General Introduction

Acidithiobacillus ferrooxidans (formerly, *Thiobacillus ferrooxidans*, Kelly and Wood, 2000) is a gram negative acidophilic chemolithotroph, deriving energy and electrons from the oxidation of ferrous iron and/or sulfur and other various reduced sulfur compounds. The organism is actively involved in the acid production in copper and coal mine and certain other Acid Mine Drainage (AMD) environments and hence being utilized in the solubilization of copper and gold in bioleaching operations (Holmes, 1998; Rawlings, 1997). The organism is an obligate autotroph and fixes CO_2 by Calvin-Bassham pathway (Drobner *et al.*, 1990). Complete genome sequence of *A. ferrooxidans* has been recently published (Valdes *et al.*, 2008)

1.1 Geological description of the sampling site: the original habitat of the *Acidithiobacillus ferrooxidans* strains used in the present study

The geological formations of Darjeeling District consist 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).

The minerals of the Darjeeling district Himalaya include coal, graphite, iron and copper ores but none has been found to be economically profitable. Iron ore, varying from strong ferruginous clay to an impure brown hematite, is found at Lohagarh. High grade iron ores free from sulfur and phosphorus is found in Samalbong lying towards the east of river Teesta. Copper ore chiefly chalcopyrite, occur in the rocks of the Daling series near Ranihat, Baffupani etc (Dash, 1947). Saha, Chakraborty and Bandopadhyaya have done the detailed investigation for lead-zinc ore in the area around Garubathan (26° 59': 88° 42') in Kalimpong sub-division whereby the probable reserve estimated is 0.602 million tones with an average of 3.54% Pb, and 2.70% Zn, and that of float ore is 2,910 tones, with an average of 7.14% Pb and 2.87% Zn. (records of GSI, vol. 109, Part I, 1982).

1.2 Microbial ecology of mineral rich acidic environments

Mineral rich acidic environments are of especial interest because, in general, the acidic condition of the habitat is the result of microbial metabolism and not a condition imposed by the system as in case of other extreme environments (Gonzalez-Toril *et al.*, 2001). The microorganisms that are capable of oxidizing sulfur and iron usually produce

sulfuric 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).

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; Lopez-Archilla *et al.*, 2001). Recently molecular approaches to examine the microbial diversity of these habitats have also been performed (Goebel and Stackebrandt, 1994; 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).

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 sulfur and/or iron-oxidizers, such as *Sulfolobus, Acidianus, Metalosphaera, Sulfurisphaera* 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).

1.3 Physiology of *Acidithiobacillus ferrooxidans*

Acidithiobacillus ferrooxidans is a gram-negative, rod shaped bacterium belonging to γ-proteobacterium that has been shown to be active in the solubilizatin of copper and in the processing of refractory gold ores in bioleaching operations (Rawlings, 1997; Kelly and Wood, 2000; Rohwerder *et al.*, 2003). The bacterium thrives optimally at 30°C and pH 2.0, but can grow at pH 1.0 or below (Rohwerder *et al.*, 2003). It is a chemolithotroph, deriving energy and electrons from the oxidation of ferrous iron and/or sulfur and reduced sulfur compounds using oxygen as the ultimate electron acceptor (Ingledew, 1982). A number of early reports regarding its ability to grow heterotrophically are considered to be false. Actually heterotrophic acidophile *Acidiphilium* that remains associated with *A. ferrooxidans* was mistaken as *A. ferrooxidans* (Harrison, 1984).

CO_2 fixation

A. ferrooxidans is obligately autotrophic and fixes carbon dioxide via the Calvin-Benson-Bassham reductive pentose phosphate cycle (Calvin cycle) using energy and reducing power derived from the oxidation of iron and sulfur (Gale and Beck, 1967; Kelly and Harrison, 1989; Drobner *et al.*, 1990). Several enzymes of Calvin cycle, including D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) have been described for *A. ferrooxidans* (Gale and Beck, 1967). However one strain has been reported to grow mixotrophically in presence of iron and glucose but in general, organic compounds inhibit the growth of *A. ferrooxidans* (Barros *et al.*, 1984; Alexander *et al.*, 1987). Moreover, low concentration formic acid can replace carbon dioxide as carbon source in most but not all the *A. ferrooxidans* strains (Pronk *et al.*, 1991b).

