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Detection of Insertion Sequences in the Chromosome of an *Acidocella* Strain

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Abstract: The acidophilic heterotrophs, *Acidocella* sp. GS19h strain, *Acidiphilium symbioticum* H8 and *Acidiphilium multivorum* JCM 8867, exhibit high resistance to several metals including zinc and all of them harbour several plasmids. The plasmid-cured derivative of the GS19h strain (GS19h/pl$^-$) shows very low level of metal resistances compared to the native strain. Attempts to transform this cured strain with plasmids of the parent strain as well as with those of *A. symbioticum* and *A. multivorum* strains yielded Zn-resistant transformants only with the plasmids of GS19h strain. The transformants, however, was found to possess no plasmid. This observation suggested chromosomal integration of the metal resistance gene(s) from the plasmid-probably through the action of insertion sequences (ISs) present either in the chromosome or onto the transforming plasmid of the *Acidocella* strain. To investigate this probability, identification of IS in the chromosome of *Acidocella* GS19h was undertaken first due to its large size (hence higher probability) compared to the plasmids. Chromosomal DNA preparation from *Acidocella* GS19h strain was subjected to especial treatment for the detection of IS. Agarose-gel electrophoresis of the treated DNA showed two bands of ca. 1000 and 570 bp sizes representing two ISs in the *Acidocella* GS19h chromosome.

Key words: *Acidocella*, *Acidiphilium*, insertion sequences, plasmids, transformation

INTRODUCTION

The microbial consortia, which cause leaching of sulfide ores, consist of acidophilic chemolithoautotrophic species of genera *Acidithiobacillus*, *Leptospirillum* etc. as well as acidophilic heterotrophs of *Acidiphilium* and *Acidocella* genera (Hallberg and Johnson, 2001; Rawlings, 2002). These bacteria, in general, exhibit resistance toward several heavy metals (Mahapatra and Banerjee, 1996; Ghosh *et al*., 1997; Leduc *et al*., 1997) and majority of the strains harbour plasmid(s) in laboratory cultures without any selective pressure like exposure to high heavy metal concentrations (Banerjee, 2004). These stable plasmids can be developed as potential vectors for carrying novel genes in this group of bacteria for achieving greater success in biomining. Since metal resistance is regarded as the most suitable phenotype for selecting genetically engineered biomining strains (Shiratori *et al*., 1991; Rawlings and Silver, 1995; Banerjee, 2004), we searched for the conferring genes in these bacteria (Ghosh *et al*., 1997, 1999; Mahapatra *et al*., 2002). In our first attempt in this direction (Ghosh *et al*., 1997), we established that metal resistance in *Acidocella* GS19h strain is plasmid-mediated, but could not identify the specific plasmid(s). In all probability, this was due to the integration of metal resistance genes into the chromosome of *E. coli* transformants; in case of *Acidiphilium multivorum* transformants, metal resistance conferring plasmid(s) of the strain could not be identified because of sequence similarity among the plasmids of two strains. In the mean time, a metal-sensitive plasmid-cured derivative of *Acidocella* sp. GS19h, the strain GS19h/pl$^-$, was isolated (Ghosh *et al*., 2000). The present attempt was therefore made to induct metal resistance in this plasmid-less, metal-sensitive

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derivative with the plasmids of the parent strain as well as of the metal-resistant acidophilic heterotrophs, *Acidiphilium symbioticum* H8 and *Acidiphilium multivorum* JCM 8867 (Mahapatra and Banerjee, 1996), with an aim to identifying metal resistance genes carried by specific plasmid or plasmids. However, the mildly Zn-resistant transformants of the strain GS19h/pl$^-$, which we obtained, did not contain any plasmid suggesting again chromosomal integration of resistance genes-probably through the action of insertion sequences (Siguier *et al*., 2006). Search for these sequences in the chromosome of GS19h strain led to the detection of two such sequences. Thus present study shows that like the chemolithoautotrophic *Acidithiobacillus ferrooxidans* (Yates *et al*., 1988; Chakraborty *et al*., 1997), the acidophilic heterotrophic strain *Acidocella* GS19h possesses insertion sequences in its chromosome.

