General Discussion & Summary

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The study of acidophilic heterotrophs of AMD has become very important because they are regular inhabitants of mineral sulphide environments (Berthelot *et al.*, 1997). Several characteristic features of these heterotrophs have been suggested to have beneficial effect on the bioleaching as well as for the sustenance of the iron-oxidising organisms in the environments. Earlier studies have shown that both sulfur- and iron-oxidizing bacteria such as *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* were present in rather high numbers (Baker and Banfield, 2003). *A. ferrooxidans* and *Ferroplasma* has been considered principally responsible for the extreme conditions of AMD systems (Bond *et al.*, 2000; Okabayashi *et al.*, 2005). The presence of heterotrophs associated with chemolithotrophs like *A. ferrooxidans* has been observed (Wichlacz and Unz, 1981; Bond *et al.*, 2000), but studies of such heterotrophs are largely restricted to obligately acidophilic bacteria like *Acidiphilium* sp. (Johnson and McGinness, 1991; Bruneel *et al.*, 2003; Okabayashi *et al.*, 2005). It was observed that a significant symbiosis exists between heterotrophic and certain autotrophic species. Acidophilic autotrophs produces organic materials as their by product. Heterotrophs makes a way by utilizing/removing organic compounds toxic for the autotrophs (Pronk *et al.*, 1990; Das and Mishra, 1996). Mixotrophic growth was evidenced by certain facultative chemolithotrophs where limited amount of both inorganic and organic substrates are present in AMD (Clark and Norris, 1996). Assuming larger degree of metabolic plasticity of facultative acidophilic heterotrophs over obligately acidophilic heterotrophs, in the present study, cultivation and characterization of acidtolerant heterotrophs from AMD samples from Garubathan ore reserves of West Bengal, India, has been attempted to study their physiology, to understand their role in nature and to identify their potential in biotechnological applications.

The enrichment techniques, isolation, purification and preliminary screening of acid-tolerant heterotrophic isolates from AMD sites of Garubathan have been described in this chapter1. Isolation and identification of acid-tolerant heterotrophs from AMD samples from Garubathan, India, has partially revealed diversity of the strains in terms

of pH tolerance, growth at a wide range of temperature, and chemolithotrophy. Primarily, eighty one acid-tolerant heterotrophic isolates were obtained from the AMD samples. Out of which ten heterotrophic isolates were purified from the autotrophic culture of *Acidithiobacillus ferrooxidans* colonies. Earlier authors (Johnson and Kelso, 1983; Mishra *et al.*, 1983) have also detected acidophilic heterotrophs in *A. ferrooxidans* cultures. It has been found that some organic substrates such as pyruvate, glutamate, aspartate, serine, glycine, and other amino acids excreted by *A. ferrooxidans* can be utilized by the heterotrophs for their growth (Schnaltman and Lundgren, 1965; Arkesteyn *et al.*, 1980; Ingledew, 1982). Several types of acidophilic heterotrophs (*Acidiphilium cryptum*, *A. acidophilum*, *A. organovorum* etc.) that are gram negative in nature were recovered from *A. ferrooxidans* enrichment cultures (Guay and Silver, 1975; Harrisson *et al.*, 1980; Lobos *et al.*, 1986). An important molecular technique has proved to be useful in typing bacterial strains is Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of whole cell bacterial proteins of the isolates, wherein differences seen in protein bands have been successfully used to group bacteria (Krech *et al.* 1988; Huey and Hall, 1989; Maiti *et al.*, 2009). In this study too, SDS-PAGE of total protein isolated from the strains was used to screenout the similar banding patterns and finally eighteen of them showing differential banding patterns of protein was selected for further studies. Twelve of the eighteen acid-tolerant isolates were found to be Gram negative. All the isolates showed growth at diverse range of pH, (from 1 to 12) and temperature (from 4 °C to 42 °C). The isolates showed best growth at pH 3 and temperature (28-30) °C except GMX1, GMX7, and GAH1which showed psychro-tolerant nature growing best at 16 °C. Nine acid-tolerant heterotrophic isolates were tentatively found to be mixotrophic (facultative sulfur-oxidizing) strains (Table1.1). Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas must have favoured abundant microbial populations of sulfur-oxidizers (both mesophilic and psychro-tolerant), including autotrophic *Acidithiobacillus* species as well as heterotrophic sulfur-oxidizing strains. These strains may provide genetic resources for the development of novel biotechnological processes. Hence, in the following chapter i.e chapter 2 contained the detailed study of facultative sulfur lithoautotrophy, organotrophy and biochemical characterization of these isolates.

