CHAPTER 5

Characterization of an indigenous plasmid in *Acidiphilium* sp. DK2AH2 and attempt to ascribe its function in relation to metal tolerance

5.1 Introduction

Plasmids are circular double stranded molecule of DNA that exists independently of chromosomal DNA in bacterial cell. These can range in size from several hundred base pairs to several thousand kilobases (Cook *et al.*, 2001). Studies on bacterial plasmids have contributed significantly to our understanding of the mechanisms and regulation of DNA replication, gene expression, and recombination, as well as other areas of prokaryotic biology. To date, many species of bacteria isolated from diverse habitats are known to contain plasmid DNA. Some plasmids are stable and can be maintained through successive generations by being partitioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy (Yoo *et al.*, 2001).

5.1.1 Types of Plasmid

There are several types of plasmid present in bacterial cell, which are as follows:

- **Conjugative or F- Plasmid:** This type of plasmids has the ability to transfer the sex factor, gene pickup and production of Hfr Strain. This plasmid helps in bacterial conjugation.
- **R-Plasmid:** This plasmid is a drug resistant plasmid, generally they are antibiotic plasmid. The R- plasmid was first discovered in a strain of *Shigella* in late 1950s in Japan (Partridge and Hall, 2004). Plasmid mediated drug resistant has serious medical implications.
- **Enteric Plasmid:** This type of plasmids produces either heat-stable toxin (ST) or both heat stable or heat labile (ST and LT) toxin. No plasmid coding for LT along has been found.
- **Hly Plasmid:** It codes for the production of α-heamolysins compounds which causes the lyses of the red blood cells.
- **K-Plasmid:** This plasmid specifies the production of species specific antigens, which are large filamentous protein covering the fine pili.

- **Col Plasmid:** Bacteriocins are protein antibiotics produced by bacteria. They are lethal to other bacteria of the same genus or related genus. Certain strains of *E.coli* synthesize bacteriocin (colicins).

- **Degradative Plasmid:** Some microbes have the ability to degrade the organic matter. This degradative enzyme may sometimes be coded by plasmids (Top and Springael, 2003). This type of plasmid is called degradative plasmid. It is found mainly in *Pseudomonas.*

- **Heavy Metal Resistant Plasmid:** Some strains of bacteria, have plasmids which enable the host to tolerate toxic concentration of heavy metal ions. Eg., *Pseudomonas* and *Staphylococcus aureus.*

- **Tumor inducing Plasmid:** A large plasmid in *Agrobacterium tumefaciens* is found to cause the disease crown gal in dicotyledonous plant. These type of plasmids are called Ti-plasmid.

- **Cryptic Plasmid:** Low molecular weight circular DNA molecule has been reported in many genera of bacteria for which no biological role has been established. Therefore these are regarded as Cryptic plasmids.

5.1.2 Plasmid Curing

Bacterial plasmids have a major impact on metabolic function. Bacterial plasmids can be eliminated from bacterial species grown as pure or mixed bacterial cultures in the presence of sub-inhibitory concentrations of non-mutagenic heterocyclic compounds (Spenglar *et al.*, 2006). The anti-plasmid action of the compounds depends on the chemical structure of amphiphillic compounds having a planar ring system with substitution in the L-molecular region. A symmetrical pi-electron conjugation at the highest occupied molecular orbitals favours the anti-plasmid effect. The anti-plasmid effect of heterocyclic compounds is expressed differentially in accordance with the structural form of the DNA to which they bind (Molnár *et al.*, 2003). In this manner "extrachromosomal" plasmid DNA that exists in a superhelical state binds more compound than its linear or open-circular form; and least to the chromosomal DNA of the bacterium, that carries the plasmid. It can also be noted that these compounds are

not mutagenic and their anti-plasmid effects correlate with the energy of HOMO-orbitals.

Plasmid elimination is considered also to take place in ecosystems containing numerous bacterial species. This opens up a new perspective in rational drug design against bacterial plasmids. The inhibition of conjugational transfer of antibiotic resistance plasmid can be exploited to reduce the spread of antibiotic resistance plasmid in the ecosystem. Inhibition of plasmid replication at various stages, as shown in the "rolling circle" model (replication, partition, conjugal transfer) may also be the theoretical basis for the elimination of bacterial virulence in the case of plasmid mediated pathogenicity and antibiotic resistance. The large number of compounds tested for anti-plasmid effects provides opportunities for QSAR studies in order to find a correlation between the anti-plasmid effect and the supramolecular chemistry of these plasmid curing compounds (Molnár *et al.*, 2003). Plasmid elimination *in vitro* provides a method of isolating plasmid free bacteria for biotechnology without any risk of inducing mutations.

Bacterial plasmids are known to harbor genes for: (i) resistances to antibiotics and metals (Foster, 1983; Silver, 1996), (ii) catabolic pathways such as lactose utilization and degradation of hydrocarbons (Chassy *et al.*, 1978; Ghosal *et al.*, 1985), and (iii) biosynthesis of certain antibiotics, etc (Stanisich, 1984). In many cases the characteristics of the host organism conferred by the plasmids remain vague, and such cryptic plasmids are plentiful in nature. Curing of this cryptic plasmid from a bacterial strain is a method to substantiate the relationship between a genetic trait and carriage of that specific trait in the plasmid. Various methods involving chemical and physical agents have been developed to eliminate plasmids. Protocols for curing plasmids consist frequently of exposure of a culture to sub-inhibitory concentrations of some chemical agents, e.g. acridine orange, acriflavine, and sodium dodecyl sulfate or to a super-optimal temperature followed by selection of cured derivatives.

The DNA intercalating agents such as Acridine orange and ethidium bromide are the most commonly used because they are found to be effective against plasmids in a wide variety of genera (Trevors, 1986). Although all of these agents have been used to enhance the recovery of plasmid less derivatives of various bacteria, they are

individually effective only against some plasmids and their likely response is unpredictable. The elimination of a plasmid (curing) from a bacterial culture is the best method to substantiate the relationship between a genetic trait and carriage of specific plasmid by the culture as the phenotypic characters which are associated with the plasmid are not expressed in cured derivatives but on the re-introduction of the plasmid in to the cured strain the lost phenotype is re-appeared. The efficiency of curing can also vary widely depending on the plasmid and the particular bacterial host carrying it. In most instances, the underlying mechanism of curing is not known. The agent may interfere directly with plasmid replication as occurs with the heat induced curing of certain temperature sensitive palsmids or curing of them by acridines or ethidium bromide (Terawaki *et al.*, 1976). Alternatively, curing may interfere with the growth of plasmid carrying bacteria thereby allowing spontaneously arising plasmidless segregants to become predominant. This occurs in certain instances of curing by acridines, sodium dodecyl sulfate and urea.

Methylene blue enhanced the plasmid curing efficiency of chlorpromazine, imipramine and amitriptyline with strains of *Escherichia coli* K12 carrying F-prime *lac* or the resistance factor R-144 (Molnár *et al.*, 1980). In contrast, methylene blue inhibited the elimination of plasmids by acridine orange and ethydium bromide at all concentrations tested. Two metabolic derivatives of chlorpromazine, chlorpromazine sulfoxide and 7.8-dioxochlorpromazine had no plasmid curing effect even in the presence of methylene blue. Amitriptyline, 7,8-dioxochlorpromazine and acridine orange were effective inhibitors of the conjugal transfer of the resistance plasmid, R-144, whilst methylene blue, chlorpromazine sulphoxide, and imipramine had only slight effects (Molnár *et al.*, 1980). A mechanism of plasmid curing by surface action of the drugs is suggested as an alternative to direct intercalation of the drugs into plasmid DNA.

5.1.3 Advantages of plasmid curing methods

- Plasmid displacement methods allow researchers to understand the basis for pathogenicity and antibiotic resistance, resulting in better treatment, vaccination and prevention of infection.
- Pathogenic traits that cause infection and disease are often plasmid encoded.

- Plasmid displacement methods are used to determine whether specific traits are plasmid mediated.
- Bacterial plasmids contain genes that code for additional traits such as antibiotic resistance, heavy metal resistance and biofilm formation.

5.1.4 Plasmid mediated metal resistance

Over the ages, all living systems have evolved to use some metals as vital constituents while they have learned to grapple with some others, which are toxic (Ghosh *et al.*, 1997). The primary source of metals in all ecosystems is the underlying bedrock of the planet. Though many metals are essential, all metal ions are toxic at some level. Heavy metal cations with high atomic masses tend to bind strongly to sulfide groups. The divalent cations of cobalt, nickel, copper and zinc are medium 'sulfur lovers' and these metals (known as trace elements) have essential functions at low concentrations but are toxic at high concentrations (Nies *et al.*, 1989).

