General Introduction

Bacteria, the simple living entities, are ubiquitous and more abundant than any other life form in the entire biosphere. It is because of their diversity, rapid reproducibility, ratio of surface area to weight which is high and adaptability in any environmental niche. In contrast to the eukaryotic system, in general, the bacterial cells are small, anucleate and rely solely on diffusion for their livelihood (Beveridge, 1989). They are completely exposed to the components of the biosphere. With the simpler physiology they efficiently respond to the changes of any of these components through genetically programmed processes. This is necessary because they cannot selectively reach to their essential nutrient component nor can they fling away a toxic component, or cannot outswim their local aqueous environment to a totally distinct environment (Beveridge, 1988). The extreme heterogeneity of nutrient components present in a given environment supports the microbial world, for the sustenance of diverse populations which enrich and colonize in their favourable sites and further modify and develop specialized ecological niches. Hence, the meaning of the word "diversity" is not really unknown. Biodiversity is an attribute of an area and specifically refers to the varieties within and among living organisms, assemblage of the living organisms, biotic communities and biotic processes, whether naturally occurring or modified by humans. The vast majority of microbial diversity (>95%) remains to be discerned. Very little is known about microbial species and functional diversity, and decisions about the role of microorganisms and their influence on sustainable ecosystems are being made on the basis of very incomplete information.

Based on rRNA trees, it may be said that the main extents of Earth's biodiversity is microbial (Hugenholtz *et al.*, 1998). Our perspective on microbial diversity has improved enormously over the past few decades. The microbial diversity is somewhat different from the macro-organisms. Establishing the diversity of microorganisms present by relying on morphological features alone is not possible; instead, specific physiological capabilities also need to be deciphered in order to establish the different types of microbes present. The specific biochemical and physiological activities of microbial cells are of profound significance for the habitat. The enormous success of enrichment culture and related isolation approaches and considerations (van Neil, 1995), along with applications of new analytical techniques (Amann *et al.*, 1995;

Hugenholtz *et al.*, 1998) has enabled us to see that we understand very little about so many of the presumptive microbes present in nearly every habitat we explore.

Much effort has been expended in the isolation and description of individual micro-organisms. Investigations on pure cultures are important to judge the potential abilities and functions of individual species in nature. Ecology and diversity study deals with interactions between organisms and relations between organisms and environments. Microbial ecology deals only with a segment of the total ecological system.

I. Mineral rich acidic habitats

Extreme environments such as acidic, thermophilic, hypersaline are important 'hot spots' of microbial 'megadiversity'. These are habitats of microorganisms which have the genetic and physiological capacity to survive and grow under these harsh or extreme conditions through which they have evolved while shaping the environment as we know it today. Extremophiles, organisms capable of thriving under extreme conditions, have become the interest from both an academic and biotechnology perspective because of their interesting ecology and physiology and thus have recently attracted considerable attention.

Acidic environments are especially fascinating because, in general, the low pH of the habitat is the consequence of microbial metabolism and not a stipulation imposed by the system as is the case in many other extreme environments like temperature, ionic strength, high pH, radiation, pressure, etc. (Lo´pez-Archilla *et al.*, 2001; Gonzalez-Toril *et al.*, 2001). Acidophiles are organisms that can withstand and even thrive in acidic environments where the pH values range from 1 to 5. Acidophiles or acid-tolerants include certain types of bacteria and archaea that are found in a variety of acidic environments, including ic pools, areas polluted by acid mine drainage, and even our own stomachs. Acidophiles have potential importance in evolution because metabolic processes might have originated on the surface of sulfide minerals (Wächtershäuser, 2006) and structuring of the genetic code could have taken place at acidic pH (Di Giulio, 2005). These organisms are also used to recover metallic minerals lost during the mining of coal and to reduce levels in coal (Marcus, 1997). Probably

first bacteria were isolated from acidic (pH <4.5) environments (Powell and Parr, 1919). Colmer and Hinkle, 1947 found that killing the bacteria stopped production of acids and dissolution of metals in acid mine drainage (AMD), thereby establishing the positive relationship between micro-organisms and environmental acidification. Bacteria contribute in many different ways to the acidic environments in which they cohabit (Robbins, 2000, Bond *et al.*, 2000a).

