Plasmidome of *Acinetobacter baumannii* DS002: Characterization and development of tools for genetic manipulation

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CERTIFICATE

This is to certify that **Mr. C. Toshisangba** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis entitled **"Plasmidome of *Acinetobacter baumannii* DS002: Characterization and development of tools for genetic manipulation"** for submission for the degree of Doctor of Philosophy in Animal Sciences of this University.

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Declaration

I hereby declare that the work embodied in this thesis entitled entitled **"Plasmidome of *Acinetobacter baumannii* DS002: Characterization and development of tools for genetic manipulation"** has been carried out by me under the supervision of Prof. S. Dayananda and this has not been submitted for any degree or diploma of any other university earlier.

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INTRODUCTION

Acinetobacter sp. are of great interest owing to the diverse habitats they colonize, their involvement in epidemic outbreaks in hospitals and their various metabolic abilities like catabolism of aromatic compounds and degradation of hydrocarbons in oil spills (Reams and Neidle, 2003; Minas and Gutnick, 1993; Fischer et al., 2008). The genus *Acinetobacter* comprises gram-negative, strictly aerobic, nonfermenting, nonfastidious, nonmotile, catalase-positive, oxidase-negative bacteria and belongs to family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genera *Moraxella*, *Acinetobacter*, *Psychrobacter*, and related organisms (Rossau et al., 1991). Many studies within the genus *Acinetobacter* have been performed with clinical isolates. However, Acinetobacters are also ubiquitous organisms in soil, water and sewage (Towner, 1996). It has been estimated that Acinetobacter may constitute as much as 0.001% of the total heterotrophic aerobic population of soil and water (Baumann, 1968). They have been found at densities exceeding 104 organisms per 100 ml in freshwater ecosystems and 106 organisms per 100 ml in raw sewage (LaCroix and Cabelli, 1982). They can be isolated from heavily polluted water, such as that found in wastewater treatment plants, but are found more frequently near the surface of fresh water and where fresh water flows into the sea (Droop and Jannasch, 1977). A significant population difference between the Acinetobacters found in clinical and other environments has been observed, such that the vast majority of clinically significant isolates belong to the *A. calcoaceticus-A. baumannii* complex, whereas genomic species 7 (*A. johnsonii*), 8 (*A. lwoffii*) and 9 seem to predominate in foods and the environment (Towner 2006).

Acinetobacter baumannii is known to be an opportunistic pathogen and is implicated in nasocomial infections, bacterimea, secondary meningitis, urinary tract

infections and ventilator associated pneumonia etc., (Towner, 2009). Since 1970's, Acinetobacters were known to have an evident role in multi drug resistance. The increasing resistance patterns of *Acinetobacter baumannii* against many clinically important antibiotics were attributed to its proclivity to acquire and spread the drug resistance genes among microbial community (Woodford et al., 2011).

In terms of application, an important advantage is that Acinetobacters are easy to isolate, cultivate and manipulate genetically in the laboratory. *Acinetobacter* sp. has received a considerable attention as possible industrial microorganisms due to the general biochemical versatility shown by members of this genus (Towner, 2006). Particularly, the normal soil and water habitats of many strains, combined with their ability to degrade a wide range of organic compounds, has suggested that Acinetobacters can be used for bioremediation of numerous hazardous and unpleasant waste and residue pollutant compounds produced as by-products of commercial processes (Towner, 2006). Thus, aromatic compounds which are toxic to most microorganisms, such as salicylate, halogenated aromatics and phenol, are degraded by Acinetobacters (Schirmer et al., 1997). In many cases, total degradation occurs by the synergic action of complex microbial communities in which Acinetobacters form an important component. Plasmids contribute for survival of bacterial communities under adverse physiological conditions. In *Acinetobacter baumannii* sp. multiple plasmids have been detected (Minas and Gutnick, 1993) due to their natural competence for acquiring both circular and linear DNA molecules (Ramirez et al., 2010; Woodford et al., 2011).

Though it is considered to be a rare event, existence of multiple plasmids in a single bacterial cell is not an unusual phenomenon (Velappan et al., 2007). Plasmids of different sizes, genetic information and those, which differ with respect to their ability to mediate

horizontal genetic transfer, have been shown to exist in a single bacterial cell (Valenzuela et al., 2007). Existence of multiple plasmids, if seen in view of plasmid incompatibility, is an interesting aspect of plasmid biology and suggests prevalence of diversity in their replication and segregation (Willetts, 1972). In view of its clinical significance, the complete genome sequence was determined for a number of *A. baumannii* strains isolated from different geographical regions and in most of them existence of multiple plasmids was reported (Minas and Gutnick, 1993). Several studies have reported that more than 80 % of *Acinetobacter* isolates carry multiple indigenous plasmids of variable molecular size (Gerner-Smidt et al., 1989; Seifert et al., 1994). Though reports of detecting indigenous plasmids are plenty, the detailed study have purely confined to plasmids associated with resistance to antibiotics. Apart from antibiotic resistance, genes encoding resistance to heavy metals (Kholodii et al., 1993) and important metabolic steps in the degradation of organic compounds and environmental pollutants, such as polychlorinated biphenyls (PCBs), have been shown to be carried on plasmids in Acinetobacter (Towner, 1991; Fujii et al., 1997).

Study of plasmid biology is an essential component to gain clear insights into physiology, genetics and evolution of bacteria. Plasmids are extrachromosomal DNA elements with characteristic copy numbers within the host, which range in size from approximately 300 bp to 2400 kb. The three living world representatives, namely, the domains *Archaea*, *Bacteria*, and *Eukarya* harbour plasmids (Woese et al, 1990). Plasmids mostly exist as covalently closed circular molecules, while some plasmids occur as linear molecules capped at both ends with protein (Clarence, 1998). Plasmids occur as different copies in cells, some occur in only one or a few copies per cell, while others occur in several copies (Pritchard et al., 1969; Novick, 1987). Copy number is a fixed characteristic

of any plasmid under constant conditions and is controlled by a plasmid-coded system that determines the rate of initiation of replication (Novic, 1987). The low-copy-number plasmids require a tighter regulation of replication and of segregation for its maintenance than that of multicopy plasmids (Scott, 1984; Mann, 1985). Plasmid incompatibility is generally defined as the failure of two coresident plasmids to be stably inherited in the absence of external selection (Novick et al, 1976). Plasmid incompatibility is due to the sharing of one or more elements of the plasmid replication or partitioning systems, and plasmid loss due to incompatibility is often a consequence of interference with the ability of the plasmid to correct stochastic fluctuations in its copy number (Novic, 1987). Many plasmids can replicate only in one or a few closely related hosts (Tardif and Grant, 1983), in contrast, promiscuous plasmids are adapted to a wide range of bacteria and can be stably inherited in distantly related hosts (Krishnapillai, 1986; Pinkney and Thomas, 1987; Thomas and Smith, 1987).