The intracellular concentrations of these metals must be finely adjusted to avoid either metal deprivation or metal toxicity and careful homeostasis is necessary (Silver and Phung, 1996). In contrast, homeostasis of the purely toxic metals is simple; the cell quickly eliminate them. Of all life forms, bacteria are not only the oldest, but they also inhabit the greatest diversity of habitats, form a major proportion of the earth's global biomass and have the greatest capacity to sorb metals from the solution, on a biomass to dry weight metal basis (μg/mg dry wt.). Thus they provide the ideal system to study the metal-microbe interactions (Nies, *et al.*, 1989; Springael, *et al.*, 1993; Suzuki *et al.*, 1997). Like all other living organisms microbial communities adapt themselves to the metal concentrations encountered. Two major strategies adopted by the microorganisms to protect themselves against metal toxicity are *avoidance* and *sequestration.* These strategies are reflected in resistance phenotype to one or several metals. A report on plasmid curing from acidophilic bacteria *Acidocella* strain GS19h of acidic mine environment is available (Ghosh *et al.*, 2000). The culture resulted in the elimination of plasmids, suggesting that curing may be achieved in the case of acidophilic bacteria

applying physical or chemical methods. Plasmid-mediated inheritance of metal resistance in this bacterium is also proved.

The obligate acidophilic, metal resistant bacteria *Acidiphilium* sp. DK2AH2 can resist about 450 mM of nickel. The aim of this study is to find the relationship between the metal resistance of DK2AH2 with its plasmid. The molecular characterization of plasmid would certainly reveal considerable information regarding metal resistance mechanisms and their use in constructing genetically engineered high metal tolerating bioleaching bacteria.

5.2 Materials and methods

5.2.1 Determination of metal tolerance profile of DK2AH2

The detailing of the metal tolerance profile is described in chapter 3 under section 3.2.3 and MTC of DK2AH2 is presented in Table 3.2.

5.2.2 Reagents used for plasmid preparation

- Alkaline Solution I:

  50 mM glucose
  25 mM Tris-Cl (pH-8.0)
  10 mM EDTA (pH-8.0)

  Sol. I was prepared from standard stocks in batches of a 100 ml; it was autoclaved for 15 min, at 15 Psi on liq-cycle and stored at 4 ^{0}C.

- Alkaline solution II:

  0.2 (N) NaOH (freshly diluted)
  1% (w/v) SDS

  It was prepared freshly and stored at room temperature.

- Alkaline Solution III:

5(M) KCH_3COO	60.0 ml
CH_3COOH	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 (M) with respect to potassium and 5(M) with respect to acetate. It was stored at 4 ^{0}C.

5.2.3 Isolation of plasmid DNA (Maxi preparation)

Plasmids were routinely isolated by the procedure of Brinboim and Dolly (Brinboim and Dolly, 1979) with slight modifications. Cells of AMD strains harbouring plasmids were grown for 48-56 hours in 1.5 L modified DSMZ 269 medium. The cells were then harvested and resuspended in solution I (1 ml), mixed vigorously and kept at room temperature for 30 min. Solution II (2 ml) was added to it and mixed gently by inverting the tube several times and was kept at room temperature for 15 min. for complete lysis. Chilled solution III (1.5 ml) was added to it and mixed gently by inverting the tube. The mixture was incubated in ice for 30-35 min and then centrifuged at 10, 000 rpm for 20 min at 4 °C. The clear supernatant was taken in a fresh tube. RNaseA (20 μg ml^{-1}) was added and incubated at 37 °C for 2 hours. It was then extracted once by equal volume of phenol: chloroform (1:1) and chloroform respectively. To the aqueous phase 0.7 volume of isopropanol was added and mixed well. The tube was incubated at room temperature for 15 min followed by centrifugation at 12, 000 rpm for 20 min at room temperature. The pellet thus obtained was washed with 70 % ethanol, air-dried and suspended in minimum volume of TE (10 mM: 0.1 mM) buffer.

5.2.4 Isolation of plasmid DNA (Mini preparation)

For screening the recombinant clones from a transformation experiment, individual colonies were incubated in 3 ml LB broth (mini scale isolation) with appropriate antibiotics and culture were grown to saturation at 37 °C overnight. 1.5 ml of culture was taken in individual microfuge and pelleted at 10, 000 rpm for 5 min. The pellet was suspended in 100 μl of solution I, followed by 200 μl of solution II. The suspension was mixed by inverting the tubes and was incubated at room temperature for 5 min for complete lysis. Chilled solution III (150 μl) was added to the tubes and mixed gently. Tubes were chilled on ice for 10 min followed by centrifugation at 10, 000 rpm for 15-20 min at 4 °C. The clear supernatant was transferred to the fresh eppendorfs and 0.7 volume isopropanol was added. Plasmid DNA was pelleted immediately by

centrifugation at 10, 000 rpm for 20 min at room temperature. Pellets were rinsed with 70 % alcohol, air-dried and suspended in 30 µl of TE buffer (pH 8.0) each.

5.2.5 Agarose gel electrophoresis

0.8 % agarose (or less based on the increasing size of DNA fragment to be resolved) in 1X TAE buffer supplemented with 1µg ml^{-1} ethidium bromide was melted in a microwave or boiling water bath and then cooled to 50-60 °C before pouring in a casting tray fitted with a Teflon comb forming wells. DNA loading buffer (1X) was mixed to the sample DNA prior to loading in the wells. Electrophoresis was performed in horizontal electrophoresis tank using 1X TAE buffer containing 1µg ml^{-1} ethidium bromide for 1-2 hours. DNA could be directly viewed in a UV transilluminator.

5.2.6 Plasmid curing by acridine orange treatment

The curing of plasmid using acridine orange was carried out as described by Caro *et al.*, 1984. The concentration range of mutagen was 50 µgml^{-1} to 500 µgml^{-1} with increments of 50 for acridine orange. The protocol for plasmid curing, using the acridine orange is as follows:

- 25ml of modified DSMZ 269 medium was inoculated with the *Acidiphilium* sp. DK2AH2 from fresh slant in a 250 ml flask and incubated on a rotary shaker (120 rev min^{-1}) at 30 ^{0}C for 24 hr.
- To 100 ml of modified DSMZ 269 medium containing 100 µg ml^{-1} acridine orange, the overnight grown pure DK2AH2 culture was inoculated so as to obtain an initial cell density of $10^4 \pm 10^5$ cells ml^{-1}.
- This was incubated on a rotary shaker (120 rev min^{-1}) at 40 °C for 48 hr.
- Cell broth was then suitably diluted and spread on modified DSMZ 269 agar plates and incubated at 28-30 °C for 48 hr till generation of the colonies.

5.2.7 Recovery of plasmid cured colonies

- Well grown discrete colonies of acridine orange treated DK2AH2 strain were selected using sterile toothpicks and placed on master plates which was prepared of modified DSMZ 269 medium. The master plates were then incubated at 30 °C for 48 hr.

- Colonies on master plates were replica-plated onto modified DSMZ 269 agar plates supplemented with metal salts [Zn(II), As(III), Ni(II), Co(II), and Cu(II)] and incubated for 48 hr at 28-30 °C. Colonies unable to grow in metal containing plates were picked up from the master plate and checked further for the disappearance of plasmid DNA cured (by alkaline lysis method described previously). Plasmid-containing (uncured) culture as positive control was included in every batch of plasmid isolation.

5.2.8 Protein profiling of cured (DK2C) and uncured (DK2AH2) cultures

Protein profiling by SDS-PAGE was done following the protocol described in details in the chapter 1 under section 1.2.5.1.

5.2.9 Detection of biofilm formation in DK2AH2 and DK2C cultures

In the present study biofilm formation of DK2AH2 and DK2C cultures were investigated. The screening was done by flask method (Southey-Pillig *et al.*, 2005). The strains formed biofilm in 100 ml flasks.

Materials used:-

- DK2C culture
- DK2AH2 culture
- Crystal Violet stain
- Decolourizer (70% alcohol)

Procedure:-

- In two separated 500 ml conical flasks [containing modified DSMZ 269 medium (pH 3)]the cultures DK2AH2 and DK2C were inoculated and incubated for 4-5 days.

- After proper heavy growth the cultures were discarded and the conical flasks were heated in hot air oven for 15 min. .

- After drying, the bottom of the flasks were flooded with 1% crystal violet to stain the cells that were present on the biofilm attached to the bottom of the flasks.

- After 10 mins of incubation stains were washed with distilled water and the flasks were air dried.

- To the dried flasks decolourizer (70% alcohol) was added.

- After a short incubation period the cells were examined in colorimeter at 470 nm.

