CHAPTER 4

GENERAL DISCUSSION
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4.1 BIODIVERSITY OF CULTURABLE BACTERIA FROM CYANOBACTERIAL MAT SAMPLES FROM MCMURDO, ANTARCTICA

Identification and characterization of psychrophilic microorganisms such as bacteria and yeasts from the continent of Antarctica has progressed relatively slowly compared to microorganisms from other continents due to various problems such as: difficulty in reviving them from their natural habitats, difficulty in transportation of the samples to the place of work before the bacteria lose their viability, inability to culture and maintain the microorganisms under laboratory conditions and their slow growth rate.

As of now, only 63 novel species of Gram-negative bacteria belonging to 34 different genera and 34 novel species of Gram-positive bacteria belonging to 16 different genera (Tables 1.4 and 1.5) have been identified from various habitats of Antarctica. Therefore, there exists a need to study the bacterial diversity of Antarctica.

In the present study, a polyphasic approach (Colwell, 1970) was used for characterization of psychrophilic bacteria up to the species level. This approach is a holistic approach in which species is defined as a group of strains sharing 70% or greater DNA-DNA relatedness, 5°C or less difference in the melting temperature of DNA (Stackebrandt and Goebel, 1994; Wayne et al., 1987) and possess similar phenotypic and chemotaxonomic features. Using this polyphasic approach, 76 bacteria which were isolated from 12 different cyanobacterial mat samples collected from various ponds and lakes of McMurdo region in Antarctica were identified up to the species level. The results led to the identification of 8 new species of Gram-positive bacteria [Kocuria polaris (CMS 76or), Arthrobacter flavus (CMS 19y), Arthrobacter roseus (CMS 90r), Planococcus
antarcticus (CMS 26or), Planomicrobium psychrophilus (CMS 53or), Leifsonia rubea (CMS 76r), Leifsonia aureus (CMS 81y), Sporosarcina mcmurdo (CMS 21w)] and 4 new species of Gram-negative bacteria [Psychrobacter psychrophilus (CMS 30), P. Psychrobacter vallis (CMS 39), Psychrobacter aquaticus (CMS 56) and Pseudomonas polaris (CMS 38)] that belonged to 2 different genera (Fig. 4.1). This study also reports for the first time species belonging to the genera Leifsonia, Sporosarcina and Kocuria from Antarctica. It is interesting to note that all the Antarctic isolates of bacteria were psychrophilic and could be differentiated from the closely related species of the genera based on their phenotypic and chemotaxonomic characteristics and also at the 16S rDNA sequence level.

Bacterial biomass is known to vary depending on the nature of the habitat with respect to its nutrient and mineral content, pH, dissolved oxygen and organic carbon content. The lakes and ponds of McMurdo region, which were sampled, varied with respect to the concentration of salts (ranged from 320 to 3200 mg/L water), pH (ranged from 5.5 to 10.8), dissolved oxygen (ranged from 5.5 to 35 ml/L water), inorganic nutrients such as nitrate, nitrite, phosphate and ammonia, total organic carbon (TOC) and dissolved organic carbon (DOC) which ranged from 0.14 to 110 mg/L of water (Matsumoto, 1993; Matsumoto and Hanya, 1977; Matsumoto et al., 1989, 1993; Parker et al., 1974, 1977, 1978, 1985).