Lithoautotrophic sulfur oxidizers are found in environments rich in H_2S, such as AMD. Particularly most sulfur-oxidizing bacteria are both acidophilic and autotrophic (Pronk *et al.*, 1991). Nine of the acid-tolerant isolates could grow chemolithoautotropically on thiosulfate (Fig. 2.1) however four isolates, GAH2, GAH5, GMX1, GMX6 grew relatively poorer than the others; whereas, elemental sulfur medium could not support the growth of any of the acid-tolerant isolates. Concurrent utilization of organic (succinate) and inorganic (thiosulfate) substrates, termed as mixotrophic nutritional ability (Matin, 1978), were shown by ten of the isolates reported in this study (Fig. 2.4). GMX4 which could not sustain in MST medium was able to grow when yeast extract was added to the medium (MSTSY). These mixotrophic behaviours of the isolates were also evident during the study of *Thiobacillus* A2 which showed mixotrophic metabolism when in the natural environment both inorganic sulfur compounds and organic substrates were available simultaneously (Gottschal and Kuenen, 1980). All nine isolates have demonstrated growth by utilizing thiosulfate as the energy source (Fig. 2.3). Rate of depletion of thiosulfate in the medium with GMX7 and GMX5 was highest among all the nine isolates. Chemolithotrophic growth of acid-tolerant isolates on thiosulfate in MST medium (Fig. 2.1J) and the data regarding consumption of thiosulfate (% consumption) by these isolates (Fig. 2.3) supported each other which means cell growth was coupled with the utilization of thiosulfate. The diversity of physiology was evident from the wide range of physicochemical requirements like temperature, pH tolerance etc. Similarly autotrophic growth of different isolates in this study in batch cultures on thiosulfate produced different growth yields. It was inferred from the growth experiment that external physical parameters do not influence the growth yield on thiosulfate for different isolates. Concurrently it also enabled to set up a working hypothesis that there may be difference in the mode of dissimilar sulphur compounds oxidation pathways, electron transport mechanisms and modes of energy conservation. Abundance of reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas could be the reason of the prevailing diversity amongst culturable microbial populations of sulfur-oxidizers (both mesophilic and psychrotolerant), including autotrophic *Acidithiobacillus* species (Gurung and Chakraborty, 2009) as well as heterotrophic sulfur-oxidizing strains. Similar diversity

of thiosulfate-oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000). Chemolithoautotrophy on thiosulfate is also known in non-thiobacilli of facultative lithotrophs like *Paracoccus pantotrophus*, hydrogen oxidizing heterotrophs, and heterotrophic marine pseudomonads (Ruby *et al.*, 1981; Kelly, 1989). The final product of chemolithotrophic sulfur compounds oxidation is sulfate. The role and production of polythionates as intermediates in oxidations of sulfur, sulfide and thiosulfate to sulfite had been addressed starting from the initial studies (Kelly, 1982, 1989). Peck (1960) first proposed a separate view of thiosulfate oxidation independent of tetrathionate formation, in which $S_2O_3^{2-}$ was cleaved to S^{2-} and SO_3^{2-} by the enzyme thiosulfate reductase. He showed that S^0 was produced from sulfide (S^{2-}) oxidation, while the SO_3^{2-} was oxidized to sulfate forming APS (adenylyl sulfate) as the intermediate product. Kelly (1989) proposed a 'common pathway", a hypothetical scheme proposed on the basis of the discrete available data generated with the various strain, viz., *T. concretivorus* (strain *T. thiooxidans*), *T. thiooxidans*, *T. thioparus, T. novellus, T. ferrooxidans, T. denitrificans*, and *T. neapolitanus.* All these strains oxidize thiosulfate. The differences on the oxidative metabolism of thiosulfate by different members of sulfur lithotrophs were primarily due to the differences in the mechanism of initial cleavage of S-S bond of thiosulfate. Tabita *et al.*, (1969) proposed the involvement of two different enzymes in the metabolism of thiosulfate- (i) Rhodanese and (ii) thiosulfate oxidizing enzyme. Rhodanese cleaves the S-S bond, thereby producing membrane associated (S^0) and sulfite (SO_3^{2-}). The thiosulfate oxidizing enzyme, thiosulfate cytochrome c oxidoreductase catalyzes union of two molecules of thiosulfate to form one molecule of tetrathionate ($S_4O_6^{2-}$), which could be further oxidized to produce thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}). The oxidation of thiosulfate without the formation and accumulation of polythionates has been best studied with facultative species *T. versutus*. Cytochrome-dependent complete oxidation of thiosulfate to sulfate was demonstrated with enzyme preparations (Kelly, 1989). A multi-enzyme system that performs the sequence of oxidation reactions of thiosulfate was identified in some greater detail (Kelly, 1989). Thiosulfate oxidation pathways operating in sulfur-oxidising bacteria have been tentatively grouped in three categories (Meyer *et al.*, 2007): (a) pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which is