5.2.10 Restriction digestion of the plasmid DNA

The reaction buffer used for the restriction enzymes in both single and double digestions were as specified by the supplier. A typical 50 µl reaction mixture contained: DNA of interest, 1-2 µg (1-5 µl or more); 10X digestion buffer (5 µl, 1X); 100X BSA (0.5 µl, 1X when required); restriction enzyme 10 units; sterile double distilled water upto 50 µl. The digestion mixture was mixed well and briefly centrifuged. It was incubated at optimum temperature (generally 37 °C) as specified by the vendors for 2-4 hours. The reaction was stopped by heat inactivation at 65 °C for 10 min. In addition to inhibiting the activity of the present enzyme, this step is necessary to minimize the negative effects on the performance of the following enzymatic reactions.

5.2.11 Elution of plasmid DNA from low melting agarose gel

1.5% low melting agarose purchased from SRL Company, India was used to isolate restricted fragments of plasmid DNA of about 1 µg or less. The gel was run for a required period of time under cold condition. After completion of the run, required DNA band was sliced with the help of sterile scalpel and taken in an eppendorf tube. The gel was melted by keeping in the water bath at 65 °C for 5 minutes to which an equal volume 1X TE was added. The mixture was freeze-thawed thrice and the agarose particles were then removed by phenol extraction. Finally, sliced plasmid DNA was precipitated in isopropanol and was re-suspended in 0.1 X TE buffer and stored at -20 °C.

5.2.12 Ligation of the restricted fragments into pBS vector

0.1 µg of the vector DNA (pBS) was transferred to a sterile microfuge tube. Equimolar amount of restricted fragments of DNA was added to it. Sterile double distilled water was added to make the volume to 8.5 µl and the solution was warmed to 45 °C for 5 minutes to melt any cohesive termini that have re-annealed. Then the mixture was chilled to 0 °C. To the mixture 1 µl of 10X T4 DNA ligase buffer [Tris.HCl - 600 mM; $MgCl_2$ – 50 mM, Dithioerythritol (DTT) - 10 mM and ATP - 10 mM (pH 7.5)] and 0.1 Weiss unit T4 DNA ligase was added. The reaction was incubated for 4 hours at 16 °C. 1-2 µl of each of the ligation mixture was used to transform competent *E. coli* cells.

5.2.13 Preparation of competent cells and transformation of *E. coli* cells

Detailed description of the method is given in chapter 4 under section 4.2.5.1 and 4.2.5.2.

5.2.14 Identification of bacterial colonies that contain recombinant plasmids by α-complementation method

To a pre-made Luria–Bartani agar plate containing 50 $\mu g ml^{-1}$ ampicillin, 0.5 mM isopropylthiogalactosidase (IPTG) and X-gal (80 $\mu g \mu l^{-1}$) transformed competent cells were plated as described earlier. It was possible to recognize colonies that carry

putative recombinant plasmids by blue-white screening of the colonies. Insertion of foreign DNA into the polycloning site of plasmid pBluescript KS(+) leads to the incapability of complementation. Bacteria carrying recombinant colonies are therefore white colonies. Recircularized vectors containing bacteria could utilize chromogenic substrate X-Gal and thus form blue colonies.

5.2.15 Checking of clones for metal tolerance

E. coli cells containing recombinant plasmids were selected and purified. LB plates supplemented with metal salts [Zn(II), 10 mM; As(III), 0.25 mM; Ni(II), 25 mM; Co(II), 2 mM; and Cu(II), 0.3 mM] were prepared. Clones of *E. coli* cells were streaked on respective metal containing LB plates. Clones showing positive growth on these plates were selected and plasmid isolation was done by alkaline lysis (mini prep.).

5.2.16 Sequencing of the clones

The recombinant plasmids were directly used for sequencing of the inserts using primers for T7 and T3 promoters. Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) using specific primers and the reaction was analyzed in an 'AB1 PRISM 377 DNA Sequencer'.

5.2.17 Analysis of the sequences

Tools and techniques used for the analysis of the sequences is described in chapter 4 under section 4.2.7

5.3 Results

5.3.1 Metal resistance profile of *Acidiphilium* sp. DK2AH2

Strain	Co(II)	Ni(II)	Cu(II)	Zn(II)	As(III)
	mM	mM	mM	mM	mM
DK2AH2	5.0	450.0	0.3	30.0	1.0

5.3.2 Detection of Plasmids in *Acidiphilium* sp. DK2AH2 and restriction analysis of the plasmid pDK2AH2

Plasmid was detected in only one bacterial isolate viz., *Acidiphilium* sp. DK2AH2 among the fifteen bacterial acid-tolerant heterotrophic isolates tested [subject to experimental limitation in following only one method of plasmid extraction for all test strains (by alkaline lysis method, both maxi and mini preparation)]. The obligate acidophilic, metal resistant bacteria *Acidiphilium* sp. DK2AH2 contained multiple (five) plasmids. The molecular sizes of different plasmids were determined by using size markers of standard plasmids of known molecular sizes (Macrina *et al.*, 1982). The size of the largest plasmid detected and isolated was named pDK2AH2; the size of which was determined to be 55 kb. In descending order, the sizes of the plasmids obtained were approximately 55 kb, 23kb, 4 kb, 2 kb, and 1 kb (Fig. 5.1). The biggest plasmid (approx. 55 kb) was sliced off from low melting point agarose gel (after separation of plasmids), purified, checked, and used as stock plasmid DNA (pDK2AH2) for further analysis. The restriction patterns of the **plasmid,** pDK2AH2, by using different restriction enzymes were studied. *Eco*RI restricted fragments were cloned in pBS (described later in the section).

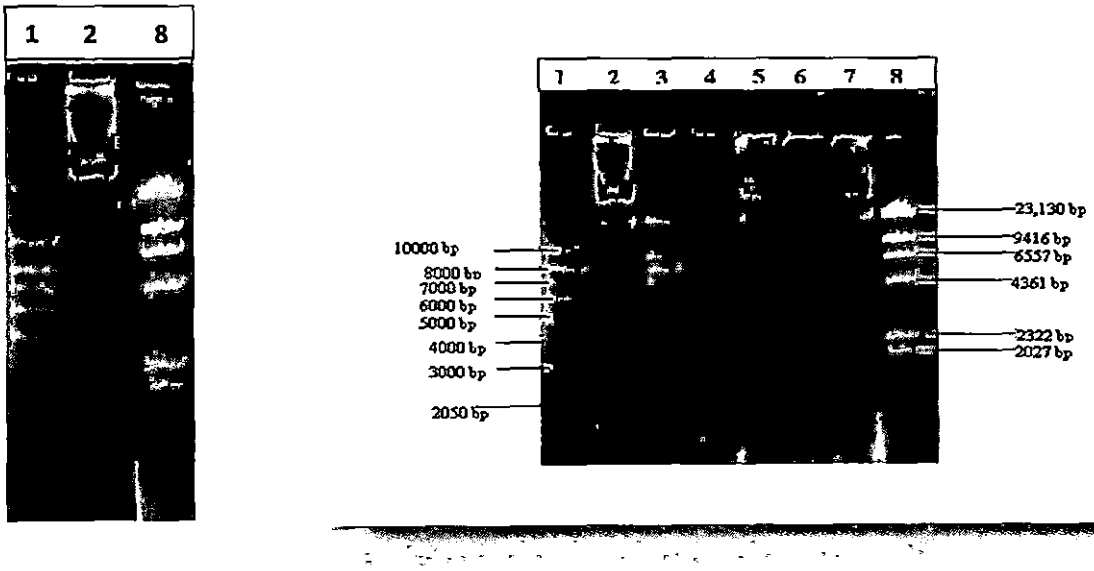


Fig. 5.1a: size determination and restriction digestion patterns of DK2AH2 plasmids.

Lane 1: super coiled plasmid DNA ladder

Lane 2: plasmid profile of DK2AH2

Lane 3: λDNA *Eco*RI digest (marker)

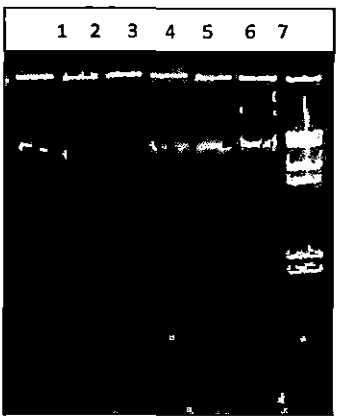
Lane 4: blank

Lane 5: *Pst*I digestion pattern of DK2AH2 plasmids

Lane 6: *Bam*HI digestion pattern of DK2AH2 plasmids

Lane 7: *Pvu*I digestion pattern of DK2AH2 plasmids

Lane 8: λDNA *Hind*III digest (marker)



Lane 1- *Not* I digested pattern of DK2AH2 plasmids

Lane 2- *Xho* I digested pattern of DK2AH2 plasmids

Lane 3- *Nco* I digested pattern of DK2AH2 plasmids

Lane 4- *Sma* I digested pattern of DK2AH2 plasmids

Lane 5- *Spe* I digested pattern of DK2AH2 plasmids

Lane 6- DK2AH2 plasmids

Lane 7- λ *Hind* III digest (marker)

Fig. 5.1b: size determination and restriction digestion patterns of DK2AH2 plasmids.