In the present study, Antarctic bacterial medium (ABM), a low nutrient medium (contains 0.5% peptone and 0.2% yeast extract), was used to ascertain the number of bacteria per gram of each of the 12 cyanobacterial mat samples collected from the ponds and lakes of McMurdo region, Antarctica. The culturabale bacterial number (colony
Fig. 4.1 Phylogenetic relationship between the 14 bacterial species from Antarctica isolated in the present study with the closely related species based on 16S rDNA sequence analysis using DNAPARS
forming units) on ABM medium at 4°C ranged from $10^3$ to $10^7$ per gram mat sample. This number is comparable to the values, reported earlier by epifluorescence microscopic studies for Antarctic lake and pond sediments, glacial ice, oceanic water and soil samples (Abyzove et al., 2001; Bruni et al, 1999; Kochikina et al, 2001; Urzi et al, 2001) and by colony counts for culturable bacteria from soils and lakes of Schirmacher oasis (Shivaji et al, 1988, 1989a, 1989b, 1991 and 1992). The results indicated that the variation in the number may depend on the nature of the habitat. However, no correlation could be established between the nutrient content of the pond / lake or the type of cyanobacteria associated with the mat sample, although it was interesting to note that the type of bacteria associated with a mat sample varied and this implied intrinsic differences in the mats in addition to the differences in the habitat i.e., the lakes and ponds. In the present study, only a few bacterial colonies from each mat sample which differed with respect to colony morphology were selected for detailed taxonomic studies. Therefore, it is very likely that though bacteria belonging to only one or two types of genera are reported from each cyanobacterial mat sample, the presence of bacteria belonging to other genera cannot be ruled out. Further, it is well documented that certain bacteria are viable but non-culturable (VBNC) (Chattopadhyay, 2000; Jiang and Chai, 1996; Oliver et al., 1991; Weichart et al., 1992) and this could be the reason for the low bacterial count in some samples.

All the 76 bacteria that were isolated from the 12 cyanobacterial mat samples were psychrophilic in nature with an optimum growth temperature of 20 - 22°C. The optimum pH for the growth for all the bacteria was observed to be 7.2. None of the bacteria could grow below pH 5 and this could be due to their adaptation to the alkaline pH of the ponds / lakes that range from pH 5.5 to 10.8 (Matsumoto, 1993). Further, all the bacteria could tolerate an average salt concentration of 3% with the exception of
bacteria belonging to *Planococcus* and *Psychrobacter* which could tolerate up to 10% of NaCl.

All the Gram-negative bacteria were found to be non-pigmented whereas all the Gram-positive bacteria were pigmented with the exception of bacteria belonging to the genus *Sporosarcina*. These results are in accordance with our earlier observations which indicated a preponderance of pigmented bacteria in Antarctic habitats (Shivaji et al., 1988, 1989a, b, 1991, 1992). Subsequent studies also demonstrated that pigments may be playing a role in adaptation of bacteria to cold (Chattopadhyay et al., 1997; Chauhan and Shivaji, 1994; Jaganadham et al., 1991, 1996a, 1996b, 2000). The seventy-six isolates could be grouped into 17 groups on the basis of phenotypic characteristics, RAPD or SDS-PAGE analysis, of which 10 were Gram-positive bacteria (CMS 19y, CMS 90r, CMS 3or, CMS 26or, CMS 84or, CMS 53or, CMS 76or, CMS 76r, CMS 81y, CMS 21w) and 7 were Gram-negative bacteria (CMS 30, CMS 39, CMS 56, CMS 35, CMS 38, CMS 45 and CMS 64) and together they represented 12 new species (*Kocuria polaris*, *Arthrobacter flavus*, *Arthrobacter roseus*, *Planococcus antarcticus*, *Planomicrobiium psychrophilus*, *Leifsonia rubea*, *Leifsonia aureus*, *Sporosarcina mcmurdo*, *Psychrobacter psychrophilus*, *Psychrobacter vallis*, *Psychrobacter aquaticus* and *Pseudomonas polaris*) and 2 new isolates of the species of genus *Pseudomonas* (*Pseudomonas orientalis* and *Pseudomonas brennerii*) which were already described (Fig. 4.1). Thus, Antarctica appears to harbor a number of new species and sampling the hitherto untouched habitats would result in the identification of many more new species.

All the bacterial isolates were aerobic and catalase positive, but varied with respect to the activity of various enzymes, utilization of carbon source, sensitivity to antibiotics, and secretion of extra-cellular enzymes (such as proteases, lipases,
amyloses and phosphatases) clearly indicating a great degree of diversity. This is further evidenced by the fact that they exhibit chemotaxonomic differences with respect to the type of menaquinone (ranging from MK-6 to MK-11), phospholipid (PI, PE, PG and/or DPG), nature of the carotenoid pigment, fatty acid composition and type of peptidoglycan present.