Though plasmids are not essential for growth they may give selective advantage to the bacteria empowering them to survive and grow under certain adverse physiological conditions. Due to their existence, bacteria acquire resistance to antibiotics and heavy metals, ability to survive on alternate carbon sources, and can even reduce atmospheric dinitrogen to ammonia (Cotter & Gunsalus, 1989; Collard et al., 1994). In addition to having vital genetic information, plasmids being prime components of horizontal gene pool, play a predominant role in dissipating genetic information among microbial community (Trevors, 1999). The mobilizable and self-transmissible plasmids by virtue of their ability to cross species barriers, serve as potentially effective vehicles for horizontal gene transfer (Chopade et al., 1985; Nemec et al., 2008). Considering the fact that in nature microbes typically coexist in a tightly knit communities such as in biofilms,

plasmids mediate horizontal gene transfer among bacteria, archaea, eukarya, including plants and fungi (Sarand et al., 1998).

The factors that have contributed to the development of plasmid research are; the genetic organization is quite simple, they can be easily isolated and manipulated *in vitro*, and manipulation of plasmid does not have adverse consequences to the hosts. Plasmids replicate autonomously and in self-controlled manner. Research on plasmid replication and its control has led to milestone discoveries, such as the existence of antisense RNAs, and has contributed to the unravelling of mechanisms of DNA replication, macromolecular interactions, and control of gene expression (del Solar et al., 1998). The studies on the ability of some of plasmids that could move to different living organisms have unravelled the mechanism involved in communication between plasmid replication components and the host machinery involved in DNA replication. Plasmid host range studies also have clear implications in clinical microbiology and in biotechnology. Therefore, study of plasmid replication is an important aspect in understanding its existence in its surrounding.

1.1 Mechanism of plasmid replication

There are three mechanisms by which plasmids of different organisms replicate; they are theta type, strand displacement, and rolling circle (RC) mode of replication. The theta mode of replication is predominantly described from gram negative bacteria, while rolling circle mode of replication is initially identified in gram positive bacteria. Strand displacement replication on the other hand has been widely associated with broad-host-range plasmids from the IncQ family (Sakai and Komano, 1996; Gloria et al., 1998). Plasmids have an essential region in its genetic structure which contains the genes or loci involved in replication and its control. In addition, plasmids can carry some genes which

can be considered dispensable such as antibiotic resistance genes, genes involved in plasmid transfer and spread among bacteria, transfer to higher eukaryotes and resistance to heavy metal and radiations. Within a plasmid there are some essential region, several genes and sequences that are important for replication; these are origin of replication (*ori*), Rep protein involved in initiation of replication and the plasmid-borne genes involved in the control of replication (Gloria et al., 1998). The mechanisms of plasmid replications are briefly described below.

1.1.1 Replication by the Theta mechanism

Circular plasmids of gram-negative bacteria represent the mostly studied theta mechanism of replication, although plasmids from gram-positive bacteria have also been found to follow this mode of replication. The theta mechanism of DNA replication involves melting of the parental strands, synthesis of a primer RNA (pRNA), and initiation of DNA synthesis by covalent extension of the pRNA (Fig. 1) (Kornberg and Baker, 1992).

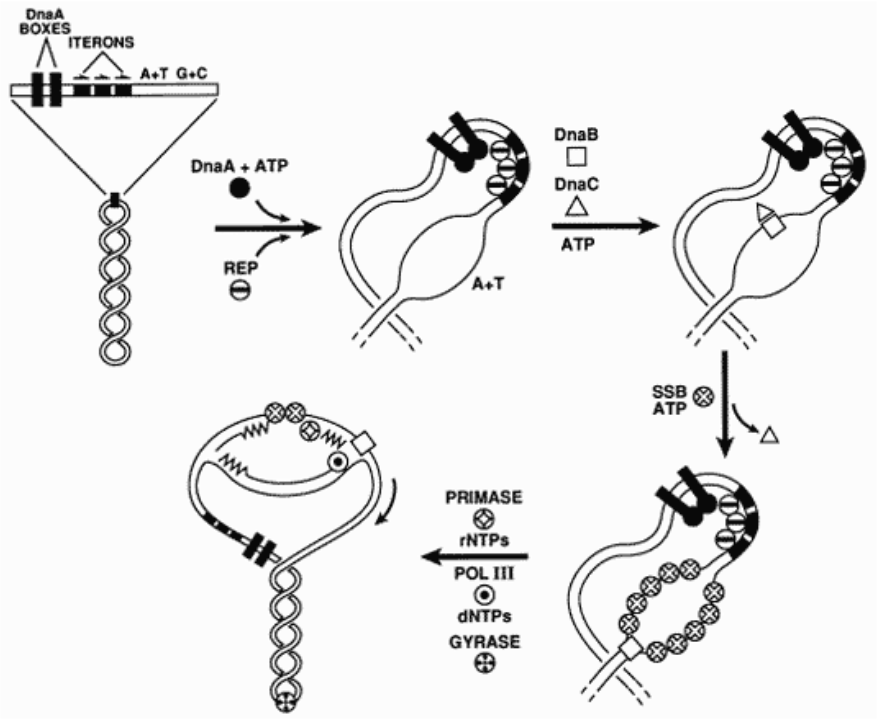

Fig. 1. Model for initiation of replication in an iteron-containing plasmid (Helinski et al, 1996).

DNA synthesis is continuous on one of the strands (leading strand) and discontinuous on the other (lagging strand), although synthesis of the two strands seems to be coupled (Kelman and O'Donnell, 1995; Zavitz and Marians, 1991). The DNA synthesis can start from one or several origins and replication can proceed in one or both the directions around the plasmid. Most of the plasmids that replicate through theta mechanism require plasmid encoded Rep initiator protein while some replicon may require host DNA polymerase I during the early stages of leading strand synthesis. The origin of replication can be defines as the minimal *cis*-acting region that can support autonomous replication of plasmid, the region where DNA strand are melted to initiate the replication process, or the base(s) at which leading strand synthesis starts. The replication origin contains sites that are required for interaction with proteins involved in replication. The plasmid encoded Rep initiator protein is required for the initiation of replication, which recognizes a specific sequence at the origin of replication to which it interacts. Some other features that are present in origin of theta replicating plasmid are AT-rich containing repeat sequence, where opening of the strands and assembly of host initiation factors occur, and one or more sites (*dnaA* boxes) where the host DnaA initiator protein binds (Bramhill and Kornberg, 1988; Kornberg and Baker, 1992). Binding sites for some factors such as the integration host factor (IHF), or the factor for inversion stimulation (FIS) can also be found at the origin of replication. Directly repeated sequences called iterons are also found in the replication origin in many cases and it is the sites where plasmid encoded Rep proteins binds (Filutowicz et al., 1994). Certain theta-replicating plasmids like R1 and ColE1 as well as plasmid pLS20 from *B. subtilis* does not contain iterons at their origin of replication (Tomizawa et al., 1974; Ortega-Jime´nez et al., 1992; Meijer et al., 1995). Initiation of replication of R1 plasmid is dependent on a