Nine different restriction enzymes were used to digest the pDK2AH2. The detailed calculations for the fragments generated by the restriction enzymes are given in Table 5.1. Six restriction enzymes viz., *Pst* I, *Bam* HI, *Pvu* I, *Sma* I, *Spe* I, and *Not* I could not digest the pDK2AH2. Three restriction enzymes *Eco* RI, *Xho* I, and *Nco* I could generate fragments of different sizes (Table 5.1).

Table. 5.1: Size and number of fragments generated after digestion of plasmid DNAs with different restriction endonucleases

Restriction enzyme	*Number of fragments*	*Number of sites*	*Size of fragments (kb)*	*Total length (kb)*
Eco RI	**6**	**6**	**17,15,9,6,5,3**	**55**
Pst I	---	---	---	---
Bam HI	---	---	---	---
Pvu I	---	---	---	---
Xho I	**6**	**6**	**14,12,10,8,6,3**	**55**
NCO I	**5**	**5**	**15,12,10,8,6**	**51**
Sma I	---	---	---	---
Spe I	---	---	---	---
Not I	---	---	---	---

5.3.3 Curing of plasmids in *Acidiphilium* sp. DK2AH2 [using Acridine Orange (AcOr)]

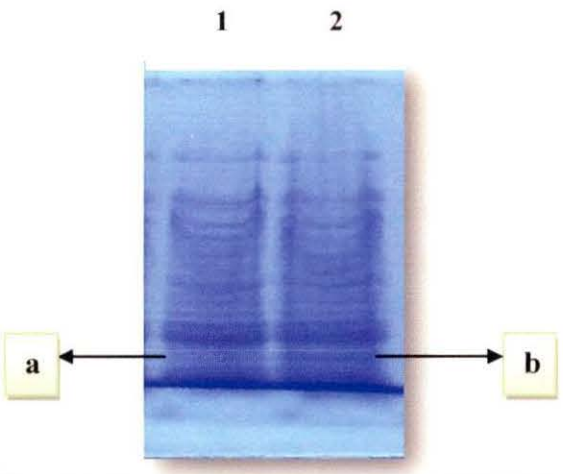
The culture of *Acidiphilium* sp. DK2AH2 was subjected to curing of the plasmids from the strain DK2AH2 using AcOr (100 μgml^{-1}). The plasmid-less isolate was obtained and was named as DK2C. The absence of plasmid DNA in the cured isolate, DK2C was verified by alkaline lysis method. Interestingly, losses of all five plasmids were observed. This indicates a curing frequency of almost >80% for plasmids in DK2AH2 strain using this method, which is comparatively higher than the frequencies normally obtained using traditional curing techniques.

Two of the metal resistant properties of the strain DK2C were found to be lost. The metals, Cu(II) and Zn(II) supplemented modified DSMZ 269 agar plates showed no growth of the DK2C strain. Besides the loss of Cu(II) and Zn(II) resistance phenotype, DK2C was also found incapable of biofilm formation (result has been described in 5.3.4)

Lane 1 : pDK2AH2

Lane 2 : AcOr treated pDK2AH2

Fig. 5.2: Plasmid profile of cured DK2C and uncured DK2AH2 strains of *Acidiphilium* sp.



Lane 1: strain DK2C

Lane 2: strain DK2AH2

a- absent, b- present

Fig. 5.3: Protein profile of DK2C and DK2AH2 strains

Whole cell protein profile of the two strains, DK2AH2 and DK2C was found to be identical, except the absence of one band in DK2C. It confirmed the purity of the strain (i.e DK2C is directly derived from DK2AH2 by dispensing the plasmids only); presence of identical protein bands except absence of one single band may signify loss of expression of certain plasmid encoded proteins.

5.3.4 Biofilm formation phenotype of *Acidiphilium* sp. DK2AH2 and DK2C

Biofilm formation character was found to be associated with plasmid genes. This finding was confirmed by checking the biofilm formation in the plasmid bearing DK2AH2 and plasmidless DK2C culture and the percentage of formation of biofilm in DK2C was nearly negligible.

Fig. 5.4: Production of biofilm by DK2AH2

Fig. 5.5: No biofilm Production by DK2C

5.3.5 Cloning of the pDK2-5 and pDK2-9 fragments of pDK2AH2

The restriction fragments of pDK2AH2 generated by *Eco* RI were subjected to shotgun cloning purpose (Sambrook *et al.*, 1989), using the vector pBS KS^{+}. The recombinant molecule (vector+insert) was transformed into *E.coli* XL1blue. Three positive clones viz., pDK2-3, pDK2-5, and pDK2-9 were found and were subjected to restriction digestion by *Eco* RI. The purified clones were sequenced and analysed.

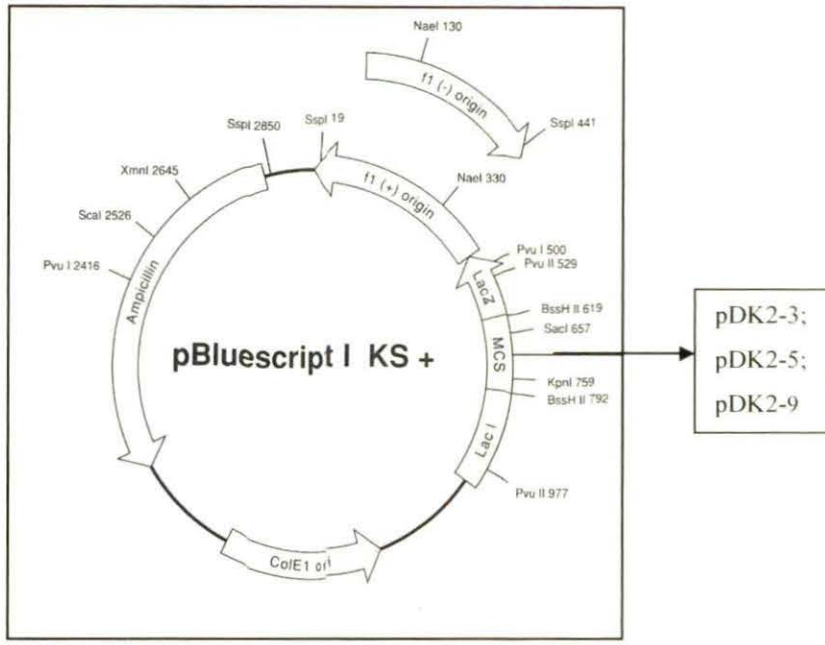


Fig. 5.6: Diagrammatic representation of pBS KS+ vector containing 5 kb and 9 kb insert DNA of pDK2-3, pDK2-5, and pDK2-9

pBluescript II KS(-), pBluescript II KS(+)

M13/pUC sequencing primer (-20), 17-mer (#S0100) — T7 promoter — T7 transcription start

```
5′ G TAA AAC GAC GGC CAG TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT
3′ C ATT TTG CTG CCG GTC ACT CGC GCG CAT TAT GCT GAG TGA TAT CCC GCT TAA
LacZ ←— Val Val Ala Leu Ser Ala Arg Tyr Tyr Ser Glu Ser Tyr Pro Ser Asn
```

653 — Ecl136II, SacI, BstXI, BtgI, Cfr42I, OliI, Eco52I, NotI, XbaI, BcuI, BamHI, Cfr9I, SmaI, PstI, EcoRI

```
GGA GCT CCA CCG CGG TGG CGG CCG CTC TAG AAC TAG TGG ATC CCC CGG GCT GCA GGA ATT C
CCT CGA GGT GGC GCC ACC GCC GGC GAG ATC TTG ATC ACC TAG GGG GCC CGA CGT CCT TAA G
Ser Ser Trp Arg Pro Pro Pro Arg Glu Leu Val Leu Pro Asp Gly Pro Ser Cys Ser Asn
```