Acid drainage is a low pH, iron and sulfate bearing water usually formed when rocks containing sulfide minerals (eg. Pyrite, pyrrhotite etc.) are exposed to the atmosphere or an oxidizing environment, and subsequently leached by water. Environments containing high levels of dissolved metals include active and disused mines, where the production of acid mine drainage (AMD) and acid rock drainage (ARD) is catalyzed by the action of microorganisms. The microorganisms that are capable of oxidizing iron usually produce acid resulting into the formation of Acid Rock Drainage (ARD) or Acid Mine Drainage (AMD) systems. ARD is the result of spontaneous oxidation of surface rock outcrops of sulfide masses whereas AMD is the result of the appearance of effluents produced by mining operations (Grande *et al.*, 2005). They are formed when the sulfide ore of a mineral comes in contact with oxygen and atmospheric humidity which leads to a complex set of reactions resulting in the production of acid. The reaction is greatly accelerated in presence of ferric iron and by the action of bacteria that oxidizes ferrous iron to ferric iron (Sand *et al.*, 2001; Rohwerder *et al.*, 2003).

Acid drainage is a major issue affecting the metal mining and coal industry throughout the world. Many old mining sites have a legacy of acid drainage long after the completion of mining (Helms and Heinrich, 1997). As water percolates through the exposed sulfide mineral waste rock, chemical and then microbiological oxidation occur, causing acid production known as acid mine drainage (AMD or ARD). Acid leaching solutions are characterized by high metal concentrations that are toxic to most life. It is well known that AMD solutions are far from "sterile" and those acidophilic microorganisms not only tolerate, but also thrive in these acidic metal rich solutions (Hallberg and Johnson, 2001). However, the biogeochemical mechanisms associated with the production of acid rock drainage are complex and more research will be

necessary to determine if the induced beneficial microbial interactions would dominate in the natural ARD environment (Marchand and Silverstein, 2000).

II. Geological description of the sampling site: the original habitat of the acidophilic autotrophic and heterotrophic strains used in the present study

The geological formations of Darjeeling District comprised of unaltered sedimentary rocks, confined to the hills on the south. A characteristic feature of this area is that the older formation rest on the younger, showing a complete reversal of the original order of superposition. The great range of Himalaya was elevated during the Tertiary period, and the area has accumulated sediments of different geological ages (Dash, 1947).

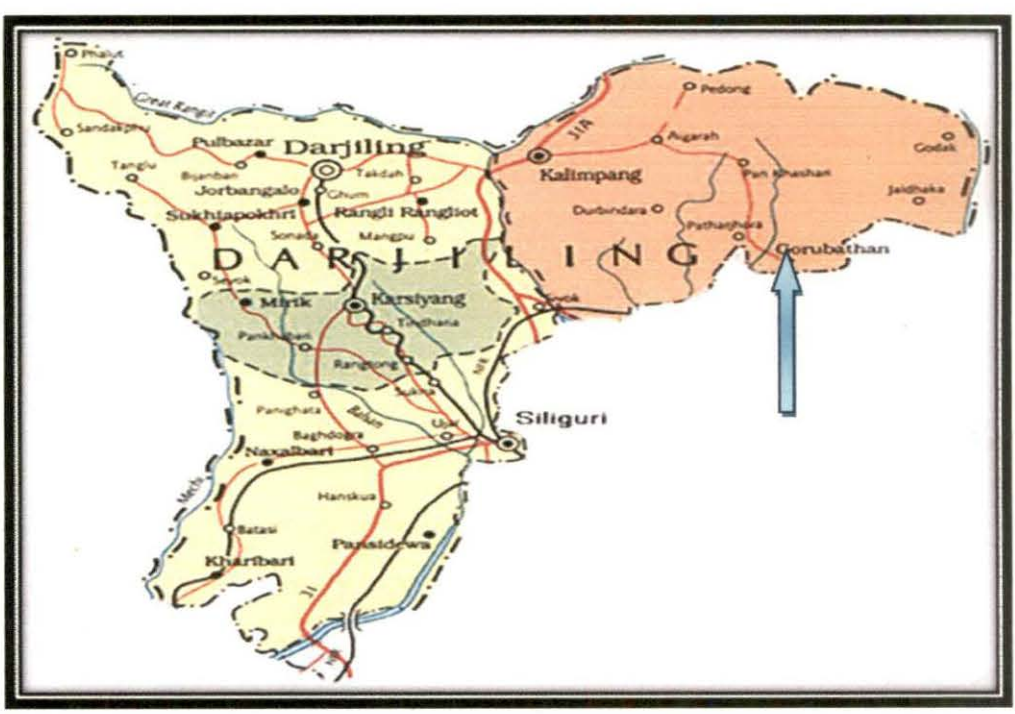

Fig. A: Location of Garubathan (marked with blue arrow) in Darjeeling Map.