plasmid-encoded initiator protein, RepA. The minimal region required for RepA-dependent replication (*oriR*) is included within a 188-bp DNA region (Masai et al., 1983) and comprises a 9-bp *dnaA* box, a contiguous 100-bp region where RepA interacts, and an adjacent AT-rich region containing three 9-bp sequences. The presence of the *dnaA* box optimizes the action of the DnaA protein at the origin, both *in vivo* and *in vitro*, but it is not absolutely required for the DnaA-dependent replication of R1 (Ortega-Jime´nez et al., 1992). ColE1 plasmid is the prototype of a class of small multicopy plasmids that replicate by a theta-type mechanism. Initiation of replication of ColE1 requires DNA Pol I and the origin of ColE1 replication spans a region of about 1 kb. The feature includes; (i) sequences promoting the synthesis of RNA II, the primer of the leading strand (Tomizawa et al., 1975; Tomizawa, 1975); (ii) sequences that allow a stable hybridization of RNA II to DNA (Itoh and Tomizawa, 1980; Tanaka et al., 1994); (iii) sequences that favour specific processing of this coupled complex by RNase H, which generates the 3' end needed to prime leading strand synthesis (Hillenbrand and Staudenbauer, 1982; Itoh and Tomizawa, 1980); (iv) a primosome assembly site (*pas* or *ssiA*) that allows the loading of the DnaB helicase and DnaG primase to initiate the discontinuous priming of the lagging strand (Bo¨ldicke et al., 1981; Masukata and Tomizawa, 1990; Nomura and Ray, 1980). A *dnaA* box that is close to *pas* is also used as a DnaA-dependent DnaB-DnaC assembly site (Seufert et al., 1988; Seufert and Messer, 1987); and (v) a sequence for termination of lagging strand synthesis, *terH*, which determines unidirectional replication (DasGupta et al., 1987; Minden and Marians, 1985). The *B. subtilis* plasmid pLS20, replicates by the theta mechanism, and its replication is independent of DNA Pol I and of a Rep initiator protein. Several palindromes flanking a putative *dnaA* box are located within the origin of replication of pLS20.

1.1.1.1 Rep proteins

Rep proteins of theta mode of replicating plasmid recognize specific sequences at the origin of replication. They generate a nucleoprotein initiation complex which is shown to be a centre for essential macromolecular interactions such as Rep-DNA, Rep-Rep, and Rep with other initiation proteins of the host (Bramhill and Kornberg, 1988). A protein-protein interaction motif resembling the leucine zipper (LZ) is present in several plasmid-encoded Rep proteins. LZ-like motifs have been detected in the N-terminal region of the Rep proteins of several plasmids (Giraldo et al, 1989, Nieto et al, 1992). Mutational studies have revealed that the LZ-like motif is a protein-protein interaction interface that regulates the equilibrium between monomers and dimmers of the RepA protein (Garcı´a de Viedma et al., 1996). Existence of RepA in mono and dimeric form has physiological relevance. The RepA monomers bind to the iterons of the origin of replication whereas dimers of the protein bind to the *repA* promoter region, to act as a repressor (Ingmer et al., 1995). Overexpression of RepA protein inhibits replication due to dimerization of the RepA, thereby hindering the interaction with iteron (Ingmer and Cohen, 1993; Vocke and Bastia, 1983).

1.1.1.2 Initiation and elongation of replication

The assembly of the complete replication machinery including DNA polymerase III holoenzyme (DNA Pol III-HE), DnaB helicase, and primase at the plasmid origin, are required for the initiation of replication. During the initiation replication, the primase vents all the opening of the strands at oriV and the synthesis of RNA primers. Replication initiator proteins such as Rep and DnaA catalyze the opening of the strand. Once oriV closed complex is converted to oriV open complex transcription is carried out by RNA polymerase (RNAP). The main replicative helicase catalyzes further unwinding of the

strands. RNA primers are synthesized either by RNAP or by bacterial or plasmid primases. Once the checkpoint corresponding to the initiation of leading strand synthesis is past, replication continues until completion, following a process catalyzed by DNA Pol III and other host proteins (Bramhill and Kornberg, 1988).

1.1.1.3 Termination of replication

The first replication arrest sequence, *ter*, was identified in plasmid R6K as a barrier to the unidirectional replication initiated in either *ori*-α or *ori*-β of this plasmid. Replication starts then from the initial origin in the opposite direction and progresses to completion (Lovett et al., 1975). The R6K terminus acts as a temporal barrier to replication initiated in other replicons (Kolter and Helinski, 1978). The *ter* sequence is the binding site of Tus, a monomeric protein of *E. coli* that promotes the termination of plasmid replication (Nordstro¨m, 1983; Sista et al., 1989). Sequences arresting lagging-strand synthesis, called *terH*, have been found upstream of and close to the *pas* site of ColE1; the arrest seems to be caused by the nonhybridized portion of RNA II (DasGupta et al., 1987; Nakasu and Tomizawa, 1992). During the final stages of plasmid replication, catenates containing gaps in both daughter strands can be originated (Nakasu and Tomizawa, 1992). These catenates can be resolved by either type I or type II topoisomerases. Involvement of a specific type II topoisomerase, Topo IV, in the segregation of plasmids and bacterial nucleoids has been revealed through genetic analysis (Kato et al., 1988). Maturation of the open-circular forms arising from the decatenation by Topo IV into supercoiled molecules can be efficiently carried out by DNA gyrase.

1.1.2 Strand displacement replication

Plasmids replicating by the strand displacement mechanism are best studied from

the promiscuous plasmids of the IncQ family, whose prototype is RSF1010 (Fig. 2). Initiations of DNA replication by the members this family require three plasmid-encoded proteins. These proteins promote initiation at a complex origin region, and replication then proceeds in either direction by a strand displacement mechanism (Scherzinger et al., 1991; Sakai and Komano, 1996). The origin of replication of plasmid RSF1010 has been defined as the minimal region able to support bidirectional replication when the RSF1010 replication proteins (RepA, RepB, and RepC) are supplied in *trans* by a second plasmid (Scherzinger et al., 1991). The minimal *ori* region includes three identical 20-bp iterons plus a 174-bp region that contains a GC-rich stretch (28 bp) and an AT-rich segment (31 bp). The origin extends further with a nonessential region and two small palindromic