MATERIALS AND METHODS

Chemicals

Medium and medium components were purchased from HiMedia (India), Qualigens (India), Merck (India) and DIFCO (USA). Most of the molecular biology grade chemicals like lysozyme, sodium perchlorate, RNAaseA, agarose, ethidium bromide and ampicillin were the products of Sigma (USA); a few others, such as Tris, EDTA, SDS, etc. were procured from SRL (India). S1 nuclease was the product of Roche Pharmaceuticals (Switzerland). Alcohol used for DNA precipitation was purchased from E. Merck (Germeny). Phenol was supplied by Qualigens (India).

Bacterial Strains and Growth Conditions

Table 1 shows the acidophilic heterotrophic bacterial strains used in this study. These bacteria were grown in MGY medium of pH 3 at 30°C under submerged condition (Bhattacharyya *et al*., 1991). Zn-resistant transformants were selected on $ZnSO_4$ containing MGY-agarose medium (Ghosh *et al*., 1997). *Escherichia coli* W3110 was cultured in Luria-Bertani (LB) broth under submerged condition or on LB-agar (Sambrook *et al*., 1989) at 37°C. *Acidithiobacillus ferrooxidans* strain was grown in a mineral salt medium containing ferrous sulfate (Bounds and Colmer, 1972).

Transformation of Bacterial Cells

Cells of *Acidocella* strain GS19h/pl$^-$ and *E. coli* W3110 were made competent by calcium chloride treatment and the competent cells were transformed with purified plasmid preparations by the heat shock method (Ausubel *et al*., 2001). Transformants of strain GS19h/pl$^-$ were selected on MGY-agarose containing several concentrations ($\geq$ 5 mM) of $ZnSO_4$. Metal-resistant transformants were repeatedly cultured on MGY-agarose; the metal resistance phenotype was checked frequently on Zn-supplemented (10 mM) MGY-agarose. *E. coli* W3110 cells transformed with the plasmid Bluescript (pBS) were selected on LB-agar supplemented with 50 μg mL^{-1} ampicillin.

Isolation of Plasmid DNA

A modified alkaline lysis method (Sambrook *et al*., 1989) was mainly followed for the preparation of plasmids from acidophilic heterotrophs. In practice, cell pellet was suspended in 1/50th of culture volume of Solution I and the suspension was kept on ice for 15 min. It was then rapidly warmed

Table 1: Acidophilic heterotrophic bacteria used in this study

Bacterial strain	Reference
Acidiphilium multivorum JCM 8867	Wakao *et al*., 1994
Acidiphilium symbioticum strain H8	Bhattacharya *et al*., 1991
Acidocella sp. strain GS19h	Banerjee *et al*., 1996
Acidocella sp strain GS19h/pl$^-$ (plasmid-cured derivative)	Ghosh *et al*., 2000

to 37°C in a water bath. Lysozyme (final concentration: 1 mg mL^{-1}) was added to the suspension, which was incubated for 1 h at 37°C followed by 10 min at 60°C in a water bath. Two volumes of Solution II were added drop-wise to the suspension, which was incubated at 60°C for another 10 min to lyse the cells. The lysate was cooled to room temperature, neutralized with 1.5 volume of Solution III and kept on ice for 15 min. The preparation was purified with phenol and chloroform and incubated with dehydrated ethanol for 30 min at -20°C prior to centrifugation of DNA. Precipitated DNA was dissolved in buffer, treated with RNAaseA and purified as above; final concentration of DNA in ddH_2O was ~0.5 mg mL^{-1}.

Preparation of Chromosomal DNA

Genomic DNA was prepared following the method of Marmur (1961) with modifications as described. Cells of *Acidocella* GS19h were harvested at late log phase, washed twice with saline-EDTA (0.15 M NaCl; 0.1 M EDTA, pH 8.0) and suspended in the same (1/25th culture volume). The suspension was incubated with lysozyme (0.5 mg mL^{-1} final concentration) at 37°C for 2 h with occasional swirling and then kept at 60°C for 2-3 min. One-tenth volume of 20% SDS was added to the suspension and incubation was allowed to continue for another 10 min. The suspension was cooled to room temperature and requisite volume of 5 M sodium perchlorate was added to a final concentration of 1 M. The mixture was placed on a shaker (100 rpm) with 1:1 (v/v) phenol-chloroform for at least 30 min and then centrifuged at 9500 x g for 8 min. The supernatant was again extracted with phenol-chloroform to remove traces of protein and once with chloroform. It was kept at 4°C for 30 min; then layered with 2 volumes of 95% ethanol. The spooled DNA was treated with DNAase-free RNAaseA, dissolved in a volume of SSC to have DNA concentration of ~1.5 mg mL^{-1} and stored at 4°C.