Nitrogen fixation

A. ferrooxidans is capable of fixing atmospheric nitrogen also, the property which is likely to be a general property of these bacteria (Mackintosh, 1978). An interesting feature is that the enzyme nitrogenase is oxygen labile and therefore obligate aerobic bacterium like *A. ferrooxidans* must have an extensive system to protect nitrogenase (Postgate, 1982). Mackintosh (1978) demonstrated that nitrogen fixation in *A. ferrooxidans*

growing in presence of ferrous iron is possible when oxygen supply is critical. Oxygen had to be provided in a condition, sufficient to allow enough iron oxidation to supply energy for nitrogen fixation but insufficient to inhibit nitrogenase activity. A putative nitrogenase gene cluster (*nifH-D-K-fer1-fer2-E-N-X*) that potentially encode the nitrogenase complex and proteins involved in the synthesis of the nitrogenase MoCo cofactor has been reported (Valdes *et al.*, 2003).

1.4 Metabolism of Iron, Elemental Sulfur and Reduced sulfur compounds

Iron oxidation

A. ferrooxidans obtains energy by oxidizing ferrous ions or elemental sulfur and/or reduced sulfur compounds to ferric ion or sulfuric acid respectively. Since in aerobic condition ferrous ion is available only in acidic pH condition, the organism grows best at pH 1.5 to 2.5 with oxygen as an electron acceptor.

There are several models for the iron oxidation pathway but none has yet been proven unequivocally (Appia-Ayme *et al.*, 1999; Yamanaka and Fukumori, 1995). Recently with the help of bioinformatics analysis of the genome sequence of *A. ferrooxidans* it has been possible to identify the components of electron transport chain involved in iron and sulfur oxidation (Valdes *et al.*, 2008). Genes encoding for iron oxidation are organized in two transcriptional units, the *petI* and *rus* operons. The *petI* operon encodes the three subunits of cytochrome bc_1 complex, a predicted short chain dehydrogenase (Sdr) of unknown function and a *cytochrome* c_4 that receive the electron from rusticyanin and pass them to the bc_1 complex (Levican *et al.*, 2002; Valdes *et al.*, 2008; Bruscella *et al.*, 2007). The *rus* operon encodes two c-type cytochromes (Cyc1 and Cyc 2), whereby the former is a part of aa_3-type cytochrome oxidase (CoxBACD) and the latter is a component of blue copper protein rusticyanin (Appia-Ayme *et al.*, 1999). On the basis of transcriptional, biochemical and genetic studies it has been proposed that electrons from ferrous ion flow through Cyc2 to rusticyanin from where some of the electrons pass through c-cytochrome Cyc 1 to aa_3 cytochrome oxidase and some electrons are used to regenerate NADH through reverse electron flow (Holmes and Bonnefoy, 2006).

The levels of rusticyanin, which is a 16.5 kDa soluble, blue copper protein and found in the periplasm of *A. ferrooxidans* growing in iron, have been found to be reduced in the bacterium growing on the medium containing elemental sulfur as energy source

(Jedlicki *et al.,* 1986), suggesting the differential expression of the proteins (Ramirez *et al.,* 2004).

Another protein, Iron oxidase (Iro) (63 kDa) related to iron oxidation was also isolated and purified from *A. ferrooxidans* (Fukumori *et al.,* 1988). The enzyme was found to reduce *A. ferrooxidans* ferricytochrome c_{552} in presence of Fe^{2+} ions under acidic condition. The protein containing 90 amino acids has a high relationship with the high-redox-potential iron-sulfur proteins (HiPIP) of *Rhodopseudomonas marina, R. globiformis and Rhodomicrobium vannielii* (Kusano *et al.,* 1992). It was postulated that the protein encoded by *iro* gene is the first electron acceptor from Fe (II) ions (Bruscella *et al.,* 2005; Cavazza *et al.,* 1995). However transcriptional studies of *iro* gene in *A. ferrooxidans* ATCC 33020 suggested that it may be involved in sulfur oxidation and is related to *petII* operon which is believed to be involved in sulfur oxidation (Kusano *et al.,* 1992; Quatrini *et al.,* 2005).