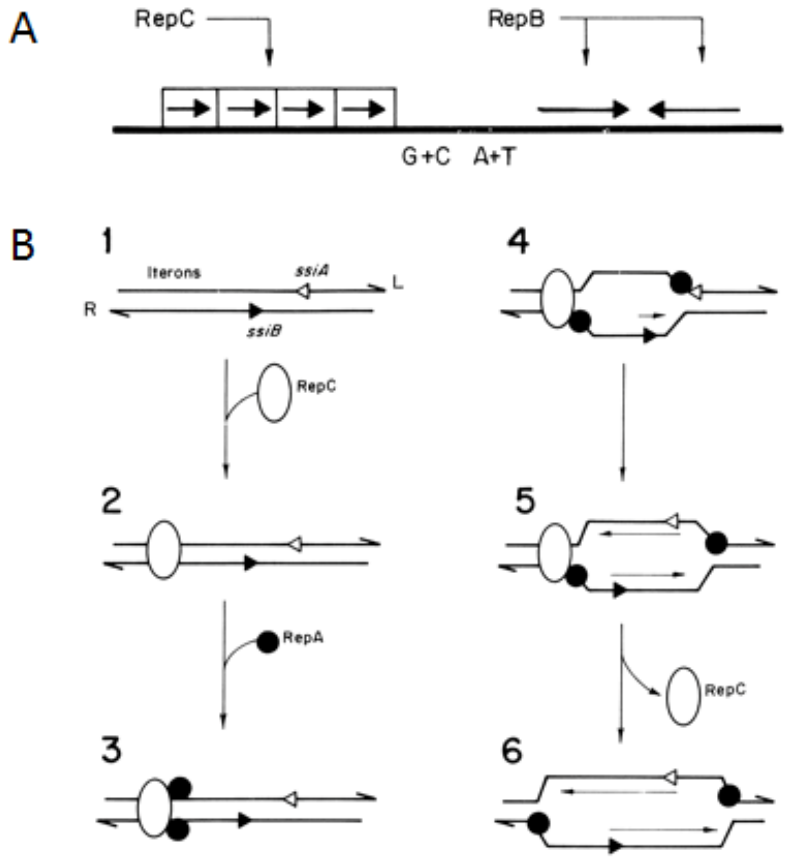

Fig. 2. Model of plasmid replication by strand displacement (del Solar, 1998). Panel A: Origin of replication and related regions. Panel B: Model for initiation of replication by the strand displacement mechanism in plasmid RSF1010.

sequences containing the *ssiA* and *ssiB* sites located in opposite strands. Iterons are the RepC-binding sites (Haring and Scherzinger, 1989; Haring et al., 1985). The inverted repeats at the *ssi* sites could favor the formation of hairpins. In these hairpins, base complementarity in the upper part of the putative stem is essential for replication, while base complementarity and sequence specificity in the lower part of the stem are important for primer synthesis (Miao et al., 1993). The *ssiA* and *ssiB* sequences are specifically recognized by the plasmid-encoded RepB primase, which primes continuous replication from these sequences (Haring and Scherzinger, 1989; Honda et al., 1989; Honda et al., 1991).

1.1.2.1 Rep proteins

The three plasmid-encoded proteins, RepA, RepB, and RepC, jointly acts together and promotes replication of RSF1010. These three proteins have helicase, primase, and initiator activity respectively (Haring and Scherzinger, 1989; Sakai and Komano, 1996). The RepC protein is a dimer of 31-kDa subunits which interacts specifically with the iterons of the origin (Haring and Scherzinger, 1989; Haring et al., 1985) and probably with the RepA helicase, promoting the exposure of the *ssi* sites in a single stranded DNA (ssDNA) configuration (Honda et al., 1991; Scherzinger et al., 1991; Tanaka et al., 1994). The RepA protein is a hexamer of 30-kDa subunits, and it contains two activities: an ssDNA-dependent ATPase and a 5' to 3' DNA helicase. Expression of *repB* from two in-frame alternative start codons results in two polypeptides of 36 and 38 kDa, which correspond to two functional forms of the RepB primase: RepB and RepB9 (Haring and Scherzinger, 1989; Scholz et al., 1989).

1.1.2.2 Mechanism of Replication

Replication of RSF1010 DNA is dependent on the combined action of the plasmid-

encoded RepA, RepB, and RepC proteins and is independent of the host-encoded DnaA, DnaB, DnaC, and DnaG proteins (Frey and Bagdasarian, 1989; Haring and Scherzinger, 1989; Scherzinger al., 1984). The template for initiation of RSF1010 replication is super-coiled plasmid DNA (Dı´az and Staudenbauer, 1982; Kramer et al., 1997). Replication of RSF1010 occurs from two symmetrical and adjacent single stranded origins (*ssiA* and *ssiB*) positioned one on each DNA strand. Replication starts when these origins are exposed as single-stranded regions. DNA Pol III-HE and SSB are involved in replication process (Nordstro¨m and Wagner, 1994). Initially, the RepC protein binds to the iterons at the origin. It is assumed that the RepA helicase binds to both DNA strands in the AT-rich region, close to the site of interaction of RepC. The plasmid-specific primase (RepB) catalyzed the priming of DNA synthesis at the origin. The exposure of the stem-loop structure in the *ssi* sites is probably required for the assembly of the RepB-primase to initiate replication (Miao et al., 1993). Synthesis of each one of the strands occurs continuously and results in the displacement of the complementary strand. Replication of this displaced strand is initiated at the exposed *ssi* origin.

1.1.3 Rolling-circle mode of replication

Rolling-circle- (RC) replication plasmids are most commonly found in gram-positive bacteria, they have also been reported in many gram-negative bacteria and in archea (del Solar et al., 1993a, 1998; Khan, 1997, 2000, 2004). Plasmids of many RC replication families also share homology in their initiator (*rep*) genes and double stranded origin of replication (DSO) with the single-stranded (ss) DNA bacteriophages of *Escherichia coli*. Usually, the size of RC replication plasmids are less than 10 kb, have multiple copies and are tightly organized. RC replication plasmids can be cryptic, while others can carry antibiotic and heavy metal resistance genes. Genes for mobilization and

transfer, and genes encoding site-specific recombinases are also known to be found in some RC replication plasmids. Furthermore, many RC replication plasmids contain insertion sequences and transposons (Khan, 2005). Both broad and narrow host range RC replication plasmids have been described and there is evidence for their horizontal transfer (Khan, 2005). All RC replication plasmids encode initiator proteins that have origin-specific binding and nicking-closing activities (Khan, 2005). The Rep proteins bind to the nick sites and creates nick at the nick site present on the DSO's of RCR. In all the family of RCR plasmids the nick site is highly conserved in the DSO's, while the Rep binding sites are less well conserved. Both the Rep binding and nicking sites are required for initiation of replication, while termination of plasmid RC replication can be promoted by a smaller sequence containing only the nick site. During the RC replication the displaced leading strand is converted to the double stranded (ds) form using the single strand origin (SSO) of RC plasmids. The SSO's are not necessarily conserved in RCR plasmids belonging to the same family, unlike the DSO's (Khan, 2005).

1.1.3.1 Mechanism of replication

The model for the mechanism of plasmid RC replication is represented in figure 3, based on studies with the plasmids of the pT181 family (Koepsel et al., 1985a,b). The Rep protein interacts with its specific bind sequence located within the plasmid DSO. A sharp bend in the DNA and/or generation of a hairpin occurs as a result of the Rep-DSO interaction in which the Rep nick site is located in the single-stranded loop. The Rep protein then nicks the DSO at the nick sequence and becomes covalently attached to the 5' phosphate through a tyrosine residue present in its active site. Through a specific protein-protein interaction, the Rep proteins recruit a DNA helicase (PcrA in the case of Gram-positive bacteria). Then the unwinding of DNA takes place by the helicase and the

