Characterization of Novel Plasmid and Proteome Analysis of a Natural Isolate *Bacillus cereus* GC subgroup A

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Characterization of Novel Plasmid and Proteome Analysis of a Natural Isolate *Bacillus cereus* GC subgroup A"** submitted by **Pankaj Kumar Jain, ID No. 2004PH29088** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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ABSTRACT

The emergence of multiple metal/antibiotic resistance among bacterial populations poses a potential threat to human health. The co-existence of metal/antibiotic resistance in bacterial strains suggests the role of metals as a factor, which can also contribute to such a phenomenon. The objective of this study was to characterize multiple metal/antibiotic resistant bacteria from the soil and water samples of a copper mining industry. 24 strains (12 Gram-positive and 12 Gram-negative bacteria) were identified by the 16s rRNA gene sequencing. Out of the 24 bacterial isolates, 9 isolates showed multiple metal and antibiotics resistance. These strains were screened to find the presence of an endogenous plasmid DNA. One strain, *Bacillus* sp. (PRS3) was found to have a plasmid (pPRS3a) of 4.2 kb. This strain was identified to be *Bacillus cereus* GC subgroup A, by 16s rRNA sequencing (EU744603), phenotypic & biochemical characterization (MTCC10207) and fatty acid methyl esters (FAMEs) analysis. This bacterium has an average size of 2.93μm, as determined by SEM. To characterize plasmid from *Bacillus cereus* GC subgroup A, it was cloned into pET-28c(+) and sequenced through primer walking. The sequences of pPRS3a were submitted to NCBI GenBank under the accession no. GQ404376.1. The sequence analysis confirmed the size of plasmid to be 4126bp, presence of Amp^r gene, unique restriction site for 30 restriction endonuclease enzymes, strong promoters and origin of replication. It was also found that pPRS3a has origin of replication (ori) which can be recognized by both Gram's positive and Gram's negative bacteria, therefore, pPRS3a can stably be maintained in both bacterial systems. Attempts were made to cure pPRS3a with curing agents; Ethidium Bromide and Proflavin, but up to 25^{th} generation, it could not be cured and pPRS3a could maintain its normal functional properties especially, ampicillin resistance. Two genes arsGFP and cat were cloned in pPRS3a and expressed successfully. The proteome of *Bacillus cereus* GC subgroup A was studied by 2D PAGE (611 spots), MudPIT (135 proteins) and under the combination of stress (copper(II) 1.5mM & temperature 40°C), a differentially expressed protein, Formate acetyltransferase (84.6kDa) was identified by MALDI-TOF/MS. Based on the above properties, pPRS3a is a potential candidate for the construction of shuttle cloning and expression vector. Formate acetyltransferase upon stress of copper(II) and elevated temperature, 40°C in *Bacillus cereus* GC subgroup A, is the first report. Thus, it can be predicted that metal pollution results in selective pressure that leads to the development of multiple metal/antibiotic resistance among bacterial populations, probably through horizontal gene transfer.

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List of Abbreviations

2D PAGE	Two dimensional Polyaccrylamide Gel Electrophoresis
AAS	Atomic Absorption Spectrophotometer
Amp	Ampicillin
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cat	Chloramphenicol acetyltransferase
CBB	Coomassie Brilliant Blue
CIAP	Calf Intestinal Alkaline Phosphatase
ddNTPs	Dideoxy Nucleotide Tri Phosphates
DTT	Dithiothreitol
EGTA	Ethylene Glycol Tetra Acetic acid
EtBr	Ethidium Bromide
FAMEs	Fatty Acid Methyl Esters
FAT	Formate Acetyl Transferase
GC	Gas Chromatography
GFP	Green Fluorescence Protein
IEF	Iso Electric Focusing
IPG	Immobilized pH Gradient
LB	Luria Bertani
LC	Liquid Chromatography
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight
MCS	Multiple Cloning Site
MICs	Minimum Inhibitory Concentrations
MS	Mass Spectrometry
MTCC	Microbial Type Culture Collection
MudPIT	Multi Dimensional Protein Identification Technology
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
Ori	Origin of Replication
PBS	Phosphate Buffer Saline
pfl	Pyruvate formate lyase
pI	Isoelectric point
PMSF	Phenylmethanesulfonyl fluoride
ROS	Reactive oxygen species
SDS PAGE	Sodium Dodecyl Sulphate Polyaccrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SI	Similarity Index
TAE	Tris Acetate EDTA
TCA	Tri-Chloro Acetic acid
TDS	Total Dissolved Solute
TEM	Transmission Electron Microscopy
TIC	Total Ion Current

Chapter I

General Introduction

Chapter-I

General Introduction

The environmental stress leads to the development of various unique genetic mechanisms in prokaryotes. Such stress could be metals, antibiotics, temperature, salt, organic & inorganic chemicals etc. This study aims to explore a copper mine industry for the presence of multiple metal and antibiotic resistant bacterial strains and their characterization for stress tolerance. To carry out this study. a metal mining and handling industry, Khetri copper complex (KCC) was selected, which is located 50KM from Pilani and works under the HCL.

Hindustan Copper Limited (HCL), a public sector undertaking under the administrative control of the Ministry of Mines, was incorporated on 9th November 1967. The Company markets copper cathodes, copper wire bar, continuous cast copper rod and by-products, such as anode slime (containing gold, silver, etc.), copper sulphate and sulphuric acid. Presently, KCC comprises two underground mines (capacity 1 million tons of ore per annum) viz. Khetri and Kolihan. Together with a concentrator plant, a metallurgical complex and a refinery, the company has in this sector a capacity to produce 31,000 TPA of refined copper in the form of cathodes.

Heavy metals are metals with a density above 5 g/cm^3, thus the transition elements from V (but not Sc and Ti) to the half-metal As, from Zr (but not Y) to Sb, from La to Po, the lanthanides and the actinides can be referred to as heavy metals. Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast 1984). Most heavy metals are transition elements with incompletely filled 'd' orbitals. These d orbitals provide heavy-metal cations with the ability to form complex compounds, which may or may not be redox-active. Thus, heavy-metal cations play an important role as trace-elements in sophisticated biochemical reaction (Nies, 1999).

Many metal ions are essential as trace elements, but at higher concentrations, they form unspecific complex compounds in the cell, which leads to toxic effects. Because it is difficult to remove heavy metals from the environment and even the low levels observed pose a high risk of heavy metal accumulation in the food chain, many heavy metals constitute a global environmental hazard. At high concentrations, these essential heavy metals, as well as the non-essential metals, not only can become extremely toxic, causing abnormal symptoms, but the entry of heavy metals into the food chain is also of concern because it can cause chronic health problems. Therefore, the possibility of transfer of these contaminants to humans through the food chain has led researchers to pay considerable attention to this issue (Yang et al. 2005).

Heavy metals have their industrial applications due to their technological importance and metal handling industries represent major sources of metal contamination into the environment (Rehman et al. 2008). In the recent past, it has been well documented that metal contamination in the environment could have an important role in the maintenance and proliferation of antibiotic resistance. Metal contamination, therefore, represents a long-standing, widespread and recalcitrant selection pressure with both environmental and clinical importance that potentially contributes to the maintenance and spread of antibiotic resistance factors. In addition, the structural and functional characteristics of antibiotic resistance share common features with those of metal resistance (Austin et al. 2006).

Among heavy metals, copper is required in trace amount for the growth and development of an organism but it is considered as a sword with two edges (Nies, 1999) if present above permissible limits (2 mg/L, as per WHO guidelines). The electrochemical potential of Cu^{2+}/Cu^{+} is -268 mV, well within the physiological range. Copper easily interacts with radicals, best with molecular oxygen. Its radical character makes it very toxic and many organisms are more sensitive to copper (Gordon et al. 1994) than others. The copper toxicity is due to the production of hydroperoxide radicals (Rodriguez et al. 1993) and their interaction with cell membrane to interfere membrane processes like, signal transduction (Suwalsky et al. 1998). Cells need to maintain certain cytoplasmic concentrations of copper if it has to meet their physiological requirements. To this end, microorganisms use a number

of mechanisms to maintain the correct equilibrium, including the uptake, chelation and extrusion of metals (Silver, 1996a; Robinson et al. 2001). Many systems have already been identified in bacteria that involve metallothioneins, P-type ATPases, cation/proton antiporters, redox enzymes etc. for homeostasis of copper ions and for acquiring resistance against metals and antibiotics.

An increasing number of studies have documented an additional mechanism for maintaining antibiotic-resistant bacteria in the environment through co- or cross- resistance to metals or co-regulation of resistance pathways (Timoney et al. 1978; Wireman et al. 1997; Rasmussen and Sorensen, 1998; McArthur and Tuckfield, 2000; Perron et al. 2004; Berg et al. 2005; Stepanauskas et al. 2005). Therefore, it is apparent that metal exposure can directly select for metal resistant bacteria while co-selecting for antibiotic-resistant bacteria. The resistance to an antibiotic is due to the presence of antibiotic resistance gene and its transfer to the other organisms by horizontal gene transfer mechanisms. The genes for antibiotic resistance are frequently located on mobile genetic elements such as plasmids and transposons (Summers et al. 1993).

In order to gain an insight into the interactions of metal ions with bacterial system and its association with antibiotic resistance, a schematic model of the biological system is illustrated in figure 1.

Antibiotic resistance in bacterial pathogens is a major public health challenge worldwide, with increased morbidity and mortality due to bacterial infections and associated costs in billions of dollars in the United States alone (Neu, 1992; Levy, 2001). While commensal bacteria are thought to function as reservoirs of antimicrobial resistance genes for pathogens in host-associated communities (Schwarz, 2001; Salyers, 2004; Yates, 2006), further research is needed to address questions about the origin, presence and persistence of antimicrobial resistance genes in the environment and their potential transfer from environmental to clinical settings.

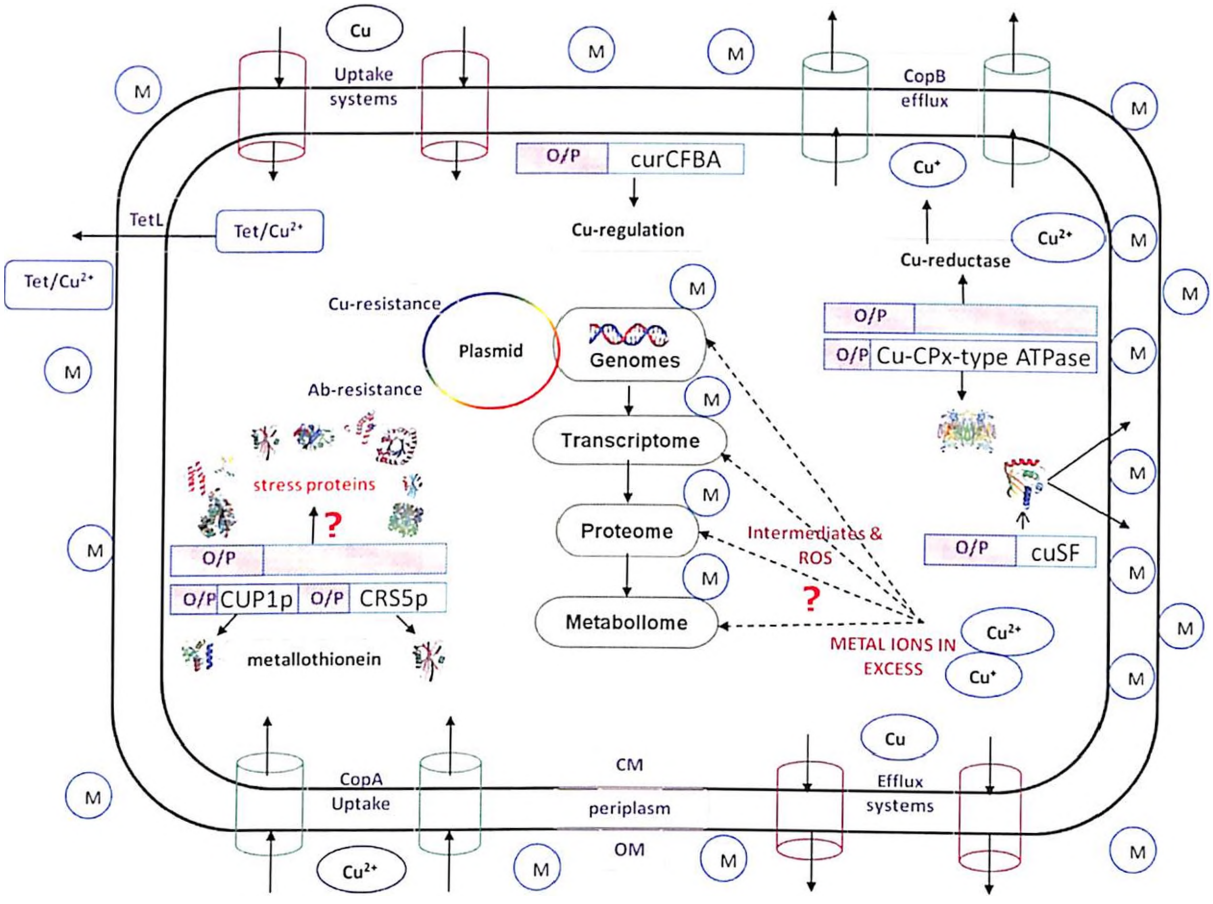


Fig. 1. The schematic diagram shows metal-microbe interactions. Copper is an essential trace metal ion for normal growth and developments of bacterial cell. Copper ions from the extracellular environment enter the cell via specific and non-specific transporters. They are incorporated into metalloproteins and excess metal ions are transported out by the highly efficient efflux systems. The cysteine rich low molecular weight protein metallothioneins sequester essential metal ions to maintain total metal content of the cell. Metallothioneins are over expressed under metal stress conditions to overcome metal toxicity. The antibiotic resistance also co-exists with metal resistance on plasmid. Generally, the metal stress in a cell leads to formation of reactive oxygen species (ROS) and other intermediates, which disrupt normal cellular processes via interaction with the genome and proteome. The "stress proteins" are expressed in response to excess metals and ROS, which play a major role in protein folding and detoxification mechanisms. The cell wall proteins, polysaccharides and fatty acids offer charged functional groups like phosphate, sulfate, amino, hydroxyl groups that favor adsorption of metals at the cell wall. Extracellular or intracellular enzymes are

also involved in the reduction of highly toxic metal ions to less toxic forms (biotransformation). M-copper ions; OM-outer membrane; CM-cytoplasmic membrane; O/P-operator-promoter.

The spread of antibiotic resistance among pathogens is a major public health concern and a striking example of evolution in action (Neu, 1992). While the use of antibiotics in medicine and agriculture clearly stimulates the proliferation of antibiotic resistance, heavy metals and other toxicants have also been suggested to play an important role. Multiple genes encoding for metal and antibiotic resistance are commonly found on the same plasmids and/or transposons, conferring co-resistance (Summers, 2002). In some cases, single enzymes function as efflux pumps for multiple metals and antibiotics; this is defined as cross-resistance (Hayashi et al. 2000). In both cases, the exposure of microbial assemblages to one toxicant could result in an indirect selection for bacteria with resistances to multiple, chemically unrelated toxicants (Austin et al., 2006). While concentrations of antibiotics in aquatic environments are typically below levels likely to impact microorganisms, except near animal and fish farms (Kolpin et al. 2002), metal contamination is ubiquitous and is still rapidly increasing (Nriagu, 1996). Most antibiotics are readily degraded in the environment (Kolpin et al. 2002), but metals are not, and so may represent a long-term selective pressure, depending on the chemical form and bio-availability. Many human pathogens and commensals are common in aquatic environments. Furthermore, antibiotic resistance genes are often located on plasmids and transposons, which may be transferable among phylogenetically diverse microorganisms (Summers, 2002). Horizontal gene transfer from environmental organisms to human commensals may be an important pathway for the acquisition of antibiotic resistance by human pathogens (Wilson and Salyers, 2003). Thus, if indirect selection for microorganisms and mobile genetic elements carrying antibiotic resistance genes takes place in metal contaminated environments, this process may facilitate the spread of antibiotic resistance in human pathogens. Elevated frequencies of microbial resistance to various antibiotics have been observed in metal-contaminated freshwater streams (McArthur and Tuckfield, 2000).

The evolution of antibiotic resistance is as much an effect of antibiotics acting on the environment and physiology of bacteria, as it is a Darwinian phenomenon of the selection of pre-existing resistant strains (Amábile-Cuevas et al. 2004). Antibiotics can promote gene

transfer by creating environments that concentrate resistance genes and by conditioning bacterial physiology to be more receptive to foreign DNA (Heinemann, 1999). Mutations and the acquisition of new genes explain some resistance phenotypes (Heinemann et al. 2000), but they do not satisfactorily explain the pace, range and physiological diversity of resistance mechanisms (Heinemann and Silby, 2003). The resistance genes are found so commonly on plasmids and other horizontally mobile vectors, such as conjugative transposons. Horizontally mobile vectors transcend the boundaries of the organism in which the resistance gene initially appeared and exceed the limitations imposed on replication by clonal inheritance. Resistance was probably introduced into most taxa and individuals by horizontal gene transfer. There is no doubt that microbial participation in the broad network of organisms formed through the horizontal exchange of genes has driven the dispersal of resistance (Amábile-Cuevas and Chicurel, 1992; Davies, 1992; Levy, 1998; Cruz et al. 2002).

Horizontal gene transfer (HGT), by transduction, conjugation, or natural transformation, is one of the driving forces of bacterial adaptation and evolution with significant numbers of bacterial genomes showing evidence of HGT (Gogarten and Townsend, 2005; Smalla and Sobecky, 2002). DNA exchange between gram-positive and gram-negative bacteria has been well described and documented (Alonso et al. 2000). Chromosomal recombination in bacteria is too infrequent to account for adaptive evolution; bacteria are thought to use the vast, phylogenetically diverse gene pool available to them by HGT (Levin and Bergstrom, 2000). The roles of plasmids in the dissemination of genes for antibiotic resistance (Taylor et al. 2004), metal resistance (Nucifora et al. 1989; Lebrun et al. 1994; Borremans et al. 2001) have been extensively studied (Sayler et al. 1990; Top et al. 2000). However, most of the studied plasmids reside within a few restricted bacterial clades; nearly 70% of the sequences in the Plasmid Genome Database (http://www.genomics.ceh.ac.uk/plasmiddb/) are from Proteobacteria and Firmicutes. It is not clear that plasmid maintenance functions from other clades (e.g., Actinobacteria) will be similar to previously identified genes.

Plasmids are extrachromosomal DNA elements with characteristic copy numbers within the host. These replicons have been found in species from the three representatives of the living

world, namely, the domains *Archaea*, *Bacteria* and *Eukarya* (Woese et al. 1990). Plasmids may constitute a substantial amount of the total genetic content of an organism, representing more than 25% of the genetic material of the cell in some members of the *Archaea* (Zillig et al. 1994; Holmes et al. 1995). They can incorporate and deliver genes by recombination or transposition, thus favoring genetic exchanges in bacterial populations. Since plasmids can be introduced into new hosts by a variety of mechanisms, they can be considered a pool of extrachromosomal DNA, which is shared among populations. The wealth of genetic information carried by plasmids, their impact in the microbial communities and the potential of these elements to act as natural cloning vectors have stimulated research into plasmids not only from the fundamental but also from the clinical, biotechnological and environmental points of view. Three main factors have contributed to the development of plasmid research: (i) the genetic organization of these elements is apparently simple, (ii) they can be easily isolated and manipulated in vitro and (iii) since plasmids are dispensable, their manipulation does not appear, in principle, to have adverse consequences to the hosts.

The feature that better defines plasmids is that they replicate in an autonomous and self-controlled way. The analysis of plasmid replication and its control has led to milestone discoveries, such as the existence of antisense RNAs, and has contributed to the unraveling of mechanisms of DNA replication, macromolecular interactions and control of gene expression. The ability of some plasmids to pass across the so-called genetic barriers among different living organisms has posed questions about general mechanisms governing replication and about the communication between plasmid replication components and the host machinery involved in DNA replication. This plasmid-host communication has attracted the attention of researchers working in environmental and in evolutionary fields. Plasmid host range studies also have clear implications in clinical microbiology and in biotechnology. Despite their autonomous replication, plasmids extensively use the replication machinery of the host, and therefore plasmid replication studies facilitate the exploration of the mechanisms involved in chromosome replication. Plasmids are genetic mosaics and often carry genes that confer novel phenotypic properties upon cells; most attention is paid to these accessory genes (Jerke et al. 2008). However, stable persistence within individual cells requires a set of core maintenance genes. Plasmids also need to

ensure that they are segregated between dividing cells; whereas high-copy number plasmids appear to rely on random segregation, large low-copy number plasmids utilize active partitioning systems. Circular plasmids can form multimeric structures (Hallet et al. 2004); to ensure proper segregation, multimer resolution systems are required.

Plasmids are ubiquitous in bacteria (Burian et al. 1997; Fredrickson et al. 1988) and occupy a different evolutionary space from those genes that primarily evolve in association with hosts adapting to environmental change (Souza and Eguiarte, 1997; Cooper and Heinemann, 2000). The space, or reproductive niche, that plasmids occupy is not as sensitive to the effects of antibiotics as is the viability, or reproduction, of the host (Heinemann et al. 2000). Understanding how plasmids evolve to carry resistance genes could enable us to develop the kinds of drugs that prevent plasmids from serving as the messengers of resistance (Levin and Bergstrom, 2000; Heinemann, 2001).

The introduction of new antibiotics into clinical practice has decreased the mortality caused by bacterial infectious diseases but the HGT of antibiotic resistance genes (as mediated by plasmid) again rising it. Emerging pathogens, poverty and multidrug resistance are contributing to this change. Many people die each year due to infections from antibiotic-resistant bacteria, and several billions of dollars are lost because of the need for additional therapies, extended hospitalization and other medical services, lost labor hours and other complications. The pace at which new antibiotics are being developed is, so far, slower than the emergence and spread of resistance traits by horizontal gene transfer (Amábile-Cuevas and Heinemann, 2004). It has been proposed that drugs that act to eliminate or inhibit the replication of resistance-gene vectors (such as plasmids) might, under some circumstances, add years of life to current and pending antibiotics. Antibiotic resistance determinants constitute a privileged system for the study of naturally occurring DNA transfer among bacteria, because they are easy to trace and because of the massive selective pressure exerted by antibiotic use for human therapy and animal feeding. The finding of inconsistencies in molecular data led to the notion of horizontal gene flux between these distantly related microorganisms (Courvalin, 1994). Plasmids exhibit diverse mechanisms that enable their survival and horizontal transfer, to the extent that, with the advent of more sophisticated

detection techniques, the distinction between chromosome and plasmid has become 'blurred' (Sedgley et al. 2004 and Thomas, 2004).

To gain an insight into various determinants responsible for multiple metal and antibiotic resistances, it is important to look into mechanism of homeostasis of these heavy metals in the living cell. It is imperative to understand the copper metabolism in different organisms as organism under study has been isolated from a copper rich environment. In the gram-positive bacterium, copper metabolism is better understood than in gram-negative bacteria. *Enterococcus hirae* contains a cop operon with two structural genes, both encoding a P-type ATPase. While CopA is probably responsible for copper uptake and copper nutrition, CopB (35% identical to CopA) is responsible for copper efflux and detoxification (Odermatt et al. 1992; Odermatt et al. 1993). Both proteins seem to transport silver as well as copper (Odermatt et al. 1994). Copper-transporting P-type ATPases have been found in a variety of organisms, in cyanobacteria (Phung et al. 1994; Kanamaru et al. 1995) and in eukaryotes; however, in *S. cerevisiae*, the copper P-type ATPase does not transport copper across the cytoplasmic membrane. In addition, Cu^{2+} is accumulated by the CorA-related transporters ALR1p and ALR2p (Dancis et al. 1994a; Dancis et al. 1994b; Hassett and Kosman 1995). *E. coli* also harbors a P-type ATPase, probably required for copper homoeostasis, besides the plasmid mediated copper-resistance determinant, which is homologous to the *Pseudomonas* system. In *Enterococcsus faecium*, tcrB gene (confer resistance to copper) and vanA gene (responsible for macrolide resistance) are located on a single transferable plasmid. So the characterization of an endogenous plasmid may provide worth of information about metal and antibiotic resistance genes.

The proteome analyses of microbes represent a powerful tool for the identification of differentially expressed proteins under environmental stress conditions (Frausto da Silva and Williams, 2001; Barton et al. 2007). In particular, the proteomic data is vital, as the observed phenotype is a direct result of the action of the proteins rather than the genome sequence (Bhurja et al. 2003). For identification of metal induced proteins and analysis of the proteome of the bacterium, techniques like, SDS PAGE, 2D PAGE, MALDI-TOF/MS and MudPIT have been used.

MudPIT has been used in a wide range of proteomics experiments, including large-scale catalogues of proteins in cells and organisms, profiling of organelle and membrane proteins, identification of protein complexes, determination of posttranslational modifications and quantitative analysis of protein expression (Florens et al. 2002; Fujii et al. 2004; Cagney et al. 2005; Yates et al. 2005; Cantin et al. 2006; Speers and Wu, 2007). The success of MudPIT for proteomics is a result of the two-dimensional resolution of peptides and the ability of database searching programs to identify proteins based on a search with one or more peptides. By using peptides for identification, unbiased identification of proteins can be made; even proteins of relatively low abundance, extreme hydrophobicity or pI and large molecular weight can be identified. MudPIT has been proven robust for the resolution of complex mixtures of peptides (Claire and John, 2007).

The present work focuses on the characterization of multiple metal and antibiotic resistance bacterial strains and understanding the genetic basis for their stress tolerance by plasmid and metal induced proteins characterization. The thesis has been organized into five chapters. Apart from general introduction, second chapter describes identification and characterization of multiple metal and antibiotic resistant bacterial strains. Third chapter illustrates the characterization of plasmid, pPRS3a and its applications in cloning and expression. Complete proteomic analysis & copper(II) induced protein of *Bacillus cereus* GC subgroup A are described in chapter four whereas chapter five summarizes the overall results of the present investigation.

Chapter II

Identification and characterization of multiple metal and antibiotic resistant bacterial strains

Chapter II

Identification and characterization of multiple metal and Antibiotic resistant bacterial strains

2.1 Introduction

Heavy metals are transition metal elements with incompletely filled d orbital, which provide them the ability to form complex compounds. Heavy metal cations play an important role in vital biochemical reactions. However, at high concentrations heavy metal ions form non-specific complex compounds in the cell, which lead to toxic effects. Though bacterial exposure to metals predates human history, anthropogenic-derived sources of metals represent a major source of contamination in the environment. In recent past, due to industrial revolution their concentration have dramatically increased as they have many industrial applications due to their technological importance and the heavy metal and radionuclide pollution from mining industries, electroplating industries, nuclear power plants and agricultural runoffs is a major cause of concern to public health, animals and ecosystem (Rehman et al. 2007). Heavy metal ions and metalloids are chemical species that can be very toxic to cells in a variety of ways, e.g. through binding to essential respiratory chain proteins, oxidative damage via the production of reactive oxygen species, DNA damage, etc. It can be anticipated that soil organisms are likely to bear systems to cope with toxic metals in their environment. Therefore, to survive, soil microorganisms have developed a variety of mechanisms to cope up with the toxicity of these heavy metals.

As metal cannot be degraded just like any other chemical species therefore, metal persist in the environment once they have accumulated. In addition, more severe problem is that, metal contamination represents a long-standing, widespread and recalcitrant selection pressure with both environmental and clinical importance that potentially contributes to the maintenance and spread of antibiotic resistance factors (Austin et al. 2006). A substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Summers et al. 1993; Alonso et al. 2001; Summers, 2002).

This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics. Unlike antibiotics, metals are not subject to degradation and can subsequently represent a long-term selection pressure (Stepanauskas et al. 2005). Thus, there are concerns regarding the potential of metal contamination to maintain a pool of antibiotic-resistance genes in both natural and clinical settings. In addition to metals, other toxicants are implicated in the co-selection of antibiotic resistance, including quaternary ammonium compounds and antifouling agents and detergents (Sidhu et al. 2001; Chapman, 2003).

The persistence and proliferation of antibiotic resistance in bacterial pathogens represents a considerable public health concern. Subsequent measures to control the emergence and propagation of antibiotic resistance have encountered limited success, and it persists in spite of the restricted use of several key antibiotics, which indicates that there are components governing the evolution, dissemination and perpetuation of these resistance systems that have yet to be understood. Resistance to antibiotics can be conferred by chromosomal or mobile genetic elements (e.g. plasmids) and achieved using four main strategies: (i) reduction of membrane permeability to antibiotics; (ii) drug inactivation; (iii) rapid efflux of the antibiotic; and (iv) mutation of cellular target(s) (Krulwich et al. 2005). In addition, antibiotic sequestration has also been suggested as a potential resistance strategy. It has been known for several decades that metal- and antibiotic resistance genes are linked, particularly on plasmids, because the evidence for co-resistance as a mechanism of antibiotic–metal co-selection came from studies that used transformation, plasmid curing and plasmid sequencing approaches (Novick et al. 1968; Foster, 1983) that may be the reason for co-selection of metal and antibiotic resistance. Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance (Table 1).

Table 1. Well characterized examples of shared structural and functional characteristics of prokaryotic antibiotic and metal resistance systems.

Resistance mechanisms	Metal ions	Antibiotics	References
Reduction in permeability	As, Cu, Zn, Mn, Co, Ag	Ciprofloxacin, Tetracycline, Chloramphenicol, β-lactams	Silver, 1996b; Ruiz, 2003
Drug and metal alteration	As, Hg	β-lactams, Chloramphenicol	Mukhopadhyay et al. 2002; Wright, 2005
Drug and metal efflux	Cu, Co, Zn, Cd, Ni, As	Tetracycline, Chloramphenicol, β-lactams	Levy, 2002; Nies, 2003
Alteration of cellular target(s)	Hg, Zn, Cu	Ciprofloxacin, β-lactams, Trimethoprim, Rifampicin	Barkay et al. 2003; Roberts, 2005
Drug and metal sequestration	Zn, Cd, Cu	Coumermycin A	Castillo et al. 1991; Bontidean et al. 2000

In the present study, we have explored a metal mining & handling industry, Khetri copper mine complex, for the isolation and characterization of bacterial strains.

2.2 Materials and Methods

2.2.1 Sample site and Sample collection

To achieve the objective of characterization of multiple metal and antibiotic resistance bacterial strains at molecular level, the sample site, Khetri copper complex, Rajasthan, India, a metal mining and handling industry was selected. As the site is used for extraction of copper from the ore to finished product so the possibility of finding a bacterial strain with desired characteristics were enormous. Khetri is located at 27.98° N & 75.8° E with an average elevation of 484 meters (1587 feet) and undergoes seasonal temperature variations of +1.0 to 45°C.

Soil and water samples were collected from five sites. Soil samples from the surface layer (0-25 cm) of cultivated soils and water samples from copper mine effluent tailing pond were taken in a sterile sample collection vial (HIMEDIA, Mumbai). Samples were transported to

laboratory for analysis. The soil samples were air dried, ground for further use and water samples were left undisturbed so that major suspended particles settle down.

Physio chemical characterization of both sample were done under the parameters like, color, smell, pH, temperature and total dissolved solute (TDS). Now these samples were subjected to metal ion analysis followed by isolation of bacterial strains.

2.2.2 Metal ion analysis of soil and water

For the metal ion analysis of soil samples, a quantity of 2gm of soil from each sample was taken in 250ml glass beaker and digested with 8ml aqua regia (1:3 mixtures of HNO_3 and HCl) on sand bath for 2hrs (Devêvre and Horwáth, 2001). After evaporation of aqua regia, the samples were dissolved in 10 ml of 2% HNO_3 followed by dilution with distilled water to 50ml (Lark et al. 2002). This resultant extract and copper mine effluent (water samples) were filtered and analyzed by Atomic Absorption Spectrophotometer (AAS) using AAnalyst 300, Perkin Elmer, USA.

Metal salts and stock solutions

Metal stock solutions were prepared using analytical grade metal salts in the form of chlorides or sulphates of Cd, Co, Cr, Cu, Mn, Ni and Zn dissolved in MilliQ water. If required, metal solutions were acidified with 1-2% nitric acid and stored in dark conditions. Metal solutions and working standards were prepared freshly from the stock. Atomic spectral grade standards were procured from National Physical Laboratory (NPL), New Delhi.

Atomic Absorption Spectrometry (AAS)

Metal ion concentrations were determined in a Perkin Elmer (AAnalyst 300) atomic absorption spectrophotometer using air-acetylene flame. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. AAS was first calibrated with standard metal solutions in the range covering concentrations of metals likely to be found in samples. Samples were analyzed in triplicates to maintain

reproducibility. The peak wavelength (nm) for determination of metals by AAS: Cd 288.8; Co 240.7; Cr 357.9; Cu 324.8; Mn 279.5; Ni 232.0; and Zn 213.9 nm.

2.2.3 Isolation and purification of microorganisms

For the isolation of bacterial strains from soil and water samples, a quantity of 1.0 gm of soil and 1 ml of water from each of the collected samples was dissolved in 9 ml sterile distilled water and serial dilutions from 10^{-1} to 10^{-7} were made. Each dilution was plated on Nutrient agar plate without antibiotics and incubated at 37°C. After growth of different microorganisms on plate, each bacterial colony based on their morphological characteristics was picked up and further purified by repeated streaking on Nutrient agar plate (Aneja, 2001). Each bacterial culture was inoculated in Nutrient broth, incubated at 37°C, 200rpm for 15h and glycerol stocks were made, frozen at -70^{0}C.

2.2.4 Identification of bacterial strains

The isolated bacterial strains from soil and water samples were further characterized by Gram's staining, 16s rRNA sequencing and fatty acid methyl esters (FAMEs) analysis for their phenotypic and genotypic identification.

2.2.4.1 Gram's staining

The technique developed by Hans Christian Gram, a Danish physician in 1884, it helps to determine: gross morphology of bacteria & differentiation of bacteria into two groups, Gram's positive and Gram's negative. This staining procedure is achieved by heat fixing a smear of bacterial culture (mid-log phase) onto glass slide, staining with crystal violet (primary stain) for 60 sec, treatment with iodine (mordant) for 60 sec followed by alcohol wash for 30 sec and finally staining with safranin (counter stain) for 60 sec (Godkar, 1994). Air dried slides were visualized under oil immersion microscope (Olympus BX41) at magnification of 1000x. Image was captured using Olympus C-4000 camera.

2.2.4.2 Morphological, Physiological and Biochemical analysis

Morphological, physiological and biochemical analysis was carried out for the identification of isolated bacterial strains at genus and species level. All these analyses were performed as

per the standard microbiological methods. Following parameters were used for each analysis, for morphological tests: colony morphology, Gram's reaction, spores, position, shape, sporangia bulging and motility; for physiological tests: growth at different temperatures, growth at different pH, growth on NaCl and growth under anaerobic conditions; and biochemical tests: growth on MacConkey, indole test, methyl red test, Voges Proskauer test, citrate utilization, H_2S production, gas production from glucose, casein hydrolysis, esculin hydrolysis, gelatin hydrolysis, starch hydrolysis, urea hydrolysis, nitrate reduction, ornithine decarboxylase, lysine decarboxylase, catalase test, oxidase test, tween 20 hydrolysis, tween 40 hydrolysis, tween 60 hydrolysis, tween 80 hydrolysis, xanthane hydrolysis, acid production from dextrose, lactose, sucrose, maltose, manitol and xylose. Based on these analyses bacterial strain was identified and strain was deposited at IMTECH, Chandigarh, India and MTCC accession no. was obtained.

2.2.4.3 16s rRNA sequence analysis

16s rRNA sequencing, analysis and submission to GenBank of all bacterial isolates was done using the following steps.

Genomic DNA isolation

For genomic DNA isolation from bacterial cell, QIAmp DNA isolation kit (QIAGEN, 51304) was used. Bacterial cultures (~25mg) were harvested from rapidly growing cells (14-15h old) on NB plate without antibiotics, in a 1.5 ml microcentrifuge tube. Cells were suspended in 180 µl of the enzyme solution (20 mg/ml lysozyme; 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton) and incubated for 30 min at 37°C. Then 20µl proteinase K (20mg/ml) was added and mixed by vortexing followed by incubation at 56°C for 2h. A 200 µl Buffer AL was added to the sample, mixed by pulse-vortexing for 15 sec and incubated at 70°C for 10 min. Finally, 200 µl of ethanol (96–100%) was added to the sample and mixed by pulse-vortexing for 15 sec. After mixing, tubes were briefly centrifuged to remove drops from inside the lid. This mixture was applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at 8000 rpm for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and filtrate was discarded. Then 500 µl of Buffer AW1 was added to the QIAamp Mini spin column and

centrifuged at 8000 rpm for 1 min. Finally, 500 µl of Buffer AW2 was added and centrifuged at 14,000 rpm for 3 min, the filtrate was discarded and centrifuged again for 1 min. Elution of the genomic DNA was carried out by adding 200 µl of Buffer AE followed by incubation at room temperature for 5 min and centrifugation at 8000 rpm for 1 min. This step was repeated once to obtain final volume of 400 µl containing genomic DNA.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was carried out using eubacterial primers targeted for 16s rRNA, to amplify a specific region (Therese at al. 1998). Assay conditions of PCR for a 50 µl reaction were 8 µl of 200 µM dNTPs, 5 µl of 10x PCR buffer (5 mM $MgCl_2$, 500 mM KCl, 100 mM Tris Cl, 0.01% gelatin), 0.36 µM of primers for eubacterial genome: U1 5' TTGGAGAGTTTGATCCTGGCTC 3', rU4 5' GGACTACCAGGGTATCTAA 3' (Bagyalakshmi et al. 2006). The primers and PCR reagents were obtained from Bangalore Genei, India. Amplification of the genome was carried out using 10 µl of template DNA (~500ng) in Perkin Elmer thermal cycler (Model 2700) with the following thermal profile: Initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min.

DNA Sequencing: ABI Prism 3100 AVANT genetic analyzer

The sequence of the PCR amplified DNA is deduced with the help of the ABI Prism 3100 AVANT genetic analyzer that works based on the principle of Sanger dideoxy sequencing. The fluroscent based detection by automated sequencer adopts the Sangers method and incorporates the fluorescent dyes into DNA extension products using 5'-dye labeled primers or 3'-dye labeled ddNTPs (dye terminators called commercially as RRMIX). Each dye emits light at a different wavelength when excited by an argon ion laser. All four colours and therefore all four bases can be detected and distinguished in a single capillary injection (Therese at al. 1998). The four nucleotode bases with the respective aceptor dyes and colour emission have been shown in Table 2.

Table 2. The four nucleotode bases with the aceptor dyes and colour emission.

Terminator	Acceptor dye	Colour of raw data on ABI PRISM3100 electrophoretogram
A	dR6G	Green
C	dROX	Red
G	dR110	Blue
T	dTAMRA	Black

The PCR reaction was carried out for cycle sequencing for generating different length fragments with 5'-dye labeled primers or 3'-dye labeled ddNTPs. Assay conditions for PCR for 10μl reaction were 1μl of amplified product, 3μl of sequencing buffer, 4 pM (picomoles) of primers, 1μl of RRMIX and 3μl of water. Amplification was carried out with the following thermal profile: Initial denaturation at 96°C for 1 min followed by 25 cycles of denaturation at 94°C for 10 sec, annealing at 50°C for 5 sec, extension at 60°C for 4 min.

Before subjecting this mixture into capillary electrophoresis for DNA sequencing it needs to be purified.

Purification of Extension Products

The products were purified to remove the unincorporated dye terminators before subjecting the samples to capillary electrophoresis. 2μl of 125mM EDTA and 10μl of 3M sodium acetate (pH 4.8) were mixed to the cycle sequenced products followed by the addition of 50μl of absolute ethanol and incubation at room temperture for 15 minutes. Centrifugation at 8000rpm for 20 min was done to precipitate the amplified product and remove the unutilized ddNTPs, primer (short length molecules) etc. The pellet was washed twice with 75% ethanol followed by air drying.

The purified samples with the dye at the terminated 3'end were suspended in fomamide and subjected to capillary electrophoresis by an automated sample injection in ABI PRISM 3100 genetic analyser. The emitted flurorescence from the dye labels was collected as counts per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing that are analysed by software known as the seq scape manager. 16s rRNA sequencing was done at Sankara Nethralaya, Chennai.

DNA sequence analysis and GenBank submission

The obtained DNA sequences in form of charomatogram were analysed with the bioinformatic tools. Chromas Lite 2.01 was used to view the chromatograms and to deduce DNA sequences. Chromatograms having considerable peaks were taken for deducing the DNA sequence. These obtained sequences were converted in to FASTA file format using BioEdit version 7.0.0. To know the identity of organism, obtained sequences were compared with nucleotide databases like GenBank (Benson et al. 2000) EMBL (Kanz et al. 2005) and the Ribosomal Database Project (RDP) (Cole et al. 2003) etc, through BLAST (Basic Local Alignment Search Tool) program under parameters like, percentage query coverage and percentage identity. Finally, all these sequences were submitted to NCBI GenBank and accession nos. were obtained.