Records of the GSI, vol. 109 part I 1982, showed the occurrence of magnetite in Khanikhola, south of Siyokbir (27° 02'00" N Lat. and 88° 30'45" E Long.). Lead-zinc

ore has been found to occur in the area around Garubathan Kalimpong sub-division (Shah *et al.*, 1974-75). An area of 0.247 sq.km on scale 1:2000 was covered by plane table mapping in the Kharkhola Block and Mal Khola block in the East and West of Garubathan. A total of 780.80 m in 4 boreholes were drilled in Mal Khola block which showed an average of 1.49% Pb, 0.01% Zn and 0.33% Cu. A total of 131 samples, comprising of surface channel samples, float-ore samples, borehole samples, composite samples, selective grab samples etc. were collected which showed the probable reserves estimated with an average of 3.54% Pb and 2.7% Zn and that of float ore is 2,910 tons, with an average of 7.147% Pb and 2.87% Zn. The average Cu and Ag in the ore, on the basis of 150 channel sample amount 1.14% and 42 g/ton, respectively (records of GSI, vol. 109, part I, 1982). A systematic geological mapping in parts of Kalimpong sub-division of Darjeeling district, covering an area of 180 sq.km on 1: 25000 scale in parts of toposheet No. 78A/8 and 12 was carried out by Roy, Chowdhury and Ghosh (1974-75). An occurrence of wolframite associated with base metal mineralization was observed at a place (27° 09'55" N Lat. and 88° 34'40" E Long.) near Mansong during the course of mapping. mineralization has been located near Pedong (27° 12'55" N Lat. and 88° 37'00" E Long.), Rishi (27° 11'30" N Lat. and 88° 38'00" E Long.), and Rorathang (27° 12'00" N Lat. and 88° 37'00" E Long.).

III. Microbial ecology of AMD/ARD

Acidophiles grow in environments of low pH (<3) and include bacteria, archaea and eukaryotes that are capable of growing chemolithoautotrophically, chemomixotrophically, and chemoheterotrophically (Hallberg and Jhonson, 2001). Bacterial populations indigenous to acidic drainage streams are heterotrophs such as the fungi *Aspergillus* sp. and *Penicillium* sp.; the bacteria *Acidiphilium* sp., *Flavobacterium acidurans*, and *Bacillus* sp. (Hallberg and Jhonson, 2001).

Traditionally, through culture-dependent methods, *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* were recognized as the major chemolithotrophic bacteria responsible for acid production in AMD (Johnson, 1998; Hallberg and Johnson, 2001). Nowadays, however cultivation-based analysis is not considered a suitable method rather 16S rRNA sequences analysis is considered as more reliable method for

characterizing microbial diversity (De Wulf-Durand *et al.*, 1997; Baker and Banfield, 2003). In the study of Tinto River the most representative bacterial species were found to be *A. ferrooxidans* (23%) and *L. ferrooxidans* (22%) and *Acidiphilium*. Other prokaryotes that were found to be the dominant microflora of Tinto River are *Ferrimicrobium acidiphilum* and closely related archaea *Ferroplasma acidiphilum* (Gonzalez-Torril *et al.*, 2003). 16S rRNA-based analysis has revealed that there are other microorganisms also that occur as dominant microflora of the AMD. *Thermopasmoles, Sulfobacillus, Acidimicrobium* etc.can remain as dominant flora of the AMD (Bond *et al.*, 2002b). The presence of archaebacteria including a group of and/or iron-oxidizers, such as *Sulfolobus, Acidianus, Metalosphaera,* has been reported from acidic environments (Edwards *et al.*, 2000; Fuchs *et al.*, 1995). Beside these, the presence of *Verrucomicrobia* and *Chlorobi* in ARD has also been confirmed by the 16S rRNA analysis of the ARD system (Okabayashi *et al.*, 2005).