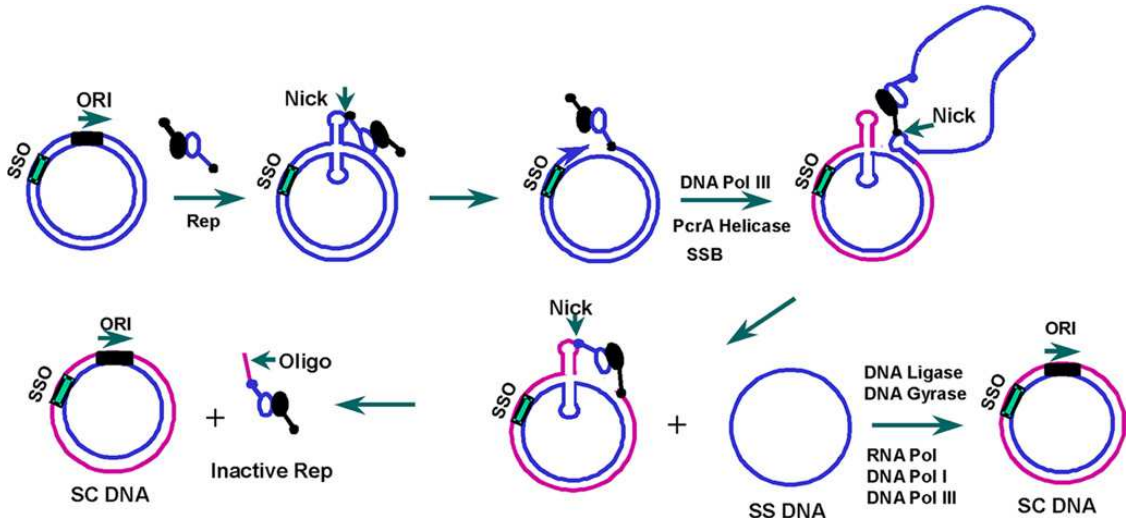

Fig. 3. Model of plasmid RC mode of DNA replication (Khan, 2005).

single-stranded DNA binding (SSB) protein coats the displaced ssDNA. Using the free 3' OH end at the nick site, the DNA polymerase III catalyses synthesis of leading strand during replication initiation and proceeds until the leading strand has been fully displaced. DNA synthesis proceeds to approximately 10 nucleotides (nt) beyond the Rep nick site after the replication fork reaches the termination site, i.e., the regenerated DSO, (Fig. 3). The displaced ssDNA is then cleaved by the second, free monomer of the Rep protein. The circular, leading strand is released along with a relaxed, closed circular DNA containing the newly synthesized leading strand, following a series of cleavage/rejoining events. The host DNA gyrase catalyse the supercoiling of dsDNA. The active tyrosine of one monomer of Rep protein which is covalently attached to the 10-mer oligonucleotide is released (Fig. 3). This form of Rep, termed RepC/RepC* for pT181, which has catalyzed one round of leading strand replication is inactive in further replication. Once the leading strand synthesis has been completed, the ssDNA released is converted to dsDNA utilizing the SSO and host proteins. During this process RNA primer is synthesized by the RNA polymerase followed by extension of the primer by DNA polymerase I and subsequent DNA synthesis by DNA polymerase III. Finally, DNA ligase joins the DNA ends and the

resultant dsDNA is converted to the supercoiled (SC) form by DNA gyrase.

1.1.3.2 Rep Proteins

According to structure-function analyses the Rep proteins of the pT181 family have a sequence-specific DNA binding domain and a nicking domain (Dempsey et al., 1992a,b; Wang et al., 1993). A tyrosine residue is involved in nicking at the origin, and the initiator becomes covalently attached to the 5' end of the DNA through a phosphotyrosine linkage as observed in several plasmids (Dempsey et al., 1992b; Thomas et al., 1988). The nicking domains of the initiator proteins belonging to a particular family are found to be highly conserved (del Solar et al., 1998; Ilyina and Koonin, 1992; Khan, 1997; Projan and Novick, 1988). The DNA binding domains, on the other hand, are quite divergent (Projan and Novick, 1988).

The initiator proteins of the plasmids of the pT181 family act as dimers during replication (Chang et al., 2000; Rasooly and Novick, 1993; Rasooly et al.,1994). However, it was shown that one monomer of the dimeric initiator is sufficient for sequence-specific DSO binding and nicking (Chang et al., 2000). Furthermore, the monomer that promotes sequence-specific binding to the DSO was also found to nick the DNA to initiate replication (Chang et al., 2000). In case of the pT181 initiator protein, it is found that whereas the Tyr-191 is required for nicking at the origin, to initiate replication, it was dispensable for termination, suggesting that alternate amino acids in the initiator may promote termination but not initiation (Chang et al., 2000).

1.1.3.3 The double stranded origin

The DSO's of RC replication plasmids contain both the bind and nick sequences for the Rep proteins. In all plasmids belonging to a particular family, the nick site is highly conserved in the DSO's, while the Rep binding sites are less well-conserved. Structural

features such as cruciforms and hairpins were also present in the DSO's of many RCR plasmids (Gros et al., 1987; Moscoso et al., 1995a; Noirot et al., 1990; Wang et al., 1993), and static and initiator protein-enhanced bending of the DSOs of the pT181 and pMV158 family members has also been reported (Koepsel and Khan, 1986; Perez-Martin et al., 1988). In case of some RC replication plasmid families, the DSO's were found to be located adjacent to the *rep* genes, while in others they were present within the sequence encoding the initiator proteins (del Solar et al., 1998; Khan, 1997). Plasmids containing two copies of the DSO in a direct orientation were used to identify the sequence requirements for the initiation and termination of replication of plasmids of the pT181 and pC194 families (Gennaro et al., 1989; Gros et al., 1987, 1989; Iordanescu and Projan, 1988; Murray et al., 1989). While the signals for initiation and termination of replication were found to overlap, a larger region of DSO containing both the initiator bind and nick sequences were required for initiation (Gros et al., 1987; Zhao and Khan, 1996). In case of termination, it was found that the nick sequence was necessary but the major Rep binding site was dispensable (Iordanescu and Projan, 1988; Zhao and Khan, 1997).

1.1.3.4 Single stranded origin

During the RC mode of replication the parental leading strand of plasmid DNA that is displaced upon synthesis of the new DNA strand is converted to the double stranded (ds) form utilizing the SSO. The SSO, which was first identified in plasmid pT181, have shown to contain sequences that can form a folded structure that was important for its function (Gruss et al., 1987). The importance of SSO was determined by deletion study which caused plasmid instability, a marked reduction in copy number, and resulted in the accumulation of large quantities of circular, leading strand DNA (Gruss et al., 1987). Based on structural and/or sequence similarities, different classes of SSO's, namely *sso*A, *sso*T,

*sso*U, and *sso*W, were identified in several RC replication plasmids (Andrup et al., 1994; Boe et al., 1989; del Solar et al., 1993b; Kramer et al., 1995; Madsen et al., 1993; Meijer et al., 1995; Seegers et al., 1995; Zaman et al., 1993). The SSO's are found in non-coding regions, are strand specific and the sequence conservation is low in the plasmids of the same family. In most cases, the location of SSO's are just immediately upstream of the DSO's, which is important in order not to remain in ss form until the leading strand has been almost fully synthesized. It was found that the *sso*A and *sso*W type origins function efficiently only in their native hosts while the *sso*T and *sso*U were found to be able to support lagging strand synthesis in several different hosts.