common in extremophilic sulfur oxidisers (*Acidithiobacillus*, *Thermothiobacillus*, and *Halothiobacillus*) (Kelly *et al.*, 1997); (b) pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by multienzyme complex (Sox) system, active in photo- and chemotrophic alphaproteobacteria (Friedrich *et al.*, 2001; Mukhopadhyaya *et al.*, 2000); (c) branched thiosulfate oxidation pathway involving formation of sulfur globules operating in sulfur-storing bacteria (Hensen *et al.*, 2006). Sox enzyme system is present in diverse thiosulfate oxidizing bacteria. Activity of thiosulfate dehydrogenase, which occurs in a wide range of chemolithotrophic bacteria, was observed in the cell-free extracts of nine of the thiosulfate oxidizing strains, with GAH2 showing the least activity; GMX1, GMX5 and GMX6 showing no activity. (Table 2.1). Even if cell free extracts produces tetrathionate, doubts prevail about the nature of the intermediate; as tetrathionate may not always be a normal intermediate in thiosulfate oxidation by normal cells (Dam *et al.*, 2007).

Studies in energy conservation in sulfur oxidation constitute electron transport (ETS) and translocation of proton. Electrons generated from sulfur substrates oxidations may couple the redox reaction at the level of cytochrome *c* or electron enters at cytochrome *b* or more higher status in the electron transport chain for energy generation (Kelly, 1989). The terminal reaction, $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$, is common in all the sulfur compound oxidation to produce sulfate from sulfite. In almost all the members of thiobacilli the enzyme responsible for the catalysis of this reaction, sulfite cytochrome *c* oxidoreductase, has been found to be actively present. The electron negative redox couple of SO_3^{2-}/ SO_4^{2-} is adequate to couple to the electron transport system at the level of flavin-quinone-cytochrome *b*. In some species it has been shown that cytochrome *c* is the physiological electron acceptor and the start point where the energy is coupled to the electron transport chain. Alternatively, adenosine phosphosulfate (APS) reductase has been shown to be operative in thiosulfate metabolism in several strains of *Thiobacillus*. It catalyzed the same terminal oxidation reactions of sulfite to sulfate. The electrons enteringthe chain at level of cytochrome *b*, are benefited in two ways- by conserving energy synthesizing ATP at the site of transfer in between cytochrome *b* to cytochrome *c* sector in the ETS and also at the cytochrome *c* / oxidase sector. These differences in major electron transport chain cause

the differences in growth yields e.g., *T. denitrificans* and *T. tepidarius*, produce better yields of growth when compared to the growth of *T. versutus* and *T. neapolitanus* (Kelly, 1989). Sulfite dehydrogenase activity was observed in cell-free extracts of all of the nine test strains (Table 2.1). *soxC* encodes a sulfite dehydrogenase, the requirement for which in thiosulfate-dependent lithotrophic growth, experimentally verified in *Paracoccus denitrificans* (Mukhopadhyaya *et al.*, 2000).

Organotrophic growth for the acid-tolerant isolate was done on MSSY and R2A medium. Thirteen of the acid-tolerant isolates showed good growth on both the media (Fig. 2.4 and Table 2.2). R2A medium was greatly preferred (better growth within 24 h) by them. The isolates also displayed a wide range of substrate utilization as well (Table 2.3). R2A is a low-nutrient containing medium. Oligotrophic bacteria are generally tested for their ability to grow in R2A medium (Bhowal and Chakraborty, 2011). The isolates DK1AH1 and DK2AH2 were excluded from biochemical characterization study as they could not survive in pH more than 5.5. The rest of the acid-tolerant heterotrophic isolates showed varying differential properties from each other biochemically. The results of S_{SM} values scored between the isolates have reflected similar behaviour as obtained from total protein profile. Growth of acid-tolerant isolates in R2A medium has impelled us for the identification of oligotrophic property of these isolates (elaborated in chapter 3).

Acid mine drainage contains relatively low concentration (10 mg/l) of dissolved organic carbon (Kolmert and Johnson, 2001), and, as such, is called an oligotrophic environment. For the purposes of enumeration, oligotrophic aquatic bacteria have been tentatively defined as bacteria that develop on first cultivation on media with a minimal content of organic matter of either 1 mg or 1 to 15 mg of C per liter, usually supplied as complex mixtures of peptone, Trypticase, and other nutrients. Eutrophic bacteria, on the other hand, have been considered to be organisms able to grow on similar nutrients but at levels supplying 2 or more g of C per liter (Akagi *et al.*, 1977). The two types of media, differing widely in nutrient level, have been used to determine the distribution of oligotrophic and eutrophic bacteria in the sea (Akagi *et al.*, 1977) and in lake water

