General Discussion

7. GENERAL DISCUSSION

Acidithiobacillus ferrooxidans (formerly *Thiobacillus ferrooxidans*) was first isolated by Temple and Colmer from AMD water in 1951. The ability of the organism to grow in mineral rich environment and oxidize ferrous iron as well as sulfur and reduced sulfur compounds to produce acid makes this organism ideal for the bioleaching processes. In different parts of the world bioleaching is carried out on commercial basis (Cerro Colorado, Chile; Cerro Verda, Peru; Fairview, South Africa; Wiluna, Australia etc.) (Olson *et al.,* 2003). Therefore, to exploit this ability of the microorganisms scientists are trying to explore and understand the biology of this microorganism.

The organism is obligately autotrophic and is very difficult to get in pure form to understand its true physiology. The bacterium often remains associated with other heterotrophic acidophile like *Acidiphilium acidophilum, A. cryptum* etc. In the present study *A. ferrooxidans* culture was found to be co-habitated by gram negative bacterium *A. cryptum*. The heterotroph was found to play an important role in scavenging the organic compounds in the natural as well as under laboratory environments. The presence of the heterotroph was found to enhance the efficiency of colony formation of *Acidithiobacillus ferrooxidans* in ferrous iron plate as well as the rate of bioleaching of copper from pyrite probably by scavenging the organic products produced due to acid hydrolysis of agar or excretion of metabolic waste products by the autotroph. On the other hand thiosulfate, one of the key intermediates of pyrite leaching was found to have a toxic effect on *A. cryptum* at the concentration above 0.7 mM. *A. cryptum* in turn was however able to survive in thiosulfate even at the concentration above 2.0 mM when grown in presence of *Acidithiobacillus ferrooxidans*. These experimental data showed that these organisms live together in mutual association benefiting each other.

A. ferrooxidans has a great application in bioleaching, however several practices to improve the strain by mutation and selection in most cases have proven to be futile due to its recalcitrant nature to form colonies in agar plates. However Schrader and Holmes (1988) isolated large spreading colony morphology variants of *A. ferrooxidans* which were lacking the property of oxidizing ferrous iron. Later it was found that the loss of iron oxidizing property was because of the insertion of IS*Afe*1 insertion element in the *resB* gene (Holmes *et al.,* 2001). Chakraborty *et al.* (2002) also found large spreading variants which were capable of ferrous oxidation. They found that the generation of colony

morphology variants was related to the movement of insertion elements, IS*T2* and IS*T445*, as southern hybridization revealed that the distribution of IS elements to be different in wild and variant type of *A. ferrooxidans.* Since then several IS elements have been detected in the genome of *A. ferrooxidans.* In recently completed genome sequence of type strain *A. ferrooxidans* ATCC23270 forty one different IS elements have been detected (Valdes *et al.,* 2008).

It is very difficult to explain the reason for the generation of colony morphology variants in *A. ferrooxidans.* However one of the plausible explanations may be the activation or inactivation of some of the genes that may be caused by the movement of the insertion sequences. There are several reports in other bacteria with regard to the movement of IS elements playing roles in the generation of the variants. It has been suggested that the effect of stress due to starvation and increased oxygen levels or any adverse condition activate IS elements (Naas *et al.,* 1994; Mahillon and Chandler, 1998; Hall, 1999) thereby helping the cells to adapt in these adverse conditions. Therefore IS elements can be seen as adaptive mutator genes, since the mutations caused by them occur at increased rates in relation to the expected rate of mutation (Chao *et al.,* 1983; Moxon *et al.,* 1994; Cooper *et al.,* 2001). In this study all the colonies of morphology variants did not emerge at the same time. Instead initially only a few colonies emerged and after that the number started to increase till it reached to a maximum level, indicating that the cells had to undergo some kind of adaptation in the given environment before forming the colonies; and only those cells which succeeded in undergoing adaptation formed the colonies. Beside that the frequency of occurrence of colony morphology variants was found to be around 27% which could not be co-related with the spontaneous mutation rate. Therefore it has been suggested that the emergence of colony morphology variants may be the case of adaptive mutation.