2.2.4.4 Fatty acid methyl esters (FAMEs) analysis

FAME analysis is used for the characterization of genera and species of microorganisms. This identification is based on the Gas Chromatographic (GC) analysis of extracted fatty acid methyl esters of microorganisms. For the species level identification the strain was grown on TSBA plates for 24h. Bacterial cells (~40mg) were harvested from a rapidly growing quadrant of a quadrant streak plate. To liberate the fatty acids from the cellular lipids saponification of the harvested cells was performed by addition of 1.0 ml of reagent A (45g sodium hydroxide, 150ml methanol and 150ml distilled water) and incubation at 100°C for 30 min. This step was followed by methylation with 2.0 ml of reagent B (325ml HCl (6.0N) and 275ml methyl alcohol) and incubation at 80°C for 10 min to give the respective fatty acid methyl esters (FAMEs). Now these FAMEs were extracted from aqueous phase by addition of 1.25 ml of reagent C (200ml hexane and 200ml methyl tert-butyl ether) and incubated at 25°C for 10 min. Finally, an aqueous wash with 3.0 ml of reagent D (10.8g sodium hydroxide dissolved in 900ml distilled water) was given to organic extract prior to Gas Chromatographic (Agilent 6890 GC, Agilent Technologies) analysis (Sasser, 2006).

The analysis of obtained peaks was done using the Sherlock pattern recognition software (Kunitsky et al. 2006). FAME profile of the sample was generated. Identification was then produced based on profile similarity when compared to a database of known species. Finally

results were expressed in terms of similarity index (SI). The SI is the expression of the relative distance of the unknown sample from the population mean.

2.2.5 Metal and Antibiotic resistance

All the bacterial strains were isolated from a copper mining industry, so its point of interest to know their resistance level for heavy metals. This resistance pattern was observed in terms of Minimum Inhibitory Concentrations (MICs). It has long been established that there is a clear association between heavy metal resistance & antibiotic resistance and the genes responsible for these properties are linked together and particularly resides on extrachromosomal molecules like plasmid, transposon or integron. Therefore, antibiotic resistance profile was generated for all the isolated and identified bacterial strains. Metal and antibiotic resistance profiles were determined in following manners.

Metal resistance profile

For metal resistance profile, overnight grown cultures of bacterial strains, *Bacillus cereus* and *E. coli* K12 were inoculated and incubated at 37^0C under shaking conditions (150 rpm). The cultures were incubated for 14-16 hrs and growth in each concentration was recorded and Minimum Inhibitory Concentration (MIC) was determined at millimolar level (Vajiheh et al. 2003). Metals used in this study were copper(II), cadmium(II), zinc(II), chromium(VI), arsenic(II), manganese(II) and uranium(II). All metal solutions were prepared in milli-Q water using respective metal salts.

Antibiotic resistance profile

Overnight grown cultures of bacterial strains were used for antibiotic resistance/susceptibility. *Bacillus cereus* and *E. coli* K12 were used as a reference for gram positive and negative bacterial strain respectively. Disc diffusion method was used to check the resistance/sensitivity of bacterial strain towards given antibiotics (Bauer et al. 1966). The bacterial cultures were incubated for 14-16 hrs and zone of inhibition was measured in millimeter. The resistance/sensitivity of the bacterial strains toward any antibiotic was calculated as per the guidelines of the manufacturer (Himedia Laboratories Pvt. Limited, Mumbai, India). Antibiotics used in this study were Amikacin, Ampicillin/Sulbactam,

Metronidazole, Cloxaicillin, Oxacillin, Meropenem, Bacitracin, Vancomycin, Erythromycin, Clindamycin, Gentamycin, Oxy tetracycline, Ampicillin, Tetracycline, Chloramphenicol, Kanamycin and Streptomycin.

2.2.6 Plasmid DNA isolation

Plasmid DNA, an extrachromosomal genetic element, imparts very special properties like metal resistance (Mergeay et al. 2003), antibiotic resistance (Chen et al. 2007; O'Connor et al. 2007; Murray et al. 2007), etc. to the host bacterium. As all the isolated and identified bacterial strains having a good degree of resistance for the given metal and antibiotics, therefore, plasmid DNA of all the bacterial strains was isolated. The collection of these 24 bacterial strains posses both Gram's negative and Gram's positive, so the alkaline lysis method of plasmid DNA isolation was used with minor modification to suit all the bacterial strains.

Plasmid DNA was isolated with a Qiagen plasmid isolation kit (QIAGEN Plasmid Mini Kit, 12123) with minor modifications. A single bacterial colony from an overnight grown Nutrient agar plates was inoculated in 10 ml of NB without antibiotic. The flasks were incubated overnight at 37°C, 200 rpm in an orbital shaker incubator. 2 ml of this overnight culture was transferred to micro centrifuge tubes and spun at 10000 rpm for one minute. The supernatant was discarded and the pellet was resuspended in 300 µl of resuspension buffer Pl and lysozyme was added to the final concentration of 2mg/ml and incubated at 37°C for 30 min. 300 µl of Lysis buffer P2 was then added and the contents were mixed gently by inversion and incubated at RT for 5 min. 300 µl of pre-chilled buffer P3 was added, followed by incubation on ice for 8 min. The contents were then spun at 10000 rpm for 10 min at 4 °C and the supernatant was transferred to the Qiagen column equilibrated with buffer QBT. After washing the column with buffer QC, the DNA was eluted using 800 µl of buffer QF in a fresh 2 ml micro centrifuge tube. DNA was precipitated by the addition of 0.75 volume of isopropanol and centrifuged. The DNA pellet was washed with 1 ml of 70% ethanol and spun again as before. The supernatant was discarded and tubes were air dried for 30 min. The DNA was resuspended in 30 µl of 10 mM Tris-Cl, pH 8.0 and stored at -20 °C until use.

2.2.7 Agarose gel electrophoresis of DNA

Electrophoresis of DNA was carried out on 1% agarose gels prepared in 1x Tris Acetate EDTA Buffer (TAE buffer) and electrophoresed with 1x TAE buffer in the gel tank at 90 Volts (V) for 1h (Sambrook et al. 2001). The resolved gels were visualized and documented with a Syngene Genesnap Gel Documentation system.

2.2.8 Growth properties

Bacteria display a characteristic four-phase pattern of growth in liquid culture. The initial Lag phase is a period of slow growth during which the bacteria adapt to the conditions in the fresh medium. This is followed by a Log phase during which growth is exponential, doubling every replication cycle. Stationary phase occurs when the nutrients become limiting and the rate of multiplication equals the rate of death. Logarithmic Decline phase is the period when cells die faster than they are replaced. Growth properties of the bacterial cells were monitored by growth curve, protein determination and total biomass contents.

2.2.8.1 Growth curve

Growth curve is used for the determination of generation time of cells and to know the phase of culture at a particular time point. To obtain growth curve overnight grown culture of bacterial strain was inoculated in both Nutrient broth (NB) & Luria Bertani (LB) broth at 0.1 OD_{600} and incubated at 37°C in an orbital shaker set at 200 rpm. The growth was monitored by measuring the cell density at every 1h interval at OD_{600} (Vries et al. 2004). A graph for OD_{600} versus time was plotted and generation time and growth pattern were determined.

2.2.8.2 Determination of proteins

To determine total protein content of the bacterial cell, overnight grown culture of bacterial strain was inoculated in Nutrient broth (NB) & Luria Bertani (LB) broth at 0.1 OD_{600} and incubated at 37°C in an orbital shaker set at 200rpm. The protein content was monitored by measuring the total protein of cells by Lowry et al. (1951) and modified by Herbert et al. (1971) method at every 1h interval. 1 ml culture was withdrawn for every hour and used for protein determination. The culture was centrifuged at 6000 rpm for 8 min and washed once with Millipore water to remove all interfering components of medium. Finally, the culture

was resuspended in 1 ml Millipore water and transferred to a clean test tube 1 ml of 1N NaOH was added and placed in a boiling water bath for 5 min. The tubes were cooled in a running tap water and 5 ml of alkaline copper tartrate solution was added and incubated for 10 min at RT. 1 ml of Folin's reagent was added and the intensity of blue color formed was measured after 20 min at 650 nm (JASCO V630 Spectrophotometer). The concentration of the protein was estimated from a calibration curve prepared using lysozyme as standard. A graph for protein in μg/ml versus time in hours was plotted.

2.2.8.3 Determination of Biomass

To determine total biomass of the bacterial cell, overnight grown culture of bacterial strain was inoculated in Nutrient broth (NB) & Luria Bertani (LB) broth at 0.1 OD_{600} and incubated at 37°C in an orbital shaker set at 200 rpm. To determine total biomass contents 2ml cells were taken and processed at every 6h. For processing, cells were centrifuged at 6000rpm for 8 min, washed once with Millipore water and kept for drying at 80°C for 8-10h. After that dried tubes were allowed to cool at room temperature and biomass was weighed. Biomass was expressed in g/L and plotted against time.

2.2.9 Scanning and Transmission electron microscopy

To understand the morphology, average size & intracellular structure, Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) were carried out.

Bacterial strain was grown in LB broth for 12h (log phase) at 37°C and harvested by centrifugation at 6000 rpm for 10min at 4°C. Cells were washed several times with 100 mM phosphate buffer (pH 7.4) and fixed by submerging in 2.5% glutaraldehyde in phosphate buffer for 6h at 4°C. Samples were washed repeatedly with the same buffer, dehydrated in a graded ethanol (50-100% v/v) and acetone to critical point drying. These were then placed on a carbon coated aluminium stub and gold coated at 0.06-0.08 mbar for 45 sec and viewed under a scanning electron microscope (PHILIPS, LEO 435 VP).

Bacterial strain was grown in LB broth for 12h (log phase) at 37°C and harvested by centrifugation at 6000 rpm for 10min at 4°C. For TEM studies, the cells of *Bacillus cereus*

GC subgroup A were washed with 100 mM phosphate buffer (pH 7.4). Primary fixation of the sample was done by submerging in 2.5% glutaraldehyde in phosphate buffer for 6h at 4°C followed by post fixation with 1.0% OsO_4 in 0.1 M phosphate buffer for 2h at 4°C. The samples were washed repeatedly with the same buffer, dehydrated in a graded ethanol (50-100% v/v) and acetone to critical point drying. It was embedded on araldite and thin sections of 60-70 nm were cut with an ultramicrotome (Reichart Ultracut E, Germany) with diamond knives, which were then mounted on copper grids, contrasted with uranyl acetate and lead citrate and viewed under transmission electron microscope at 80kV (PHILIPS Morgagni 268).

2.3 Results and discussion

2.3.1 Soil and water samples characterization

To achieve the objective of identification and characterization of multiple metal and antibiotic resistant microorganisms, Khetri copper mine complex was selected as sample collection site. Soil and water samples were collected from different places of copper mining industry, as shown in fig 2 & 3, to obtain mixed populations of bacterial strains. Soil samples were been collected from different sites. The collected soil samples have been shown in figure 4. The physio-chemical properties of the soil and water samples were determined and have been shown in Table 3.

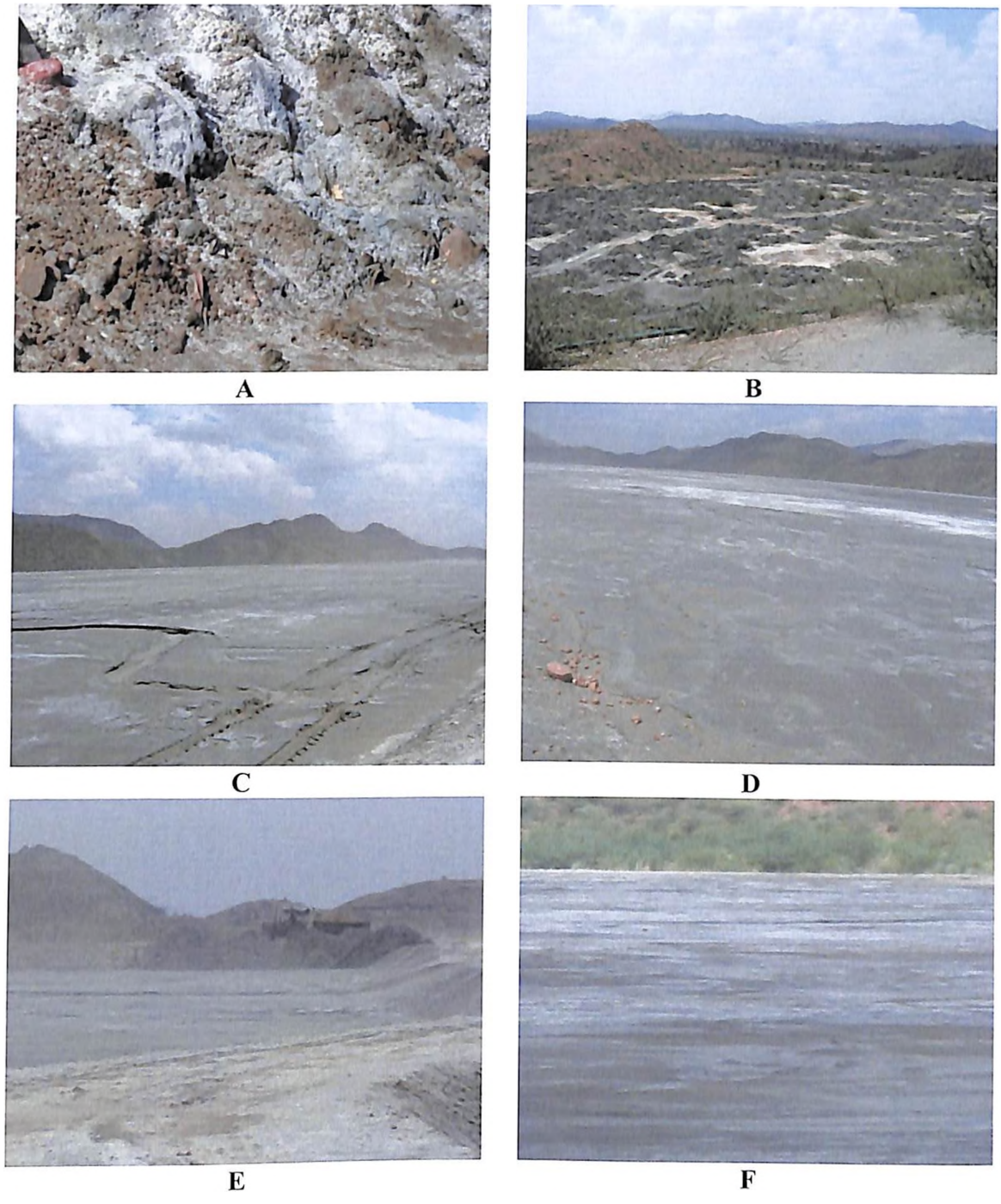


Fig. 2. Soil sample sites. Site A-F, showing different stages of copper mine discharge materials later on mixed with soil. These sample sites depicts the spread of antibiotic resistance bacterial population from contaminated waste through soil erosion.

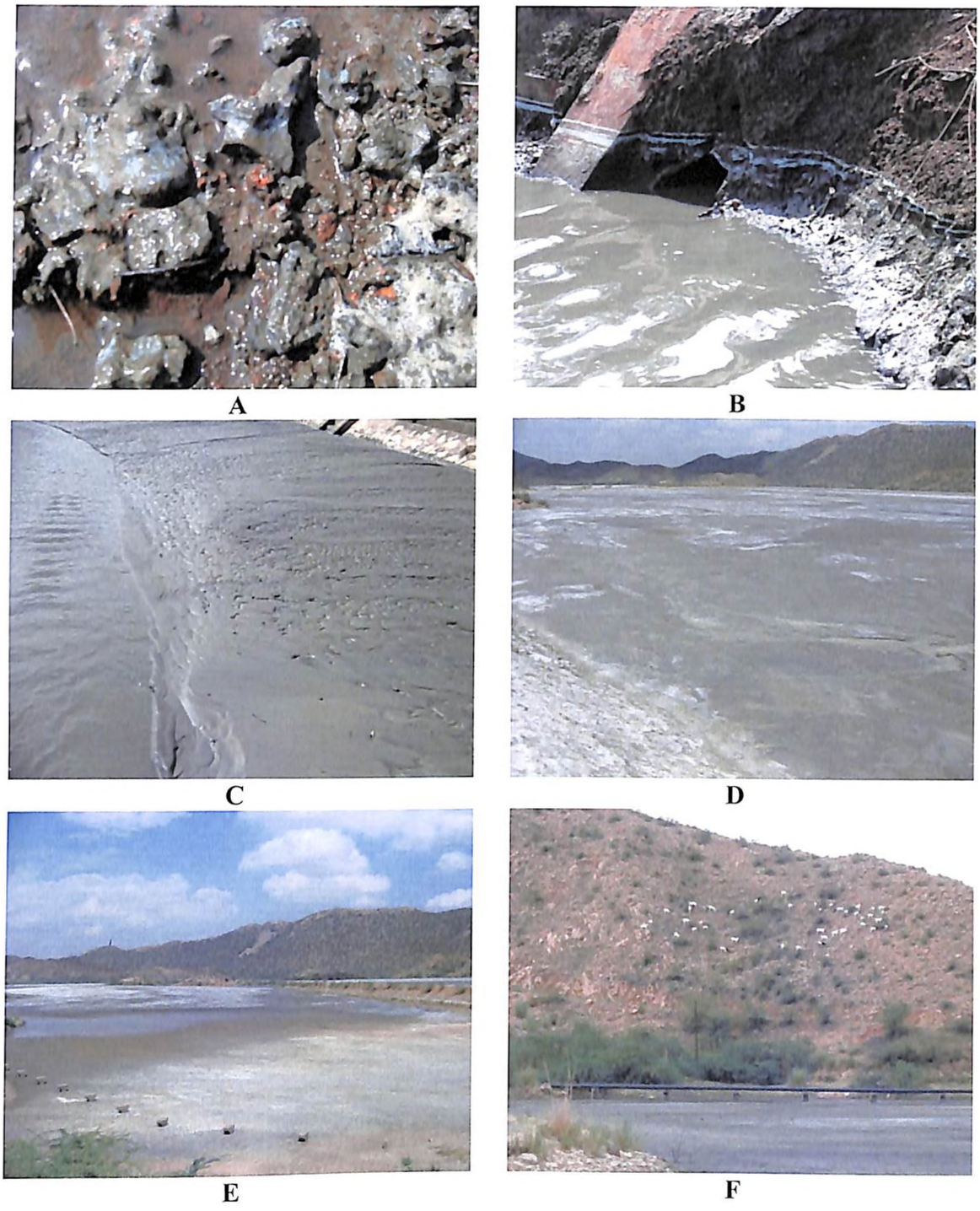


Fig. 3. Water sample sites. Site A-F, showing different stages of copper mine discharge effluent. These sample sites signifies the spread of antibiotic resistant bacterial population through contaminated waste erosion by rain water.

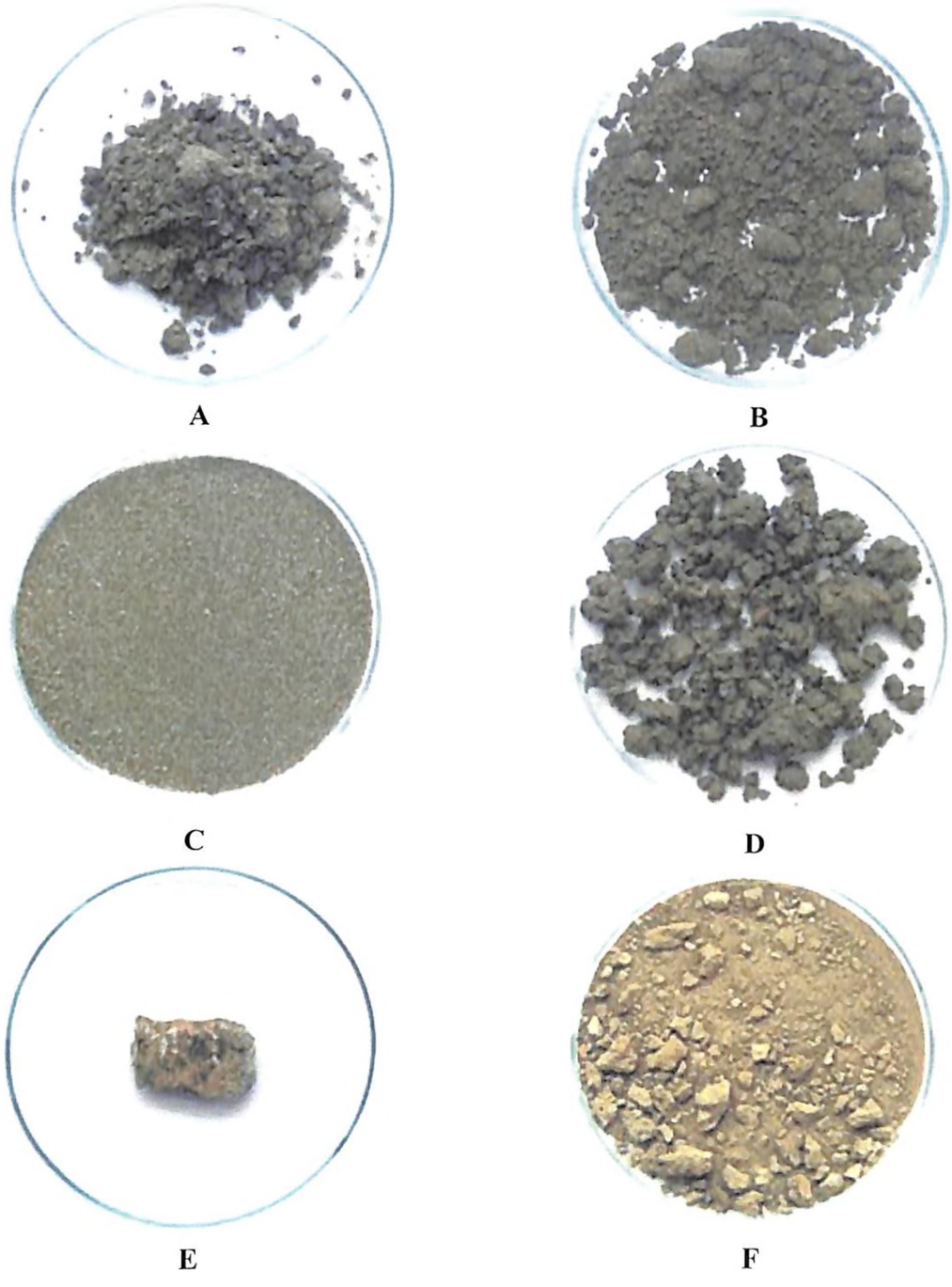


Fig. 4. Soil samples. Soil samples (A-F) were collected from different samples sites of copper mine. (A), Collected from 10 cm deep trench, near by running effluent stream (close to source); (B), Collected from 5 cm deep trench, far away from settling pond of effluent; (C), collected from surface of heap of discharged material, very close to dump-yard; (D), collected from 25cm deep trench, near by settling pond of effluent; (E), A portion of ore (Chalcopyrite; $CuFeS_2$), near to discharge line; (F), Collected from dried surface of dump near to effluent pond.

Table 3. Physio chemical characteristics of soil and water samples, collected from Khetri copper mine complex.

Parameters	Soil	Water (effluent)
Temperature	40°C	40°C
Color	Brown	Bluish green
Smell	Earthy/muddy	Fishy/rancid
pH	8.20 ±0.1	4.81 ±0.1
Total dissolved solute	3.56 g/L	-

These properties show that both the soil and water samples are showing the deviation from the normal properties of soil and water, so there is a probability of obtaining different types of microorganism, which may not be present under normal conditions.

Metal ion analysis

The samples have been collected from a copper mining industry, which is expected to have high concentration of metal. Thus, the heavy metal ion analysis becomes significant. Both the samples were prepared for metal ion analysis and analyzed using Atomic Absorption Spectrophotometer (AAS) using AAnalyst 300, Perkin Elmer, USA, as described in section 2.2.2.

The metal ions analysis was done for the metals like, copper, manganese, zinc, cobalt, nickel, chromium and cadmium. The obtained results were expressed in ppm (mg/L) and have been shown in Table 4.

Table 4. Metal ion analysis of soil and water samples collected from Khetri copper mine complex.

Metal ion	Soil (ppm)	Water (ppm)	Permissible limits (ppm)
Copper	961.87	141.5	0.5
Manganese	90.28	6.86	0.3
Zinc	87.94	5.15	2.0
Cobalt	61.37	1.83	0.05
Nickel	31.84	1.55	0.1
Chromium	3.49	1.20	0.5
Cadmium	1.86	1.18	0.01

Metal ion analysis of both soil and water samples was done in triplicates to maintain reproducibility. The high concentration of copper in both soil and water samples indicates that the site is rich in copper. The site was found to have high concentration of Copper (Cu) followed by Manganese (Mn), Zinc (Zn), Cobalt (Co), Nickel (Ni), Chromium (Cr) and Cadmium (Cd).

2.3.2 Isolation of bacterial strain

Upon the completion of physio-chemical characterization and metal ion analysis, samples were taken for isolation of microorganisms, especially bacteria. For the isolation Nutrient broth was used as a bacterial culture medium to allow the growth of all possible bacterial strain present in a particular sample. The isolation was carried out from soil and water samples as described in section 2.2.3.

24 bacterial strains were isolated from soil and water samples. Among these bacterial strains, 17 (strain no. 1-17) were taken from soil samples and 7 (strain no. 18-24) from water (effluent) samples. These were then distinguished based on their colony morphology on nutrient agar plates. Bacterial strains were preserved in glycerol (*cf* 15%) at -70°C.

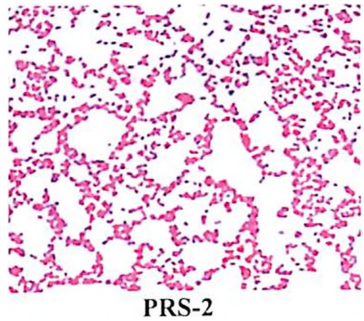
2.3.3 Identification of bacterial strains

24 bacterial strains were selected for this study. The first step towards the characterization was their identification, which was done with Gram's staining followed by 16s rRNA sequencing and fatty acid methyl esters (FAMEs) analysis.

2.3.3.1 Gram's staining

Selected strains were subjected to Gram's staining and results obtained have been shown in figure 5. Under oil immersion microscopic examination, Gram's positive organisms appear purple and Gram's negative organisms appear pink-red in color. Based on the Gram's staining, the classification of bacterial strain for Gram's positive/negative was done. The observed morphology have been summarized in table 5.

PRS-1



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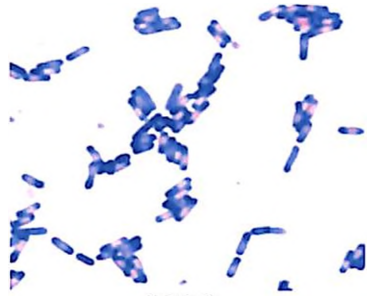

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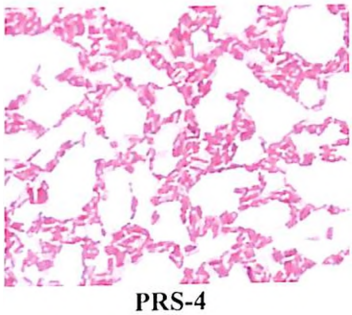
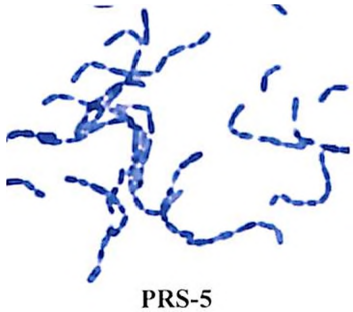
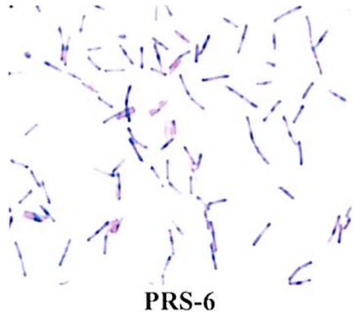


PRS-4



PRS-5



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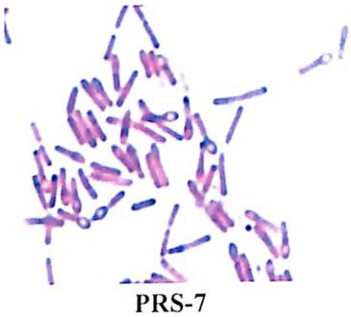
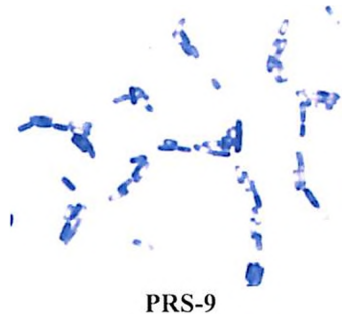


PRS-7

PRS-8



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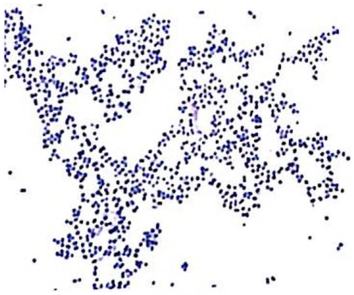

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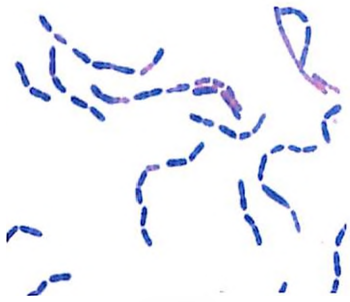
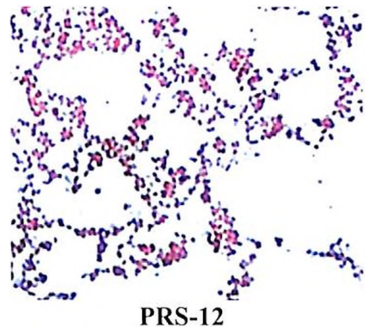


PRS-11



PRS-12

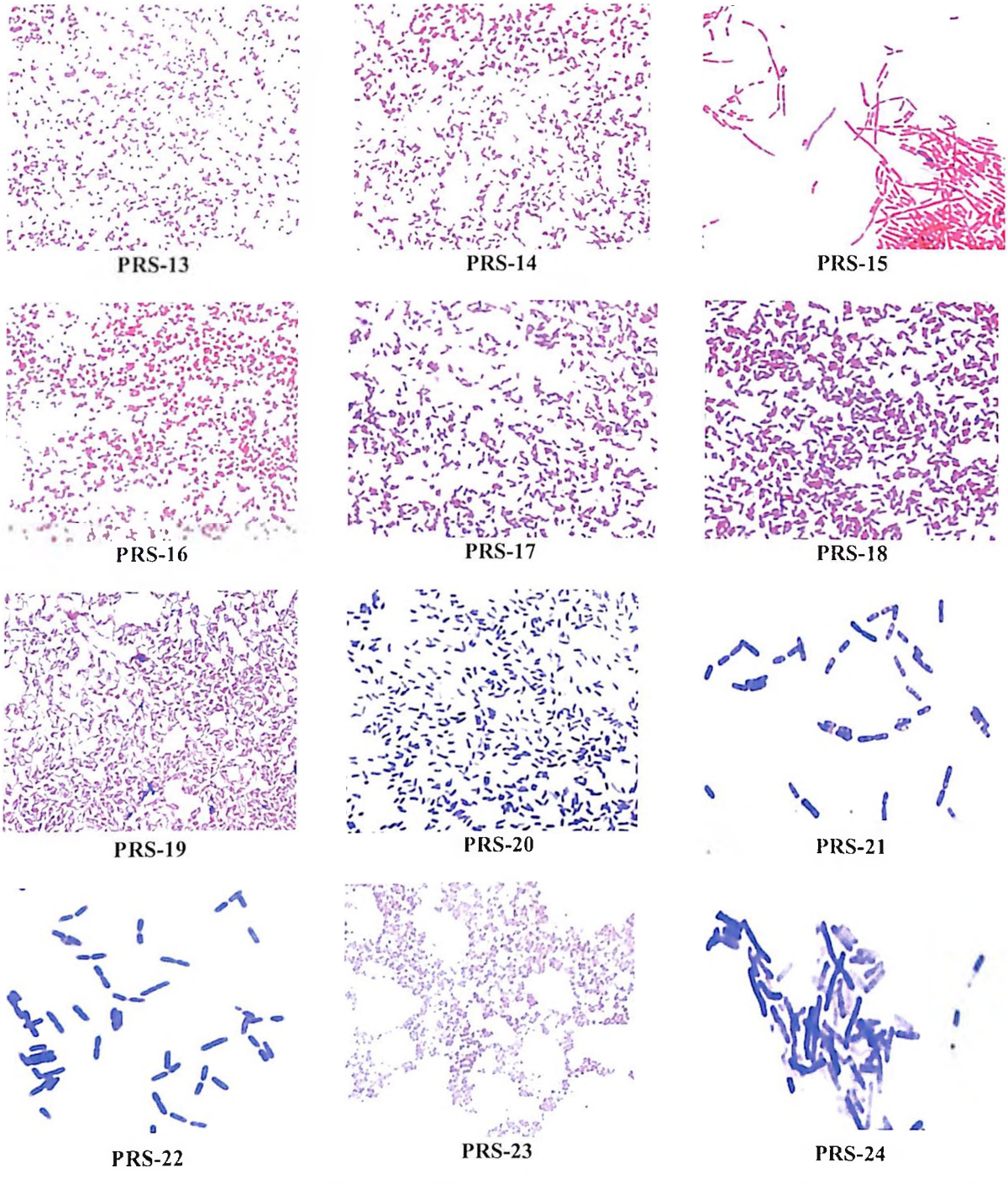


Fig. 5. Gram's staining of bacterial strains PRS1 to PRS24: Gram's positive bacterial strains appeared purple in color and Gram's negative in pink color.

Table 5. Morphology of bacterial isolates (PRS-1 to PRS-24).

Bacterial strain no.	Gram's reaction	Colony morphology
PRS-1	Positive	Rod shape, moderate length and arranged in bee hive like manner
PRS-2	Negative	Rod shape, small in size and arranged in bee hive like manner
PRS-3	Positive	Rod shape, thick cells with moderate length seemingly having bipolar ends
PRS-4	Negative	Rod shape, moderate length and arranged in bee hive like fashion
PRS-5	Positive	Rod shaped, solid, moderate length and cells look like transparent forming chain like structure
PRS-6	Positive	Needle like, long rods and scattered
PRS-7	Positive	Thick Needle like rods and having bipolar staining
PRS-8	Negative	Rod shape, tiny size and clusterd in some unusual shapes
PRS-9	Positive	Rod shape, moderate length, bipolar and small chains
PRS-10	Positive	Cocci, small and clustered at some locations
PRS-11	Positive	Rod shape, moderate length and arranged in chain like fashion
PRS-12	Positive	Rod shape, very small and arranged in bee hive like fashion
PRS-13	Negative	Rod shape, very small length and no clustering
PRS-14	Negative	Rod shape, small in length and scattering
PRS-15	Negative	Rod shape, thin, long and small chain formation
PRS-16	Negative	Rod shape, very small length and no clustering
PRS-17	Negative	Rod shape, small in length and scattering
PRS-18	Negative	Rod shape, moderate in length and scattered
PRS-19	Negative	Rod shape, small niddle and clustered
PRS-20	Negative	Rod shape, small and scattered
PRS-21	Positive	Rod shape, moderate in size with middle spots like structure
PRS-22	Positive	Rod shape, solid and chains of 2-3 cells
PRS-23	Negative	Rod shape, very small and clustered
PRS-24	Positive	Rod shape, moderate length in chain and cluster like arrangement

Gram's staining has extensively been used for the preliminary identification of bacterial strains. It classifies bacterial strains into two major groups of bacteria; Gram's positive and Gram's negative. Here we have reported that out of 24 bacterial strains, 12 appeared to be Gram's positive in nature and rest 12 were Gram's negative. These results confirmed the

presence of diverse population in soil and water samples of Khetri copper mine complex, Rajasthan.

2.3.3.2 16s rRNA sequence analysis

16s rRNA was done for characterization of bacterial strains at genotypic level. 16s rRNA is the highly conserved region and often used for the identification of bacterial strains at genotypic level. This identification procedure involves, genomic DNA isolation, amplification of a specific region of 16s rRNA using eubacterial primers, DNA sequencing of amplified PCR product in ABI Prism 3100 AVANT genetic analyzer followed by analysis of obtained DNA sequence through Chromas Lite 2.01, BioEdit version 7.0.0 and BLAST as described in section 2.2.4.3. Finally the sequences were submitted to NCBI GenBank and accession numbers were obtained. The identification parameters for 24 bacterial strains including strain name, GenBank accession no., % Query coverage and % Identity have been shown in Table 6.

Table 6. A preliminary identification of bacterial strains by 16s rRNA sequencing. Identification was done based on comparison of 16s rRNA sequences in different data bases using bioinformatics tool BLAST.

Bacterial strain no.	Name	% Query coverage	% Identity	NCBI Accession No.
PRS-1	*Bacillus thuringiensis*	94	99	EU723845
PRS-2	*Bacterium*	94	99	EU723846
PRS-3	*Bacillus* sp.	87	100	EU744603
PRS-4	*Bacterium*	87	100	EU744601
PRS-5	*Bacillus megaterium*	96	98	EU744602
PRS-6	*Bacillus* sp.	93	98	EU744604
PRS-7	*Lysinibacillus sphaericus*	95	99	EU744605
PRS-8	*Pseudomonas aeruginosa*	95	98	EU744606
PRS-9	*Bacillus cereus*	86	100	EU744607
PRS-10	*Acinetobacter junii*	91	92	EU744608
PRS-11	*Bacillus megaterium*	93	95	EU744609
PRS-12	*Comamonas* sp.	96	97	EU744610
PRS-13	*Alcaligenes* sp.	100	99	EU744611
PRS-14	*Stenotrophomonas* sp.	99	99	EU744612
PRS-15	*Alcaligenes* sp.	99	100	EU744613
PRS-16	*Stenotrophomonas maltophilia*	99	96	EU744614
PRS-17	*Achromobacter* sp.	99	100	EU744615
PRS-18	*Stenotrophomonas* sp.	99	100	EU744616
PRS-19	*Bacillus pumilus*	99	99	EU744617
PRS-20	*Pseudomonas stutzeri*	99	99	EU744618
PRS-21	*Bacillus flexus*	99	100	EU744619
PRS-22	*Bacillus thuringiensis*	99	100	EU744620
PRS-23	*Acinetobacter junii*	99	100	EU744621
PRS-24	*Bacillus benzoevorans*	100	100	EU744622

Though the sequences (16s rRNA) were compared in different databases but results obtained were quite ambiguous. Although some strains were found to be gram positive during Gram' s staining but 16s rRNA analysis using databases [GenBank (Benson et al. 2000), EMBL (Kanz et al. 2005) and the Ribosomal Database Project (RDP) (Cole et al. 2003)] indicated it to be a gram negative. The similar findings were also obtained for gram negative bacterium. In addition, some of them were exhibited different morphological characters including different shape and size. These findings have also been supported by Ashelford et al. (2005),

which states that "At least 1 in 20 16s rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies".

2.3.4 Metal and antibiotic resistance profile

For metal resistance profile, minimum inhibitory concentrations (MIC) for all isolates were determined at millimolar level. The growth of the bacterial strains in the varying concentration of metals was determined. *Bacillus cereus* and *E. coli* K12 were used as a reference bacterial strain for gram positive and gram negative respectively. Except few, most of the strains were found to have higher MIC as compared to *Bacillus cereus* and *E. coli* K12 at millimolar level for the metals like copper(II) and uranium(II). Some of the bacterial strains like PRS-2, PRS-3, PRS-8, PRS-13 and PRS-18 have significantly higher MICs for most of the metals as compared to *Bacillus cereus* and *E. coli* K12 (Table 7).

For antibiotic resistance/susceptibility profiling, the disc diffusion method, as discussed in material and methods, was used. *Bacillus cereus* and *E. coli* K12 were used as a reference bacterial strain for gram positive and gram negative respectively. Zone of inhibition was measured in millimeter and the resistance and sensitivity of the bacterial strains toward any antibiotic was calculated as per the guidelines of the manufacturer (Himedia Laboratories Pvt. Limited, Mumbai, India). Except few, most of the strains were resistance to Metranidazol, Meropenem, Bacitracin, Vancomycin and Ampicillin. Some of the strains like PRS-8, PRS-10, PRS-12, PRS-13, PRS-17 and PRS-18 were resistant to most of the antibiotics (Table 8).

Currently, metal and antibiotic resistances among bacterial population have been posed as a major global concern. There is a clear association between heavy antimicrobial consumption within a population and the frequent recovery of antibiotic resistant bacteria (Enne et al. 2001). In addition, a correlation between the resistance to high level of Cu(II), Pb(II), Zn(II) and antibiotic in bacterial species found in drinking water has long been established (Calomiris et al. 1984). Multiple metal resistance bacterial isolates have exhibited high resistance towards a group of antibiotics (Vajiheh et al. 2003). However, the increase in the metal concentrations in the ambient environment has also shown a decrease in antibiotic resistance in natural isolates (Tuckfield et al. 2007). In our results, we have reported that if a

bacterial isolate has high MIC value for a set of metals, it also exhibits high resistance pattern towards a group of antibiotics as reported by Vajiheh et al. (2003).

Table 7. Metal resistance profile of bacterial isolates. The minimum inhibitory concentration (MICs) was measured after 18h and expressed as millimolar (mM).