(Ishida *et al.*, 1980). Oligotrophic bacteria were considered to be those organisms which grew at the lower nutrient level but failed to grow when transferred to the higher level. In another study organisms able to grow only at the lower concentration of nutrients were called obligate oligotrophs, whereas those which grew at both low and high concentrations of nutrients were termed facultative oligotrophs (Ishida *et al.*, 1980, 1982). When one detects growth by measuring changes in numbers of viable cells in a liquid medium by using the plate count technique (Van der Kooij *et al.*, 1980; ZoBell and Grant, 1942), the concentrations of nutrients required to produce detectable increases in cell numbers are at least two orders of magnitude smaller than those needed to produce turbidity in a liquid medium or colonies on a solid medium. Thus, using plating techniques, ZoBell and Grant in 1943 were able to show that *Escherichia coli*, *Staphylococcus citreus*, *Bacillus megat erium*, *Proteus vulgaris*, and *Lactobacillus lactis* multiplied in solutions containing 0.1 mg of glucose (supplying 40 µg of C per liter) (ZoBell and Grant, 1942). Lower concentrations were not tested for technical reasons. Even *Pseudomonas aeruginosa* has been shown to grow in tap water at 25 µg of C per liter supplied by any one of a number of compounds (Van der Kooij *et al.*, 1982), and *Aeromonas hydrophila* multiplied when C supplied as glucose was added at 10 µg/liter (Van der Kooij *et al.*, 1980). These organisms are ordinarily not considered to be oligotrophs, yet they more than qualify when the current definition of an oligotroph as an organism which can grow in a medium containing nutrient supplying 1 to 15 mg of C per liter is applied. Oligotrophic bacteria are generally tested for their ability to grow in R2A medium. Very recently, diluted Luria-Bertani broth has been used to detect oligotrophic bacteria from environmental water samples (Kumar *et al.*, 2010; Oh *et al.*, 2009). Sixteen acid-tolerant heterotrophic strains from Garubathan AMD are facultatively oligotrophic capable of growing in R2A and diluted Luria-Bertani as well as in diluted modified DSMZ 269 media (Table 3.1). There are numerous reports of the isolation of acidophilic heterotrophs from extensibly pure culture of *Acidithiobacillus ferrooxidans* (Guay and Silver, 1975; Harrison *et al.*, 1980) and they are the part of the consortium present in the sulfide mineral occurrence sites (Harrison, 1981, Berthelot *et al.*, 1997). Although these environments are very poor in organic materials, the heterotrophs probably survive by scavenging on the low concentration of organic compounds excreted by *A. ferrooxidans* and other autotrophic

chemolithotrophs. Heterotrophic acidophiles are able to utilize organic materials produced by acidophilic autotrophs. The culture filtrate from the autotroph *A. ferrooxidans* contained sufficient organic matter to support heterotrophic growth of *Sulfobacillus thermosulfidooxidans* TH1 (Norris and Kelly, 1980) and *Acidiphilium* sp. DKAP1.1 (Gurung and Chakraborty, 2009). Of the seven strains reported in this study, two strains, which tolerated pH to the extreme of 1.0, GAH1 and GAH4, were able to grow in sterile acid mine water (pH 1.0-1.5) and elemental sulfur spent medium of *A. ferrooxidans* (pH 1.5-2.0) while the others (lesser acid-tolerant; Table 1.1 of chapter 1) have shown growth in the said media only when pH was increased to 3.0 - 4.0. DOC measurements of AMD samples from Garubathan (17-22 mg l^{-1}) and elemental sulfur spent medium of *A. ferrooxidans* (65-71 mg l^{-1}) have confirmed very low carbon content which supported the oligotrophic growth of the acid-tolerant strains. This demonstrated the adaptation of these acid-tolerant heterotrophs to a low nutrient condition that usually prevails in the mineral rich environments. The ability to grow in low nutrient condition, on the other hand is highly beneficial to the autotrophic partner *A. ferrooxidans.*

Microorganisms surviving in AMD environment meet substantial selective pressure to develop resistance mechanism to metal ions, supporting them with a competitive selective advantage. As a result, in shaping the characteristics of microbial communities in acidic environments in terms of both structure and function, the efficacy of diverse heavy metal resistance mechanisms would play a significant role (Dopson *et al.*, 2003). Acidophilic heterotrophic bacteria representing *Acidiphilium* and *Acidocella* genera were found to resist high levels of Cd, Zn, Ni, and Cu (Ghosh *et al.*, 1997; Mahapatra and Banerjee, 1996). Distinct patterns of heavy metal resistance in isolates from coal mining environments of Brazil were evidenced, being the Zn and Ni resistance the most widespread (Castro-Silva *et al.*, 2003). The AMD sites selected in this study is restricted to an area of 0.247 sq.km where zinc ore has been found to occur (Shah *et al.*, 1974-75). Not surprisingly, the strains isolated from Garubathan had been able to resist the heavy metal ions. Acid-tolerant heterotrophic isolates from AMD samples could be classified into nine groups on the basis of distinct patterns of metal resistance (Table 3.2). Isolates of Group III, strains GAH2 and GAH5, tolerated Co(II),