Eco32I, HindIII, Bsu15I, HincII, SalI, XmiI, PspXI, XhoI, Eco0109I, ApaI, Bsp120I, Acc65I, KpnI — 760

```
GA TAT CAA GCT TAT CGA TAC CGT CGA CCT CGA GGG GGG GCC CGG TAC CCA
CT ATA GTT CGA ATA GCT ATG GCA GCT GGA GCT CCC CCC CGG GCC ATG GGT
Ser Ile Leu Ser Ile Ser Val Thr Ser Arg Ser Pro Pro Gly Pro Val Trp
```

```
GCT TTT GTT CCC TTT AGT GAG GGT TAA TTG CGC GCT TGG CGT AAT CAT GGT CAT AGC TGT TTC CTG 3′
CGA AAA CAA GGG AAA TCA CTC CCA ATT AAC GCG CGA ACC GCA TTA GTA CCA GTA TCG ACA AAG GAC 5′
```

T3 transcription start — T3 promoter — M13/pUC reverse sequencing primer (-26), 17-mer

```
Ser Lys Asn Gly Lys Thr Leu Thr Leu Glu Ser Ser Pro Thr Ile Met Thr Met
```

5.3.6 Analyses of pDK2-3, pDK2-5, and pDK2-9 sequence

BlastN was performed using NCBI tool which revealed that the sequence of pDK2-5(Fig. 5.9 and 5.11) and pDK2-9 (Fig. 5.13 and 5.15) are >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). Similarly, further sequence analysis 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 was done with T3 primer (Fig. 5.7) which 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).

```
CATTACCCTGCACTGGCCGACTGTTTACCCCGACCGGACTGGCAAAACCCTGCCGTTACCCGGCTT
AATCGCCTTGAACCACATCCCCCTTTCGCCAGCTGGAGAAATACCGAAGAGGCCCGCACCGATCCC
CCTTCCCAACATTTGCGCCCCCTGAATGGCGAATGGGACGCGCCCTGTAGATCCGCATTAAGCGCG
GCGGGCGGGGTGCTTACACGCAGAGTGACCGCTACATTTTGGAGCGCCGTACCGCCTGCTACTTTA
TATTTCTTACCTTCGTTTGTCGCCCCGTTCGCCAGCTTTTCGGTCACCTCTAAATACTGCCCTCCC
TTTACGATGACGATTGTGGCTTCACGGCCCTGCGACCCACACAACTTGATTATCTGATGGTTGCGT
ATGATATCATCGGCTGATACCCGTATCTCCACGTTTGATTGTGGGACACGTCTGCCAGGGCGGATC
GTGTGCGATTGCGCAAACTCAGCTTATGACGTCTATTATTTGATTCATCAGGTATTTGCCGATGCA
GCCGATTAGGTTGAGATGATCTGATTCAGC (558)
```

Fig. 5.7: Partial nucleotide sequence of pDK2-3; sequence done with T3 primer.

```
  2 attaccctgcactggccgactgtttaccccgaccggactggcaaa
      I  T  L  H  W  P  T  V  Y  P  D  R  T  G  K
 47 accctgccgttacccggcttaatcgccttgaaccacatccccctt
      T  L  P  L  P  G  L  I  A  L  N  H  I  P  L
 92 tcgccagctggagaaataccgaagaggcccgcaccgatccccctt
      S  P  A  G  E  I  P  K  R  P  A  P  I  P  L
137 cccaacatttgcgccccctga 157
      P  N  I  C  A  P  *                    (a)
```

Fig. 5.8a: ORF obtained from pDK2-3 insert. **a**, 51 amino acid long at +2 frame showing 74% homology with hypothetical protein of uncultured Sphingobacteria (1) and glycosyl transferase protein of uncultured Flavobacteria (2).

1. hypothetical protein S18_1049_0001 [uncultured Sphingobacteria]

```
pDK2-3    1  ITLHWPTVYPDRTGKTLPLPGLIALNHIPLSPAGEIPKRPAPIPLPNICA  50
             IT+HWP+ Y   TGKTL LP LIAL HIPLSPAG I KRPAPI LPN CA
hypothet 18 ITIHWPSFYNVVTGKTLALPNLIALQHIPLSPAGVIAKRPAPIALPNSCA  67
```

2. glycosyl transferase protein,family 1 [uncultured Flavobacteria]

```
pDK2-3 1    ITLHWPTVYPDRTGKTLPLPGLIALNHIPLSPAGEIPKRPAPIPLPNICA  50
            IT+HWP+ Y   TGKTL LP LIAL HIPLSPAG I KRPAPI LPN CA
gly tra 212 ITIHWPSFYNVVTGKTLALPNLIALQHIPLSPAGVIAKRPAPIALPNSCA  261
```

```
144 atgttgggaaggggatcggtgcgggcctcttcggtatttctcca
    M  L  G  R  G  I  G  A  G  L  F  G  I  S  P
 99 gctggcgaaaggggatgtggttcaaggcgattaagccgggtaac
    A  G  E  R  G  M  W  F  K  A  I  K  P  G  N
 54 ggcagggttttgccagtccggtcggggtaa 25
       G  R  V  L  P  V  R  S  G  *        (b)
```

Fig. 5.8b: ORF obtained from pDK2-3 insert. **b**, 39 amino acid long at -1 frame showing 71% homology with conserved domain protein of *E. coli* MS 69-1.

conserved domain protein [Escherichia coli MS 69-1]

```
pDK2-3    1   MLGRGIGAGLFGISPAGERGMWFKAIKPGNGRVLPVRS  38
              +LGR IGAGLF I+PAGERGM  KAIK GN RV PV +
cons dm   30  LLGRAIGAGLFAITPAGERGMCRKAIKLGNARVFPVTT  67
```

```
GCGATGGGAGACCATAGAGACAGAGGAAAGAAAAAAAAAAACACACACGAACAACACGCCAAACAA
TGACACCGGGGGAGTACGAAAAAGAGAGAGAAGAGGAAGAAGAGAAAAAAAAACGGGCTGACATTC
TTATCCTCCTTCTCAGCTGAAATGTGATACCCTCCTGATCGAGGCAGGGGCGCGGGCGAAGGCGGT
TTGATGCGCACGAACTGTGACAGTATGACTTGCTCCCTTCCTCGTGCCATGCGCGCCGGCGATTTC
AAGCCGATGAACATGCCGAATAACCGACACCGCGATCGAGACAGAAAATCACGCGCCGCCTTCGGC
CACGAGCCGTCCATCTCGGCGAGGTCTTGCCCCGCCTCAACAGAATACTCCACTCTTTGCTATTGT
CTCGAAACCCGGATGGCGGTCCTCCCGCGGATCGAGGTGTGGGTGACTGATCTGGAGACTCTGCAC
ACTCGCCACCGCGCCGGACTCCCCCTCCCTGCCTCCGTCCCGAAAAATCC    (512)
```

Fig. 5.9: Partial nucleotide sequence of pDK2-5; sequence done with T7 primer.

```
pDK2-5     155     TGTGATACCCTCCTGATCGAGGCAGGGGCG-CGGGC-GA-AGGCGGTTTGATGCG-CACG  210
                   ||||||||||| | || | | |||||||||| ||||| || |||||||||||||| ||||
Ac mult 205021     TGTGATACCCAC-TG-TAG-GGCAGGGGCGTCGGGCCGAGAGGCGGTTTGATGCGGCACG
205077

pDK2-5     211     AACTGTGACAGTATGACTTGCTCC--CTTCCTCGTGCCATGCGCGCCGGCGATTTC-AAG  267
                   ||   |||| || ||||| |||||  ||||||||||||||||||||||||||||||| |||
Ac mult 205078     AA---TGACGGT-TGACTAGCTCCAGCTTCCTCGTGCCATGCGCGCCGGCGATTTCAAAG
205133

pDK2-5     268     CC-GATGAAC-ATGCC-GAATAACCGAC---ACCGCGATCGAGACA--GAAAATCA--CG  317
                   || ||||||| | ||| ||||||| |||    ||||||||||||||   |||||||   |
Ac mult 205134     CCAGATGAACAACGCCAGAATAACGGACCAGGCCGCGATCGAGACAAGAAAAATCAACAG
205193

pDK2-5     318     CGCCGCCTTCGGCCA--CGAGCCGTCCA  343
                   ||||||||||||||||  |||||||| ||
Ac mult 205194     CGCCGCCTTCGGCCAGGCGAGCCGTTCA  205221                    (a)
```

```
pDK2-5     225       GACTTGCTCC-CTTCCTCGTGCCATGCGCGCC----GGCGATTTCAAGCC--GATGAACA  277
                   |||| ||||  | |||||| ||| ||||||||    || ||  |||  ||  ||||| ||
Gly tra    2065010   GACTGGCTCGGCCTCCTCGCGCCGTGCGCGCCAAACGGAGACCTCAGACCCAGATGATCA  2065069

pDK2-5     278       TGCCGA--ATAACCGACA---CCGCGATCGAGAC-AGAAAA-TCAC--GCGCCGCCTTCG  328
                   |||| || || | |    |||||| |||||| |||||| |||   ||||||||||||
Gly tra    2065070   ATCCGAGAATGACGGCCCAGGCCGCGACCGAGACGAGAAAAATCAGCAGCGCCGCCTTCG  2065129

pDK2-5     329       GCCA--CGAGCCGTCCA  343
                     ||||  |||||||| ||
Gly tra    2065130   GCCAGGCGAGCCGTTCA  2065146                              (b)
```