The 16S rDNA sequence analysis indicated that the distance between the new species identified in this study to their nearest reported species ranged from 0.1 to 5% in case of Gram-positive bacteria and 0.05 to less than 2.5% in case of Gram-negative bacteria. Dissimilarity of less than 2.5% among Gram-negative bacteria is expected, as the number of nucleotides conserved in 16S rDNA sequence of Gram-negative bacteria (713 out of 1542 nucleotides) is more when compared to Gram-positve bacteria, where only 568 nucleotides out of 1542 are conserved (Fig. 4.1) (Ludwig et al, 1998). Therefore, in resolving a species status based on 16S rDNA sequence homology, it may not always be accurate to set > 2.5% difference as a bench-mark difference for species status (Stackebrandt and Geobel, 1994). In resolving the taxonomic status of species which had less than 2% difference at the 16S rDNA sequence level with the already reported species such as species of the genera *Kocuria* (CMS 76or), *Arthrobacter* (CMS 19y,CMS 90r), *Planococcus* (CMS 26or), *Planomicrobium* (CMS 53or), *Psychrobacter* (CMS 30, CMS 39 and CMS 56) and *Pseudomonas* (CMS 38), phenotypic and chemotaxonomic characteristics were compared along with the DNA-DNA hybridization (Stackebrandt and Geobel, 1994) to establish the species identity. Further, the phylogenetic position of the isolate was established using distanced based methods such as UPGMA, KITCH and FITCH and sequence based method such as DNAPARS of PHYLIP program.
Survival of extremophiles is dependent on the ability of these microorganisms to produce enzymes which can catalyse biological reactions under conditions under which they survive such as extremes of temperature, pH, salt concentration, etc. Such enzymes are known as extremozymes and there is a need to characterize them. Extremophiles are normally the producers of such enzymes and therefore, screening for extremozymes in extremophiles would add to the current database on extremozymes and help in deciphering the mystery (Russell, 2000; Sheridan et al., 2000; Tsigos et al., 2001) behind their activity. Psychrophilic microorganisms produce cold-adapted enzymes which show high catalytic activity at low temperatures and are normally cold-active and heat-labile. Such enzymes have been used as additives to detergents used at low temperatures, as biocatalysts for biotransformation of volatile or labile compounds at cold temperatures, and in the baking and brewery industry.

The present work is focused on classifying the psychrophilic bacteria isolated from cyanobacterial mat samples of McMurdo, Antarctica into physiological groups on the basis of 4 enzymes namely, amylases, lipases, proteases and phosphatases. The 76 bacteria could be classified into 11 physiological groups (Table 3.34). Out of the 11 groups, four groups secreted more than one enzyme as indicated below:
4.2.1 Characterization of Amylases from Antarctic Psychrophilic Bacteria

Amylase is an enzyme that hydrolyzes starch and exists in two forms, α-amylase and β-amylase. α-amylase is an endo-glycosidase and hydrolyzes the internal α-1,4 linkage to yield maltose, maltotriose and α-dextrin where as β-amylase hydrolyzes starch into maltose by sequential cleaving of the disaccharide units from the non-reducing end of the starch. Though quite a number of α-amylases have been purified and characterized from different organisms including mesophilic and thermophilic bacteria, not much is known about amylases from psychrophilic bacteria. Demot and Verachtert (1987) were the first to purify an extracellular α-amylase and a β-glucoamylase from an Antarctic yeast, Candida antarctica. Subsequently, α-amylase was purified from Alteromonas species (Chessa et al., 1999; Feller et al., 1992, 1994, 1996, 1998a, 1998b, 1999) and a chloride dependent α-amylase from Alteromonas haloplanctis was characterized with respect to its thermal stability, structural determinants and its active site which was mapped to a triad of amino acid residues made up of Aspartic acid 174, Glutamic acid 200 and Aspartic acid 264 (Aghajari et al. 1996, 1998a, 1998b, 2002; D'Amico et al. 2000, 2001). However, no attempt was made, thereafter, to characterize the amylases from other species of Antarctic bacteria. In the present study, an attempt was made to screen for the presence of amylases in psychrophilic bacteria from cyanobacterial mat samples from McMurdo region in Antarctica.
The amylase of CMS 21w and CMS 76or is cold-active and exhibited activity in the temperature range of 4°C to 30°C. The pH optimum was around 6 to 7 and salt had no effect on the activity. The present enzyme is unique in that it could be heat inactivated at 50°C, which is close to the optimum activity reported for one of the amylases from Antarctic yeast (Demot and Verachtert, 1987).

