as i-13:0/13:0 and 14:0/14:0 appeared to be associated with EPA in PE phospholipid species only, whereas the association of n-17:1 and acyl-18:0 chains with EPA was specific to PG in strain ACAM 456 (Bowman et al., 1997b). Yazawa also reported the presence of NEFA in the PUFA producing strain SCRC-2738, with 5–10 % of total EPA reported to be in the non-esterified form. This may be explained by the contribution of most EPA-producing bacterial genera possessing PG as their major

phospholipid type, MUFAs components and EPA were mostly concentrated in PG, while the proportion of branched-chain fatty acids was elevated in PE. Furthermore, the above mentioned five PUFA-producing bacteria all contain appreciable amounts of NEFA or FFA, of which EPA appears as a particular role of NEFA in PUFA metabolism. This remains an area for further investigation.

6.6 Conclusions

Our study demonstrated that *Shewanella* sp. strain MAR441 isolated from Mid-Atlantic Ridge (MAR) deep-sea sediments, could be grown easily under different conditions with high production of EPA. The EPA of the strain is contained within phospholipids (PL), mainly PE and PG, with high percentage level. For optimization EPA production, variable conditions such as temperature, time period, Na^+, antibiotic and NTG mutation have been investigated. We also found that the synthesis of middle-chain fatty acids and the synthesis of EPA would be independently functioning by using a common starting material(s) as a primer. Therefore, strain MAR441 could be used as a source of oils rich in omega-3 PUFAs production commercially or used as feedstock for organisms, such as rotifers, as a way of introducing them into a marine food web for producing PUFA-rich oils by aquaculture.

It will be interesting to examine the heterogenous expression of the polyketide synthase genes responsible for EPA production in Gram positives, such as *Bacillus subtilis*, rather than the homologous expression in *E. coli*, which should also be aided by our ongoing experiments including genomic analysis. Models of EPA production of wide type strain MAR441 under different conditions, and a better understanding of the synergistic corporation of each individual polyketide synthase gene (*pfa*A, *pfa*B, *pfa*C, *pfa*D and *pfa*E) will help us to better predict the maximum EPA production.

Chapter 7. Biosynthesis of polyunsaturated fatty acids by polyketide synthases in *Gammaproteobacteria*

7.1 Abstract

Microbes capable of producing omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are of great interesting due to their vital roles in marine food webs and dietary importance as an alternative resource. In this study, we reported the highest yield of EPA at 21 % of TFA (or 25 mg g^{-1}) from a novel deep-sea *Shewanella* species, *Shewanella* sp. strain MAR441; investigated EPA production from sponge-associated bacterium *Vibrio* sp. strain NSP560; and for the first time described shallow-sea algae associated *Photobacterium* sp. strain MA665, of this genus, could be cultured easily under atmospheric conditions with appreciable levels of EPA (up to 25 % of TFA or 10.6 mg g^{-1}). All these three *gammaproteobacteria* were defined as psychrotrophiles, they showed relative high contents of EPA (up to 15-21 % of total fatty acids at 4 °C) at low temperatures. Furthermore, in the cerulenin-treated cells, decreases in levels of middle-chain fatty acids and increased levels of EPA were observed at 4 °C, 15 °C and 25 °C (by up to 2 fold at 15 °C). This study indicated that the synthesis of EPA and short-chain fatty acids was separated from the synthesis of middle-chain fatty acids. The putative EPA synthesis gene clusters were mined from the genomic DNA from these three species by fosmid library construction and/ or PCR cloning. The gene cluster of strain NSP560 encodes a PKS-like pathway that consists of six open reading frames (ORFs): *pfaABCDE*, whereas *pfaE* was separated from *pfaABCD* in the genomic DNAs of strains MAR441 and MA665. The deduced amino acid sequences encoded by these genes reveal large multidomain proteins, with high similarity to those polyketide synthases capable of catalysing EPA biosynthesis by a novel polyketide synthesis mechanism. These findings reveal a common distribution of the novel PUFA pathways among psychrotrophilic marine microorganisms.

7.2 Introduction

High fish intake is associated with a decreased risk of colorectal cancer according to epidemiological studies (Norat et al., 2005; Geelen et al., 2007; Hall et al., 2008). Most of the beneficial effects have been linked to the high content of the omega-3

polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) in some fish. They were well documented for their role as precursors for many hormone and hormone like regulatory molecules, such as the eicosanoids signaling molecule (Braden and Caroll, 1986; Abbey et al., 1990; Lauritzen et al., 2001; Sauer et al., 2001). Furthermore, these fatty acids provide beneficial effects on the heart of healthy people (Prokofeva et al., 2005), people at high risk of cardiovascular disease and patients with cardiovascular disease (Angerer and von Schacky, 2000; Kris-Etherton et al., 2003b), high blood pressure (Tresguerres et al., 1989), inflammation (Fiocchi et al., 1994), Alzheimer's disease (Connor and Connor, 2007) and certain types of cancer (Sauer et al., 2001). However, the fish original omega-3 oils may have potential problems, such as fishy odor, coextracted contaminants, and the market is limited to the non vegetarian society (Arts et al., 2001). Nevertheless, the crucial problem of those oils is their sustainability due to the worldwide decline of fish stocks (Garcia and Rosenberg, 2010). Therefore, the development of alternative lipid sources such as marine microbial oils as a possible source for EPA and DHA is urgently required.

So far, only limited bacterial species, such as genera *Shewanella*, *Moritella*, *Colwellia*, *Alteromonas*, *Photobacterium*, *Flexibacter* and *Psychroserpens* are reported to be able to produce PUFAs (Nichols and McMeekin, 2002). Most of these bacteria are psychrophiles or psychrotrophiles isolated from the polar regions and the deep sea (Delong and Yayanos, 1986; Bowman et al., 1997b; Nogi et al., 1998a; Nichols et al., 1999; Kato and Nogi, 2001; Gentile et al., 2003; Wang et al., 2009). And some of them are mesophiles obtained from a temperate estuary and shallow seawater samples (Ivanova et al., 2001; Skerratt et al., 2002; Ivanova et al., 2003b; Frolova et al., 2005). Furthermore, psychrotrophilic bacteria could be used as a primary PUFA-rich feedstock in artificial food chains in the aquaculture industry, thereby protecting natural fish stocks (Watanabe et al., 1992; Nichols et al., 1996a). Meanwhile, levels of EPA produced by prokaryotes may significantly influence the efficiency of energy transfer between primary and consumer trophic levels in aquatic ecosystems (Muller-Navarra et al., 2000). Therefore, prokaryotic PUFA production in marine food webs is important.

Increasing production of EPA and/ or DHA responses to temperature decrease by some bacteria lead to the hypothesis that these molecules may be important for growth at low temperatures (Delong and Yayanos, 1986; Valentine and Valentine, 2004; Amiri-Jami

et al., 2006). However, EPA was not required for low-temperature growth in the deep-sea bacterium *Photobacterium profundum* (Allen et al., 1999), but it may be required for low-temperature growth in *Shewanella* (Valentine and Valentine, 2004; Sato et al., 2008; Wang et al., 2009). Thus, the function of PUFAs in the microbial membrane remains enigmatic.

The aims of the present study were to characterize three gammaproteobacterial strains *Shewanella* sp. MAR441, *Vibrio* sp. NSP560 and *Photobacterium* sp. MA665, isolated from deep-sea sediments, marine sponge and micro algae respectively; to examine the modulation of EPA production related to temperature and cerulenin-treatment; and to characterize their PUFA synthesis gene clusters responsible for EPA biosynthesis based on fosmid library construction and PCR cloning.

7.3 Materials and methods

7.3.1 Bacterial strains and cultivation

Strains MAR441, NSP560 and MA665 were isolated from Mid-Atlantic Ridge (MAR) "non-vent" site at the depth of 2,500 m, North-sea sponge and micro algal culture respectively. Strains were routinely cultured in marine 2216E broth (MB) (Difco) according to the methods mentioned in Chapter 2 and Chapter 6.

7.3.2 Cultivation conditions based on supplying with antibiotics

The 10 ml MAR441, NSP560 and MA665 seed cultures were respectively used to inoculate 90 ml volumes of marine broth contained in 500 ml conical flasks pre-rinsed in chloroform, where the antibiotic, cerulenin (MERCK), in 50 % (v/v) ethanol was added at various concentrations (0, 0.5, 1, 2.5, 5 and 7.5 μg ml^{-1}) prior to cultivation. Flasks were incubated at 4 °C, 15 °C and 25 °C respectively. The growth of cells was monitored turbidometrically at 600 nm.

7.3.3 Fatty acid analysis as methods mentioned in Chapter 2

7.3.4 Construction of genomic fosmid libraries

Preparation of genomic libraries of *Shewanella* sp. MAR441, *Vibrio* sp. NSP560 and *Photobacterium* sp. MA665 using CopyControl™ HTP Fosmid Library Production Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA) according to the manufacturer's protocol. Briefly, high-molecular-mass genomic DNA was isolated from all these three strains respectively following the published method (DiLella and Woo, 1987). Aliquots (50 μl) of extracted DNA were randomly sheared through a 25 gauge needle with a series of draws. The optimal size of sheared DNA was selected for end-repairing and then separated by pulse-field gel electrophoresis (PFGE). PFGE was performed for 12 h in a 0.8 % low melting point (LMP) agarose gel at a constant voltage of 30-35 V. The appropriate size of DNA ranging from 35–40 kb were isolated, electroeluted and dialyzed against 0.5×TE buffer, and then ligated into the vector pCC1FOS. After *in vitro* packaging into lambda phages and transfection into the phage T1-resistant *Escherichia coli* EPI300-T1^{R}, the bacterial cells were plated onto LB plates containing 25 mg ml^{-1} chloramphenicol and incubated at 37 ºC for ~24 h. Approximately 600 fosmid clones were obtained for each library, all the clones were individually picked into 96-well microtiter plates containing LB medium plus 20 μg chloramphenicol ml^{-1}, and grown at 37 ºC for 24 h, and then 10 % (v/v) glycerol was added and stored at -80 ºC until further analysis.

To estimate the insert sizes, 15 clones were randomly selected from each fosmid library. Fosmid DNA was isolated via mini-prep using the FosmidMAX™ DNA Purification Kit (Epicentre Biotechnologies, Madison, WI), and treated with Plasmid-Safe™ ATP-Dependent DNase (Epicentre Biotechnologies, Madison, WI), by following the manufacturer's instructions. The inserts were released from the vector by complete digestion with *NotI*. The insert sizes were estimated using PFGE and Quantity One. We used the following formula to check the library capacity/quality:

$$N = \ln(1-P) / \ln(1-f)$$

Where P is the desired probability, f is the proportion of the genome contained in a single clone, and N is the required number of fosmid clones.

7.3.5 PCR screening of the fosmid libraries

Colony PCR was employed for the first around screening of PUFA synthase gene on 10 % of the clones of each library randomly by using the primer PCR primers targeting the keto-acyl synthase (KS) domain of the *pfaA* and *pfaB* genes involved in omega-3

polyunsaturated fatty acid (PUFA) biosynthesis were used to construct clone libraries to investigate KS sequence. *pfa*B 1240F: 5`- GGTGAAGCATCRATGTGGGC-3` and *pfa*B 1840R: 5`-TCSGCRCCAATTTCAACAA-3`, to detect *pfa*B gene which located in the middle position of the PUFAs synthesis gene cluster (Gentile et al., 2003). Positive clones from the first round screening were further sequenced by sequencing primers for pCC1FOS™: HTFP061, 5`- GTACAACGACACCTAGAC-3` and HTRP062, 5`-CAGGAAACAGCCTAGGAA-3` to get the clones containing full PUFA synthase gene cluster, positive clones from the second screening were further cloned by primer working methods and sequenced by Eurofins MWG Operon.

7.3.6 Phylogenetic analysis -16S rRNA gene and EPA gene clusters analysis

The above mentioned high-molecular-mass genomic DNA samples isolated from strains MAR441, NSP560 and MA665 were used as templates for PCR amplification of the 16S rRNA gene fragments according to the methods described previously (DeLong, 1992; Rainey et al., 1996). The EPA genes from NSP560 and MA665 were PCR cloned by degenerate primers, which were designed based on known PfaA KS sequences in NCBI's GenBank and the Joint Genome Institute's (JGI) Integrated Microbial Genomes (IMG) database (http://img.jgi.doe.gov) (Table 7.1). The PCR products were sequenced by Eurofins MWG Operon after purification with PureLink™ PCR Purification Kit (Invitrogen Ltd, Paisley, U.K) following the manufacturer's protocol.

The 16S rRNA and PUFA synthase gene sequences determined were checked for similarities to DNA sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) and RDPII (http://rdp.cme.msu.edu) database using the Basic Local Alignment Search Tool (BLAST). Deduced amino acid sequences of PUFA synthase enzymes were subjected to protein phylogenetic analysis. The alignment and phylogenetic analysis of sequences were achieved with the neighbour-joining method of Saitou and Nei (Saitou and Nei, 1987), by DNAMAN software package (Version 5.1), cluster and molecular evolutionary genetics analysis (MEGA) Version 4.0 (Tamura et al., 2007). The length of each branch pair represents the evolutionary distance between the sequences.

The nucleotide sequences of 16S rRNA gene and polyunsaturated fatty acid (*pfa*) synthase gene sequences have been deposited in EMBL under the accession numbers: FR744784, FR744874 and FR750952 (strains MAR441, NSP560 and MA665

respectively); FR837656-FR837670 (*pfaA, pfaB, pfaC, pfaD* and *pfaE* from strains MAR441, NSP560 and MA665 respectively).

Table 7.1 Primers for PCR screening on PUFAs genes in the fosmid library and genomic DNA

Primer	5′→3′	nucleotide sequence accession No./source	Gene
HTFP061	GTACAACGACACCTAGAC	Sequencing primer for pCC1FOS™	
HTRP062	CAGGAAACAGCCTAGGAA	Sequencing primer for pCC1FOS™	
pfaA 422F	GGTGTSGGYGGTGGTCAR	(Gentile et al., 2003)	
pfaA 2100R	CTCACCRAARCTRTGRCC	(Gentile et al., 2003)	
pfaB 1240F	GGTGAAGCATCRATGTGGGC	(Gentile et al., 2003)	
pfaB 1840R	TCSGCRCCAATTTCAACAA	(Gentile et al., 2003)	
pfaC 3525F	TTGATGGTCARATCCCTTGG	(Gentile et al., 2003)	
pfaC 5075R	GTTMCGGAAGAACAGCTC	(Gentile et al., 2003)	
pfaB 1240F	GGTGAAGCATCRATGTGGGC	(Gentile et al., 2003)	
pfaB 1840R	TCSGCRCCAATTTCAACAA	(Gentile et al., 2003)	
MAR441-pfaE(P0F1)	GTNAGRTTRGGNTTRAGRGCNTTRCT	This study	
MAR441-pfaE(P3R1)	TTRATRTANGAYTCYTT	This study	
560pfaA-420F	GGTGTTGGTGGTGGTCAG	This study	
560pfaA-2082R	CTCACCAAAGCTGTGGCC	This study	
560pfaB-1182F	GGTGAAGCCTCCATGTGGGC	This study	
560pfaB-1756R	TCGGCACCGACTTCTACAA	This study	
560pfaC-3613F	TTGATGGTCAGATCCCTTGG	This study	
560pfaC-5136R	GTTGCGGAAAAACAGTTC	This study	
560pfaD-545F	GGTTGAGGCTTCTGCCTTCTTA	This study	
560pfaD-1095R	CACGCCCATTTCGAACATGTC	This study	
560pfaEF	ATGAAAACTCCCGTTGTTGATT	This study	
560pfaER	TTAAAGGAGATGAGACGACGA	This study	
665pfaA-420F	GGTGTGGGTGGTGGTCAG	This study	
665pfaA-2082R	CTCACCGAAGCTGTGACC	This study	
665pfaB-1236F	GGTGAGGCATCGATGTGGGC	This study	
665pfaB-1819F	TTGTTGAAGTTGGTGCCGA	This study	
665pfaC-3523F	TTGATGGTCAAATCCCTTGG	This study	
665pfaC-5070R	GTTCCGGAAGAACAGCTC	This study	
665pfaD-542F	CGTTGAAGCGTCTGCATTCTT	This study	
665pfaD-1092F	GACATGTTCGAAATGGGTGTA	This study	
665pfaEF	ATGAATCCCCCTTATATTTCGC	This study	
665pfaER	TCAACTCTGAGTGAATGTTCT	This study	

7.4 Results and discussion

7.4.1 Identification of the EPA producing Gamma-Proteobacteria

The three gammaproteobacterial strains were isolated under aerobic conditions and grew well by fermentation of amino acids or carbohydrates. Aerobically grown, they utilized a wide range of carbon sources, including biopolymers, carbohydrates, and fatty and amino acids (Table 7.2). All strains grew with nitrate as electron acceptor. Strain MAR441 also reduced manganese oxide, thiosulfate and ferric compounds (Fe(III)).

The temperature range for growth was similar for all strains ranging from 4 °C to 28-35 °C, with optimal grow temperatures at 15-20 °C, defining them as psychrotrophiles.

Table 7.2 Morphological and physiological properties of the EPA-containing isolates, their isolation substrates

Phenotypic characteristic	*Shewanella* sp. MAR441	*Vibrio* sp. NSP560	*Photobacterium* sp. MA665
Isolated from	Deep-sea sediments	North-sea sponge	North-sea microalgae
Shape	Rod	Oval-rod	Oval-rod
Temperature range (°C)	4-28	4-30	4-30
Size (μm)	0.5×1.5–4.5	0.6–0.7×1.5–4.5	0.6 × 1.5–2.8
Requires Na^+ ions for growth	+	+	+
Substrates utilized			
Peptone	+	+	+
Cellulose	+	-	+
Chitin	-	+	+
Laminarin	+	+	+
Monosaccharides	+	+	+
Fatty acids	+	+	+
Alcohols	-	+	+
Mono- and dicarboxylic acids	+	+	+
Amino acids	+	+	+
Fermentation of			
Carbohydrates	+	+	+
Amino acids	+	+	+
Electron acceptors			
Nitrate	+	+	+
Nitrite	-	-	-
MnO_2	+	-	-
Fe(III)	+	-	-
Thiosulfate	+	-	-
Production of			
DNase	-	-	-
Amylase	+	-	-
Lipase	-	+	+
Gelatinase	+	+	-
Chitinase	-	-	-
Protease	-	+	+
Utilization of:			
D-Glucose	+	+	+
Maltose	+	+	+
Sucrose	-	+	-
Motile	-	+	+

Analysis of partial 16S rRNA gene sequences revealed that the three strains belonged to the *Gammaproteobacteria* and were closely related to those species already described. Strains MAR441 turned out to be a member of the genus *Shewanella* within *Altermonadaceae*, with 97.9 % the highest similarity with *Shewanella olleyana*, which isolated from a temperate estuary (Skerratt et al., 2002), 97.6 % to *Shewanella japonica* isolated from sea water (Ivanova et al., 2001), and 97.1 % to *Shewanella donghaensis*

isolated from deep-sea sediments of the East Sea (Yang et al., 2007); whereas strains NSP560 and MA665 affiliated with genus *Vibrionaceae*, strain NSP560 being most closely related to *Vibrio tasmaniensis* (pairwise similarity 99.4 %) isolated from Atlantic Salmon (Thompson et al., 2003) and 99.2 % to *Vibrio cyclitrophicus* isolated from marine sediments (Hedlund and Staley, 2001), and strain MA665 showed homology to *Photobacterium frigidiphilum* (pairwise similarity 99.2%) and to *Photobacterium profundum* (pairwise similarity 98.2 %) isolated from deep-sea sediments (Nogi et al., 1998c; Seo et al., 2005) (Figure 7.1). The cells of these three gammaprobacteria were rod-shaped, Gram-negative, 2.0–4.5 μm in length, 0.5–1.5 μm in diameter.

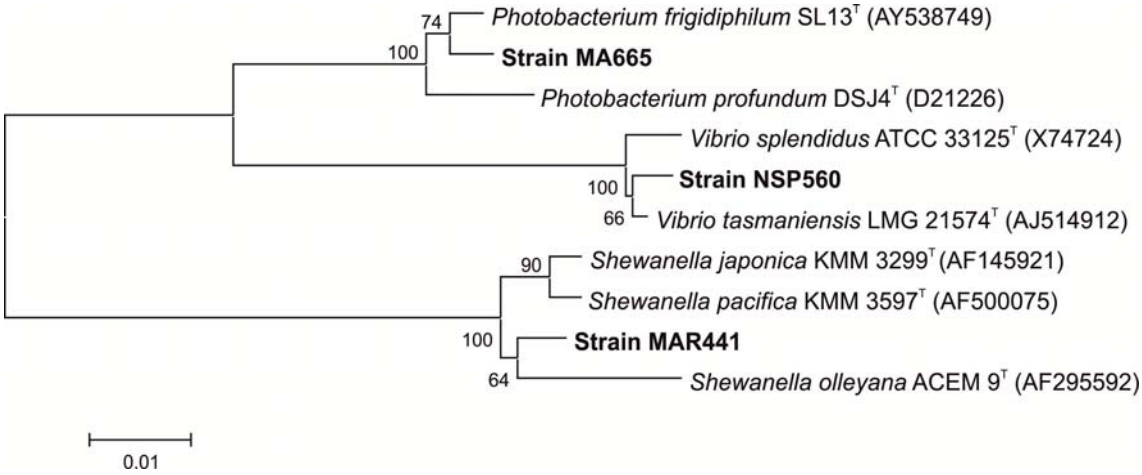

Figure 7.1 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of strains MAR441, NSP560, MA665 and their nearest type strains in the database. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.01 nucleotides substitution per site.

7.4.2 Fatty acid composition

Generally, the fatty-acid compositions of the three strains agree well with those of their closest relatives (Nogi et al., 1998c; Skerratt et al., 2002; Seo et al., 2005), with n-16:0 and n-16:1ω7/n-16:1ω9 being the dominant fatty acids (Table 7.3). All strains contained relatively high amounts of EPA (up to 24 %). The fatty-acid patterns of *Vibrio* sp. NSP560 and *Photobacterium* sp. MA665 were very similar to each other but differed from that of *Shewanella* sp. MAR441 by containing higher amounts of n-13:0 and n-

15:0 fatty acids, and much higher amount of branched-chain fatty acids (16–26 %), with i-13:0 and i-15:0 being the most prominent BCFAs. Trans-fatty acids (mainly trans-16:1ω7) were found in *Vibrio* sp. NSP560, with the highest relative abundance of about 40 % in the culture grown at 4 °C (Table 7.3); while, trans-16:1ω9 presented at the highest level of 37-41 % in *Photobacterium* sp. MA665 culture at temperatures from 4-25 °C.

7.4.3 Temperature dependence of whole-cell fatty-acid patterns

All three strains changed their fatty-acid patterns with changing temperatures (Table 7.3, Figure 7.2A). In. *Shewanella* sp. MAR441 and *Vibrio* sp. NSP560, the proportion of monounsaturated fatty acids (MUFAs) increased at lower temperatures, with the most pronounced change occurring between cells grown at 15 °C or 25 °C. In contrast, in *Photobacterium* sp. MA665, only relatively minor changes in saturated straight-chain fatty acids (SCFAs) and MUFAs were observed, and the ratio of MUFAs to SCFAs remained almost constant at higher temperatures.