A variety of chemolithotrophic and heterotrophic microorganisms are responsible for the solubilization of metals from sulfide minerals in acidic environments (Goebel and Stackebrandt, 1994a; Espejo and Romero, 1997). Although *Acidithiobacillus ferrooxidans* and *A. thiooxidans*, in the presence of heterotrophic *Acidiphilium* bacteria, were commonly regarded as the principle biological catalysts, recent analysis showed the important role played by other acidophilic heterotrophy also (Bhattacharya *et al.*, 1991; Hallberg and Lindstrom, 1994; van Niel, 1995). There is a lack of completed investigations into acidophile metal resistance mechanism (Hallberg and Johnson, 2001; Dopson *et al.*, 2003). Studying acidophiles one can learn about general biological resistance mechanisms too. Inducing biological competition for oxygen by adding a degradable organic carbon substrate has the potential to change the microbial ecology of ARD sites such that bacteria like *A. ferrooxidans* no longer dominate the environment. Furthermore, dominance of heterotrophs may alter the ARD environment sufficiently to allow in situ remediation take place (Marchand and Silverstein, 2000). The singular role of acidophilic heterotrophic bacteria in coupling iron reduction and glucose oxidation is demonstrated in anoxic batch reactor. As addition of organic substrates promote the dominance of heterotrophic bacteria in biofilm that coat rock surfaces, the solubilization of iron resulting from bacterial iron oxidation might cease altogether. On the other hand, investigators have found that several species of

heterotrophic or mixotrophic acidophile are also capable of leaching and oxidizing iron from pyrite (Edwards *et al.*, 1999; Marchand and Silverstein, 2000). Industrial leaching processes use several acidophilic bacteria, including *A. thiooxidans*, *A. ferrooxidans* etc. (Bruins *et al.*, 2000). It is somewhat analogous that commercial bioprocessing of sulfidic minerals has developed into an important and successful area of biotechnology with (in most cases) very limited data on microbial populations that are present in bioleaching systems. One reason for this has been the lack of accurate and appropriate methods for analyzing populations of the diverse acidophiles that are active in the metal enriched, acidic environments that constitute commercial leach liquors. Molecular techniques (gene libraries constructed from extracted DNA, amplification of 16S rRNA gene, restriction enzyme analysis, fluorescence in situ hybridization etc.) have been used to some effect in recent years (Goebel and Stackebrandt, 1994b). To study the ecological relationships, of these microorganisms and the population dynamics during the bioleaching processes, specific methods for their identification and enumerization are required. Molecular methods based on the detection of genomic diversity, such as GC content, DNA-DNA hybridization, and rRNA analysis, have been used to obtain a phylogenetic survey of the iron-oxidizing bacteria (De Wulf-Durand *et al.*, 1997).

Since the acidophilic microorganisms growing in these systems have important biotechnological applications many conventional microbial ecological studies of such acid laden metal rich environments have been performed (Norris, 1990; Hallberg and Johnson, 2001; Lo´pez-Archilla *et al.*, 2001). Recently molecular approaches to examine the microbial diversity of these habitats have also been performed (Goebel and Stackebrandt, 1994a,b; De Wulf-Durand *et al.*, 1997; Bond *et al.*, 2000a). Because of the limited types of substrates available in such environments, the microbial diversity was initially expected to be extremely poor. Cultivation-based studies have however revealed a great diversity of the microbial community in AMD (Johnson, 1998; Hallberg and Johnson, 2001).

Even though AMD environments are diverse and variable microbiologically, relatively little work has been done on their microbial ecology (Leduc *et al.*, 2002). Moreover, very few investigations have examined seasonal variations of bacterial numbers in an AMD environment. On the other hand many studies have been done on one of the key

organism *A. ferrooxidans* (Leduc *et al.*, 2002). The purpose of this study is to explore the acidophilic heterotrophic diversity indigenous to ARD/AMD samples. It has been postulated that their have been large scale swapping of genes amongst organism that share the same ecological niche. The tolerance towards high inorganic acidity and metal concentration has been a common phenotype of acidophilic autotrophs and heterotrophs. The useful genes of the heterotrophs can be manipulated to bring out better leaching microorganisms. The understanding of the genetic basis of tolerance to heavy metals in heterotrophs would therefore be an important area of investigation. The characterization of plasmids from acidophilic heterotrophs might serve as repositories of uncharacterized ORFs for future application in the field of mineral biotechnology.