Detection of Insertion Sequences

The method of Chakraborty *et al*. (1997) was mainly followed to detect the insertion sequences (ISs). In practice, genomic DNA (200 μg in 800 μL TE buffer) was sheared by passing through a 29-gauge needle. It was collected after precipitation with ethanol and centrifugation at 4°C. Sheared DNA was dried in vacuum, dissolved in 120 μL sterile water and distributed as 15 μL aliquots. Each aliquot was denatured with 5 μL 1 N NaOH at room temperature for 5 min and then neutralized with 5 μL 1 N HCl. DNA of each sample was precipitated with ice-cold ethanol sequentially at an interval of 30 seconds, i.e. from 0.5 to 4 min, centrifuged and vacuum-dried. It was dissolved in 25 μL S1 nuclease buffer and treated with the enzyme for an hour at 37°C to remove single stranded portions. Samples were extracted sequentially with phenol, phenol-chloroform (1:1, v/v) and chloroform and precipitated with ethanol. Each pellet was dissolved in 50 μL sterile water and 8 μL of each sample was loaded on agarose (1.4%, w/v) gel for electrophoresis.

Gel Electrophoresis of DNA

For electrophoresis of DNA, agarose gel [0.6% (w/v) for plasmid DNA and 1.4% (w/v) for IS] and TAE buffer (40 mM Tris-acetate; 1 mM Na_2EDTA, pH 7.6) were used. Electrophoresis was run at 4 V cm^{-1}; gels were stained with ethidium bromide (0.5 μg mL^{-1}); DNA bands (plasmid and IS elements) were detected on a transilluminator. Molecular sizes of plasmids and ISs were determined by comparing their mobility in the gel with that of the molecular size markers.

RESULTS AND DISCUSSION

This study was conducted at Indian Institute of Chemical Biology, Kolkata, India during 2004.

The plasmid-cured derivative of *Acidocella* GS19h could not grow in MGY medium containing more than 0.5 mM zinc; competent cells of the same, however, could grow at higher concentrations of the metal (MIC, 2 mM). Therefore, transformants were selected on media containing different concentrations of zinc from 5 mM upwards.

Transformation of the GS19h/pl$^-$ strain with plasmids of *Acidiphilium multivorum*, *Acidiphilium symbioticum* and the parent strain yielded Zn-resistant colonies on 10 mM (and less) Zn-containing medium only in the case of parent strain, i.e. *Acidocella* strain GS19h. No colony was observed with untransformed competent cells at the same concentration of zinc under identical conditions (Fig. 1). That the cells gained competence after the treatment was checked with *E. coli* W3110 cells, which were transformed with pBS to develop ampicillin resistance. *Acidocella* transformants retained the level of Zn-resistance after repeated transfer on metal-free medium suggesting this phenotype to be a stable characteristic of the transformants. However, no plasmid could be detected in any of the transformants on several attempts indicating the property to be chromosomal. Since the Zn-resistant colonies were obtained after transformation with the plasmid preparation of the parent strain, it may be concluded that the zinc resistance gene(s) was transferred from a plasmid into the chromosome. Such recombination event is possible if ISs are present in either of the genomes (plasmid and/or chromosome).

The level of Zn-resistance in the transformed colonies was quite low compared to the parent strain. It may be suggested that high level Zn-resistance in this strain is attained through co-operative activity of several proteins encoded by different genes; in all probability only a portion of the total operon was transferred and integrated into the chromosome endowing partial resistance to the metal. This observation (as well as previous observations) further indicates that natural plasmids of this bacterium (and probably others of the same and related species) are maintained through mutual participation of each DNA element, which becomes unstable in absence of others. This phenomenon of mutual stability might be the reason why no transformant was obtained with the plasmids of *Acidiphilium* strains.