Bacterial Strain no.	As (mM)	Cu (mM)	Cd (mM)	Zn (mM)	U (mM)	Cr (mM)	Mn (mM)
PRS-1	3.5	1.5	1.0	6.0	22.5	37.5	6.5
PRS-2	12.5	22.5	1.0	37.5	24.0	40.0	40.0
PRS-3	11.5	7.0	4.5	22.5	7.0	37.5	27.5
PRS-4	3.5	1.0	1.5	6.5	13.5	15.0	6.5
PRS-5	7.5	1.0	0.25	1.0	9.0	11.0	12.5
PRS-6	17.5	1.0	1.0	1.5	12.5	12.5	5.0
PRS-7	7.5	1.5	1.5	3.5	7.5	32.5	5.0
PRS-8	9.0	18.5	4.5	27.5	7.5	32.5	10.0
PRS-9	3.5	2.5	1.0	4.5	2.5	32.5	5.0
PRS-10	12.5	15.0	1.0	4.5	2.5	5.0	7.0
PRS-11	7.5	1.5	1.0	5.0	7.5	27.5	32.5
PRS-12	12.5	22.5	0.5	5.0	7.5	12.5	7.5
PRS-13	12.5	22.5	1.5	12.5	20.5	10.0	37.5
PRS-14	7.5	9.0	1.0	8.0	9.0	11.0	5.5
PRS-15	7.5	7.0	2.0	3.0	9.0	3.0	4.5
PRS-16	15.0	17.5	1.0	13.0	12.5	32.5	29.5
PRS-17	20.0	15.5	1.5	12.5	12.5	12.5	30.0
PRS-18	1.5	7.5	1.5	42.5	15.0	37.5	42.5
PRS-19	3.5	1.5	1.0	3.5	13.0	13.5	9.5
PRS-20	10.0	3.5	0.75	2.0	9.0	2.0	9.0
PRS-21	8.5	2.5	1.0	3.5	9.0	32.5	5.0
PRS-22	10.0	2.5	1.5	4.5	7.5	32.5	7.5
PRS-23	22.5	15.0	1.0	5.0	7.5	5.0	5.0
PRS-24	7.5	15	1.0	5.0	9.0	15.0	5.0
Bacillus cereus	2.0	10.0	2.0	10.0	6.0	37.5	27.5
E. coli K12	11.5	9.5	3.0	9.5	6.0	37.5	20.0

Table 8. Antibiotic resistance profile of bacterial isolates. The zone of inhibition was measured after 18h and expressed as millimeter (mm). The resistant and sensitive are denoted in the table as R & S respectively.

Bacterial Strain No.	Am	As	Mt	Cx	Ox	Mr	B	Va	E	Cd	Gm	O	Amp	Tet	Cm	Km	Sm
PRS-1	R	R	R	S	S	R	R	R	R	R	S	S	R	R	S	R	R
PRS-2	S	S	R	S	S	R	R	R	R	R	S	S	R	R	S	R	R
PRS-3	S	R	R	S	R	R	R	R	S	S	S	S	R	S	S	S	S
PRS-4	S	S	R	S	S	R	S	R	S	S	S	S	S	S	S	S	S
PRS-5	S	R	R	S	R	R	R	R	R	R	S	R	R	S	S	S	S
PRS-6	S	S	S	S	S	R	S	R	S	S	S	R	S	S	S	S	S
PRS-7	S	S	R	S	S	R	R	R	S	S	S	R	S	S	S	R	R
PRS-8	R	R	S	R	R	S	R	R	R	R	R	R	R	R	R	R	R
PRS-9	R	R	R	R	R	R	S	R	R	S	S	R	R	S	S	S	S
PRS-10	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R
PRS-11	R	S	R	R	S	R	S	S	S	R	S	R	S	S	S	S	S
PRS-12	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
PRS-13	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
PRS-14	S	R	R	R	R	R	R	R	R	R	S	R	R	R	S	S	S
PRS-15	S	R	R	R	R	S	R	R	R	R	S	R	R	S	S	S	S
PRS-16	S	S	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R
PRS-17	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	R	R
PRS-18	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	R	R
PRS-19	S	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PRS-20	S	R	R	R	R	R	R	R	S	R	S	S	R	S	S	S	R
PRS-21	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PRS-22	R	R	R	R	R	R	R	R	S	S	S	R	R	S	S	S	S
PRS-23	R	R	R	R	S	R	R	R	S	R	S	R	R	S	S	S	S
PRS-24	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
Bacillus cereus	R	S	R	R	R	R	S	R	S	S	S	S	S	S	S	S	S
E. coli K12	S	S	R	R	R	R	R	R	S	R	R	R	S	S	S	S	S

** Amikacin (Ak), Ampicillin/Sulbactam (As), Metronidazole (Mt), Cloxaicillin (Cx), Oxacillin (Ox), Meropenem (Mr), Bacitracin (B), Vancomycin (Va), Erythromycin (E), Clindamycin (Cd), Gentamycin (Gm), Oxytetracycline (O), Ampicillin (Amp), Tetracycline (Tet), Chloramphenicol (Cm), Kanamycin (Km) and Streptomycin (Sm).

2.3.5 Plasmid DNA analysis

Most of the strains were analyzed under present study, demonstrated a good degree of resistance for metals and antibiotics. As reported earlier, metal and antibiotic resistance properties generally reside on extra chromosomal DNA molecules like plasmid. Plasmid DNA was isolated and analyzed. It has found that most of the strains contain plasmid DNA of size ranging from 50-200 kb or mega plasmids, except PRS-3, as shown in gel picture (fig. 6). Multiple bands in PRS-3 confirm the presence of plasmid DNA (labeled as pPRS3a) which possesses various forms like covalently closed circular, open circular, linear, etc.

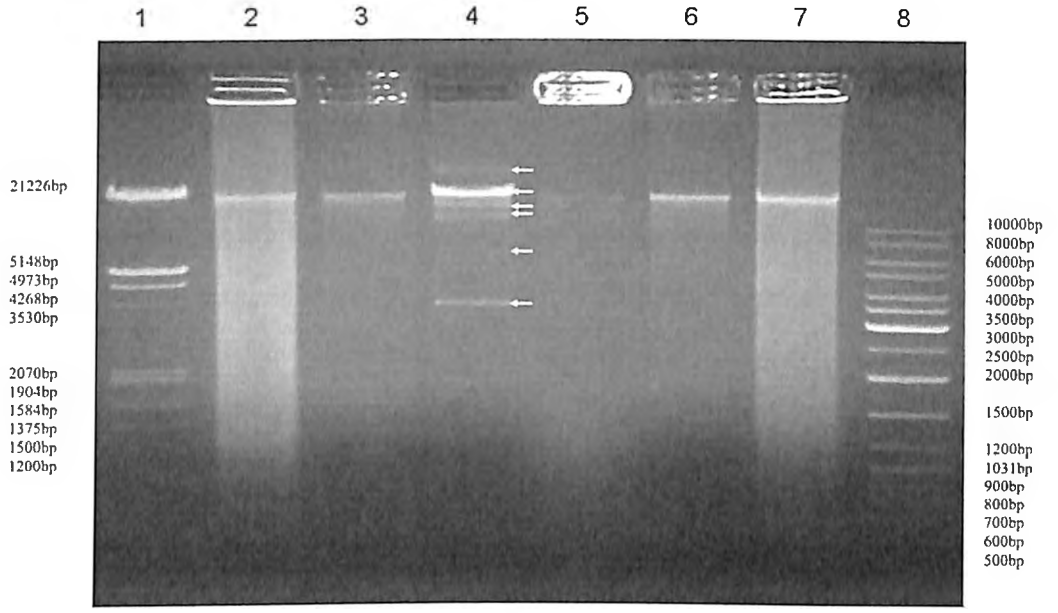


Fig. 6. Plasmid DNA profile of bacterial strains.

- lane 1, Lambda DNA marker (double digest of EcoR1 & HindIII);
- lane 2-7, plasmid DNA of PRS-2, PRS-8, PRS-3, PRS-10, PRS-13 & PRS-18 respectively; lane 8, DNA marker (MBI Fermentas, SM0331, 1kb ladder).
- Arrows show multiple bands of PRS-3.
- The DNA samples were separated on a 1% agarose gel.

It has been reported that large plasmids are responsible for encoding resistance to antibiotics and heavy metals (Mergeay et al. 1985; Vajiheh et al. 2003; Mergeay et al. 2003). In addition, transferable plasmid encoding resistance to various heavy metals and antibiotics from *Salmonella abortus equi* (Vajiheh et al. 2003) was reported. However, the metal and

antibiotic resistance in *Bacillus* spp. [*B. thuringiensis* (350 kb & 105 kb), *B. anthracis* (208 kb) and *B. cereus* (208 kb)] is reported to reside on larger plasmids (Andrup et al. 2008).

Among these 24 bacterial strains, PRS3 strain showed resistance to metals (copper, cadmium, zinc, chromium, arsenic, manganese and uranium), antibiotics (Ampicillin/Sulbactam, Metronidazole, Oxacillin, Meropenem, Bacitracin, Vancomycin, Ampicillin) and presence of a plasmid DNA with multiple bands. Based on the observations of metal resistance, antibiotic resistance and smaller size plasmid DNA, the PRS3 was selected for further characterization.

2.3.6 Characterization of PRS3

Identification of PRS3 was done at species level by morphological, physiological, biochemical analysis and fatty acid methyl ester (FAMEs) analysis. Growth properties in different culture were studied to optimize the media for further analyzed. To delineate surface morphology and internal structure, ultra structural studies like scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were carried out. Plasmid DNA sequencing and analysis (as described in chapter-III) followed by proteomic studies (as described in chapter-IV) were performed to know the molecular basis for multiple metal and antibiotic resistance.

2.3.6.1 Identification of PRS3 by morphological, physiological, biochemical analysis

For the identification of PRS3 at species level morphological, physiological and biochemical analysis was carried out as described in section 2.2.4.2. The results obtained for each of the analysis have been shown in Table 9, 10 & 11.

Table 9. Morphological tests on PRS3

Test	Results
Colony morphology	
Configuration	Circular
Margin	Irregular
Elevation	Convex
Surface	Rough
Pigment	Off white
Opacity	Opaque
Gram's reaction	+
Cell shape	rods
Size(μm)	2-4μm
Arrangement	Scattered
Spores	+
Position	Sub terminal
Shape	Ellipsoidal
Sporangia bulging	Non bulged
Motility	-

** (+) positive and (-) negative

Table 10. Physiological tests on PRS3

Test	Results
Growth at temperatures (°C)	
4°C	-
10°C	-
25°C	+
30°C	+
37°C	+
42°C	+
55°C	-
Growth at pH	
pH4.0	-
pH5.0	-
pH6.0	+
pH7.0	+
pH8.0	+
pH9.0	+
pH10.0	+
pH11.5	+
Growth on NaCl (%)	
2.0	+
4.0	+
6.0	+
8.0	+
10.0	-
Growth under anaerobic conditions	+

** (+) positive and (-) negative

Table 11. Biochemical tests on PRS3

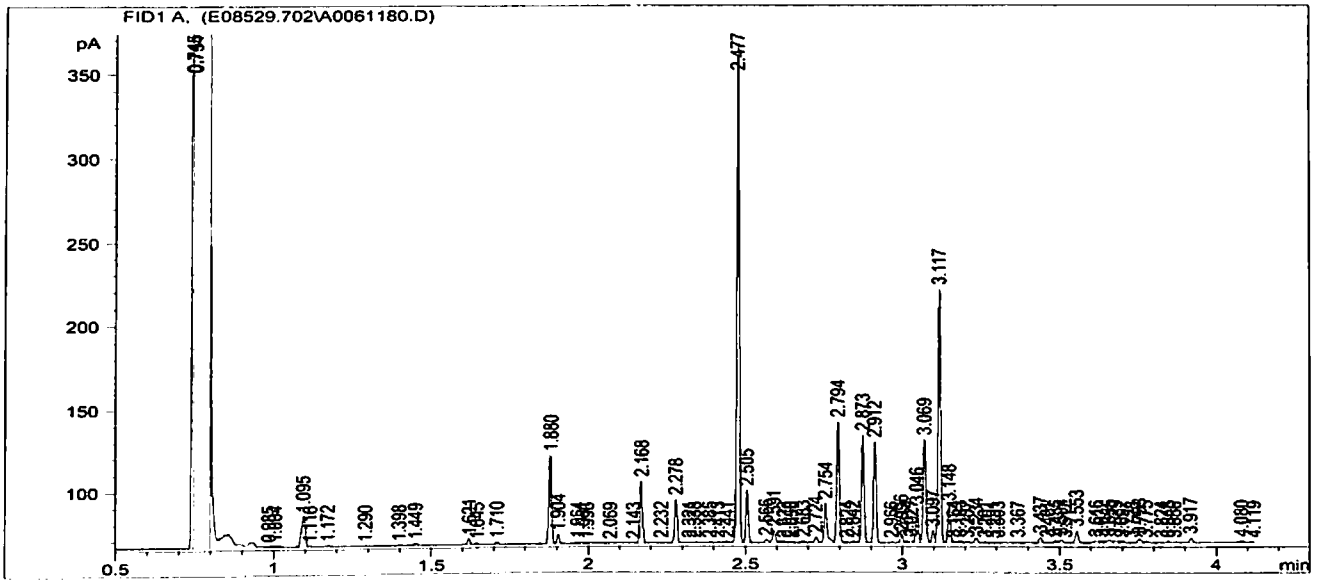
Test	Results
Growth on MacConkey	-
Indole test	-
Methyl red test	+
Voges Proskauer test,	+
Citrate utilization	-
H_2S production	-
Gas production from glucose	-
Casein hydrolysis	+
Esculin hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis,	-
Nitrat reduction	+
Ornithine decarboxylase	+
Lysine decarboxylase	+
Catalase test	+
Oxidase test	+
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
Tween 60 hydrolysis	+
Tween 80 hydrolysis	+
Xanthane hydrolysis	-
Acid production from	
Dextrose	+
Lactose	+
Sucrose	+
Maltose	-
Manitol	-
Xylose	+

** (+) positive and (-) negative

Based on these analyses bacterial strain was identified as *Bacillus cereus* and strain was deposited at IMTECH, Chandigarh, India under the accession no. MTCC 10207 on 20th October, 2009 in the name of Prof. S. K. Verma, BITS-Pilani (appendix III).

2.3.6.2 Identification of PRS3 by fatty acid methyl esters (FAMEs) analysis

PRS3 was initially identified as *Bacillus* sp. by 16s rRNA sequencing. The sequence was submitted to GenBank under the accession no. **EU744603** (Jain et al. 2009). For further identification, PRS3 was subjected to Fatty Acid Methyl Esters (FAMEs) analysis, which has been reported to be more confirmatory tool for identification (Lawrence et al. 1984; Beverly et al. 1996; Kim et al. 2000). The FAMEs analysis was performed as described in section 2.2.4.4. A chromatographic run and sample composition have been shown in figure 7. The similarity index (SI) for different species of *Bacillus* have been shown in Table 12. Based on the high value of SI (0.407), this bacterium was identified as *Bacillus cereus* GC subgroup A. The FAMEs analysis for PRS3 was performed at MIDI Sherlock microbial identification system, Secundrabad, India



RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.7446	1.479E+6	0.005	----	6.5590		----	< min rt	
0.7536	1.113E+9	0.020	----	6.6167	SOLVENT PEAK	----	< min rt	
0.9854	1084	0.011	----	8.1130		----	< min rt	
1.0041	2437	0.018	----	8.2339		----	< min rt	
1.0952	33493	0.013	----	8.8262		----	< min rt	
1.1156	422	0.009	----	8.9578		----	< min rt	
1.1720	1699	0.020	----	9.3214		----		
1.2904	336	0.009	----	10.0654		----		
1.3983	681	0.011	1.077	10.6148	11:0 iso	0.06	ECL deviates -	Reference 0.002
1.4491	2514	0.013	----	10.8730		----		
1.6205	5029	0.010	1.047	11.6181	12:0 iso	0.46	ECL deviates -	Reference 0.002
1.6451	1255	0.010	1.045	11.7219	12:0 anteiso	0.11	ECL deviates 0.012	Reference 0.017
1.7102	1846	0.009	1.037	11.9967	12:0	0.17	ECL deviates -	Reference 0.002
1.8796	59945	0.009	1.022	12.6229	13:0 iso	5.36	ECL deviates 0.000	Reference 0.005
1.9041	7039	0.010	1.020	12.7135	13:0 anteiso	0.63	ECL deviates -	Reference 0.004
1.9642	1133	0.009	----	12.9358		----		
1.9813	1303	0.010	1.013	12.9987	13:0	0.12	ECL deviates -	Reference 0.003
1.9955	349	0.008	----	13.0467		----		
2.0688	655	0.015	----	13.2935		----		
2.1427	395	0.009	----	13.5425		----		
2.1681	41939	0.009	1.000	13.6280	14:0 iso	3.67	ECL deviates 0.000	Reference 0.004
2.2322	716	0.013	----	13.8440		----		
2.2782	30527	0.009	0.993	13.9989	14:0	2.65	ECL deviates -	Reference 0.003
2.3214	628	0.011	0.991	14.1369	13:0 iso 3OH	0.05	ECL deviates 0.005	
2.3384	499	0.010	----	14.1913		----		
2.3848	1213	0.018	----	14.3398		----		
2.4130	906	0.013	----	14.4300		----		
2.4412	1400	0.015	0.984	14.5203	unknown 14.502	----	ECL deviates 0.004	
2.4766	341181	0.009	0.982	14.6334	15:0 iso	29.31	ECL deviates 0.001	Reference 0.005
2.5051	35807	0.009	0.980	14.7245	15:0 anteiso	3.07	ECL deviates -	Reference 0.003
2.5661	2926	0.011	0.977	14.9196	15:1 w5c	0.25	ECL deviates -	
2.5909	10630	0.010	0.976	14.9990	15:0	----	ECL deviates -	
2.6230	414	0.006	----	15.0989		----		
2.6399	755	0.011	0.974	15.1515	14:0 iso 3OH	0.06	ECL deviates 0.005	
2.6497	927	0.017	----	15.1823		----		
2.6834	2543	0.017	----	15.2871		----		
2.7244	4875	0.010	0.970	15.4148	16:1 w7c alcohol	0.41	ECL deviates 0.001	
2.7544	31825	0.010	0.969	15.5085	Sum In Feature 2	2.70	ECL deviates -	14:0 3OH/16:1 iso
2.7944	87371	0.009	0.967	15.6331	16:0 iso	7.39	ECL deviates 0.000	Reference 0.002
2.8239	772	0.011	0.966	15.7248	16:0 anteiso	0.07	ECL deviates -	

2.8425	1241	0.010	0.965	15.7828	16:1 w11c	0.10	ECL deviates 0.001	
2.8732	74712	0.009	0.964	15.8786	Sum In Feature 3	6.30	ECL deviates 0.004	16:1 w6c/16:1
2.9119	72305	0.009	0.962	15.9991	16:0	6.09	ECL deviates -	Reference 0.001
2.9657	600	0.012	0.960	16.1664	15:0 iso 3OH	0.05	ECL deviates 0.004	
2.9962	5864	0.010	0.959	16.2613	15:0 2OH	0.49	ECL deviates 0.006	
3.0137	1052	0.008	----	16.3156		----		
3.0266	1607	0.009	----	16.3556		----		
3.0460	24918	0.009	0.957	16.4161	17:1 iso w10c	2.09	ECL deviates 0.002	
3.0690	80553	0.010	0.956	16.4874	17:1 iso w5c	6.74	ECL deviates 0.004	
3.0967	8628	0.009	0.955	16.5738	17:1 anteiso A	0.72	ECL deviates 0.003	
3.1171	182092	0.009	0.955	16.6371	17:0 iso	15.21	ECL deviates 0.000	Reference 0.001
3.1478	26399	0.009	0.954	16.7326	17:0 anteiso	2.20	ECL deviates 0.000	Reference 0.000
3.1642	689	0.007	0.953	16.7835	17:1 w9c	0.06	ECL deviates 0.000	
3.1833	1726	0.011	0.953	16.8429	17:1 w7c	0.14	ECL deviates 0.007	
3.2069	954	0.009	0.952	16.9163	17:0 cyclo	0.08	ECL deviates 0.001	
3.2339	3666	0.011	0.951	17.0002	17:0	0.30	ECL deviates 0.000	Reference 0.000
3.2614	864	0.011	----	17.0861		----		
3.2907	372	0.007	0.949	17.1777	16:0 iso 3OH	0.03	ECL deviates 0.004	
3.3029	635	0.009	----	17.2161		----		
3.3667	1483	0.013	0.947	17.4155	17:0 10-methyl	0.12	ECL deviates 0.001	
3.4375	4664	0.010	0.945	17.6367	18:0 iso	0.39	ECL deviates 0.001	Reference 0.000
3.4609	856	0.011	----	17.7100		----		
3.4862	2262	0.012	0.944	17.7890	18:1 w9c	0.19	ECL deviates -	
3.5038	2653	0.010	0.943	17.8439	Sum In Feature 8	0.22	ECL deviates -	18:1 w7c
3.5167	994	0.009	----	17.8843		----		
3.5534	10239	0.010	0.942	17.9992	18:0	0.84	ECL deviates -	Reference -0.003
3.6158	3734	0.020	0.940	18.1993	17:0 iso 3OH	0.31	ECL deviates 0.006	
3.6319	1069	0.009	----	18.2510		----		
3.6551	3952	0.016	----	18.3252		----		
3.6787	2429	0.014	0.939	18.4010	18:0 10-methyl,	0.20	ECL deviates 0.006	
3.6971	2292	0.019	----	18.4600		----		
3.7285	568	0.008	0.938	18.5608	17:0 3OH	0.05	ECL deviates -	
3.7526	3756	0.013	0.937	18.6380	19:0 iso	0.31	ECL deviates 0.000	Reference -0.003
3.7750	2314	0.019	----	18.7101		----		
3.8238	839	0.017	0.936	18.8666	Sum In Feature 7	0.07	ECL deviates 0.010	19:1
3.8451	1093	0.012	0.935	18.9351	19:0 cyclo w8c	0.09	ECL deviates 0.003	
3.8655	689	0.012	0.935	19.0005	19:0	0.06	ECL deviates 0.000	Reference -0.004
3.9171	4317	0.012	----	19.1691		----		
4.0802	1427	0.012	----	19.7027		----		
4.1189	435	0.010	----	19.8294		----		
----	31825	---	----	----	Summed Feature 2	2.70	12:0 aldehyde ?	unknown 10.9525
----	-----	---	----	----		----	16:1 iso I/14:0 3OH	14:0 3OH/16:1 iso
----	74712	---	----	----	Summed Feature 3	6.30	16:1 w7c/16:1 w6c	16:1 w6c/16:1
----	839	---	----	----	Summed Feature 7	0.07	19:1w7c/19:1 w6c	19:1
----	-----	---	----	----		----	19:0 cyclo	
----	2653	---	----	----	Summed Feature 8	0.22	18:1 w7c	18:1 w6c

ECL Deviation: 0.004
Total Response: 1210943
Percent Named: 97.02%

Reference ECL Shift: 0.005
Total Named: 1174830
Total Amount: 1154634

Number Reference Peaks: 20

Fig. 7. The FAMEs analysis: Chromatographic run and composition report of *Bacillus cereus* GC subgroup A. Chromatogram shows peaks of fatty acids and table shows composition of each.

Table 12. Bacterial identification using FAME analysis: Fatty acid methyl ester composition of the PRS3 was compared to a standard database using a covariance matrix, principle component analysis and pattern recognition software. Library used and the Similarity Index of the best matches have been shown.

Library	Similarity Index (SI)	Entry Name
RTSBA6 6.00	0.407	*Bacillus cereus* GC subgroup A
RTSBA6 6.00	0.222	*Bacillus thuringiensis-israelensis*
RTSBA6 6.00	0.184	*Bacillus cereus* GC subgroup B
RTSBA6 6.00	0.128	*Bacillus thuringiensis-kurstakii*

Strain tracking via cluster analysis

The FAMEs analysis is a confirmatory analysis for the identification of microorganisms and has proven authentic so far (Osterhout et al. 1991; Kim et al. 2000), as it utilizes the unique property of total fatty acids of an organism and fatty acid profile (Sasser, 2006). Strain tracking, a technique in which the data obtained from fatty acid analyses of organisms are used to establish relationships between the organisms via cluster analysis. The identified bacterial strains by FAME versus Euclidian distance are plotted in a Dendrogram, which uses cluster analysis techniques to produce pair matching based on fatty acid compositions. The results are displayed graphically to depict the relatedness between organisms. A comparison of fatty acid analysis data of *Bacillus cereus* GC subgroup A with other organisms like *Bacillus thuringiensis-israelensis, Bacillus cereus* GC subgroup B and *Bacillus thuringiensis-kurstakii* through strain tracking (fig. 8) shows that Euclidian distance of 3.4 for *Bacillus cereus* GC subgroup A and *Bacillus thuringiensis-israelensis*, which establishes a clear relatedness between the two. The Euclidian distance of 4.8 and 16.6 for *Bacillus thuringiensis-kurstakii* and *Bacillus cereus* GC subgroup B respectively, shows the less relatedness of the species to *Bacillus cereus* GC subgroup A.

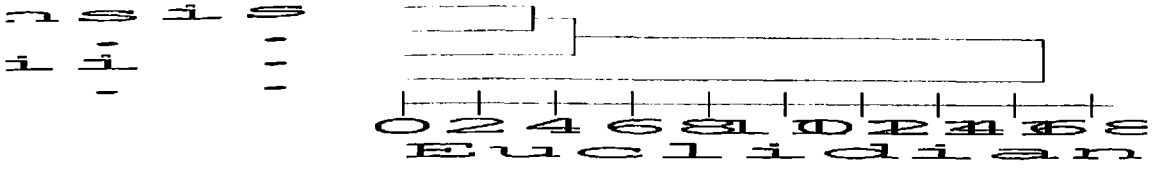


Fig. 8. Dendrogram: FAMEs analysis of *Bacillus cereus* GC subgroup A and its relatedness with other *Bacillus* spp.

2.3.6.3 Growth properties of *Bacillus cereus* GC subgroup A: Growth curve, protein and biomass

The growth, protein contents and biomass of *Bacillus cereus* GC subgroup A were observed in Nutrient broth (NB) and Luria bertani (LB) broth. The obtained results were plotted in graphs and have been shown in figure 9. All the experiments were performed as described in section 2.2.8 and results were taken in triplicate to maintain the reproducibility.

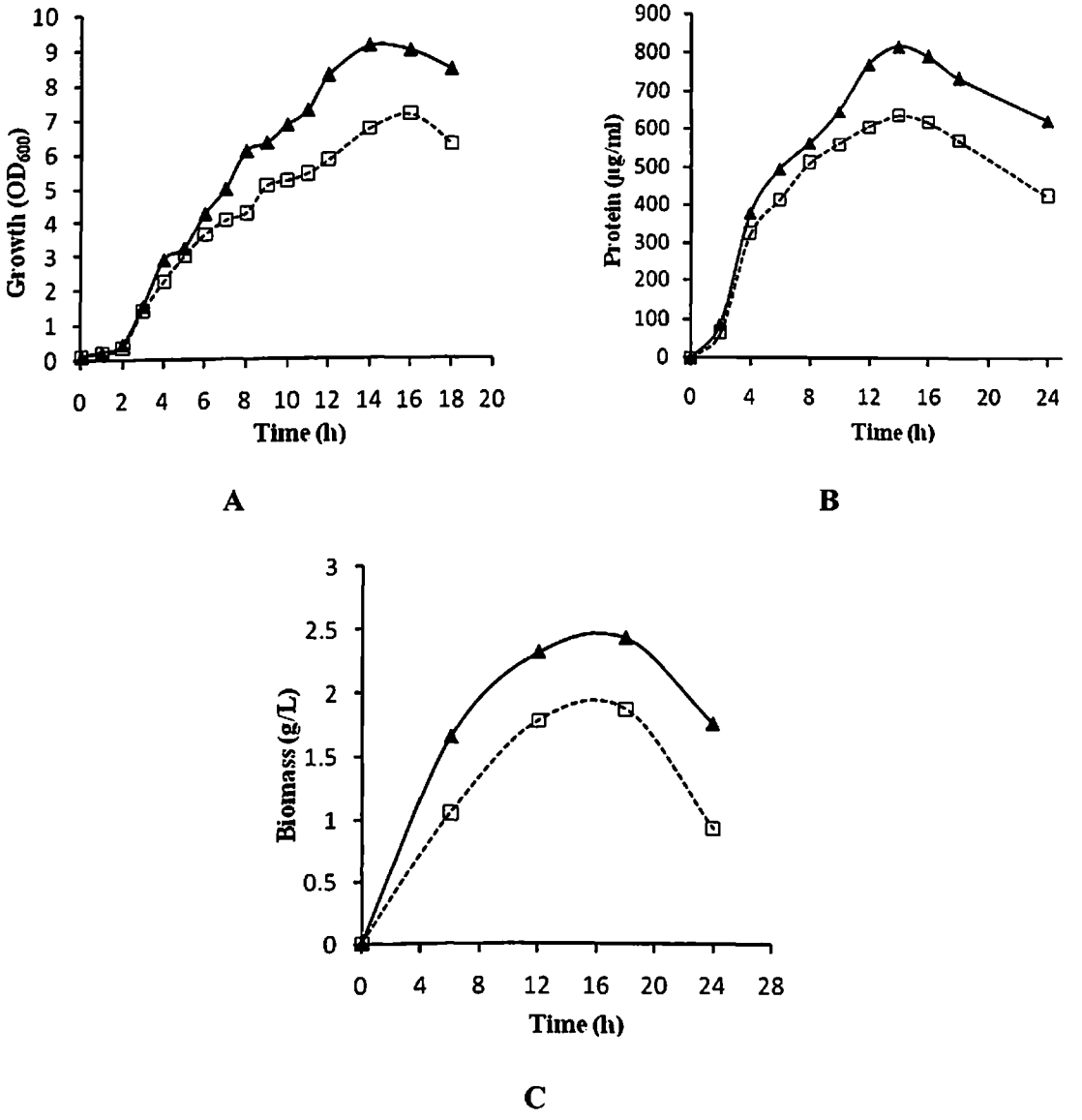


Fig. 9. The growth of *Bacillus cereus* GC subgroup A in NB (□) and LB (▲). (A) Growth curve, (B) total protein contents and (C) biomass.

The growth curve of *Bacillus cereus* GC subgroup A shows all three phases of the growth (log, lag, stationary and decline phase). In different bacterial culture medium like, LB and NB it shows same patterns of growth with different yield (fig. 9A). The calculated generation times for the LB and NB were 22 min and 24 min respectively. The maximum culture density was observed at 14h and 16h for LB and NB respectively followed by stationary phase after 18h. The similar pattern was obtained for total protein content and biomass production (fig. 9B & C) in LB & NB respectively. The growth of *Bacillus* sp. is known to dependent on culture conditions and the composition of growth medium. Spira et al. (1979) have reported a better growth under aerobic condition (generation time 24-31 min) than anaerobic conditions (generation time 51 min). In the present study, the log phase in *Bacillus cereus* GC subgroup A showed to exist up to 14-16 h followed by stationary phase. A similar growth pattern was also reported for *Bacillus cereus* ATCC14579 by Vries et al. (2004).

A comparison of growth using all the three parameters clearly establishes superiority of LB medium than NB for *Bacillus cereus* GC subgroup A. Thus, for further experiments like, Plasmids and proteomic analysis, *Bacillus cereus* GC subgroup A was grown in LB medium.

2.3.6.4 Electron microscopy of *Bacillus cereus* GC subgroup A

Electron microscopy of *Bacillus cereus* GC subgroup A was done to understand the surface morphology by scanning electron microscopy (SEM) and intracellular organization by transmission electron microscopy (TEM). Electron microscopy was performed as described in section 2.2.9.

SEM revealed the smooth surface of *Bacillus cereus* GC subgroup A as shown in fig 10. Average size of the *Bacillus cereus* GC subgroup A was found to be 2.93μm.

The *Bacillus* cereus is a well known spore forming bacterium (Granum, 1997). Single cell, dividing cells, cell adhesion, spores, different stages of spore formation, cell's and spore's internal structure, cell wall, genetic material and intracellular organization have been

visualized at various magnification in TEM (figure 11). This figure clearly shows the characteristics of a typical *Bacillus cereus*. Presence of spores indicates the dormant stage of *Bacillus cereus* and thick cell wall indicates the presence of high amount of peptidoglycan without any void space. Adjacent and dividing cells show the cellular growth. Intracellular organization support for its Gram's positive nature.

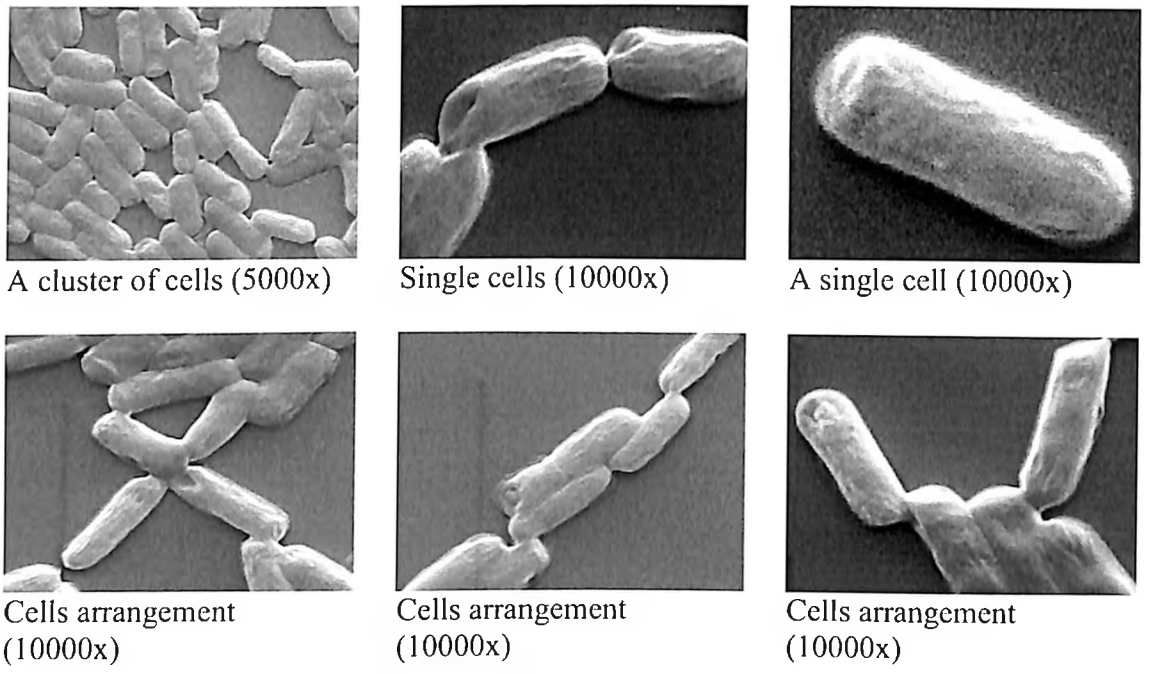


Fig. 10. Scanning Electron Microscopy of *Bacillus cereus* GC subgroup A. organization of cells and a single cell at high magnifications.

A total of 24 bacterial strains were isolated from a metal polluted site, Khetri copper mine complex, Rajasthan and identified by Gram's staining and 16s rRNA sequence analysis. These all bacterial strains were characterized for their metal and antibiotic resistance properties. Among these a *Bacillus* sp. (PRS3) was further identified by phenotypic, biochemical and fatty acid methyl esters (FAME) analysis and found to be *Bacillus cereus* GC subgroup A. The *Bacillus cereus* GC subgroup A was characterized by growth, biomass and total proteins in different medium (NB & LB), ultra-structural studies and for the presence of plasmid DNA. The plasmid DNA was further characterized by sequence analysis, as discussed in chapter-III.

Chapter III

Characterization of plasmid DNA pPRS3a from Bacillus cereus GC subgroup A

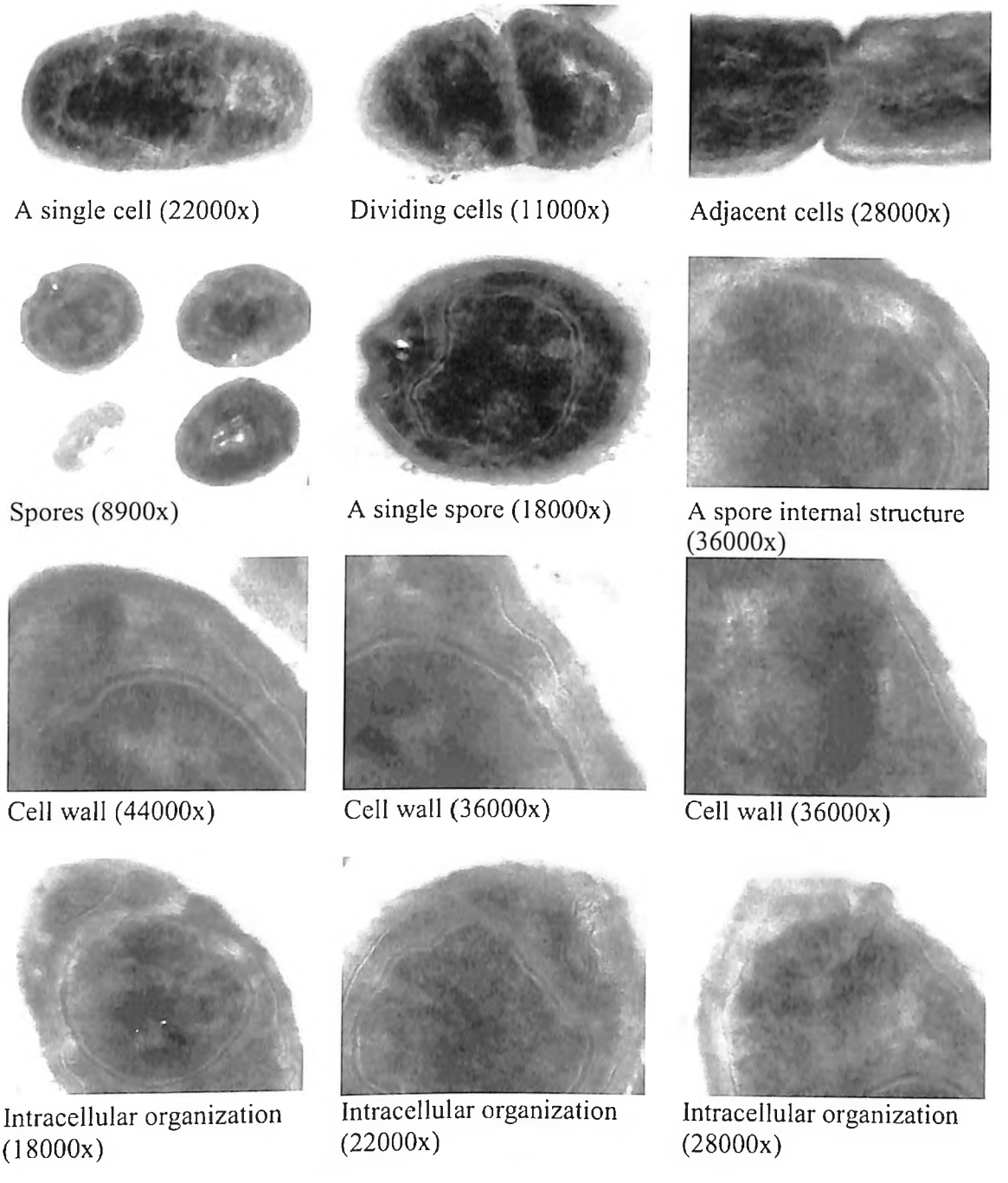


Fig. 11. Transmission Electron Microscopy of *Bacillus cereus* GC subgroup A. different structural and internal organization at high magnifications.

Chapter III

Characterization of plasmid DNA pPRS3a from Bacillus cereus GC subgroup A

Chapter III

Characterization of plasmid DNA, pPRS3a from *Bacillus cereus* GC subgroup A

3.1 Introduction

During the past few decades, it has become increasingly apparent that extra-chromosomal plasmids contribute largely to the phenotypic diversity of bacterial communities in environmental settings (Smit et al. 1998; Droge et al. 2000; Bergstrom et al. 2004; Hansen et al. 2004). Extensive anthropogenic use of antibiotics in clinical settings, aquaculture and farming has drastically increased the selective pressure of these compounds and consequently led to an increase in the occurrence and diversity of the corresponding bacterial resistance determinants, which are often associated with self-transmissible plasmids or transposons (Levy, 2001).