Oxidation of reduced sulfur compounds

The oxidative and electron transport pathways for oxidation of reduced inorganic sulfur compounds are more complicated than those for Fe (II) oxidation, making their prediction and elucidation more difficult (Pronk *et al.,* 1990). Beside that some steps of inorganic sulfur oxidation occur spontaneously, without enzymatic catalysis which makes the study more difficult.

The bacteria use any of the major forms of reduced sulfur, including sulfide (S^{2-}), elemental sulfur (S^0 or cyclo-octa-sulfur S_8), thiosulfate ($S_2O_3^{2-}$), and sulfite (SO_3^{2-}) which will be ultimately oxidized to sulfate (SO_4^{2-}).

Genes encoding enzymes and electron transfer proteins predicted to be involved in the oxidation of reduced inorganic sulfur compounds (RISCs) were detected in the genome. Based on the genome analysis some of the missing as well as novel enzymes are predicted (Valdes *et al.,* 2008).

In *A. ferrooxidans* the components involved in RISC metabolism have been recently detected. Those which has been experimentally validated are: *petII* operon and quinol oxidases; a sulfide/quinine oxidoreductase (SQR) encoded by *sqr* gene and a tetrathionate hydrolase (Tth) encoded by *tetH* gene. SQR is suggested to be involved in the

oxidation of sulfide to sulfur (Brasseur *et al.*, 2004; Bruscella *et al.*, 2007) and it is believed to be involved in the oxidation of tetrathionate (Kanao *et al.*, 2007). Another protein, rhodanese enzyme, first reported by Tabita *et al.* (1969), has been postulated to be involved in thiosulfate metabolism. The protein is 21 kDa and its expression has been found to be accelerated during growth in pyrite, thiosulfate, elemental sulfur etc. but greatly repressed during growth in ferrous iron (Ramirez *et al.*, 2002). The enzyme is thought to be involved in the splitting of thiosulfate into sulfur and sulfite however, experimental proof is yet to be established for its activity in the sulfur metabolism (Valdes *et al.*, 2008). Nevertheless five genes have been predicted to encode rhodanese (thiosulfate sulfur transferase) enzyme that remain dispersed in the genome (Acosta *et al.*, 2005). Another protein homologous to rhodanese is thiosulfate/quinine oxidoreductase (TQR) which is encoded by *doxDA* gene. The other proteins involved in RISC metabolism whose functions have been experimentally demonstrated are sulfur dioxygenase (SDO) and sulfite oxidase (SO) that oxidizes sulfite to sulfate in *Acidithiobacillus ferrooxidans* (Rohwerder *et al.*, 2003; Silver and Lundgren, 1968; Vestal and Lundgren, 1971). However, the genes for these enzymes have not been detected in the *A. ferrooxidans* type strain ATCC 23270 (Valdes *et al.*, 2008).

Oxidation in anaerobic condition

Several strains of *A. ferrooxidans* have been reported to grow anaerobically. Some derive energy by utilizing ferric iron as the electron acceptor and formate, sulfur or hydrogen as electron donor while some use sulfur, reduced sulfur compounds as electron acceptor obtaining the electron from hydrogen (Das *et al.*, 1992; Pronk *et al.*, 1991a; Sugio *et al.*, 1985; Ohmura *et al.*, 2002).

The reduction of sulfur to hydrogen sulfide was predicted to be accomplished by the enzyme called, sulfur reductase (Ng *et al.*, 2000). A gene cluster for the probable sulfur reductase has been detected in *A. ferrooxidans* ATCC 23720, the amino acid sequence of which shows a great similarity with the gene cluster to be responsible for sulfur reduction in *Acidianus ambivalens* (Laska *et al.*, 2003). It has been postulated that the enzyme hydrogenase and sulfur oxidase can remain associated to form a super-complex which facilitates the use of both hydrogen as well as sulfur as electron donor and electron acceptor respectively (Valdes *et al.*, 2008). There are reports of utilizing hydrogen as a source of energy. Based on the classification by Vignais *et al.* (2001) in *A. ferrooxidans*