4.2.2 Characterization of Lipases from Antarctic Psychrophilic Bacteria

Lipases catalyze the hydrolysis of acylglycerides and other fatty acid esters. They resemble esterases, but differ markedly from them in their ability to act on water-insoluble esters (Brockerhoff and Jensen, 1974). Lipases and esterases have been recognized as very useful biocatalysts because of their wide-ranging versatility in industrial applications. A variety of microbial lipases with different enzymological properties and substrate specificities have been found (Jaeger et al., 1994). The temperature stability of lipases has been regarded as the most important characteristic for use in industry (Herbert, 1992).

Recently, the genes of cold-adapted lipases from psychrophilic bacteria *Moraxella* TA144 (Feller et al., 1991) and *Psychrobacter immobile* B10 (Arpigny et al., 1993) isolated in Antarctica were cloned and sequenced. The enzymes showed high activities at temperatures as low as 3°C. None of these recombinant enzymes, however, have been purified to homogeneity due to strong interaction of the enzymes with lipopolysaccharides secreted by the bacterial cells (Arpigny et al., 1993). Choo et al., (1998) have also isolated cold-adapted microorganisms producing cold-adapted lipases of molecular weight 33714 da (contained 308 aa residues and 924 bp gene sequence)
from Alaskan and Siberian soils and cloned the lipase gene, lipP, from an Alaskan psychrotroph, *Pseudomonas* sp. strain B11-1. A 29 kDa lipase was purified from *Aspergillus nidulans* by Mayordomo et al (2000) and the optimum temperature and pH for the activity was found to be 40°C and 6.5, respectively and high activity was also seen from 0 to 20°C.

In the present study, cold-active lipase activity was detected in *Kocuria polaris* (CMS 76or), in species of *Psychrobacter* (CMS 30, CMS 39 and CMS 56) and *Pseudomonas* (CMS 64). The enzyme from all the isolates exhibited activity between 4°C to 30°C, pH 6 – 11 and salt did not have any effect and the enzyme could be heat-inactivated at 65°C. Earlier studies have also indicated the presence of cold adapted lipases in the genus *Psychrobacter* (Feller et al, 1991) and *Pseudomonas* (Choo et al, 1998).

4.2.3 Characterization of Proteases from Antarctic Psychrophilic Bacteria

Proteases are enzymes that hydrolyze the peptide bond and have wide application in industry such as in cheese manufacturing, baking, meat tenderization, brewing, detergents industry (Cavichioli and Thomas, 2000) etc. (Table 1.2). Proteases are also known to exhibit a wide range of diversity and therefore, it is required to characterize as many proteases as possible to understand their function and to exploit them for industrial use. Though, quite a number of proteases have been characterized from various sources, very little is known about the cold adapted proteases. So far the cold-active proteases have been characterized from 11 isolates (Adams et al., 1975; Birk et al., 1983; Chessa et al. 2000; Irwin et al. 2001; Kristjansson et al. 1999; Kulakova et al. 1999; Morita et al. 1998; Oh et al. 1999; Ray et al. 1992; Secades et al. 2001;
purified and characterized an 85113 kDa serine alkaline protease from *Shewanella* strain Ac10 and mapped the active sites to Asp-30, His-65 and Ser-369 residues. Out of the 11 known cold-active enzymes, only three have been purified from Antarctic organisms (Chessa *et al*., 2000; Ray *et al*., 1992; Villeret, 1997).

In the present study, an attempt was made to characterize the protease from Antarctic bacteria. Cold-active and heat-labile proteases were characterized in two species of the genus *Planococcus* (CMS 26 or, CMS 53 or), two species of the genus *Arthrobacter* (CMS 19 y and CMS 90) and one species each of *Sporosarcina* (CMS 21 w) and *Pseudomonas* sp (CMS 64). The enzyme from all the isolates showed activity between 4-30°C, pH 6 – 11 and did not depend on the salt concentration. The protease identified was highly heat-labile and could be heat-inactivated at above 45°C. The heat-inactivation studies also indicated that the protease from the Antarctic isolates of present study is different from the earlier reported proteases, since the present enzyme gets heat-inactivated below 50°C. Though, the nature of protease is not know, it appears from the pH studies that it is a neutral protease.