Relative abundances of EPA increased with decreasing growth temperature in all strains. In *Shewanella* sp. MAR441, the relative increase in EPA was the highest between cells grown at 15 °C to 4 °C (max. 21.4 % of total fatty acids). In *Vibrio* sp. NSP560 (maximum, 8 % of total fatty acids) and *Photobacterium* sp. MA665 (maximum, 14 % of total fatty acids), largest differences in relative abundances of EPA were observed between cultures grown at 15 °C and 4 °C. These findings agree with data for other *Vibrio* (Ringo et al., 1992; Hamamoto et al., 1995; Jostensen and Landfald, 1996) and *Shewanella* species (Nichols et al., 1997; Skerratt et al., 2002; Wang et al., 2004). As in *Photobacterium* sp. MA665, the relative abundances of MUFAs in cells grown at 4 °C, 15 °C, or 25 °C vary only slightly; the relative increase of EPA appears to be the main mechanism of adaptation to low temperatures as indicated on this species (Freese et al., 2009). Likewise, a mutant strain of *Photobacterium profundum* produced little MUFAs but elevated levels of EPA and was neither low temperature nor high-pressure sensitive (Allen et al., 1999). *Shewanella* sp. MAR441 was seldom grown at 30 °C, and with very limited production of EPA above 25 °C. It may be that temperature-sensitive enzymes are involved in biosynthesis in that EPA production of species related to some *Shewanella* species was limited to a maximum growth temperature of approximately 30 °C (Hirota et al., 2005; Freese et al., 2009). It may be also that the amount of EPA produced exclusively depends on

temperature, and little affected by other environmental parameters like salinity (Henderson et al., 1995), anoxia (Nichols et al., 1992; Metz et al., 2001), or variations in nutrient composition and availability, although these were shown to influence the composition of other fatty acids (Jostensen and Landfald, 1996; Yumoto et al., 2004).

Branched fatty acids (BCFAs) have repeatedly been reported to promote cold adaptation (Suutari and Laakso, 1994; Annous et al., 1997; Chattopadhyay and Jagannadham, 2001). However, the results obtained with *Shewanella* sp. MAR441 are ambiguous. Although the relative contribution of BCFAs is higher at 4 °C than that at 15 °C, the highest values were observed in cells grown at 25 °C, due to the great change of i-13:0 acid. In the latter case, however, it appears that BCFAs compensate for a strong decrease in MUFAs. Similarly, increasing contribution of BCFAs at lower and higher temperatures were observed for *Shewanella gelidimarina* ACAM 456 (Nichols et al., 1997), and *Desulfobacterium autotrophicum* (Rabus et al., 2002). For other bacteria, no clear changes in BCFAs with varying growth temperature were observed (Henderson et al., 1993; Nichols and McMeekin, 2002).

The values of average chain length (ACL) and quantitative level of EPA production of these three strains were decreased with increasing growth temperature in that much longer chain fatty acids and higher percentage of EPA were produced. The yield of EPA from strain MAR441 was 24.7 mg g^{-1} (cells dry weight) and showed the highest quantitative yield of 11 % TFA of dry cell weight at 4 °C, values which compare favourably with literature reports for other EPA-producing bacteria, such as *Shewanella putrefaciens*-like strain SCRC-8132, *Shewanella gelidimarina* ACAM 456^T and *Shewanella pneumatophori* SCRC-2738, up to the highest production of 15-16 mg g^{-1} of EPA (cells dry weight) (Yazawa et al., 1988b; Akimoto et al., 1990; Yazawa, 1996; Nichols et al., 1997; Hirota et al., 2005).

Table 7.3 Temperature dependence of the fatty acid composition of strains MAR441, NSP560 and MA665 grown in marine broth medium without or with adding 1 µg ml^{-1} cerulenin

Fatty acids	*Shewanellas* sp. MAR441						*Vibrio* sp. NSP560						*Photobacterium* sp. MA665					
	MB			+ 1 µg ml^{-1} Cerulenin			MB			+ 1 µg ml^{-1} Cerulenin			MB			+ 1 µg ml^{-1} Cerulenin		
Temperatures	4°C	15°C	25°C	4°C	15°C	25°C	4°C	15°C	25°C	4°C	15°C	25°C	4°C	15°C	25°C	4°C	15°C	25°C
n-10:0	-	-	-	-	-	-	2.7	3.1	5.8	6.5	7.2	7.7	-	-	-	-	-	-
n-12:0	1.3	2.2	0.3	2.5	5.7	8.6	5.4	7.5	9.8	13.2	15.7	17.1	0.5	2.1	0.8	6.9	10.1	11.7
n-13:0	13.5	23.6	32.1	17.1	19.4	35.6	-	-	-	-	-	-	0.2	0.3	0.2	1	1.0	1.7
n-14:0	3.9	2.9	2.7	4.1	4.2	4.5	6.5	11	13.7	17.8	18.7	19.5	1.7	2.8	3.5	12.8	10.9	15.2
n-15:0	3.5	4.5	1.9	8.5	11.6	10.2	-	-	-	-	-	-	0.5	1.3	2.1	0.5	1.9	1.8
n-16:0	5.3	14.5	12.4	5.1	1.2	5.0	15.9	20.2	25.6	14.5	13.7	16.8	27.6	24.3	26.5	23.4	16.6	18.5
n-17:0	0.3	0.8	5.7	-	-	-	-	-	-	-	-	-	0.2	0.1	0.3	-	-	-
n-18:0	0.5	1.3	1.0	0.1	0.0	0.0	0.6	0.8	1.5	-	-	-	0.8	2.4	3.5	-	-	-
Σ SCFA	28.3	49.7	56.0	37.4	42.1	63.9	31.1	42.6	56.4	52	55.3	61.1	31.5	33.3	36.9	45.3	40.5	48.9
i-13:0	11.6	4.9	11.2	20.6	8.0	7.5	-	-	-	-	-	-	-	-	-	-	-	-
i-14:0	0.7	0.4	0.2	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	-	-	-
ai-15:0	1.2	0.4	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
i-15:0	7.2	10.2	14.2	5.5	6.8	8.6	1.2	3.5	3	0.5	1.2	2.7	-	-	-	-	-	-
i-16:0	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.2	0.2	-	-	-
i-17:0	0.5	0.2	0.2	-	-	-	-	-	-	-	-	-	-	0.1		-	-	-
Σ BCFA	21.2	16.2	26.6	26.1	14.8	16.1	1.2	3.5	3	0.5	1.2	2.7	0.4	0.4	0.3	-	-	-
n-14:1ω5c	-	-	-	-	-	-	-	-	-	-	-	-	1.6	1	0.5	6.1	8.5	8.0
n-15:1ω6	0.1	0.9	0.6	0.6	0.6	0.5	-	-	-	-	-	-	1.4	1	0.6	2.2	4.1	5.4
i-16:1ω6	-	-	-	-	-	-	1	1.2	1.1	0.4	0.7	1.2	-	-	-	-	-	-
n-16:1ω7	16.9	11.2	9.3	12.6	6.3	2.3	39.5	36.7	31.2	32.3	29.8	28	1	1.3	0.6	0.8	-	-
n-16:1ω9	-	-	-	-	-	-	-	-	-	-	-	-	37	40.1	41.2	21.1	26.2	28.9
n-16:1ω11	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-
n-17:1ω8	0.2	2.8	4.0	-	0.1	0.1	-	-	-	-	-	-	0.1	0.1	-	-	-	-
n-17:1ω6	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.3	0.4	-	-	-
n-18:1ω9c	-	-	-	-	-	-	-	-	-	-	-	-	11.3	13.2	14.5	-	0.3	0.1
n-18:1ω7c	5.2	4.2	1.1	-	-	-	17.9	6.7	4.4	1.5	1.2	0.6	0.5	0.4	0.6	-	-	-
Σ MUFA	23.3	19.7	15.5	13.2	7.0	2.9	58.4	47.1	36.7	34.2	31.7	29.8	53.2	57.4	58.4	30.2	40.3	42.4
n-18:2ω6t	2.8	1.5	0.3	0.4	9.1	6.3	-	-	-	-	-	-	-	-	-	-	-	-
n-18:3ω3	0.3	0.6	0.2	0.7	5.4	3.2	-	-	-	-	-	-	-	-	-	-	0.5	-
n-20:5ω3	21.4	9.5	0.6	20.9	18.3	6.5	8	5.8	2.5	12.3	10.5	5.5	14	7.5	3.2	24.5	17.9	7.7
n-22:1ω9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-
n-22:5ω3	0.8	0.3	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Σ PUFA	27.8	14.4	2.0	22.0	22.0	16.0	8	5.8	2.5	12.3	10.5	5.5	14.0	7.5	3.2	24.5	18.4	7.7
Others	3.9	3.0	1.2	1.3	1.3	1.1	1.3	1	1.4	1	1.3	1.2	0.4	0.9	1	0.7	0.8	1.0
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100.0
ACL	16.3	15.5	14.7	15.2	15.5	14.4	16.0	15.1	15.0	15.1	14.8	14.5	16.6	16.2	16.2	16.3	15.89	15.1
EPA (mg g^{-1})	24.7	9.7	0.6	25.5	18.7	6.5	5.4	4.0	1.7	8.4	7.2	3.7	5.8	3.0	1.3	10.6	8.2	3.5
TFA (mg g^{-1})	115.2	102.5	96.5	122.0	102.0	99.6	67.3	68.5	67	68	69	67	41.5	40.5	41	43.1	46	45.7
Cells(g l^{-1}) [a]	2.25	2.6	0.6	2.95	3.2	0.75	1.8	1.9	1.8	1.8	1.9	1.8	2.1	2.2	2.1	2.15	2.42	2.1

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length calculated after reference (White et al., 2005a); SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, Eicosapentaenoic acid (n-20:5ω3); and (–), not detectable.

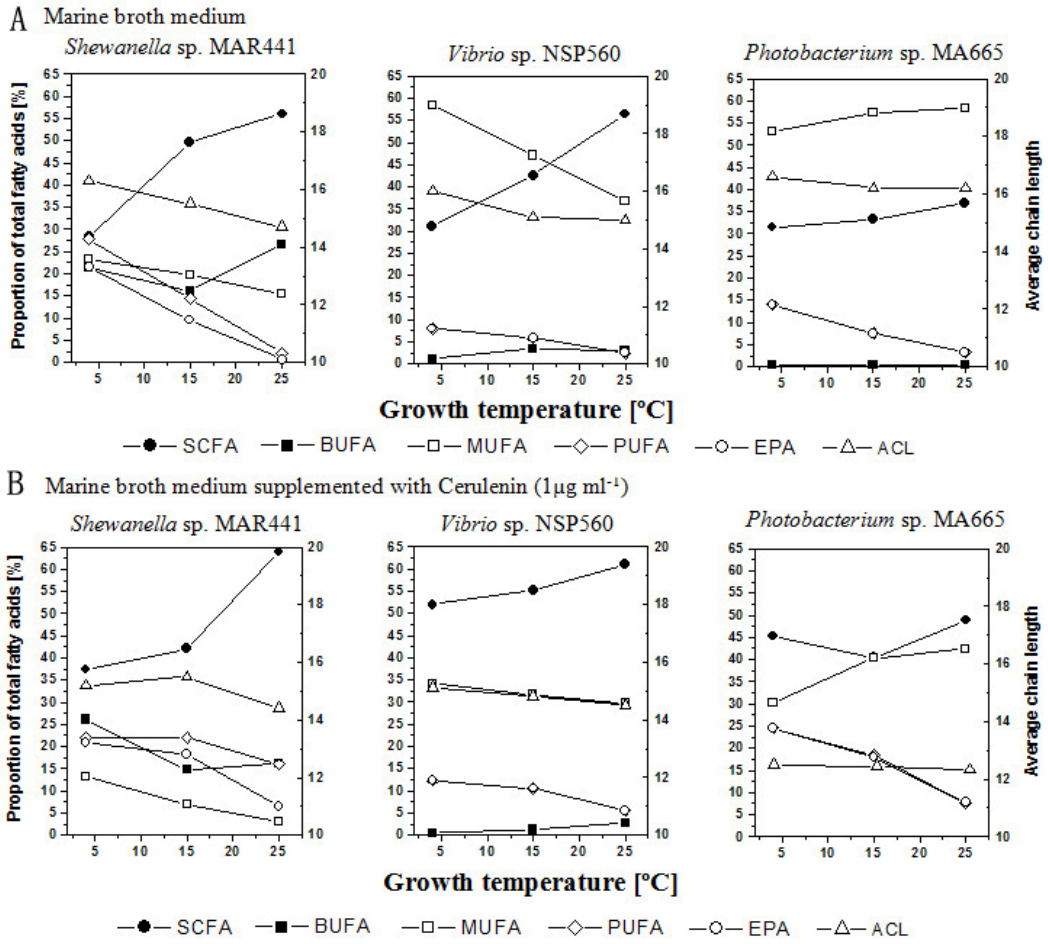

Figure 7.2 Change in average chain length (ACL) (triangles) and relative proportion of whole cell FAs in *Shewanella* sp. MAR441, *Vibrio* sp. NSP560 and *Photobacterium* sp. MA665 grown in marine broth medium at 4, 15 and 25 °C without (A) and with (B) 1 µg ml^{-1} of cerulenin. Saturated straight-chain fatty acids (SCFAs, filled circles), branched-chain fatty acids (BCFAs, filled squares), monounsaturated fatty acids (MUFAs, open boxes), polyunsaturated fatty acids (PUFA,open diamonds) and eicosapentaenoic acid (EPA, open circles) as percentage of total fatty acids in the isolates investigated. The experiments were carried out in triplicate and values are means of three samples based on Table 7.3. The growth of cells was monitored turbidometrically at 600 nm and Cells were collected for fatty acid analysis during the exponential phase as methods mentioned in Chapter 6.

7.4.4 Effect of cerulenin treatment on the amount of biomass, lipids and EPA

Biomass production, lipid and EPA yields obtained from these three gammaproteobacterial cells grown in the medium containing 1 µg ml^{-1} of cerulenin at 4 °C, 15 °C and 25 °C are summarized in Figure 7.2B and Table 7.3. Addition of 1 µg ml^{-}

[1] cerulenin in marine broth improved the production of cell biomass and EPA of *Shewanella* sp. strain MAR441, comparing to those from the medium with different concentration of cerulenin at 0.5, 1, 2.5, 5 and 7.5 μg ml^{-1} (data not shown in this study). In *Shewanella* sp. MAR441, EPA was increased to 18.3 % and 6.5 % respectively at 15 °C and 25 °C, by cerulenin treatment, whereas not apparently changed at 4 °C (max. 20.9% of total fatty acids). In *Vibrio* sp. NSP560 (maximum, 12.3% EPA of total fatty acids) and *Photobacterium* sp. MA665 (maximum, 24.5 % EPA of total fatty acids) at 4 °C, and higher levers of EPA were also observed between cultures grown at 15 °C and 25 °C. The result supports the other studies on enhancement of DHA production from *Moritella marina* strain MP-1 (Morita et al., 2000), and EPA production from *Shewanella marinintestina* strain IK-1 (Morita et al., 2005), by cerulenin treatment. The biomass was slightly influenced by adding cerulenin to the medium, with 20 % increased in strain MAR441, and the lever of lipid yield (g l^{-1}) was unchanged, although it was reported that the antibiotic could inhibit the growth of a variety of yeasts, fungi and bacteria (Matsumae et al., 1964), and by inhibiting the incorporation of acetate into the lipids (Nomura et al., 1972; Goldberg et al., 1973). Cerulenin treatment enhanced EPA or PUFAs production as well as the short-chain fatty acids, such as n-13:0 and n-15:0, and most of the middle-chain FAs, such as n-16:0, n-18:0, i-15:0, n-15:1ω6, n-16:1ω7, n-17:1ω8 and n-18:1ω7c acids were inhibited in these three species.

Cerulenin specifically blocks the activity of β-keto acyl thioester synthetase, which may account for the inhibition of overall fatty acid synthesis (Vance et al., 1972). However, our results suggest that cerulenin commonly inhibited the *de novo* synthesis of middle-chain fatty acids but not the synthesis of EPA and short-chain fatty acids. Since the gross amount of EPA per dry weight of cells increased (e.g. from 9.7 to 18.7, 4 to 7.2, and 3 to 8.2 mg g^{-1} dry weight of cells at 15 °C for MAR441, NSP560 and MA665 respectively), partially at the expense of the total amount of n-16:1 and n-18:1 (e.g. from 15.8 to 6.4, 6.3 to 0 and 21.6 to 12.1 mg g^{-1} dry weight of cells at 15 °C for MAR441, NSP560 and MA665 respectively), in the presence of cerulenin, it is considered that these three gammaproteobacterial strains use a common starting material(s) as a precursor in their two fatty acid-biosynthetic systems.

7.4.5 Construction and characterisation of the fosmid library

The purified DNA ranged in size from 24–145 kb, but was concentrated in the 40–80 kb range (Figure 7.3 A), suggesting that additional shear was necessary. The extracted

DNA was therefore randomly sheared through a 1 ml pipet tip with 65 times (optimal). After 50 repetitions, the DNA fragments were primarily in the 30–50 kb size range (Figure 7.3B). The sheared DNA was processed to end-repair and the 30–50 kb fragments were recovered by PFGE (Figure 7.4A) and confimed by PCR amplification on pfaB gene (Figure 7.4B). The selected DNA was ligated with the vector pCC1FOS, then transformed into *E. coli* EPI300-T1R. A total of 600 clones were isolated and transferred to ten 96-well microtiter plates.

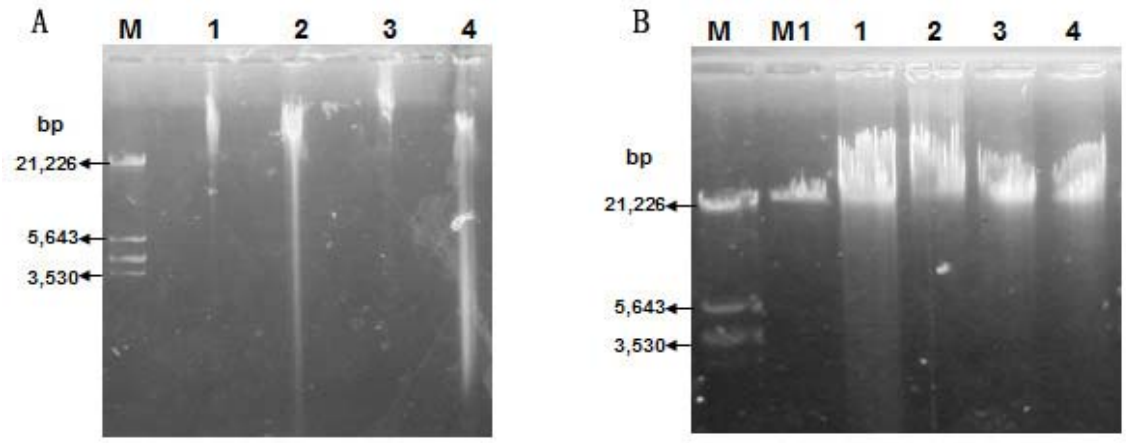

Figure 7.3 (A) High molecular genome DNA extracted from strains MA66, NSP560, MAR441 and MA665 (lanes 1-4); (B) Analysis of the size of sheared DNA fragments by pulsed-field gel electrophoresis (PFGE). M: Lambda DNA/EcoR I Marker; M1: the 35 kb control DNA; Lane 1, the genome DNA of *Photobacterium* sp. strain MA65; 2, the genome DNA of *Vibrio* sp. strain NSP560; 3, the genome DNA of *Shewanella* sp. strain MAR441 and 4, the genome DNA of *Photobacterium* sp. strain MA665.

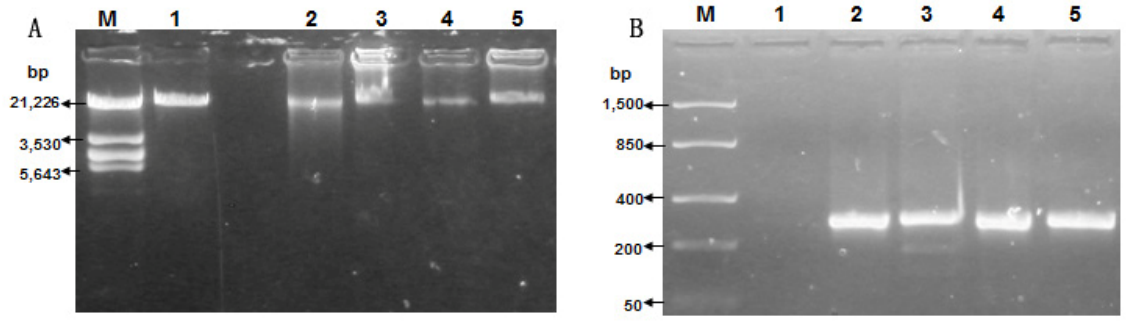

Figure 7.4 (A) Genome DNA purified from pulsed-field gel electrophoresis (PFGE). M: Lambda DNA/EcoR I Marker; Lane1: the 35 kb control DNA; 2, the genome DNA of *Photobacterium* sp. strain MA66; 3, the genome DNA of *Vibrio* sp. strain NSP560; 4, the genome DNA of *Shewanella* sp. strain MAR441 and 5, the genome DNA of *Photobacterium* sp. strain MA665. **(B)** PCR amplification of *pfaB* partial gene from the purified genome DNA samples. M, FastRuler™ Low Range DNA Ladder; Lane 1, negative control; 2, PCR product from the genome DNA of strain MA66; 3, PCR product from the genome DNA of strain NSP560; 4, PCR product from the genome DNA of strain MAR441 and 5, PCR product from the genome DNA of strain MA665.

Primer pairs showed in Table 7.1 were used for library screening by a matrix two-step PCR system. For example, the first PCR screening on the fosmid clones in the library was conducted by using the pfaB primer pair (pfaB 1240F and pfaB 1840R) (Figure 7.5), with a positive clone of MAR441-1.

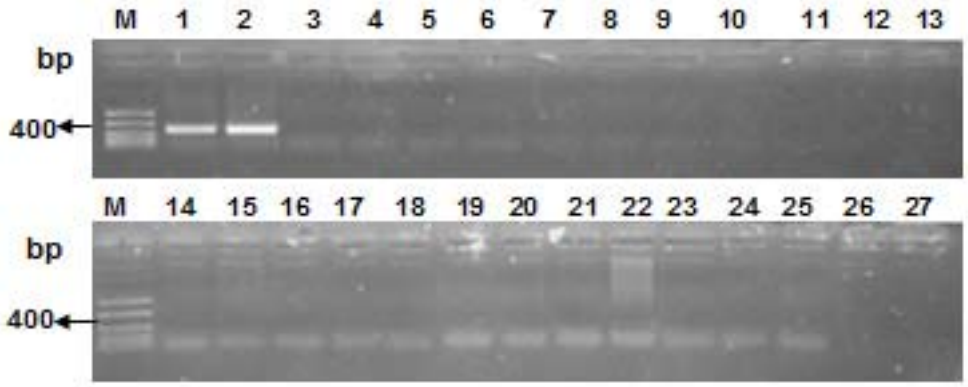

Figure 7.5 PCR screening of the *pfaB* partial gene from the fosmid library of *Shewanella* sp. strain MAR441 with the primer pair pfaB 1240F and pfaB 1840R. M, DNA size ladder; Lane 1, MAR441 genome DNA used as the positive control. Lanes 2-27 are PCR products from fosmid clones in the library constructed by using MAR441 genome DNA.