Ni(II), and Zn(II) as high as 35, 30, and 40 mM respectively. Similarly, isolates of Group IX, strains DK1AH1 and DK2AH2 tolerated Ni(II) and Zn(II) at its most 90, 20; and 450, 30 mM. The maximum tolerance of 100 mM Zn (II) was shown by GAH4. They could tolerate cobalt, nickel, copper, zinc, and arsenite concentration up to the level of 35 mM, 450 mM, 5 mM, 100 mM, and 10 mM respectively. However, maximum tolerable concentration of chromium, cadmium, and mercury was found to be only 1.5 mM, 1 mM, and 0.1 mM respectively. The strains have shown multiple metal tolerance as well as higher metal tolerance ability due to the selective pressure as the site from where these strains were obtained was rich in mineral occurrences.

For further characterization, it was important to test the sensitivity of the acid-tolerant isolates towards several antibiotics. The antibiotics tested were selected to represent 7 different classes: Aminoglycosides, Antifolates, Cephalosporins, Penicillin, Quinolones, and Others. The detailed antibiotic tolerance profiles of the acid-tolerant isolates were given in Table 3.3. Four (GAH4, GAH44, DK1AH1, and DK2AH2) out of eighteen strains were found to be sensitive towards antibiotics tested. However five (GAH1, GAH3, GAH8, GAH9, and GAH10) of them were resistant to all the 12 panel of antibiotics tested. The rest of the strains showed resistance towards two or more than two antibiotics. The resistance to a particular heavy metal has been correlated to antibiotics and other heavy metal resistance in a variety of organisms (Austin and Colwell, 1977; Luli *et al.*, 1983; Sabry *et al.*, 1997) and the role of plasmids in confering resistance to both antibiotics and metals has been previously demonstrated (Foster, 1983; Lyon and Skurray, 1987). Hence, research followed a natural tract to uncover phylogenetic affiliations of the AMD isolates (elaborated in Chapter 4).

The outcome of the 16S rRNA sequence comparisons was presented in Chapter 4, indicating phylogenetic diversity among the isolates studied. The 16S phylogeny of the fifteen acid-tolerant, thiosulfate metabolising bacteria isolated from AMD samples of Garubathan, India, fell into the genus *Burkholderia* , *Comamonas*, *Serratia*, *Psychrobacter*, *Acidiphilium* and *Bacillus* (Fig. 4.3-4.17) of the sub-class *Beta-proteobacteria, Gamma-proteobacteria, Alpha-proteobacteria* and *Firmicutes*. Despite extremities, a diverse range of bacteria, including representatives of *Proteobacteria*, *Nitrospirae*, *Firmicutes*, and *Actinobacteria*, inhabit AMD environments. Besides the

most studied group of bacteria under *Gamma-proteobacteria*, bacteria under *Alpha-* and *Beta-proteobacteria* were also identified in the acid mine environments (Baker and Banfield, 2003; Valverde *et al.*, 2006; Xiao *et al.*, 2009). Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas favor abundant microbial populations of sulfur oxidizers (both mesophilic and psychrotolerant), including autotrophic *Acidithiobacillus* species as well as heterotrophic sulfur-oxidizing strains. Similar diversity of thiosulfate oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000).

High sequence similarities of the 16S rRNA gene sequence of the two strains with *Burkholderia* species and clustering of them with *Burkholderia pyrrocinia* in the phylogenetic tree (Fig. 4.18) suggest that the isolates GAH2 and GAH5 can be assigned as novel acid- and metal-tolerant (elaborated in chapter 3) thiosulfate-oxidizing strains of *Burkholderia pyrrocinia*. In the phylogenetic trees, isolates GMX5 and GMX8 clustered with *Bacillus megaterium* with a bootstrap support of 100%. Another Gram positive heterotrophic strain, GMX6, clustered with *B. cereus* with a bootstrap support of 78%.