**4.2.4 Characterization of Phosphatases from Antarctic Psychrophilic Bacteria**

Phosphatase is an enzyme that hydrolyzes phosphodiester bonds and is involved in triggering biological reactions, mineralization of phosphate and degradation of DNA and RNA molecules. The extracellular phosphatases secreted by bacteria play an important role in degradation and mineralization of phosphate. Though extracellular phosphatases have been purified from various sources including bacteria, only few enzymes have been purified from psychrophilic bacteria. The phosphatases so far
purified from psychrophilic bacteria (Chattopadhyay et al., 1995; DeBacker et al., 2002; Hauksson et al., 2000; Hoffmann and Jendrisak, 1990; Kobori et al., 1984; Mavromatis et al., 2002; Murakawa et al., 2002; Rina et al., 2000; Tsuruta et al., 1998), were observed to be cold-active and heat-labile.

Maximum production of phosphatase was found in species of the genus Psychrobacter (CMS 30, CMS 39 and CMS 54) and were observed to be cold-active, active between 4°C to 30°C, and exhibited activity in the pH range of 5 to 11, indicating that it could be a neutral phosphatase. The heat-inactivation studies indicated that the enzyme is either heat-resistant or the heat-inactivation process is reversible. Thus, the enzyme appears to be unique, since the phosphatases purified so far from other psychrophilic enzymes could be irreversibly inactivated above 65°C (Chattopadhyay et al., 1995; Hoffmann and Jendrisak, 1990; Kobori et al., 1984; Rina et al., 2000; Tsuruta et al., 1998). To our knowledge, this is the first characterized enzyme from a psychrophile which is cold-active and heat-resistant.

4.3 BIOTECHNOLOGICAL POTENTIAL OF ANTARCTIC ISOLATES

Microorganisms and their products have potential applications in a broad range of industrial, agricultural and medical processes. Recently, Cavicchioli et al. (2002) and Margesin and Schinner (1994) reviewed the applications of psychrophilic microorganisms (Table 1.2) in industry and highlighted that microbial derived products such as their unique lipids, fatty acids, pigments and enzymes find a prominent application in the biotechnology industry. In the present study, the specific product and its application have not been evaluated but attempts have been made to theoretically project the possible applications of the biochemical products produced by these unique psychrophiles (Table 3.37).
4.3.1 Significance of the Genus *Kocuria*

Species of *Kocuria* are known to have significant economic value. Basaglia *et al.* (2002) implicated *K. kristinae* as the causative agent of recurrent bacteremia in human beings. Mahony *et al.* (2001) isolated an antibiotic “variacin” from *K. varians* and used it in controlling the growth of psychrophilic *B. cereus* on chilled dairy foods. Wenzel *et al.* (2002) have isolated cellulolytic bacteria belonging to genus *Kocuria* from the mid gut of the termite *Zootermopsis angusticollis*. In the present study, one isolate, which was identified as *K. polaris* (CMS 76or) produces four different types of menaquinones (MK-6(H$_2$), MK-7 (H$_2$), MK-8(H$_2$) and MK-9(H$_2$) and thus could serve as a model organism for characterization of menaquinones. It could also be used as a source of anteiso-C$_{15:0}$ which constitutes 70% of the total fatty acids. Apart from this, the bacterium produces cold-active and heat-labile amylase and lipase that have wide applications in industry (Table 1.3).