The replication of ssDNA containing *sso*A-type origin requires *sso*A sequence and solely the host proteins (Birch and Khan, 1992). It has been shown that DNA polymerase I is required for the ss to ds synthesis, and both its polymerase and 5' to 3' exonuclease activities were involved in this function (Diaz et al., 1994; Kramer et al., 1997). Two conserved elements such as RS-B and the CS-6 sequences were found to exist in the *sso*A-type origins (Dempsey et al., 1995; Kramer et al., 1995). In an *in vitro* experiment, it was demonstrated that *sso*As and *sso*U contained ssDNA promoters and RNA primers of approximately 20 and 45 nt long are synthesized by the host RNA polymerase, that are used for the initiation of lagging strand DNA synthesis (Kramer et al., 1997; 1998a, 1999). Further, the RNA polymerase binds to *sso*A sequences, and the RS-B sequence is important for this binding while the conserved CS-6 sequence of *sso*A s was found to serve as the termination site for primer RNA synthesis (Kramer et al., 1997; 1998a, 1998b).

1.1.3.5 The host proteins

As mentioned above, for replication of RC plasmid, RNA polymerase and DNA

polymerase I are involved. It is also assumed that SSB is required for both leading and lagging strand DNA synthesis. The *pcrA* gene of pT181 plasmid is required for its replication and predicted to encode a helicase (Iordanescu, 1993; Iordanescu and Basheer, 1991). Although not directly demonstrated, it is likely that PcrA is also required for the replication of all RCR plasmids in Gram-positive bacteria (Khan, 2005). The PcrA is absent in Gram-negative bacteria, instead, the UvrD helicase was shown to promote the replication of RC replication plasmids (Bruand and Ehrlich, 2000). The involvement of DNA polymerase III in plasmid RC replication was suggested previously (Alonso et al., 1988). Recently two essential DNA polymerases, PolC and DnaE, have been identified in Gram-positive bacteria which are also thought to be involved in RC replication (Dervyn et al., 2001; Inoue et al., 2001).

1.2 Xenobiotics detoxification

Both natural and anthropogenic activities result in accumulation of wide ranges of toxic xenobiotic compounds in the environment, and thus cause a global concern (Gienfrada and Rao, 2008). Primarily, xenobiotics are those compounds that are alien to a living individual and have a propensity to accumulate in the environment. Principal xenobiotics include pesticides, fuels, solvents, alkanes, polycyclic hydrocarbons (PAHs), antibiotics, synthetic azo dyes, pollutants (dioxins and polychlorinated biphenyls), polyaromatic, chlorinated and nitro-aromatic compounds (Sinha et al., 2009). The main concern with xenobiotic compounds is the toxicity threat they pose to public health. It is quite shocking that some xenobiotic compounds (phenols, biphenyl compounds, phthalates, etc.) act as endocrine disruptors (Nagao, 1998; Borgeest et al., 2002).

Biodegradation is one of the natural processes that help to remove xenobiotic chemicals from the environment by microorganisms. This is primarily a strategy for the

survival of the microorganisms (Singh, 2008). Bacterial glutathione S-transferases (GSTs) are part of a superfamily of enzymes that play a key role in cellular detoxification. Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a protein superfamily that is involved in cellular detoxification against harmful xenobiotics and endobiotics (Sheehan et al., 2001; Hayes et al., 2005; Oakley, 2005). One common feature of all GSTs is their ability to catalyse nucleophilic attack by the tripeptide glutathione (GSH) on the electrophilic groups of a wide range of hydrophobic toxic compounds, thus promoting their excretion from the cell (Hayes et al., 2005). GSTs are also involved in several other cell functions, and are capable of binding non-catalytically to a large number of endogenous and exogenous compounds (Hayes et al., 2005). GSTs are widely distributed in nature and are found in both eukaryotes and prokaryotes. GSTs are divided into at least four major families of proteins, namely cytosolic GSTs, mitochondrial GSTs, microsomal GSTs and bacterial fosfomycin-resistance proteins (Armstrong RN, 1997; Hayes et al., 2005). The cytosolic GSTs (cGSTs) have been subgrouped into numerous divergent classes on the basis of their chemical, physical and structural properties (Sheehan et al., 2001; Hayes et al., 2005). The mitochondrial GSTs, also known as kappa class GSTs, are soluble enzymes that have been characterized in eukaryotes (Robinson et al., 2004). The third GST family comprises membrane-bound transferases called membrane-associated proteins involved in ecosanoid and glutathione metabolism (MAPEG), but these bear no similarity to soluble GSTs (Jakobsson et al., 1999). Representatives of all three families are also present in prokaryotes. The fourth family is found exclusively in bacteria. Crystallographic studies of GSTs from several species (Dirr et al., 1994; Ji, X et al., 1995; Wilce et al.,1995), including bacteria (Rossjohn et al., 1998), have elucidated the subunit architecture and quaternary structure of these enzymes. Despite their low inter-class

sequence identity, the GST monomers show a basically similar structural organization, i.e. two domains joined by a short linker of six or seven amino acid residues. Domain I (GSH-binding site), which is mainly located in the N-terminal region of the protein, is an alpha and beta structure. GST domain II provides most of the hydrophobic binding site (H-site), and is formed by alpha-helices.

In prokaryotes, GST activity is very low and hence it has only recently been discovered. Although very little is known about the properties of GSTs in prokaryotes, they seem to be implicated in biodegradation of xenobiotics (Orser et al., 1993; McCarthy et al., 1996), including antibiotics (Arca et al.,1988; Piccolomini et al., 1989; Arca et al., 1990). In addition to the GSTs of *Escherichia coli* (Nishida et al., 1994) and *Proteus mirabilis* (Di Ilio et al., 1988), the prokaryotic members of the GST family also include the dichloromethane dehalogenase of *Methylophylus* sp. (Bader et al., 1994) and the *pcpC*-encoded enzyme involved in the reductive dechlorination of pentachlorophenol by *Sphingomonas paucimobilis* (Mueller *et al*., 1990). In *Pseudomonas* sp. strain LB400, a GST-encoding gene, with an unclear function, was found within the *pbh* locus, which involves aromatic machinery for degradation of polychlorinated- biphenols (Hofer et al., 1994). In insects, studies suggest that GSTs play an important role in resistance against several classes of insecticides including organophosphates (OP) (Syvanen et al., 1996; Wei et al., 2001). Methyl parathion (MeP), a widely used OP insecticide, is biotransformed via a glutathione-dependent pathway in rat and mouse liver fractions (Clark et al., 1973; Benke et al., 1974; Benke and Murphy, 1975). In the case of MeP, GSTs catalyze *O*-dealkylation of the parent compound with formation of S-methylglutathione (reviewed in Fukami, 1980). *O*-Demethylation of MeP prevents activation via oxon formation.

Radulovic and coworkers have shown that human GSTs expressed in fetal liver and placenta catalyze *O*-dealkylation of MeP (Radulovic et al., 1986, 1987).