Although plasmid may not be essential for bacterial survival, in many cases they play important roles in bacterial virulence, drug resistance and conjugation. Plasmids and extrachromosomal elements are essential tools for elucidating fundamental mechanisms such as DNA replication, recombination and gene regulation. Isolation and molecular characterization of plasmids are thus necessary to gain new insights into the field of biochemistry and molecular biology. Furthermore, knowledge of the fine structure of extrachromosomal DNA elements paves the way for cloning/expression systems for biotechnological applications. (Santina et al. 2006)

In recombinant DNA technology, plasmids form the foundation for gene cloning and expression. While plasmid systems are available for a variety of prokaryotic and eukaryotic hosts, most vectors are used in combination with *Escherichia coli* K-12 strains (Peterson and Phillips, 2008). *E. coli* plasmid vectors have been modified in numerous ways to enable selection and screening for DNA inserts, for regulation of gene expression by inclusion of a variety of promoters, to facilitate protein purification by addition of epitope tags to gene products and allow the choice of multiple antibiotic resistance markers (Lu, 2004). Despite the large variety of plasmid derivatives, most vectors have been constructed from only a few

different replicons. Most plasmids used for recombinant DNA utilize the replication origin from the ColE1-like plasmids pMB1 (including pBR322 and pUC18/19-derivatives) (Hershfield et al. 1974) and p15A (Chang and Cohen, 1978) or pSC101 (Cohen et al. 1973). *Bacillus subtilis* and related species (*Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens* and *Bacillus mojavensis*) during the last decades have been largely employed by industry for their ability to produce carbohydrases, proteases and other enzymes (Guglielmetti et al. 2007). The natural plasmids in this group of bacteria have attracted the interest of researchers since they represent the elective starting point for the development of genetic engineering molecular tools. Both large (>50 kb) and small plasmids (<10 kb) have been found, often co-resident with one another, in *B. subtilis* and its related species (Tanaka and Koshikawa, 1977; Uozumi et al. 1980; Parini et al. 1991; Manachini et al. 1998). The large plasmid is replicated through a *theta* mechanism (Meijer et al. 1995; Titok et al. 2003), while the small plasmid belongs to the class of rolling circle (RC) replicons, for which their small size has represented a convenient model for genetic research devoted to understanding their physiology and to developing molecular tools for the genetic modification of their hosts.

In *B. anthracis*, *B. thuringiensis* and the emetic *B. cereus* major virulence factors are located extrachromosomally on large plasmids (Gonza´lez et al. 1981; Hoton et al. 2005; Rasko et al. 2005). These species are thought to have emerged from a common ancestor following a series of genetic rearrangements mediated by mobile DNA elements, in synergy with various mechanisms of horizontal gene transfer. Horizontal DNA transfer, mediated by large conjugative plasmids, between members of the B. cereus group has been documented in both insects (Jarrett and Stephenson 1990), mammals, foodstuff (Auwera et al. 2007), soil (Thomas et al., 2000) and rhizosphere (Saile and Koehler, 2006). Recently, new pathogenic isolates have been described which apparently have acquired crucial virulence factors via plasmid transfer. (Lars et al. 2008)

Use of curing agents could be sufficient to remove those genes necessary for virulence and resistance because both types are frequently carried by plasmids. The virulence of *Bacillus anthracis* is related to the number of virulence-plasmid copies a strain carries (Coker et al.

2003). Exploiting the biochemical differences between plasmids and chromosomes might lead to a new sophistication in curing technology. Plasmids are cured by: intercalating compounds, such as Ethidium bromide or acridine orange (Hahn and Ciak, 1976); agents that indirectly damage DNA, such as quinolones (Michel-Briand et al. 1986); and compounds that affect the architecture of cell membranes, thereby disrupting plasmid partition, such as SDS or phenothiazines (Molnár et al. 1982). Curing agents can be applied at concentrations that do not kill bacteria but do destabilize plasmids, enabling continued chromosomal replication and gene expression. The chromosome seems to be less susceptible to these agents, and/or more readily repaired, than plasmids. For example, quinolone antibiotics (Michel-Briand et al. 1986), rifampicin (Bazzicalupo and Tocchini-Valentini, 1972) and the anticancer drugs bleomycin (Attfield and Pinney, 1985) and *cis*-dichlorodiamine platinum(II) (Lakshmi and Polasa, 1991) all eliminate several kinds of plasmids at sub-inhibitory concentrations.

Bacillus cereus has been explored for the presence of endogenous plasmid DNA of size 3-200 kb (Lars et al. 2008). The advantages of using an endogenous plasmid DNA for the expression of foreign genes into *Bacillus cereus* includes, recognition of the promoter by the host RNA polymerase, protection from the host methylation and restriction system, easy replication and ease of transformation. *Bacillus cereus* plasmids have successfully been used for the construction of shuttle expression vectors, which can function in both Gram-positive as well in as Gram-negative bacteria (Noriyuki et al. 1992; Mesrati et al. 2005).

Among the 24 bacterial strains isolated, identified as described in chapter-II, a *Bacillus cereus* GC subgroup A was selected for further studies. This bacterium was characterized morphologically & biochemically and it possesses a plasmid DNA. Characterization and sequencing of a plasmid is the first step toward the finding of genes responsible for antibiotic resistance and the development of a cloning & expression vector in bacteria. Plasmid DNA sequencing provides comprehensive information about the basic mechanisms of its replication and inheritance, presence of insertion elements to the phenotypic markers carried on it.

In this chapter the characterization of *Bacillus cereus* GC subgroup A for the presence of an endogenous plasmid DNA, pPRS3a; plasmid DNA sequencing for the presence of antibiotic resistance marker and other components; and the cloning and expression characteristics of this plasmid for biotechnological applications will be discussed.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

Bacillus cereus GC subgroup A was used as a source bacterial strain for plasmid DNA characterization. *E. coli* DH5α was used a general host for most recombinant DNA protocols. *E. coli* HB101 and *E. coli* DH10B were also used as host for the transformation of plasmid DNA and their axenic cultures were procured from Bangalore GENEI, Bangalore. *Bacillus cereus* GC subgroup A and all the strains of *E. coli* were grown in Luria Bertani (LB) broth or LB supplemented with 1.5% agar under standard culture conditions. All the bacterial strains and plasmid used in this study have been listed in Table 13.

Table 13. Bacterial strains and plasmid used.

S. No.	Bacterial strains/Plasmid	Properties
1.	*B. cereus* GC subgroup A	Source of pPRS3a
2.	*E. coli* DH5α	Host for recombinant plasmids
3.	*E. coli* DH10B	Host for recombinant plasmids; F^- conjugal strain
4.	*E. coli* HB101	Host; F^- conjugal strain; $mcrB^-$
5.	pET-28c(+)	A cloning and expression vector, T7 promoter, kan^r
6.	pJSKV51	Source for arsGFP (PvuII & EcoRI)
7.	pKT-210	Source for cat (HindIII & PstI)

3.2.2 Enzymes and chemicals

Luria Bertani bacterial culture medium was procured from Himedia Pvt. Ltd., Mumbai. All the enzymes used for DNA manipulations and cloning were purchased from New England Biolabs or MBI Fermentas (USA). The metal salts used were of analytical grade and these were purchased from Sigma, USA. Metal stock solutions were prepared in Milli-Q water.

Atomic spectral standards for all the metals were purchased from National Physical Laboratory, New Delhi, and were used for preparing working standard for atomic absorption spectroscopy and metal estimations. All other required chemical for SDS PAGE were also obtained from Sigma, USA. Glassware and plastic wares were cleaned with cedepol before use, followed by overnight soaking in 50% nitric acid.

3.2.3 Plasmid DNA isolation

For plasmid DNA isolation from *Bacillus cereus* GC subgroup A, it was grown in LB supplemented with/without ampicillin (50μg/ml) at 37°C under shaking condition of 200rpm. Plasmid DNA (pPRS3a) was isolated as described in section 2.2.6.

Plasmid DNA from recombinant stains of *E. coli* was isolated using Qiagen plasmid isolation kit (QIAGEN Plasmid Mini Kit, 12123) as per the manufacturer instructions. A single bacterial colony from an overnight grown LB agar plates was inoculated in 10 ml of LB with respective antibiotics. The flasks were incubated overnight at 37 °C, 200 rpm in an orbital shaker incubator. 2 ml of this overnight culture was transferred to micro centrifuge tubes and spun at 10000 rpm for one minute. The supernatant was discarded and the pellet was resuspended in 300μl of buffer Pl and mixed by vortexing. 300μl of lysis buffer P2 was then added and the contents were mixed gently by inversion and incubated at RT for 5 min. 300μl of pre-chilled buffer P3 was added, followed by incubation on ice for 8 min. The contents were then spun at 10000 rpm for 10 min at 4 °C and the supernatant was applied to the Qiagen column equilibrated with buffer QBT. After washing the column with buffer QC, the DNA was eluted using 800μl of buffer QF in a fresh 2 ml micro centrifuge tube. DNA was precipitated by the addition of 0.75 volume of isopropanol and centrifuged. The DNA pellet was washed with 1 ml of 70% ethanol and spun again as before. The supernatant was discarded and tubes were air dried for 30 min. The DNA was resuspended in 30μl of 10 mM Tris-Cl, pH 8.0 and stored at -20 °C until use.

3.2.4 Agarose gel electrophoresis of DNA

Electrophoresis of DNA was carried out on 1 % agarose gels prepared in 1x Tris Acetate EDTA Buffer (TAE buffer) and electrophoresed with 1x TAE buffer in the gel tank at 90

Volts (V) for 1h. The resolved gels were visualized and documented with a Syngene Genesnap Gel Documentation system.

3.2.5 Transformation

Preparation of competent cells

For the preparation of competent cells of *E. coli* DH5α, HB101 and DH10B, each strain was streaked separately on LB agar plates without any antibiotics and incubated at 37°C for 12h. A single colony was transferred to 5 ml LB broth in a 20 ml conical flask and was incubated at 37°C with 200 rpm in an orbital incubator shaker for 14h. 1 ml of this starter culture was used to inoculate 50 ml of LB broth in a 500 ml conical flask and was incubated at 37°C, 200 rpm, till OD_{600} reached 0.5. The flask was then chilled on ice for 10 min and culture was transferred to a 50 ml oakridge tube. This was centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 12 ml of pre-chilled 0.1 M $CaCl_2$ solution. The cell suspension was incubated on ice for 30 min and centrifuged at 8000 rpm for 8 min at 4°C. The supernatant was discarded and pellet was resuspended in 1 ml of 0.1 M $CaCl_2$ and pre-chilled glycerol was added to a final concentration of 15%. The competent cells were than frozen in a mini cooler and stored at -70 °C until use (Sambrook et al. 2001).

Transformation

10μl (~1 μg) of DNA (plasmid DNA or ligation mixture) was added to a 0.5 ml micro centrifuge tube and was allowed to chill on ice for 10 min. 90μl of competent cells were added to this tube and incubated on ice for 30 min. The mixture was subjected to heat shock at 42°C for 60 – 90 sec in a water bath followed by incubation on ice for 5 min. 300μl of LB broth was added to the cell suspension and the tube was incubated at 37°C for 1 h. The tube was then centrifuged at 8000 rpm for 6 min and 300μl of supernatant was removed. Cell pellet was resuspended in remaining 100μl of supernatant and the cell suspension was then plated on LB agar plates containing respective antibiotics. The plates were incubated at 37°C for 14h and screened for transformants.

Screening of the transformants for recombinants

After incubation, the resulting colonies were replica plated in duplicates on LB agar plates containing respective antibiotics. The bacterial colonies were picked at random, sub-cultured in 10 ml LB broth and incubated at 37°C, 200rpm for 12h in an orbital shaker. 2 ml of the grown culture was used to isolate plasmids as described in section 3.2.3. The plasmid DNA was electrophoresed as described in section 3.2.4. The recombinants were selected based on the gel mobility shift and plasmid DNA without the insert DNA was taken as control. Recombinant plasmid showing gel mobility shift were selected and the presence of insert was confirmed by digestion with respective restriction enzymes.

3.2.6 Restriction endonuclease digestion

From *E. coli* DH5α-pPRS3a, plasmid DNA (pPRS3a) was isolated by alkaline lysis method and subjected to restriction enzyme digestion (Sambrook et al. 2001). Restriction enzymes used in this study were: BamHI, BglII, SalI, XhoI, PstI, PvuII, SmaI, NcoI, EcoRI, EcoRV, NotI (MBI Fermentas). The compositions for the digestion reaction have been shown in Table 14. This reaction was carried for 1h at 37°C.

Table 14. Restriction endonuclease digestion reaction components.

S. No.	Components	Stock	Volume
1	Autoclaved Milli-Q water	-	24 µl
2	Plasmid DNA	500ng/µl	2 µl
3	Restriction enzyme	10U/µl	1 µl
4	Reaction buffer	10x	3 µl
	Total		**30 µl**

Digested Plasmid DNA was electrophoresed and separated on a 1% agarose gel as described in section 3.2.4.

3.2.7 Plasmid DNA (pPRS3a) sequencing

The sequencing of pPRS3a was done by Primer walking method (Bjorland et al. 2007). To achieve this, pPRS3a was cloned into pET-28c(+). The sequencing of plasmid DNA (pPRS3a) was carried out using the following steps.

3.2.7.1 Cloning of pPRS3a into pET-28c(+)

To sequence pPRS3a, this plasmid DNA was cloned into at EcoRI restriction enzyme site under a defined strategy, as shown in figure 12. To construct the recombinant plasmid DNA molecule (pPRS3a- pET-28c(+) series of steps were used, as described below.

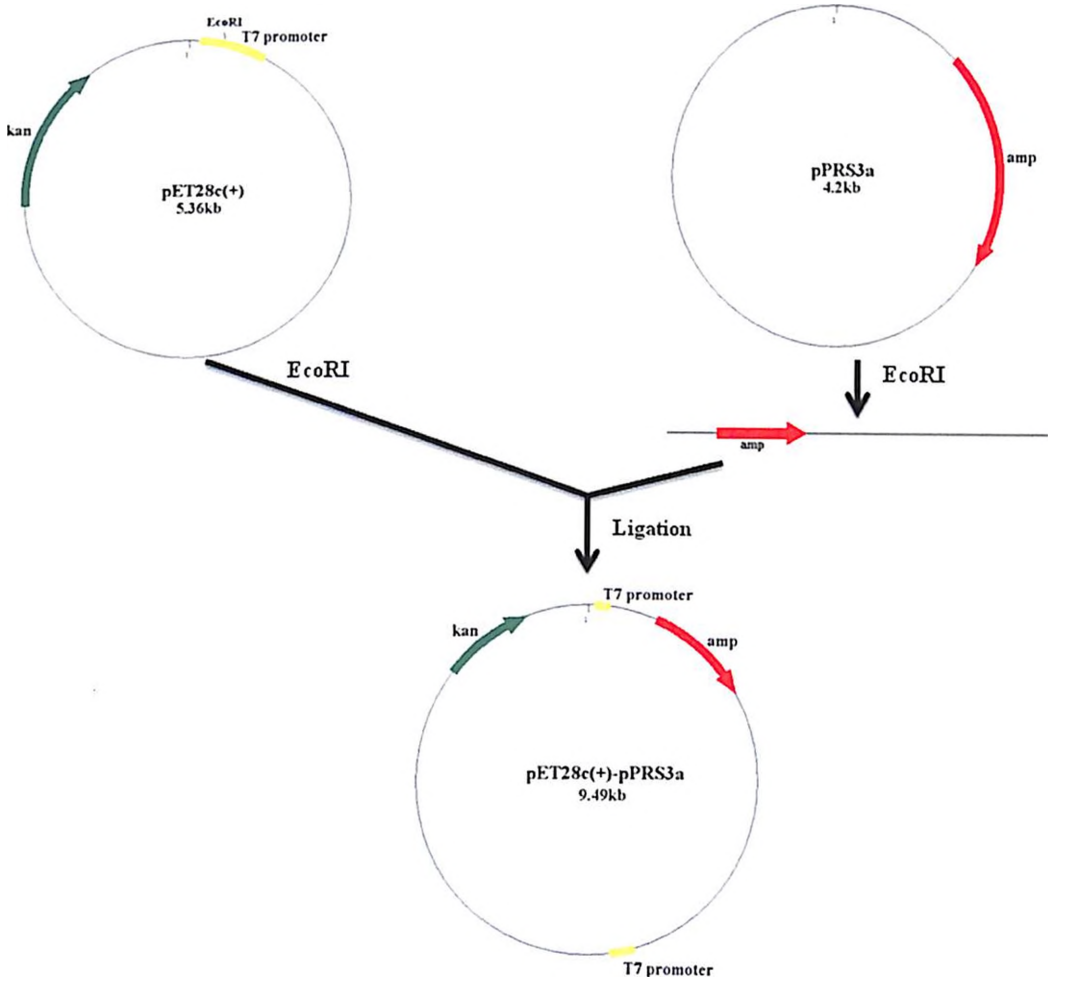


Fig. 12. Cloning strategy for construction of the plasmid pET-28c(+)-pPRS3a. pPRS3a was digested with EcoRI and cloned into pET-28c(+) at EcoRI site. The resulting pET-28c(+)-pPRS3a was transferred into *E. coli* DH5α for sequencing of pPRS3a through primer walking method.

Plasmid DNA isolation

Plasmid DNA from recombinant *E. coli* DH5α pPRS3a and *E. coli* DH5α pET-28c(+) were isolated as described in section 3.2.3. Isolated plasmid DNA was electrophoresed on 1% agarose gel as described in section 3.2.4.

Restriction endonuclease digestion

Plasmids DNA pPRS3a and pET-28c(+) were digested with the enzyme EcoRI. The components of this restriction endonuclease digestion reaction have been shown in Table 11. This reaction mixture was incubated at 37°C in water bath for 1h. The digested plasmid DNA was electrophoresed on 1% agarose gel as described in section 3.2.4.

Purification of DNA from enzymatic reactions

This purification reaction was carried out using QIAquick Gel Extraction Kit (QIAGEN, 28704). Digested DNA of pPRS3a and pET-28c(+) was loaded into a 1% agarose gel in TAE and was electrophoresed at 90 Volts (V) for 1h; and DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The slice was weighed in a micro centrifuge tube and 3 volumes of buffer QG was added to 1 volumes of gel slice (100mg=100μl). Gel slice with the buffer was incubated at 50°C until the gel is completely dissolved. After the dissolution of the gel fragment 1 volume of absolute isopropanol was added and mixed. The QIAquick column was placed in the 2 ml collection tube. To bind DNA, the sample was applied to the column and was centrifuged at 13000 rpm for 1 min in an Eppendorf micro centrifuge. The flow-through was discarded and the column was placed back in the same collection tube. Column was further washed twice by adding 0.75 ml of buffer PE and centrifuged as before. The flow-through was discarded and column was centrifuged for additional 1 minute at 13000 rpm. To elute DNA the column was transferred on top of a 2 ml micro centrifuge tube and 20μl of autoclaved Millipore water was added to the center at the QIAquick membrane inside the column and the assembly was centrifuged at 13000 rpm for 1 minute. The purified DNA collected inside the 2 ml micro centrifuge tube was stored at -20°C until use.

Dephosphorylation of vector

To reduce the probability of self-ligation of the linearised vector pET-28c(+), after digestion with EcoRI restriction enzyme, 1 unit of Calf Intestinal Alkaline Phosphate (CIAP) was added to the restriction endonuclease digestion mixture and was incubated for another 30 minutes at 37°C in a pre-adjusted circulating water bath. The dephosphorylated DNA was then purified using a QIAquick column as described above.

Ligation

Ligation of the restriction enzyme digested insert DNA (pPRS3a) and dephosphorylated pET-28c(+) vector DNA was carried out with T4 DNA ligase. A 10µl reaction mixture was set up as per the composition given in Table 15.

Table 15. Composition of reaction mixture used for ligation of pPRS3a & pET-28c(+)

S. No.	Components	Stock	Volume
1	Autoclaved Milli-Q water	-	4.9 µl
2	pET28c(+) Vector DNA	10ng/µl	1 µl
3	pPRS3a Insert DNA	20ng/µl	1.6 µl
4	T4 DNA ligase	1U/µl	1.5 µl
5	T4 DNA ligase buffer	10x	1 µl
	Total		**30 µl**

For obtaining the maximum number of recombinants, a vector: insert molar ratio of 1:2 was maintained in the reaction mixture. The above mixture was pre-chilled in a 0.5 ml micro centrifuge tube and the tube was incubated at 16°C for 14h in a preset circulating water bath and the reaction mixture was used to transform competent cells of *E. coli* DH5α.

Transformation and screening of recombinants

The ligated mixture of pPRS3a-pET-28c(+) was transformed into *E. coli* DH5α as described in section 3.2.5. The transformants were selected on LB agar plate supplemented with ampicillin and kanamycin. One positive and one negative control were also performed to minimize the transformation errors. After 16h of incubation at 37°C, transformants were picked up and streaked in duplicate on another LB agar plate containing ampicillin and kanamycin antibiotics. The bacterial colonies were picked at random, sub-cultured in 10 ml

LB broth and incubated at 37°C for 12h in an orbital shaker with 200 rpm. 2 ml of the grown culture was used to isolate plasmids as described in section 3.2.3. The plasmid DNA was electrophoresed as described in section 3.2.4. The recombinants were selected based on the gel mobility shift and plasmid DNA without the insert DNA served as control. Recombinant plasmids showing gel mobility shift were selected and the presence of insert was confirmed by digestion with EcoRI restriction enzyme. After the confirmation of true recombinants by gel mobility shift assay and restriction enzyme digestion, plasmid DNA from recombinant *E. coli* DH5α (containing a fusion plasmid of pPRS3a-pET-28c(+)) was isolated as described in section 3.2.3.

This purified plasmid DNA was used for sequencing of insert through Primer Walking method using universal primers for pET vectors; PET Fwd (5'-TAATACGACTCACTATAGGG-3') and PET Rev (5'-GCTAGTTATTGCTCAGCGG-3'). These primers are very specific to the T7 promoter region of pET vectors.

3.2.7.2 pPRS3a sequencing

Sequencing of pPRS3a, this is cloned into pET-28c(+) was performed by primer walking method (Bjorland et al. 2007). In primer walking, initial primers are used based on vector DNA sequence. Later in subsequent reaction, internal primers are designed and used. This amplification and sequencing is carried out until the vector DNA sequences are obtained. The sequence of pPRS3a was done in both the directions to eliminate the errors and to get a good quality sequence of DNA. Here, the plasmid DNA sequencing through primer walking was performed at "The DNA facility of The Iowa State University, Office of Biotechnology", Iowa, USA. Sequencing samples were run on the DNA Facility's Applied Biosystems 3730xl DNA Analyzer. The AB 3730xl uses a four-color dye system and provides up to 900 bases of usable sequence data per reaction.

3.2.8 pPRS3a sequence analysis

The obtained chromatogram of each fragment was viewed and analyzed using bioinformatics software Chromas Lite version 2.01. The sequences were deduced from this chromatogram using BioEdit version 7.0.5.2 (Hall, 1999). The obtained sequences of each

fragment were aligned to obtain the over-lapping regions. Each fragment of the sequence was called as contig. All the obtained contigs were aligned in such a way that a complete sequence in both the directions could be obtained.

To find out structural and functional components in the obtained sequence of pPRS3a, bioinformatics tool were used. The presence of following components were been observed.

3.2.8.1 Open reading frames (ORFs)

To find out open reading frames, ORF finder from NCBI was used (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). In finding of ORF bacterial genetic code was used as a standard. The ORF Finder is a graphical analysis tool, which finds all open reading frames of a selectable minimum size in a given sequence or in a sequence already in the database. This tool identifies all open reading frames using the standard or alternative genetic codes. The deduced amino acid sequence can be saved in various formats and searched against the sequence database using the WWW BLAST server.

3.2.8.2 Promoters

To find out the promoters, bacterial promoter finder (BPROM) (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) and NNPP version 2.2 (http://www.fruitfly.org/seq_tools/promoter.html) were used BPROM is bacterial sigma70 promoter recognition program with about 80% accuracy and specificity. It is best used in regions immediately upstream from ORF start for improved gene and operon prediction in bacteria.

3.2.8.3 Origin of replication (Ori)

For origin of replication prediction, Ori-Finder was used (http://tubic.tju.edu.cn/Ori-Finder) (Gao and Ting, 2008). Ori-Finder is an online system for finding oriCs in bacterial genomes based on an integrated method comprising the analysis of base composition asymmetry using the Z-curve method, distribution of DnaA boxes and the occurrence of genes frequently close to oriCs. Output of the predicted results is exported to an HTML report,

which offers convenient views on the results in both graphical and tabular formats. In origin of replication region, direct repeats and inverted repeats were found out

3.2.8.4 Restriction endonuclease enzymes sites

To find out all the restriction endonuclease enzyme sites BioEdit version 7.0.5.2 (Hall, 1999) software was used. BioEdit is a biological sequence editor that runs in Windows 95/98/NT/2000/XP and is intended to provide basic functions for protein and nucleic sequence editing, alignment, manipulation and analysis. BioEdit is a powerful sequence analysis program, which offers many quick and easy functions for sequence editing, annotation and manipulation, as well as a few links to external sequence analysis programs. The search for restriction enzyme sites was confined to parameters like, sites that cut less than five times for 4-6 base cutters, large recognition sites (<6 bases) and enzymes with degenerate recognition.

3.2.8.5 GC/AT rich regions

To find out GC/AT rich region in plasmid DNA sequence, a MATLAB based program was used (Baker and Rogers, 2005). Searching cutoff was given 70% & 75% for GC and AT rich region respectively. Apart from this analysis the base composition was also included in the pPRS3a sequence analysis.

3.2.8.6 Plasmid map

After complete sequence analysis, BioEdit version 7.0.5.2 was used to draw the map of the plasmid DNA pPRS3a. The plasmid DNA map shows each of its components in different color along with their positions and directions.

3.2.9 Nucleotide sequence accession no.

The annotated nucleotide sequence of pPRS3a is available in the NCBI GenBank under the accession no. GQ404376.1.

3.2.10 Cloning of arsGFP and cat gene into pPRS3a

To construct a recombinant expression vector system, arsGFP and cat genes were cloned into pPRS3a at PvuII restriction enzyme site under a defined strategy, as shown in figure 13 & 14. For the construction of this recombinant plasmid DNA molecule (pPRS3a-arsGFP-cat), series of steps was used, as described below.

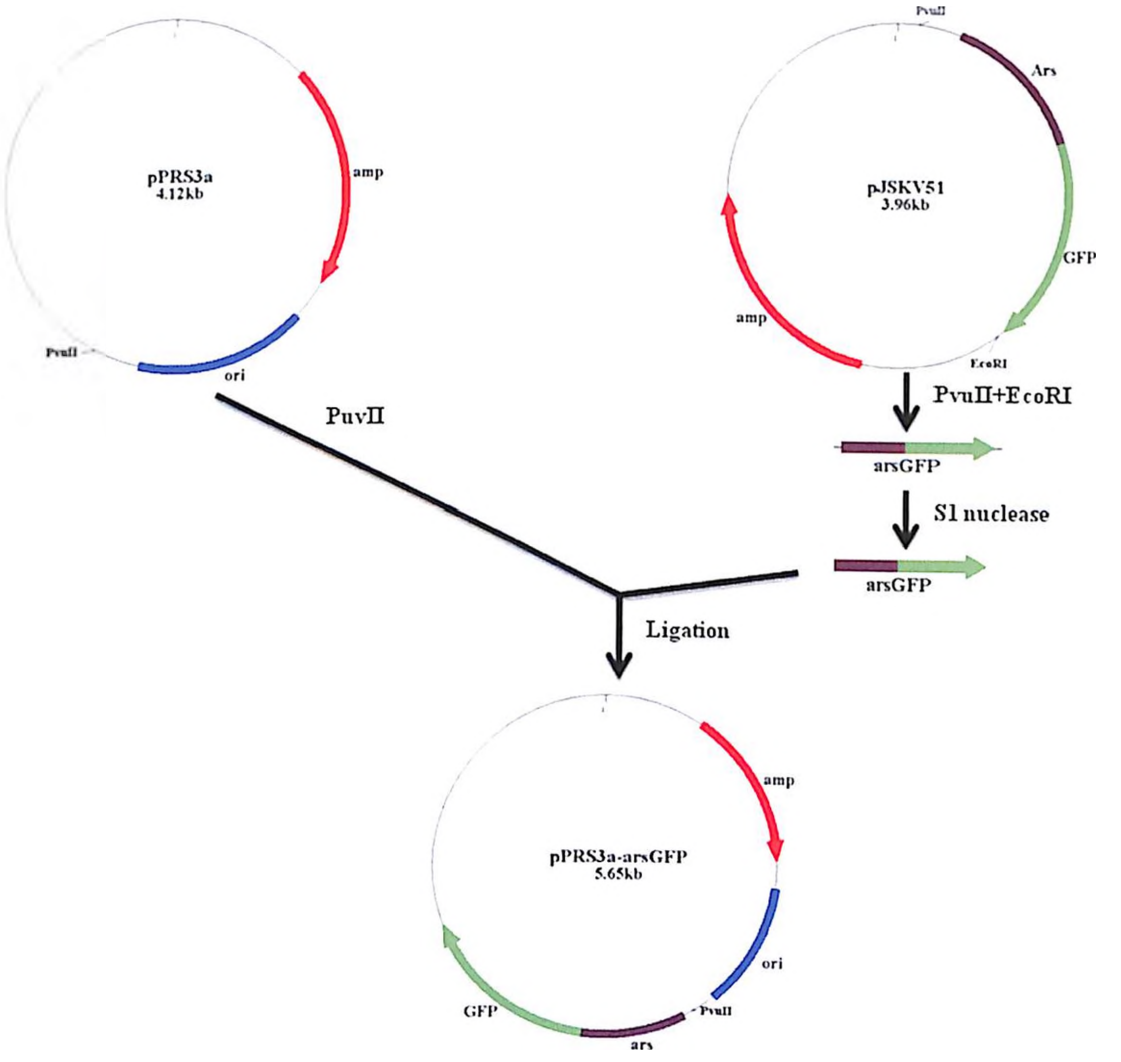


Fig. 13. Cloning strategy for construction of the plasmid pPRS3a-arsGFP. arsGFP gene from pJSKV-51 was digested with PvuII/EcoRI, treated with S1 nuclease and cloned into the PvuII site on plasmid pPRS3a. The resulting pPRS3a-arsGFP, which contains the arsGFP gene, was transferred into *E. coli* DH5α for the next step of cloning.

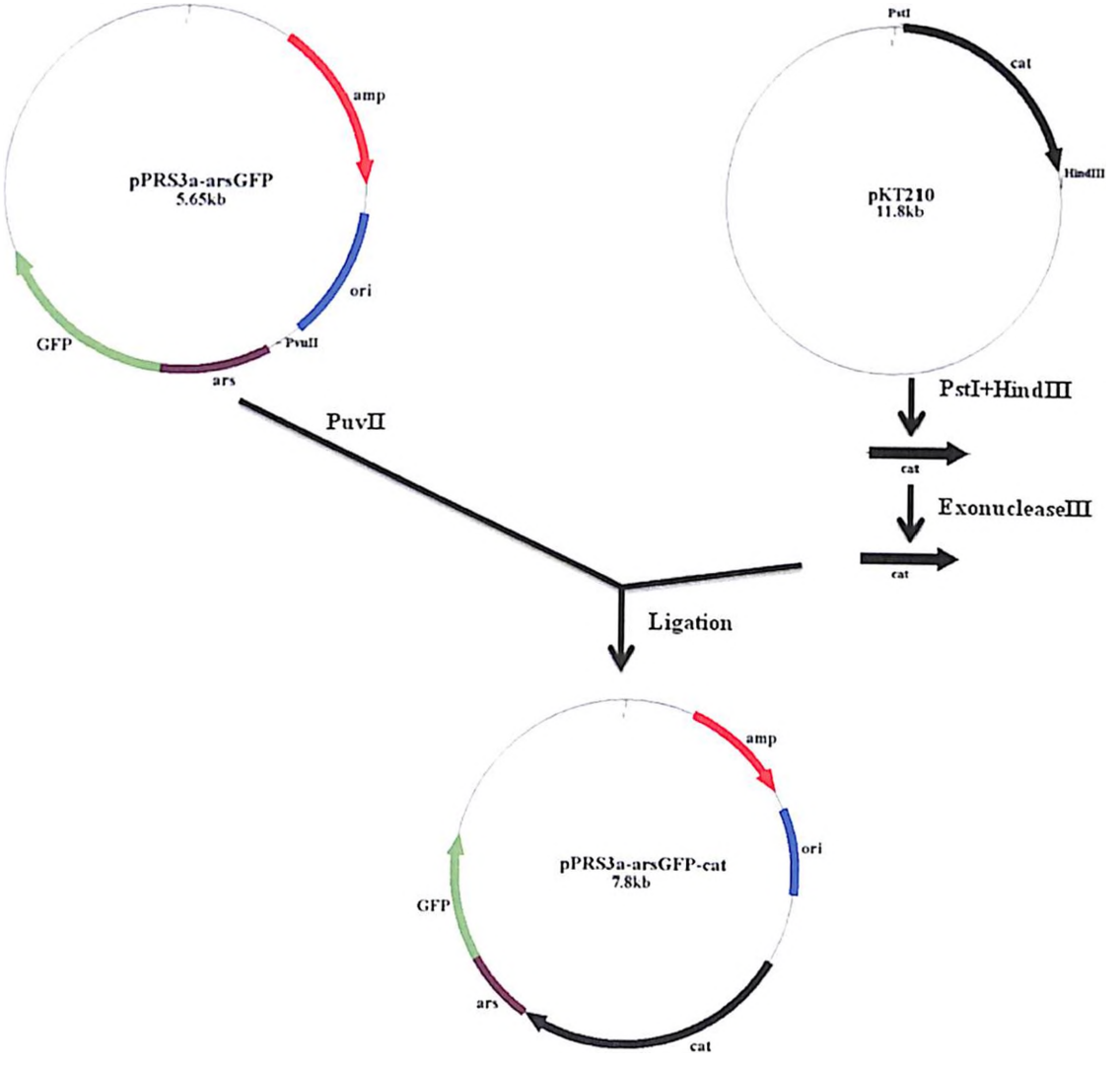


Fig. 14. Cloning strategy for construction of the plasmid pPRS3a-arsGFP-cat. cat gene from pKT-210 was digested with PstI/HindIII, treated with ExonucleaseIII and cloned into the PvuII site on plasmid pPRS3a-arsGFP. The resulting pPRS3a-arsGFP-cat, which contains the arsGFP & cat gene, was transferred into *E. coli* DH5α for the expression of cloned genes.

Plasmid DNA isolation

Plasmid DNA from *E. coli* DH5α-pJSKV51 (source for arsGFP) and *E. coli* DH5α-pKT-210 (source for cat gene) were isolated as described in section 3.2.3. The isolated plasmid DNA was run on 1% agarose gel as described in section 3.2.4.

Restriction enzyme digestion

Plasmid DNA (template) was digested with PvuII, EcoRI, HindIII and PstI at different stages of cloning. This digestion reaction was performed at 37°C for 1h and components have been described in Table 11. The restriction exonuclease digestion for enzymes S1 Nuclease and Exonuclease III was carried out at 37°C for 20 min on template DNA at different stages of cloning. The reaction components for exonuclease digestion have been shown in Table 16.

Table 16. The exonuclease digestion reaction components.

S. No.	Components	Stock	Volume
1	Autoclaved Milli-Q water	-	24 µl
2	Template DNA	500ng/µl	2 µl
3	Restriction exonuclease enzyme	10U/µl	1 µl
4	Reaction buffer	10x	3 µl
	Total		**30 µl**

Purification of DNA from enzymatic reactions

This purification reaction was carried out using QIAquick Gel Extraction Kit (QIAGEN, 28704) as described previously.

Dephosphorylation of vector

To reduce the probability of self-ligation of the linearised vector pPRS3a, 1 unit of Calf Intestinal Alkaline Phosphatase (CIAP) was added to the restriction endonuclease digestion mixture after digestion with EcoRI restriction enzyme, and was incubated for another 30 minutes at 37°C in a pre-adjusted circulating water bath. The dephosphorylated DNA was then purified using a QIAquick column as described previously.

Ligation

Ligation of the restriction enzyme digested insert DNA and the restriction enzyme digested, dephosphorylated vector DNA was carried out with T4 DNA ligase as described in previously.

Transformation and screening of recombinants

The ligated mixture was transformed into *E. coli* DH5α as described in section 3.2.5. The transformants were selected on LB agar plate supplemented with ampicillin or ampicillin and chloramphenicol. One positive and one negative control were also performed to minimize the transformation errors. After 16h of incubation at 37°C, transformants were picked up and streaked in duplicate on another LB agar plate containing ampicillin or ampicillin and chloramphenicol antibiotics. The bacterial colonies were picked at random, sub-cultured in 10 ml LB broth and incubated at 37°C, 200 rpm for 12h in an orbital shaker. 2 ml of the grown culture was used to isolate plasmids as described in section 3.2.3. The plasmid DNA was electrophoresed as described in section 3.2.4. The recombinants were selected based on the gel mobility shift and DNA without the insert DNA served as control. Recombinant plasmids showing gel mobility shift were selected and the presence of insert was confirmed by digestion with restriction enzymes. After the confirmation of true recombinants by gel mobility shift assay and restriction enzyme digestion, plasmid DNA from recombinant *E. coli* DH5α (containing a fusion plasmid of pPRS3a-arsGFP) was isolated as described in section 3.2.3 and subjected for next step of cloning experiment as shown in figure 10.

3.2.11 Expression studies

The constructed recombinant strain of *E. coli* DH5α containing plasmid DNA molecule (pPRS3a-arsGFP-cat) was used for the expression studies of inserted genes. The expression profile of arsGFP and chloramphenicol resistance gene was checked. The final products of both the genes are the protein molecules. Therefore, the protein molecules were assayed using qualitative and quantitative methods. For the qualitative measurements of the gene product sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

Fluorescence microscopy were used and for the quantitative measurements of the gene product, Multi label counter was used.

3.2.11.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

In order to gain an insight into the changes in protein expression of recombinant *E. coli* DH5α (containing a fusion plasmid of pPRS3a-arsGFP-cat) upon exposure to arsenic (an inducer of arsGFP) and chloramphenicol (an inducer of cat), the total cell lysate was analyzed by separating total protein on SDS-PAGE. To achieve it, cells were grown in stress conditions of ampicillin & arsenic and ampicillin, arsenic & chloramphenicol along with the control, for 18h under shaking condition (150rpm). After incubation, cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C and washed once with phosphate buffered saline (pH 7.4), resuspended in PBS containing 2mM EDTA (pH 8.0) and then a protease inhibitor, 0.2 mM phenyl methyl sulphonyl fluoride (PMSF) was added. Finally, the cells were disrupted by sonication on ice bath and cell debris was removed by centrifugation at 15000 rpm for 15 min at 4°C (Wang et al. 2005). A fraction of the obtained protein supernatant was used for protein determination by BCA method (Brown et al. 1989). To quantified protein mixture, 2x sample buffer (SIGMA) was added in equal volume. For denaturing polyacrylamide gel electrophoresis, the samples were prepared as per the standard protocol and SDS PAGE was carried out (Sambrook et al. 2001). 80μg of total protein in each lane was electrophoresed on 10%, 12% and 15% of 0.75 mm thick gel using BioRad Mini Protean gel electrophoresis apparatus at 80/100 V. A protein molecular weight marker (SM0431, MBI Fermentas) was also loaded along with protein samples. The proteins separated on polyacrylamide gel were visualized by staining the gel with 0.25% Coomassie brilliant blue G-250 in 45% methanol and 10% acetic acid and destained in 35% methanol with 10% acetic acid.

3.2.11.2 Fluorescence microscopy

For fluorescence microscopy, cells were grown in stress conditions of ampicillin (50μg/ml), arsenite (0-1000ppb) & chloramphenicol (30μg/ml) along with the control, for 12h under shaking condition (150 rpm). EGFP expression in recombinant *E. coli* DH5α was analyzed and documented with an Olympus BX41 epifluorescence microscope fitted with an

Olympus Camedia C-4000 digital camera. Bacterial cells were spread on a gelatin coated glass slide and were viewed by exciting with a 100W mercury arc lamp and filter block fitted with a 465-495 nm excitation filter and a 500-540nm emission filter. Photomicrographs were captured with 11X digital zoom, ISO200 light sensitivity and automatic capture mode.

3.2.11.3 Fluorescence Assay

For fluorescence assay, cells were grown in stress conditions of ampicillin (50µg/ml), arsenite (0-1000ppb) & chloramphenicol (30µg/ml) along with the control, for 12h under shaking condition (150rpm). EGFP expression levels in recombinant *E. coli* DH5α were estimated with a Wallac Victor^3 Multilabel counter. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. To minimize the background fluorescence, low background 96 well plates (Tarsons, Mumbai) were used. For measurements, 200µl of bacterial suspension was pipetted into each well and fluorescence was estimated by exciting the cells at 580nm and emission was detected using a 510nm filter set. All measurements were performed in triplicates.

3.2.12 Plasmid curing

This is a technique in which plasmid DNA is subjected to some DNA intercalating agents to lose its structural and functional properties or to eliminate it. This technique was used to differentiate characteristics features (imparted by plasmid) of *Bacillus cereus* GC subgroup A, which contain pPRS3a, by using two different DNA intercalating agents named as Ethidium bromide and Proflavin. The curing of plasmid using Ethidium bromide & Proflavin was carried out as described by Caro et al. (1984) and Singh and Yadava (1988). To determine MIC of Ethidium bromide and Proflavin this bacterium was grown in LB medium supplemented with the increasing concentration of curing agent from 5µg/ml to 1mg/ml keeping the interval of 5µg/ml each. The minimum concentration of Ethidium bromide and Proflavin in which no growth observed was considered as minimum inhibitory concentration (MIC). After determination of MIC, *Bacillus cereus* GC subgroup A was grown in LB supplemented with sub-lethal concentration of Ethidium bromide and Proflavin. This culture was grown at 37°C for 18h under shaking condition (150 rpm). After

every 18h, the culture was sub cultured aseptically, using 1% of inoculums into a fresh medium containing respective curing agents. This sub culturing was continued until 25th generations. After every generation, cells were checked for their sensitivity for ampicillin on LB agar plate. In addition, plasmid was isolated as described in section 2.2.6 and compared with control, a culture of *Bacillus cereus* GC subgroup A without any curing treatment.