End sequencing by pCC1FOS™ sequencing primers confirmed that fosmid clone, MAR441-1 contained full length of polyunsaturated fatty acid gene clusters (*pfaA-D*), similar to *Photobacterium profundum* strain SS9 pfa gene cluster (*pfaA-D*) responsible for EPA synthesis. MAR441-1 fosmid DNA was digested by restriction enzymes, e.g. BamHI, EcoRI, NdeI, SalI and Xhol I, confirmed that the size of insert DNA from MAR441is about 42,000 bp (Figure 7.6).

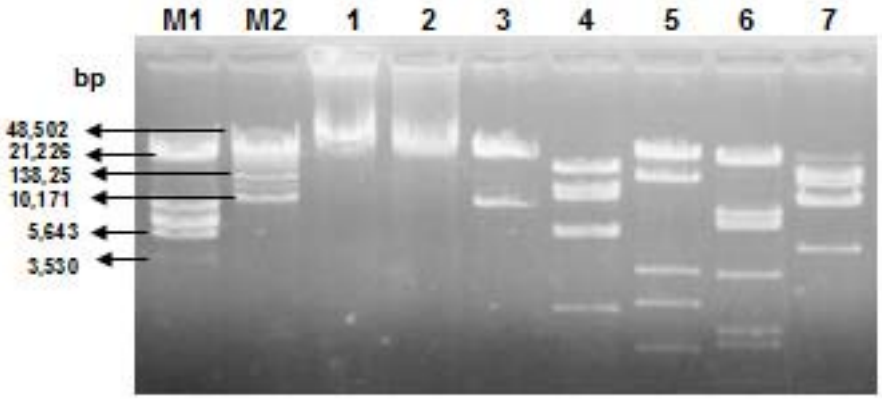

Figure 7.6 Restricted enzymes digestion patterns of recombinant fosmid MAR441-1. M1, DHA EcoRI ladder; M2, Lamda DNA ladder; Lane 3, MAR441-1 Fosmid DNA (2.5 μg); 4, MAR441 genome DNA; lanes 5 to 9, MAR441-1 Fosmid DNA digested by BamHI, EcoRI, NdeI, SalI, XholI respectively.

7.4.6 Analysis of the strains MAR441, NSP560 and MA665 pfa gene cluster

The isolation of *Shewanella* sp. MAR441 genes required for EPA synthesis, designated as *pfaABCD*, involved the generation of a large insert fosmid library of MAR441 genomic DNA and the subsequent probing of this library using published PUFA primers targeting conservative regions. The MAR441 *pfaA-D* genes span a region of 17313 bp (Table 7.4).

Two large insert fosmid libraries of NSP560 and MA665 genomic DNA were also constructed, however, no positive clones were found containing PUFA gene clusters. Thus, the full EPA synthesis gene clusters were cloned based on the primers designed according to the genomic sequences published on *Vibrio splendidus* strain LGP32 and *Photobacterium profundum* strain SS9 from NCBI database (Table 7.3). The NSP560 *pfaABCD* genes span a region of 17442 bp. The *Photobacterium* sp. MA665 *pfaABCD* genes span a region of 17359 bp (Table 7.4).

Table 7.4 The similarity of the deduced Pfa amino acid sequences from the strains MAR441, NSP560 and MA665 *pfa* genes to those from referenced species

Pfa enzymes	Size (bp)	Identity to the protein sequences from the referenced species (%)		
		Photobacterium	*Vibrio*	*Shewanella*
Pfa from *Shewanella* sp. strain MAR441				
PfaA	7709	98	78	66
PfaB	2077	96	67	51
PfaC	5877	97	79	71
PfaD	1638	98	89	83
PfaE	924	58-98	35-45	
Pfa from *Vibrio* sp. strain NSP560				
PfaA	7815	98	79	66
PfaB	2034	93	67	49
PfaC	5943	99	80	71
PfaD	1638	99	89	85
PfaE	828	41-47	91-99	
Pfa from *Photobacterium* sp. strain MA665				
PfaA	7722	99	78	66
PfaB	2100	95	67	50
PfaC	5877	96	79	71
PfaD	1635	99	89	85
PfaE	693	93	39	

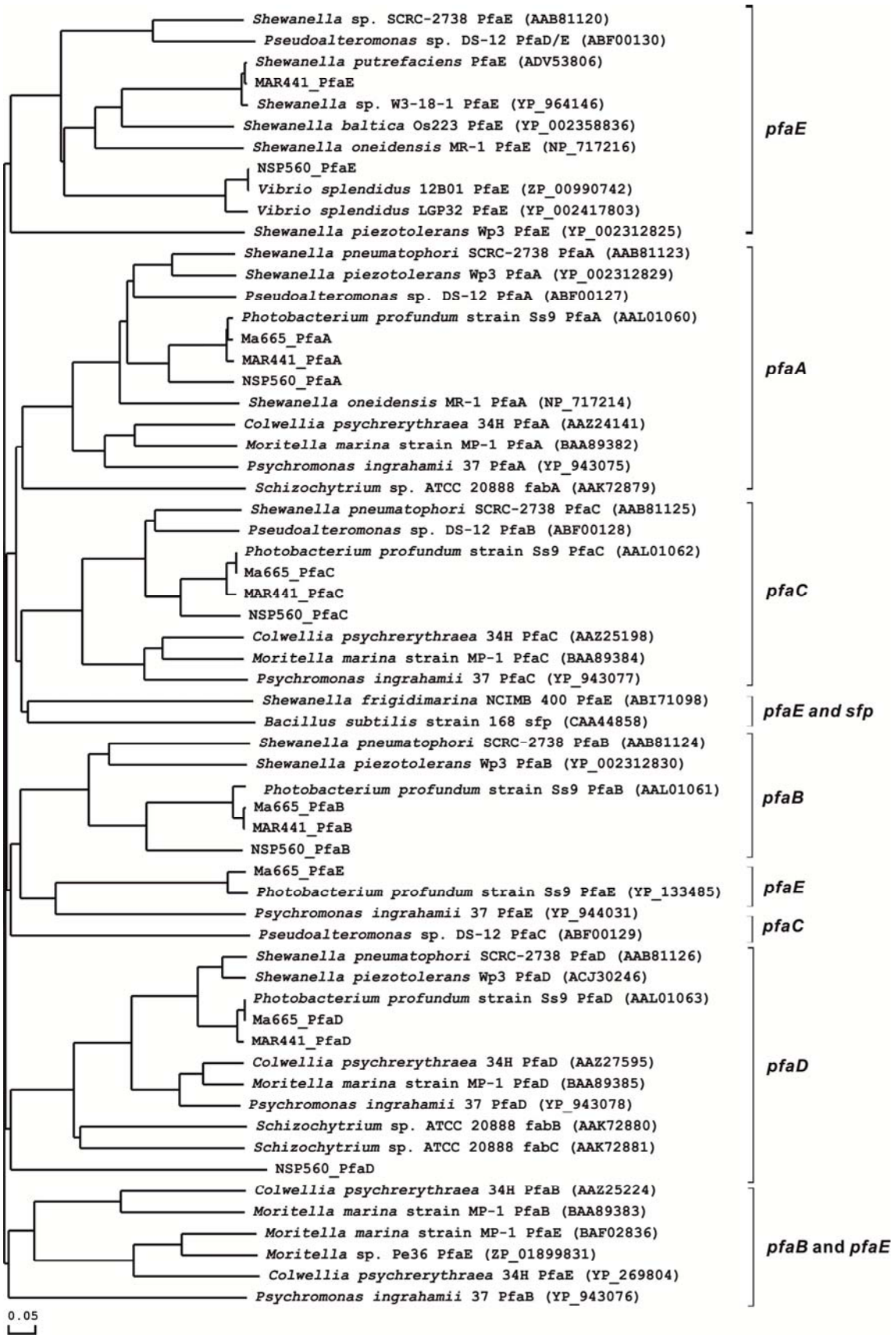

Figure 7.7 Phylogenetic analysis of domains of PUFA polyketide synthase and closely related proteins. Phylogenetic analysis was performed using the program DNAMAN 5.1. The referenced protein sequences with their accession numbers were retrieved from GenBank http://www.ncbi.nlm.nih.gov. Bars: 0.05 nucleotides substitution per site.

Furthermore, phosphopantetheinyl transferase gene (PPTase: *pfaE*) from MAR441 (924 bp), NSP560 (828 bp) and MA665 (693 bp) were PCR cloned by primers designed according to the conserved regions (Nishida et al., 2006). The deduced amino acid sequence of the MAR441, NSP560 and MA665 *pfaE* genes had a high degree of identity to PfaE homologues in *Photobacterium*, *Vibrio* and *Shewanella* (% identity): MAR441 PfaE– 58-98 % *Shewanella,* 35-45 % *Vibrio/ Moritella*; NSP560 PfaE– 91-99 % *Vibrio,* 41-47 % *Shewanella*; MA665 PfaE – 93 % *Shewanella*, 39% *Vibrio* (Table 7.4).

In between from MAR441, NSP560 and MA665, PfaA, PfaB, PfaC and PfaD, except for PfaE, were with high homology respectively, according to the phylogenetic analysis of domains of PUFA polyketide synthase and closely related proteins (Figure 7.7). MA665 PfaD along with *Photobacterium profundum* SS9 PfaD and *Psychromonas ingrahmii* 37 PfaE showed close to PfaB phylogenetically. PfaE and PfaB are similar in *Moritella* and *Colwellia* species. Interestingly, 4'-phosphopantetheinyl transferase from *Bacillus subtilis* strain 168 is similar to PfaE from *Shewanella frigidimarina* NCIMB 400. PfaB in *Pseudoalteromonas* sp. DS-12 was grouped in the phylum of PfaC. In *Schizochytrium* sp. ATCC 20888, fabB and fabC was grouped into PfaD.

Five deduced proteins from *pfaABCD* genes involved in EPA biosynthesis from these three gammaproteobacterial bacteria had domains that were conserved in fatty acid synthetase and/or polyketide synthases (PKSs) (Metz et al., 2001). These five *pfaABCDE* genes are generally necessary for the biosynthesis of EPA and DHA, and the basic structures of all *pfa* genes for EPA and DHA biosynthesis are very similar, the domain structures of some of the individual genes are slightly different (Figure 7.8 and Table 7.5). The *pfa* genes cluster from *Vibrio* sp. NSP560 was similar to those from *Shewanella pneumatophori* SCRC-2738 (Orikasa et al., 2004), belonging to type I *pfa* gene cluster, which includes all five *pfa* genes in a similar vicinity. Whereas, *pfa* genes clusters from *Shewanella* sp. MAR441 and *Photobacterium* sp. MA665 were similar to those type II *pfa* gene clusters from *P. profundum* SS9 (Allen and Bartlett, 2002), and *M. marina* MP-1 (Morita et al., 2000), which consists of a cluster of the four genes *pfaABCD*, with *pfaE* separate from the other genes. In the *pfa* gene clusters, *pfaA* encode a multifunction protein that includes domains for 3-ketoacyl synthase (KS), five repeats of malonyl coenzyme A: acyl carrier protein (ACP) acyltransferase in strains MAR441 and MA665, six repeats of ACP in strain NSP560, and 3-ketoacyl-ACP

reductase (KR). The growing acyl chains are presumably bound covalently to these ACP groups as thioesters with AT domains being required for the loading of the starter and extender units (Valentine and Valentine, 2004). ACPs contain a conserved Ser residue that is post-translationally modified with the phosphopantetheinyl moiety of CoA by a phosphopantetheinyl transferase (PPTase) (Rahman et al., 2005), providing a free thiol for tethering the starter and extender units and channeling the intermediates during fatty acid and polyketide biosynthesis (Lambalot et al., 1996; Sanchez et al., 2001).

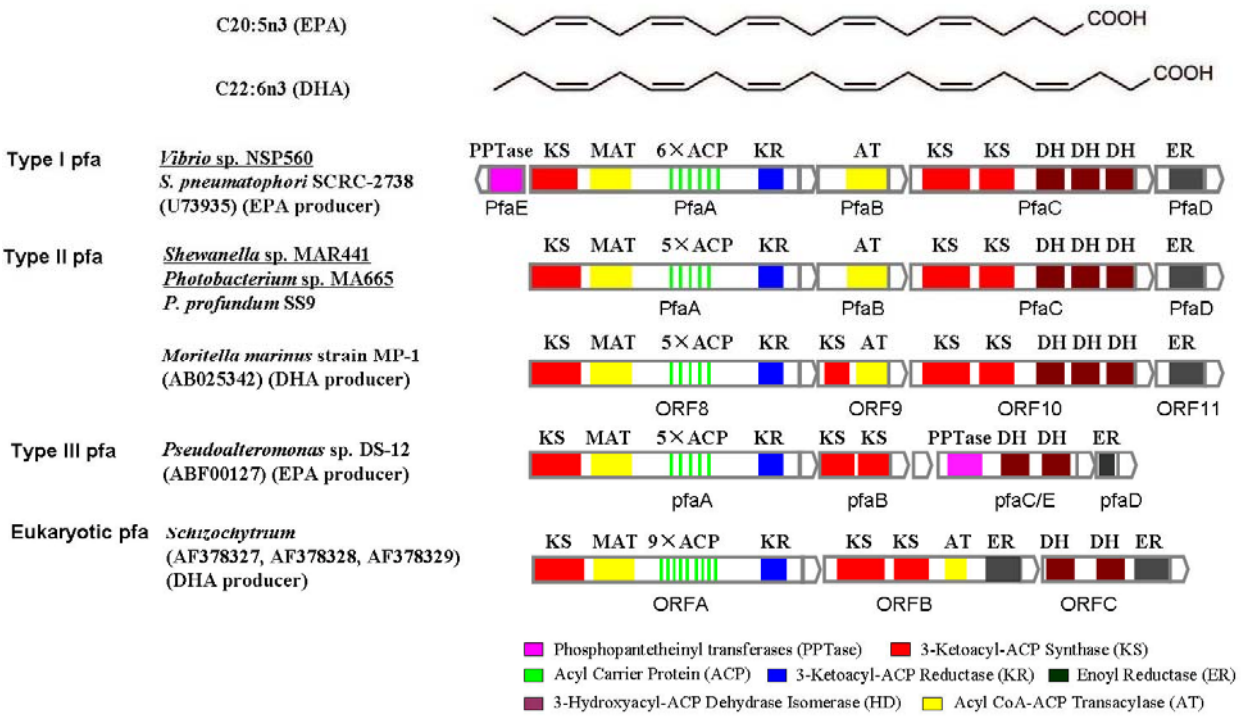

Figure 7.8 Comparison genes responsible for bacterial EPA biosynthesis and genes responsible for bacterial EPA from strains MAR441, NSP560 and MA665 with those gene clusters published.

The *pfaC* gene encodes a protein with two KS repeats and two or three 3-hydroxydecanoyl-ACP dehydratases (HD), which were homologous to FabA when in PfaC for EPA, and had two HD domains similar to that of FabA and one domain in the centre, similar to that of FabZ/FabA when PfaC for DHA. The second KS domain in PfaC of was considered to be a chain length factor (Okuyama et al., 2007). Genes *pfaB* and *pfaD* encode proteins with acyltransferase domains (AT) and an enoyl reductase (ER) domain, respectively, similar to those in SCRC-2738 (Orikasa et al., 2004). However, in DHA derived strains *M. marina* MP-1 (Tanaka et al., 1999) and *C. psychrerythraea* 34H (Methe et al., 2005), KS domain is also included in PfaB. The KS

domain of PfaB in *M. marina* MP-1 lacked an active-site sequence (Allen and Bartlett, 2002). The *pfa* genes of *Pseudoalteromonas* sp. strain DS-12 was defied as type III *pfa* gene clusters, in which PfaB was found to have two KS domains, and PfaC/E (which was registered as a product of the *pfaD* gene in the database) (Dai and Zhang, unpublished) has one PPTase domain and two HD domains (Figure 7.8).

Table 7.5 List of known microbial *pfa* genes[a]

Organism	Product[b]	*pfa* genes in the cluster		No. of ACP repeats in PfaA	Domain(s) in PfaB	HD domains in PfaC or PfaC/E	*pfaE* (PfaE)		Recombinant synthesis of the product	Reference or source
		Type[c]	Cloning				Group[d]	Cloning		
Shewanella sp. MAR441	EPA	II	Yes	5	AT	FabA-FabA[e]	I	Yes	No	This study
Vibrio sp. NSP560	EPA	I	Yes	6		FabA-FabA-FabA	I	Yes	No	This study
Photobacterium sp. MA665	EPA	II	Yes	5	AT	FabA-FabA[e]	II	Yes	No	This study
S. pneumatophori SCRC-2738	EPA	I	Yes	6	AT	FabA-FabA-FabA	I	Yes	Yes	(Orikasa et al., 2004)
S. oneidensis MR-1	EPA	I	No	4	AT	FabA-FabA[e]	I	No	Yes	(Venkateswaran et al., 1999)
P. profundum SS9	EPA	II	Yes	5	AT	FabA-FabA[e]	II	No	No	(Allen and Bartlett, 2002), (Vezzi et al., 2005)
Pseudoalteromonas sp. strain DS-12	(EPA)	III	Yes	5	KS, KS	FabA-FabA[f]	I[g]	Yes	No	ABF00130
M. marina MP-1	DHA	II	Yes	5	KS, AT	FabA-FabZ/FabA-FabA	I	Yes	Yes	(Orikasa et al., 2006a)
C.psychrerythreae 34H	(DHA)	I	No	6	KS, AT	FabA-FabA[e]	I	No	No	(Methe et al., 2005)

[a] Data format adapted and updated from reference (Okuyama et al., 2007). Nucleic acid and deduced amino acid sequences were retrieved from databases (DDBJ/GenBank/EMBL) (http://www.ddbj.nig.ac.jp/Welcome-j.html). The name of each domain in individual *pfa* genes is described in the legend of Figure 7.4.

[b] In cases of (EPA) and (DHA), the production of EPA or DHA is expected but not confirmed.

[c] Type I, II, and III *pfa* genes are defined in references (Orikasa et al., 2006a; Okuyama et al., 2007).

[d] Group I and II PPTases are defined in reference (Okuyama et al., 2007).

[e] Unannotated 900- to 1,000-bp sequences are present between the two FabA-like sequences.

[f] Unannotated 360- to 450-bp sequences are present between the two FabA-like sequences.

[g] *pfaE* is included in *pfaC/E*.

In order to understand microbial production of EPA/DHA via the PKS-like pathway, insight into the molecular genetics of EPA/DHA biosynthesis were gained by the cloning, sequencing, and complementation analysis (Yazawa, 1996; Tanaka et al., 1999; Allen and Bartlett, 2002; Gentile et al., 2003; Orikasa et al., 2004; Okuyama et al., 2007). *Escherichia coli* has a powerful genetic tool system and is widely used in the

fermentation. Therefore, *pfa* genes were further expressed for finial EPA/DHA production in *Escherichia coli*, with 2-6 % EPA/DHA recombinant production in TFA (Valentine and Valentine, 2004; Lee et al., 2006; Orikasa et al., 2006b; Amiri-Jami and Griffiths, 2010). And, the production of EPA could be improved to 16-22 % when high copy-number plasmid carrying the EPA gene cluster was transformed into *E. coli* cell (Orikasa et al., 2004). However, the EPA/DHA recombinant does not behave like a typical *E. coli* cell, such as growth occurs around 0.2 M NaCl and restricted to about 12–22 °C (Valentine and Valentine, 2004).

Bacteria mostly produce fatty acids via the type II fatty acid synthase (FAS) in which discrete enzymes encoded by separate genes catalyse specific steps of the biosynthetic pathway (White et al., 2005b). The dominant cellular fatty acids produced via the type II FAS typically contain between 14 and 18 carbons, such as via iterative reactions of the elongation and desaturation on the saturated fatty acid palmitic acid (PA, 16:0) (Wallis et al., 2002; Qiu, 2003; Chung et al., 2005). Whereas, type I FAS systems, multifunctional enzyme complexes harbouring catalytic activities as discrete functional domains, is found in certain coryneform bacteria of the order *Actinomycetales* (Schweizer and Hofmann, 2004). Then, a novel alternative pathway for the biosynthesis of C_{20+} PUFAs has been suggested in a narrow group of predominately marine *Gamma-Proteobacteria* that includes species of the *Shewanella*, *Photobacterium*, *Moritella*, *Colwellia* and *Vibrio* genera (Yazawa, 1996; Morita et al., 2000; Kaulmann and Hertweck, 2002; Nichols and McMeekin, 2002; Nichols, 2003). This system is responsible for the specific *de novo* synthesis of the long-chain omega-3 PUFAs EPA and DHA, and the omega-6 PUFA arachidonic acid (AA, 20:4ω6) (Russell and Nichols, 1999). The mechanism of Pfa Synthase for PUFA synthesis proceeds via a novel type I iterative fatty acid synthase/polyketide synthase (FAS/PKS) enzyme complex (Metz et al., 2001), as shown in this study strains *Shewanella* sp. MAR441 and *Shewanella* sp. MA665 with *pfaEABCD* and *pfaE* separated, and *Vibrio* sp. NSP560 of with *pfaEABCD* in the gene cluster dedicated to PUFA production. The PKS-like modules were assumed to directly condense acetyl units to C_{20+} polyketides and have an advantage over the desaturase-elongase pathway due to the structural simplicity of enzymatic complexes. Each step of the two-carbon extension consists of sequential catalysis by ketoreductase, dehydratase, and/or enoyl reductase, resulting in the partial or complete reduction of the keto group. In this unique process, a *cis*-double bond is inserted by aerobic desaturation

after the complete reduction of keto group, thus conserving cellular reduction energy (1 NADPH every double bond) (Metz et al., 2001).

7.5 Conclusions

Most of the deep-sea *Shewanella* species, such as *S. benthica, S. abyssi*, *S. kaireitica*, *S. violacea*, *S. peizotolerans* and *S. psychrophila* were reported to produce EPA at the level of 2-14 % of total fatty acids (TFA) (Deming et al., 1984; Delong and Yayanos, 1986; Delong et al., 1997; Nogi et al., 1998b; Miyazaki et al., 2006; Xiao et al., 2007). *Shewanella* sp. MAR441 from Mid-Atlantic Ridge deep-sea sediments, taxonomically, was identified as a novel species (unpublished data), producing EPA up to 21 % of TFA (or 25 mg g^{-1}) under atmospheric conditions, which is the highest yield published so far from deep-sea *Shewanella*.