Fig. 5.10: BlastN result of pDK2-5 nucleotide sequence **(a)** showing 83% homology with + strand of *Acidiphilium multivorum* AIU301 plasmid pACMV1, Features flanking this part of subject sequence: 63 bp at 5' side: hypothetical protein; 484 bp at 3' side: hypothetical protein and **(b)** showing 72% homology with + strand of *Acidiphilium multivorum* AIU301, Features flanking this part of subject sequence: putative glycosyltransferase and hypothetical protein.

```
TGGATTCCGGAACTGAGCCCCAGGAAGGTCGCCGTACCCAACTGGGTGGCCATTCCGTTCAGCGCA
TTGCTCGCGTCGTAGTAAGTGGTCTCGGTTACACTCGATAGCGCGCCCGAGGGCTGGTTCAGCGTG
AACCCGCTCAGGAAGGACTGGATGCCGGTCGGCGACGTGATTCCGGTTTCCGACGCGTAGAGATAC
AGCGTGCCACCCGCAGACGTGGAGGTATTGATGCTGTTGGTGCTAAAGTTGTCTGCAGGCAGAAGA
TCGCCGCTGCTACCGATCTTGTTGATGGTGAACGTACCGAAAGAGCCATTGTAGTAGGGTCCGCCC
GGGGCCGGAGACTGCGTGACATAATTCGCGTAACCGGCCTCCTGCAGGGTGATGGTGATCACAGGC
GTCGCATGGGCGACCGGCGTTCCGACCAGAGCAATGGCGCCGCATATCGCTGCGCCGAGTAATGTC
GATTTCAGTGATTTACGTTTCATTGTCCTCATTTCCTAGTCGCCTTGGCCACGAAGTTGCTGCCGT
TTGCCGTAAAATTTGATGATGCGCGTTTACCGCTAAATTTTGAAAGCAAAAACAATGCCATTTTTA
TTAGCTTTTGTTTTCAATACCTTATCATTACGTTTCGGCACG     (636)
```

Fig. 5.11: Partial nucleotide sequence of pDK2-5; sequence done with T3 primer.

```
494 atgaggacaatgaaacgtaaatcactgaaatcgacattactcggc
    M  R  T  M  K  R  K  S  L  K  S  T  L  L  G
449 gcagcgatatgcggcgccattgctctggtcggaacgccggtcgcc
    A  A  I  C  G  A  I  A  L  V  G  T  P  V  A
404 catgcgacgcctgtgatcaccatcaccctgcaggaggccggttac
    H  A  T  P  V  I  T  I  T  L  Q  E  A  G  Y
359 gcgaattatgtcacgcagtctccggccccgggcggaccctactac
    A  N  Y  V  T  Q  S  P  A  P  G  G  P  Y  Y
314 aatggctctttcggtacgttcaccatcaacaagatcggtagcagc
    N  G  S  F  G  T  F  T  I  N  K  I  G  S  S
269 ggcgatcttctgcctgcagacaactttagcaccaacagcatcaat
    G  D  L  L  P  A  D  N  F  S  T  N  S  I  N
224 acctccacgtctgcgggtggcacgctgtatctctacgcgtcggaa
    T  S  T  S  A  G  G  T  L  Y  L  Y  A  S  E
179 accggaatcacgtcgccgaccggcatccagtccttcctgagcggg
    T  G  I  T  S  P  T  G  I  Q  S  F  L  S  G
134 ttcacgctgaaccagccctcgggcgcgctatcgagtgtaaccgag
    F  T  L  N  Q  P  S  G  A  L  S  S  V  T  E
 89 accacttactacgacgcgagcaatgcgctgaacggaatggccacc
    T  T  Y  Y  D  A  S  N  A  L  N  G  M  A  T
 44 cagttgggtacggcgaccttcctggggctcagttccggaatcca 1
    Q  L  G  T  A  T  F  L  G  L  S  S  G  I
```

Fig. 5.12: ORF obtained from pDK2-5 insert. 164 amino acid long at -2 frame showing 100% homology with hypothetical protein APM_0575 of *Acidiphilium* sp. PM.

```
                hypothetical protein APM_0575 [Acidiphilium sp. PM]

 pDK2-5 4    MKRKSLKSTLLGAAICGAIALVGTPVAHATPVITITLQEAGYANYVTQSPAPGGPYYNGS  63
             MKRKSLKSTLLGAAICGAIALVGTPVAHATPVITITLQEAGYANYVTQSPAPGGPYYNGS
 hypoth 1    MKRKSLKSTLLGAAICGAIALVGTPVAHATPVITITLQEAGYANYVTQSPAPGGPYYNGS  60

 pDK2-5 64   FGTFTINKIGSSGDLLPADNFSTNSINTSTSAGGTLYLYASETGITSPTGIQSFLSGFTL  123
             FGTFTINKIGSSGDLLPADNFSTNSINTSTSAGGTLYLYASETGITSPTGIQSFLSGFTL
 hypoth 61   FGTFTINKIGSSGDLLPADNFSTNSINTSTSAGGTLYLYASETGITSPTGIQSFLSGFTL  120

pDK2-5  124  NQPSGALSSVTETTYYDASNALNGMATQLGTATFLGLSSGI  164
             NQPSGALSSVTETTYYDASNALNGMATQLGTATFLGLSSGI
hypoth  121  NQPSGALSSVTETTYYDASNALNGMATQLGTATFLGLSSGI  161
```

```
ATCAGGCTGGTTGTGATACCCACTGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTTTGATGCGGC
ACGAATGACGGTTGACTTGCTCCAGCTACTCGGTGCCATGCGCGCCGGCGATTTCAAAGCCAGATG
AACAATGCCAGAATAACCGACCAGGCCGCGATCGAGACAAGAAAAAATCAACAGCGGCGGTTTTCG
GCAAGGCAAGCTTTTCAGCACTCCTCGTGGCGTAATGTATTCTGGCCCCCGGCACTCAGAAGCGAA
ATAACTTCCCACAACCAATTTGCATCAATTGTATCCATACGGCGAAGAACTCCGCGTGATTGGGGG
GCGGTGTTCAACATCTCCCGGGTCCGTTGATCAGGTGCCTGGGCGGCTGTACTTGATTCTTGCTTG
AGGATCAGTCATGCCATCAGTCCCCGCCGATCCGCCTGCCTGAAGAACTTTTGAACCGCCCGGGCC
TCTCTTTGCCGAAGTCAATCTCGTCCATTGCCCCATGGGGGAAGGGCGAAACTTAAACCTCCTAAT
TTCGCCCAAGTTTTGTGAAGTTGGGAACAAACTATAAAGGGCGGGTTGGTCTCAAAATTCGGGAAA
AGGGCCTCCTTTTTTGGGAAGAGGCC    (620)
```

Fig. 5.13: Partial nucleotide sequence of pDK2-9; sequence done with T7 primer.

```
pDK2-9   1       ATCAGGCTGGTTGTGATACCCACTGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTTTGA  60
A mul    205010  ATCAGGCTGGTTGTGATACCCACTGTAGGGCA-GGGGCGTCGGGCCGAGAGGCGGTTTGA  205068

pDK2-9   61      TGCGGCACGAATGACGGTTGACTTGCTCCAGCTACTCGGTGCCATGCGCGCCGGCGATTT  120
A mul    205069  TGCGGCACGAATGACGGTTGACTAGCTCCAGCTTCCTCGTGCCATGCGCGCCGGCGATTT  205128

pDK2-9   121     CAAAGCCAGATGAACAATGCCAGAATAACCGACCAGGCCGCGATCGAGACAAGAAAAAAT  180
A mul    205129  CAAAGCCAGATGAACAACGCCAGAATAACGGACCAGGCCGCGATCGAGACAAG-AAAAAT  205187

pDK2-9   181     CAACAGCGGCGGTTTTCGGCAAGGCAAGCTTTTCAGCACTCCTCGTGGCGTAATGTATTC  240
A mul    205188  CAACAGC-GCCGCCTTCGGCCAGGCGAGCCGTTCAGCA-TCCTCGTGGCGT-ATGTATCT  205244

pDK2-9   241     TGGC----------------------CCCCGGCACTCAGAAGCGAAATAACTTCCCACAAC  279
A mul    205245  TGCCGCGGCACTCAGATGTATCTTGCCGCGGCACTCAGACGCGAAATAAC-TCCGAC-AT  205302

pDK2-9   280     CAATTTGCATCAATTGTATCCATACGGCGAAGAACTCCGCGTGATTGGGGGGCGGTG-TT  338
A mul    205303  CAATTTGCATCGATTGTATCCATATGGCGAAGACCTCGGCGTGATTGGGGGGCGGTGTTT  205362

pDK2-9   339     CAACATCTCCCGGGTCCGTTGATCAGG-TGCCTGGGCGGCTGTACTTGATTCTTGCTTGA  397
A mul    205363  CAGCATCCCCTGGGTCCGTTGATCAGGCTGCCTGGGCGGCTGTACTTGGATCTTGC-TGA  205421

pDK2-9   398     G-GAT-CAGT--CATGC-CATCA  415
A mul    205422  GCGATCCAGTTCCATGCGCATCA  205444
```