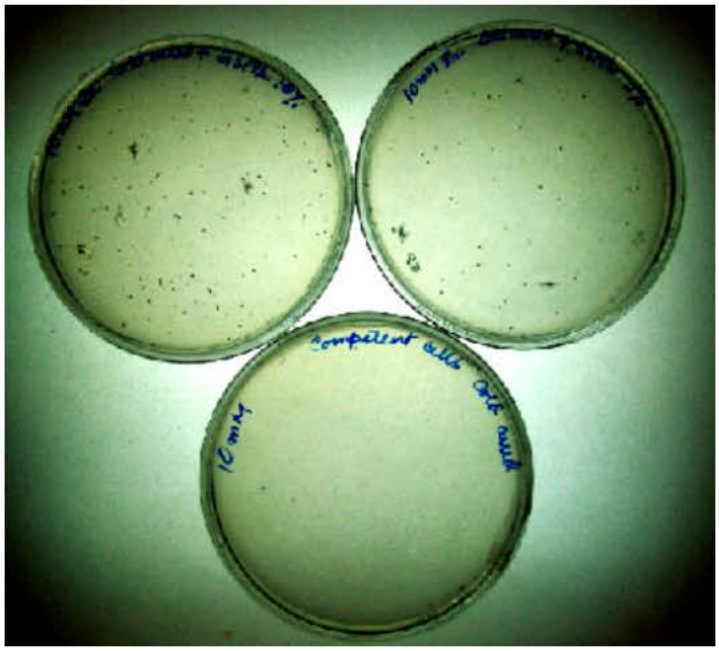

Fig. 1: Growth of Zn-resistant transformed colonies of *Acidocella* GS19h/pl$^-$ on MGY medium containing 10 mM $ZnSO_4$ inoculated with 100 and 50 μL cell suspension of OD_{590} ~0.7 (upper right and left plate, respectively); the lower plate is the control set inoculated with 100 μL untransformed cells on the same medium

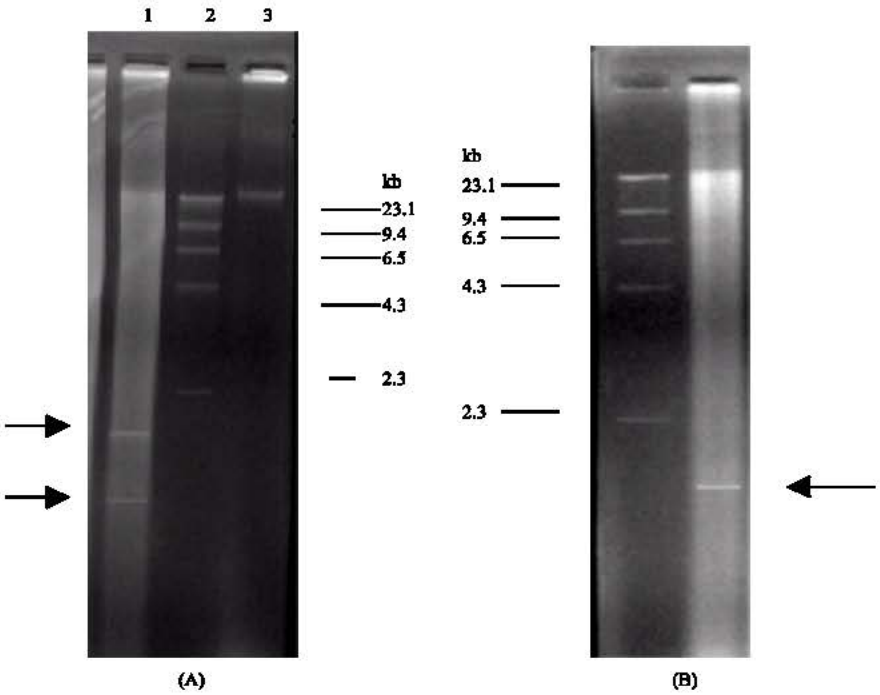

Fig. 2: Detection of IS (i.e., heteroduplexes) on agarose gel electrophoretogram. (A) Lane 1, genomic DNA heteroduplexes of *Acidocella* GS19h strain after S1 nuclease treatment: lane 3, untreated genomic DNA of the strain; lane 2, *Hin*dIII digest of λDNA. (B) Lane 2, *Acidithiobacillus ferrooxidans* genomic DNA heteroduplexes after S1 nuclease treatment; lane 1, *Hin*dIII digest of λDNA. Arrows indicate the positions of heteroduplexes