Photobacterium profundum and *Ph. frigidiphilum* are the only two species within *Photobacterium* genus known to be able to produce EPA at the level of 2-14 % of TFA, however, they require pressure for growth (Nogi et al., 1998c; Seo et al., 2005). *Photobacterium* sp. MA665, phylogenetically with high similarity with these two psychropiezophiles, isolated from shallow-sea algal plant from North Sea, was first time described of this genus could be cultured easily under atmospheric conditions with appreciable levels of EPA (up to 25 % of TFA or 10.6 mg g^{-1}).

Some *Vibrio* species may contain only silent copies of the PUFA genes, which unable to synthesize EPA successfully. North-sea sponge associated bacteria *Vibrio* sp. NSP560 was able to produce up to 12 % EPA of TFA (or 8.4 mg g^{-1}), while its closest type strains, *Vibrio tasmaniensis* and *V. cyclitrophicus* were reported no produce EPA (Hedlund and Staley, 2001; Thompson et al., 2003).

Therefore, varied proportion of EPA from bacteria may reflect nutrient requirement of their host or the food web characteristics of the environment where they were isolated. Furthermore, EPA production greatly enhanced by cerulenin treatment has two indications: (i), two fatty acid-biosynthetic systems involved in PUFA producing bacteria, including synthesis of EPA and short-chain fatty acids, and synthesis of middle-chain fatty acids; (ii) the environments where the strains isolated may not be the primary controlling factor in PUFA synthesis, but mostly may impact the growth.

Therefore it suggests that the necessary of rethinking the conventional concepts/ways of screening strains with highly accumulate PUFAs, as well as the mechanisms of cold adaptation, such as the increasing levels of EPA and/or DHA responses to cold temperatures.

EPA gene clusters (*pfaA*, *pfaB*, *pfaC*, *pfaD* and *pfaE*) from *Vibrio*, *Photobacterium* and *Shewanella* in this study, along with those published data from EPA/DHA derived prokaryotic and eukaryotic species, such as *S. pneumatophori* SCRC-2738, *P. profundum* SS9 and *Pseudoalteromonas* sp. DS-12, *Moritella* marina MP-1 and *Schizochytrium*, which demonstrated that EPA or DHA polyketide biosynthesis gene clusters from different genera showed a high degree of gene sequence similarity. The result also provided evidence of the common distribution of the novel PUFA synthease pathways among marine microorganisms regardless of their biogeographic variability, which has recently been further testified by the investigation of genetic capacity for production of long-chain fatty acids using a culture-independent approach (Shulse and Allen, 2011). Therefore, the study suggests the possible involvement of horizontal gene transfer in the acquisition of the *pfa* gene clusters in the marine environments, although no flanking genes possessing functions which could facilitate horizontal transfer have been observed, so far.

Therefore, in some way, PUFA genes may contribute to the bacterial taxonomy by redefining old classifications, creating new groupings, and tracing back evolution (Nichols et al., 1997; Nichols et al., 1999). New strains with active and silent copies of the PUFA genes can be tracked by using molecular biology tools, and more might be learned about the world-wide distribution of strains with PUFA-biosynthesis gene clusters and their ecological importance (DeLong and Yayanos, 1985; Nichols et al., 1996a). The genes for PUFA biosynthesis are distinct from previously recognized PKSs in both structure and mechanism as well as the novel putative dehydrases/isomerases, and may thus provide new tools for combinatorial biosynthesis of polyketide antibiotics (Leadlay, 1997; Chartrain et al., 2000; Rohlin et al., 2001).

Chapter 8. Outdoor production of eicosapentaenoic acid by marine microalgae in the UK

8.1 Abstract

Microalgae contain large quantities of eicosapentaenoic acid (EPA) and are therefore considered a potential alternative source of this important fatty acid, which currently comes mainly from fish oil. We investigated the natural populations of algae from North Sea. Among fifty strains, a green microalgae strain M1 and a diatom strain M7 were chosen, for their ability to grow well and produce high level of EPA. These isolates were identified by molecular biological and morphological methods as *Tetraselmis* sp. and *Phaeodactylum tricornutum* respectively. Cells of strain M7 were able to grow indoors and outdoors under local natural weather months. *P. tricornutum* strain M7 had a lipid content of 10 % dry wt biomass and 22-30 % EPA of total fatty acids (TFA), whereas *Tetraselmis* sp. M1 produced 5 % oil content (dry wt) and 6 % EPA of TFA. The results indicate the potential of growing marine microalgae in temperate regions, rather than in tropical and subtropical areas.

8.2 Introduction

Omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) have received much attention due to their therapeutic significance. Remarkably, they have a wide range of physiological functions in the human body, providing beneficial effects in the prevention and treatment of heart disease, Alzheimer's disease, high blood pressure, inflammation, and certain types of cancer (Tresguerres et al., 1989; Angerer and von Schacky, 2000; Sauer et al., 2002; Connor and Connor, 2007). These findings have led to considerable interest in developing commercial processes for EPA production from microalgae for food and pharmaceutical markets (Belarbi et al., 2000; Molina Grima et al., 2003). Heightened consumer awareness of the value of omega-3s has increased the growth in demand for the omega-3 products.

Currently, the main sources of dietary EPA and DHA are marine fish, and fish oil is used for commercial production of EPA and DHA. However, recovery of EPA from fish oil is expensive (Belarbi et al., 2000), declining fish populations (Garcia and

Rosenberg, 2010), an undesirable fishy flavor and odor in fish oil (Arts *et al.*, 2001), have led to an extensive search for alternative sources such as microalgae. It has been assumed that fatty acids in marine food webs come only from microalgae, such as diatoms (Pohnert, 2005). Fish usually obtain EPA via bioaccumulation from microalgae in the food chain (Wen and Chen, 2003). Most diatoms contain high content of EPA, such as diatoms *Phaeodactylum tricornutum* and *Nitzschia laevis* have been intensively investigated for their EPA production potentials. However, most of these EPA production processes investigated to date have been based on photoautotrophic (Sa´nchez Miro´n et al., 2002; Molina Grima et al., 2003). Unfortunately, photoautotrophic growth require strictly controlled growth conditions in terms of nutrients, light quantity and quality, oxygenation and carbon dioxide levels (Seto et al., 1984; Chen, 1996). Consequently, photosynthetic systems are not easy to achieve high level of EPA productivity (Barclay *et al.*, 1994), and can result in considerable expense (Seto et al., 1984). Therefore, it would be desirable to isolate specific algal species which are capable of producing high level of EPA via a heterotrophic growth process under natural weather conditions, especially on cheap organic substrates.

Currently, algal cultivation has been focused on tropical and subtropical regions, very little work has been done on outdoor cultures in cold climate. In this study, we investigated species composition and diversity from fifteen sea water samples from the coast of North Sea. Better algal candidates were chose for further identification by molecular biological, morphological methods and fatty acid compositions analysis. We also demonstrated the feasibility of growing these marine microalgae indoors and outdoors for lipid production under natural weather conditions in the northeast of the United Kingdom.

8.3 Materials and methods

8.3.1 Marine algae isolation and purification

The sea water algal samples were collected from Cullercoats coast near the Dove Marine Laboratory (latitude 55°04' N and longitude 1°28'W) (North of England) in October 2008 and cultured in filtered sea water with or without adding F/2 medium (Sigma). The algae were subjected to purification by serial dilution followed by plating. The microscopic observations of the isolated algae revealed its colonial existence. The individual colonies were isolated and inoculated into F/2 medium and incubated at 15 ±

1 °C under natural light-night period in UK, for biomass accumulation. The purity of the culture was ensured by repeated plating and by regular observation under microscope. The stock culture was maintained on F/2 medium.

8.3.2 Algal cultivation conditions

Algal cells were subcultured in 50 ml F/2 medium in 250-ml conical flasks every 3 weeks at 15 ± 1 °C, on a rotary shaker (100 rev. min^{-1}), under natural light and dark cycle of North-east England. Cells in the exponential growth phase were used as inoculum for following experiments in shake flasks and open raceway ponds. Inoculum size, temperature and light intensity were denoted as in the results. Productivity (in mg l^{-1} day^{-1}) was defined as the final yield of EPA and PUFA (in mg l^{-1}) divided by the total culture period (in days).

A time course study was carried out on marine micro algae and diatoms growth in conical flasks of 1500 ml capacity, containing 500 ml F/2 medium for a period of 3 weeks. The culture flasks were inoculated (5 % v/v) and incubated at 15 ± 1 °C under natural light and dark cycle. Cultures were harvested and dry biomass was estimated at 1-3 days of intervals for fatty acids analysis. All the experiments were carried out in triplicate.

A time course study was carried out on marine diatoms growth in a open raceway pond. The equipment was facilitated with a pump pushed by two solar panels (BP Solar, model SX305M), to economically supply an attached battery with the needed power to control the motor to which the paddlewheel of the raceway is attached, creating a current of 0.5 m s^{-1}. The experiment was carried out in open raceway ponds of 40 L capacity, containing 35 L F/2 medium for a period of 3 weeks. The raceway ponds were inoculated (5 % v/v) and incubated at natural conditions in summer (June and August 2010) and winter (October and November 2010) respectively. Cultures were harvested and dry biomass was estimated at 1-3 days of intervals for fatty acids analysis. All the experiments were carried out in triplicates.

8.3.3 Biomass estimation

Algal cell density was recorded daily for 10–20 days by reading the culture absorbance at a wavelength of 600 nm. To combine the optical density and the dry biomass a

calibration curve was made. The cultures were harvested and the cells were washed with distilled water after centrifugation at 5000 rpm. Then the pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight.

8.3.4 Electron microscopy as methods mentioned in Chapter 5

8.3.5 Fatty acid analysis as methods mentioned in Chapter 2

8.3.6 18S rRNA gene PCR amplification

Genomic DNA was extracted from the strains using the PureLink™ Genomic Plant DNA Purification Kit (Invitrogen Ltd, Paisley, U.K) and used as templates for PCR amplification of the 18S rRNA gene fragments by using the primers pair: 18S-F1 (5'-CCAACCTGGTTGATCCTGCCAGTA-3') and 18S-R1 (5'-CCTTGTTACGACTTCACCTTCCTCT-3') (Goff and Coleman, 1988). The PCR reactions were performed on Eppendorf Master thermocycler with the program of 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1min at 55 °C, 6 min at 72 °C and a final hold at 72 °C for 10 min with DreamTaq™ Green PCR Master Mix (Fermentas). The PCR products were sequenced by Eurofins MWG Operon after purification with PureLink™ PCR Purification Kit (Invitrogen Ltd, Paisley, U.K) following the manufacturer's protocol. Phylogenetic analysis and diversity estimates were conducted according to the methonds mentioned in Chapter 2. The nucleotide sequences of 18S rRNA gene have been deposited in EMBL under the accession numbers FR744744-FR744763.

8.4 Results and Discussion

8.4.1 Phylogeny, morphology and classification

From a total of 15 sea water algal samples, 54 pure isolates of marine algae were isolated and chose for identification based on morphological and microbiological experiments. They were found mainly green micro algae and diatoms (Table 8.1). Of these 54 isolates, 20 algal strains were identified by 18S rRNA gene sequences and subjected to phylogenetic analysis (Figure 8.1). From these studied, green algae and

diatoms are the main populations, and *Tetraselmis* is the dominant in the group of green algae with following different species: *Tetraselmis striata*, *Tetraselmis suecica*, *Tetraselmis convolutae* and *Tetraselmis chuii*; while *Phaeodactylum tricornutum* is the dominant in diatoms group with species of *Phaeodactylum tricornutum* and *Amphora Montana*.

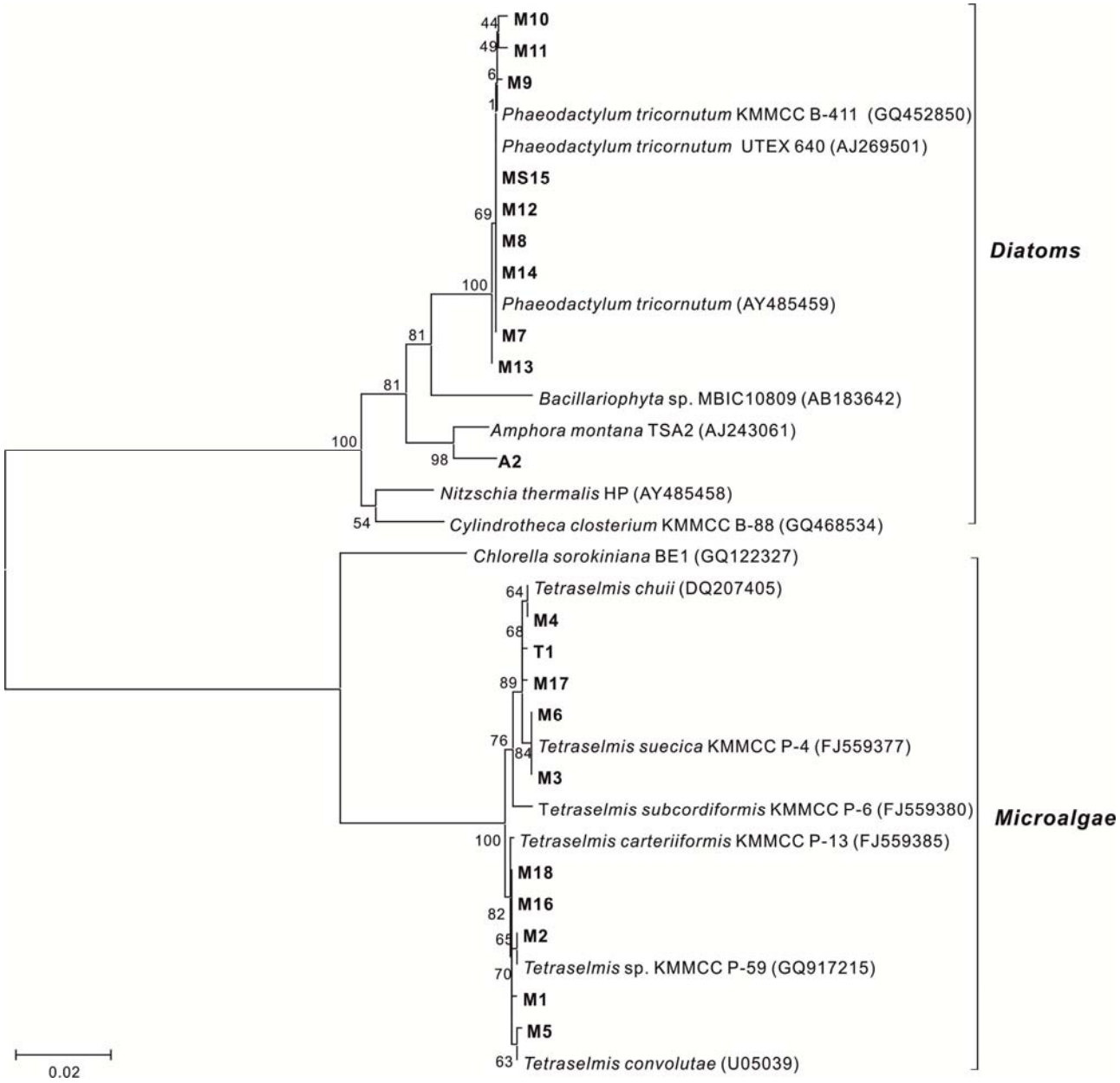

Figure 8.1 Neighbour-joining distance tree based on the 18S rRNA gene sequences of 20 representative micro algal strains observed in this study and their nearest similar strains. The phylogenetic tree was constructed by neighbour-joining method using the programs of MEGA package. 1000 trials of bootstrap analysis were used to provide confident estimates for phylogenetic tree topologies. Bars: 0.02 nucleotides substitution per site.

Table 8.1 List of marine micro algal isolates from North Sea

Phylogenetic group (family)	Representative isolates	Closest identified strain in the database (accession no.)	Similarity (%)
Green micro algae			
Prasinophyceae	M1 ; M2	*Tetraselmis sp.* KMMCC P-59 (GQ917215)	99.2-99.4
	M3	*Tetraselmis suecica* KMMCC P-4 (FJ559377)	99.5
	M4; T1	*Tetraselmis chuii* (DQ207405)	99.1-99.6
	M5	*Tetraselmis convolutae* (U05039)	99.0
	M6	*Tetraselmis suecica* KMMCC P-4 (FJ559377)	99.2
	M16; M18	*Tetraselmis striata* KMMCC P-43 (FJ559403)	99.6
Diatoms			
Bacillariophyceae	M7; M8	*Phaeodactylum tricornutum* (AY485459)	99.5-99.8
	M9	*Phaeodactylum tricornutum* UTEX 640 (AJ269501)	99.765
	M10; M11; M12; M13; M14; M15	*Phaeodactylum tricornutum* KMMCC B-411 (GQ452850)	99.95-99.9
	M17	*Phaeodactylum tricornutum strain* SJ (GQ118681)	99.6
Catenulaceae	A2	*Amphora montana isolate* TSA2 (AJ243061)	98.5

The green microalgal strain M1 is unicellular (Figure 8.2). Cells are solitary, non-flagellate, spherical or elongate, occasionally ovoid or subspherical, widely oval before division and after division hemispherical, 7.0-9.0 μm in width and reaching 15 μm in cell length, and yellow-green in color (Figure 8.2. light microscopic picture). The pyrenoid, a feature of taxonomic significance in *Tetraselmis* (Hori *et al.*, 1982), was penetrated by cytoplasmic channels and the eyespot was present (Figure 8.2. M1-TEM). M1 broth culture was observed with gliding movement but with not flagella, having two cell walls, the color of the cell is bright green and turn to greenish yellow on the fifth day of growth. Reproduction by solitary cells, dividing into two morphologically equal, hemispherical daughter cells (binary fission), which reach the original globular shape before next division, cells divide in two planes in successive generations in broth media, the envelopes around cells will split together with dividing cells.

The algal strain M7 is also unicellular. Cells are fusiform, 5-18 μm long, 1.5-5 μm wide, rounded, margin entire, with two more or less blunt, slightly bent arms, silica wall absent, non-motile and golden-brown in color (Figure 8.2); in sea water F/2 medium culture, cells separate and tend to remain suspended, which is agreeable to the morphology description of the genus (Lewin, 1958). Valve weakly silicified, with a thin ragged margin. Girdle bands absent. Valve flat, linear lanceolate, slightly curved,

symmetrical about the transapical plane; one edge convex, the other more or less straight with a median bulge. Ends broadly rounded but not capitate. Raphe slightly curved. Central area small, frequently with a pore on the side of the bulge (Figure 8.2. M7-SEM).

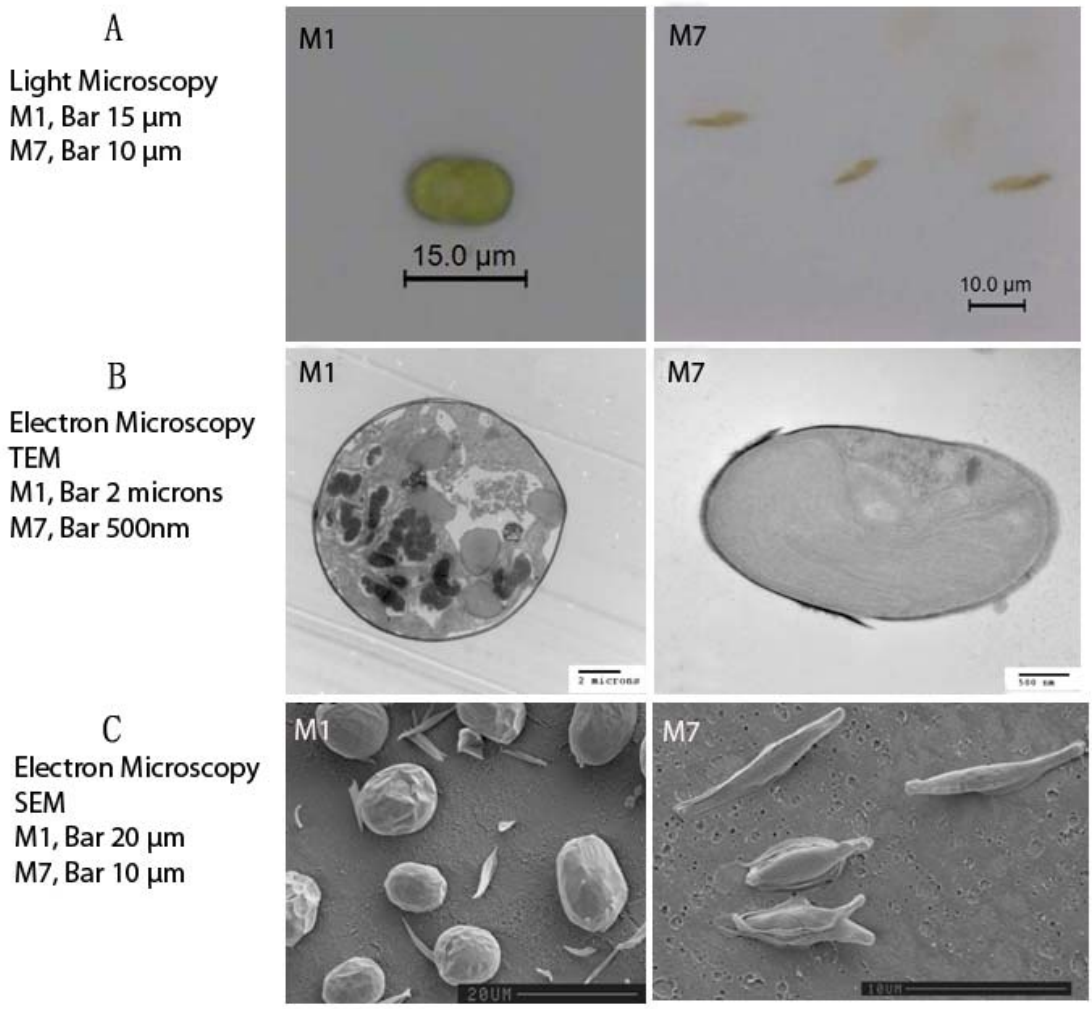

Figure 8.2 Light microscopic (A), transmission electron microscopic (B) and scanning electron microscopic (C) pictures of green micro algae M1 and diatom M7

8.4.2 Ecophysiological characterization of the local algal isolates

Ecophysiological characterization of the local isolates of green algae and diatoms were considered by growth and factor influencing growth including temperature, salinity and light. These local algal isolates exhibit ranges of tolerance for ecophysiological factors that determine their limits of growth. When the culture was tested in survival on the extreme temperature (30 °C and -20 °C) and without light, they still able to live. Although low temperatures restrict the rates of growth and enzymatic activities of organisms, it does not kill these organisms. Therefore, when the culture is thawed, they

can grow. Diatoms isolated from North Sea, such as strain M7 showed great growth under temperatures range from about -4 °C to 28 °C, and could still survive under severe temperatures, where temperatures range from about 0 °C on the bottom to -15 °C or less on the top.