(a)

```
pDK2-9 1         ATCAGGCTGGTTGTGATACCCAC---TGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTT  57
Acryp  174018    ATCAGGCTGGTTGTGATACACACGTTTGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTT  173959

pDK2-9  58       TGATGCGGCACGAATGACGGTTGACTTGCTCCAGCTA------CTCGGTGCCATGCGCGC  111
Acryp  173958    TGATGCGGCGCGAATGATGGTTGACTAGCTGTAGCTTAGCTTCCTCG-TGCCAATCGCGC  173900

pDK2-9 112       CGGCGATTTCAAAGCCAGATGAACAATGCCAGAATAACCGACCAGGCCGCGATCGAGACA  171
Acryp  173899    CGGCGAATTCAAAACCAGATGAACAACGCCAGAATAACGGCCCAGGCCGCGATCGAGACA  173840

pDK2-9  172      AGAAAAAATCAACAGCGGCGGTTTTCGGCAAGGCAAGCTTTTCAGCACTCCTCGTGGCGT  231
Acryp  173839    AGA-AAAATCAACAGC-GCCGCCTTCGGCCAGGCGAGCCGTTCGGCA-TCCTCGTGGCGT  173783

pDK2-9  232      AATGTATTCTGGCCCCCGGCACTCAGAAGCGAAATAACTTCCCACAACCAATTTGCATCA  291
Acryp  173782    -ATGTATCTTG--CCGCGGCACTCAGACGCGAAATAAC-TCCGAC-ATCAATTTGCATCA  173728

pDK2-9  292      ATTGTATCCATACGGCGAAGAACTCCGCGTGATT-GGGGGGCGGTG-TTCAACATCTCCC  349
Acryp  173727    ATTGTATCCATACGGCGAAGACCTCGGCGTGGTTGGGGGGGCGGTGTTTCAGCATCCCCT  173668

pDK2-9  350      GGGTCCGTTGATCAGG-TGCCTGGGCGGCTGTACTTGATTCTTGCTTGAG-GAT-CAGT-  405
Acryp  173667    GGGTCCGTTGATCAGGCTGCCTGGGCGGCTCTACCTGGATCTTGC-TGAGCGATCCAGTT  173609

pDK2-9  406      -CATGC-CATCA  415
Acryp  173608    CCATGCGCATCA  173597
```

(b)

Fig. 5.14: BlastN result of pDK2-9 nucleotide sequence **(a)** showing 85% homology with + strand of *Acidiphilium multivorum* AIU301 plasmid pACMV1, Features flanking this part of subject sequence: 52 bp at 5' side: hypothetical protein; 261 bp at 3' side: hypothetical protein and **(b)** showing 85% homology with - strand of *Acidiphilium cryptum* JF-5 plasmid pACRY01, Features flanking this part of subject sequence: hypothetical protein.

```
TGGATTCCGGAACTGAGCCCCAGGAAGGTCGCCGTACCCAACTGGGTGGCCATTCCGTTCAGCGCA
TTGCTCGCGTCGTAGTAAGTGGTCTCGGTTACACTCGATAGCGCGCCCGAGGGCTGGTTCAGCGTG
AACCCGCTCAGGAAGGACTGGATGCCGGTCGGCGACGTGATTCGGGTTTCCGACGCGTAGAAGATA
CAGCGTGCCACCCGCAGACGTGGAGGTATTGATGCTGTTGGGTGCTAAAGTTGTCTGCGAGCAGAA
GATCGCCGCTGCTACCGATCTCTGTGATGGTGGACGTACCGAAAGAGCCACTGTAGTAAGGTCCGC
CCAGGGGCCGGAGACTGCGTGACATATTCGCCGTAACCCGCCTCCTGCAAGGGTGATTGTGAATAC
CAGGCGCCCCAGGGCGAACCGCGTCCCACCAAAACAATGGCGCCCCCATCCCTGCCCGAGAAAGGC
GAATCCCGGGATAACGTTCATTGGCTCATTCAATCGCTGGCCCAAGTGCGGTCGTTGCCGTAAAAT
TGGTAAAGCGCGTTACGCCTATTTTGAAGCAAACATGCATTTTACCCTGGTTTTAAAACTATAATA
ATTCGCCCACCAGGACATGAAGTTTAACCTTC  (626)
```

Fig. 5.15: Partial nucleotide sequence of pDK2-9; sequence done with T3 primer.

```
445 atgggggcgccattgttttggtgggacgcggttcgccctggggcg
    M  G  A  P  L  F  W  W  D  A  V  R  P  G  A
400 cctggtattcacaatcacccttgcaggaggcgggttacggcgaat
    P  G  I  H  N  H  P  C  R  R  R  V  T  A  N
355 atgtcacgcagtctccggcccctgggcggaccttactacagtggc
    M  S  R  S  L  R  P  L  G  G  P  Y  Y  S  G
310 tctttcggtacgtccaccatcacagagatcggtagcagcggcgat
    S  F  G  T  S  T  I  T  E  I  G  S  S  G  D
265 cttctgctcgcagacaactttagcacccaacagcatcaatacctc
    L  L  L  A  D  N  F  S  T  Q  Q  H  Q  Y  L
220 cacgtctgcgggtggcacgctgtatcttctacgcgtcggaaaccc
    H  V  C  G  W  H  A  V  S  S  T  R  R  K  P
175 gaatcacgtcgccgaccggcatccagtccttcctga 140
    E  S  R  R  R  P  A  S  S  P  S  *
```

Fig. 5.16: ORF obtained from pDK2-9 insert. 101 amino acid long at -2 frame showing 81% homology with hypothetical protein APM_0575 of *Acidiphilium* sp. PM.

hypothetical protein APM_0575 [Acidiphilium sp. PM]

```
pDK2-9   39  GGPYYSGSFGTSTITEIGSSGDLLLADNFSTQ  70
             GGPYY+GSFGT TI +IGSSGDLL ADNFST
hypoth   53  GGPYYNGSFGTFTINKIGSSGDLLPADNFSTN  84
```

5.4 Discussion

Plasmids of acidophilic heterotrophs of the genera *Acidiphilium* and *Acidocella,* were demonstrated 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). A cryptic plasmid pAM5 (5161bp) bearing approximately copy number of 50-60 of *Acidiphilium multivorum* JCM-8867, which was completely sequenced, revealed five putative open reading frames of which two ORFs, ORF1 and ORF3 showed significant identity with various plasmid encoded mobilization (Mob) and replication initiation (Rep) proteins, respectively. One of the recombinant clones, pSK1 bearing pAM5 mob region was mobilized to *Escherichia coli* in presence of conjugative plasmid pRK2013 (Singh and Banerjee, 2007). 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,

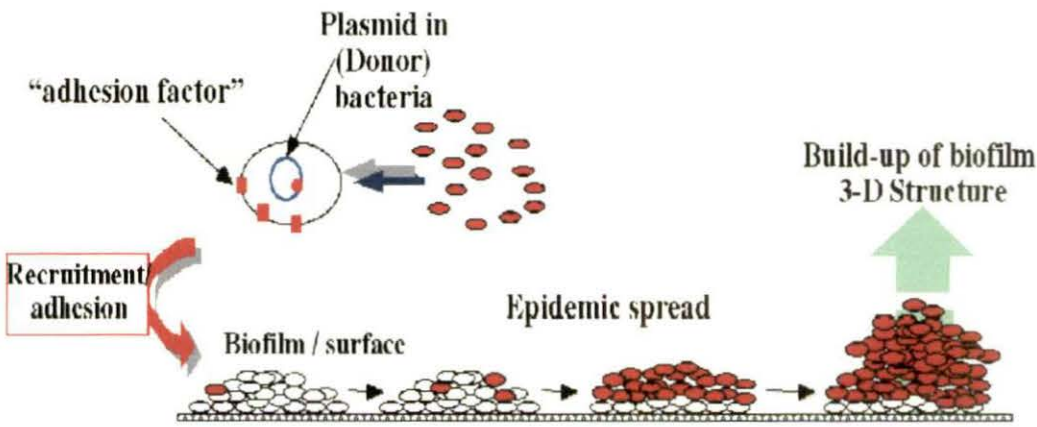
3,449 ORF) and **eight circular plasmids** pACMV1 (271,573 bp, 62.9% G+C, 284 ORF), pACMV2 (65,564 bp, 61.9% G+C, 69 ORF), pACMV3 (54,248 bp, 61.2% G+C, 61 ORF), pACMV4 (40,588 bp, 60.1% G+C, 44 ORF), pACMV5 (14,328 bp, 59.0% G+C, 19 ORF), pACMV6 (12,125 bp, 59.6% G+C, 14 ORF), pACMV7 (5,178 bp, 57.6% 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). In the present study, five plasmids, sizes approximately 55 kb, 23kb, 4 kb, 2 kb, and 1 kb, were reported from the strain DK2AH2 (Fig. 5.1). The largest plasmid detected and isolated 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* JF-5 (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).