IV. Physiology of Acidophilic Chemolithoheterotrophs

Acidophilic microorganisms are mainly of two types: Heterotrophs and Chemoautotrophs. Acidophilic chemoautotrophs have in common the ability to use CO_2 as their sole source of carbon and to derive energy from the oxidation of inorganic chemicals. *Acidithiobacillus thiooxidans* grows between the pH values 1.0 and 4.0 with an optimum at pH 2.5 (Rohwerder *et al.*, 2003) and uses elemental or reduced compounds as substrates (Ingledew, 1982). *A. thiooxidans* is an obligate chemoautotroph. *A. ferrooxidans* is probably the most widely studied acidophile. Further, there is a report that *A. ferrooxidans* grows heterotrophically at pH 7 with glucose as carbon and energy source (Harrison, 1984). This finding has been disputed and it has been suggested that *A. ferrooxidans* cultures are contaminated with the facultatively heterotrophic *Thiobacillus acidophilus*, which has since been isolated in axenic culture (Jhonson and Kelso, 1983). *T. acidophilus* is a member of the group of acidophilic thiobacilli capable of both chemoautotrophic and heterotrophic growth. This species, recently isolated from a culture of *A. ferrooxidans*, grows optimally at pH 3.0 to 3.5. The chemoautotrophic ability of this organism is limited to the oxidation of elemental; sugars, amino acids, and carboxylic acids support heterotrophic growth. Other organisms, such as species of *Plantomyces, Pseudomonas, Leptothrix, Aquabacterium, Caulobacter, Ralstonia, Achromobacter,* and *Microcyclus* which have been associated with acid environments, are probably only acid-tolerant (Yang *et al.*, 2008). The bacterium *Caulobacter crescentus* is known for the distinctive ability to

live in low-nutrient environments, a characteristic of most heavily metal-contaminated sites.

The use of acidophilic, chemolithotrophic microorganisms capable of oxidizing iron and in industrial processes to recover metals from minerals containing copper, gold and uranium is a well established biotechnology (Torma, 1983; Brierley, 1982, 1997; Acevedo, 2000; Rawlings, 2002; Olson *et al.*, 2003; Suzuki, 2001). The insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being the re-oxidation of the generated ferrous iron to obtain additional ferric iron (Rohwerder *et al.*, 2003; Rawlings, 2002; Olson *et al.*, 2003). Currently, there are operations using mesophilic and thermophilic microorganisms (Torma, 1983, 1988; Brierley, 1982, 1997; Lindstrom *et al.*, 1992; Acevedo, 2000; Rawlings, 2002, 2005a, b; Olson *et al.*, 2003). Biomining has distinctive advantages over the traditional mining procedures. For example, it does not require the high amounts of energy used during roasting and smelting and does not generate harmful gaseous emissions such as dioxide (Rawlings, 2002). Nevertheless, acid mine drainage can be generated, which if not properly controlled, pollutes the environment with acid and metals (Rohwerder *et al.*, 2003; Rawlings, 2002, 2005a,b; Olson *et al.*, 2003). Biomining is also of great advantage since discarded low-grade ores from standard mining procedures can be leached in an economically feasible way. There are complete previous reviews regarding methods of bioleaching and their implementation in several countries (Torma, 1983; Brierley, 1982, 1997; Acevedo, 2000; Lindstro¨m *et al.*, 1992; Rohwerder *et al.*, 2003; Rawlings, 2002, 2005a, b; Olson *et al.*, 2003).

Recently, thiosulfate has been postulated as a key compound in the oxidation of the moiety of pyrite (Schippers and Sand, 1999). Iron (III) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would be consequently degraded in a cyclic process to sulfate, with elemental being a side product. Lithotrophic oxidation is an ancient metabolic process. Ecologically and taxonomically diverged prokaryotes have differential abilities to utilize different reduced compounds as lithotrophic substrates (Ghosh and Dam, 2009). While the mechanisms of oxidation in obligately chemolithotrophic bacteria, predominantly belonging to *Beta-* (e.g. *Thiobacillus*) and

Gamma-proteobacteria (e.g. *Thiomicrospira*), are not well established, the Sox system is the central pathway in the facultative bacteria from *Alpha-proteobacteria* (e.g. *Paracoccus*) (Rother *et al.*, 2001).