8.4.3 Fatty acid content

The fatty acid profiles of the selected microalgal isolates are presented in Table 8.2. The highest lipid content was observed in *Phaeodactylum tricornutum* followed by *Amphora montana* and *Tetraselmis* sp., while *Tetraselmis chuii* had the lowest lipid content. The fatty acid profiles varied between the classes. In *Prasinophyceae*, *Tetraselmis* sp. had n-16:0, n-18:1ω9 and n-18:3ω3 as the dominating fatty acids, which accounted for 47-55 % of the total fatty acids, with lower amounts of n-16:1ω7, while n-16:0, n-16:2ω4, n-18:1ω9 and n-20:5ω3 were the most abundant fatty acids (71 %) in *Amphora montana*. The diatom *Phaeodactylum tricornutum* was dominated by n-14:0, n-16:0, n-16:1ω7, n-16:3 and n-20:5ω3 acids (70-73 %). The average chain lengths of these five species were similar (17.2-17.6).

These findings reveal that n-16:0 was the major saturated fatty acid in all the algae. The major monosaturated fatty acids were n-16:1 in *P. tricornutum*; n-18:1 in *Tetraselmis* sp., *Tetraselmis chui*; n-16:1 and n-18:1 in *Amphora Montana.* The abundance of PUFAs showed pronounced variation between algal species and classes. The highest amount of EPA was found in *P. tricornutum* (29.7 mg g^{-1}), followed by *Amphora Montana* (16.1 mg g^{-1}), *Tetraselmis* sp. (4.2 mg g^{-1}) and *Tetraselmis chuii* (3.3 mg g^{-1}), while DHA was not found among these isolates. In general, our data were in good accordance with results from the literature.

8.4.4 Fatty acids of Chlorophyceae

Both species of *Tetraselmis* (M1 and T1) generally showed a pattern of fatty acid distribution similar to each other, and distinguished from *Bacillariophyceae* by dominated with high percentage of n-18:1ω7, n-18:2ω6 and n-18:3ω3 acids (8-20 %), and with only considerable amounts of n-20:5ω3 (up to 6.9 %). The fatty acid profiles were in accordance with those in earlier reports (Ackman et al., 1968; Volkman et al., 1989; Reitan et al., 1994). However, in contrast to members of *Chlorophyceae*, the two *Tetraselmis* species examined, had a little different from other representatives of this

class, which have a high concentration of n-16:4ω-3 and n-18:4ω-3 (Dunstan et al., 1992; Zlmkova and Aizdaicher, 1995). Other main components, such as n-16:0, n-18:3ω3 and the amount of n-20:5ω3 were similar.

Table 8.2 Fatty acid (FA) profile of the micro algal isolates from North Sea

Fatty acid	*Tetraselmis* sp.	*Tetraselmis chuii*	*Amphora* sp.	*Phaeodactylum tricornutum*	*Phaeodactylum tricornutum*
	M1	T1	A2	M7	M13
n-14:0	2.7	2	1.4	7.8	8.9
n-16:0	17.2	19.6	19.3	15.5	17.4
n-16:1ω7	1.4	1.6	2.2	16.4	15.7
n-16:2ω4	4.9	5.5	18.4	4.4	3.3
n-16:3ω4	1.7	2.4	2.2	12.9	11.7
n-16:4	3.2	5.6	2.4	-	-
n-18:0	0.4	0.7	7.5	1.3	1.6
n-18:1ω9	16.3	12.5	15.3	2.1	3.2
n-18:1ω7	10.9	6.9	0.9	1.1	2.9
n-18:2 ω6	7.7	9.2	2.5	3.5	4.3
n-18:3 ω3	20.9	14.9	2.6	1.3	1.6
n-18:4 ω3	3.2	5.9	0.6	0.6	0.6
n-20:1 ω9	1.6	3.1	2.1	1.2	0.2
n-20:2 ω6	-	-	0.5	0.7	0.1
n-20:4 ω6	0.8	1.6	0.5	0.6	0.8
n-20:4 ω-3	0.6	0.4	1.8	2.2	2.1
n-20:5 ω3	6.5	6.9	18.7	28.4	25.6
n-22:5 ω3	-	1.2	1.1	-	-
Σ TFA	100	100	100.0	100	100
Σ SCFA	20.3	22.3	28.2	24.6	27.9
Σ MUFA	30.2	24.1	20.5	20.8	22
Σ PUFA	49.5	53.6	51.3	54.6	50.1
EPA	6.5	6.9	18.7	28.4	25.6
ACL	17.5	17.5	17.6	17.4	17.3
EPA (mg g^{-1})	4.18	3.27	16.1	29.65	27.70
TFA (mg g^{-1})	64.4	47.5	85.7	104.40	108.20
Cells(g l^{-1}) [a]	1.8	1.5	1.3	1.75	0.62

[a] Cellular dry weight; Values are means of three samples; ACL, average chain length calculated after White et al., 2005; SCFA, straight chain fatty acids; BCFA, branched chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids; EPA, Eicosapentaenoic acid (n-20:5ω3); and (–) not detectable.

8.4.5 Fatty acids of Catenulaceae

Catenulaceae is a Family within the order of *Bacillariophyceae*. The fatty acids of the

Amphora montana (*Catenulaceae*) (A2) showed different from *Tetraselmis* species by presenting high amount of n-16:2ω4, n-18:0 and n-20:5ω3 acids, and low levels of n-18:1ω7 and n-18:3ω3 acids, whereas distinguished from *Phaeodactylum tricornutum* species with high content of n-16:2ω4, n-18:0 and n-18:1ω9 acids, and small amount of n-14:0, n-16:1ω7 and n-16:3ω4.

8.4.6 Fatty acids of Bacillariophyceae

The fatty acids of the *Bacillariophyceae* have been studied more extensively than other micro algal Classes. This interest is connected with the wide use of diatoms in mariculture. Moreover, diatoms have a worldwide distribution. The characteristic features of the fatty acids *of Bacillariophyceae*, which often represent the main food source in marine ecosystems, are of particular interest. The two species (M7 and M11) examined had a similar fatty acid composition. However, comparison of the component fatty acid revealed some differences. The most abundant fatty acids were n-14:0, n-16:0, n-16:1ω7, n-16:3ω4 and n-20:5ω3 acids (Table 8.2), which accounted for 70-73% of the total fatty acids. The fatty acid patterns were in agreement with previous results (Kates and Volcani, 1966; Ackman et al., 1968; Volkman et al., 1989; Yongmanitchai and Ward, 1991b; Reitan et al., 1994; Patil et al., 2007). The predominance of n-16:1ω7 over n-16:0 has been reported with the ratio of n-16:1ω7/16:0 varied from 1.3 to 2.0 in previous studies (Ackman et al., 1968; Alvarez and Zarco, 1989; Volkman et al., 1989; Zlmkova and Aizdaicher, 1995), although Volkman et al. reported somewhat lower concentrations of n-16:1ω7 for *Pavlova* species (Volkman *et al.*, 1991). However, our study showed similar value between n-16:1ω7 and n-16:0.

The acid n-20:5ω3 was most abundant among PUFAs; its content varied from 12.8 (*Chaetoceros constrictus*) to 28.4 % (*Phaeodactylum tricornutum*). A wide range of relative amounts of n-20:5ω3 have been reported for different diatom species (Volkman et al., 1989; Dunstan et al., 1994). Several factors cause this variability in diatoms. Variation of the nutritional and physical factors results in a change in n-20:5ω3 production by *Phaeodactylum tricornutum* from several per cent to 30-40 % of the total fatty acids (Yongmanitchai and Ward, 1991a). The variation in fatty acid composition and especially n-20:5ω3 content is a function of silicate availability, light and temperature (Mortensen *et al.*, 1988).

8.4.7 Time course of cell growth and PUFA production in shake flasks

A typical time course of PUFA production showed by *Tetraselmis* sp. M1 and *P. tricornutum* strain M7 suspension culture in F/2 medium in flask indoor (Figure 8.3A and 8.3B). The increase in PUFA content parallels that of cell growth in doors at 15±2 °C. After 7 days of cultivation, the M1 cells entered the late exponential or early stationary phase and the content of PUFA reached its maximum, from which 1.6 g l^{-1} freeze dried biomass was obtained, and the maximum TFA and PUFA concentration reached about 101.6 mg l^{-1} (63.5 mg g^{-1}) and 47 mg l^{-1} respectively, with ca. 5.3 mg l^{-1} of EPA (3.3 mg g^{-1}, 0.5 mg $g^{-1}day^{-1}$). For strain M7, after 6 days of cultivation, the cells entered the late exponential or early stationary phase and the content of PUFA reached its maximum, from which 1.65 g l^{-1} freeze dried biomass was obtained, and the maximum TFA and PUFA concentration reached about 170 mg l^{-1} (103 mg g^{-1}) and 54 mg l^{-1} respectively, with ca. 40.4 mg l^{-1} of EPA (24.4 mg g^{-1}, 4.1 mg $g^{-1}day^{-1}$).

8.4.8 Time course of cell growth and PUFA production in open raceway ponds

The culture of *P. tricornutum* strain M7 in the open raceway ponds during the summer days showed a typical time course of PUFA production. The increase in PUFA content parallels that of cell growth in both seasons (Figure 8.3C and 8.3D). According to the UK climate summaries by Met Office (www.metoffice.gov.uk), the months of July and August 2010 in Notheast of England, a maximum temperature of 13.8-21.8 °C and a minimum temperature of 4.6-15.1 °C were recorded (Figure 8.3E and 8.3F).

Under this weather conditions, the cells entered the late exponential or early stationary phase and the content of PUFA reached its maximum, after 9 days of cultivation. In this phase 1.55 g/l freeze dried biomass was obtained, and the maximum TFA and PUFA concentration reached about 160 mg l^{-1} (103 mg g^{-1}) and 50.4 mg l^{-1} respectively, with ca. 37.7 mg l^{-1} of EPA (24.3 mg g^{-1}, 2.7 mg $g^{-1}day^{-1}$). Whereas the months of October and November 2010 in Notheast of England, a maximum temperatures of 5.2-14.8 °C and a minimum temperature of -1.2-8.7 °C were recorded. During the weather conditions, after 13 days of cultivation, the cells entered the late exponential or early stationary phase and the content of PUFA reached its maximum, from which 1.55 g l^{-1} freeze dried biomass was obtained, and the maximum TFA and PUFA concentration reached about 160 mg l^{-1} (103 mg g^{-1}) and 58.4 mg l^{-1} respectively, with ca. 52 mg l^{-1} of EPA (33.3 mg g^{-1}, 2.6 mg $g^{-1}day^{-1}$).

The instantaneous productivity of PUFA (mg l^{-1} day^{-1}) is not a constant during the course of the culture. As indicated in Figure 8.3, maximum productivity is seen during the exponential growth phase (day 7–14). For comparative purposes, the productivity data reported in this study were the average value based on the entire cultivation cycle.

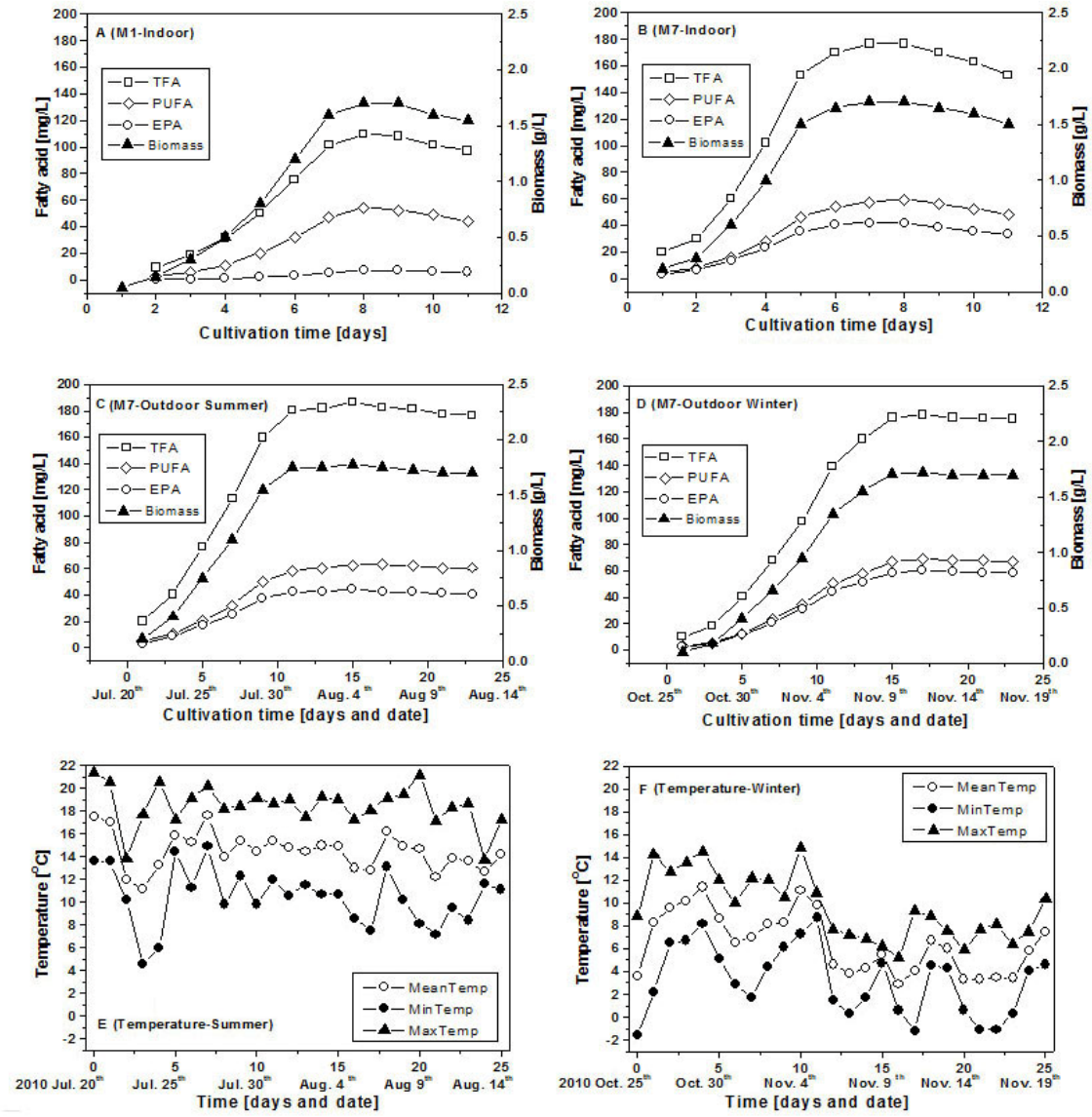

Figure 8.3 Algal cell growth and fatty acids productivity of *Tetraselmis* sp. M1 in flasks indoor (A), *Phaeodactylum tricornutum* strain M7 in flasks indoor (B), strain M7 in open raceway pond outdoor during the summer days (C and E) and strain M7 in open raceway pond outdoor during the winter days (D and F) in Nothern England. The weather data was provided by Met Office UK. The experiments were carried out in duplicate and values are means of two samples.

8.5 Discussion

Algae are an extremely diverse group of organisms in the aqua system (Radmer, 1996), however lots of work needed to get most of the single algal cultures from the aquatic environment. At least 13 species of *Botryococcus* were reported based on morphological differences by omitting the chemical analyses (Komárek and Marvan, 1992), while within each chemical race and for the same strain the morphology of the algae could vary in relation to age and culture conditions (Metzger and Largeau, 2005). However, in the marine water samples, there are still lots of different algal species could be seen under microscope. As for successful isolation onto agar, the algae must be able to grow on agar. Most diatoms and chlorarachniophytes grow very well on agar; some cryptophytes do, whereas others do not, and dinoflagellates rarely grow on agar (Anderson and Kawachi, 2005). For example, some flagellates (e.g., *Heterosigma*, *Pelagomonas*, and *Peridinium*) do not grow agar, but others (e.g., *Chlamydomonas*, *Pavlova*, *Synura* and *Tetraselmis*) grow very well on agar. Coccoid cells frequently grow well on or in agar, but some (e.g., *Aureococcus*, *Aureoumbra*) do not. In this study, we are, for the first time, reported the identification and distribution of marine micro algae species in North England, with limited species *Tetraselmis*, *Phaeodactylum tricornutum* and *Amphora Montana* able to grow in agar.

The typical forms of green micro algae and diatoms could be easily distinguished from each other with light microscopes. However, the identification of micro algae or diatoms to species level almost always requires scanning electron microscopy (SEM)/ transmission electron microscopy (TEM) and more often than not the development of species-specific molecular probes, in order to get details of morphological features from external surface to inside the cell, for precise identification of species especially on deformed cells (Drum, 1969). For example, the use of SEM can clearly present the special characteristics of their cell structure known as frustules as has been observed (Tesson and Hildebrand, 2010), as shown in this study. Molecular techniques on the other hand can be used for species (Beszteri et al., 2001), and for quantification of micro algae abundance (Beja *et al.*, 2000), however its development may rely on established and identified cultured material for probe validation. The morphological identification was further confirmed by molecular method based on 18S rRNA gene sequences, resulting in the separation all the strains hierarchically with two main groups. Mostly, strains with same molecular identity might be different species, and vice versa. Therefore, combining microscopic analysis with molecular technique may provide

precise results (Auinger et al., 2008).

To our knowledge, this is the first report on the fatty acid composition of a species *Amphora Montana*. The diatoms *Amphora* sp. *Amphora Montana* and *Amphora coffeaeformis* were mainly isolated from the marine growing as biofilms (Wigglesworth-Cooksey et al., 2007; Desai, 2008; Khodse and Bhosle, 2010). As observed from *Amphora montana* strain A1, the cells were seldom suspended in the liquid culture and formed biofilms on the wall of the flasks. However, fatty acid profiles of the diatom *P. tricornutum* were in agreement with previous results (Kates and Volcani, 1966; Ackman et al., 1968; Volkman et al., 1989; Yongmanitchai and Ward, 1991a; Reitan et al., 1994). The fatty acid profiles for *Tetraselmis* sp. and *Tetraselmis chuii* were in accordance with those in earlier reports (Ackman et al., 1968; Volkman et al., 1989; Reitan et al., 1994). These species are able to produce EPA only, which could minimize problems associated with downstream processing (Yongmanitchai and Ward, 1991b).

During algal cultivation and its scale up, surface area is a very important factor to be considered (Chisti, 2007). Open ponds with a large surface area are cost less to build and operate and have been in use since the 1950s (Borowitzka, 1999). Various types of outdoor open ponds have been employed for algal culture. For example, raceway ponds, circular ponds and thin layer inclined ponds, which are shallow and lined with impermeable or concrete materials (Chaumont, 1993; Borowitzka, 1999; Borowitzka, 2005a). Whereas unmixed open ponds are shallow and using only wind or convention as mechanical means (Benemann and Oswald, 1996). The depth the ponds need to be limited, usually shallow, at which the radiation of the sunlight can access and therefore support the growth of the diatoms. These tanks are the most popular ones and are found to be used for mass culture of *Spirulina, Haematococcus* and *Dunaliella* (Benemann and Oswald, 1996; Borowitzka, 2005b).

Algal cultures have been focused in tropical and subtropical areas, such as Spain, Hawaii, California, Roswell and New Mexico, grown outdoors on a commercial scale (Borowitzka, 1999; Moreno-Garrido and Canavate, 2001; Voltolina et al., 2008; Mata et al., 2010). Pennate diatoms, such as *Phaeodactylum tricornutum*, *Navicula* sp., *Amphora* sp., unicellular microalgae, for example *Tetraselmis marina*, were the most commonly observed species and been successfully grown in outdoors (Olaizola et al.,

1991; Kudo et al., 2000; Lee, 2001). However, the fluctuations in temperature were cited as one of the main reasons for hampering productivity rates (Sheehan et al., 1998), as study found that *Isochrysis* sp. and *Tahitian Isochrysis* grew very slowly at 35 °C, whereas *Nitzschia closterium* did not grow at temperatures higher than 30 °C (Renaud *et al.*, 1995). It was explained that photoinhibition of the algae could have occurred due to high intensity and longer durations of day light hours (Lee, 2001). Therefore, tropical and subtropical areas are not the best places for algal outdoor culture, and the species chose for these areas need to be screened very carefully. Furthermore, little has been reported on algal culture outdoor in temperate regions. In this study, the raceway pond was designed with a paddle, powered by solar panel, attached for inducing agitation or mixing of algal cultures. The growth of a *Phaeodactylum tricornutum* strain M7 was monitored outdoors (North of England) in the raceway with sand-filtered seawater as a medium and no other nutrients was added though silica is one of the essential nutrients in seawater which controls the environment as a result of its uptake by diatoms (Tanaka *et al.*, 2009).

Diatoms are known to be one of the most successful class inhabiting all kinds of habitats (Pentecost, 1984). The distribution and abundance of diatom species in water column are dependent on environmental factors, such as current, light, nutrients, and others. Diatoms isolated from North Sea showing well adaptation to local light levels, temperature fluctuations and local water in a small scale raceway pond outdoors throughout the year, with high lipid/PUFAs content under low temperatures as described on those isolated from polar region (Maykut, 1986; Eicken, 1992). These characteristics made it possible for our diatom isolates for farming marine micro algae in large scare levels outdoors used only seawater, sunlight and carbon dioxide as their feedstocks with sustainable production of biomass (1.55 g l^{-1}) containing high content of lipid and EPA (25-35 % EPA of TFA). The outdoor cultivation of *Phaeodactylum* sp. in 1000 l open ponds was studied in 1963 and gave a good insight in the potential of this species (Ansell et al., 1963). Logically, the production of biomass may be improved by grow the diatoms on various nitrogen substrates such as ammonium nitrate and urea according to other studies on this species. For example, by increasing the concentration of nitrate and urea the EPA content of the total fatty acid also increased. Vitamin B12 addition to the growth medium increased EPA production with 65 %. The highest yield of EPA was obtained at 1 % CO_2 in the air gas inlet (Yongmanitchai and Ward, 1992). The outdoor cultivation of *Phaeodactylum tricornutum* in bioreactors resulted in a

steady state of ~1 kg m^{-3} and a biomass yield of 0.3 kg m^{-3} (Miróna et al., 2003). The mixotrophic cultivation of *Phaeodactylum tricornutum* as a batch culture in airlift photo bioreactors resulted in a 25 g l^{-1} maximum biomass, and EPA production was boosted to 3 % of the dry weight with the addition of glycerol (Mann and Myers, 1968). Silicate in sea water may not contribute the production of biomass as *Phaeodactylum tricornutum* does not require silicate for growth (Martino et al., 2007). In contrast, most of the diatom species are dependent on silicate for growth. In microalgal heterotrophic cultures, the accumulation of triglycerides may result in a decrease or increase in fatty acid unsaturation, depending on the algal strains employed (Day et al., 1991; Tan and Johns, 1996). Therefore, optimization of culture conditions with different carbon sources for biomass and lipid production by this diatom should be aided by our ongoing experiments including genomic analysis on its cold adaption mechanism.