four different types of hydrogenase have been grouped. Group 1 [NiFe]-hydrogenase is a membrane-bound respiratory enzyme which is used by the cells to derive energy from molecular hydrogen. Group 2 [NiFe]-hydrogenase is the cytoplasmic enzyme and is induced during nitrogen fixation. The enzyme use the molecular hydrogen as source of energy, released during the fixation of nitrogen (Appel *et al.*, 2000). Group 3 hydrogenase is also a cytoplasmic protein which in association with other protein binds to soluble cofactors such as NAD, cofactor 420, and NADP (Vignais *et al.*, 2001). The enzyme possibly plays a role in recycling of redox cofactors as has been suggested for *Alcaligenes eutrophus* (Lenz and Friedrich, 1998). Group 4 hydrogenase of *A. ferrooxidans* shows great similarity with the H_2-evolving hydrogenase complex of other microorganisms like *Methanococcus barkeri* (Hedderich, 2004). In *Escherichia coli* it has been reported that group4 hydrogenase binds with formate dehydrogenase to form formate dehydrogenase complex that catalyzes the oxidation of formate with the evolution of oxygen (Andrew *et al.*, 1997).

A genome-based model for the oxidation of iron and reduced sulfur compounds has been constructed (Valdes *et al.*, 2008) (Fig 1).

1.5 ATP production and uphill electron transport in *A. ferrooxidans*

Unlike the other chemolithotrophic organisms, *A. ferrooxidans* while growing on ferrous iron under acidic condition does not derive H^+ from its oxidizable substrate but obtains it from the environment. Since the cytoplasmic pH is maintained at 6.5, while growing in the environment of 2.0, a natural pH gradient of 4.5U is maintained in *A. ferrooxidans* cells (Cox *et al.*, 1979). This gradient is then utilized to synthesize ATP molecules with the help of Mg^{2+} dependent ATPase (Apel *et al.*, 1980). The fact was substantiated by the increase in the pH of suspending medium due to uptake of the H^+ from the medium with the production of ATP when ferrous iron was supplied as oxidizable substrate (Apel *et al.*, 1978; Cox *et al.*, 1979). Therefore it was suggested that the electrons from Fe^{2+} are taken up by the cells to neutralize the H^+ coming from the environment.