The movement of IS elements in several strains of *A. ferrooxidans* has been reported (Holmes *et al.,* 2001; Chakraborty *et a.,l* 2002). In this study the presence of IS*T2* and IS*T445* has been shown in the *A. ferrooxidans* strains isolated from Garubathan by amplifying the IS elements using PCR technology. The internal primers for these IS elements were designed from their conserved terminal inverted repeats. In the strains of Garubathan when PCR amplification was conducted with these primers, in addition to IS*T445* and IS*T2* two other DNA fragments got co-amplified. Both the non-IS fragments were found to contain the same conserved inverted repeats at their ends.

The non-IS sequence, that co-amplified with IS*T445* internal primers, was found to contain the conserved domain sequence for glutamyl-tRNA-reductase gene. The sequence was compared with the representative molecule of glutamyl-tRNA-reductase (isolated from ***Methanoparus kandleri***) (Moser *et al.,* 2001) and was found to be very similar. 3D model of both these molecules have also been presented (fig 5.15 and 5.16). The other non-IS amplicon which was generated with the internal primers of IS*T2* when sequenced and analyzed was found to contain the conserved domain for Pirin_C molecule of *A. ferrooxidans.*

Tracing the movement of IS elements was performed with a novel PCR-based strategy. Once the presence of the IS*T2* and IS*T445/* IS*Afe1* was confirmed in the strains of Garubathan, primers were designed from the same conserved inverted sequence but outwardly directed. The outwardly directed primer pairs allowed to amplify DNA region spanning two IS elements. The variously amplified fragments of different sizes were then analyzed by gel electrophoresis. The basic idea was that the unique DNA band(s) which would be present or absent in either of the wild or variant strain(s) will be cloned and sequenced to analyze the genes that got affected by the movement of IS elements.

In the present investigation the amplification of the inter IS regions could be successfully performed using different combinations of outwardly directed primers (Fig.5.25 and 5.26). Although 16 different combinations of primers were theoretically possible for the inter IS amplification, only two specific combinations of primers (RP1-RP4 and RP2-RP3) could be used considering the similarities in their annealing temperatures and relatively very small difference in T_m values of the primers (Table 5.1).

The electrophoretic profile of amplified DNA fragments revealed similar banding profile in DK1 and DK1S1 strains when RP2-RP3 combination of primer sequence was used. The use of same set of primers in strains GBVI produced a distinctly different DNA banding pattern which differed remarkably from DK strains in their Dice Coefficient value. However the two GB strains showed very similar DNA profile when GB strains were amplified using RP 2- RP3 primers. Similar results were obtained when CMO strains (CMO I and CMOS2) and CMI strains (CMI and CMI S 2) were amplified with the same primer combination (Fig 5.25). The change in the primer set (RP 1-RP 4) again produced very similar profile in the two DK strains with the appearance of one unique band of interest in DKS1 strain which was purified and cloned for further analysis. The fragment

of interest was found to be completely lacking in all other strains when amplified with the same primer sequence (RP1–RP4). The differences in banding patterns arising in different isolates of *A. ferrooxidans* may perhaps be attributed to the difference in the distribution of IS elements and differences in their habitats owing to their occurrence in different geographical locations.

The unique inter IS fragment generated in DKIS1 was 420bp in length (pAR1) and was found to contain the partial ORF of MntH transporter protein which is responsible for the transport of bivalent cations such as Mn^{2+}, Zn^{2+} and Fe^{2+}. The nucleotide sequence showed 95% identity with *A. ferrooxidans* ATCC 53993 strain. However BlastN result did not show any similarity with the terminal sequence of pAR1 which contained the terminal inverted repeats of IS*T445* and IS*T2* indicating the event of insertion of IS elements in MntH gene. The total gene sequence for MntH gene of *A. ferrooxidans* ATCC 53993 was retrieved and pair-wise alignment was performed. The pair-wise alignment result showed that the terminal repeat sequences of IS elements present in the inter IS fragment was partially similar to the sequence present in the flanking regions of the *A. ferrooxidans* sequence. The non specific amplification of this inter IS fragment with RP1-RP4 primer pair however was ruled out because the same fragment was not amplified in DK1 strain. Therefore, it can be inferred that the presence of such sequences which are partially homologous to the inverted repeat sequence of the IS elements may act as a substrate for recombination with terminal repeats of IS elements leading to the integration of IS elements or active transposition during the growth in the stressed condition.