An efficient large-scale cultivation system is needed in order to explore a process for commercial production of EPA (Lebeau and Robert, 2003). Microalgae are often considered obligate photoautotrophs that require light for growth. Nevertheless, a number of microalgae are capable of heterotrophic growth with one or more organic substrates as their sole carbon and energy source (García *et al.*, 2000). Scalable cultivation of micro algae for commercial heterotrophic production of EPA will be further studied by employing some continual systems using the above mentioned isolates.

8.6 Conclusions

A local isolate of *Phaeodactylum tricornutum* strain M7, was able to adapt to grow under local climatic conditions coupled with the less need for nutrients makes them available to be cultured in areas inappropriate for agricultural uses autonomous of changes in weather, local light levels, temperature fluctuations and local water in a small scale raceway pond throughout the year. The strain was stable in a continuous biomass production system, with high production of biomass and lipid content in an outdoor open culturing system in the temperate climate of the UK.

Chapter 9. Enhanced electricity production using reconstituted artificial consortia of estuarine bacteria grown as biofilms

9.1 Abstract

Microbial fuel cells (MFCs) can convert organic compounds directly into electricity by catalytic oxidation and, although MFCs have attracted considerable interest, there is little information on the electricity generating potential of bacterial biofilms enriched from estuarine sediments. We have used acetate-fed MFC inoculated with sediment, with two-chamber bottles and carbon cloth electrodes to deliver a maximum power output of ~175 mW/m^2 and a stable power output of ~105 mW/m^2. Power production was by direct transfer of electrons to the anode from bacterial consortia growing on the anode, as confirmed by cyclic voltammetry (CV) and scanning electron microscopy (SEM). Twenty different species (74 strains) of bacteria were isolated from the consortium under anaerobic conditions and cultured in the laboratory, of which 34 % were found to be exoelectrogens in single species studies. Exoelectrogenesis by members of the genera *Vibrio*, *Enterobacter* and *Citrobacter*, and by *Bacillus stratosphericus* is confirmed, using culture based methods, for the first time. The bacterial consortia MFC showed higher power densities than those obtained using single strains isolated from the original biofilm. In addition, the maximum power output could be further increased to ~200 mW/m^2 when an artificial consortium consisting of twenty-five preselected exoelectrogenic isolates was used. Demonstrating the potential for increased performance of microbial fuel cells in the future.

9.2 Introduction

Microbial fuel cells (MFCs) can convert biodegradable and reduced compounds, such as glucose, acetate, lactate or waste water, directly into electricity, which offers a clean and renewable source of energy that could potentially contribute to current environmentally friendly power sources (Grant, 2003; Rabaey and Verstraete, 2005; Shinnar and Citro, 2006; Scott and Murano, 2007a; Scott and Murano, 2007b; Lovley, 2008). In addition, use of MFCs may also assist environmental protection, for example through waster water treatment. In MFC devices, bacterial cells in the anode chamber

play a key role in catalyzing the oxidation of an organic substrate (i.e. fuel) and transferring electrons derived from metabolic processes to the electrode (Logan et al., 2006). Thus, one primary aim of MFC research is to isolate electrogenic bacteria or communities with high electrochemical activity.

Electricity can be produced by naturally existing consortia of bacteria without addition of exogenous mediators in the fuel cell systems (Kim et al., 2003; Phung et al., 2004). Sufficient current can also be generated in order to power subsurface devices by placing an anode into anoxic sediment and a cathode into overlying water (Reimers et al., 2001). The diversity of bacteria capable of exoelectrogenic activity in anodophillic biofilms has been well studied (Phung et al., 2004; Logan and Regan, 2006) and some pure cultures that exhibit strong electrogenic activity in the MFC environment have been characterised (Logan et al., 2006). Work has mainly focused on pure isolates of two dissimilatory metal reducing genera *Shewanella* and *Geobacter* (Reguera et al., 2005; Gorby et al., 2006). The present list of confirmed exoelectrogenic bacteria includes representatives of five classes of *Proteobacteria*, such as *Rhodopseudomonas palustris* DX-1 (Xing et al., 2008), *Ochrobactrum anthropi* YZ-1 (Zuo et al., 2008) (α-*Proteobacteria*); *Rhodoferax ferrireducens* (β-*Proteobacteria*) (Chaudhuri and Lovley, 2003); *Escherichia coli* (Lowy et al., 2006), *Pseudomonas aeruginosa* (Rabaey et al., 2004), *Shewanella putrefaciens* (γ-*Proteobacteria*) (Kim et al., 1999); *Geobacter sulfurreducens* (Tender et al., 2002; Bond and Lovley, 2003), *Desulfobulbus propionicus* (δ-*Proteobacteria*) (Tender et al., 2002), *Arcobacter butzleri* ED-1 (ε-*Proteobacteria*) (Fedorovich et al., 2009), as well as representatives of the *Firmicutes* and *Acidobacteria*, such as *Clostridium butyricum* Eg3 (Peng et al., 2003), *Thermincola* sp. strain Jr (Wrighton et al., 2008); and *Geothrix fermentans* (*Acidobacteria*) (Coates et al., 1999). However, community analysis of electrochemically active biofilms in MFCs suggests a far greater diversity of exoelectrogens in these biofilms than was previously suspected and novel electrogenic bacteria remain to be discovered. In addition, biofilm communities may also produce greater power densities than individual strains, for example, two species were better than one in a cellulose-fed fuel cell, in which *Clostridium cellulolyticum* ferments cellulose while *Geobacter sulfurreducens* acts as an electroactive bacterium (Ren et al., 2007; Logan, 2009).

We were interested in using artificially reconstituted consortia of exoelectrogens to enhance electricity production. Such an approach has been used previously, for

example, to enhance oil biodegradation (Piedad Diaz et al., 2000). In the present study, highly electrogenic anodophilic biofilms and their culturable microbial communities are described, and several novel electricity generating strains were isolated. Furthermore, we could enhance electricity production by deliberately choosing and reconstituting biofilms containing the best electricity producers.

9.3 Materials and methods

9.3.1 Sediment samples and growth media

Estuarine sediments used for inoculating the microbial fuel cells were collected at low tide from the River Wear (54°54'25"N, 1°21'35"W). The sediment was obtained using a sterilized stainless steel corer and transported at ambient temperature intact to the laboratory within 60 minutes and placed in an anaerobic container system (GasPak™ EZ, BD, Maryland). Before inoculating sediments into the anode chambers of the MFCs, sedimentary bacteria were anaerobically cultured in fumaric acid medium (FAM)(Izallalen et al., 2008): 10 mM fumaric acid, 10 mM sodium acetate, 0.05 % yeast extract in 1:1 diluted sea water, at room temperature. The medium was adjusted to pH 7.0, and was flushed with N_2 to remove oxygen before autoclaving in sealed bottles. The sediment samples were inoculated into FAM medium for two weeks to promote bacteria growth and then 10 ml of the mixed cultures was used to inoculate the sterile anaerobic chambers of the MFC containing a carbon cloth anode and 200 ml of growth medium. The growth medium was 10 mM sodium acetate, 0.05 % yeast extract in 1:1 diluted sea water. Fresh sea water was collected from the Dove Marine Laboratory, Cullercoats, North Shield, Tyne and Wear, North of England (near North Sea) and passed through a 0.2 μm pore-size filter, for medium preparation. Acetate (5, 10 mM, pH adjusted to 7.0) was provided as the electron donor, and no electron acceptors other than the electrode were present.

9.3.2 MFC construction and operation

The glass dual-chamber MFC was constructed from two 250 ml bottles (Corning Inc.) with H2315 carbon cloth (4×5 cm) (Freudenberg FCCT KG, Germany) electrodes (Zhang et al., 2006b) (Figure 9.1). A proton exchange membrane (inner diameter: 1.3 cm, Nafion^R 117, Dupont Co., Wilmington, USA) was installed between the two chambers. The membrane was equilibrated by incubating in 0.1 M NaCl solution for 2

hours prior to use. New electrodes were pretreated by soaking in 1 M HCl to eliminate possible metal ion contamination. Before inoculation, the anode chamber was filled with 200 ml media and flushed with pure N_2 gas for 10 min to create anaerobic conditions. The cathode chamber was filled with 200 ml electrolyte solution containing 50 mM $K_3Fe(CN)_6$ and 100 mM KH_2PO_4 (pH adjusted to 7.0 with 1 N NaOH). All experiments were conducted at room temperature (23±2 °C), and one set-up with no inoculum was also operated in parallel as a control.

Figure 9.1 Two-chamber Microbial Fuel Cell design with membrane chamber, organic substrate is oxidized in the anodic compartment and electrons were transferred via an external resistor to the cathode. The voltage output was measured continuously by a high impedance multimeter.

9.3.3 Electrochemical measurements

MFCs were operated in batch mode and the circuit was operated with a fixed external resistance of 1000 Ω. The voltage across the known resistance was continuously measured by using a digital multimeter (SkyTronic UK). Polarization curves for MFCs were measured using using linear sweep voltammetry (scan rate: 1 mV s^{-1}) from the open circuit potential to 0 V with a potentiostat (Autolab PGSTAT302) when a stable voltage production was achieved. Power and maximum power were calculated using data from stable voltage production and measured polarization curves, respectively, and then normalized to the total area (40 cm^2) of the anode surface. Internal resistance was determined by electrochemical impedance spectroscopy. To characterize anodophilic biofilms in-situ, cyclic voltammetry measurements were carried out with Autolab

PGSTAT302, with the anode in the anodic chamber as the working electrode, a Ag/AgCl electrode, connected to anodic solution through a lugin capillary, as reference electrode and a 30 mm×10 mm×0.2 mm platinum foil as counter electrode in the cathode chamber. Other electrochemical measurements were conducted in 50 ml three-electrode cells with a 3 mm diameter glassy carbon electrode as the working electrode, a Ag/AgCl electrode as reference electrode and a Pt wire as the counter electrode.

9.3.4 Scanning electron microscopy (SEM)

Biofilms on the anode surfaces were examined by scanning electron microscope (SEM) (Cambridge Stereoscan 240) according to the methods published previously (Zhang et al., 2006b).

9.3.5 Strain isolation

An anaerobic container system (GasPak™ EZ, Becton Dickinson, Maryland) was employed to isolate bacteria from the anode carbon paper and sediments. Samples were processed in an anaerobic jar under a N_2 atmosphere, and were inoculated onto different marine isolation media (MA1 to MA3), which consisted of the following: MA1, Difco™ Marine agar; MA2 (Fumaric Acid Medium (FAM)), 10 mM sodium fumarate, 10 mM sodium acetate, 0.05 % yeast extract, 1 % agar, made up with 50 % sea water and 50 % fresh water; and MA3 (Ferric Citrate Medium (FCM)), 5 mM Ferric Citrate, 10 mM glucose, 0.05 % yeast extract, 1 % agar, made up with 50 % sea water and 50 % fresh water.

9.3.6 16S rRNA gene analysis as methonds mentioned in Chapter2

Strains were grown for 3 days at room temperature in liquid medium anaerobically, using stoppered bottles which had been flushed with N_2. Cell mass from broth cultures was collected by centrifugation at 4500 g for 15 min. Preparation of genomic DNA and 16S rRNA gene analysis, phylogenetic analysis and diversity estimates were conducted according to the methods mentioned in Chapter 2. The nucleotide sequences of 16S rRNA genes have been deposited in the EMBL database under the accession numbers: from FN997605 to FN997642.

9.4 Results

9.4.1 Power generation

Generally, a long lag phase (over 10 days) occurred before voltage started to increase. Figure 9.2A shows the voltage output of a fuel cells inoculated with sediment bacteria, with a lag phase of ~13 days. The voltage began to increase exponentially, and reached higher stable electricity generation (> 0.6 V) which was maintained for 10 days.

The recovered electrode, coated with biofilm, was used to provide inocula for new microbial fuel cells with fresh media. The voltage increased exponentially to a high stable level (> 0.6 V) after a much shorter lag phase (< 2 days). The current density at this point was around 175 mA m^{-2}. High electricity generation was maintained for over 2 weeks and subsequently fell rapidly, probably due to acetate depletion (Figure 9.2B). When these anodes were transferred to fresh growth medium, the voltage was rapidly restored to the previous stable level within a few hours. The medium replacement and voltage restoration of the present MFCs could be repeated without decay in electricity generation.

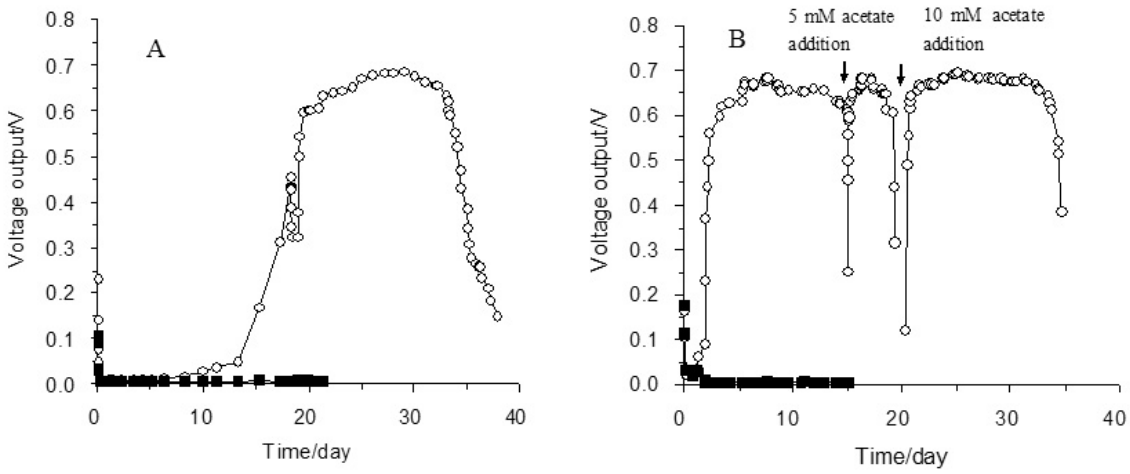

Figure 9.2 Voltage output produced by sedimentary bacteria activated MFC (A), and anodophilic bacteria activated MFC (B). Symbol (■) the voltage output by controlling MFCs without bacteria. Arrows in figure B indicate the replacements of anodic solution by fresh growth medium. The experiments were carried out in duplicate and values are means of two samples.

The presence and appearance of anodophilic biofilms was confirmed by SEM which confirmed coverage by biofilms consisting of different coccoid (~0.5 μm diameter),

and rod-shaped bacterial cells (2.0-4.0 μm long and 0.5-0.7 μm). Pilus-like appendages connecting the cells were also observed (Figure 9.3C and 9.3D).

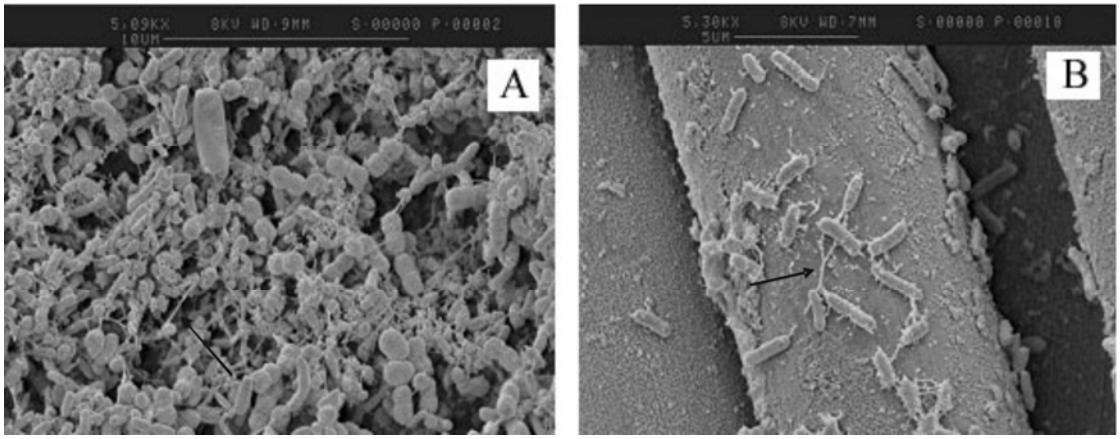

Figure 9.3 (A and B). SEM images of acetate-induced bacteria morphology on the anode surface recovered from the river sediment. Arrows indicate microbial pilus-like appendages connecting bacterial cells.

The restoration of voltage after replacement of growth medium suggested that anodophilic biofilms could directly transfer electrons derived from acetate metabolism to the anode surface in the absence of any soluble electron shuttles. This characteristic was also supported by cyclic voltammetry measurements. Figure 9.4A shows cyclic voltammograms using a glassy carbon electrode for the fresh sterile medium without inoculum, the MFC-derived cell suspension and the cell-free suspension. No obvious electroactive species were detected in the above solutions within the electrode potential range where possible soluble electron shuttles would be detectable as redox peaks (Rabaey et al., 2004; Marsili et al., 2008a).

9.4.2 Performance of the MFCs

The open-circuit potentials of the double-bottle MFCs reached 0.8 V when sufficient quantities of acetate were present. When the electrical current became stable for acetate-fed MFCs (voltage output 0.65 V to 0.7 V), slow cyclic voltammetry was carried out to determine the polarization curves of the MFC. Figure 9.4B shows a representative cell voltage current density polarization measurement of the MFC. The voltage fell almost lnearly with increase in current density which indicates that Ohmic (transport of ionic species through the medium) was predominant in the MFCs, and no obvious voltage drop caused by charge transfer resistance was observed at a lower rate of current flow. The voltages achieved in the MFC tests agree with values obtained during the biofilm

development stage (Figure 9.1A), where a voltage of around 0.65 V to 0.7 V occurred at current densities around 162-175 mA m^{-2}.

Electrochemical impedance spectroscopy (EIS) (data not shown) showed that the internal resistance (IR) of the MFCs used in this work was in the range of 120 to 145 ohms; greater than higher specification devices reported previously (Liang et al., 2007). From the voltage current density behaviour of the MFC (Figure 9.4) the internal resistance was approximately 175 ohm in reasonable agreement with the EIS data. At low cell voltages there was an indication of a mass transport limitation, which may be associated with the oxygen reduction reaction or possibly acetate mass transport limitation. The acetate-fed MFC used in this study could generate a maximum power density of 175 mW m^{-2}, and the maximum current density reached ~1600 mA m^{-2} during stable current production (Figure 9.4B). Maximum power density and stable power generation were higher than similar marine and fresh water sediment-inoculated MFCs reported recently (Table 9.1). This could be attributed to the use of added acetate as a feedstock and also to use of anodophilic microbial biofilms in transporting electrons to the electrode, considering the high internal resistance of the MFCs. In fact the IR free peak power and current densities were about 130 ohms and 175 mW m^{-2} respectively, indicating the potential of relatively high power generation with higher specification MFCs using minimal electrode separation and improved cathodes.

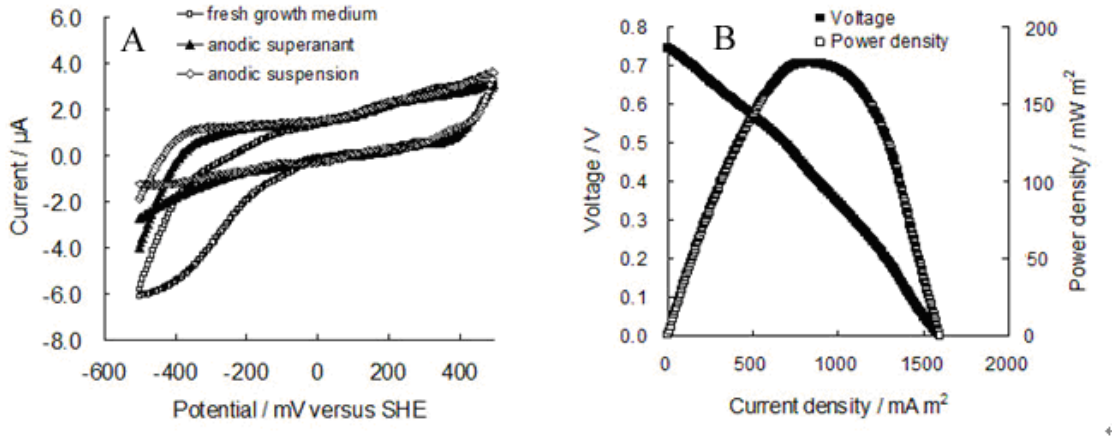

Figure 9.4 Cyclic voltammetry measurement (scan rate: 50 mV s^{-1}) with a glassy carbon electrode on fresh growth medium, anodic supernatant and anodic suspension during stable electricity generation. 2B. Current density–voltage and current density–power density relationships for acetate-fed microbial fuel cells during stable electricity generation (measured using slow scan cyclic voltammetry, scan rate: 1 mV s^{-1}). The experiments were carried out in duplicate and values are means of two samples.

Table 9.1 Comparison of power density from different MFCs

Maximum or sustained power density mW/m^2	Location of Inoculum used	Electron donor	Anode material	Cathode material	Reference
2.2	Freshwater sediment	Organic matter in sediments	Bare graphite felt	Bare graphite felt	(Hong et al., 2009)
10	Marine sediment	Organic matter in sediments	Pairs of platinum mesh or graphite fiber-based electrodes	Pairs of platinum mesh or graphite fiber-based electrodes	(Reimers et al., 2001)
24-36	Marine sediment	Organic matter in sediments	Graphite discs	Connector-silver epoxy-graphite union	(Tender et al., 2008)
30	Marine sediment	Organic matter in sediments	Graphite discs	Connector-silver epoxy-graphite union	(Tender et al., 2002)
39	Marine sediment	Cysteine	Carbon cloth	graphite paper contained Pt catalyst	(Logan et al., 2005)
55	Marine sediment	Organic matter in sediments	Graphite sponge	Carbon cloth	(Scott et al., 2008)
70	Fresh water sediment	Acetate and glucose	Carbon cloth	Carbon cloth	(Zhang et al., 2006b)
100	Marine sediment	Organic matter in sediments	Carbon cloth	Proximal seawater	(Lowy et al., 2006)
100	Coastal sediment	Organic matter in sediments	Carbon cloth	Stainless steel	(Dumas et al., 2008)
27-140	Marine sediment	Organic matter in sediments	Solid graphite	Carbon-fiber brush electrode	(Nielsen et al., 2008)
233	Marine sediment	Organic matter in sediments	High surface area and semi-enclosed anode	1-m long graphite bottle brush electrode	(Nielsen et al., 2007)
150-175	Estuarine sediment	Acetate	Carbon cloth	Carbon cloth	This study

9.4.3 Electrochemical properties of anodophilic biofilms

Cyclic voltammetry (CV) was performed to characterize the catalytic properties of the anodophilic biofilm on the carbon cloth anodes. When a new carbon cloth anode was placed in anodic growth medium containing excess electrode donor (10 mM acetate) and bacterial cells, no obvious catalytic current was observed (curve *a* in Figure 9.5A), indicating that the anode did not directly catalyze the oxidation of acetate. During exponential increase in voltage output, slow scan cyclic voltammetry produced potential waves (curves *b* and *c* in Figure 9.5A) in the range of -200 mV to 0 mV (vs SHE). The peak/limiting current of the wave increased with the development of the MFC voltage output, and eventually reached a maximum value of ~ 425 mA m^{-2} (curves *d* in Figure 9.5A) when the MFC achieved its highest stable electricity generation (V_{cell} > 0.65 V). A mature biofilm on the anode was necessary for high stable electricity generation, consistent with the observations using growth medium replacement (Figure 9.2B). When the acetate in the anodic growth medium was depleted, the limiting current of the catalytic wave also decreased (Figure 9.5B).