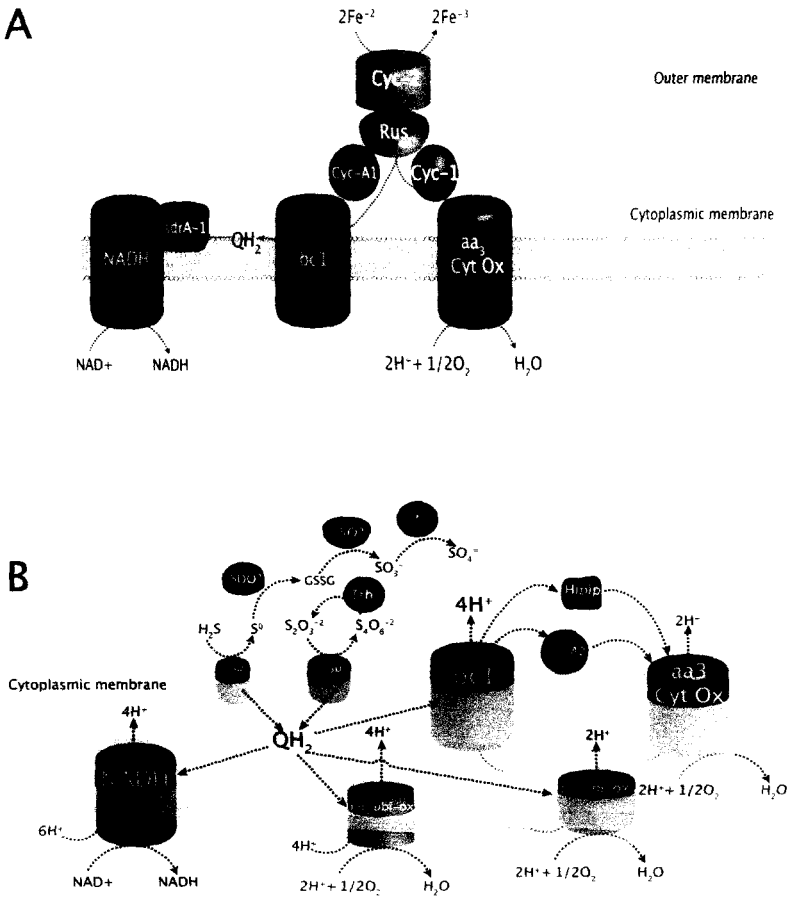

Fig 1: Schematic representation of enzymes and electron transfer proteins involved in the oxidation of (A) ferrous iron and (B) reduced inorganic sulfur compounds (RISCs) (Valdes *et al.*, 2008). NAD: Nicotinamide adenine dinucleotide; sdrA-1: Short chain dehydrogenase of unknown function; QH_2: Quinole; bc1: Cytochrome bc1; Cyc: Cytochrome; Rus: Rusticyanin; Cyt aa_3 Ox: Cytochrome oxidase; SQR: sulfide/quinine oxidoreductase; TQR: thiosulfate/quinine oxidoreductase; SDO: Sulfur dioxygenase; SO: sulfite oxidase.

Though very little energy is available when the bacterium is grown in ferrous iron, the organism fixes CO_2 during the growth. Therefore the oxidation of ferrous iron must be coupled to the reduction of $NAD(P)^+$ required for the fixation of CO_2 and other anabolic processes. Since the electron reduction potential of Fe^{2+}/Fe^{3+} is 770 mV at cytoplasmic pH of 6.5 and that of $NAD(P)^+/NAD(P)H$ is -305 mV, the electron cannot move from Fe^{2+} to NAD(P) without the expense of energy (Cox *et al.*, 1979). The experimental results showed that this uphill pathway involves a bc_1 and an NADH-Q oxidoreductase complex functioning in reverse order, using the energy derived from ATP hydrolysis (Elbehti *et al.*, 2000). A tentative model for the reverse electron flow has been proposed. According to the

model the electrons arising from Fe^{2+} pass through cytochrome oxidase to oxygen and establish the proton motive force which is utilized by ATP synthase in the ATP synthesis when the ATP/ADP ratio is low. The ratio is maintained low as long as the cells are involved in active anabolic processes. However when the carbon availability is limited and ATP is no longer used, ATP/ADP ratio increases. It has been hypothesized that this increased ATP/ADP ratio then auto-regulates the cytochrome *c* oxidase by decreasing its activity. As a result proton motive force is decreased. This causes ATP synthase to function like ATPase and ATPs are hydrolyzed, the energy released during the hydrolysis is utilized in the generation of proton motive force which will then be used for the reverse electron transfer through bc_1 to $NAD(P)^+$ leading to the formation of NAD(P)H required for CO_2 fixation (Elbehti *et al.*, 2000)

1.6 Bioleaching activity

The mechanism for the oxidation and dissolution of metal sulfides has been determined (Schippers and Sand, 1999; Sand *et al.*, 2001). It has been proposed that the bacterial leaching of metal sulfides proceeds in two indirect mechanisms via thiosulfate or via polysulfide and sulfur pathway ultimately producing sulfuric acid (SO_4^{2-}) that directly or indirectly dissolves the metal ions helping in the bioleaching.

Thiosulfate pathway

This pathway occurs during the oxidation of acid non-soluble metal sulfides (pyrite FeS_2, Molybdenite MoS_2, and tungstenite WS_2). In this mechanism the main sulfur intermediate is thiosulfate. The bond between the metal sulfides is broken by six successive transfer of one electron from Fe (III) ions, producing thiosulfate and Fe (II) (Luther, 1987; Moses *et al.*, 1987; Schippers *et al.*, 1996):

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \longrightarrow S_2O_3^{2-} + 7\,Fe^{2+} + 6\,H^+$$

Thiosulfate so produced is oxidized to sulfate via tetrathionate and other polythionates. It has also been reported that significant amount of elemental sulfur may be produced in absence of sulfur oxidizing bacteria (Schippers *et al.*, 1996).