3.3 Results and Discussion

3.3.1 Plasmid DNA

Plasmid DNA from *Bacillus cereus* GC subgroup A was isolated as described in methods section 3.2.3 and has been shown in figure 15.

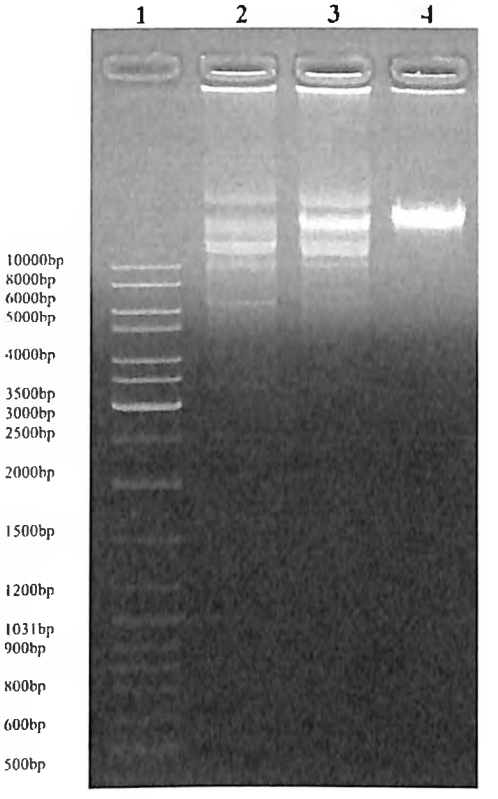


Fig. 15. Plasmid DNA of *Bacillus cereus* GC subgroup A.

- lane 1, DNA marker*;
- lane 2, pPRS3a grown in LB;
- lane 3, pPRS3a grown in ampicillin 50µg/ml;
- lane 4, lambda DNA (500ng).
- DNA marker (*MBI Fermentas, SM0331, 1kb ladder).
- The DNA samples were separated on 1% agarose gel.

This bacterium was isolated from a copper mining industry and exhibit property of resistance to multiple metal and antibiotics. So, its point of interest to study the plasmid DNA profile in detail. As per the literature, properties like metal & antibiotic resistance are generally imparted by extrachromosomal genetic elements, so plasmid DNA isolation was carried out.

The presence of multiple bands on the agarose gel confirmed the presence of plasmid DNA. The plasmid DNA was named as pPRS3a, because it was isolated from strain PRS3, as initially identified by 16s rRNA sequencing (Jain et al. 2009). Later on, the identification of this bacterium was carried up to species level by biochemical and fatty acid methyl esters analysis (FAMEs). Figure 15 shows that the nature of the plasmid DNA in the presence & absence of ampicillin. This observation confirms that for the maintenance and proliferation of pPRS3a in host bacterium does not require any external molecule as a maintenance factor and signify the fact that this is a naturally occurring plasmid DNA molecule.

3.3.2 Transformation and Restriction endonuclease digestion

The isolated plasmid DNA from *Bacillus cereus* GC subgroup A was subjected to transformation as described in methods section 3.2.5. The resultant transformants were selected on to all possible antibiotics to which *Bacillus cereus* GC subgroup A was showing resistance. True transformants were obtained only from the LB agar plate containing ampicillin and they were further confirmed by gel-mobility shift assay. The above step of selection was done to confirm the presence of resistance marker on the plasmid DNA. The growth of true transformants onto ampicillin confirmed that the plasmid (pPRS3a) is having ampicillin resistance gene as marker.

The transformation was done into a Gram's negative bacterium *E. coli*, a widely used host in the molecular biology. Different strains of *E. coli* like DH5α, HB101 and DH10B were used to ensure the maintenance of pPRS3a into a Gram's negative bacterium as this plasmid is of Gram's positive origin. In all the strains of *E. coli*, transformants were obtained on LB agar plate supplemented with ampicillin (50μg/ml) but in *E. coli* DH5α especially good numbers of transformants were obtained. Due to the ease of isolation and better yield of plasmid

DNA from recombinant *E. coli* DH5α (Ausubel et al. 1991), this strain was used for further studies. In the subsequent experiment the transformants were selected on to all antibiotics to which *Bacillus cereus* GC subgroup A was resistant. True transformants were obtained only from the LB agar plate containing ampicillin (50μg/ml) and they were further confirmed by plasmid DNA isolation. The above step of selection was done to confirm the presence of resistance marker on the plasmid DNA. The growth of true transformants onto ampicillin confirmed that the plasmid (pPRS3a) is having only ampicillin resistance gene as marker.

The transformation of this plasmid DNA into different strains of *E. coli* also suggests that pPRS3a may have an origin of replication that can be recognized by Gram's positive as well as Gram's negative bacterial systems. This assumption can only be confirmed after the sequencing & analysis of pPRS3a. The transformed recombinant strain of *E. coli* like DH5α was subjected to plasmid DNA isolation and restriction endonuclease digestion as described in methods section 3.2.6. The digestion patterns of pPRS3a have been shown in figure 16.

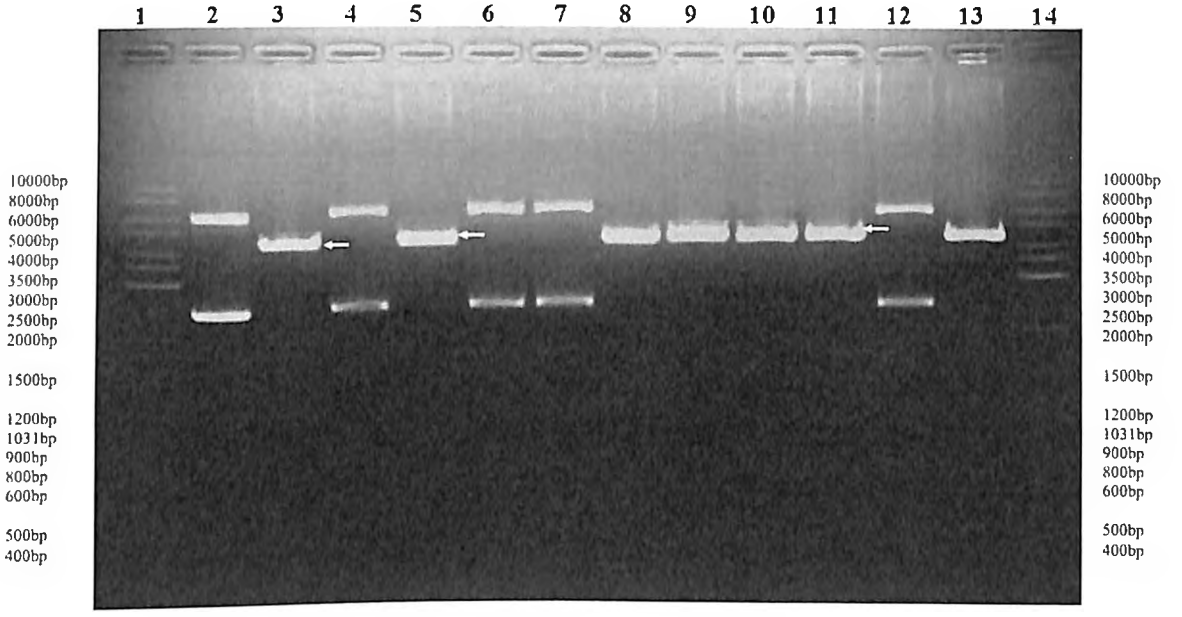


Fig. 16. Restriction endonuclease digestion profile of pPRS3a.

- lane 1, DNA marker; lane 2, pPRS3a from *E. coli* DH5α-pPRS3a;
- lane 3-13, pPRS3a digested with BamHI, BglII, SalI, XhoI, PstI, PvuII, SmaI, NcoI, EcoRI, EcoRV, NotI respectively (4.2 kb);
- lane 14, DNA marker
- Arrows show a 4.2 kb band
- The DNA samples were separated on a 1% agarose gel.

This enzymatic digestion was carried out with routinely used enzymes in molecular biology laboratory. Upon digestion a single band was observed without any smear or trailing of bands that confirmed the size of the plasmid DNA to be 4.2kb. Among restriction enzymes used, a clear band of 4.2kb was observed for BamHI, SalI, PvuII, SmaI, NcoI, EcoRI and NotI but not for BglII, XhoI, PstI and EcoRV. The digestion with number of enzymes suggests that it can be easily manipulated. There may be many more enzymes for which restriction sites may be present. This possibility can only be ruled out after pPRS3a DNA sequencing & analysis.

3.3.3 Plasmid DNA (pPRS3a) sequencing

To unveil many facts about this plasmid DNA, pPRS3a was subjected to DNA sequencing through primer walking. Primer walking is used for the sequencing of large DNA molecules (>1.5kb), for which a single run is not recommended. The sequencing of pPRS3a was carried using a series of steps as earlier described in methods section 3.2.7.

To start the project of sequencing, pPRS3a was cloned into pET-28c(+) vector at EcoRI site. The pET-28c(+) was selected for cloning because it is having kanamycin resistance marker (pPRS3a already having ampicillin resistance marker) and T7 promoter region (for which universal primers are available). After the cloning obtained transformants were selected on LB agar plate containing a mixture of ampicillin & kanamycin. The true transformants were confirmed by gel-mobility shift assay and restriction endonuclease digestion with EcoRI. Upon digestion with EcoRI, the release of insert (pPRS3a) from the recombinant DNA molecule (pET-28c(+)-pPRS3a) was observed. The size of the pPRS3a & pET-28c(+) is 4.2kb and 5.3kb respectively. The fusion of both at EcoRI site was observed to be of the size 9.5kb, as shown in figure 17.

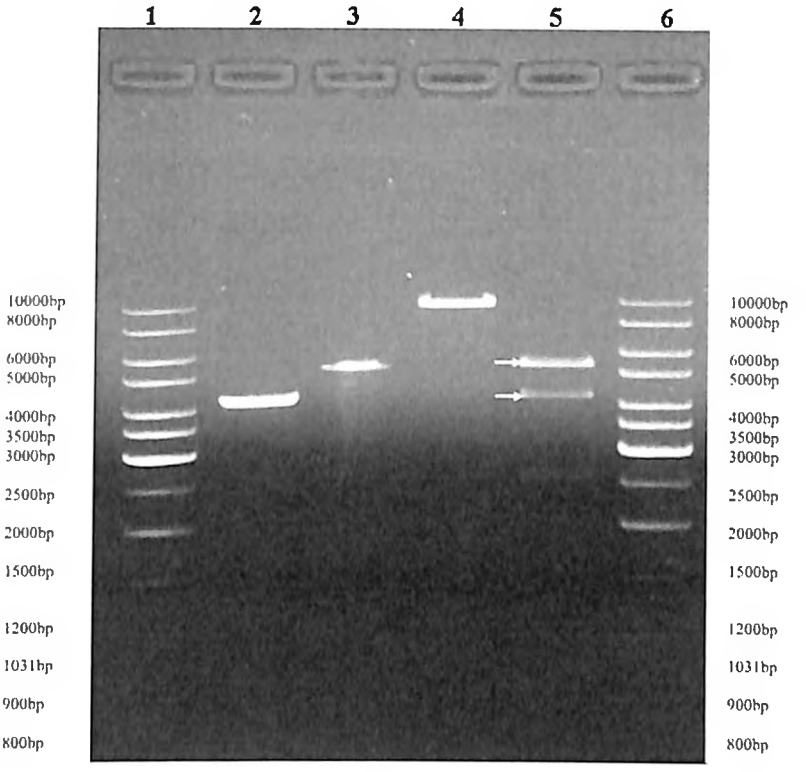


Fig. 17. Cloning of pPRS3a into vector pET-28c(+).

- lane 1, DNA marker;
- lane 2, pPRS3a plasmid DNA with EcoRI (4.2kb);
- lane 3, plasmid DNA vector pET-28c(+) digested with EcoRI (5.3kb);
- lane 4, pET-28c(+)-pPRS3a (9.5kb);
- lane 5, pET-28c(+)-pPRS3a digested with EcoRI (4.2kb, 5.3kb);
- lane 6, DNA marker.
- The DNA samples were separated on a 1.0% agarose gel.
- Arrow shows the vector & insert.

The ultra pure plasmid DNA was isolated by QIAGEN column and subjected to sequencing through primer walking. To start the sequencing, first set of primers (PET Fwd & PET Rev) on the basis of T7 promoter region of vector DNA, pET-28c(+) were used. Then in the subsequent steps, internal primers were designed based on the obtained sequence using previous primers. All the used primers for sequencing of pPRS3a have been listed in Table 17 & 18. The sequencing was carried out in both directions to get a good signal and to eliminate chance of errors.

Table 17. Reverse primers: Primers used for the sequencing of pPRS3a in the reverse direction along with their sequence and positions.

Name	Sequence	Coordinates
PET Rev	5' GCT AGT TAT TGC TCA GCG G 3'	-
Rev 1	5' TTA AGG GCC TCG TGA TAC 3'	335-352
Rev 2	5' AAC GTT TTC CAA TGA TGA GCA CTT 3'	716-739
Rev 3	5' GCA AAC TAT TAA CTG GCG AAC TAC 3'	1097-1120
Rev 4	5' GAG CGT CAG ACC CCG TAG AAA AGA 3'	1526-1549
Rev 5	5' CAC CGC CTA CAT ACC TCG CTC TG 3'	1758-1780
Rev 6	5' CTT GAG CGT CGA TTT TTG TGA TGC 3'	2102-2125
Rev 7	5' CGC AAC GCA ATT AAT GTG AG 3'	2429-2448
Rev 8	5' GAT CAA TCG TCA CCC TTT CTC G 3'	2629-2650
Rev 9	5' CGT CGG CCG TTT CCA TCT GC 3'	2939-2958
Rev 10	5' GGC TGC TGA ACC CCC AAC C 3'	3167-3185
Rev 11	5' ATG GTG AGC AAG GGC GAG GAG 3'	3460-3480
Rev 12	5' CAG CCG CTA CCC CGA CCA C 3'	3676-3694

Table 18. Forward primers: Primers used for the sequencing of pPRS3a in the forward direction along with their sequence and positions.

Name	Sequence	Coordinates
PET Fwd	5' TAA TAC GAC TCA CTA TAG GG 3'	
Fwd 1	5' CTC CTC GCC CTT GCT CAC 3'	647-664
Fwd 2	5' CCA TGC TGG CGG AGA ATC 3'	1017-1034
Fwd 3	5' GGC GGC ACC GTT GAA CAG 3'	1320-1337
Fwd 4	5' ACA AAA ATC GAC GCT CAA G 3'	2007-2025
Fwd 5	5' GCA GAG CGA GGT ATG TAG 3'	2346-2363
Fwd 6	5' TAA GGG ATT TTG GTC ATG AG 3'	2622-2641
Fwd 7	5' GGG AAG CTA GAG TAA GTA GTT CGC CA 3'	2992-3017
Fwd 8	5' TGT CAT GCC ATC CGT AAG 3'	3248-3265
Fwd 9	5' AAG TCA TTC TGA GAA TAG TG 3'	3297-3316
Fwd 10	5' AGA AAC CAT TAT TAT CAT GAC 3'	3734-3754

Each sequenced fragment was called as contig. After the completion of sequence all the obtained chromatograms were viewed and analyzed in Chromas Lite version 2.01 and true DNA sequences (A, T, G & C) were deduced using BioEdit version 7.0.5.2 (Hall, 1999). All the contigs (11 in Fwd direction and 13 in Rev direction) were aligned in such a way that a continuous sequence can be obtained with overlapping regions. This task was performed using bioinformatics tool as described in methods section 3.2.8. The assembly of contigs has been shown in figure 18.

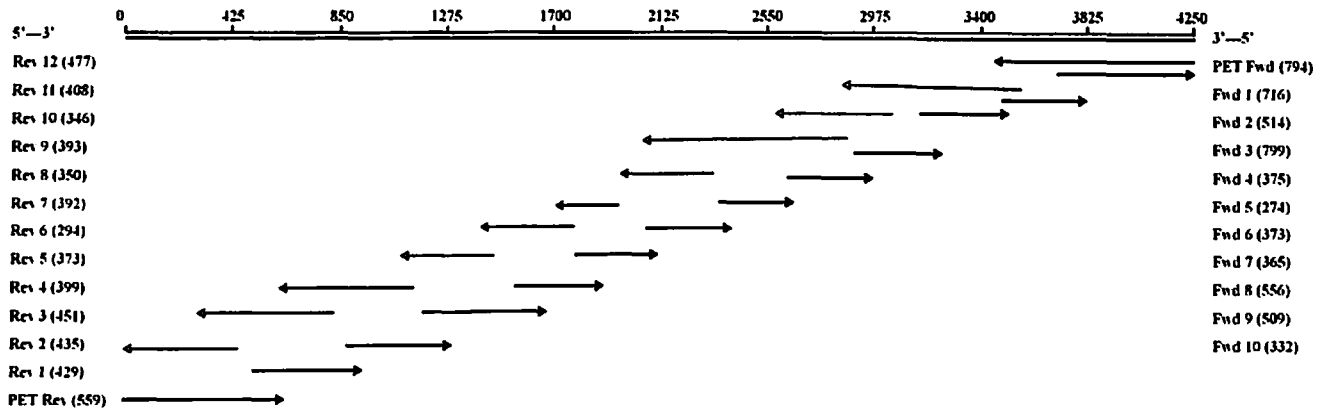


Fig. 18. Assembly of overlapping contigs to get a complete pPRS3a DNA sequence. For Fwd (3'-5') and Rev (5'-3') direction, red and black color arrows are depicted respectively. Each arrow showing approx length mentioned for respective contig.

3.3.4 pPRS3a sequence analysis

A complete sequence length of 4126bp was obtained and subjected to sequence analysis. The major analysis of this sequence includes, open reading frames (ORFs), promoters, origin of replication (ori) & restriction enzymes sites and minor analysis includes, direct & inverted repeats in ori region, % AT/GC rich region & sequence composition. The bioinformatic software and tools were used for analysis of pPRS3a as described in methods section 3.2.8. Analyses of each component have been discussed below.

3.3.4.1 Open reading frames (ORFs)

Upon analysis using NCBI ORF finder, four ORFs (cutoff 450NTs) were found. All ORFs with their putative proteins and accession no. have been shown in Table 19.

Table 19. Predicted open reading frames (ORFs): Open reading frames are shown with their reading frame, position, length of ORF, putative protein with its GenBank accession no.

Frame	Position (5'-3')	Length	G+C (%) contents	Protein	Accession No.	Reference
+1	532-1392	861	49.13	Beta-lactamase (*S. aureus*)	NP052129.1	Needham *et al.*,1995
-1	3485-4125	642	61.78	TRAP dicarboxylate transporter, DctM subunit (*Enterobacter* sp. 638)	YP001178580.1	unpublished
+2	3515-4125	612	61.37	pBI-SS (Tom) (TP) 101-EGFP (synthetic construct)	ABG78037.1	Estévez *et al.*, 2006
-2	2842-3309	468	61.54	Tetracycline resistance repressor protein tetR (m MN_gF3SD01_16)	ACT97616.1	Sommer *et al.*, 2009

The 100% identity of Beta-lactamase gene (*bla*) from *Staphylococcus aureus* confirmed that property of ampicillin resistance conferred by the *Bacillus cereus* GC subgroup A is due to the plasmid pPRS3a. In addition to *bla* gene, three ORFs of putative DNA homologs to TRAP dicarboxylate transporter, DctM subunit (*Enterobacter* sp. 638), pBI-SS (Tom) (TP) 101-EGFP (synthetic construct), tetracycline resistance repressor protein *tetR* (m MN_gF3SD01_16) were found on pPRS3a and none of them code for any functional protein. The presence of these truncated fragments could be due to the horizontal gene transfer from the diverse bacterial community at the site (Jain *et al.*, 2009). Such horizontal gene transfer mechanisms are common at polluted sites in the environment (Frost *et al.*, 2005, Berde *et al.*, 2010). The transfer of these resistance determinants might have occurred in environmental conditions among the organisms that share the same environmental habitat. Antibiotic resistance determinants constitute a privileged system for the study of naturally occurring DNA transfer among bacteria, because they are easy to trace and because of the massive selective pressure exerted by antibiotic used in clinical practices. The finding of inconsistencies in molecular data led to the notion of horizontal gene flux between these distantly related microorganisms (Courvalin, 1994). Plasmids exhibit diverse mechanisms that enable their survival and horizontal transfer, to the extent that, with the advent of more

sophisticated detection techniques, the distinction between chromosome and plasmid has become 'blurred' (Sedgley et al. 2004 and Thomas, 2004).

3.3.4.2 Promoters

For the prediction of promoters in the obtained sequence of pPRS3a, BPROM and NNPP version 2.2 were used as described in methods section 3.2.8.2. Promoter prediction was done on both the strands with the help of BPROM and NNPP version 2.2. A total of 19 & 15 promoters were predicted in forward and reverse directions respectively as shown in Table 20 & 21.

Table 20. Promoters: promoter predictions for pPRS3a in forward direction (5'-3') with score cutoff 0.80.

Start	End	Score	Promoter sequence (5'-3')
257	302	0.88	ATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGT
283	328	0.88	GTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGGC
327	372	0.91	GGCGGCCTGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCA
340	385	0.90	GGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGT
352	397	0.83	CGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTC
456	501	0.82	AATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGC
477	522	0.85	GCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGA
735	780	0.99	CACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCG
1387	1432	0.94	TGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAA
1404	1449	0.81	AGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTA
1423	1468	0.94	TAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGAT
1438	1483	0.95	CTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCT
1475	1520	0.85	TTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCAC
1557	1602	0.92	ATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAA
1630	1675	0.92	GTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGG
1830	1875	0.89	CCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGC
2471	2516	0.97	CAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAG
2526	2571	0.92	AACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCT
3280	3325	0.95	CATGCTTGACACTTTATCACTGATAAACATAATATGTCCACCAACTTATC

Table 21. Promoters: promoter predictions for pPRS3a in reverse direction (5'-3') with score cutoff 0.80.

Start	End	Score	Promoter sequence (5'-3')
3951	3906	0.90	AGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTG
3356	3311	0.87	GGTCCGATTGAACGCGCGGATTCTTTATCACTGATAAGTTGGTGGACATA
3342	3297	0.80	CGCGGATTCTTTATCACTGATAAGTTGGTGGACATATTATGTTTATCAGT
3324	3279	0.95	GATAAGTTGGTGGACATATTATGTTTATCAGTGATAAAGTGTCAAGCATG
1751	1706	0.96	TACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGTAACA
1502	1457	0.95	GTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC
1453	1408	0.95	CCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
1438	1393	0.98	ATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACA
951	906	0.89	CCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGT
922	877	0.83	CGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTG
515	470	0.97	TTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATAC
495	450	0.90	TATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAA
410	365	0.91	GAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACC
380	335	0.83	TTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTT
291	246	0.90	GCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGAT

Among these predicted promoters, one promoter was found to be functional in pPRS3a as it is located just upstream of ORF. For with score of 0.85. The presence of cis-acting regulatory DNA elements for bla gene promoter (GAGA, ACGG, GATA, AAAG, ACGT and GACGTC) confirmed functional significance of this promoters.

The functional promoter of β-lactamase (bla) gene, is located at 477-522 region. As this plasmid is of natural origin, then it is significant to have the similarity with other natural strains especially at the functional promoter region. The similarities with other natural strains have been shown in Table 22.

Table 22. Similarity of β-lactamase (bla) gene promoter with other natural strains.

Position	Organism & plasmid name	Size (bp)	% similarity	Reference
515-470	*Klebsiella oxytoca* plasmid pFP10-1	6964	100	HQ651092.1
515-470 & 477-522	*Baculovirus* shuttle vector pBacHTS	6205	100	JN029539.1
515-470 & 477-522	*Proteus mirabilis* strain 7001324 plasmid beta-lactamase TEM variant gene, complete cds.	1069	100	HM246246.1
515-470 & 477-522	*Enterobacter* sp. W001 plasmid pR23	10497	100	JF703130.1
477-522	Plasmid pGEX-5G/LIC expression vector(*E. coli/S. japonicium*) encoding glutathione S-transferase gene, complete cds.	4968	100	M97937.1
477-522	*Bacillus intermedius* plasmid pET3xa-barn36, gene encoding barnase	4753	100	AJ006407.1

3.3.4.3 Origin of replication (Ori)

Origin of replication is the most important element of any plasmid DNA molecule, as it governs the segregation ability of a plasmid DNA. In pPRS3a, ori was predicted by ORI finder as described in methods section 3.2.8.3. This region was 669bp long and located at 1540-2208 as shown in figure 19. Ideally the base numbers of a plasmid DNA should start from ori. In our studies for pPRS3a, the numbers have started from the cloning site (at EcoRI) because it's the first base that was sequenced by primer walking. This predicted ori is found to have 100% similarity with Shuttle vector pMK4 (EU549778.1, 5585bp, *Bacillus subtilis-E. coli* shuttle vector) and Shuttle vector pLES003 (AB370338.1, 6134bp, *Lactobacillus brevis-E. coli* shuttle vector).

Upon detailed analysis of ori, direct & inverted repeats were also been found as shown in Table 23. The presence of the repeats is the characteristic feature of an origin of replication (Romero et al. 2007).

```
1       GTAGAAAAGA TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG
        CATCTTTTCT AGTTTCCTAG AAGAACTCTA GGAAAAAAAG ACGCGCATTA GACGACGAAC
61      CAAACAAAAA AACCACCGCT ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT
        GTTTGTTTTT TTGGTGGCGA TGGTCGCCAC CAAACAAACG GCCTAGTTCT CGATGGTTGA
121     CTTTTTCCGA AGGTAACTGG CTTCAGCAGA GCGCAGATAC CAAATACTGT TACTTCTAGT
        GAAAAAGGCT TCCATTGACC GAAGTCGTCT CGCGTCTATG GTTTATGACA ATGAAGATCA
181     GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT ACCTCGCTCT
        CATCGGCATC AATCCGGTGG TGAAGTTCTT GAGACATCGT GGCGGATGTA TGGAGCGAGA
241     GCTAATCCTG TTACCAGTGG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA
        CGATTAGGAC AATGGTCACC GACGACGGTC ACCGCTATTC AGCACAGAAT GGCCCAACCT
301     CTCAAGACGA TAGTTACCGG ATAAGGCGCA GCGGTCGGGC TGAACGGGGG GTTCGTGCAC
        GAGTTCTGCT ATCAATGGCC TATTCCGCGT CGCCAGCCCG ACTTGCCCCC CAAGCACGTG
361     ACAGCCCAGC TTGGAGCGAA CGACCTACAC CGAACTGAGA TACCTACAGC GTGAGCTATG
        TGTCGGGTCG AACCTCGCTT GCTGGATGTG GCTTGACTCT ATGGATGTCG CACTCGATAC
421     AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA GGCGGACAGG TATCCGGTAA GCGGCAGGGT
        TCTTTCGCGG TGCGAAGGGC TTCCCTCTTT CCGCCTGTCC ATAGGCCATT CGCCGTCCCA
481     CGGAACAGGA GAGCGCACGA GGGAGCTTCC AGGGGGAAAC GCCTGGTATC TTTATAGTCC
        GCCTTGTCCT CTCGCGTGCT CCCTCGAAGG TCCCCCTTTG CGGACCATAG AAATATCAGG
541     TGTCGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTTG TGATGCTCGT CAGGGGGGCG
        ACAGCCCAAA GCGGTGGAGA CTGAACTCGC AGCTAAAAAC ACTACGAGCA GTCCCCCCGC
601     GAGCCTATGG AAAAACGCCA GCAACGCGGC CTTTTTACGG TTCCTGGCCT TTTGCTGGCC
        CTCGGATACC TTTTTGCGGT CGTTGCGCCG GAAAAATGCC AAGGACCGGA AAACGACCGG
661     TTTTGCTCA
        AAAACGAGT
```

Fig. 19. Origin or replication of pPRS3a: Complete ori of 669bp along with its complementary sequence.

Table 23. Inverted repeats & Direct repeats in ori: Inverted and Direct repeats in the origin of replication with their position and respective sequence.

Inverted repeats				Direct repeats			
Size (bp)	Pos1	Pos2	Repeat Sequence	Size (bp)	Pos1	Pos2	Repeat Sequence
9	13	27	AAAGGATCT	8	148	490	AGAGCGCA
9	60	92	GCAAACAAA	8	254	267	CCAGTGGC
11	70	84	AAACCACCGCT	8	628	657	GGCCTTTT
9	145	235	AGCAGAGCG	8	628	646	GGCCTTTT
8	155	524	AGATACCA	13	644	655	CTGGCCTTTTGCT
8	287	464	CTTACCGG				
10	314	461	TTACCGGATA				
8	617	652	GCCAGCAA				

Replication origins of several well studied bacterial plasmids have array of direct and inverted repeats called iterons, which are sites for binding a plasmid-specific replication initiator protein. The iteron-initiator protein interactions are essential for plasmid replication as well as for inhibition of plasmid over-replication (Chattoraj, 2000). Due to the presence

of ori which is recognized by both Gram's negative/positive bacterial system, this study suggests that pPRS3a can be used as a stable cloning shuttle vector between *Bacillus cereus* and *E. coli*.

3.3.4.4 Restriction endonuclease enzymes sites

The sites on the DNA for the enzyme that are very specific in their recognition, methylation and cleavage are called as restriction enzyme sites. The search for restriction sites was done by BioEdit version 7.0.5.2 as described in methods section 3.2.8.4. In the pPRS3a, >200 sites for restriction enzymes were found. Later on the search was confined to the restrictions sites for >5bp cutters and 30 unique restriction sites and a cluster located in pPRS3a were obtained, as shown in Table 24.

Table 24. Restriction Enzyme sites on pPRS3a: A total of 30 enzymes listed which are having unique restriction enzymes.

S. No.	Enzyme	Recognition (5'-3')	Position	S. No.	Enzyme	Recognition (5'-3')	Position
1	EcoRI	G'AATT_C	2	16	PvuII	CAG'CTG	2389
2	StuI	AGG'CCT	62	17	HindIII	A'AGCT_T	2568
3	SpeI	A'CTAG_T	66	18	EcoICRI	GAG'CTC	2624
4	BsiWI	C'GTAC_G	76	19	SacI	G_AGCT'C	2626
5	PspOMI	G'GGCC_C	81	20	BclI	T'GATC_A	2629
6	ApaI	G_GGCC'C	85	21	BmgBI	CAC'GTC	3388
7	NdeI	CA'TA_TG	277	22	SalI	G'TCGA_C	3424
8	ZraI	GAC'GTC	399	23	XbaI	T'CTAG_A	3430
9	AatII	G_ACGT'C	401	24	BamHI	G'GATC_C	3436
10	SspI	AAT'ATT	515	25	XmaI	C'CCGG_G	3441
11	ScaI	AGT'ACT	839	26	SmaI	CCC'GGG	3443
12	PvuI	CG_AT'CG	951	27	Acc65I	G'GTAC_C	3445
13	FspI	TGC'GCA	1097	28	AgeI	A'CCGG_T	3448
14	BseYI	C'CCAG_C	1905	29	KpnI	G_GTAC'C	3449
15	PciI	A'CATG_T	2209	30	NcoI	C'CATG_G	3459

Apart from the major analysis, some minor analyses were also done. A MAT-LAB based program was used to find out AT/GC rich region (Baker and Rogers, 2005). AT rich region is located at 498-548 & 1396-1485 with 76.5% & 76% AT contents respectively (cutoff for searching was 75%). Similarly GC rich region is located at 2761-3048 with ~70% GC content in this region (cutoff for searching was 70%). Total base compositions of the

pPRS3a have been shown in figure 20. This plasmid DNA is having 54.34% GC contents. The presence of high GC contents is the characteristic feature of any plasmid DNA of a prokaryotic origin. This observation once again strengthens its characterization as plasmid of bacterial origin.

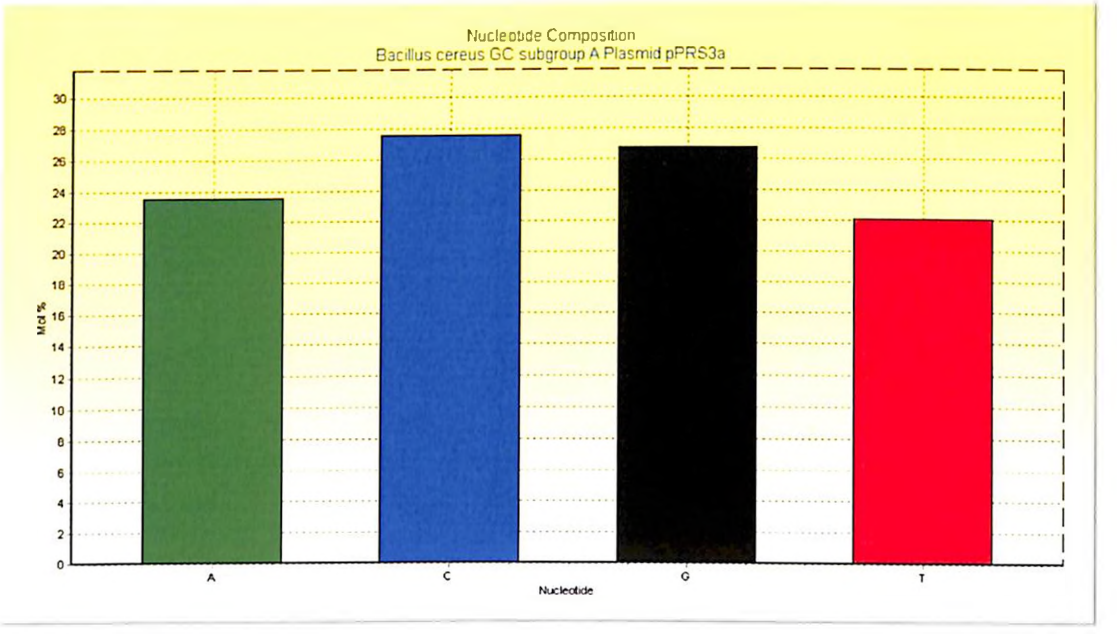


Fig. 20. Nucleotide composition of pPRS3a: Total G+C content=54.34% & A+T content=45.66%. A=973 (23.58%), C=1139 (27.61%), G=1103 (26.73%) and T=911 (22.08%).

After the complete analysis of pPRS3a, the plasmid DNA sequences were submitted to NCBI GenBank under the accession no. GQ404376.1 (appendix-IV). Based on the information, a map was drawn using BioEdit version 7.0.5.2, as shown in figure 21.

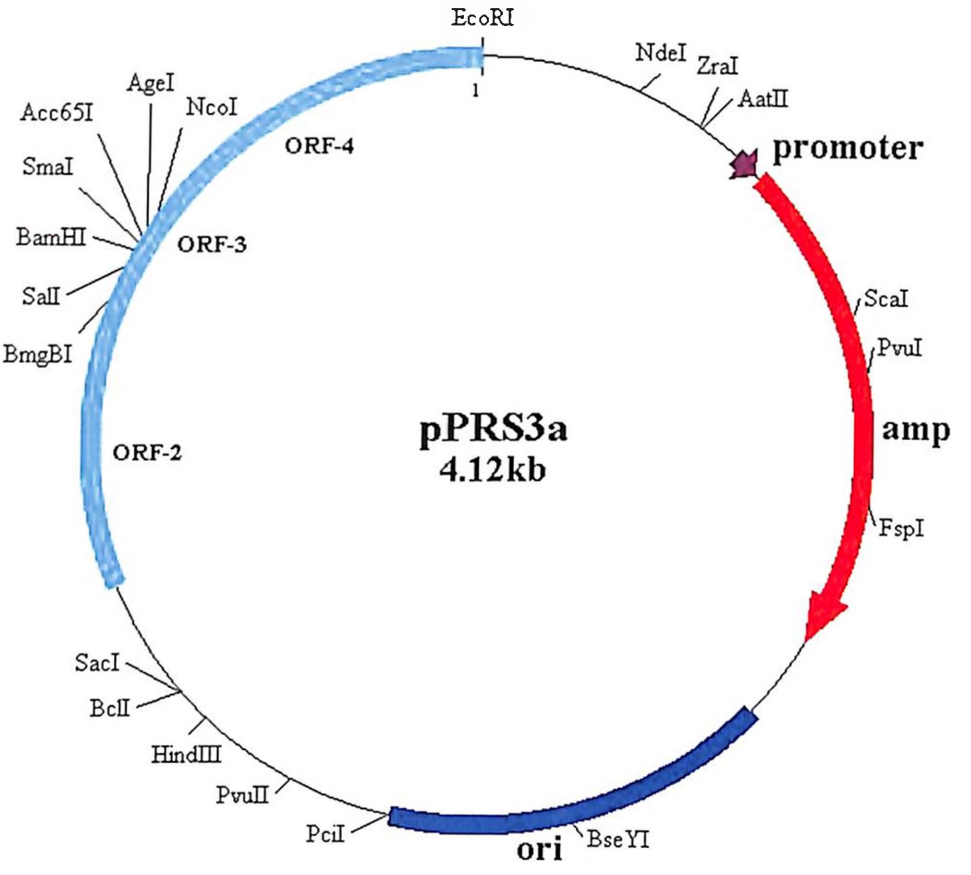


Fig. 21. pPRS3a plasmid DNA map (4.12kb): Map showing ORFs, Promoters, Origin of replication and Restriction enzyme sites.

The complete analysis of pPRS3a includes ORFs, promoters, ori, restriction sites, base composition etc. Based on this analysis some general conclusions can be made like, presence of a functional ampicillin resistance gene, number of strong promoters and origin of replication (recognized by both Gram's positive/negative bacterial systems). Based on the presence of variety of components, we can say that this plasmid may be useful in cloning and expression of foreign genes for industrial application.

As the pPRS3a is of natural origin, therefore similarity with other naturally occurring plasmid can be supportive for its natural origin. To confirm this each fragment of pPRS3a (β-lactamase (bla) gene, origin of replication and backbone) have been compared with other natural plasmids. The comparison and similarity have been shown in Table 25, 26 and 27.

Table 25. Simialrity of beta lactamse region of pPRS3a with other natural plasmids.

Position	Organism, plasmid name and source	Size	% similarity	Reference
335-2564	*Staphylococcus aureus* plasmid J3356::pOX7;3	8007	100	NC_002157.1
335-1539	*Enterobacter cloacae* plasmid pEC-IMP	318782	100	NC_012555.1
335-1540	*Escherichia coli* SMS-3-5 plasmid pSMS35_130: multidrug-resistant environmental isolate	130440	100	NC_010488.1
335-1539	*Klebsiella pneumoniae* plasmid pCTXM360	68018	100	NC_011641.1
335-1539	*Neisseria gonorrhoeae* plasmid pSJ5.2	5161	100	NC_010881.1
335-1539	*Providencia rettgeri* plasmid R7K	39792	100	NC_010643.1
340-1539	*Citrobacter freundii* plasmid pCTX-M3	89468	100	NC_004464.2
335-1539	*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SC-B67 plasmid pSC138	138742	100	NC_006856.1
706-1259	*Yersinia pestis* biovar Orientalis str. IP275 plasmid pIP1202	182913	64	NC_009141.1

Table 26. Simialrity of origin of replication region of pPRS3a with other natural plasmids.

Position	Organism, plasmid name and source	Size	% similarity	Reference
335-2564	*Staphylococcus aureus* plasmid J3356::pOX7;3	8007	100	NC_002157.1
1516-2334	*Pseudomonas fulva* IF-4 plasmid pNI10	5128	100	NC_004951.1
1540-2009	*Klebsiella pneumoniae* plasmid 15S: Isolated in a NYC Hospital	23753	70	NC_011382.1
1552-2339	*Klebsiella pneumoniae* 2kI plasmid pKPN2	4196	98	NC_005018.1
1540-2336	*Yersinia pestis* Antiqua plasmid pPCP	10777	100	NC_008121.1
1552-2332	*Endophytic bacterium* LOB-07 plasmid pLK39: isolated from *Solanum lycocarpum*	4029	98	NC_013090.1
1698-2339	*Citrobacter rodentium* DBS100 plasmid pCRP3	3172	76	NC_003114.1
1671-2175	*Pantoea citrea* 1056R plasmid pUCD5000	5229	75	NC_001898.1
1809-2337	*Salmonella enterica* subsp. enterica serovar Virchow str. SL491 plasmid pSL491_5	5880	60	NC_011214.1

Table 27. Simialrity of backbone region of pPRS3a with other natural plasmids.

Position	Organism, plasmid name and source	Size	% similarity	Reference
335-2564	*Staphylococcus aureus* plasmid J3356::pOX7;3	8007	54	NC_002157.1
2575-3422	*Edwardsiella tarda* EIB202 plasmid pEIB202	43703	20	NC_013509.1
2575-3422	*Escherichia coli* plasmid pFL129	6464	20	NC_005923.1
2575-3422	*Klebsiella pneumoniae* plasmid pKP96	67850	20	NC_011617.1
2575-3422	*Laribacter hongkongensis* plasmid pHLHK22	15665	20	NC_010370.1
2575-3422	*Pseudomonas aeruginosa* plasmid pBS228	89147	20	NC_008357.1
2575-3422	*Salmonella enterica* subsp. enterica serovar Choleraesuis plasmid pOU7519	127212	20	NC_010119.1
2575-3422	*Aeromonas punctata* HGB5 plasmid pFBAOT6	84749	20	NC_006143.1
2575-3422	*Shigella sonnei* Ss046 plasmid pSS046_spA	8401	20	NC_009345.1
2575-3422	Activated sludge plasmid pRSB101: isolated from a wastewater treatment plant	47829	20	NC_006385.1

The overall similarity of pPRS3a with other naturally occurring plasmids, with respect to percentage similarity, their size and NCBI accession numbers, have also been demonstrated and shown in Table 28.