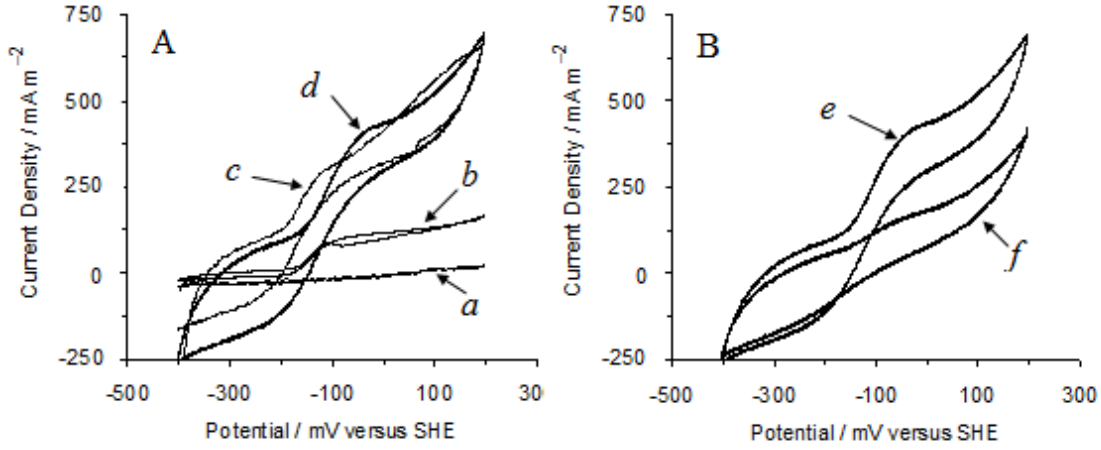

Figure 9.5 Cyclic voltammetry curves (scan rate: 1 mV s^{-1}) obtained on carbon cloth anode with bacterial biofilms at different electricity-generating stages. *a* : using a newly prepared carbon cloth anode in growth medium containing 10 mM acetate and suspended anodophilic bacteria, *b* and *c:* results with carbon cloth anode partially covered by anodophilic bacteria during exponential increase in voltage, *d*, and *e:* carbon cloth anode with a mature biofilm during stable current generation (V_{cell}: ~0.65 V), *f:* with acetate-depleted medium. The experiments were carried out in duplicate and values are means of two samples.

The potential wave observed with the anodophilic bacterial biofilm was similar to results obtained with carbon-attached biofilms formed using a pure strain, of *Geobacter*

sulfurreducens (Marsili et al., 2008a). *Geobacter sulfurreducens* may directly transfer electrons from bacterial cells to the anode via several different redox active outer-membrane cytochromes (Marsili et al., 2008b; Srikanth et al., 2008). It is unknown which microbial species or proteins in the present complex biofilm play these roles. The redox potentials observed (-200 ~ 0 mV versus SHE) are similar to those of reported strains or purified outer-membrane combined cytochromes (Magnuson et al., 2001; Lloyd et al., 2003; Marsili et al., 2008b).

9.4.4 Phylogenetic analysis of anodophilic bacteria

Bacteria colonizing the electrode surface were isolated and cultured under anaerobic conditions, 74 isolates were obtained and their 16S rRNA genes sequenced. Thirty-eight distinct sequences were chosen for phylogenetic analysis (Table 2 and Figure 9.6). NCBI nucleotide BLAST searches using the partial 16S rRNA gene sequences of these 74 strains revealed that 44 (59.5 %) of the isolates fell into the *Gamma-Proteobacteria* group and shared a phylogenetic affiliation with members of the *Enterobacteriaceae, Aeromonadaceae, Vibrionaceae, Shewanellaceae, Moraxellaceae* and *Alteromonadaceae*. These results were further confirmed by Gram staining and microscopy. Twenty-two strains fell into the class *Enterobacteriaceae* and showed similarity to 4 taxonomic units: *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Citrobacter freundii* and *Serratia proteamaculans*; 6 strains fell into the class *Aeromonadaceae* including 2 taxonomic units: *Aeromonas salmonicida* and *Aeromonas hydrophila*; while *Shewanellaceae* contained *Shewanella haliotis* and *Shewanella algae*. Isolates similar to *Vibrio azureus*, *Psychrobacter nivimaris* and *Marinobacter lipolyticus* were also found.

The Firmicutes represented 16.2 % of the sequences with strains similar to *Bacillus stratosphericus, Bacillus altitudinis* and *Exiguobacterium mexicanum*. Within the *Enterococcaceae*, one strain similar to *Vagococcus fluvialis* was recovered. The remaining strains fell into the *Alpha-Proteobacteria* (6.8 %)*, Beta-Proteobacteria* (10.8 %), *Epsilon-Proteobacteria* (2.7 %) and C*ytopahga-Flexibacteria-Bacteroides* group (CFB) (1.4 %). Most of the strains isolated were found to have a high homology (98.3-99.9 %) to their closest neighbours, while MS22, MS30 and TRS1-A1 showed lower similarities of 96.7, 95.3 and 93.3 % respectively to *Rhodobacter maris*, *Arcobacter nitrofigilis* and *Marinifilum fragile*. These strains are taxonomically unusual and worthy of more detailed characterisation.

Figure 9.6 Neighbour-joining distance tree based on the nearly complete and aligned 16S rRNA gene sequences of 38 representative strains observed in this study and their nearest neighbours. The phylogenetic tree was constructed using the neighbour-joining method using the programs of the MEGA package. 1000 trials of bootstrap analysis were used to provide confidence estimates for phylogenetic tree topologies. Bar- 0.05 nucleotides substitution per site.

Table 9.2 Anodophilic bacterial isolates and their individual power generation abilities

Phylogenetic group (family)	Representative isolates	Nearest type strains in the GenBank (accession number)	Similarity (%)	Max power density mW/m^2
Firmicutes				
Bacillaceae	**MS10**[a]	*Bacillus stratosphericus* 41KF2a^T (AJ831841)	99.859	87.5 (±1.3)
	MS28; MS27	*Bacillus altitudinis* 41KF2b^T (AJ831842)	99.930	6 (±0.7)
	MS34; MS4; MS1	*Exiguobacterium mexicanum* 8N^T (AM072764)	99.636	0
Enterococcaceae	**MS23**; MS7; MS51; MS53; MS60; MS69	*Vagococcus fluvialis* CCUG 32704^T (Y18098)	99.853	0
Alpha-Proteobacteria				
Rhodobacteraceae	**MS22**; MS9; MS45; MS46; MS65	*Rhodobacter maris* JA276^T (AM745438)	96.726	0
Beta-Proteobacteria				
Alcaligenaceae	**MS11**; MS13; MS14; MS50; MS62	*Alcaligenes faecalis subsp. faecalis* IAM12369^T (D88008)	99.430	0
	MS16; MS47; MS48; MS52; MS61	*Alcaligenes faecalis subsp. parafaecalis* G^T (AJ242986)	99.571	0
Gamma-Proteobacteria				
Enterobacteriaceae	**MS2**	*Klebsiella oxytoca* JCM 1665^T (AB004754)	98.517	9 (±1)
	MS6	*Klebsiella oxytoca* JCM 1665^T (AB004754)	98.593	12.5(±1.7)
	MS25	*Klebsiella oxytoca* JCM 1665^T (AB004754)	98.742	5 (±0.6)
	MS5	*Enterobacter aerogenes* NCTC 10006^T (AJ251468)	98.655	15 (±0.8)
	MS8	*Citrobacter freundii* DSM 30039^T (AJ233408)	99.514	37.5 (±0.7)
	MS17	*Citrobacter freundii* DSM 30039^T (AJ233408)	99.789	30 (±0.5)
	MS18	*Citrobacter freundii* DSM 30039^T (AJ233408)	99.220	10 (±0.6)
	MS36; MS39	*Citrobacter freundii* DSM 30039^T (AJ233408)	99.507	14 (±0.5)
	TRS1-B4	*Citrobacter freundii* DSM 30039^T (AJ233408)	99.578	9 (±1.0)
	TRS1-WB; MS31; MS49; MS59	*Serratia proteamaculans* DSM 4543^T (AJ233434)	99.647	0
Aeromonadaceae	**MS24**; MS20; MS21; MS58; MS66	*Aeromonas salmonicida subsp. masoucida* ACC 27013^T (X74680)	99.956	0
	TRS1-B1; MS15; MS19; MS33; MS70	*Aeromonas hydrophila subsp. hydrophila* ATCC 7966^T (CP000462)	99.719	15 (±1.0)
	MS12	*Aeromonas hydrophila subsp. hydrophila* ATCC 7966^T (CP000462)	99.859	13.5 (±1.5)
Vibrionaceae	**MS26**	*Vibrio azureus* LC2-005^T (AB428897)	99.127	30 (±1.0)
Shewanellaceae	**MS32**	*Shewanella haliotis* DW01^T (EF178282)	99.788	5 (±0.25)
	MS43	*Shewanella algae* ATCC 51192^T (AF005249)	99.531	15 (±0.7)
Moraxellaceae	**TRS1-R2**; MS42; MS56; MS64; MS67	*Psychrobacter nivimaris* 88/2-7^T (AJ313425)	99.929	0
Alteromonadaceae	**MS3**; MS37; MS54; MS55; MS63	*Marinobacter lipolyticus* SM19^T (AY147906)	98.379	0
	MS38; MS40; MS44; MS57; MS68	*Marinobacter lipolyticus* SM19^T (AY147906)	98.310	0
Epsilon-Proteobacteria				
Campylobacteraceae	**MS30**; MS35	*Arcobacter nitrofigilis* CCUG 15893^T (L14627)	95.345	8.7 (±0.4)
CFB group bacteria				
Bacteroidetes	**TRS1-A1**	*Marinifilum fragile* JC2469^T (FJ394546)	93.309	12.5 (±0.5)

[a] Strains chosen for phylogenetic analysis in bold text. The experiments on electricity production were carried out in duplicate and values are means of two samples.

9.4.5 Electrochemical properties of the isolated anodophilic bacteria

Of the 44 *Gamma-Proteobacteria* isolates, 19 strains could produce electricity. Strains MS8 and MS17 similar to *Citrobacter freundii* and strain MS26 similar to *Vibrio azureus* produced the highest power density of 30-40 mW/m^2 (Table 9.2). Three out of 15 strains in the *Firmicutes* group were able to produce electricity. Strain MS10 simlar to *Bacillus stratosphericus* was found with 87.5 mW/m^2 power density production under the above mentioned conditions, while *Bacillus altitudinis* similar strains MS 27 and MS 28 generated lower power of ~6 mW/m^2.

In the *Alpha-, Beta-*, *Epsilon-Proteobacteria* and CFB group bacteria phylum, strain MS11 homology to *Alcaligenes faecalis* was unable to produce electricity, whereas strain MS30 homology to *Arcobacter nitrofigilis* and strain TRS1-A1 tentatively identified as *Marinifilum fragile* showed relatively small power generation (3.5-17.5 mW/m^2).

9.4.6 Electrogenic properties of artificial consortia

When 74 strains were re-mixed and cultured as an artificial biofilm consortium (TRS2) in the MFCs, power output remained similar to the wild type consortium (TRS1). However, the maximum power output was increased to ~200 mW/m^2 when the consortium biofilm (TRS3) was reconstituted using only the exoelectrogenic strains (Table 9.2 and Figure 9.7).

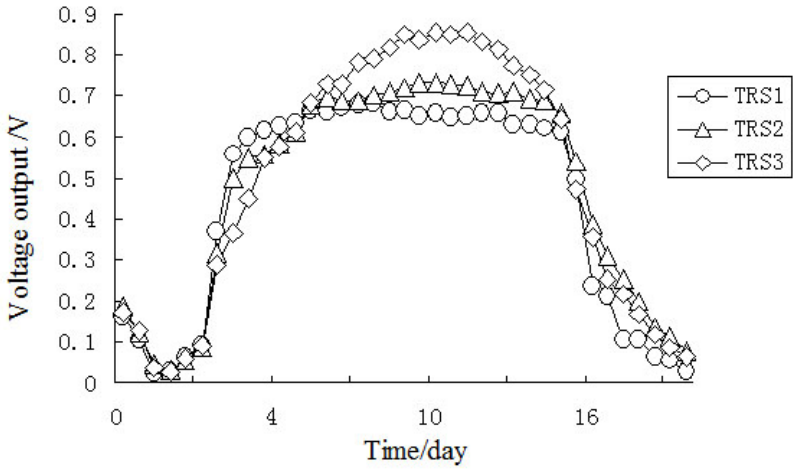

Figure 9.7 Voltage output produced by MFCs containing: wild type biofilm (TRS1, open circles), an artificial consortium of all pure strains isolated from the original biofilm (TRS2, open triangles), an artificial consortium composed using all exoelectrogenic bacteria (TRS3, open diamonds). The experiments were carried out in duplicate and values are means of two samples.

9.5 Discussion

Using a two-chamber microbial fuel cell inoculated with estuarine sediments, we isolated anodophilic biofilms which selectively proliferated in the presence of acetate on carbon cloth electrodes. Our approach involved allowing the microbial community to adapt to this environment and as a result a power density of 175 mW/m^2 was achieved, the highest level reported for sediment inoculated MFCs (Table 9.1A). In 2001, Reimers

et al. (Reimers et al., 2001) first utilized marine sediment-seawater interfaces in situ and obtained power generation of 10 mW/m^2 using graphite fiber-based electrodes. By using different marine sediments and specific anodes, a range of power densities (20-100 mW/m^2) were obtained (Tender et al., 2002; Lowy et al., 2006; Dumas et al., 2008). By using high surface area, semi-enclosed anodes, the power generation was improved to 140 mW/m^2 (Nielsen et al., 2008), and 233 mW/m^2 (Nielsen et al., 2007). However, in the laboratory electricity production from sedimentary MFCs remains low, between 40-70 mW/m^2 (Logan et al., 2005; Zhang et al., 2006b).

Much analysis of the composition of bacterial communities inhabiting fuel cell anode chambers has been carried out by culture independent methods such as denaturing gradient gel electrophoresis (DGGE) or restriction fragment length polymorphism (RFLP) of the amplified 16S rRNA gene fragments and sequencing of the dominant bands (Tender et al., 2002; Lowy et al., 2006; Dumas et al., 2008; Tender et al., 2008). However, such analysis provides no information about whether these species are electrogenic. A culture dependent study, though more difficult, allows relative contributions to the overall power generation of the various strains to be estimated. This is important since our work demonstrates that a significant proportion (67 %) of bacteria isolated from an electrogenic biofilm are not electrogenic when tested in isolation.

Cytophaga/Flexibacter/Bacteroides, one of the major groups of the MFC microbial community has been detected using molecular methods in MFCs (Kim et al., 2004; Choo et al., 2006; Martins et al., 2010). However, there are fewer studies based on cultured CFB representatives, we report for the first time, successful isolation of a *Bacteroides* representative, strain TRS1-A1 similar to *Marinifilum fragile*, and we have demonstrated that this isolate can act as an exoelectrogenic bacterium. *Epsilon-Proteobacteria* bacteria, such as *Arcobacter* sp. were first isolated from an MFC in 2008 (Ha et al., 2008), and the ability of members of this genus to produce electricity was demonstrated (Fedorovich et al., 2009). In this study, strain MS30 was also confirmed as an electrogen supporting these observations.

The majority of *Gram-negative* bacteria can produce electricity (Lovley, 2006; Logan, 2009). However, relatively few *Gram-positive* strains are exoelectrogens (Logan, 2009). However, we report power generation by *Bacillus stratosphericus* (strain MS10), with sustained generation of electricity of 87.5 mW/m^2 in acetate-fed MFC. This is novel in

the phylum *Firmicutes*. Furthermore, we have shown that members of the genera *Vibrio*, *Enterobacter* and *Citrobacter* can act as exoelectrogens. Although *Vibrio* has been detected from a cysteine-fed MFC using DGGE, strains of *Vibrio* were not isolated or shown to produce electricity (Logan et al., 2005). *Citrobacter* was reported in a bacterial consortium of a glucose/glutamate-fed MFC based on RFLP analysis (Park et al., 2008), and *Enterobacter* was first reported as part of a power generating consortium digesting cellulose (Rezaei et al., 2009). The genus *Klebsiella* has been recently reported as power producer in MFCs (Xia et al., 2010; Zeng et al., 2010), and also has been demonstrated in this study by strains MS2, MS6 and MS25.

Non-exoelectrogenic bacteria were detected in the original biofilms (Table 9.2), and these have been suggested to play a role as helper strains for fermenting organic matter (Ren et al., 2007). Interestingly, maximum power output was increased and maintained at ~200 mW/m^2 when artificial consortia were reconstituted using only exoelectrogenic bacteria. It may be that the presence of non-exoelectrogenic bacteria can disrupt or reduce the overall electrical conductivity of the biofilm.

No detectable redox species were involved in electron transfer by the anodophilic biofilms, and isolates similar to *B. stratosphericus*, *C. freundii* and *V. azureus* isolated from the biofilms could generate considerable power in the same MFC devices using acetate as electron donor. Therefore, power production could be confirmed by direct transfer of electrons to the anode by the bacterial consortia growing on the anode as demonstrated by cyclic voltammetry (CV). Scanning electron microscopy of the biofilms showed numerous pilus-like appendages, connecting cells to form an integrated community (thick biofilm) on the surface of the electrode. It has been reported that similar appendages may serve as “nanowires” and be directly responsible for electricity conduction (El-Naggar et al., 2010). However, the precise biochemical composition of the observed nano wires observed in *Shewanella oneidensis* MR-1 remains unknown, while rigorous direct measurement of electricity conduction by pili based nanowires in *Geobacter* species has not been reported.

It will be interesting to examine how bacteria cooperate with each other to efficiently metabolise organic carbon sources for electricity production, and to investigate whether the lateral appendages produced by strains isolated in this work, contribute to electron transfer. Models of consortium biofilm structure and its activities, and a better

understanding of the synergistic cooperation of each individual strain in the biofilm will help us to better predict the maximum power densities achievable using MFCs.

Chapter 10. Final discussion and conclusion

This final discussion chapter will attempt to draw together the data presented in this thesis to further understand the diversity and biotechnological application of the culturable marine microbes producing omega-3 fatty acids, their importance in marine food web and energy research.

10.1 Final discussion

Fish oils are the most important source of omega-3 fatty acids, however the contamination of fish due to pollution, as well as unstable fish catches, has created a need for alternative ways to provide those PUFAs (Qi et al., 2004). Heightened consumer awareness of the value of omega-3 fatty acids has increased the growth in demand for the omega-3 products, which have led to considerable interest in developing commercial processes for EPA/DHA production from marine biomass rather than from marine fish for food and pharmaceutical markets (Belarbi et al., 2000; Molina Grima et al., 2003). However, one enormous challenge is to develop a range of bioprocesses, which can convert chemically complex biological mixtures into pure chemicals that industry needs. Nature already processes biological materials using complex arrays of enzymes, which form carefully regulated biosynthetic pathways, such as polyketide synthases, responsible for the production of EPA and/or DHA (Metz et al., 2001; Okuyama et al., 2007). The majority of known industrial enzymatic processes, which produce pure chemicals, are based on enzyme systems from terrestrial organisms (Robertson and Steer, 2004; Alcalde et al., 2006). However, not only is the sea a major source of biomass (for food and energy), it is becoming an increasingly significant source of biomass for the production of fine chemicals (Trincone, 2011), including EPA or DHA (Kaulmann and Hertweck, 2002). In addition, the diversity of marine organisms is known to be greater than any other environment and remains relatively untapped. Thus, this thesis has been focused on the natural processing of fatty acids in the marine habitat and specifically by marine microorganism.

10.1.1 Ecologically study microbial communities and their fatty acid composition

Ecologically, PUFA producing bacteria are mainly characterised as being psychrophilic, halophilic and predominantly piezophilic or piezotolerant as most of them were isolated from deep sea or polar zones (Russell and Nichols, 1999; Kato and Nogi, 2001), and few of them are identified as mesophiles isolated from a temperate estuary or shallow seawater (Ivanova et al., 2001; Skerratt et al., 2002; Ivanova et al., 2003b; Frolova et al., 2005). Therefore, these physiological characteristics may contribute to the ecosystem diversity of bacterial PUFA producers in the marine environment.

Sediments from the Mid-Atlantic Ridge (MAR) north and south east of the Charlie-Gibb Fracture Zone (CGFZ) (2,400 m and 2,750 m) are remote from any islands and seamounts, with no known hydrothermal activity, and are proposed to be good locations for isolating cold-adapted bacteria which are capable of producing PUFAs. This thesis reported, for the first time, the diversity of culturable microorganisms in this area, finding Gram-positive bacteria to be the most abundant, followed by *Gamma-Proteobacteria* and *Alpha-Proteobacteria*. A *Bacillus* strain (MAR019) was observed to produce squalene, which is the first report squalene production in marine Gram-positive following by that of the marine Gram-negative bacteria *Rubritalea squalenifaciens* (Kasai et al., 2007). Strains MAR441 and MAR445 are phylogenetically unusual species of the genus *Shewanella*, and showed stable growth and production of EPA (15-21 % EPA of TFA) under atmospheric conditions, which is the highest level reported among the deep-sea *Shewanella* species (2-14 % EPA of RFA) (Deming et al., 1984; Delong and Yayanos, 1986; Delong et al., 1997; Nogi et al., 1998b; Miyazaki et al., 2006; Xiao et al., 2007). Thus, strain MAR441 was identified as a new species, designated as *Shewanella dovemarina* sp. nov. (Type strain MAR441^T). This strain also had the ability to produce electricity at a considerable level (~150-200 mW/m^2).

Marine sponges are excellent models for the study of marine host-associated bacteria (Wilkinson, 1978; Taylor et al., 2007), from which novel biological active chemically compounds could be found (Berge and Barnathan, 2005; Ding et al., 2006). Biogeographic variability of bacterial communities and fatty acid compositions between temperate and tropical sponges by isolating bacteria from the temperate North-sea sponge *Halichondria panicea* and the tropical Caribbean sponge *Agelas clathrodes*, was investigated. The study showed that the temperate sponge associated bacterial

communities with abundance of *Gamma-Proteobacteria* (90%) and Gram-positive bacteria mainly occured in tropical sponge. Fatty acids analysis showed that *Vibrio* and *Shewanella*, isolated from *Halichondria panicea*, were able to produce EPA (2-10% of TFA), and no strains capable of producing EPA were isolated from *Agelas clathrodes.* Some *Vibrio* species may contain only silent copies of the PUFA genes, which unable to synthesize EPA successfully. North-sea sponge associated bacteria *Vibrio* sp. NSP560 was able to produce up to 12 % EPA of TFA (or 8.4 mg g^{-1}), while its closest type strains, *Vibrio tasmaniensis* and *V. cyclitrophicus* were reported no produce EPA (Hedlund and Staley, 2001; Thompson et al., 2003).