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \longrightarrow 2SO_4^{2-} + 8\,Fe^{+2} + 10\,H^+$$

Polysulfide pathway

This pathway is operated during the oxidation and dissolution of acid soluble metal sulfides (sphalerite ZnS, galena PbS, arsenopyrite FeAsS, Chalcopyrite $CuFeS_2$, and Haurite MnS_2). This mechanism does not necessarily require the presence of Fe (III) ions rather the bond between metal and sulfur moiety is broken by proton attack and hydrogen sulfide is formed. However in presence of Fe (III) ions the sulfur moiety is oxidized in a one-electron step concomitantly with the proton attack. Therefore the first sulfur compound that is produced is likely to be sulfide cation (H_2S^-) which can spontaneously dimerize to form disulfide (H_2S_2). Further dimerization results in the formation of polysulfide (H_2S_n) (Steudal, 1996). Polysulfide nevertheless is finally oxidized to sulfate. The mechanism follows the following reactions:

$$MS + Fe^{3+} + H^+ \longrightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+} \quad (n \geq 2)$$

$$0.5\ H_2S_n + Fe^{3+} \longrightarrow 0.125\ S_8 + Fe^{2+} + H^+$$

$$0.125\ S_8 + 1.5\ O_2 + H_2O \longrightarrow SO_4^{2-} + 2\ H^+$$

1.7 Genetics of *Acidithiobacillus ferrooxidans*

The sequencing of *A. ferrooxidans* type strain ATCC 23720 has been recently completed in TIGR (The Institute for Genomic Research now called J. Craig Venter Institute). The total genome size of the bacterium has been found to be 2,982,397 bp with G+C content of 58.77%. A total of 3217 protein-coding genes have been predicted of which 64.3% (2070) have been assigned to have putative functions. The genome contains two ribosomal operons and 78 tRNA genes. There was no plasmid detected in the type strains (Valdes *et al.*, 2008).

Several chromosomal genes of *A. ferrooxidans* have been isolated, cloned and expressed in other bacteria such as *E. coli*, *Salmonella* etc. After getting the total genome sequence of the organisms and expression of different genes in other bacteria it has been

found that *A. ferrooxidans* has a complete set of genes required for living as chemolithoautotrophic organism . Genes encoding for three different types of RuBisCO have been determined for *A. ferrooxidans*. The genes coding for form-I type and form-II type of RuBisCO have been found to be *rbcL1-rbcS1* and *rbcL2-rbcS2* respectively (Kusano *et al.,* 1991). These two types of RuBisCO have different catalytic properties, and have been shown to be differentially expressed depending on whether the organism was grown on iron- or sulfur-containing medium (Quatrini *et al.,* 2006). Another gene for RuBisCo-like protein has been identified which is suggested to be involved in stress response.

Earlier it was predicted that out of several genes required for the biosynthesis of most amino acids, ten genes are missing in *A. ferrooxidans*. Out of these ten genes seven have now been detected. They are: 6-phosphofructokinase, pyruvate dehydrogenase, shikimate kinase, homoserine kinase, N-acetyl-gamma-glutamil-1-phosphate reductase, pirroline-5-carboxilate reductase and asparagine synthase. The unidentified three genes that have been identified in *E. coli* are ornithine cyclodeaminase, aromatic-amino-acid transaminase and arogenate dehydrogenase (Valdes *et al.,* 2008).

The genes involved in nitrogen fixation in *A. ferrooxidans* have been isolated and characterized. Under the conditions of limited nitrogen supply the enzyme responsible for ammonia assimilation is glutamine synthase encoded by *glnA* that converts l-glutamate to L-glutamine is activated (Tyler, 1978). Other genes predicted to be responsible for ammonia transfer located in *A. ferrooxidans* genome is class-I glutamine amidotransferase encoded by *amt1* and *amt2* genes. The enzyme in other organisms has been shown to transfer ammonia derived from the hydrolysis of glutamine to other substrates (Valdes *et al.,* 2008). The nitrogenase gene from *A. ferrooxidans* has been isolated and characterized.

Another important gene that has been isolated from *A. ferrooxidans* is *recA* gene. The gene function was studied by cloning and expressing in *E coli,* and it was found that RecA protein can exhibit both DNA repair associated and recombination activities (Ramesar *et al.,* 1989). Similarly *A. ferrooxidans* has been found to contain genes for two glutamyl-t-RNA synthetase which are required as an intermediate in protein synthesis in many organisms (Valdes *et al.,* 2008). Beside that rRNA genes encoding 16S and 23S rRNAs have been isolated (Salazar *et al.,* 1989). The operon of rRNA genes has been found to be similar to that of rrnB operon of *E. coli.* The spacer region between 16S and