To the best our knowledge, till date, no plasmid from *Bacillus cereus* has been reported with the properties similar to pPRS3a. Complete identification and characterization of pPRS3a from *Bacillus cereus* GC subgroup A confirms the presence of a plasmid DNA of size 4.2kb that has not been reported so far. The complete specifications of all reported plasmid DNA from *Bacillus cereus* till date have been shown in Table 29.

Table 28. Similarity of *Bacillus cereus* GC subgroup A plasmid DNA (pPRS3a) with other naturally occurring plasmids.

% similarity	Organism, plasmid name and source	Size	Reference
61	*Staphylococcus aureus* plasmid J3356::pOX7;3	8007	NC_002157.1
60	*Salmonella enterica* subsp. enterica serovar Choleraesuis plasmid pOU7519	127212	NC_010119.1
53	*Pseudomonas aeruginosa* plasmid pBS228	89147	NC_008357.1
53	*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SC-B67 plasmid pSC138	138742	NC_006856.1
52	*Escherichia coli* SMS-3-5 plasmid pSMS35_130: multidrug-resistant environmental isolate	130440	NC_010488.1
40	*Shigella sonnei* Ss046 plasmid pSS046_spA	8401	NC_009345.1
37	*Enterobacter cloacae* plasmid pEC-IMP	318782	NC_012555.1
33	*Providencia rettgeri* plasmid R7K	39792	NC_010643.1
31	*Citrobacter freundii* plasmid pCTX-M3	89468	NC_004464.2
30	*Edwardsiella tarda* EIB202 plasmid pEIB202	43703	NC_013509.1
30	*Klebsiella pneumoniae* plasmid pCTXM360	68018	NC_011641.1
30	*Neisseria gonorrhoeae* plasmid pSJ5.2	5161	NC_010881.1
29	*Klebsiella pneumoniae* plasmid 15S: Isolated in a NYC Hospital	23753	NC_011382.1
29	*Yersinia pestis* Angola plasmid pMT-pPCP	114570	NC_010158.1
28	*Pseudomonas fulva* IF-4 plasmid pNI10	5128	NC_004951.1
28	*Salmonella enteritidis* plasmid pC	5269	NC_003457.1
27	*Escherichia coli* plasmid pFL129	6464	NC_005923.1
27	*Shigella sonnei* colicin type 7 plasmid ColJs	5210	NC_002809.1
25	*Klebsiella pneumoniae* 2kI plasmid pKPN2	4196	NC_005018.1
25	*Salmonella enterica* subsp. enterica serovar Virchow str. SL491plasmid pSL491_5	5880	NC_011214.1
25	*Yersinia pestis* Antiqua plasmid pPCP	10777	NC_008121.1
25	*Yersinia pestis* biovar Microtus str. 91001 plasmid pPCP1	9609	NC_005816.1
25	Activated sludge plasmid pRSB101: isolated from a wastewater treatment plant	47829	NC_006385.1
23	*Aeromonas punctata* HGB5 plasmid pFBAOT6	84749	NC_006143.1
23	*Klebsiella pneumoniae* plasmid pKP96	67850	NC_011617.1
22	*Laribacter hongkongensis* plasmid pHLHK22	15665	NC_010370.1
22	Endophytic bacterium LOB-07 plasmid pLK39: isolated from *Solanum lycocarpum*	4029	NC_013090.1
22	*Yersinia pestis* biovar Orientalis str. IP275 plasmid pIP1202	182913	NC_009141.1
18	*Citrobacter rodentium* DBS100 plasmid pCRP3	3172	NC_003114.1
18	*Escherichia coli* plasmid pBHRK19	5721	NC_005569.1
18	*Neisseria gonorrhoeae* plasmid pCmGFP	6062	NC_011521.1
16	*Pantoea citrea* 1056R plasmid pUCD5000	5229	NC_001898.1

Table 29. *Bacillus cereus* plasmids DNA: A list of *Bacillus cereus* plasmids repository from NCBI GenBank database, excluding pPRS3a.

Organism	Name	Accession	Length (bp)	Proteins	Genes	Create date
Bacillus cereus	pBC210	NC_010933	209385	201	201	Jun 11 2008
Bacillus cereus	pBCXO1	NC_010934	190861	174	186	Jun 11 2008
Bacillus cereus	pCER270	NC_010924	270082	235	250	Jun 11 2008
Bacillus cereus	pPER272	NC_010916	272145	266	271	Jun 11 2008
Bacillus cereus	pPER272	NC_010921	272145	264	271	Jun 11 2008
Bacillus cereus 03BB102	p03BB102_179	NC_012473	179680	208	209	Mar 30 2009
Bacillus cereus AH187	pAH187_12	NC_011654	12481	22	23	Dec 11 2008
Bacillus cereus AH187	pAH187_270	NC_011655	270082	273	277	Dec 15 2008
Bacillus cereus AH187	pAH187_3	NC_011657	3091	5	5	Dec 11 2008
Bacillus cereus AH187	pAH187_45	NC_011656	45173	59	60	Dec 11 2008
Bacillus cereus AH820	pAH820_10	NC_011771	10915	11	11	Dec 28 2008
Bacillus cereus AH820	pAH820_272	NC_011777	272145	320	321	Dec 28 2008
Bacillus cereus AH820	pAH820_3	NC_011776	3091	5	5	Dec 28 2008
Bacillus cereus ATCC 10987	pBc10987	NC_005707	208369	241	242	Feb 27 2004
Bacillus cereus ATCC 14579	pBClin15	NC_004721	15274	21	21	Apr 16 2003
Bacillus cereus E33L	pE33L466	NC_007103	466370	430	447	Jun 9 2005
Bacillus cereus E33L	pE33L5	NC_007104	5108	5	5	Jun 9 2005
Bacillus cereus E33L	pE33L54	NC_007105	53501	54	57	Jun 9 2005
Bacillus cereus E33L	pE33L8	NC_007106	8191	8	8	Jun 9 2005
Bacillus cereus E33L	pE33L9	NC_007107	9150	10	10	Jun 9 2005
Bacillus cereus G9842	pG9842_140	NC_011774	140001	118	120	Dec 28 2008
Bacillus cereus G9842	pG9842_209	NC_011775	209488	251	251	Dec 28 2008
Bacillus cereus H3081.97	pH308197_10	NC_011341	10077	12	12	Oct 6 2008
Bacillus cereus H3081.97	pH308197_11	NC_011340	11567	16	16	Oct 6 2008
Bacillus cereus H3081.97	pH308197_258	NC_011339	258484	257	257	Oct 6 2008
Bacillus cereus H3081.97	pH308197_29	NC_011342	29189	40	40	Oct 6 2008
Bacillus cereus H3081.97	pH308197_3	NC_011338	3424	5	5	Oct 6 2008
Bacillus cereus H3081.97	pH308197_73	NC_011337	72792	101	101	Oct 6 2008
Bacillus cereus Q1	pBc239	NC_011973	239246	228	228	Jan 27 2009
Bacillus cereus Q1	pBc53	NC_011971	52766	69	69	Jan 24 2009

Further to strengthen the cloning and expression characteristics of pPRS3a, two foreign genes were cloned and expressed successfully.

3.3.5 Cloning of arsGFP and cat genes into pPRS3a

The characterization of pPRS3a was carried out by cloning and expression of arsGFP (arsenic biosensor) & cat (chloramphenicol resistance) genes. Both the genes/cassette was taken from different sources. The source for arsGFP was an arsenic biosensor (pJSKV51, developed in our lab) and cat gene was taken from pKT-210, a shuttle vector (Ren et al. 1998). Upon induction of arsGFP by arsenite molecule, it codes for a functional enhanced green fluorescent protein due to the presence of EGFP gene downstream to regulatory region of arsenic operon. Similarly, upon induction of cat gene it codes for chloramphenicol acetyl transferase. Chloramphenicol acetyltransferase (cat) is a bacterial enzyme that detoxifies the antibiotic chloramphenicol and is responsible for chloramphenicol resistance in bacteria. This enzyme covalently attaches an acetyl group from acetyl-CoA to chloramphenicol, which prevents chloramphenicol from binding to ribosomes.

The strategy for cloning of arsGFP & cat genes into pPRS3a have been depicted in figure 9 & 10. This procedure was carried out as described in methods section 3.2.10. In the first step, arsGFP was cloned into pPRS3a at PvuII site. The S1 nuclease treatment of the insert (digested with PvuII & EcoRI) facilitated the ligation. After construction of pPRS3a-arsGFP, the cat gene was cloned at PvuII site, as shown in figure 22. The ExonucleaseIII treatment of insert (digested with PstI & HindIII) facilitated the ligation. Essentially, these two special enzymes S1 nuclease & ExonucleaseIII were used in cloning experiment to make the insert blunt ended. S1 nuclease & ExonucleaseIII are very specific for the degradation of single stranded DNA and removal of nucleotides from 5' overhangs and 3' overhangs respectively.

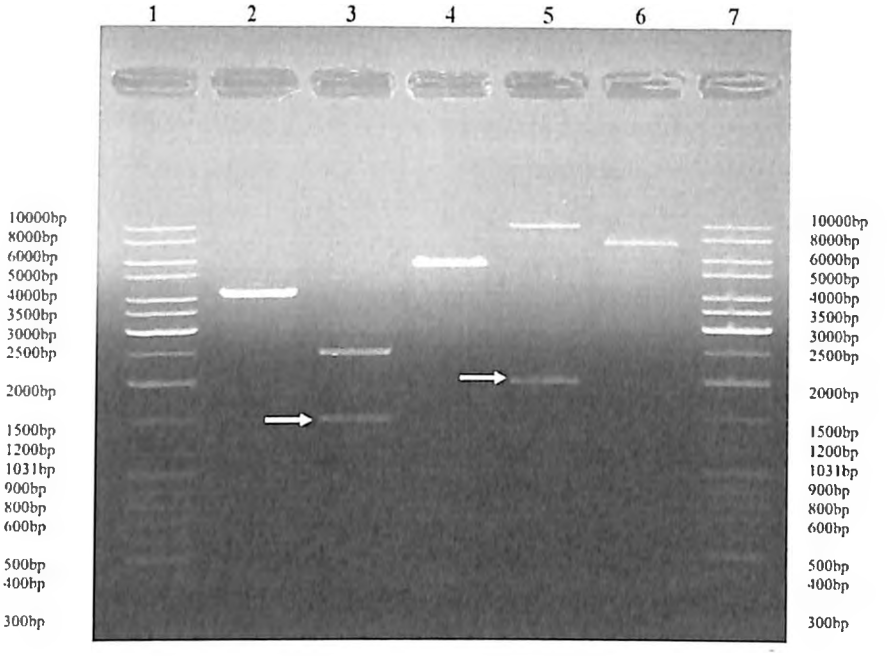


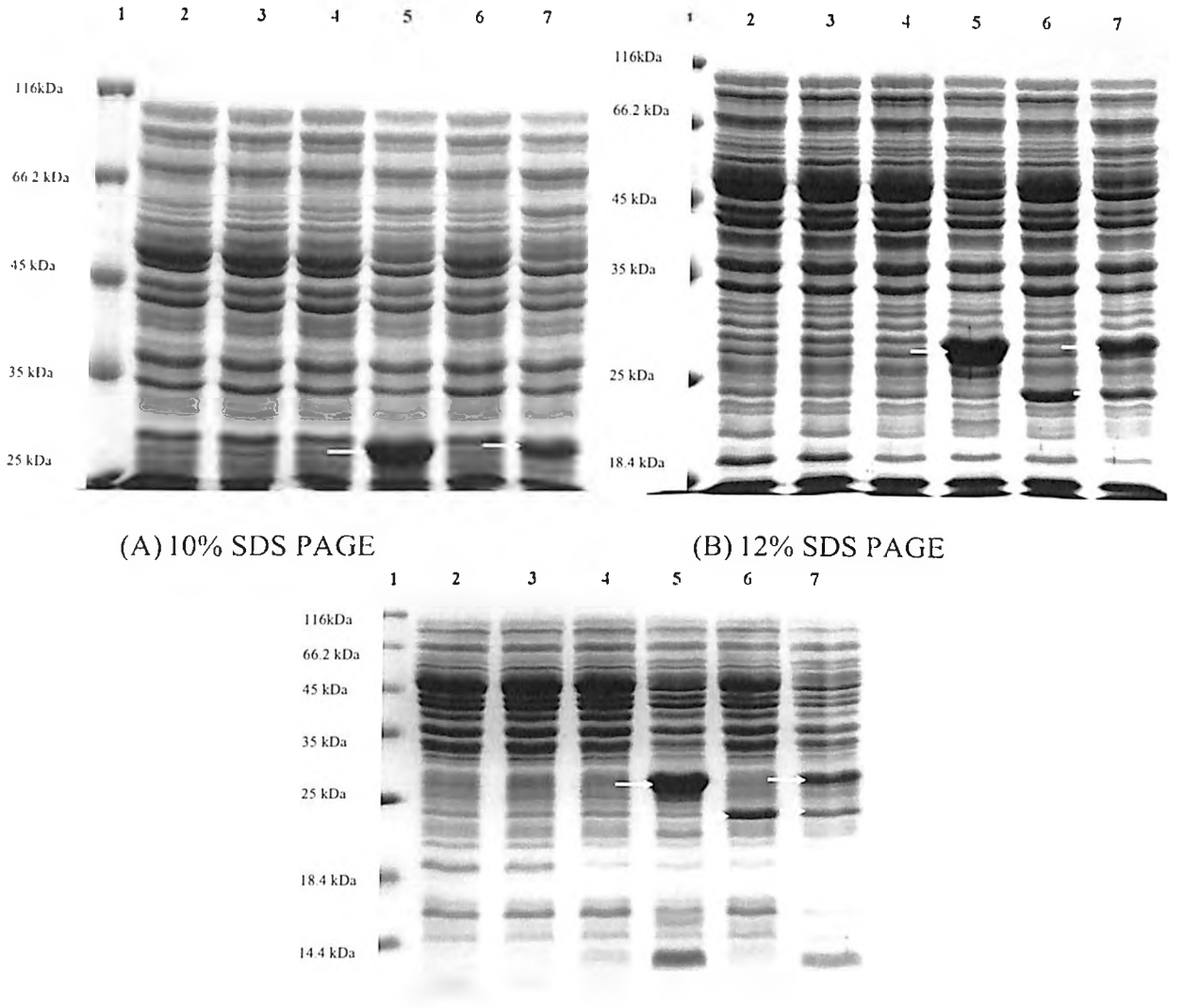
Fig. 22. Cloning of arsGFP and cat genes into plasmid pPRS3a.

- lane 1, DNA marker;
- lane 2, pPRS3a digested with EcoRI (4.2kb);
- lane 3, pJSKV51 digested with EcoRI & PvuII (1.5kb, 2.4kb);
- lane 4, pPRS3a-arsGFP (5.7kb);
- lane 5, pKT-210 digested with HindIII & PstI (2.1kb, 9.7kb);
- lane 6, pPRS3a-arsGFP-cat (7.8kb);
- lane 7, DNA marker.
- The DNA samples were separated on a 1.0% agarose gel.
- Arrow shows the insert for subsequent cloning.

Once the final construct of pPRS3a-arsGFP-cat was ready, the expression of both the genes was tested. The qualitative and quantitative measures were taken in consideration to assay the gene product as described in methods section 3.2.11.

3.3.6 Expression of arsGFP and cat genes

The total cell lysate of recombinant *E. coli* DH5α carrying pPRS3a-arsGFP-cat construct was used for SDS-PAGE analysis. The recombinant strain was grown in the different set of conditions like, LB supplemented with ampicillin, ampicillin & arsenite, ampicillin & chloramphenicol and ampicillin, arsenite & chloramphenicol. This cell lysate was run on different percentages of gels (10%, 12% and 15%) to visualize wide range of separation of proteins of different molecular mass. The gel images for all percentages have been shown in figure 23.



(A) 10% SDS PAGE (B) 12% SDS PAGE

(C) 15% SDS PAGE

Fig. 23. SDS PAGE of the cell lysate of *E. coli* DH5α carrying pPRS3a-arsGFP-cat at different percentages of protein gel.

- Lane 1, Protein molecular weight marker;
- lane 2, *E. coli* DH5α (~80μg of total protein);
- lane 3, *E. coli* DH5α with pPRS3a grown in ampicillin (50μg/ml);
- lane 4, *E. coli* DH5α with pPRS3a-arsGFP grown in ampicillin (50μg/ml);
- lane 5, *E. coli* DH5α with pPRS3a-arsGFP grown in ampicillin (50μg/ml) and supplemented with arsenite (500ppb);
- lane 6, *E. coli* DH5α with pPRS3a-arsGFP-cat grown in ampicillin (50μg/ml) and chloramphenicol (30μg/ml);
- lane 7, *E. coli* DH5α with pPRS3a-arsGFP-cat grown in ampicillin (50μg/ml), chloramphenicol (30μg/ml) and supplemented with arsenite (500ppb).
- Arrow shows the expression of EGFP (~31kD) and cat (~24kD) gene.
- Samples were electrophoresed on a (A) 10%, (B) 12% and (C) 15% polyacrylamide gel and stained with Coomassie brilliant blue G-250.

A protein band of ~31kDa from arsGFP and ~24kDa from cat gene was observed. The cloning and expression of foreign genes tells that pPRS3a have the characteristic feature to clone and express foreign genes of different origin.

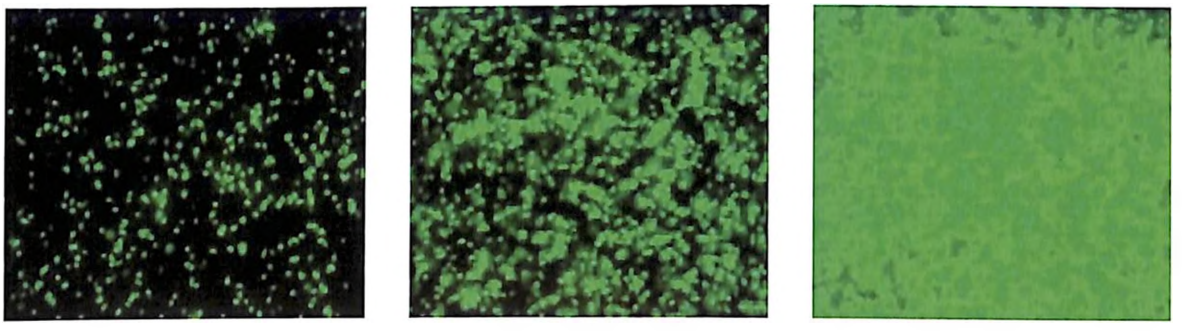
The expression of GFP was also observed by using fluorescent microscopy. The recombinant *E. coli* DH5α carrying pPRS3a-arsGFP-cat was grown in different concentration of arsenite as described in methods section 3.2.11.2. The images of obtained fluorescence were captured and have been shown in figure 23. The fluorescence obtained at 0ppb was considered as basal level expression of arsGFP. The amount of fluorescence increased with increase in the concentration of arsenite from 50ppb to 500ppb and decreased above 500ppb as illustrated by figure 24. This observation suggests the sensitivity of arsGFP to arsenite (an inducer molecule). After 500ppb, the fluorescence amount decreased because the concentration becomes toxic to the cell and higher amount of arsenite inhibits the expression of GFP. This observation was again confirmed by the quantitative method of fluorescence detection. Here we have used Wallac Victor^3 Multilabel counter as described in methods section 3.2.11.3. The fluorescence counts were found to be increased with the increasing concentration of arsenite and maxima was obtained at 500ppb. Similarly, after 500ppb there was a decrease in fluorescence counts, as shown in figure 25.

Thus all three ways of assaying the expression of gene confirmed the ability of pPRS3a plasmid DNA to clone and express the genes of foreign origin.

A. EGFP induction by 0ppb arsenite (400x)

B. EGFP induction by 50ppb arsenite (400x)

C. EGFP induction by 100ppb arsenite (400x)



D. EGFP induction by 200ppb arsenite (400x)

E. EGFP induction by 300ppb arsenite (400x)

F. EGFP induction by 400ppb arsenite (400x)

G. EGFP induction by 500ppb arsenite (400x)

H. EGFP induction by 600ppb arsenite (400x)

I. EGFP induction by 1ppm arsenite (400x)

Fig. 24. (A-I) Epifluorescence microscope view of GFP expression by various concentrations of arsenite from recombinant *E. coli* DH5α carrying pPRS3a-arsGFP-cat.

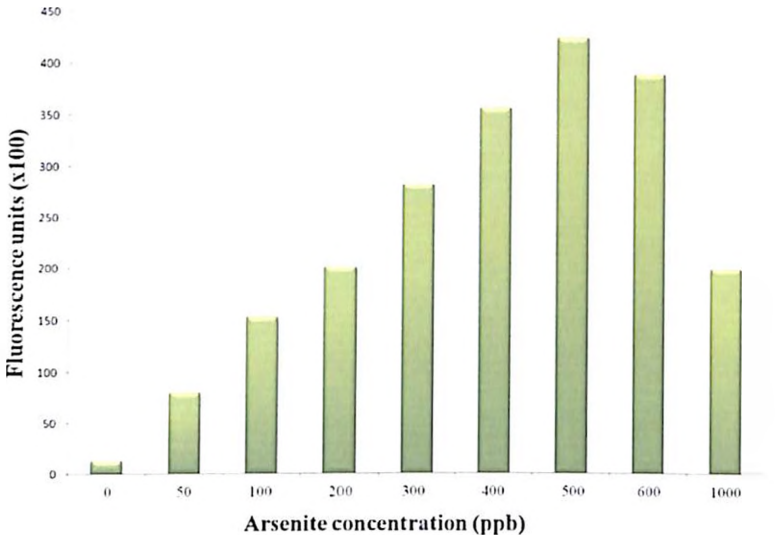


Fig. 25. Fluorescence counts of GFP expression by various concentrations of arsenite from recombinant *E. coli* DH5α carrying pPRS3a-arsGFP-cat.

3.3.7 Plasmid curing

Finally, to prove that the property of ampicillin resistance of *Bacillus cereus* GC subgroup A is due to presence of plasmid DNA and also the plasmid stability, plasmid curing experiment was conducted as described in methods section 3.2.12.

After the successive growth of *Bacillus cereus* GC subgroup A in Ethidium bromide (15μg/ml) and Proflavin (15μg/ml) till 25th generation, no plasmid curing was observed. After 25th generation, cells of *Bacillus cereus* GC subgroup A were resistance to ampicillin, this raises the possibility of the presence of ampicillin resistance marker on genomic DNA. This possibility was ruled out when no change in the quality of plasmid DNA pPRS3a was reported even after the 25th generation, as shown in figure 26. During the continuous growth of *Bacillus cereus* GC subgroup A with plasmid curing agents (Ethidium bromide and Proflavin), small changes in the morphology were reported, because of chemical stress. As both the drugs having fluorescence properties, therefore images were taken to report morphological changes, as shown in figure 27.

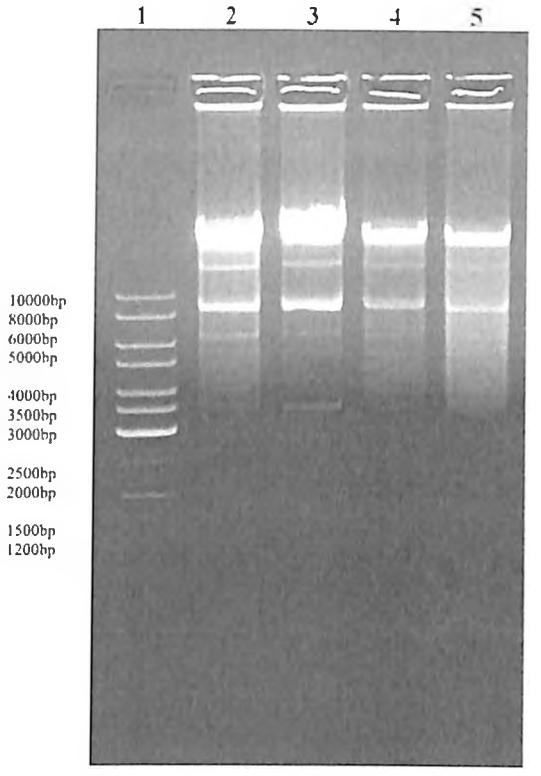


Fig. 26. Plasmid curing of pPRS3a:

- lane 1, DNA marker;
- lane 2, pPRS3a grown in LB 50µg/ml;
- lane 3, pPRS3a grown for 5th generation in ampicillin 50µg/ml with 15µg/ml of Proflavin/Ethidium bromide;
- lane 4, pPRS3a grown for 15th generation in ampicillin 50µg/ml with 15µg/ml of Proflavin/Ethidium bromide;
- lane 5, pPRS3a grown for 25th generation in ampicillin 50µg/ml with 15µg/ml of Proflavin/Ethidium bromide.
- The DNA samples were separated on 1% agarose gel.

Previously, attempts were made to cure plasmid pUK 651 from *E. coli* (R-plasmid) by the plant extract, DNA intercalating dyes (acridine orange and Ethidium bromide) and a DNA gyrase antagonizing drug (pefloxacin). All these agents could cure R-plasmid effectively at their respective sub-MIC concentrations (Arina and Ahmad, 2000). On the other hand in *Pseudomonas putida*, grown in Acridine orange, Ethidium bromide, mitomycin C and SDS failed to cure the plasmid (Kulkarni and Kanekar, 1998). Ghosh et al. (2000) have demonstrated that these curing agents are very specific to some group of plasmids as well as to some bacteria, which may or may not be applicable to all the bacterial strains. The plasmid under this study was found to be refractory to various plasmid-curing agents. One of

the reasons could be that it may be carrying unknown functions vital for cell viability for e.g., none of the mega plasmids of Rhizobium or Agrobacterium have yet been cured (Stanisich, 1984). Fenselau et al. (2007) have identified the function of beta-lactamase in *Bacillus cereus* sporulation. So if this beta-lactamase is encoded by plasmid DNA and responsible for providing antibiotic resistance than the possibility of curing this plasmid by any of chemical curing agent can be ruled out.

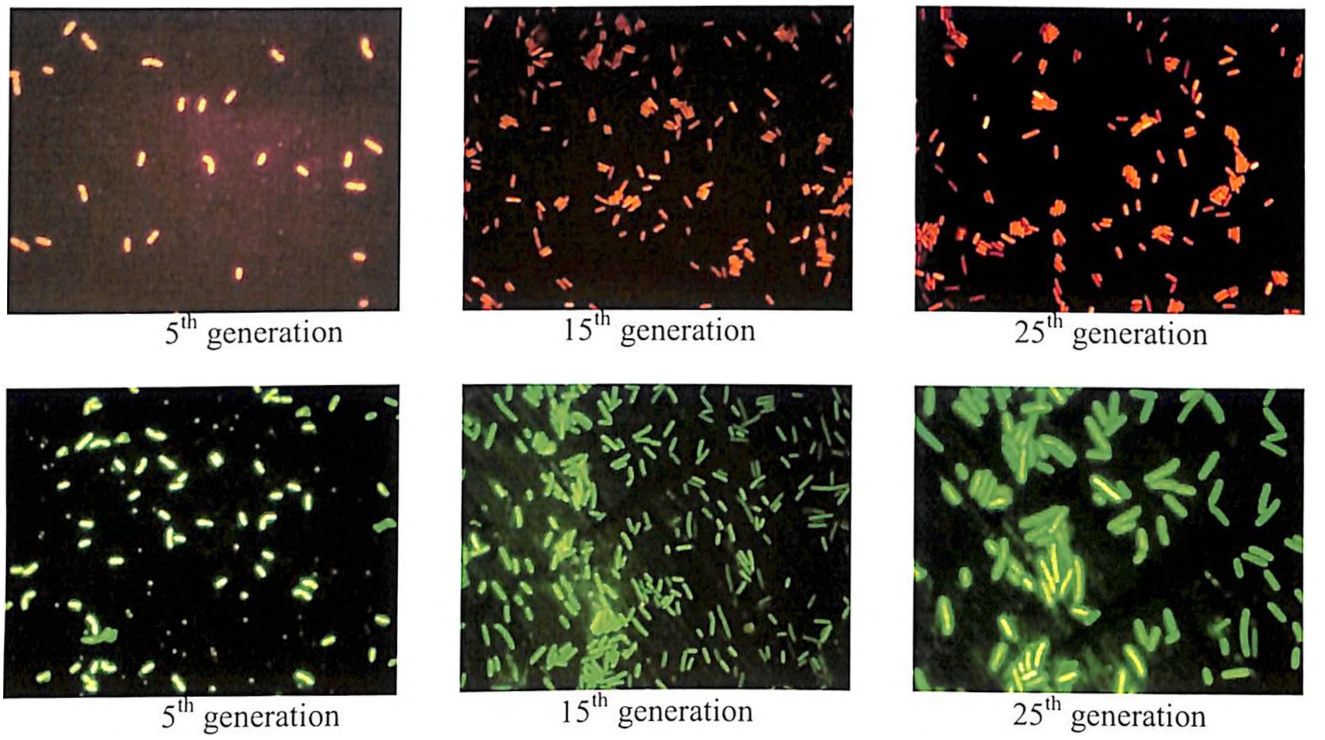


Fig. 27. Morphological changes of *Bacillus cereus* GC subgroup A during plasmid curing. Cells of *Bacillus cereus* GC subgroup A were grown in Ethidium bromide & Proflavin at concentration of 15µg/ml. Morphological changes were reported for (A) Ethidium bromide (cells are in brick-red color) & (B) Proflavin (cells are in fluorescence-green color) at 5^{th}, 15^{th} & 25^{th} generations.

Based on the observations, it became very clear that the plasmid DNA pPRS3a could not be cured. To confirm the stability of plasmid DNA pPRS3a, *Bacillus cereus* GC subgroup A was grown in LB, without any selection pressure, for several generations and the presence of pPRS3a was reported. These results support the stability of plasmid DNA in *Bacillus cereus* GC subgroup A.

Complete identification and characterization of pPRS3a from a natural isolate, *Bacillus cereus* GC subgroup A confirms the presence of a plasmid DNA of size 4126bp. The complete analysis of pPRS3a showed the presence of a functional ampicillin resistance gene, number of strong promoters, origin of replication (recognized by both Gram's positive and negative bacterial systems) and MCS. Based on the presence of variety of components, we can say that this plasmid may be useful in constructing a shuttle cloning and expression vector.

Chapter IV

Complete proteomic analysis & Copper (II) induced protein of Bacillus cereus GC subgroup A

Chapter-IV

Complete proteomic analysis & Copper(II) induced protein of *Bacillus cereus* GC subgroup A

4.1 Introduction

The increase in industrial and mining activities of heavy metals has intensified environmental pollution, deterioration of ecosystem and has become a major global concern now a days. These activities lead to release of enormous amount of heavy metals into the air, soil and water bodies. Among heavy metals, divalent transition metal cations serve as an essential cofactor of proteins involved in variety of cellular processes. Out of these, copper is required in trace amount for the growth and development of an organism but it is considered as a sword with two edges (Nies, 1999) if present above permissible limits (2 mg/L, as per WHO guidelines).

Copper(II) is found in effluents from various industries including tanning, mining, metal processing & finishing, electroplating, automobile and pharmaceutical industry (Quershi et al. 2001). Elemental copper and copper sulfate are pesticides currently and historically used as herbicides, fungicides in agriculture and control of algae in aquatic pest management (Hong et al. 1996). The high level of copper concentration is quite common at the site surrounding areas of the copper handling industry. The excess concentration of copper leads to the survival difficulties for local flora and fauna. To balance the stimulatory and inhibitory properties of copper, microorganisms are equipped with a number of homeostatic mechanisms that ensure proper accumulation, distribution and detoxification of the metal (Camakaris et al. 1999; Gaballa and Helmann, 2003). Especially, bacteria have evolved several types of mechanisms to resist high concentrations of the metal (Dameron and Harrison, 1998).

Bacteria have specific and general defense strategies to counter environmental changes including detoxification of stressor as well protection mechanisms and repair systems. Copper homeostasis has been characterized in classical model organisms like, *Escherichia coli*, *Pseudomonas syringae*, *Enterococcus hirae* and *Saccharomyces cerevisiae* (Brown et

al. 1992; Cooksey, 1993; Cooksey, 1994; Zhou and Thiele, 2001). Most commonly, Cu(II) resistance into bacteria can be achieved by rapid efflux (an active extrusion) of the metal by transporters (Verma and Singh 1991; Dwivedi et al. 1992; Nies and Silver, 1995), metal sequestration by thiol group containing proteins, metal reduction to less toxic oxidation state by reductases (Nies, 1999) and copper uptake (Singh et al. 1989). Although, considerable efforts have been made to understand the mechanisms for metal resistance but most of the mechanisms are still largely unknown.

The analysis of a proteome, which can be used to elucidate the mechanism of metal resistance, requires the resolution of the proteins in a sample followed by the identification of the resolved proteins. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by mass spectrometry (MS) is the most widely used method of protein resolution and identification (Hanash, 2000; Pandey and Mann, 2000; Washburn and Yates, 2000). In 2D-PAGE, proteins are separated in one dimension by isoelectric point (pI) and in the other dimension by molecular weight (MW). High-throughput analysis of proteomes remains challenging because the individual extraction, digestion and analysis of each spot from 2D-PAGE are tedious and time-consuming process. As a result, the largest 2D-PAGE-based proteomic study to date identified 502 unique proteins for the *Haemophilus influenzae* proteome (Langen et al. 2000). Portions of proteomes such as proteins with extremes in pI and molecular weight (Oh-Ishi et al. 2000; Corthals et al. 2000), low-abundance proteins (Fountoulakis et al. 1999a; Fountoulakis et al. 1999b; Gygi et al. 2000) and membrane-associated or bound proteins (Molloy, 2000; Santoni et al. 2000) are rarely seen in a 2D-PAGE study. While efforts to alleviate the current shortcomings in 2D-PAGE continue, we are exploring non-gel-based chromatography systems to resolve and identify thousands of proteins from a biological sample (Eng et al. 1994; McCormack et al. 1997; Link et al. 1999).

An alternative two-dimensional separation system subject proteins or peptides to two independent separation methods and maintain the separation of two components after they have been resolved in one-step (Giddings, 1987). Varieties of efforts are underway to utilize multidimensional chromatography coupled with mass spectrometry to characterize

proteomes (Washburn and Yates, 2000). Link *et al.* developed an online method coupling two-dimensional liquid chromatography (LC) to tandem mass spectrometry (MS/MS) (Link et al. 1999). In this method a microcapillary column is packed with two independent chromatography phases. Once a complex peptide mixture is loaded onto the system, no additional sample handling is required because the peptides are eluted directly off the column and into the mass spectrometer (Link et al. 1999). This system is largely unbiased, proteins from all subcellular portions of the cell with extremes in pI, MW, abundance and hydrophobicity can be identified.

A largely unbiased method for rapid and large-scale proteome analysis by multidimensional liquid chromatography, tandem mass spectrometry and database searching by the SEQUEST algorithm, called multidimensional protein identification technology (MudPIT) have been developed to analyze the highly complex samples necessary for large-scale proteome analysis (Claire and John, 2007). MudPIT couples a two-dimensional liquid chromatography (2D-LC) for separation of peptides on a microcapillary column with detection in a tandem mass spectrometer (Link et al. 1999).

In the chapter III, we have discussed about the characterization of plasmid, pPRS3a. The presence of pPRS3a, in *Bacillus cereus* GC subgroup A has confirmed the genetic basis of antibiotic (ampicillin) resistance of this bacterium. The gene for ampicillin resistance is found to be located on plasmid. To further carry out the characterization of *Bacillus cereus* GC subgroup A, the genetic basis for heavy metal (copper(II)) resistance was elucidated by finding the proteins (enzymes) responsible for copper(II) resistance. This chapter deals in detail about the separation of the total protein of *Bacillus cereus* GC subgroup A, by 1D PAGE, 2D PAGE and their identification by MudPIT. Copper(II) stress induced protein were identified and characterized to understand Cu(II) resistance mechanisms by 1D PAGE followed by MALDI-TOF/MS.

4.2 Materials and methods

4.2.1 *Bacillus cereus* GC subgroup A and culture conditions

Bacillus cereus GC subgroup A (EU744603 & MTCC 10207) was isolated from Khetri copper mine complex, Rajasthan, India, a metal handling industry. This bacterium was grown in Luria Bertani (LB) medium at 37°C, 200 rpm for 18h. Stock cultures of axenic *Bacillus cereus* GC subgroup A were preserved as glycerol stocks and frozen at -70^{0}C.

4.2.2 Enzymes and chemicals

Luria Bertani (LB) bacterial culture medium was procured from Himedia Pvt. Ltd., Mumbai. The metal salts used were of analytical grade and these were purchased from Sigma, USA. Metal stock solutions were prepared in Milli-Q water. Atomic spectral standards for all the metals were purchased from National Physical Laboratory, New Delhi, and were used for preparing working standard for atomic absorption spectroscopy and metal estimations. All other required chemical for SDS PAGE, 2D PAGE, etc. were also obtained from Sigma, USA. Glassware and plastic wares were cleaned with cedepol before use, followed by overnight soaking in 50% nitric acid.

4.2.3 Determination of proteins

Protein concentrations for electrophoretic separation and protein sequencing were determined by using Bicinchoninic acid (BCA) method using QuantiPro BCA assay kit (SIGMA, BCA1) (Brown et al. 1989). 1 ml of cell lysate was mixed with 1 part of a protein sample followed by addition of 1 part of the QuantiPro working reagent. The working reagent was prepared by mixing 25 parts of QA (solution containing sodium carbonate, sodium tartrate and sodium bicarbonate in 0.2M NaOH, pH 11.25) with 25 parts of QB (4% bicinchoninic acid, pH 8.5); after a brief incubation at RT, 1 part of reagent QC (4% copper(II) sulfate) was added and mixed well until it gave uniform color. Protein standards (stock standard 1 mg/ml, bovine serum albumin, BSA) were prepared with a range between 5-30 µg/ml and made up to 1 ml using MilliQ water. The unknown samples of the cell lysate were made in the similar way. 1 ml of QuantiPro working reagent was added to 1 ml of each protein standard, blank (water) and unknown samples. The contents were mixed well and incubated at 60°C water bath for 1h. The absorbance was measured at 562 nm after cooling

the tubes at RT. The net absorbance of the samples was found by subtracting absorbance of the reagent blank. Concentration of the protein was estimated from a calibration curve prepared using known concentrations of BSA.

4.2.4 Metal salts and stock solutions

Metal stock solutions were prepared using analytical grade metal salts in the form of sulphates of copper and dissolved in Milli-Q water. If required, metal solutions were acidified with 1-2% nitric acid and stored in dark conditions. Metal solutions and working standards were prepared freshly from the stock. Atomic spectral grade standards were procured from National Physical Laboratory (NPL), New Delhi.

4.2.5 Determination of metal resistance in *Bacillus cereus* GC subgroup A

Sensitivity of *Bacillus cereus* GC subgroup A towards copper(II) was determined by growing the cells in the varying concentration of Cu(II) and MIC was calculated, as described in section 2.2.5.

4.2.6 Two dimensional gel electrophoresis (2D PAGE)

Bacillus cereus GC subgroup A was grown in LB medium for 18h at 37°C, 200 rpm. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. The cell pellet was homogenized in 5 ml lysis buffer (100mM Tris (pH 7.5), 2% SDS, 2% β-Mercaptoethanol) followed by centrifugation at 20000 rpm for 30min at 4°C. The resulting supernatant (4ml) was precipitated using TCA/acetone method (Brown et al. 1989). To this supernatant 400µl of DOC, 200µl of Triton X-100, 640µl of TCA (13% TCA) were added and kept on ice for 2h. Centrifugation at 15000 rpm, 4°C for 30 min and supernatant was discarded. 4ml of chilled acetone was added and above step repeated twice. After air-drying the pellet at room temperature the pellet was reconstituted in 300µl suspension buffer (8M urea, 100mM Tris.Cl (pH 8.5), 0.4% SDS, 5mM TBP). The protein concentration was measured by Bio-Rad RC-DC assay kit (Lowry et al. 1951). 62.5µg protein solution was diluted with sample buffer (9M Urea, 4% CHAPS, 1% DTT, 0.5% Triton X-100, 1mM EGTA) to 125µl and loaded onto Bio-Rad IPG strip pH 4-7, 7cm. Isoelectro-focusing (IEF) was done on Amersham Ettan IPGphor for about 28000kwhr at 20°C. SDS-PAGE (12%) was conducted

on Bio-Rad Mini-Protean Dodeca Cell for 1.5h at 60v and 160v. The gel was stained with Bio-Rad SYPRO Ruby protein stain. First the gel was fixed with 10% methanol, 7% acetic acid and 83% H_2O for 60 min and then kept in SYPRO Ruby solution for at least 3 hour with gentle shaking. Finally the gel was washed with 10% methanol, 7% acetic acid and 83% H_2O for 30-60 min to decrease background fluorescence followed by 3-5 washing with Milli-Q water. Stained gel was imaged with Perkin Elmer proXPRESS 2D proteomic imaging system. The spots were detected automatically using the Nonlinear Progenesis Workstation version 2005.