1.3 Organophosphates

Organophosphorus compounds are usually esters, amides or thiol derivatives of phosphonic acid. They form a large family of ~50,000 chemical agents with biological properties that have important and sometimes unique implications for man. This family of organophosphate (OP) pesticides effectively eliminates pests owing to its acute neurotoxicity. The effectiveness of OP compounds as pesticides and insecticides also makes them hazardous to humans and to the environment. OPs and their family of compounds are potent neurotoxins that share structural similarities to chemical warfare agents such as sarin, soman and VX [Oethyl-S-(2-diisopropylaminoethyl) methylphosphonothioate]. They act as cholinesterase inhibitors and in turn disrupt neurotransmission in both insects and humans.

The first step in parathion degradation is its hydrolysis to the metabolites diethyl thiophosphate (DETP) and *p*-nitrophenol (PNP). The metabolite DETP is essentially non-toxic, and it has been shown that DETP can be used as source of phosphorous, sulphur, and carbon by some isolates (Cook et al., 1978). The bacterial organophosphate hydrolase (OPH) catalyzes the hydrolysis of parathion as well as other organophosphorus compounds such as the pesticides coumaphos and diazinon and chemical warfare agents soman, tabun, and sarin (diSioudi et al., 1999). The OPH has been isolated from diverse group of bacteria (Serdar et al., 1982; Mulbry and Karns 1989a; Singh et al., 1999). The most thoroughly studied OPH are from *Pseudomonas diminuta* and *Flavobacterium* sp. ATCC 27551. This enzyme hydrolyzes the compounds that are structurally related to parathion exhibiting broad substrate, temperature range, pH optima and high stability

(Brown, 1980). The organophosphate degrading (*opd*) genes of *Flavobacterium* sp. ATCC 27551 and *B. diminuta* has been studied extensively. These identical genes are localized on large indigenous dissimilar plasmids (Harper et al., 1988). Further, the *opd* gene found on pPDL2 plasmid of *Flavobacterium* sp. ATCC27551 was shown to exist as transposon-like organization (Siddavattam et al., 2003).

The PNP, hydrolysis product of parathion, must be metabolized by the organism as it is considered carcinogen and an Environmental Protection Agency priority pollutant (Anonymous, 1992). Several organisms have been found capable of using PNP as a carbon and energy source, including *Bacillus* (Kadiyala et al., 1998), *Arthrobacter* (Bhushan et al., 2000; Hanne et al., 1993; Jain et al., 1994), *Pseudomonas* (Prakash et al., 1996), *Burkholderia* (Bhushan, et al., 2000), and *Moraxella* (Spain, et al., 1979; Spain and Gibson, 1991). There are two proposed pathways for PNP degradation. In the first pathway, the PNP are degraded via hydroquinone (HQ), while the other degrades PNP through hydroxyquinol (BT). In the BT pathway, two enzymes were first characterized from *Rhodococcus opacus* SAO101: one was the two-component PNP monooxygenase NpcAB; the other was the one-component BT 1,2-dioxygenase NpcC (Kitagawa et al., 2004). In *Arthrobacter* sp. strain JS443, another two-component monooxygenase gene *NpdA1A2* has been identified (Perry and Zylstra 2007). Recently, Chauhan et al. identified two lower stream genes (*pnpCD*) encoding BT 1,2-dioxygenase and maleylacetate (MA) reductase in this pathway (Jain et al., 1994). In case of gram-positive bacterium *Rhodococcus* sp. strain PN1 there are two clusters involved in PNP degradation. Within these two clusters, two kinds of two-component PNP monooxygenase genes (*nphA1A2* and *npsA1A2*), a regulator protein gene (*npcR*) and a BT 1,2-dioxygenase gene (*npsB*) have been identified (Yamamoto et al., 2011; Takeo et al., 2008).

1.4 Importance of the present study

Our laboratory has been working to understand the molecular basis for mineralization of organophosphate compounds, especially in soil samples polluted with OP-pesticides. During the process of our study, we have also isolated a number of soil isolates that use methyl parathion as source of carbon (Somara and Siddavattam, 1995; Pakala et al., 2006). In certain isolates, the *opd* gene was localized on large indigenous plasmids (Somara and Siddavattam, 1995). One of the soil isolates, later identified as *Serratia* sp. DS001 has also displayed the ability to use methyl parathion as sole source of carbon (Pakala et al., 2006). During enrichment process, we have always obtained *A. baumannii* DS002 along with *Serratia* sp. DS001. Our previous studies have shown induction of glutathione S-transferase, along with some other catabolic proteins, in *A. baumannii* DS002 exclusively in presence of methyl parathion. In order to have further insights into the role of *A. baumannii* DS002 in degradation of OP compounds, a detailed studies were conducted with the following objectives;

(i). Plasmid profile and its involvement in degradation of OP compounds and its degradation product *p*-nitrophenol.

(ii). Role of Glutathione S-transferase in OP compound degradation and underlying mechanism that contribute for its regulation.

(iii). Generation of cloning/expression vectors for manipulation of *A. baumannii* strains by exploiting one of the indigenous plasmids identified in *A. baumannii* DS002.

MATERIALS AND METHODS

2.1 Tables

2.1.1 Antibiotics

Name of the antibiotic	Name of the Supplier
Ampicillin sodium salt	Himedia
Chloramphenicol	Himedia
Kanamycin Sulfate	Himedia
Streptomycin	Himedia
Tetracycline hydrochloride	Himedia

2.1.2 Chemicals

Name of the Chemical	Name of the Supplier
Absolute alcohol	HAYMAN
Acetic acid (Glacial)	Qualigens
Acetylacetone	SRL
Acetonitrile	Qualigens
Acrylamide	Merck
Agar	Himedia
Agarose	SeaKem
Ammonium acetate	SRL
Ammonium persulphate	Sigma Aldrich Chemicals
Ammonium nitrate	Qualigens
Ammonium sulfate	Qualigens
Ascorbate	SRL
Boric acid	Qualigens
Bovine serum albumin	GE Healthcare Lifesciences, USA
Bromophenol blue	GE Healthcare Lifesciences, USA
Butanol	Qualigens
Calcium chloride	Qualigens
Calcium nitrate	Qualigens
1-chloro 2,4-dinitrobenzene (CDNB)	SRL