23S rRNA has also been sequenced. The region has been found to contain genes for isoleucine- and alanine-tRNA (Venegas *et al.*, 1988). The gene for RNase P encoded by *rnpB* gene has also been isolated and sequenced which shows similarity to the γ-proteobacterial group (Takeshima *et al.*, 1989; Brown *et al.*, 1991)

Natural plasmids of *A. ferrooxidans*

Although no plasmid was detected in the type strain of *A. ferrooxidans* ATCC 23270, there are number of reports of the occurrence of natural plasmids in strains of *A. ferrooxidans* isolated from different parts of the world. The occurrence of plasmid has been reported from the strains of USA, Bulgaria, South Africa, Chile, Italy etc. isolated from sites heavily laden with heavy metals (Martin *et al.*, 1981; Rawlings *et al.*, 1983; Sanchez *et al.*, 1986; Valenti *et al.*, 1990). Some workers tried to correlate the presence of plasmid with the increase in uranium resistance. It was found that the strains exhibiting highest uranium resistance were all containing 20 kbp plasmid. This resistance was found to disappear with the loss of the plasmid (Martin *et al.*, 1983). Other attempts have also been made to correlate the presence of plasmid with the metal resistance, but so far none have been successful.

There are reports of cloning and expression as well as mobilization of plasmids isolated from *A. ferrooxidans* in *Escherichia coli* (Rawlings *et al.*, 1984; Rawlings and Woods, 1985). Two *A. ferrooxidans* plasmids have been characterized in detail. Holmes *et al.* (1988) isolated a 6.7 kb mobilizable plasmid from *A. ferrooxidans* and cloned in pBR322. The plasmid was named as pTF1. Drolet *et al.* (2006) found that a 2.8 kbp region is required for the mobilization. The mobilization of plasmid was found to be dictated by two proteins, MobL (42.6 kDa) and MobS (11.4 kDa) which were found to be related with MobA (49%) and MobC (53%) of the broad-host range IncQ plasmids.

Another plasmid that has been studied in more detail is pTF-FC2. It is a 12.2 kb, broad host range plasmid, highly mobilizable and was isolated from arsenic resistant culture of *A. ferrooxidans.* The nucleotide sequence of pTF-FC2 shows that it contains three regions: a replicon region, a mobilization region and a transposon-like element (Rawlings *et al.*, 1993). The replicon region contains five or six genes and an oriV which is closely related to oriV of the broad-host range IncQ plasmids (Lin *et al.*, 1987). The mobilization region is located at 3.5 kbp DNA fragment and consists of an oriT and five

mob genes (Rohrer and Rawlings, 1992). Three mob proteins (Mob A, Mob C, and Mob D) are required for the mobilization whereas two mob proteins (Mob B and Mob E) determine the frequency of mobilization. The transposon-like regions of pTF-FC2 is bordered by two identical inverted repeats of 38 bp having a similarity to the inverted repeats of Tn*21*. The region between these two inverted repeats has been found to contain several ORFs, one of which is similar to the MerR regulatory proteins of the mercury reductase operon and another has the similarity with the *cmlA* (Chloramphenicol resistance) gene of Tb*1696* of plasmid R1033 from *Pseudomonas aeruginosa* (Bissonnette *et al.*, 1991; Silver and Walderhaug, 1992; Rawlings *et al.*, 1993). Other ORFs showed the relationship with the N-terminal region of resolvase (*tpnR*), C- terminus of Tn*21* transposase with 85% and 78% similarity respectively (Sherrat, 1989)