4.2.7 Multi Dimensional Protein Identification Technology (MudPIT)

MudPIT (multidimensional protein identification technology) is a non-gel approach for the identification of proteins from complex mixtures (Lin et al. 2001; Claire et al. 2007). The technique consists of a 2-dimensional chromatography separation, prior to electrospray mass spectrometry (Eng et al. 1994; Link et al. 1999).

4.2.7.1 Experimental procedure

Bacillus cereus GC subgroup A was grown in LB medium for 18h at 37°C and 200 rpm. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C and cell pellet was washed 3 times with PBS. To the cell pellet, acid washed glass beads, 5ml of homogenization buffer (100 mM Tris.Cl (pH 8.0), 3% SDS & 10mM DTT were added, and the mixture was vortexed until the cells were broken complete. This mixture was centrifuged at 18000 rpm to remove cell debris. To the supernatant, TCA precipitation was done using 10% TCA and acetone washing. The pellet was air-dried and protein quantification was done by BCA method (Brown et al. 1989). The quantified protein pellet was dissolved in suspension buffer (0.4% SDS, 6M urea & 100mM Tris) followed by reduction and alkylation. Tryptic digestion (1:30) was done overnight after the mixture was diluted for 10 fold with 50mM Tris.Cl (pH 7.5). Peptide mixture was acidified by addition of 10% TFA. C18 column (1ml) from Sigma was used to clean up the digests. The eluant was loaded onto a SCX column (1ml). The sample was fractionated using step gradation of KCl + 0.1% TFA as follows: 0, 20, 40, 60, 70, 80, 100, 120, 125,150, 175, 200, 250, 300, 350 and 500 mM.

The sample was concentrated to 125µl for injection. The following parameters & instrumentation were used:

- HPLC: Ultimate cap/nano HPLC(Dionex, Sunnyvale, CA, USA)
- Column: Magic C18 (5µm, 100µm ID × 150mm, Michrom Bioresources, Auburn, CA)
- Solvent A: 5% CH3CN+0.1% formic acid+0.01% TFA
- Solvent B: 85% CH3CN+10% isopropanol+5% H2O+0.1% formic acid+0.01% TFA
- Flow rate: 300nl/min
- Gradient: 78 min linear gradient from 15-50% B
- MS/MS: QSTAR XL (Applied Biosystems, USA)

4.2.7.2 Data analysis

MGF peak list file was created using the Mascot distiller 2.0 software from the Matrix Science. In house MASCOT 2.2 from Matrix Science (London, UK) was used to assistant the interpretation of tandem mass spectra against the NRDB bacteria sub-databases (3440445 sequences). Variable modifications including deamidation (N,Q), oxidation (M), and carbamidomethylation (C) were considered for the searching (Kline and Wu, 2009).

4.2.8 Analysis of copper(II) induced proteome of *Bacillus cereus* GC subgroup A

4.2.8.1 Sample preparation

For copper(II) induced protein analysis, cells were grown in each combination of stress in LB broth containing varying concentration of Cu(II) (0.5mM, 1.0mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM, 3.5mM, 4.0mM, 4.5mM and 5.0mM) at different temperatures (37°C, 38°C, 39°C, 40°C, 41°C and 42°C) for 18h under shaking condition (150rpm).The pH of the metal containing medium was adjusted to 7.5±0.1. A control was setup without metal at the similar conditions.

4.2.8.2 Preparation of whole cell lysate

In order to gain an insight into the changes in protein expression of *Bacillus cereus* GC subgroup A upon exposure to metals at different temperatures, the total cell lysate was analyzed by separating total protein on SDS-PAGE. To achieve it, cells were grown in stress conditions as described in section 4.2.8.1. After incubation, cells (metal treated and untreated) were harvested by centrifugation at 8000rpm for 10min at 4°C and washed once with phosphate buffered saline (pH 7.4), resuspended in PBS containing 2mM EDTA (pH 8.0) and then a protease inhibitor, 0.2 mM phenyl methyl sulphonyl fluoride (PMSF) was added. Finally, the cells were disrupted by sonication on ice bath and cell debris was removed by centrifugation at 15000 rpm for 15 min at 4°C (Wang et al. 2005). A fraction of the obtained protein supernatant was used for protein determination by BCA method (Brown, et al. 1989). To quantified protein mixture, 2x sample buffer (SIGMA) was added in equal volume. For denaturing polyacrylamide gel electrophoresis, the samples were prepared as per the standard protocol and SDS PAGE was carried out (Sambrook et al. 2001). 80µg of total protein in each lane was electrophoresed on 12% of 0.75 mm thick gel using BioRad Mini Protean gel electrophoresis apparatus at 80/100 V. A protein molecular weight marker (SM0431, MBI Fermentas) was also loaded along with protein samples. The proteins separated on polyacrylamide gel were visualized after staining the gel with 0.25% Coomassie brilliant blue G-250 in 45% methanol and 10% acetic acid followed by destaining in 35% methanol with 10% acetic acid.

4.2.8.3 In gel trypsin digestion

The desired protein band from gel was cut into 1 mm^3 pieces and transferred into a sterile micro centrifuge tube. The band was destained with 200µl of 50% acetonitrile and 40mM ammonium bicarbonate solution. The destained gel pieces were then dehydrated with 200µl of 100% acetonitrile for 5 min at room temperature. The micro centrifuge tube with the gel pieces was kept in a speed vac for 15 min to ensure complete removal of any residual water. The gel pieces were then immersed in 20µl of digestion buffer (25mM ammonium bicarbonate) containing 500ng of sequencing grade trypsin (Promega) and incubated on ice for 45 min. The swollen gel pieces were then covered with 50µl of digestion buffer and incubated for 18h at 37°C. After a 10min wash with 150µl nano-pure water, the peptides

were extracted with 50µl of extraction buffer (50% acetonitrile and 5% formic acid) twice, each with 60min incubation at room temperature. All the extracts were pooled and lyophilized in a speed vac until they were completely dry.

4.2.8.4 MS/MS analysis

Digested peptides were solubilized in 50% acetonitrile containing 0.1 % formic acid and loaded in a silica capillary (Proxeon Biosystem, USA) and fixed to a QSTAR XL quadrapole/time-of-flight tandem mass spectrometer (Applied Biosystem, USA). Nanospray ionization was carried out using an ion spray voltage of 900. The progress of each run was monitored by recording the total ion current (TIC) for positive ions as a function of time for ions in the m/z range of 400-1600 for MS and 140-1600 for MS/MS. Mass spectra were acquired for 10 min setting the parameters by information-dependent acquisition (IDA) method. The other parameters set were: interface temperature, 50°C; curtain gas flow, 1.13 L/min; declustering potential, 60 V; focusing potential, 280 V and declustering potential 2, 15 V (Wang et al. 2009).

4.2.8.5 Database searches

All spectra were searched against MSDB with MASCOT (Version 1.0 Matrix Science, UK) search. While searching some modifications like, oxidation of methionine and carbamidomethylation of cysteine were considered. Search was further refined to include peptides with charged state from +2 to +3 and limited to bacteria (eubacteria). The peptide mass tolerance range was ± 1.2 kDa and fragment mass tolerance was ± 0.6 kDa. The confidence limit for the protein identification was set at 99.9%. Hits with a probability based Mowse score more than the designated values were considered to be identified successfully.

4.3 Results and discussion

To gain an insight into the proteome of *Bacillus cereus* GC subgroup A, techniques like, single dimensional polyacrylamide gel electrophoresis (1D PAGE), two dimensional polyacrylamide gel electrophoresis (2D PAGE), multidimensional protein identification technology (MudPIT) and MALDI-TOF/MS have been used.

4.3.1 1D PAGE

Single dimensional polyacrylamide gel electrophoresis (1D PAGE) is well documented for the separation of total protein of an organism based on their molecular mass. We have used 1D PAGE for the visualization of all the proteins of *Bacillus cereus* GC subgroup A. The experimental procedure for 1D PAGE or SDS-PAGE was used as described in methods section 3.2.11.1 and the gel-image was obtained for 1D PAGE (figure 28).

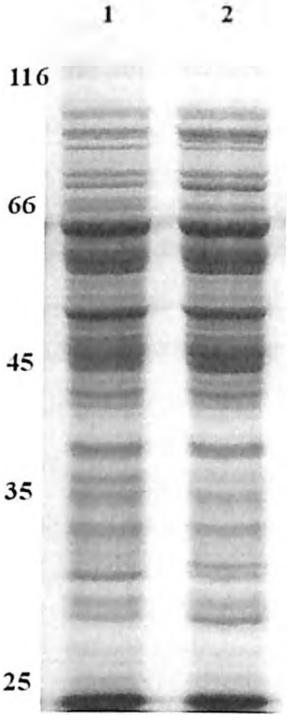


Fig. 28. 1D PAGE of the cell lysate of *Bacillus cereus* GC subgroup A. Protein molecular weight marker (kDa);

- lane 1 & 2, *Bacillus cereus* GC subgroup A (80µg of total protein) grown at 37°C without any selection pressure,
- Samples were electrophoresed on a 12% polyacrylamide gel and stained with Commassie brilliant blue G-250.

The gel-image showed that the presence of number of proteins of different molecular mass in varying amounts. In this separation, ~50-60 protein bands (minor & major) were visualized. Each band in gel represents a type of proteins, which are having similar molecular mass but may differ in their charge. Therefore, to further analyze, these proteins were separated based on their charge and mass.

4.3.2 2D PAGE

To find out all possible protein of a bacterium, two dimensional polyacrylamide gel electrophoresis (2D PAGE) is used. This technique separates the proteins based on charge and mass. First, all the protein are separated based on their charge by isoelectric focusing (IEF) on immobilized pH gradient (IPG) strips, than by mass in a regular SDS PAGE. The separation of proteins by 2D PAGE gave more significant results than 1D PAGE.

The subject bacterium, *Bacillus cereus* GC subgroup A was grown and lysed completely for loading onto the IPG strips and then on SDS PAGE, as described in methods section 4.2.6. The gel images obtained are shown in figure 29.

The gel picture shows that 611 proteins can be identified as a distinct spot when separated in a pH range of 4-7. This range was selected because when proteins were separated in the pH range of 3-10, most of the proteins clustered at pH 4-7. So to visualize the maximum number of protein pH 4-7 IPG strip was used.

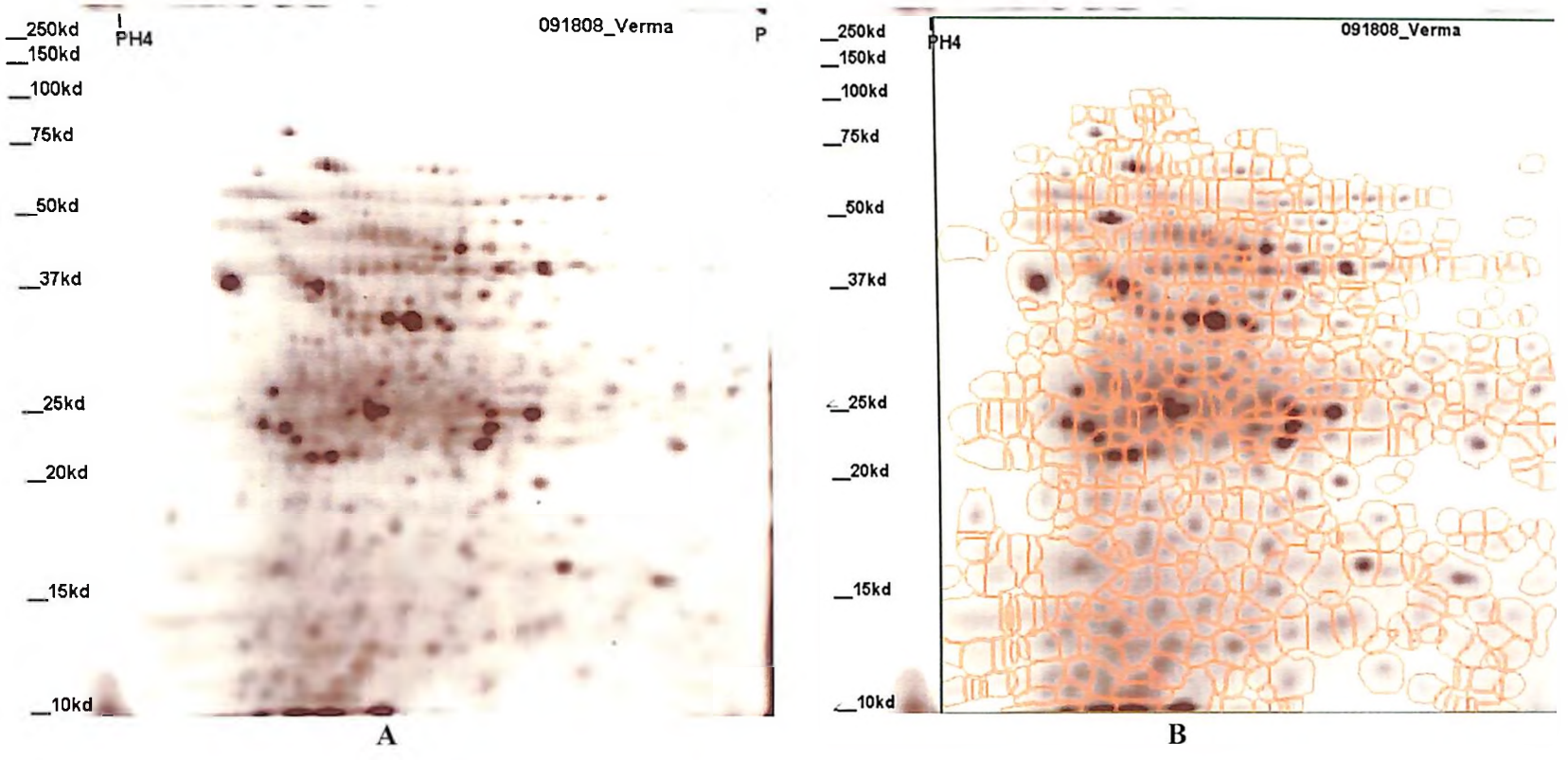


Fig. 29. 2D PAGE of the total cell lysate of *Bacillus cereus* GC subgroup A.

(A) Samples are separated on IPG strips & 12% SDS PAGE.

(B) The spots were detected automatically using the Nonelinear Progenesis Workstation version 2005. As being shown, 611 spots were detected.

As reported earlier, the total number of proteins in *Bacillus cereus* is 5240 (Ivanova et al. 2003) and maximum no. of 1300 spots were identified by 2D PAGE in *Bacillus cereus* (Martinez-Gomariz et al. 2009). In the another study, 956 distinct protein spots were detected by silver staining (Luo et al. 2007) and 525-683 protein spots were detected in the different strains of *Bacillus cereus* and analyzed by 2D PAGE (Dworzanski et al. 2010).

Theoretically, 2D-PAGE is capable of detecting more than 10,000 protein spots. Although the resolving power of this technology is excellent, the identification of individual spots requires a second analytical step such as mass spectrometry. In published reports of 2D-PAGE separations producing 1000 resolved spots from *Saccharomyces cerevisiae*, only around 300 different spots have been correlated to proteins (Garrels et al. 1997; Perrot. et al. 1999; Futcher et al. 1999). Dynamic range and protein solubility issues complicate the detection and separation of low-abundance and hydrophobic proteins by 2D-PAGE (Gygi et al. 1999b; Santoni et al. 2000; Gygi et al. 2000). In particular, the analysis of integral membrane proteins remains a critical challenge; due to their low solubility in gel electrophoresis buffer, proteins with an either low (<10 kDa) or high (>200 kDa) molecular weight (MW), as well as those with an extreme isoelectric point (pI <4 or >9) (Gygi et al. 2000), however, new detergents have been designed to enhance membrane protein solubility for analysis by 2D PAGE An additional limitation of this approach resides in the difficulty in analysis of less represented proteins (Rabilloud et al. 1997; Chevallet et al. 1998; Molloy et al. 1998).

The next step for the characterization of *Bacillus cereus* GC subgroup A through proteomics is to identify all the 611 proteins. The identification of proteins from 2D-gel has some limitations as discussed above. To overcome these difficulties an alternative approach to 2D-PAGE, a non-gel based approach, which directly interface protein and peptide separations to mass spectrometers, named multidimensional protein identification technology (MudPIT) was used for the identification of all possible proteins of *Bacillus cereus* GC subgroup A.

4.3.3 MudPIT

MudPIT (Multidimensional Protein Identification Technology) is a non-gel approach for the separation and identification of complex protein and peptide mixtures. Rather than use traditional 2D gel electrophoresis, MudPIT separates peptides in 2D liquid chromatography. In this way, the separation can be interfaced directly with the ion source of a mass spectrometer. The experimental procedure used for MudPIT has been described in methods section 4.2.7. The obtained results were in form of tandem mass spectra. In house MASCOT 2.2 from Matrix Science (London, UK) was used for interpretation of the tandem mass spectra against the NRDB bacteria sub-database (3440445 sequences). Based on this search & analysis, 135 most significant proteins were identified. The proteins have been grouped based on their factions, as shown in Table 30-34. The MudPIT was carried out at Center for Functional Genomics, University at Albany, NY, USA.

Table 30. Protein identification through MudPIT involved in Metabolism. 63 proteins were identified from *Bacillus cereus* GC subgroup A. all the proteins have been shown with their NCBI GI number, no. of peptides identified, sequence coverage, pI, molecular mass, organism and putative function.

S. No.	NCBI GI number	Putative protein	No. of Peptides identified	Sequence coverage (%)	pI	MW (Da)	Organism
1	163940058	Alcohol dehydrogenase GroES domain protein	7	28	5.13	36765	*Bacillus weihenstephanensis* KBAB4
2	166997401	Phosphatidylinositol-specific phospholipase C X region	2	25	4.90	11950	*Bacillus cereus* AH820
3	30023174	Glyceraldehyde-3-phosphate dehydrogenase	2	4	5.04	35176	*Bacillus cereus* ATCC 14579
4	30020418	2-methylcitrate dehydratase	4	8	5.41	53363	*Bacillus cereus* ATCC 14579
5	187603478	Alcohol dehydrogenase zinc-binding domain protein	2	7	6.91	37620	*Exiguobacterium* sp. AT1b
6	7225772	Alcohol dehydrogenase, propanol-preferring	3	9	5.65	36525	*Neisseria meningitidis* MC58
7	30021715	Aconitate hydratase	3	4	4.88	98936	*Bacillus cereus* ATCC 14579
8	30023031	Glycine cleavage system protein H	1	8	4.18	13978	*Bacillus cereus* ATCC 14579
9	168156208	Acyl-CoA dehydrogenase	4	7	5.1	65535	*Bacillus cereus* NVH0597-99
10	30018552	1-pyrroline-5-carboxylate dehydrogenase	5	13	5.43	56190	*Bacillus cereus* ATCC 14579
11	30021924	Succinyl-CoA synthetase subunit beta	2	4	5.00	41669	*Bacillus cereus* ATCC 14579
12	165906467	Glyceraldehyde-3-phosphate dehydrogenase	1	10	5.32	15488	*Bacillus subtilis* subsp. Spizizenii
13	27467293	Alcohol dehydrogenase	1	6	5.13	36497	*Staphylococcus epidermidis* ATCC 12228
14	15615300	1-pyrroline-5-carboxylate dehydrogenase	2	3	5.41	56607	*Bacillus halodurans* C-125

15	30021657	Aldehyde dehydrogenase	4	14	5.49	53711	*Bacillus cereus* ATCC 14579
16	30018489	4-hydroxyphenylpyruvate dioxygenase	2	6	5.04	42066	*Bacillus cereus* ATCC 14579
17	30020422	Methylmalonate-semialdehyde dehydrogenase (acylating)	5	10	5.30	52920	*Bacillus cereus* ATCC 14579
18	6561887	IMP dehydrogenase	2	5	6.35	53358	*Bacillus cereus*
19	30262538	Acyl-CoA dehydrogenase	3	9	5.54	41606	*Bacillus anthracis* str. Ames
20	89100684	Acetyl-CoA dehydrogenase	3	8	5.33	65757	*Bacillus* sp. NRRL B-14911
21	30022060	Pyruvate dehydrogenase E1 component beta subunit	4	14	4.75	35207	*Bacillus cereus* ATCC 14579
22	30020420	Acyl-CoA dehydrogenase	2	3	5.40	63712	*Bacillus cereus* ATCC 14579
23	47567343	Triosephosphate isomerase	1	6	4.74	25157	*Bacillus cereus* G9241
24	148263656	Acyl-CoA dehydrogenase domain protein	2	3	5.12	64720	*Geobacter uraniireducens* Rf4
25	30019468	Acetoacetyl-CoA reductase	4	21	5.83	24461	*Bacillus cereus* ATCC 14579
26	30018427	D-fructose-6-phosphate amidotransferase	3	9	4.99	65781	*Bacillus cereus* ATCC 14579
27	30023173	Phosphoglyceromutase	1	2	4.86	56317	*Bacillus cereus* ATCC 14579
28	72161350	Ptative acyl-CoA dehydrogenase	1	3	5.06	41279	*Thermobifida fusca* YX
29	30023172	Phosphopyruvate hydratase	2	6	4.66	46343	*Bacillus cereus* ATCC 14579
30	163793244	Phosphopyruvate hydratase	1	5	4.92	45249	*Alpha proteobacterium* BAL199
31	16800114	PdhB	2	10	4.79	35275	*Listeria innocua* Clip11262
32	75265603	3-oxoacyl-(acyl-carrier-protein) reductase-like protein	1	4	5.95	25910	*Pseudomonas fluorescens*
33	134299918	3-oxoacyl-(acyl-carrier-protein) reductase	1	4	8.64	25923	*Desulfotomaculum reducens* MI-1
34	146311372	2-deoxy-D-gluconate 3-dehydrogenase	1	4	5.28	27145	*Enterobacter* sp. 638
35	42783351	Glycine cleavage system aminomethyltransferase T	2	6	5.15	40208	*Bacillus cereus* ATCC 10987
36	30020419	Methylisocitrate lyase	4	17	4.91	32925	*Bacillus cereus* ATCC 14579

37	129061	Pyruvate dehydrogenase E1 component subunit alpha	1	2	5.44	41443	*Geobacillus stearothermophilus*
38	167938104	Cysteine synthase	4	17	6.39	32898	*Bacillus cereus* AH1134
39	30023366	Fructose-bisphosphate aldolase	1	4	5.00	30653	*Bacillus cereus* ATCC 14579
40	56420960	Glycine cleavage system aminomethyltransferase T	1	3	5.41	39738	*Geobacillus kaustophilus* HTA426
41	15644471	3-oxoacyl-(acyl carrier protein) reductase	1	4	6.43	26385	*Thermotoga maritima* MSB8
42	15806941	3-oxoacyl-acyl carrier protein reductase	1	4	6.84	26198	*Deinococcus radiodurans* R1
43	30021890	Aspartate-semialdehyde dehydrogenase	2	6	5.44	38081	*Bacillus cereus* ATCC 14579
44	52079282	Acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	2	7	4.78	36862	*Bacillus licheniformis* ATCC 14580
45	124521050	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	2	3	5.32	56650	*Bacillus coagulans* 36D1
46	30022246	Leucine dehydrogenase	3	9	5.13	39842	*Bacillus cereus* ATCC 14579
47	146340657	Putative 3-oxoacyl-[acyl-carrier-protein] reductase, related to short chain alcohol dehydrogenases	1	4	7.98	28137	*Bradyrhizobium* sp. ORS278
48	150015338	2-deoxy-D-gluconate 3-dehydrogenase]	1	4	6.85	28479	*Clostridium beijerinckii* NCIMB 8052
49	47569758	Fructose-1,6-bisphosphatase, class II	1	3	5.52	33232	*Bacillus cereus* G9241
50	29375921	Pyruvate dehydrogenase complex, E1 component, beta subunit]	2	6	4.67	35373	*Enterococcus faecalis* V583
51	30020612	Acetyl-CoA carboxylase biotin carboxylase subunit	2	5	5.29	49527	*Bacillus cereus* ATCC 14579
52	168139292	Acetyl-CoA carboxylase biotin carboxylase subunit	3	5	4.50	49557	*Bacillus cereus* G9842
53	30021923	Succinyl-CoA synthetase subunit alpha	2	9	5.23	31206	*Bacillus cereus* ATCC 14579
54	30019653	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	1	2	5.22	36669	*Bacillus cereus* ATCC 14579
55	32265991	Glyceraldehyde-3-phosphate dehydrogenase	2	4	8.12	35451	*Helicobacter hepaticus* ATCC 51449
56	94969568	Glyceraldehyde-3-phosphate dehydrogenase	1	4	8.27	35873	*Acidobacteria bacterium* Ellin345

57	38234885	Tryptophan synthase subunit beta	1	2	5.27	47425	*Corynebacterium diphtheriae* NCTC 13129
58	124522059	Glu/Leu/Phe/Val dehydrogenase, C terminal	2	6	5.48	40370	*Bacillus coagulans* 36D1
59	20808582	Glutamate dehydrogenase/leucine dehydrogenase	1	3	5.43	39218	*Thermoanaerobacter tengcongensis* MB4
60	30022058	Dihydrolipoamide dehydrogenase	2	4	5.25	49409	*Bacillus cereus* ATCC 14579
61	162455724	Dehydrogenase of unknown specificity	1	4	6.00	27446	*Sorangium cellulosum* 'So ce 56'
62	30022668	Isocitrate dehydrogenase	5	13	5.07	46943	*Bacillus cereus* ATCC 14579
63	152976974	Isocitrate dehydrogenase, NADP-dependent	3	10	5.11	46787	*Bacillus cereus* subsp. cytotoxis NVH 391-98

Most of the identified proteins were in pI range of 4-6 and for molecular mass. 12-20, 30-40 & 50-60kDa. The obtained results also confirms that the presence of extreme range of proteins in pI (<4.0) and molecular mass (<10kDa & >200kDa). The major groups taken into consideration for functions were metabolism, stress, translation, other, energy transfer, replication, gene regulation, kinase, transcription, transport and proteolysis. The functional and categorical distribution of all the identified proteins of *Bacillus cereus* GC subgroup A, showed that majority of protein are involved in metabolism (Table 30) followed by stress (Table 31), translation (Table 32), energy transfer (Table 33) and replication, gene regulation and kinase, transcription, transport, proteolysis & other functions (Table 34). The majority of proteins (63 proteins) are involved in metabolism that represents the healthy metabolic status of the *Bacillus cereus* GC subgroup A. A surprising observation is that 17% proteins are involved in stress regulation. The presence of stress regulation proteins suggest that either the bacterium *Bacillus cereus* GC subgroup A was being grown under stress conditions or it had been encountered them earlier. In this protein identification through MudPIT, the bacterium *Bacillus cereus* GC subgroup A was grown in LB medium at 37°C, without any stress like, antibiotic, metal, temperatures etc. Therefore, the identification of stress proteins shows history of stress to which the organism is subjected. The sample site is a copper mine industry, which again signify the presence of stress proteins at basal level in *Bacillus cereus* GC subgroup A. The bacterium has encountered for the stress for very long period so the genes involved in the stress regulation might have become the part of housekeeping genes and expressed always at their basal level.

Table 31. Protein identification through MudPIT involved in Stress. 23 proteins were identified from *Bacillus cereus* GC subgroup A. all the proteins have been shown with their NCBI GI number, no. of peptides identified, sequence coverage, pI, molecular mass, organism and putative function.

S. No.	NCBI GI number	Putative protein	No. of Peptides identified	Sequence coverage (%)	pI	MW (Da)	Organism
1	30022561	Trigger factor	4	14	4.53	47316	*Bacillus cereus* ATCC 14579
2	167939703	Chaperonin GroEL	4	8	4.73	57370	*Bacillus cereus* AH1134
3	152976888	Trigger factor	2	6	4.59	47639	*Bacillus cereus* subsp. cytotoxis NVH 391-98
4	16801239	Chaperonin GroEL	4	7	4.70	57266	*Listeria innocua* Clip11262
5	116250	60 kDa chaperonin (Protein Cpn60) (groEL protein) (Heat shock 61 kDa)	3	6	5.08	57147	Bacillus sp. PS3
6	89100205	Chaperonin GroEL	3	5	4.77	57270	*Bacillus* sp. NRRL B-14911
7	1682951	GroEL	3	4	4.84	57365	*Bacillus* sp.
8	30018651	Tellurium resistance protein terD	2	13	4.60	21353	*Bacillus cereus* ATCC 14579
9	138896219	Trigger factor	1	3	4.64	49051	*Geobacillus thermodenitrificans* NG80-2
10	30020148	DNA-binding protein Dps/Fe-binding ferritin-like antioxidant/Ferroxidase	2	15	4.73	16669	*Bacillus cereus* ATCC 14579
11	5921715	Chemical-damaging agent resistance protein B	1	9	4.46	15420	*Clostridium acetobutylicum*
12	584917	10 kDa chaperonin (Protein Cpn10) (groES protein)	2	22	4.89	10235	*Geobacillus stearothermophilus*
13	30018652	Tellurium resistance protein terD	2	21	4.58	20789	*Bacillus cereus* ATCC 14579
14	30022700	Universal stress protein family	3	17	5.68	16700	*Bacillus cereus* ATCC 14579
15	30022393	Molecular chaperone DnaK	5	8	4.66	65726	*Bacillus cereus* ATCC 14579
16	42783441	Molecular chaperone DnaK	5	8	4.68	65835	*Bacillus cereus* ATCC 10987
17	186681701	Chaperonin GroEL	2	5	4.90	58871	*N. punctiforme* PCC 73102
18	22298955	60kD chaperonin 2	2	4	4.90	57067	*Thermosynehococcus elongatus* BP-1

19	70780023	GroEL	4	6	4.73	56112	*Streptococcus ratti*
20	60671688	Hsf	1	1	5.54	249377	*Haemophilus influenzae*
21	30020324	Penicillin-binding protein	1	2	5.39	54575	*Bacillus cereus* ATCC 14579
22	47570164	Penicillin-binding protein	1	2	5.22	54698	*Bacillus cereus* G9241
23	138896078	Heat-shock protein GrpE	1	5	4.60	24596	*Geobacillus thermodenitrificans* NG80-2

Table 32. Protein identification through MudPIT involved in Translation. 15 proteins were identified from *Bacillus cereus* GC subgroup A. all the proteins have been shown with their NCBI GI number, no. of peptides identified, sequence coverage, pI, molecular mass, organism and putative function.

S. No.	NCBI GI number	Putative protein	No. of Peptides identified	Sequence coverage (%)	pI	MW (Da)	Organism
1	30022519	50S ribosomal protein L21	2	24	9.73	11184	*Bacillus cereus* ATCC 14579
2	30018378	Elongation factor Tu	4	11	4.93	42912	*Bacillus cereus* ATCC 14579
3	30018377	Elongation factor G	3	5	4.91	76276	*Bacillus cereus* ATCC 14579
4	30019646	30S ribosomal protein S1	4	10	4.97	42140	*Bacillus cereus* ATCC 14579
5	30018404	30S ribosomal protein S13	1	9	11.17	13811	*Bacillus cereus* ATCC 14579
6	134298090	Elongation factor G	2	3	5.18	76331	*Desulfotomaculum reducens* MI-1
7	167629464	Elongation factor G	2	3	5.26	76444	*Heliobacterium modesticaldum* Ice1
8	65317563	COG0480: Translation elongation factors (GTPases)	2	3	5.09	52291	*Bacillus anthracis* str. A2012
9	950070	Elongation factor G	1	6	6.32	22271	*Mycoplasma capricolum* subsp. capricolum ATCC 27343
10	30018386	30S ribosomal protein S3	1	7	9.99	24279	*Bacillus cereus* ATCC 14579
11	30022455	Transcription elongation factor GreA	1	8	4.84	17484	*Bacillus cereus* ATCC 14579
12	30018399	50S ribosomal protein L15	1	8	10.40	15468	*Bacillus cereus* ATCC 14579
13	30018370	50S ribosomal protein L7/L12	1	11	4.71	12510	*Bacillus cereus* ATCC 14579
14	18310681	Elongation factor Ts	1	3	5.13	33166	*Clostridium perfringens* str. 13
15	30018379	30S ribosomal protein S10	1	14	9.64	11676	*Bacillus cereus* ATCC 14579

Table 33. Protein identification through MudPIT involved in Energy Transfer. 10 proteins were identified from *Bacillus cereus* GC subgroup A. all the proteins have been shown with their NCBI GI number, no. of peptides identified, sequence coverage, pI, molecular mass, organism and putative function.

S. No.	NCBI GI number	Putative protein	No. of Peptides identified	Sequence coverage (%)	pI	MW (Da)	Organism
1	30264588	Electron transfer flavoprotein, alpha subunit	5	22	5.01	34320	*Bacillus anthracis* str. Ames
2	30022600	Electron transfer flavoprotein beta-subunit	6	30	4.33	28129	*Bacillus cereus* ATCC 14579
3	163942253	Electron transfer flavoprotein alpha subunit	4	18	5.16	34403	*Bacillus weihenstephanensis* KBAB4
4	30023098	NADH dehydrogenase	2	6	6.68	41581	*Bacillus cereus* ATCC 14579
5	169185541	Inorganic diphosphatase	2	12	5.33	33583	*Paenibacillus* sp. JDR-2
6	30018585	Alkyl hydroperoxide reductase C22	3	18	4.79	20693	*Bacillus cereus* ATCC 14579
7	30018584	Alkyl hydroperoxide reductase subunit F	2	4	4.93	54774	*Bacillus cereus* ATCC 14579
8	163938334	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	2	4	4.97	54819	*Bacillus weihenstephanensis* KBAB4
9	52140264	NADH dehydrogenase subunit C	1	2	8.46	44826	*Bacillus cereus* E33L
10	30023338	F0F1 ATP synthase subunit gamma	2	7	6.56	31586	*Bacillus cereus* ATCC 14579

Table 34. Protein identification through MudPIT involved in various functions. Replication, Kinase activity, Gene regulation, Proteolysis, RNA processing, Transcription, Transport and Other functions. 24 proteins were identified from *Bacillus cereus* GC subgroup A. all the proteins have been shown with their NCBI GI number, no. of peptides identified, sequence coverage, pI, molecular mass, organism and putative function.

S. No.	NCBI GI number	Putative protein	No. of Peptides identified	Sequence coverage (%)	pI	MW (Da)	Organism
1	92090120	UvrB/UvrC protein:AAA ATPase, central region:Clp, N terminal:ATPase associated with various cellular activities, AAA_5	2	5	6.33	91300	*Lactobacillus reuteri* 100-23
2	29376113	DNA-binding protein HU	1	15	9.75	9650	*Enterococcus faecalis* V583
3	30019658	DNA-binding protein HU	1	12	7.93	12485	*Bacillus cereus* ATCC 14579
4	116618389	Nucleoid protein Hbs	1	15	9.52	9664	*Leuconostoc mesenteroides* subsp. mesenteroides ATCC 8293
5	72162530	DNA topoisomerase IV subunit A	1	1	5.40	90359	*Thermobifida fusca* YX
6	30022835	Phosphoenolpyruvate carboxykinase	1	2	5.26	57915	*Bacillus cereus* ATCC 14579
7	149181316	Serine protein kinase	2	3	5.85	72549	*Bacillus* sp. SG-1
8	30018352	Negative regulator of genetic competence clpC/mecB	3	4	6.06	90517	*Bacillus cereus* ATCC 14579
9	67078198	Response regulator aspartate phosphatase	1	2	7.03	35362	*Bacillus cereus* E33L
10	30019432	Cell envelope-bound metalloprotease (camelysin)	1	11	4.71	21801	*Bacillus cereus* ATCC 14579
11	gi|30018407	50S ribosomal protein L17	1	12	10.48	13441	*Bacillus cereus* ATCC 14579
12	30019467	PhaR protein	2	12	5.28	19844	*Bacillus cereus* ATCC 14579
13	42782651	Oligopeptide ABC transporter, oligopeptide-binding protein, putative	2	4	8.75	64166	*Bacillus cereus* ATCC 10987
14	126653094	Hypothetical protein BB14905_12685	2	3	5.26	65314	*Bacillus* sp. B14905
15	89896113	Hypothetical protein DSY3367	1	1	5.09	63797	*Desulfitobacterium hafniense* Y51

16	16080334	Hypothetical protein BSU32820	3	6	5.31	65295	*Bacillus subtilis* subsp. subtilis str. 168
17	167757664	Hypothetical protein CLORAM_03214	1	1	4.84	76123	*Clostridium ramosum* DSM 1402
18	160894620	Hypothetical protein CLOL250_02172	2	8	8.48	26166	*Clostridium* sp. L2-50
19	30019589	Hypothetical protein BC1440	1	14	4.58	10173	*Bacillus cereus* ATCC 14579
20	30022026	Hypothetical Cytosolic Protein	1	10	4.72	14105	*Bacillus cereus* ATCC 14579
21	126664919	Hypothetical protein MELB17_17664	1	4	9.66	21677	*Marinobacter* sp. ELB17
22	56964137	Hypothetical protein ABC2372	1	7	4.58	19576	*Bacillus clausii* KSM-K16
23	74318633	Hypothetical protein Tbd_2615	1	4	5.65	19776	*Thiobacillus denitrificans* ATCC 25259
24	187779657	Hypothetical protein CLOSPO_03253	1	1	9.23	61464	*Clostridium sporogenes* ATCC 15579

MudPIT approach has been used to identify 136 proteins from *Bifidobacterium infantis* (Vitali et al. 2005). In another study by Skipp et al. (2005), total 286 proteins (35% of the predicted proteome) were experimentally verified using MudPIT in *Chlamydia trachomatis* strain L2. These proteins have been categorized based on their involvement in various cellular processes (with the number of proteins identified) like, Amino acid biosynthesis (3), Biosynthesis of cofactors (4), Cell envelope (20), Central intermediary metabolism (3), Cellular processes (23), Energy metabolism (38), Fatty acid and phospholipid metabolism (18), Purines, pyrimidines, nucleosides & nucleotides (9), Regulatory functions (3) Replication (8), Transcription (9), Translation (41), Transport and binding proteins (17), Hypothetical protein (68) and Other (22) In *Haloferax volcanii*, a combination of approaches was used to identify a total of 1296 proteins, representing 32% of the theoretical proteome of the organism. This included separation of proteins/peptides by 2D PAGE, immobilized metal affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC) and MudPIT (Kirkland et al. 2008). Using MudPIT, in *S. cerevisiae*, 1484 proteins have been identified from 5540 peptides (Washburn et al. 2001).

The identified proteins of *Bacillus cereus* GC subgroup A using MudPIT are having their role in various stages of growth and development. Based on their pI, molecular mass and functions, all the proteins have been grouped, as shown in figure 30.

In this study, the expected proteins from MudPIT were more than 135. Due to limitation in the total cellular protein isolation, most of the membrane proteins were not been identified. Although the 135 proteins we identified do not represent a complete analysis of the whole proteome of the logarithmically growing cells of *Bacillus cereus* GC subgroup A, but our method clearly provides a large-scale and global analysis of the proteome of this organism. In addition, we have developed a successful procedure for the protein sample preparation that could be useful in future proteomics studies on *Bacillus cereus* GC subgroup A. Thus, we believe that the scientific impact of our study is considerable both from a biological point of view, integrating the information derived from the genome sequence of *Bacillus cereus* GC subgroup A and from a methodological point of view, providing the first coordinates for further investigations on *Bacillus cereus* GC subgroup A proteome.

One of the most significant features of the MudPIT approach is the capability to identify high mass and basic pI proteins that are generally challenging to visualize using the 2-D PAGE. When emerging quantitative proteomic methods are combined with MudPIT, the large-scale analysis of entire proteome of an organism will be possible (Pasa-Tolic et al. 1999; Oda et al. 1999; Gygi et al. 1999a; Hatizmanikatis and Lee, 1999; Münchbach et al. 2000).