The presence of ISs in the chromosome of GS19h/pl$^-$ strain was detected on the agarose gel electrophoretogram. The stretches of inverted repeats present at the ends of ISs quickly form heteroduplexes after melting followed by partial reannealing of DNA. These reannealed portions of DNA appear on the gel as faint or strong bands (depending on the percentage in whole genome) while rest of the single stranded portions are digested by S1 nuclease. In Fig. 2A, two faint bands of 1 kb and 570 bp size corresponding to the putative IS elements may be seen. Intensity of the bands in all the sets was same showing no effect of incubation time within the range of 30 sec to 4 min. Existence of two DNA bands indicates the presence of two types of IS in the chromosome of *Acidocella* GS19h strain, albeit their percentage is low. Following this protocol, the 1.4 kb IS present in the *Acidithiobacillus ferrooxidans* genome (Yates *et al*., 1988) could be detected (Fig. 2B). However, the close 1.2 kb IS band could not be seen, most probably due to its low percentage (hence weak intensity in the gel) in the genome of this strain compared to the 1.4 kb IS. It was observed that both the bands are rarely visible in all strains (Chakraborty *et al*., 1997). Thus detection of the 1.4 kb IS of *Acidithiobacillus ferrooxidans* confirmed that the two visible bands on the gel electrophoretogram of GS19h strain are nothing but IS. Sequencing of the two IS bands could not so far been achieved by direct cloning of the bands (due to very low yield).

Plasmid genetics of acidophilic heterotrophic bacteria inhabiting sulfide mines and similar regions are at the rudimentary stage although a set of interesting reports is in hand (Glenn *et al*., 1992; Quentmeier and Friedrich, 1994; Ghosh *et al*., 1997; Suzuki *et al*., 1997; Mahapatra *et al*., 2002). Unlike the plasmids of their autotrophic co-habitant *Acidithiobacillus ferrooxidans*, these genetic

elements of acidophilic heterotrophs have been shown to bear metal resistance genes (Suzuki *et al*., 1997; Mahapatra *et al*., 2002; Ghosh *et al*., 1997, 2005) and plasmid-borne *ars* operon of the *Acidiphilium multivorum* strain used in this study was cloned and sequenced (Suzuki *et al*., 1998). The other features of these plasmid DNAs that create much interest in their genetics are the generation of novel plasmids in *E. coli* (Mahapatra *et al*., 2003), non-existence or instability of plasmids in metal-resistant transformed strains (Ghosh *et al*., 1997, 2005) and completely new ORF sequences in the cloned DNA portions conferring metal resistance (Ghosh *et al*., 1999; Mahapatra *et al*., 2002). To get an understanding on these aspects, we have already sequenced an entire plasmid of the *Acidiphilium multivorum* strain, which does not bear any known phenotype-conferring gene (Singh and Banerjee, 2007). The present study was also undertaken in this direction to identify the plasmid(s) carrying metal resistance genes. Although this particular aim was not achieved, this work ushered a new opening through identification of IS elements in the chromosome of an *Acidocella* strain.

CONCLUSIONS

Plasmid(s) of highly metal-resistant *Acidocella* strain GS19h can induce Zn-resistance in its metal-sensitive plasmid-less derivative. Since the level of metal resistance in the transformed cells was much less compared to the parent strain, it is suggested that high resistance to zinc is achieved through co-operative action of different proteins-the related genes being encoded on one or more plasmids. Absence of any plasmid in the Zn-resistant transformants indicated chromosomal integration of putative resistance genes through the action of insertion elements. Search for insertion elements in the chromosome of the strain revealed the presence of two such elements.

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