Amplification of soxB gene homologs and Phylogeny with soxB gene sequences: The *soxB* gene encodes the SoxB component of the periplasmic thiosulfate-oxidizing Sox enzyme complex, which has been proposed to be widespread among the various phylogenetic groups of sulfur-oxidizing bacteria (SOB) that convert thiosulfate to sulfate with and without the formation of sulfur globules as intermediate. Thiosulfate oxidation pathways operating in sulfur-oxidising bacteria have been tentatively grouped in three categories (Meyer *et al.*, 2007): (a) pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which is common in extremophilic sulfur oxidisers (*Acidithiobacillus*, *Thermothiobacillus*, and *Halothiobacillus*) (Kelly *et al.*, 1997); (b) pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by multienzyme complex (Sox) system, active in photo- and chemotrophic alphaproteobacteria (Friedrich *et al.*, 2001; Mukhopadhyaya *et al.*, 2000); (c) branched thiosulfate oxidation pathway involving formation of sulfur globules operating in sulfur-storing

bacteria (Hensen *et al.*, 2006). Sox enzyme system is present in diverse thiosulfate oxidizing bacteria.

Two pairs of degenerate soxB primers (Petri *et al.*, 2001; Meyer *et al.*, 2007) were used to amplify gene sequences. Two of the sequences obtained from the PCR products allowed a first view of the distribution of this gene among sulfur oxidizing *Beta-proteobacteria* and its phylogenetic relationships. Phylogenetic trees constructed with diverse *soxB* gene homologues available in the databases revealed that *Gamma-proteobacteria* and *Alpha-proteobacteria* were not monophyletic and formed at least four and two clusters respectively, while *Beta-proteobacteria* were shown to be monophyletic (Anandham *et al.*, 2008; Meyer *et al.*, 2007; Petri *et al.*, 2001). With introduction of two novel *soxB* gene homologues of *Burkholderia* spp. (strains, GAH2 and GAH4) in the database, the SoxB phylogenetic tree presented in this report has established for the first time that *Beta-proteobacteria* were not monophyletic but formed at least two distinct groups (Fig. 4.37). *Thiobacillus thioparus*, *T. denitrificans*, *Herminimonas arsenicoxydans*, *Methylibium petroleiphilum*, and *Pandorea* spp. formed a distinct cluster (cluster I), congruent to the cluster obtained in an earlier study (Anandham *et al.*, 2008) and the newly described *Burkholderia* strains GAH2 and GAH4 formed a new group among *Beta-proteobacteria* (cluster II). The *soxB* gene homologues of GAH2 and GAH4 were closest to *soxB* homologue of phototrophic sulfur-oxidising *Allochromatium vinosum* ABE01359. A partial *sox* gene cluster constituted of *soxB* and *soxXA*, detected in by PCR assay, was predicted to be essential for reduced sulfur compound oxidation in *A. vinosum* (Friedrich *et al.*, 2001). Inactivation of *fcc*A and *aprBA* genes in *A. vinosum*, which codes for cytochrome subunit of flavocytochrome c-sulfide dehydrogenase and adenosine-5′-phosphosulphate reductase respectively, left hydrogen sulfide and sulfite oxidation unaffected (Dahl, 1996; Reinartz *et al.*, 1998). Event of lateral *soxB* gene transfer is therefore the most reasonable explanation for the inferred close relationship of *soxB* from thiosulfate -metabolising *Burkholderia* spp. (strains GAH2 and GAH4) and *A. vinosum* species that are distantly related on the basis of the 16S rRNA gene phylogeny.

Interestingly, amplicons generated by degenerate soxB primers produced sequence similar to the genes for ABC transport protein (ATP binding) in GAH1,

GAH5 and GMX7 (Fig. 4.29-4.34). Part of gene sequence in GMX7 (Fig. 4.35) was found to possess truncated portion of thiosulfate binding protein. ABC systems constitute probably the largest superfamily of proteins ever detected in living organisms. Most ABC systems are primary transporters that transport unidirectionally molecules through membranes (Schneider and Hunke, 1998). The ABC-type drug transporters also belong to the ABC superfamily, the members of which all contain a highly conserved ATP-binding cassette (Higgins, 1992; Van Veen *et al.*, 2001). They utilize the energy released by ATP-hydrolysis to pump cytotoxic compounds out of the cell. The substrates handled by these transporters are extraordinarily varied, ranging from small molecules (ions, carbohydrates, amino acids, antibiotics) to macromolecules (polysaccharides, proteins). Purified ABC proteins or modules have been shown to bind and hydrolyze ATP (Dayan *et al.*, 1996; Higgins *et al.*, 1985; Ko *et al.*, 1994; Morbach *et al.*, 1993; Muller *et al.*, 1994; Shimabuku *et al.*, 1992; Thiagalingam and Grossman, 1993), making reasonable the hypothesis that they couple the energy of ATP hydrolysis to the movement of substrates by an unknown mechanism.