V. Sulfur Oxidizing Microorganisms

The sulfur-oxidizing microorganisms are primarily the gram negative bacteria currently classified as species of *Thiobacillus, Thiomicrospira* and *Thiosphaera*, but heterotrophs, such as some species of *Paracoccus, Xanthobacter, Alcaligens* and *Pseudomonas* can also exhibit chemolithotrophic growth on inorganic sulfur compounds (Buonfiglio *et al.*, 1999).

Reduced inorganic sulfur compounds are exclusively oxidized by prokaryotes and sulfate is the major oxidation product. In the domain *Bacteria* sulfur is oxidized by aerobic lithotrophs or by anaerobic phototrophs. Prokaryotes oxidize hydrogen sulfide, sulfite, thiosulfate and various polythionates under alkaline (Friedrich *et al.*, 2005) neutral or acidic conditions (Friedrich *et al.*, 2001). Aerobic oxidizing prokaryotes belong to genera like *Acidianus*, *Acidithiobacillus*, *Aquaspirillum*, *Aquifer*, *Bacillus*, *Beggiatoa*, *Methylobacterium*, *Paracoccus, Pseudomonas*, *Starkeya*, *Sulfolobus*, *Thermithiobacillus*, *Thiobacillus* and *Xanthobacter* and are mainly mesophilic ((Lu and Kelly, 1983; Ghosh and Roy, 2007). Phototrophic anaerobic sulfur oxidizing bacteria are mainly neutrophilic and mesophilic and belong to genera like *Allochromatium, Chlorobium, Rhodobacter, Rhodopseudomonas, Rhodovulum* and *Thiocapsa* (Mittenhuber *et al.*, 1991; Meulenberg *et al.*, 1993; Meyer *et al.*, 2007).

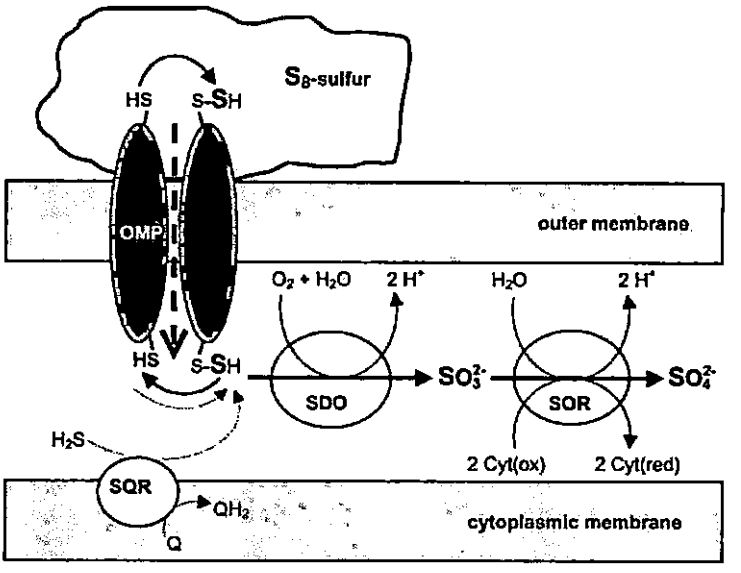

Fig. B. Proposal of a biochemical model for oxidation in *Acidithiobacillus* and *Acidiphilium* spp. In this scheme extracellular elemental (S_8) is mobilized as persulfide sulfane by special outer-membrane proteins (OMP) and oxidized by periplasmic dioxygenase (SDO). The resulting sulfite is oxidized to sulfate by sulfite: acceptor oxidoreductase (SOR), which probably uses cytochromes as electron acceptors (Cyt). Free sulfide is oxidized by a separate dehydrogenase (SQR), which uses quinones (Q) as electron acceptors (Rohwerder and Sand, 2003).