4.3.2 Significance of the Genus *Arthrobacter*

Species of the genus *Arthrobacter* are ubiquitous in their distribution and have significant economic value (Boldt *et al.*, 1997; Degtyarev *et al.*, 1992; Dubinina and Zhdanov, 1975; Ensign and Rittenberg, 1963; Ferdinandus and Clark, 1969; Gasdorf *et al.*, 1965; Graf *et al.*, 1997; Gurr and Jones, 1977; Hiyama and Okada, 1975; Hoe *et al.*, 1998; Ikuta *et al.*, 1977; Juda *et al.*, 2001; Levinson *et al.*, 1975; Lochhead, 1958; Oguma *et al.*, 1999; Phinny and Hoober, 1992; Suhayada *et al.*, 1995; Tanizawa *et al.*, 1994; Tate and Ensign, 1974; Torii *et al.*, 1976; Turnbull *et al.*, 2001; Wiese *et al.*, 2000; 2001; Yoshinaka *et al.*, 1973) since they produce a variety of enzymes of commercial value such as the hydantoin cleaving enzymes (Ragnitz *et al.*, 2001, levan fructotransferase (Lee *et al.*, 2001), acid urease (Miyagawa *et al.*, 1999), dimethylsulfone and dimethylsulfoxide reductases (Borodina *et al.*, 2002) and 2,6-dihydroxypyridine 3-
hydroxylase (Baitsch et al., 2001). Secondary metabolites were not characterized in the present thesis from the psychrophilic species of A. flavus (CMS 19y) and A. roseus (CMS 90r) but it was observed that the extra-cellular protease was cold-active and heat-labile and could thus find application either in the detergent industry or the brewery. Further, the carotenoid pigments could be used as antioxidants and in food industry.

4.3.3 Significance of the Genus Planococcus

Species of the genus Planococcus have been shown to have a potential use in biodegradation and molecular biology. Planococcus alkanolacticus was shown to have a capability to degrade linear and branched alkanes (Engelhardt et al., 2001). A restriction endonuclease, FokI, was purified from Planomicrobium okeanokoites by Sugisaki and Kanazawa (1981) and Sheridan and Brenchley (2000) have characterized a halotolerant β-galactosidase from a psychrophilic antarctic Planococcus sp. In the present study, it was observed that both Planococcus antarcticus and Planococcus psychrophiles could produce extracellular proteases which were cold-active and heat-labile (Table 1.2). The orange pigment produced by Planococcus antarcticus and Planococcus psychrophiles could find use in textile and food industry.

4.3.4 Significance of the Genus Leifsonia

Earlier it was demonstrated that two species of the genus Leifsonia namely L. poae isolated from the plant Poa annua (Evtushenko et al., 2000) and L. xyli isolated from sugar cane and the barmuda grass Cynodon dactylon (Davis et al., 1984) existed in symbiotic association with the host plant as in the case of L. poae or as a phytopathogen as in the case of L. xyli (Davis et al., 1984). In the present study, the two new species namely L. rubea and L. aureus were also in close association with cyanobacterium, Phormidium spp., therefore it is worthwhile speculating that they may be symbiotic or
CONCLUSIONS

1. From a total of 76 isolates of psychrophilic bacteria, 12 new species were identified.

2. CMS 76or, an orange pigmented Gram-positive bacterium was identified as Kocuria polaris.

3. CMS 19y and CMS 90r, an yellow and a red pigmented Gram-positive bacteria, exhibiting rod-coccus type of growth cycle were identified as Arthrobacter flavus (CMS 19y) and Arthrobacter roseus (CMS 90r), respectively.

4. CMS 26or, an orange-pigmented Gram-positive, motile, coccoid bacterium was identified as Planococcus antarcticus and another orange pigmented, rod shaped and motile bacterium, CMS 53or, was identified as Planomicrobium psychrophilus.

5. CMS 76r and CMS 81y, a red pigmented and a yellow pigmented, Gram-positive, motile and curved rods were identified as Leifsonia rubea and Leifsonia aureus, respectively.

6. CMS 21w, a rod shaped, motile and sporulating bacterium was identified as Sporosarcina mcmurdo.

7. CMS 30, CMS 39 and CMS 56, Gram-negative, non-motile, coccoid bacteria were identified as Psychrobacter psychrophilus, Psychrobacter vallis and Psychrobacter aquaticus, respectively.
8. CMS 38, a Gram-negative, motile and rod-shaped bacterium was identified as *Pseudomonas polaris*, two isolates CMS 35 and CMS 45 were identified as new isolates of the species *Pseudomonas orientalis* and CMS 64 as a new isolate of the species *Pseudomonas brennerii*.

9. Based on the activity of the enzymes amylase, lipase, protease and phosphatase, all the bacteria were divided into 11 physiological groups.

10. The biotechnological potential of the new species with respect to the enzymes produced and other biological products such as pigments, fatty acids, and menaquinones was evaluated.