As the acid-tolerant heterotrophic isolates GAH1, GAH5 and GMX7 isolated from AMD possess facultative thiosulfate chemolithoautotrophic property, the sequence obtained for ATP binding ABC transporters, probably participate in the sulfur metabolism. A number of transport and binding genes appear to play roles in sulfur metabolism of *A. ferrooxidans*. Recently in one of the study it was seen that the the expression level of transport and binding protein encoding genes for ABC transporter was up-regulated when *A. ferrooxidans* culture was grown on sulfur (Xia *et al.*, 2010). Candidate genes potentially encoding uptake of inorganic sulfate were discovered, including a sulfate permease belonging the SulP family of the major facilitator superfamily of transporters (MFS) and a possible sulfate/thiosulfate/molybdenate ATP binding cassette (ABC)-type transporter in *A. ferrooxidans* (Valdés *et al.*, 2003).

In the last and final Chapter 5, the authoress has attempted to relate heavy metal tolerance phenotype with the plasmid (if any) determinants present in any of the acid-tolerant heterotrophic isolates. In the present study, five plasmids, sizes approximately 55 kb, 23kb, 4 kb, 2 kb, and 1 kb, were reported from the *Acidiphilium* strain DK2AH2

(Fig.5.1). Plasmids of acidophilic heterotrophs of the genera *Acidiphilium* and *Acidocella*, were demonstrated by earlier authors to carry metal resistant genes. While genes conferring arsenic resistance in *Acidiphilium multivorum* are similar to those analyzed from other sources, there is no sequence similarity with the reported Cd- and Zn-resistant genes with cloned plasmid DNA fragments from *Acidiphilium symbioticum* KM2 and *Acidocella* GS19h strains which were found to have link with Cd and Zn resistance phenotype. Such observations have indicated some novel aspects of metal resistance in acidophilic bacteria. *Acidiphilium organovorum* contained at least three distinct plasmids; one of them was > 30 kb, and the other two were < 4.0 kb (Lobos *et al.*, 1986). Another AMD strain *Acidiphilium multivorum* AIU 301 was shown to harbour multiple plasmids of different sizes. One of the plasmids, pKW301A (56 kb), when transferred into *Escherichia coli* JM109 by electroporation, an *E. coli* transformant carrying pKW301 displayed resistance to sodium arsenite, sodium arsenate, and mercuric(II) chloride (Suzuki et. al., 1997). Another acidophilic bacterium, *Acidiphilium symbioticum* H8, resistant to high levels of several heavy metals, hydrophobic agents, and organic solvents, was found to host a 9.6 kb plasmid pASH8. One of the clones of the plasmid bank was found to encode a single putative open reading frame (ORF) showing significant homology to several rusticyaninA1 proteins. Another clone, pASH8, encoded for a 43-kDa protein having conserved domain homology with several outer envelope TolC proteins, functionally complemented an *Escherichia coli* tolC mutant strain, making it resistant to several toxic hydrophobic agents, earlier for which it was sensitive. The third clone encoded for a putative 318-aa AcrA (acriflavine resistance protein A) protein and the clone was resistance to plasmid curing dye acriflavine. The clone contained a truncated ORF, showing significant homology to cation-efflux pump AcrB (Singh et. al., 2010). *A. multivorum* AIU301T, on whole genome analysis, was found to contain nine replicons; one circular chromosome (3,749,411 bp, 67.6% G+C, 3,449 ORF) and **eight circular plasmids** pACMV1 (271,573 bp, 62.9% G+C, 284 ORF), pACMV2 (65,564 bp, 61.9% G+C, 69 ORF), pACMV3 (54,248 bp, 61.2% G+C, 61 ORF), pACMV4 (40,588 bp, 60.1% G+C, 44 ORF), pACMV5 (14,328 bp, 59.0% G+C, 19 ORF), pACMV6 (12,125 bp, 59.6% G+C, 14 ORF), pACMV7 (5,178 bp, 57.6% G+C, 8 ORF) and pACMV8 (1,728 bp, 60.9% G+C, 1 ORF). Several genes with putative involvement in heavy

metal-transport, and three operons (which appeared to contribute to arsenic resistance) were identified from the chromosome and circular plasmids. *A. multivorum* AIU301T chromosome showed overall high conservation of synteny with *A. cryptum* JF-5 chromosome which has been sequenced, excluding a few regions (http://www.nbrc.nite.go.jp/e/index.html). The largest plasmid detected and isolated from *Acidiphilum* strain DK2AH2 was named pDK2AH2 (55 kb); restriction digested and gene bank was constructed with *EcoR*I fragments of the same. Three gene bank clones, pDK2-3, pDK2-5 and pDK2-9, were partially sequenced. Sequence analyses of pDK2-5 (Fig. 5.9 and 5.11) and pDK2-9 (Fig. 5.13 and 5.15) revealed significant similarities (>80% homologous) to the hypothetical protein of *Acidiphilium multivorum* AIU301 plasmid and hypothetical protein of *A. cryptum* JF5 (Fig. 5.10a,b and 5.14a,b). Translated sequences of pDK2-5 and pDK2-9 revealed that -2 frame of the sequence contained an open reading frame (ORF) (164 and 101 amino acid long respectively), showing 100% and 81% homology respectively with hypothetical protein APM_0575 of *Acidiphilium* sp. PM. Partial nucleotide sequence of pDK2-3 on in-silico translation revealed that +2 frame of the sequence contained an ORF showing 74% homology with hypothetical protein of uncultured *Sphingobacteria* and glycosyl transferase protein of uncultured *Flavobacteria* (Fig. 5.8a); and -1 frame showing 71% homology with conserved domain protein of *E. coli* MS 69-1 (Fig. 5.8b).