Insertion Sequence elements of *A. ferrooxidans*

Insertion Sequences (ISs) are prokaryotic autonomous transposable elements that encode a transposase gene mediating their ability to move from one to another locus. They are the smallest and most frequent transposable elements in prokaryotes where they play an important evolutionary role by promoting gene activation and genome plasticity. Their genomic abundance varies by several orders of magnitude for reasons largely unknown and widely speculated. ISs are widespread among prokaryotic genomes. Out of 262 representative genomes surveyed, ISs are present in more than 75% of them (Touchon and Rocha, 2007). It was Holmes and his coworkers (1988) who for the first time discovered and isolated two families of insertion sequences from *A. ferrooxidans* viz. IS*T1* and IS*T2*. The representative of family 2 member IS*T2* was later sequenced and described (Yates and Holmes, 1987; Yates *et al.*, 1988). The size of IS*T2* was found to be 1408-bp containing terminal inverted repeats of 25-bp and target duplication of 9-bp, and three ORFs. Similarly IS*T1*, now referred to as IS*Afe1* was isolated and sequenced from *A. ferrooxidans* ATCC 19859 and suggested to play a role in the phenotypic switching of the *A. ferrooxidans* cells (Holmes *et al.*, 2001). In the year 1997, Chakraborty *et al.* isolated another IS element from *A. ferrooxidans* viz. IS*T445* which was 1219-bp long containing 8 bp inverted repeats. They also found that the distribution of these elements to be different in different variants of *A. ferrooxidans* strains (Chakraborty *et al.*, 2002). Till date there are forty one IS elements that have been detected in the genome of *A. ferrooxidans* type strain ATCC 23270 (Valdes *et al.*, 2008). The mobility of both IS*T1* and IS*T2* sequences has been apprehended. When the southern hybridization experiments were repeated after

several rounds on cell replication in the laboratory, the changes in banding patterns with IS*T1* and IS*T2* were noticed (Holmes *et al.,* 1988).

1.8 Implication of genomic abundance of Insertion Sequences

Insertion sequence(s) can spread within a genome by enzymatic reactions catalyzed by the transposase. These elements are transferred between genomes by all the classical mechanisms of horizontal gene transfer. IS elements can mediate the transfer of genetic information between genomes or between replicons of the same genome. They have been found to shuttle the transfer of adaptive traits, such as antibiotic resistance (Boutoille *et al.,* 2004), virulence (Lichter *et al.,* 1996), and new metabolic capabilities (Schmid-Appert *et al.,* 1997). ISs have many effects on genomes where they can also induce duplications, deletions and rearrangements (Naas *et al.,* 1995). Due to their effects, ISs are regarded as key determinants of genome plasticity (Rocha, 2004) and have been suggested to provide significant adaptive change to genomes (Capy *et al.,* 2000). Contrary to the situation of some transposable elements in eukaryotes, only few cases have been demonstrated in bacteria for which the action of insertion sequences led to the direct acquisition of an advantageous trait (Volff, 2006). Within the paradigm that insertion elements are advantageous for genomes, these sequences are thought to be partly responsible for the evolutionary breakthrough leading to the invasion of a new niche (Chain *et al.,* 2004). A variant of this idea suggests that facultative ecological associations are correlated with higher abundance of insertion sequences. Though in general the genomes of ancient obligate endosymbionts do not contain insertion sequences, but the genome of *Wolbachia* (one of the most abundant bacterial endosymbiont on Earth) is literally found to be littered with IS elements (Cordaux *et al.,* 2008). Results of Cordaux *et al.* (2008) have indicated that *Wolbachiai* genomes have experienced multiple and temporarily distinct IS invasions during their evolutionary history. In case of *A. ferrooxidans* and other bacteria *Pseudomonas cepacia, Serratia marcescens* etc. the events of phenotypic switching has been found to be related with IS-mediated genome rearrangement. This may represent the genetic and phenotypic diversity brought about by the IS-mediated genome changes. Loss of the ability to oxidize ferrous iron by *A. ferrooxidans* has been found to be due to the insertion of IS element into the *resB* gene leading to the inactivation of the gene involvedin iron oxidation (Schrader and Holmes, 1988).

In the present study, an attempt has been made to characterize *Acidithiobacillus ferrooxidans* strains isolated from Garubathan AMD to explore the reasons behind the generation of colony morphology *vis-a-vis* genetic rearrangements caused by the movement of IS elements (IS*T445* and IS*T2*).

1.9 Objective of the study

The objectives of the present study chiefly included the following:

1. Isolation and characterization of *Acidithiobacillus ferrooxidans* strains indigenous to Darjeeling Himalaya
2. To utilize IS elements of *A. ferrooxidans* in studying genotypic variability with special reference to the development of colony morphology, and iron-sulfur lithotrophy variants.
3. Tagging of IS elements and pull out the mutant gene for identifying gene function.
4. To test Fe/S oxidizing variants in developing high efficiency ore leaching strains.

1.10 References

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