Further retrieval of sequence from *A. ferrooxidans* whole genome revealed the presence of an operon where the MntH gene was followed by MgtC gene and porin gene responsible for transport of Mg^{2+} and carbohydrate transport respectively. The operon contained the promoter sequence as well as SD sequences at the upstream of the MntH gene indicating the MntH gene is the first gene among all other genes to be transcribed.

The morphological and physiological characters of the wild and colony morphology variants were studied and compared. The colony morphology of the variants on thiosulfate agar was large, white and spreading. The colonies were unicellular in thickness probably because they separated from one another as soon as they were divided. The physiology of these variants were compared with their mother (wild type) strains in terms of generation time in elemental sulfur medium, rate of oxidation of iron, sulfur, thiosulfate, tetrathionate

and leaching. The generation time for these CMVs were found to be shorter and rates of oxidation of elemental sulfur and reduced sulfur compounds were found to be significantly higher than that observed in case of the wild types. In addition to that, the rate of copper leaching from pyrite was also found to be slightly higher than the wild types.

The origin of CMVs from their respective mother strains was unequivocally established from Dice coefficient data (Table 5.3 and 5.5) The difference in inter IS amplicon profile of DKS1 with DK1 by a single polymorphic band indicated the activity of ISs in host cells when plated in thiosulfate agar medium. Some kind of stress has compelled to modify genetically to adapt and survive in the medium. Therefore, it is suggested that thiosulfate being a readily utilizable substrate for *A. ferrooxidans*, does not impose any kind of stress to the organism especially in natural environment (where the amount of thiosulfate generated is very small during pyrite leaching). However, higher concentration of thiosulfate that prevails in thiosulfate agar medium is inhibitory to the inseparable heterotrophic partner, *Acidiphilium*, and that induces stress condition. The stress is due to the accumulation of acid hydrolyzed products of agar which remain unscavenged due to suppression of the heterotroph. Under such circumstances, *A. ferrooxidans* struggles to survive. Beneficial mutations mediated by ISs may help rescue the situation by inactivating the genes that help import organic molecules from the growing environment.

In chapter 3 it has been discussed that the presence of organic molecules inhibits and sometimes kills the *A. ferrooxidans* cells. It was also shown that this inhibition can be relieved in presence of acidophilic heterotroph. However thiosulfate is toxic to the heterotroph and therefore unlike in ferrous iron double layered agar plates, same kind of enhancement of colony formation in thiosulfate double layered agar plate could not be performed. This indicates that few heterotroph cells which may remain as a co-habitant with the *A. ferrooxidans* in culture medium plays a very important role in the colony formation of *A. ferrooxidans* in the agar plates. However since thiosulfate is toxic to the heterotrophs, the *A. ferrooxidans* cells in thiosulfate agar plate directly comes in contact with the organic products produced due to acid hydrolysis of agar. This may be the cause for stress for *A. ferrooxidans* cells growing in thiosulfate agar plates. Because of this, many of the cells instead of forming a normal colony forms a spreading colony whereby cells get separated out and move away from each other as soon as they are divided so as to avoid the organic compounds produced as the metabolic waste product in absence of the scavenger like *A. cryptum*. This postulation can be substantiated with the oligotrophic

nature of the acidophilic heterotrophs which were able to grow in spent sulfur medium also.

The polymorphic inter IS band that appeared in the variant DKS1, was sequenced and analyzed using various bioinformatics tool. The sequence having terminal repeats of IS elements, was superimposed on the corresponding region of whole genome sequence of *A. ferrooxidans* ATCC 53993 to map the mutations due to insertions of IS elements in one of the genes of a transporter operon. This transporter operon was named as Mn Mg Carpo operon. The possible effect of the disruption of MntH gene, the first cistron of the operon, the polar effect of this mutation on the transcription of other three cistrons, were discussed in detail in chapter 5 of this thesis. It was theoretically interpreted that such rearrangements due to IS activity disrupting an operon function could in turn be beneficial to the host organism to colonize on thiosulfate agar medium haaving unique colony morphology. The possible reason for the spreading of DKS1 in thiosulfate agar was also discussed. However, much more research works need to be undertaken to understand the central control modes of regulation and reveal the mechanism of cross talk between transport function and chemotaxis via assimilatory and dissimilatory metabolism.

7.1 REFERENCES

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