Building up of evidences that Acidiphilium strains do possess the property of biofilm formation: 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. Loosely packed coccobacillus-type normal cells formed characteristic chains of coccoidal lenticular shape with constrictions at the junctions between them in the

presence of Cd; Cu induced transformation of cells to become round shaped; Ni caused the cells to aggregate, but Zn showed no effect. Respective metal depositions on the cell surface were confirmed by scanning electron microscopy equipped with energy dispersive X-ray analysis (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.

Association of plasmid borne genes with formation of biofilm: 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 (Dudley *et al.*, 2006). 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) and Zn(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.

5.5 References

Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic. Acid. Res. **7:** 1513-1523.

Caro, L., G. Churchward, and M. Chandler. 1984. Study of plasmid replication *in vivo*. Meth. Microbiol. **17:** 97-122.

Chakravarty, R., and P. C. Banerjee. 2008. Morphological changes in an acidophilic bacterium induced by heavy metals. Extremophiles. **12:** 279-284.

Chassy, B.M., E. M. Gibson, and A. Giu¡rida. 1978. Evidence for plasmid associated lactose metabolism in *Lactobacillus casei* subsp. *casei.* Curr. Microbiol. **1:** 141-144.

Cook, M.A., A. M. Osborn, J. Bettandorff, P. A. Sobecky. 2001. Endogenous isolation of replicon probes for assessing plasmid ecology of marine sediment microbial communities. Microbiology. **147:** 2089-2101.

Dudley, E.G., C. Abe, J. M. Ghigo, P. Latour-Lambert, J. C. Homazabal, and J. P. Nataro. 2006. An Inc 1 plasmid contributes to the adherence of the atypical Enteroaggregative *Escherichia coli* Strain C1096 to cultured cells and abiotic surfaces. Infect Immun. **74:** 2102-2114.

Foster, T. J. 1983. Plasmid determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47:** 361-409.

Ghigo, J. M. 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature. **412:** 442-445.

Ghosal, D., I. S. You, D. K. Chatterjeee, and A. M. Chakrabarty. 1985. Plasmids in the degradation of chlorinated aromatic compounds. In: Plasmids in Bacteria (Helniski, D.R., Cohen, S.N., Clewell, D.B., Jackson, D.A. and Hollander, A., Eds.), pp. 667-687. Plenum Press, New York.

Ghosh, S., N. R. Mahapatra, and P. C.Banerjee. 1997. Metal resistance in *Acidocella* strain and plasmid-mediated transfer of this characteristic to *Acidiphilium multivorum* and *Escherichia coli*. Appl. Environ. Microbiol. **63:** 4523-4527.

Ghosh, S., N. R. Mahapatra, T. Ramamurthy, and P. C.Banerjee. 2000. Plasmid curing from an acidophilic bacterium of the genus *Acidocella.* FEMS Microbiol. Lett. **183:** 271-274.

Lobos, J.H., T. E. Chisolm, L. H. Bopp, and D. S. Holmes. 1986. *Acidiphilium organovorum* . sp. nov., an acidophilic heterotroph isolated from a *Thiobacillus ferrooxidans* culture. Int. J. Sys. Evol. Microbiol. **36:** 139-144.

Macrina, F.L., J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell. 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene. **19:** 345-353.

Molnár, A., L. Amaral, and J. Molnár. 2003. Antiplasmid effect of promethazine in mixed bacterial cultures. Int. J. Antimicrob. Agents. **22:** 217-222.

Molnár, J., B. Schneider, Y. Mandi, S. Farkas, and I. B. Holland. 1980. New mechanism of plasmid curing by psychotropic drugs. Acta. Microbiol. Acad. Sci. Hung. **27:** 309-315.

Nies, A., D.H. Nies, and S. Silver. 1989. Cloning and expression of plasmid gene encoding resistance to chromate and cobalt in *Alcaligens eutrophus*. J. Bacteriol. **171:** 5065-5070.

Partridge, S.R., and R. M. Hall. 2004. Complex multiple antibiotic and mercury resistance region derived from the r-det of NR1 (R100). Antimicrob. Agents Chemother. **48:** 4250-4255.

Puttamreddy, S., N. A. Cornick, F. C. Minion. 2010. Genome-wide transposon mutagenesis reveals a role for pO157 genes in biofilm development in *Escherichia coli* O157:H7 EDL933. Infect. Immun. **78:** 2377-2384.

Sambrook, J.E., F. Fritshch, and T. Maniatis. 1989. Molecular cloning a lab manual. 2nd edition cold spring harbor laboratory press, cold spring harbor . N.Y.

Silver, S. 1996. Bacterial resistances to toxic metal ions- a review. Gene. **179:** 9-19.

Silver, S., and L. T. Phung. 1996. Bacterial heavy metal resistance; new surprises. Annu. Rev. Microbiol. **50:** 753-789.

Singh, S.K., A. Singh, and P. C. Banerjee. 2010. Plasmid encoded AcrAB-TolC tripartite multidrug-efflux system in *Acidiphilium symbioticum* H8. Curr. Microbiol. **61:** 163-168.

Singh, S.K., and P. C. Banerjee. 2007. Nucleotide sequence analysis of cryptic plasmid pAM5 from *Acidiphilium multivorum*. Plasmid. **58:** 101-114.

Southey-Pillig, C.J., D. G. Davies, and K. Sauer. 2005. Characterization of Temporal Protein Production in *Pseudomonas aeruginosa* biofilms. J. Bacteriol. **187:** 8114-8126.

Spenglar, G., A. Molnár, Z. Schelz, L. Amaral, D. Sharples, and J. Molnár. 2006. The mechanism of plasmid curing in bacteria. Curr. Drug. Targets. **7:** 823-841.

Springael, D., L. Diels, L. Hooyberghs, S. Kreps, and M. Mergeay. 1993. Construction and characterization of heavy metal-resistnat haloaromatic-degradaing *Alcaligens eutrophus* strains. Appl. Environ. Microbiol. **59:** 334-339.

Stanisich, V. A. 1984. Identification and analysis of plasmids at the genetic level. In: Methods in Microbiology, Vol. **17** (Bennett, P.M. and Grinsted, J., Eds.), pp. 5-32. Academic Press, London.

Suzuki, K., N. Wakao, Y. Sakurai, T. Kimura, K. Sakka, and K. Ohmiya. 1997. Transformation of *Escherichia coli* with a large plasmid of *Acidiphilum multivorum* AIU 301 encoding arsenic resistance. Appl. Environ. Microbiol. **63:** 2089-2091.

Tapia, J.M., J. A. Muñoz, F. González, M. L. Blázquez, M. Malki, and A. Ballester. 2009. Extraction of extracellular polymeric substances from the acidophilic bacterium *Acidiphilium* 3.2Sup(5). Water. Sci. Technol. **59:** 1959-1967.

Terawaki, Y., K. Ishizu, S. Horiuchi, N. Goto, and R. Nakaya. 1976. Control of replication and segregation of R plasmid Rts1. J. Bacteriol. **128:** 693-700.

Top, E.M., and D. Springael. 2003. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. Curr. Opin. Biotechnol. **14:** 262-269.

Trevors, J. T. 1986. Plasmid curing in bacteria. FEMS Microbiol. Lett. **32:** 149-157.

Yoo, J.S., H. S. Kim, S. Y. Chung, Y. C. Lee, Y. S. Cho, and Y. L. Choi. 2001. Characterization of the Small Cryptic Plasmid, pGD2, of *Klebsiellia* sp. KCL-2. J. Biochem. and Mol. Biol. **34:** 584-589.