As natural consequence, the *Acidiphilium* strains inhabiting AMD are often subjected to several physico-chemical stresses such as high and low temperatures and exposure to various heavy metals, etc. Stress induced changes in cell morphology has been observed with *Acidiphilium symbioticum* H8 where maximum alterations in size occurred when the bacterium was exposed to sub-inhibitory concentrations of Cu and Cd. (Chakravarty and Banerjee, 2008). Such changes in cellular morphology are generally observed with biofilm forming strains and biofilm formation is also induced by stress factors. It may be so that the authors (Chakravarty and Banerjee, 2008) have missed the evidences to relate such changes with biofilm formation; and biofilm formation is also related to metal resistance in bacteria. In one of the successive studies on *Acidiphilium* strains, extracellular polymeric substances (EPS) that constitute biofilm was reported from *Acidiphilium* 3.2Sup(5) (Tapia *et al.* 2009). Electron

microscopy (both TEM and SEM) enabled to determine the degree of attachment and the growth of the biofilm overtime on two solid supports: carbon fibre cloth and graphite rods (Tapia *et al.* 2009). In the present study it was shown that *Acidiphilium* sp.DK2AH2 is also capable of forming biofilm on glass surface. Extracellular polymeric substances (EPS) produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix. The key functions of EPS comprise the mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration. Many chromosomal genes have now been shown to be involved in different stages of biofilm development. By contrast, the contribution of the extra-chromosomal plasmid gene pool (representing as much as 10-20 % of total bacterial DNA) to biofilm biology is poorly understood. As a consequence, with the exception of biotechnology application and antibiotic resistance spread, the role of plasmids in bacterial ecology has been largely overlooked. Even though conjugation has been studied first and foremost in liquid, most natural bacterial populations are found associated with environmental surfaces in complex multispecies communities called biofilms. Biofilms are ideally suitable for the exchange of genetic material of various origins, and it has been shown that bacterial conjugation occurs within biofilms. It was shown that conjugative plasmids contribute directly to the capacity of the bacterial host to form a biofilm. Natural conjugative plasmids expressed factors that induced planktonic bacteria to form or enter biofilm communities, which favour the infectious transfer of the plasmid. This general connection between conjugation and biofilms suggested that medically relevant plasmid-bearing strains are more likely to form a biofilm (Ghigo, 2001). Though biofilms have been envisaged to be involved in persistence, the process of biofilm formation remained complex and poorly understood in *E. coli* O157:H7. A mini-Tn5 transposon insertion library was constructed in strain EDL933 and screened for biofilm-negative mutants using a microtiter plate assay in order to comprehend the genetics of this process. Of 11,000 independent insertions 95 of (0.86%) them failed to produce biofilm and transposon insertions were located in 51 distinct genes/intergenic regions that must be involved either directly or indirectly in biofilm formation. All of the 51

biofilm-negative mutants showed reduced biofilm formation on both hydrophilic and hydrophobic surfaces. Thirty-six genes were unique including genes on the virulence plasmid pO157. The type V secreted autotransporter serine protease EspP and the enterohemolysin translocator EhxD were found to be directly involved in biofilm formation. In addition, EhxD and EspP were also important for adherence to T84 intestinal epithelial cells, suggesting a role for these genes in tissue interactions in vivo (Puttamreddy *et al.*, 2010). In the present study it was shown that plasmids in *Acidiphilium* sp. DK2AH2 on getting cured the biofilm forming ability is lost to a great extent coupled to the sensitivity towards Cu(II) andZn(II). Significant advances have been made to reveal new insights into biofilms and their constituents. The expansion of knowledge in relation to molecular mechanisms involved in bacterial-mineral attachment may be relevant in the enhancement of bioleaching timing and efficiency

Finally, it may be concluded in a way that isolation and identification of acid-tolerant bacteria from AMD samples from Garubathan, India, have revealed diversity of the strains in terms of phylogenetic affiliation and thiosulfate chemolithotrophy. These strains may provide genetic resources for the development of novel biotechnological processes.

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