VI. Metal resistance/ tolerance property of acidophilic heterotrophs

Several reports had described the adverse effects of toxic metals on microbial biomass and metabolic activities in soil (Duxbury and Bicknell, 1983; Chander and Brookes, 1992). The interactions between toxic heavy metals and indigenous bacterial flora has become much significant when it was reported that pollution due to organic compounds was also associated with heavy metals like arsenic, mercury, lead, and zinc (Roane and Kellogg, 1996). Metal resistance was thought to be emerged during the early period of prokaryotic evolution in the metal contaminated environment (Ji and Silver, 1995). Environments containing high levels of dissolved metals include active and disused mines (Ledin and Pedersen, 1996). Metals may accumulate above normal physiological

concentrations by the action of unspecific, constitutively expressed transport systems, whereby they become toxic. Intracellular metals can exert a toxic effect by forming co-ordinate bonds with anions blocking functional groups of enzymes, inhibiting transport systems, displacing essential metals from their native binding sites and disrupting cellular membrane integrity (Nies, 1999). The toxicity of As (III) has been documented in *Acidiphilium multivorum*, *A. ferrooxidans*, *Ferroplasma acidarmanus* and *Metallosphaera sedula* (Harvey and Crundwell, 1996). The presence of the *arsB* gene (efflux pump) has been identified by Southern hybridization in various acidophilic micro-organisms; these include *A. caldus*, *A. thiooxidans*, *A. ferrooxidans*, *Acidiphilium acidophilum*, *Thiomonas cuprina* and *Acidocella facilis* (Dopson *et al.*, 2001). *A. ferrooxidans* strains adapted to increased levels of Cu (II) have been found to be tolerant to 800 mM (Dew *et al.*, 1999). Other acidophiles shown to be resistant to copper include *Leptospirillum ferrooxidans*, which shows growth in 5 mM Cu (II) (Johnson *et al.*, 1992). '*Acidiphilium symbioticum*' KM2 has been shown to harbour three plasmids which, when a mini-plasmid library was created and transformed into *E. coli*, conferred resistance to Zn (II) and Cd (II) (Mahapatra *et al.*, 2002). Apart from *A. ferrooxidans* and *Sulfolobus* other acidophiles resistant to Ni (II) include *Acidiphilium multivorum*, *Acidocella aminolytica* and *Acidocella* strain GS19 (Sampson and Phillips, 2001). Many other metals are found in acidic environments, including uranium, molybdenum and chromium. A number of different acidophiles have been isolated from locations containing these metals, and the following species have been isolated from environments containing uranium: *A. ferrooxidans*, *L. ferrooxidans*, *A. thiooxidans*, *Thiomicrospira cuprina*, cells resembling *Sulfolobus*/ *Acidianus* spp., *Acidiphilium* spp. and other heterotrophic iron-oxidizing acidophiles (Tuovinen and Bhatti, 1999).

Acidic waters from AMD cause the dissolution of other resident minerals increasing the concentration of heavy metals in these environments. Therefore, bacteria isolated from AMD exhibit high levels of tolerance to heavy metals. The objective of this work was to isolate and characterize acidophilic, aerobic, chemolithoheterotrophic bacteria from AMD that are tolerant to high concentrations of heavy metal divalent cations. Enrichments and isolated bacteria were characterized by tolerance to heavy metals, presence of extrachromosomal DNA, and presence of known metal tolerance-related

genes. Chemolithoheterotrophic, heavy metal tolerant bacteria were successfully isolated from Garubathan. The majority of the strains were closely related to the genera *Serratia, Burkholderia, Bacillus megaterium, B. cereus, Psychrobacter, Comamonas testosteroni, and Acidiphilium cryptum* respectively. The heavy metal tolerance profiles of all the isolated strains revealed varied tolerance of heavy metals. An attempt was made to correlate tolerance towards heavy metals to the presence of a plasmid. A large portion of the microbial diversity in these acidic metal-rich environments is yet to be cultured and a further challenge will then be to discover the secrets of metal resistance mechanisms in them.

VII. Objectives of the study

1. To study the diversity of acid-tolerant heterotrophic bacteria in ARD samples from mineral occurrences sites of Eastern Himalaya
2. To apply molecular systematics in ascertaining taxonomic status of the isolates
3. To understand the physiology of the acidophilic metal-tolerant chemolithoheterotrophs with special reference to sulfur oxidation phenotype and *sox*B genotype
4. To explore the presence of indigenous plasmids in the isolates and attempt to ascribe their function in relation to metal tolerance

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