Various species of macro/micro algal species were obtained from the coast water of North Sea, found with high content of omega-3 fatty acids, particularly EPA (10-43% of TFA). Such highly valuable nutrients could be provided as human diets. Nutritionally, their surface might also be a good place for obtaining omega-3 fatty acids bacterial producers. Bacterial communities associated with micro algae and macro algae were phylogenetically identified respectively based on 16S rRNA gene sequences, indicating that the abundance of *Gamma-Proteobacteria* (90%). Macro algae associated bacteria responsible for EPA or DHA production were mainly members of genera *Vibrio* and *Colwellia*, whereas genera *Vibrio*, *Photobacterium* and *Shewanella* were presented in the micro algal culture as bacterial EPA producers. *Photobacterium profundum* and *Ph. frigidiphilum* are the only two species within *Photobacterium* genus known to be able to produce EPA at the level of 2-14 % of TFA, however, they require pressure for growth (Nogi et al., 1998c; Seo et al., 2005). *Photobacterium* sp. MA665, phylogenetically with high similarity with these two psychropiezophiles, isolated from shallow-sea algal plant from North Sea, was first time described of this genus could be cultured easily under atmospheric conditions with appreciable content of EPA (up to 25 % of TFA or 10.6 mg g^{-1}).

It has been nearly ten years that only two bacterial phyla: the *Gammaproteobacteria* and the *Bacteroidetes*, with limited species are capable of producing PUFAs (Nichols and McMeekin, 2002). Gram-positive bacteria seldom produce PUFA, but typically terminal branched fatty acids (Kaneda, 1991). Varied proportion of EPA from bacteria reflects the geographic temperatures, nutrient content of their phytoplankton hosts or the food web characteristics of the environments where they were isolated. Marine bacteria isolated from cold marine environments, such as Mid-Atlantic Ridge deep-sea

sediments, North England coast bay water, North-sea sponges and algae, were confirmed with finding of PUFA producers, whereas strains isolated from tropical Caribbean marine water and sponges were not produced PUFAs. Therefore weather or temperature is one of the major factors concerned for screening PUFA producers.

Generally, cells must cope with decreases in temperature by modulating the lipid composition of their membrane, which can crystallize or enter nonbilayer phases at low temperatures (Russell and Nichols, 1999). According to the observation of this study, Gram positive strains, mainly *Firmicutes* affiliated with branched-chain fatty acid family, employed anteiso-15:0, iso-15:0 to adjust their membrane viscosity. The presence of these branched fatty acids has been suggested to have a functional role in piezoadaptation (Fang and Kato, 2007). Whereas other strains, such as *Alpha*, *Gamma-Proteobactria* and CFB group bacteria belong to straight-chain fatty acid family which required unsaturated fatty acids, especially PUFAs, for growth and membrane viscosity manipulation. Production of EPA by some bacteria increases as temperature decreases, leading to the hypothesis that these molecules may be important for growth at low temperatures (Delong and Yayanos, 1986; Valentine and Valentine, 2004; Amiri-Jami et al., 2006). EPA was not required for low-temperature growth in the deep-sea bacterium *Photobacterium profundum* (Allen et al., 1999), but it may be required for low temperature growth in *Shewanella* (Valentine and Valentine, 2004; Sato et al., 2008; Wang et al., 2009). Therefore, it is unclear why these bacteria produce omega-3 fatty acids.

10.1.2 Biotechnological production of EPA under various fermentation conditions

Biotechnologically, PUFA biosynthesis, speciation and the interaction of PUFA with other fatty acid types in the adaptive responses of bacteria to changing environmental conditions could be manipulated according to previous studies (Akimoto et al., 1990; Suzuki et al., 1991; Suzuki et al., 1992; Henderson et al., 1993; Hamamoto et al., 1994; Nichols et al., 1994; Bowman et al., 1997b; Nichols et al., 1997; Gentile et al., 2003). The level of EPA production of strain *Shewanella* sp. MAR441 has been optimized under various fermentation conditions, and 15-25 % EPA of TFA (or 17-30 mg g^{-1} in dried cell) could be achieved with more than 40 % improvement (Table 10.1). Thus, strain MAR441 produced higher levels of EPA than other novel bacteria, such as *Shewanella putrefaciens*-like strain SCRC-8132 produced 4-15 mg g^{-1} of EPA (Yazawa

et al., 1988b; Yazawa, 1996), *Shewanella gelidimarina* ACAM 456^T could yield 1-16 mg g^{-1} of EPA (Nichols et al., 1997), and *Shewanella pneumatophori* SCRC-2738 produced 4-17 mg g^{-1} of EPA (Yazawa et al., 1988b; Akimoto et al., 1990; Hirota et al., 2005). This study reported the highest yield of EPA from bacterial strain MAR441 will therefore bring it closer for next step of industrial commercialization.

Vibrio sp. strain NSP560 and *Photobacterium* sp. strain MA665 isolated from North-sea coast, produced lower levels of EPA than *Shewanella* sp. MAR441, in marine broth. However, the EPA production in these three species could be greatly enhanced to nearly the same levels by cerulenin treatment, which inhibited the synthesis of middle-chain fatty acids. The observation indicates that the environments where the strains isolated may not be the primary controlling factor in PUFA synthesis, but mostly may impact the growth. Therefore it suggests that the necessary of rethinking the conventional concepts/ways of screening strains with highly accumulate PUFAs, as well as the mechanisms of cold adaptation, such as the increasing levels of EPA and/or DHA responses to cold temperatures.

Marine microalgae have several advantages over conventional (energy) crops for converting carbon dioxide into biomass; a high growth rate, high CO_2 fixation rates, their lack of requirement for fertile soil and suitability for large scale production (Solovchenko *et al.*, 2008). Algal cultures have been focused in tropical and subtropical areas, such as Spain, Hawaii, California, Roswell and New Mexico, grown outdoors on a commercial scale (Borowitzka, 1999; Moreno-Garrido and Canavate, 2001; Voltolina et al., 2008; Mata et al., 2010). However, such areas with higher temperatures and fluctuations in temperature are not suitable for many algal species for outdoors culture (Renaud et al., 1995; Lee, 2001), and therefore result in hampering productivity rates (Sheehan et al., 1998). The investigation of the natural populations of algae from North Sea was conducted with finding of *Phaeodactylum tricornutum* strain M7 capable of growing indoors and outdoors under local natural weather months (Table 10.1). Strain M7 had a lipid content of 10 % dry wt biomass and 22-30 % EPA of TFA among the highest level reported (Kates and Volcani, 1966; Ackman et al., 1968; Volkman et al., 1989; Yongmanitchai and Ward, 1991a; Reitan et al., 1994; Patil et al., 2007). The results indicate the potential of growing marine microalgae in temperate regions using this local isolate M7, rather than in tropical and subtropical areas.

Table 10.1 Summary of microbes with their productivity of biomass, total fatty acids and EPA in this study

Strains	Biomass ($g\ l^{-1}\ day^{-1}$)	EPA/TFA (%)	TFA ($mg\ g^{-1}$)	TFA ($mg\ g^{-1}day^{-1}$)	EPA ($mg\ g^{-1}$)	EPA ($mg\ g^{-1}day^{-1}$)
Shewanella sp. strain MAR441 (Chapter 6)						
4 °C for 3 days	0.9	25.5	118.6	39.5	30.2	10.1
15 °C for 2 days	1.5	17.6	115.2	57.6	20.3	10.1
Photobacterium sp. strain MA665 (Chapter 7)						
4 °C for 3 days	0.7	24.5	43.1	14.4	10.6	3.5
15 °C for 2 days	0.8	18.4	46	23	8.2	4.1
Vibrio sp. NSP560 (Chapter 7)						
4 °C for 3 days	0.6	12.3	68	22.7	8.4	2.8
15 °C for 2 days	9.5	10.5	69	34.5	7.2	3.6
Phaeodactylum tricornutum strain M7 (Chapter 8)						
Indoor (15 °C, 6 days)	0.28	24	170	28.3	24.4	4.1
Outdoor (summer, 9 days)	0.17	23.5	103	11.4	24.3	2.7
Outdoor (winter, 13 days)	0.12	32	103	7.9	33.3	2.6
Tetraselmis sp. strain M1 (Chapter 8)						
Indoor (15 °C, 7 days)	0.23	5.2	63.5	9.1	3.3	0.5
Shewanella gelidimarina ACAM 456^T (Nichols et al., 1997)						
6 °C	NP	16	40	NP	4.8	NP
15 °C	NP	12.8	40.1	NP	5.2	NP
Phaeodactylum tricornutum cultured in a photobioreactor (Patil et al., 2007)						
20 °C, 2.1days	0.43	NP	37.2	17.7	28.4	13.5
Tetraselmis suecica cultured in a photobioreactor (Patil et al., 2007)						
20 °C, 3.3 days	0.27	NP	18.6	5.6	4.8	1.45

NP, Specific data not published post-February 2011.

10.1.3 Molecular analysis of bacterial PUFA biosynthesis pathway

Molecular analysis of microbial EPA/DHA biosynthesis via the PKS-like pathway was conducted by cloning, sequencing, and complementation analysis of the PUFA gene cluster (Yazawa, 1996; Tanaka et al., 1999; Allen and Bartlett, 2002; Gentile et al., 2003; Orikasa et al., 2004; Okuyama et al., 2007). Thus, *pfa* genes were further expressed for finial EPA/DHA production in *Escherichia coli*, with 2-6 % EPA/DHA recombinant production of TFA (Valentine and Valentine, 2004; Lee et al., 2006; Orikasa et al., 2006b; Amiri-Jami and Griffiths, 2010). And, the production of EPA could be improved to 16-22 % when high copy-number plasmid carrying the EPA gene cluster was transformed into *E. coli* cell (Orikasa et al., 2004). However, the EPA/DHA recombinant does not behave like a typical *E. coli* cell, such as growth occurs around 0.2 M NaCl and restricted to about 12–22 °C (Valentine and Valentine, 2004).

EPA gene clusters (*pfaA*, *pfaB*, *pfaC* and *pfaD* with *pfaE* connected or separated) from *Vibrio*, *Photobacterium* and *Shewanella* in this study, along with those published data from EPA/DHA derived prokaryotic and eukaryotic species, such as *Shewanella pneumatophori*, *Photobacterium profundum* and *Pseudoalteromonas* sp., *Moritella* marina and *Schizochytrium*, which demonstrated that EPA or DHA polyketide biosynthesis gene clusters from different genera showed a high degree of gene sequence similarity. The result also provided evidence of the common distribution of the novel PUFA synthease pathways among marine microorganisms regardless of their biogeographic variability, which has recently been further testified by the investigation of genetic capacity for production of long-chain fatty acids using a culture-independent approach (Shulse and Allen, 2011). The gene *pfaE* plays important role in EPA/DHA biosynthesis (Rahman et al., 2005), but it is still unclear why *pfaE* shift its position, as observed in this study, in the genomic DNAs of different strains/species. Furthermore, it is still speculated that the final PUFA product in the system may contributed by some cooperative interactions between domains of different Pfa proteins, rather than depending on the activity of any single Pfa protein. Therefore, the study suggests the possible involvement of gene transfer in the acquisition of the *pfa* gene clusters among different strain/species in the marine environment, although no flanking genes possessing functions which could facilitate horizontal transfer have been observed, so far.

10.1.4 Bio-electrochemically study power generation by bacteria

A shortage of oxygen easily occurs in deep-sea environments where reduced sulfur compounds or other metals are supplied constantly as final electron acceptor for microbes in their respiratory pathway (Woulds *et al.*, 2007). Anaerobic microbes inhabiting such anoxic subsurface are able to use organic matter and eventually liberate electrons as a form energy (Lovley, 2008). Effective anaerobic oxidation of complex assemblages of organic matter, such as those found in most wastes and biomass, requires the fermentation products from the metabolism of sugars, amino acids and related compounds, in addition to other constituents, such as aromatic compounds and long-chain fatty acids, to be oxidized with electron transfer to an electron acceptor (Lovley, 2006). Based on these principles, the devices, microbial fuel cells (MFCs) have been set up by employing bacterial metabolism to produce an electrical current

from various organic substrates. Several studies found that MFCs contained diverse microbial communities, which was unexpected given the apparent need for cells to be able to respire using an electrode (Logan and Regan, 2006).

In this thesis, bacteria from MAR deep-sea sediments, such as strain MAR441, could utilize peptone and deliver a stable power output of ~75-100 mW/m^2, which is competitive comparing to that from strain *Shewanella oneidensis* MR-1 (Watson and Logan, 2010). However, under such anaerobic conditions, high content of middle-chain fatty acids (e.g. n-16:1ω7 and n-18:1ω7c), but less long-chain fatty acids, such as EPA, were observed from the MAR441. This is probably due to the activation of oxygen-independent (anaerobic) pathway catalysed by a fatty acid synthetase, which contribute more middle-chain fatty acids production (Yano *et al.*, 1998), or polyunsaturated fatty acids play its cell membrane protection role as an antioxidant (Nishida et al., 2006), and therefore decreased the level of EPA.

The organic matter stored in anoxic subsurface environments and aquatic sediments represents a large potential source of energy (Bond et al., 2002). Thus, in this study, MFC was set up by adding estuarine sediment directly with acetate as electron donor, capable of producing electricity up to ~175 mW/m^2, which is the highest level reported so far among the sediment MFCs. Various levels of electricity were observed by other sediment MFCs, such as marine sediment MFCs in situ with power output of 10-230 mW/m^2 (Reimers et al., 2001; Tender et al., 2002; Lowy et al., 2006; Nielsen et al., 2007; Dumas et al., 2008; Nielsen et al., 2008; Tender et al., 2008), marine sediment MFC in laboratory with electricity production of 40-70 mW/m^2 (Logan et al., 2005; Zhang et al., 2006b). Furthermore, the bacterial consortia MFC showed higher power densities than those obtained using single strains isolated from the original biofilm. The observation supports the suggestion that biofilm communities may produce greater power densities than individual strains (Logan, 2009), as it has been proved by the fact that two species were better than one in a cellulose-fed fuel cell (Ren et al., 2007). In addition, the maximum power output could be further increased to ~200 mW/m^2 when an artificial consortium consisting of twenty-five preselected exoelectrogenic isolates was used. Non-exoelectrogenic bacteria were detected in the original biofilms, and these have been suggested to play a role as helper strains for fermenting organic matter (Ren et al., 2007). The study demonstrated the highest level of power output from estuarine

sediment in MFC, and its performance could be increased by combining different exoelectrogenic and/or non-exoelectrogenic bacteria as artificial biofilms.

10.2 Conclusions

Overall, this study helps better understand the structure of culturable bacterial community in the little studied deep-sea sediments, marine plants, and estuarine sediments, based on molecular identification and fatty acid composition; biotechnologically, provides potential bacterial candidates for industrial production of EPA, and brings it closer to the next step of commercialization on marine biomass; molecularly, elucidates the microbial EPA/DHA biosynthesis via the PKS-like pathway; and bio-electrochemically reveals the role of PUFA under anaerobic conditions and demonstrates the potential for increased performance of microbial fuel cells in the future.

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Appendices

Appendix A: Chemicals, media, kits and enzymes used in this study

A1 Organic solvents

VWR (Leicestershire, UK)

- 2 Propanol (1 L)
- 4 methoxy benzaldehyde (200 ml)
- Acetic acid (250 ml)
- Acetic acid glacial (100 ml)
- Acetone (HPLC gradient grade) (1L)
- Butylated hydroxytoluene - Solution 1 % (in Ethanol)
- Butylhydroxytoluol (100 ml)
- Chloroform (250 ml)
- Diethyl ether
- Dimethylformamide(1 L)
- Ethylacetate (2.5 L)
- Hydrogen chloride (2.5 l)
- Hydrogen peroxide (250 ml)
- Isoamyl alcohol (1 L)
- Methanol (HPLC gradient grade) (1.5 L)
- Methylated spriti (2 L)
- n-Hexane (HPLC gradient grade) (2.5 L)
- Orthophosphoric acid (500 ml)
- Phenol (0.5 L)
- Triethyl amine (500 ml)
- Triphenyl tetrazolium chloride (100 ml)
- Vanadomolybdate reagent(1 L)

A2 Media

BD Becton Dickinson UK Limited

- BD Difco™ Marine Broth 2216

BD Difco™ Marine Agar 2216

Nutrient broth

Difco™ LB Broth, Miller

Difco

Bacto®tryptone

Bacto®yeast extract

Oxoid

Bacteriological Agar No.1

A3 Chemicals

Fisher Scientific Inc., UK

Glycerol

Tween 80

Tween 60

Tween 40

Fermentas, UK

X-Gal Solution, ready-to-use 10 ml (20mg/ml)

Sigma

Ethidium bromide (10ml)

Tokyo Kasei Kogyo Co., Ltd.

N-Methyl-N'-nitro-N-nitrosoguanidine

VWR (Leicestershire, UK)

D(+)-Glucose GPR RECTAPUR® anhydrous
Bactopeptone
Meat extract
Starch soluble
Caesin hydrolysate
D-(+)-Maltose monohydrate
L(+)-Glutamic Acid
Calcium Chloride monohydrate
Disodium fumarate
Ferric citrate
Iron (iii) chloride
Iron (iii) phosphate hydrate
Lithium acetate dihydrate
Magnesium acetate

3,5 – Dinitrosalicylic acid
L-Leucine
L-Alanine
L-Proline
L-Serine
Iodine indicator
CTAB - Lysis buffer BioChemica, 2% (w/v)
EDTA trisodium salt 0.1 mol/l (0.3 N)
N,N,N',N,-Tetramethylethylene diamine (TEMED)
Isopropyl-β-D-thiogalactoside (IPTG)
Dithiothrietol (DTT)
Ethelene diamine tetra-aceticacid, disodium salt (EDTA)
Polyvinylpyrrolidone (K15) BioChemica
Ammonium Acetate HiPerSolv for HPLC
Guanidine hydrochloride - Solution (8 M) BioChemica
Triphenyltetrazolium chloride (TTC)
AccuGENE® 10X PBS, 1 L
Agarose DNA grade Electran® BDA
Coomassie Brilliant Blue G
M9 Minimal salts
Urea
Ammonium chloride
Calcium chloride
Magnesium Chloride Hexahydrate
Magnesium sulphate anhydrous
Monopotassium phosphate
Potassium acetate
Potassium chloride
Potassium chloride
Potassium dihydrogen phosphate
Potassium hydroxide
Potassium ferricyanide
Potassium nitrate
Sodium acetate
Sodium chloride
Sodium hydroxide
Sodium nitrate
Sodium phosphate
Sodium phosphate monobasic
Sodium succinate
Sodium sulphate
Zinc chloride
β-Mercaptoethanol
Streptomycin sulfate
Kanamycin sulfate
Ampicillin

A4 Enzymes

Cambio Ltd. UK

Plasmid safe DNase

Fermentas, UK

Proteinase K 1 ml (20mg/ml)

RNase T1 100000 units (1000u/µl)

T4 DNA Ligase (supplied with PEG) 1000u (5u/µl)

T4 Polynucleotide Kinase (T4 PNK) 500u (10u/µl)

Calf Intestine Alkaline Phosphatase (CIAP) 5x200u (1u/µl)

Lambda DNA/EcoRI+HindIII Marker

Lambda Mix, 19

DNA restriction endonucleases

HotStart DNA polymerase

PfuTurbo DNA polymerase

A5 Kits

bioMérieux UK

API 20 NE test strip

API 20E test strip

Cambio Ltd. UK

CopyControl™ HTP Fosmid Library Production Kit

CopyControl™ Induction Solution 400X concentrated solution

FosmidMAX DNA Purification kit

Fermentas, UK

PCR Master Mix (2X)

GeneJET(tm) PCR Purification Kit, #K0701

GeneJET(tm) PCR Purification Kit

GeneJET™ Plasmid Miniprep Kit

Rapid DNA Ligation & Transformation Kit

Invitrogen Ltd UK

PureLink™ PCR Purification Kits

PureLink™ Genomic DNA Mini Kit

PureLink™ Genomic Plant DNA Purification Kit

Qiagen

Plasmid Mini kit

Plasmid Midi kit

Qiaquik Gel Extraction kit

Qiaquik PCR Purification kit

Sigma, Aldrich

GenElute™ Bacterial Genomic DNA Kit

Appendix B: Hardware/Equipment used in this Study

Agilent Technologies Co., Ltd., Palo Alto , USA

Hewlett-Packard model 7890A gas chromatograph (Varian CP-3800)

Agilent 5975 GC/MS

HP-5ms Capillary GC-MS Column (19091S-433, 30 m x 0.25 mm, 0.25 μm),

DB225 capillary column (BPX70, 10 m x 0.1 mm, 0.2 μm)

Becton Dickinson, Maryland

Anaerobic container system (GasPak™ EZ)

Biomedical EM Unit, Newcastle University

Philips CM100 transmission electron microscopy

Scanning electron microscopy (Cambridge Stereoscan 240)

Confocal microscope (Leica INM100)

BOC Gases,UK

Standard Cylinder (W Size) of Oxygen

Cylinder Oxygen Regulator (0-10 bar)

Corning Inc.

Dual chamber glass

Eppendorf, Wesseling-Berzdorf, Germany

Mastercycler ep gradient thermal cycler

Mini Spin Eppendorf

Eco Chemie, Utrecht, Netherlands

Autolab PGSTAT302 potentiostat

Dupont Co., Wilmington, USA

Proton exchange membrane Nafion^{R} 117

Freudenberg FCCT KG, Germany

H2315 carbon cloth

Grant Instruments

Grant Water Bath SBB6

GasPak™ EZ, BD, Maryland

2.5 L anaerobic container system

Anaerobic GasPaks (Oxoid)

Invitrogen Ltd UK

Xcell SureLock™ Mini Cell

LEEC Limited (Laboratory Equipment & Mortuary Equipment)

LEEC K2 Compact Oven

Compact Microbiological Incubators

MARTIN CHRIST Gefriertrocknungsanlagen GmbH

Freeze Dryers (Alpha 1-4LD)

New Brunswicbk Scientific (UK) Ltd

BioFlo 3000 Fermentation system. 3.3L (F1001-1)

Water regulator kit (4 Mani folds)

Oxford, OX, United Kingdom

G Series Analytical and Semi Micro (G21002 and F31002)

Sigma, Aldrich

Vials, screw-top V-Vials® with solid-top cap capacity 1.0 Ml

(screw-cap size 13-425)

Sigma Laboratory Centrifuge 2-16P

Scientific Laboratory Supplies Limited, UK

Standard Vertical Gel Unit 20x20cm

Angle Rotor 6x50ml Falcon Tubes

Silca gel (TLC Plates)

Merck silica gel 60

SkyTronic UK

Digital multimeter

(SLS) Scientific Laboratory Supplies Ltd

Clifton Blockheater Thermubloc Analogue

SCHOTT UK Ltd

Dual-chamber vials

Varian Ltd, UK

VF-WAXms FS 30x.25 (.5) (CP9222)