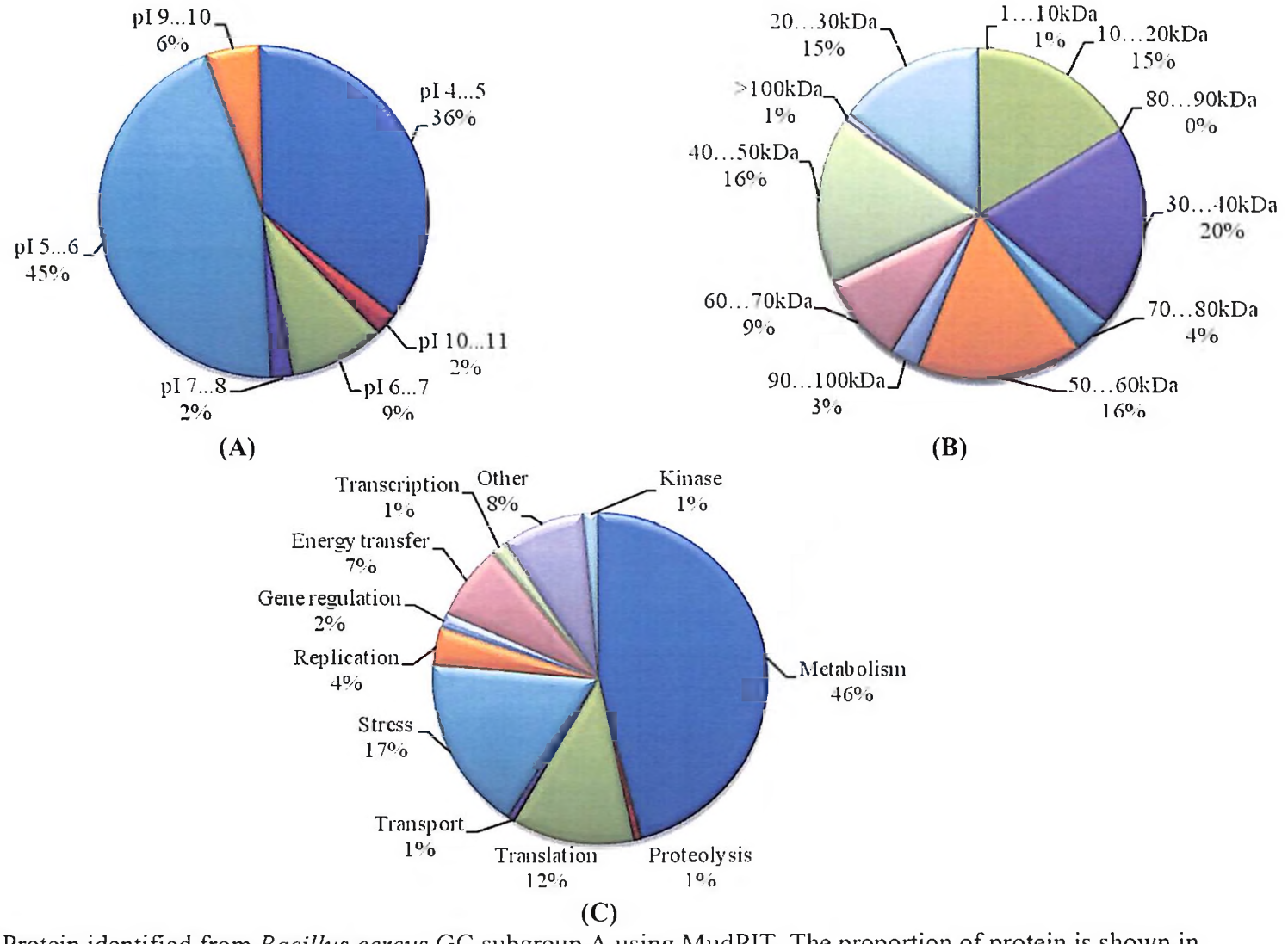


Fig. 30. Protein identified from *Bacillus cereus* GC subgroup A using MudPIT. The proportion of protein is shown in percentage. Distribution of proteins based on their (A) pI, (B) Molecular mass and (C) functions.

4.3.4 Identification and characterization of copper (II) induced protein

To understand the mechanism of heavy metal resistance, the total proteins were separated on SDS-PAGE (1D PAGE) and studied in detail by proteomic analysis. As the candidate organism (*Bacillus cereus* GC subgroup A) has been isolated from a copper rich soil so it is point of interest to study copper resistance mechanisms. To know the minimum inhibitory concentration (MIC), cells were grown in varying concentration of copper (II) and MIC was recorded to be 7.0mM (Jain et al. 2009). Copper homeostasis in bacteria is mainly by the action of active efflux systems that remove the metal ion from the cell (Nies, 2003; Rensing and Grass, 2003; Magnani and Solioz, 2005; Moore and Helmann, 2005). Among these systems, transporters of the P-type ATPases family (Kuhlbrandt, 2004; Arguello et al. 2007) are usually involved.

To study the metal induced protein in *Bacillus cereus* GC subgroup A, a combination of stress (copper(II) & elevated temperature) was given as the sample site experiences a wide range of temperature variations (+1.0 to 45°C). Therefore, cells were grown in medium containing varying concentrations of copper at different temperatures and total proteins of the cells were analyzed by SDS-PAGE. Among all these combinations of stress (as described in methods section) a clear induction of a protein band in 1.5mM of Cu (II) at 40°C was observed, as shown in figure 31. The induction of this protein was observed in varying amount in rest of the combinations, as shown in Table 35. The molecular mass of the induced protein was expected to be 82 kDa as per information from protein-gel.

For the identification and characterization of the induced protein, ingel-digestion followed by mass spectrometry was performed. The desired protein band was digested and analyzed as described in method section and for each peptide, mass spectra were obtained as shown in figure 32. The obtained results of mass spectrometry were searched in NCBInr database, as shown in Table 36. This protein was confirmed to be Formate acetyltransferase, based upon mass (84.855 kDa), sequence coverage (11%), score (168) and bacterium *Bacillus cereus* ATCC 14579 (Ivanova et al. 2003). Matched peptides for this protein have been shown in figure 33.

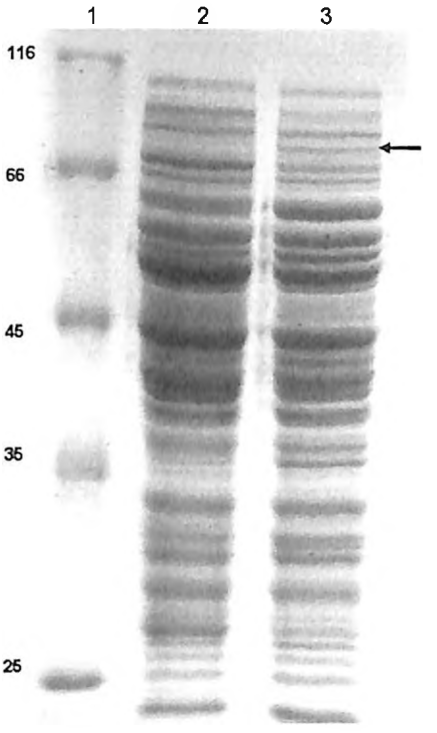
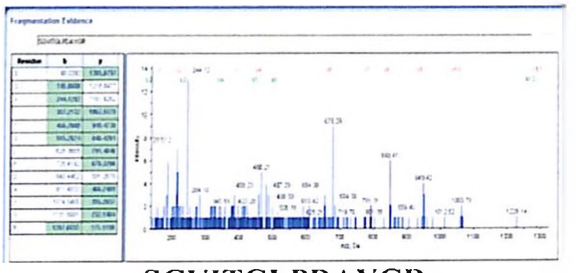


Fig. 31. Differential expression of *Bacillus cereus* GC subgroup A under stress conditions.

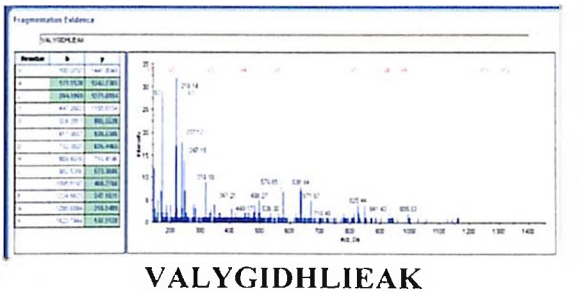
- Lane 1, Protein molecular weight marker (kDa);
- lane 2, *Bacillus cereus* GC subgroup A (80µg of total protein) grown at 40°C without Cu(II); lane 3, *Bacillus cereus* GC subgroup A grown in medium supplemented with Cu(II) (1.5mM) at 40°C.
- Samples were electrophoresed on a 12% polyacrylamide gel and stained with Commassie brilliant blue G-250.
- Arrow shows a protein band induced under stress conditions.

Table 35. The induction pattern of desired protein in *Bacillus cereus* GC subgroup A. It was grown in different concentration of copper(II) at varying temperatures. (+) shows the induction of protein and (-) shows no induction. +, ++, +++ and ++++ showing the increase in the amount of induced protein, respectively, which can be detected on SDS PAGE.

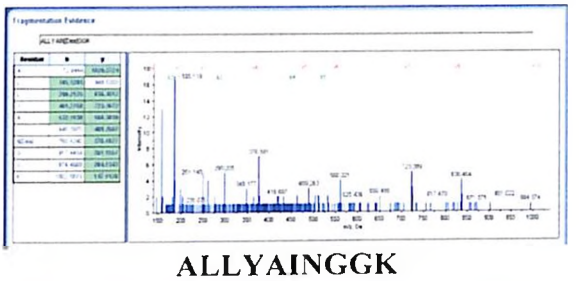
Cu(II) (mM)/Temp. (°C)	**37°C**	**38°C**	**39°C**	**40°C**	**41°C**	**42°C**
0.5mM	-	-	-	-	-	-
1.0mM	-	-	-	+	+	
1.5mM	-	+	+++	++++	++	-
2.0mM	-	+	+	++	+	-
2.5mM	-	-	-	+	-	-
3.0mM	-	-	-	+	-	-
3.5mM	-	-	-	-	-	-
4.0mM	-	-	-	-	-	-
4.5mM	-	-	-	-	-	-
5.0mM	-	-	-	-	-	-



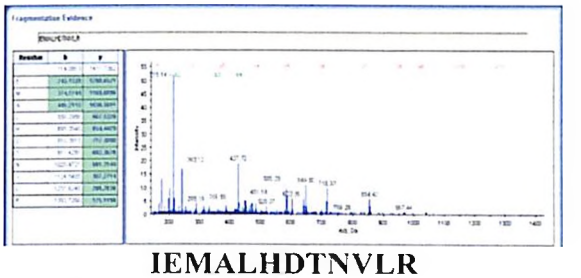
SGVITGLPDAYGR



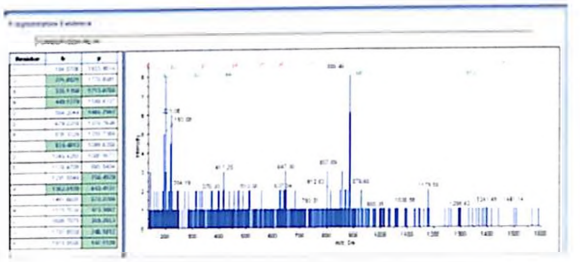
VALYGIDHLIEAK



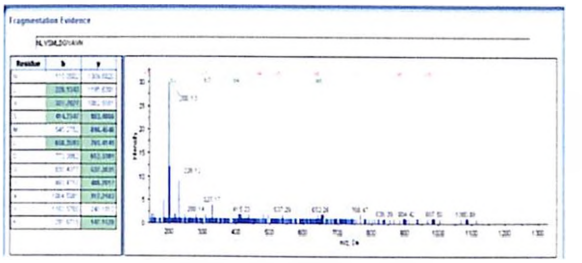
ALLYAINGGK



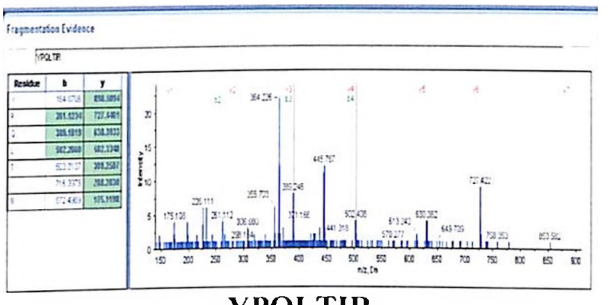
IEMALHDTNVLR



YGNNDDRVDEIAVNLVK



NLVSMLDGYAVK



YPQLTIR

Fig. 32. Identified peptides using MALDI-TOF/MS: Spectra or peptide mass fingerprints.

Table 36. Protein identification by MS/MS analysis and data base searching using Mascot. Top five matches found for the protein have been shown in detail.

Accession	Name	Species	Score	Calculated pI value	Queries matched	Mass (Da)
gi|30018697	Formate acetyltransferase	*Bacillus cereus* ATCC 14579	168	5.51	7	84855
gi|47567276	Formate acetyltransferase	*Bacillus cereus* G9241	153	5.59	6	84693
gi|6730181	Chain A, Crystal Structure of C418a,C419a Mutant Of *Pfl*	*E. coli*	57	5.69	2	85457
gi|116249795	hypothetical protein RL0029	*Rhizobium leguminosarum* bv. viciae 3841	35	5.56	1	56485
gi|67922569	DegT/DnrJ/EryC1/StrS aminotransferase	*Crocosphaera watsonii* WH 8501	33	5.30	1	41570

```
  1 MTQVLENVKN AWENFKGEKW KAEIDVRDFI LNNVNVFEGD ESFLAEATEA
 51 TKQLWDQVMD LTTKERENGG VLDMDTKIVS SITSHDPGYL NKDIEKVVGF
101 QTDKPFKRSL QPYGGIRMAE QACESYGYEM DKELSRIFRE WRKTHNQGVF
151 DAYTPEMRNA RKSGVITGLP DAYGRGRIIG DYRRVALYGI DHLIEAKKAD
201 LNLTGGVMSE DTMRLREELS EQMRALQELK EMAASHGFDI SKPATNAQEA
251 FQWLYFAYLA AIKEQNGAAM SLGRTSTFLD IYIERDLANG TLTEEEVQEI
301 VDHFIMKLRL VKFARTPDYN ELFSGDPTWV TESIGGMALD GRPLVTKNSF
351 RFLHTLDNLG PAPEPNLTVL WSKQLPENFK NYCAKMSIKT SAIQYENDDI
401 MRPEYGDDYG IACCVSAMRI GKQMQFFGAR ANLAKALLYA INGGKDEKSK
451 AQVGPEYAPI TSEVLNYEEV MHKFDMTMEW LAGLYLNTLN VIHYMHDKYS
501 YERIEMALHD TNVLRTMATG IAGLSVVADS LSAIKYAKVK PIRDENGIAV
551 DFEIEGDFPK YGNNDDRVDE IAVNLVKTFM NKLRKHTTYR NSVHTMSILT
601 ITSNVVYGKK TGNTPDGRRT GEPFAPGANP MHGRDTKGAL ASLLSVAKLP
651 YEDAQDGISN TFSIIPKALG KEDDVQVRNL VSMLDGYAVK EGHHLNINVF
701 NRETLMDAME HPEKYPQLTI RVSGYAVNFI KLTREQQIDV INRTMHESM
```

Fig. 33. Peptide sequence of Formate acetyltransferase: Matched peptides have been shown in bold-Red color

The total amino acid composition of identified formate acetyltransferase was determined using BioEdit version 7.0.5.2 (Hall, 1999) (figure 34). Structure of formate acetyltransferase was predicted using Swiss Model (http://swissmodel.expasy.org/workspace) and Modeller 9v2 (Sali and Blundell, 1993) as shown in figure 35.

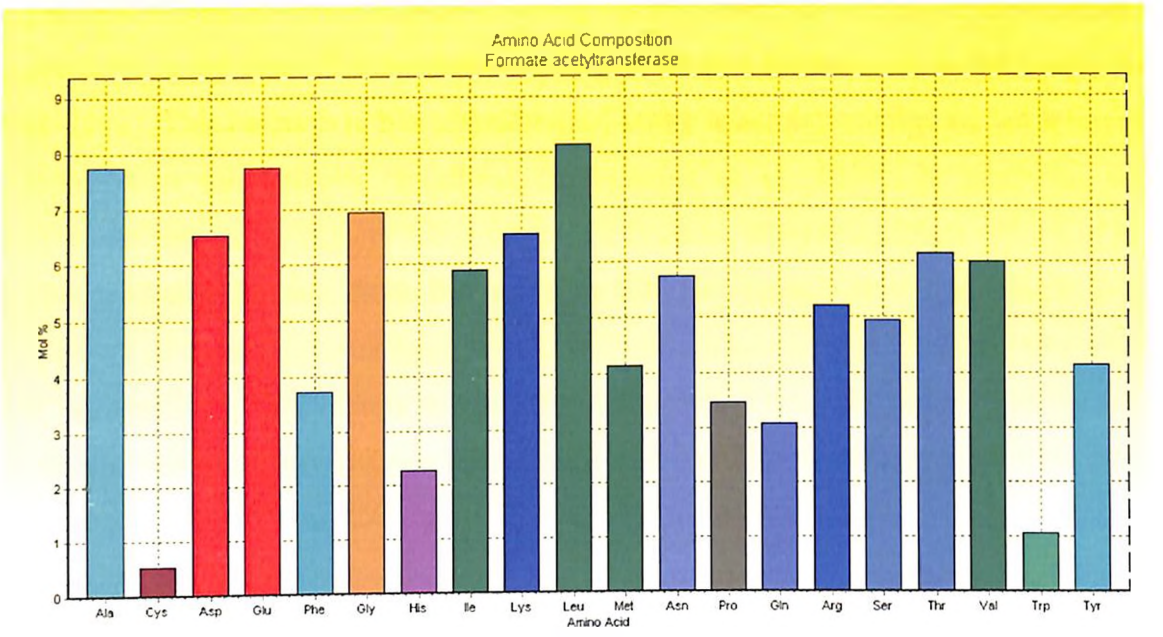


Fig. 34. The total amino acid composition of formate acetyltransferase.

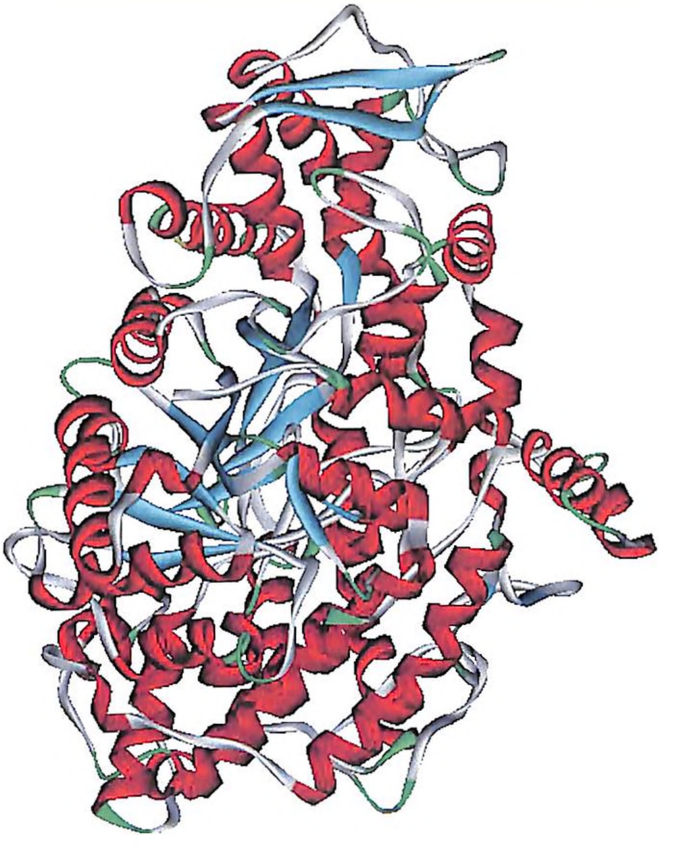


Fig. 35. Predicted protein structure of Formate acetyltransferase

Formate acetyltransferase, a carbon metabolism enzyme that is also known as Pyruvate formate lyase is encoded by *pfl* and consists of 744 amino acids. The catalytic principle of Formate acetyltransferase is unique among enzyme reactions of cellular metabolism and it is involved in the reversible breakdown of pyruvate into formate and acetyl CoA (Wagner et al. 1992). This reaction is best characterized under anaerobic conditions but it has also been reported in microaerobic condition (Kirkpatrick et al. 2001). In anaerobic condition, pyruvate acts as an inducer for synthesis of Formate acetyltransferase (Wong et al. 1989). The produced formate from this reaction is toxic organic molecule, which is a known inhibitor of 2-ketoglutarate dehydrogenase (SucB) a key enzyme of TCA cycle; therefore, it is necessary to remove this formate from the cell. There are three ways by which this formate can be removed from the cell: direct excretion, anaerobic conversion by Formate hydrogen lyase to form CO_2 and H_2O and high level of formate could also drive reversible pyruvate formate lyase making pyruvate under microaerobic conditions (Kirkpatrick et al. 2001). Thus, produced pyruvate can be utilized for energy production in aerobic conditions by TCA cycle (Alexeeva et al. 2000). In *Bacillus cereus* and *Bacillus anthracis*, Formate acetyltransferase is involved in propanoate metabolism.

Formate acetyltransferase of molecular mass 90.8kDa, pI 5.3, encoded by *pfl* and having putative function in carbohydrate metabolism have been identified in *Bifidobacterium longum* NCIMB 8809 upon stress conditions of 1.2g/L bile salt (Borja et al. 2005). But apart from the carbon metabolism, Formate acetyltransferase have been involved in copper homeostasis through negative regulation by *cpxR* and in iron homeostasis as miscellaneous function in *E. coli* K12 (Kershaw et al. 2005). This enzyme was found to be induced in *S. aureus* upon the nitric oxide stress (Hochgrafe et al. 2008). A putative Formate acetyltransferase (encoded by *pflB*) was identified as being upregulated at the protein level and found to be induced at transcription level, upon exposure to chromium in *Shewanella oneidensis* (Brown et al. 2006). In *Lactobacillus plantarum* under the conditions of lactic acid and osmotic stress (pH 6.0, 800mM NaCl, μ_{max} 0.4, dilution rate 0.3) the expression level of Formate acetyltransferase activating enzyme and Formate acetyltransferase was found to increase by 3.3 fold and 1.8 fold respectively (Pieterse et al. 2005). We have reported the induction of Formate acetyltransferase (mass 84.855 kDa; sequence coverage

11%; score 168) upon the combination of stress (copper (II) & temperature 40°C) in *Bacillus cereus* GC subgroup A, which further strengthen its role in copper stress regulation.

The synthesis of Formate acetyltransferase indicates that the cells experience oxygen-limiting conditions. For this one possible explanation is that under metal (Cu (II)) and temperature stress, the Cu (II) enters inside the cell by copper specific uptake machinery. Inside the cell, it reacts with the elemental oxygen to form cupric oxide (CuO) (Nies, 1999). Due to the formation of CuO, cells experience slightly anaerobic or microaerobic conditions momentarily. In this situation, cell triggers the synthesis of Formate acetyltransferase and this converts pyruvate into formate and acetyl CoA reversibly. As discussed earlier, the produced formate is toxic to the cell so in this situation, the Formate acetyltransferase catalyzes the reversible reaction from formate back into pyruvate, which can be utilized for energy production in later stages. By using this mechanism, cells can nullify the effect of copper and temperature stress, which lead to microaerobic condition inside the cell and thus channelizing the metabolites to energy production, which can be utilized in metabolic functions. The induction of Formate acetyltransferase and its role in copper homeostasis is an additional mechanism to the existing one for *Bacillus cereus* GC subgroup A.

The characterization of *Bacillus cereus* GC subgroup A was done by proteomics through SDS-PAGE, 2D PAGE (611 spots) and Multidimensional Protein Identification Technology (135 proteins). The proteomic analysis confirmed the induction of number of stress proteins, which are required by cell to maintain homeostasis under stress conditions. Formate acetyl transferase of a molecular mass 84kDa & pI 5.51 was identified by MADI-TOF/MS coupled with LC from *Bacillus cereus* GC subgroup A, under stress conditions of Cu (II) 1.5mM & 40°C. Formate acetyl transferase is one of the stress proteins and it is also involved in anaerobic metabolism. As *Bacillus cereus* GC subgroup A is a facultative anaerobe and had undergone stress of temperature and Cu (II), which again prove its significance. To the best our knowledge, this is the first report of induction of Formate acetyl transferase under Cu (II) & 40°C stress from *Bacillus cereus* GC subgroup A.

Chapter V

Summary

Chapter V

SUMMARY

Environmental pollutants like metals, antibiotics, organic and inorganic chemicals act as selective pressure in the evolution of bacteria, plants, animals and humans. It has been well documented that metal pollution results in selective pressure and leads to the development of multiple-metal/antibiotic resistance among bacterial pollution. Microbial flora of the metal contaminated sites alters the metal speciation, bioavailability and metal toxicity. Moreover, metal polluted environments also act as a source point for the emergence of new multiple antibiotic resistant bacteria through horizontal gene transfer. This study was aimed to explore a copper mining industry for the presence of multiple metal and antibiotic resistant bacterial strains and their characterization for stress tolerance. To carry out the study a metal mining and handling industry, Khetri copper complex was selected, which is located 50km from Pilani and works under the HCL. The major findings of this research are as follows:

1. A total of 24 bacterial strains were isolated from soil, rock, deposits and waste disposal sites of a metal polluted site, Khetri copper mine complex, Rajasthan. The soil analysis showed that this site is rich in metal ions say, Copper (Cu) followed by Manganese (Mn), Zinc (Zn), Cobalt (Co), Nickel (Ni), Chromium (Cr) and Cadmium (Cd). The waste disposal or effluent was characterized by pH (4.81), total dissolved solutes (3.56 gm/L) and the presence of very high concentration of Cu (141.5ppm) and traces of other metal ions.
2. Isolated bacterial strains were identified by Gram's staining and 16s rRNA sequence analysis. The obtained sequences were submitted to NCBI GenBank under the Accession nos.: EU723845 (PRS1), EU723846 (PRS2), EU744603 (PRS3), EU744601 (PRS4), EU744602 (PRS5), EU744604 (PRS6), EU744605 (PRS7), EU744606 (PRS8), EU744607 (PRS9), EU744608 (PRS10), EU744609 (PRS11), EU744610 (PRS12), EU744611 (PRS13), EU744612 (PRS14), EU744613 (PRS15), EU744614 (PRS16), EU744615 (PRS17), EU744616 (PRS18), EU744617 (PRS19), EU744618 (PRS20), EU744619 (PRS21), EU744620 (PRS22), EU744621 (PRS23) and EU744622 (PRS24).

3. These all-bacterial strains were characterized for their metal and antibiotic resistance properties. Out of 24, most of the strains showed several fold higher resistance to a number of metals/antibiotics as compared to *E. coli* and *B. cereus*. As properties of metal and antibiotic resistance are largely conferred by extrachromosomal elements like plasmids so bacterial strains were screened for the presence of plasmid DNA and 6 strains (PRS2, PRS3, PRS8, PRS10, PRS13 and PRS18) were found to have a plasmid DNA. Out of this only one strain PRS3 had a plasmid DNA (pPRS3a) of 4.2kb, confirmed by restriction digestion, whereas, other strains had mega plasmids ranging in size 50-200kb. PRS3 was selected for further characterization as it harbors a plasmid of manageable size, which can be easily manipulated.
4. PRS3 identified by 16s rRNA as *Bacillus* sp., further confirmed by phenotypic, biochemical and fatty acid methyl esters (FAME) analysis and found to be *Bacillus cereus* GC subgroup A. This strain was characterized by growth, biomass and total proteins in different medium (NB & LB) and ultra-structural studies by SEM & TEM were carried out. This bacterial strain was deposited at IMTECH, under the accession no. MTCC 10207, in the name of Dr. S.K. Verma, BITS-Pilani.
5. *Bacillus cereus* GC subgroup A plasmid (pPRS3a) was cloned into pET-28c(+) and sequenced through primer walking. The sequence analysis confirmed size of plasmid to be 4126bp, presence of Amp^r gene, unique restriction site for 30 restriction endonuclease enzymes, promoters and origin of replication. It also found that pPRS3a has origin of replication (ori) which can be recognized by gram positive/negative bacteria i.e. this plasmid can be stably maintained in gram positive/negative bacterium that signifies its importance to be used as shuttle vector. After the complete analysis of pPRS3a, the sequences were submitted to NCBI GenBank under the accession no. GQ404376.1.
6. To confirm expression properties of pPRS3a, two foreign genes, arsGFP with lac promoter (source: pJSKV51, an arsenic biosensor) and cat (source: pKT210, Cm^r) were ligated and successfully maintained & expressed. The expression of arsGFP and *cat* gene showed induction of ~31kDa and ~24kDa protein bands respectively, which strengthens the fact that plasmid, can have potential applications as an expression vector in molecular biology.

7. Attempts were made to cure pPRS3a with curing agents such as Ethidium Bromide and Proflavin to further confirm that property of antibiotic resistance is due to presence of plasmid DNA. However, up to 25th generation, in both the curing agents it could not be cured and pPRS3a could maintain its normal functional properties especially, Amp^r marker, which proves that it, is a naturally occurring plasmid having important physiological significance.
8. The characterization of *Bacillus cereus* GC subgroup A was done by proteomics through SDS-PAGE, 2D PAGE (611 spots) and Multidimensional Protein Identification Technology (135 proteins). The proteomic analysis confirmed the induction of number of stress proteins, which are required by cell to maintain homeostasis under stress conditions.
9. Formate acetyl transferase of a molecular mass 84kDa, pI 5.51, score 168, was identified by MADI-TOF/MS coupled with LC, from *Bacillus cereus* GC subgroup A, under stress conditions of Cu(II) 1.5mM & 40°C. Formate acetyl transferase is one of the stress proteins and it is also involved in anaerobic metabolism. As *Bacillus cereus* GC subgroup A is a facultative anaerobe and had undergone stress of temperature and Cu(II), which again prove its significance.

To the best our knowledge, this is the first report of identification and characterization of an endogenous plasmid DNA (pPRS3a) and induction of Formate acetyl transferase under Cu(II) & 40°C stress from *Bacillus cereus* GC subgroup A.

In this study, we have explored a multiple metal and antibiotic resistant *Bacillus cereus* GC subgroup A for the presence of plasmid DNA with an antibiotic resistance marker. Differential expression of proteins under stress conditions was studied and a protein formate acetyl transferase was found to have important function. Mobile genetic elements like, plasmid, gives chances of horizontal gene transfer from one population to another. This study strongly emphasizes the need for metal laden waste treatment and safe disposal to prevent passage of such potential pathogens into human population. Such comprehensive studies may give an insight into the role of environmental factors in the evolution of pathogenic strains, which can become a threat to human health and have serious clinical implications.

FUTURE SCOPE OF THE WORK

There is a need for characterization of natural plasmids for the construction of stable shuttle cloning and expression vector. These plasmids harbor stable metal/antibiotic resistance markers, stable promoters and origin of replication, which can be recognized by Gram's positive/negative bacterial systems. The identified and characterized plasmid, pPRS3a from *Bacillus cereus* GC subgroup A, can be used for the construction of stable shuttle expression vector, by utilizing the properties of ampicillin marker, promoters and origin of replication for industrial applications.

In *Bacillus cereus* little is known about the mechanisms of metal resistance and metal homeostasis. The identification of Formate acetyltransferase from *Bacillus cereus* GC subgroup A upon copper stress, suggest its role in copper homeostasis. This finding opens up the areas of research in the finding of relationship between carbon metabolism and metal stress.

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Appendix I

LIST OF PUBLICATIONS

(a) Patent

S. Ramachandran, **Pankaj Kumar Jain** and Sanjay Kumar Verma (2009) Rapid non-enzymatic process for the reduction of chromium(VI) to chromium(III) by the *Nostoc calcicola* immobilized on silica gel. Indian Patent, Application No.: 582/DEL/2008, Date of filing of Application: 10/03/2008, Publication Date: 18/09/2009, Journal No.: 38/2009: 5597

(b) Manuscripts published/submitted:

1. **Jain PK,** Kush D, Ramachandran S and Verma SK (2011) Isolation and characterization of R-plasmid pPRS3a from *Bacillus cereus* GC subgroup A PRS3. Intl J Integrative Biol 11(1): 1-7

2. **Jain PK,** Ramachandran S, Shukla V, Bhakuni D and Verma SK (2009) Characterization of metal and antibiotic resistance in the bacterial population isolated from copper mining industry. Intl J Integrative Biol 6(2): 57-61

3. **Jain PK,** Ramachandran S and Verma SK (2009) *Bacillus cereus* plasmid pPRS3a, complete sequence. NCBI GenBank Accession GQ404376.

4. Scaria J, Ramachandran S, **Jain PK** and Verma SK (2009) Construction and testing of EGFP based bacterial biosensor for the detection of residual tetracycline in milk and water. Res J Microbiol 4(3): 104-111

5. Ramachandran S, Coradin T, **Jain PK** and Verma SK (2009) *Nostoc calcicola* immobilized in silica-coated calcium alginate and silica gel for applications in heavy metal biosorption. Silicon 1: 215-223

6. **Jain PK,** Ramachandran S and Verma SK (2011) Cu(II) induced Formate acetyltransferase from *Bacillus cereus* GC subgroup A. Biometals (submitted)

(b) Publications in Proceedings/Symposia/Conferences

1. **Jain PK,** Deepshikha, Ramachandran S and Verma S K (2009) Complete sequence analysis of a plasmid DNA pPRS3a from a multiple metal & antibiotic resistance *Bacillus* sp., 29th Annual Conference of STOX, Food and Drug Toxicology Research Centre National Institute of Nutrition, Hyderabad, India, p. 114

2. **Jain PK**, Ramachandran S and Verma SK (2009) Characterization of endogenous plasmid DNA and heavy metal induced proteins of multiple metal/antibiotic resistance bacterial strains, National Conference on Emerging Trends in Life Science Research March 6-7, 2009, Department of Biological Sciences Group and Pharmacy Group, Birla Institute of Technology & Science, Pilani -333031 Rajasthan, India, p. 19

3. **Jain PK**, Ramachandran S, Rai A, Shukla V, Bhakuni D, Rai VK and Verma SK (2008) Isolation and Characterization of multiple metal/antibiotic resistant bacterial strains from rock and soil samples of a copper mining industry, 14th International Symposium on Biodeterioration and Biodegradation, Oct. 6 - 11, Hotel Capo dei Greci, Sant'Alessio Siculo, Messina, Italy.

4. Shukla V, Bhakuni D, **Jain PK**, Ramachandran S, Rai A, Rai VK and Verma S K (2008) Prevalence of antibiotic resistance in the bacterial population of a copper mining industry, International Conference on Molecular Biology & Biotechnology Oct. 19-21, Department of Bioscience and Biotechnology, Banasthali University, Banasthali Vidyapith, Rajasthan, India, p. 128

5. **Jain PK**, Ramachandran S, Shukla V, Bhakuni D and Verma SK (2008) Molecular evolution of antibiotic resistance in bacteria and their impact on human health, 28th Annual Conference of Society of Toxicology, India and International Symposium on "Monitoring and Modulating Global Resources of Environmental and Food Contaminants: Nature versus Chemicals" October 16-18, Department of Pharmacology & Toxicology College of Veterinary Science Guru Angad Dev Veterinary and Animal Sciences University Ludhiana, India, p. 36

6. **Jain PK**, Ramachandran S, Rai A and Verma SK (2008) Mesoporous silica gel immobilized multiple metal resistant bacteria expressing metallothionein for heavy metal bioremediation, 2nd International Conference on Trends in Cellular and Molecular Biology, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India, p. 138

7. Ramachandran S, **Jain PK**, Prasanna A, Verma SK (2007) Chromium(VI) and Zinc(II) regulated protein expression in the cyanobacterium *Nostoc calcicola*, ASM 107th General Meeting, May 21-25, Toronto, Canada.

8. Ramachandran S, Coradin T, **Jain PK** and Verma SK (2007) Mesoporous silica and silica-alginate composite materials for biotechnological applications, Indo-Australian Symposium on Multi Functional Nanomaterials Nanostructures and Applications, Department of Physics and Astrophysics, University of Delhi, Delhi, India, p. 65

9. **Jain PK**, Rai VK, Rai A, Ramachandran S and Verma SK (2007) Multiple antibiotic resistant bacterial strains from a copper mining industry: an environmental health hazard, 27th Annual Conference of Society of Toxicology, India, Department of Pharmacology & Toxicology, Veterinary College, Bangalore, Karnataka, India, p. 90

10. Ramachandran S, Coradin T, **Jain PK** and Verma SK (2007) Mesoporous silica and silica-alginate composite materials for biotechnological applications. Indo-Australia Symposium on Multifunctional Nanomaterials Nanostructures and Applications. Dec 19-21, University of Delhi. p. 65

11. **Jain PK**, Ramachandran S and Verma SK (2007) Chromium(VI) induced mutagenesis in the cyanobacterium *Nostoc calcicola*. Intl. Sym. on Applied Phycology and Environmental Biotechnology, Oct 29-31, BITS-Pilani. p. 28

12. Ramachandran S, **Jain PK** and Verma SK (2006) Chromium(VI) induced damage to the cytoskeleton and mutagenesis in *Nostoc calcicola,* International Conference on Toxicology, Toxicogenomic and Occupational Health, STOX, Oct 9-11, School of Studies in Zoology, Jiwaji University, Gwalior, pp. 30-31

13. Ramachandran S, **Jain PK** and Verma SK (2006) Metal biosorption by silica gel immobilized *Nostoc calcicola*, All India Seminar on Advances in Botanical, Biotechnological and Microbiological Research in India (AISBBMR), M.N. Institute of Applied Sciences, University of Bikaner, Bikaner, Rajasthan, India. pp. 43

14. **Jain PK**, Ramachandran S and Verma SK (2006) Cytotoxic and genotoxic properties of chromium(vi) in the cyanobacterium *Nostoc calcicola,* International Conference on Applied Phycology-Algae in Biotechnology and Environment, Feb 14-15, Department of Botany University of Delhi, pp. 258

15. Verma SK, **Jain PK** and Ramachandran S (2006) Biosorption technology for removal of Co^{2+}, Cu^{2+}, Zn^{2+} and Sr^{2+}, Proceedings of National Conference on Environmental Conservation, Sept 1-3, Birla Institute of Technology and Science, Pilani, pp. 543-550

Appendix II

Biography of Prof. Sanjay Kumar Verma

Prof. Sanjay Kumar Verma has obtained master's degree in Genetics from Banaras Hindu University (BHU), Varanasi with specialization in cyanobacteria and applied phycology before completing Ph.D in the area of Environmental Biotechnology from the same university. He worked as post-doctoral research fellow at University of Hyderabad in the area of microbial and molecular genetics. Prof. Verma has handled several research projects related to bioremediation and biodegradation of toxic industrial waste, development of recombinant bacterial biosensors for environmental applications, funded by Bhabha Atomic Research Centre (BARC), University Grants Commission (UGC), Council of Scientific and Industrial Research (CSIR) and Department of Science & Technology (DST). Currently, he is working as Professor, Department of Biological sciences, Birla Institute of Technology & Science, Pilani. His major research interest lies in molecular biology and environmental biotechnology.

Biography of Mr. Pankaj Kumar Jain

Mr. Pankaj Kumar Jain completed M.Sc. Biotechnology from AAIDU, Allahabad and M.E. Biotechnology from Birla Institute of Technology and Science, Pilani. He has continued for Ph.D program of this institute from 2006-2010. He was a recipient of Council Scientific and Industrial Research (CSIR) senior research fellowship. Pankaj Kumar Jain has an active interest in environmental biotechnology, genomics and proteomics. He has also been involved in teaching various courses of Biological sciences group, BITS-Pilani. He has published research articles in renowned International journals and presented papers in various National and International conferences.

Appendix III

MTCC Certificate

प्रतिरुप-मानक सूक्ष्मजीव संग्रहण एवं जीन बैंक
MICROBIAL TYPE CULTURE COLLECTION & GENE BANK
सूक्ष्मजीव प्रौद्योगिकी संस्थान
सैक्टर 39 ए, चण्डीगढ 160036, भारत
INSTITUTE OF MICROBIAL TECHNOLOGY
(A CONSTITUENT ESTABLISHMENT OF CSIR)
Sector 39-A, Chandigarh-160 036, INDIA

Dr. Suresh Korpole
Scientist

Date: 20-10-09

To,
Dr. S.K. Verma
Centre for Biotechnology
Biological Sciences Group
Faculty Division III
Birla Institute of Technology and Sciences
Pilani-333031
Rajasthan.

Dear Sir,

Please find enclosed herewith the results of identification of your strain. Based on biochemical and physiological characteristics the isolate was identical with the following strain:

Sl. No.	Strain designation	Identity	MTCC number*
1	PRS-3	***Bacillus cereus***	**MTCC 10207**

A detailed list of results is annexed for your kind information. Your strain has also been deposited with the MTCC accession number given in the above table.

Thanking you for your interest in MTCC

Best regards

Yours Sincerely

(Dr. Suresh Korpole)

Phones : +91-172-2690562, 2695215
दूरभाष : 2695216, 2695219
2695225, 2695226
2695227, 2690908
Fax : +91-172-2690632
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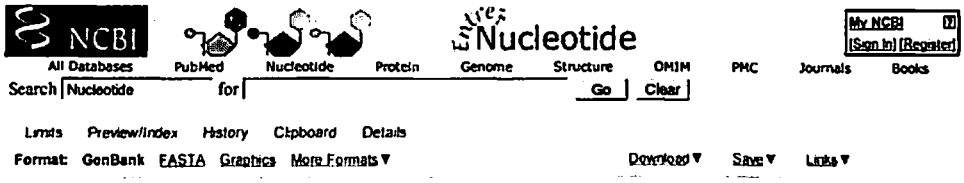
Gram तार : IMTECH
E-mail : curator@imtech.res.in
Web : http://mtcc.imtech.res.in

Appendix IV

Plasmid NCBI GenBank Submission

Nucleotide - Bacillus cereus plasmid pPRS3a, complete sequence



GenBank: GQ404376.1

Bacillus cereus plasmid pPRS3a, complete sequence

Change Region Shown
Customize View
Analyze This Sequence
Run BLAST
Pick Primers
Recent activity
All links from this record

Features Sequence

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  ORGANISM  Bacillus cereus
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Bacillus
            cereus group.
REFERENCE   1  (bases 1 to 4126)
  AUTHORS   Jain,P.K., S,R. and Verma,S.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2009) Biological Sciences Group, Birla Institute
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