Chloroform	Qualigens
Cobalt chloride	SRL
Coomassie Brilliant blue R-250	SRL
Coomassie Brilliant Blue G-250	SRL
Cumene hydroperoxide (CHP)	Sigma Aldrich Chemicals
Deoxynucleotide triphosphates	MBI Fermentas
Dichloromethane	Sigma Aldrich Chemicals
Diethyl pyrocarbonate (DEPC)	Sigma Aldrich Chemicals
Dimethyl sulfoxide (DMSO)	Sigma Aldrich Chemicals
Dipotassium hydrogen orthophosphate	Qualigens
N,N-Dimethylformamide	Merk
Formaldehyde	Merk
Glutathione (GSH)	Sigma Aldrich Chemicals
Glycerol	Qualigens
Glycine	SRL
Hydrochloric acid	Qualigens
Imidazole	SRL
Isopropanol	SRL
Isopropyl thiogalactopyranoside (IPTG)	SRL
Lysozyme	Bangalore GeneI
Manganese chloride	SRL
Magnesium chloride	SRL
Magnesium sulphate	SRL
Methanol	SRL
N,N'-Methylene bis acrylamide	SRL
Methylene blue	Sigma Aldrich Chemicals
Methyl parathion	Sigma Aldrich Chemicals
Methyl viologen (Paraquat)	Sigma Aldrich Chemicals
β-mercaptoethanol	Sigma Aldrich Chemicals
NADP (Nicotinamide Adenine Dinucleotide Phosphate)	Sigma Aldrich Chemicals
ONPG (*O*-nitrophenyl-β-D-galactopyranoside)	Sigma Aldrich Chemicals
Nickel chloride	Merk
4-Nitrocatechol	Sigma Aldrich Chemicals

p-Nitrophenol	Sigma Aldrich Chemicals
Polyethylene glycol (PEG) 8000	Himedia
Peptone	Himedia
Phenol Saturated	Bangalore GeneI
Phosphoric acid	Merck
p-phenylphenol	Sigma Aldrich Chemicals
Piperazine-N, N'-bis[2-ethanesulfinic acid] (PIPES)	SRL
Potassium acetate	Qualigens
Potassium chloride	Qualigens
Potassium dihydrogen ortho phosphate	Qualigens
Potassium tellurite	Sigma Aldrich Chemicals
PMSF (phenylmethanesulfonylfluoride)	Sigma Aldrich Chemicals
Protein molecular weight marker	MBI Fermentas
Silver nitrate	SRL
Sodium acetate	Qualigens
Sodium benzoate	Sigma Aldrich Chemicals
Sodium carbonate	SRL
Sodium citrate	SRL
Sodium chloride	SRL
Sodium dihydrogen orthophosphate	Qualigens
Sodium dodecyl sulfate	SRL
Sodium hydroxide	SRL
Sodium Sulphate	Merck
Succinic acid	SRL
Sucrose	Sigma Aldrich Chemicals
tert-butyl hydroperoxide	Sigma Aldrich Chemicals
Tetrachlorohydroquinone (TCHQ)	Sigma Aldrich Chemicals
Tetrabutylammonium phosphate (TBAP)	Sigma Aldrich Chemicals
Tetra ethyl methylene diamine (TEMED)	Sigma Aldrich Chemicals
Tris-base	Sigma Aldrich Chemicals
Trichloroacetic acid	Sigma Aldrich Chemicals
Trifluoroacetic acid	Sigma Aldrich Chemicals
Tryptone	Himedia
Tween-20	Bangalore GeneI

Uranyl acetate	Sigma Aldrich Chemicals
X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside)	Sigma Aldrich Chemicals
Xylene cyanol	GE Healthcare Lifesciences, USA
Yeast extract	Himedia
Zinc chloride	SRL

2.1.3 Restriction enzymes and other DNA modifying enzymes

Name of the enzyme	**Name of the Supplier**
*Bam*HI	MBI, Fermentas
*Bgl*II	MBI, Fermentas
*Dpn*I	MBI, Fermentas
*Eco*RI	MBI, Fermentas
*Hind*III	MBI, Fermentas
*Mlu*I	MBI, Fermentas
*Nde*I	MBI, Fermentas
*Pst*I	MBI, Fermentas
*Sac*I	MBI, Fermentas
*Xba*I	MBI, Fermentas
*Xho*I	MBI, Fermentas
Klenow fragment	MBI, Fermentas
Proteinase K	MBI, Fermentas
RNase A	Bangalore GeneI
S1 nuclease	MBI, Fermentas
T_4 DNA Ligase	MBI, Fermentas
Taq DNA polymerase	MBI, Fermentas
Pfu DNA polymerase	MBI, Fermentas
T4 Polynucleotide Kinase (T4 PNK)	MBI, Fermentas
Tn*5* Transposase	Epicenter Biotechnologies, USA

2.1.4 Bacterial strains and plasmids

Strains	**Genotype or Phenotype**	**Reference or Source**
E coli BL21 DE3	*hsdS gal(λcIts857 ind1 sam7 nin5lac UV5 T7 gene 1*	Studier and Moffat, 1986
E. coli DH5α	*supE*44 Δ*lacU*169 (ϕ80 *lacZ* Δ*M*15)	Hanahan et al., 1983

	*hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*1 *relA*1	
E coli EP 1300	*F⁻ e14⁻(McrA⁻) D(mcrC⁻mrr) (Tet^R) hsdR514 supE44 supF58 lacY1 or D(lacIZY)6 galK2 galT22 metB1 trpR55 l⁻*	Epicentre Biotechnologies, USA
E. coli pir-116	*F-* mcr Δ*(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (StrR) nupG pir-116(DHFR)*	Epicentre Biotechnologies, USA
E.coli S17.1	Sm^r, RP4-2-Tc::Mu aph::Tn7 recA	Simon et al., 1983
Acinetobacter baumannii DS002	Cm^r, Sm^r, ben$^+$	This work

2.1.5 Plasmids

Plasmids	**Genotype or Phenotype**	**Reference or Source**
pBluescript II KS	Ap^r, *lacZ*$^+$, cloning vector	Alting-Mees and Short, 1989
pGEMT-Easy	Ap^r, *lacZ*$^+$, TA cloning vector	Kobs, 1995
pTZ57R	Ap^r, *lacZ*$^+$, TA cloning vector	MBI Fermentas
pCC1FOS	Cm^r, *lacZ*$^+$, cloning vector	Epicentre Biotechnologies, USA
EZ-Tn5™<R6Kγ*ori*/ KAN-2> Insertion Kit	A mini-transposon having R6Kγ origin of replication with a Kanamycin cassette	Epicentre Biotechnologies, USA
pTS4-K	A mini-transposon having R6Kγ origin of replication with a Kanamycin cassette inserted into indigenous plasmid, pTS4	This study
pET-23b	Ap^r, C- terminal His tag, expression vector	Novagen, Germany
pMP220	Tet^r, promoter probe vector	Spaink, 1987
pSUP202	Mob$^+$, Amp^r, Cm^r, Tc^r and mobilizable suicidal vector	Simon et al., 1983.
pTRW1	Ap^r, *repA* cloned in pET23b from predicted alternative start codon TTG as *Eco*RI and *Xho*I fragment to code RepA with C-terminal His-tag	This study
pTRW2	Ap^r, *repA* cloned in pET23b from the first ATG codon as *Eco*RI and *Xho*I fragment to code RepA with C-terminal His-tag	This study
pTRM	Expression plasmid coding RepA variant RepAY265F with C-terminal His-tag	This study
pTRW1X	Ap^r, *repA* cloned in pET23b as *Eco*RI	This study

	and *Xho*I fragment to express RepA without C-terminal His-tag	
pTS4	A 2.2 Kb Indigenous plasmid of *A. baumannii* DS002	This study
pT113M	A pTS4 variant having termination codon immediately downstream to the start codons of *orf113*	This study
pT96M	A pTS4 variant having termination codon immediately downstream to the start codons of *orf96*	This study
pT96W	Ap^r, *orf96* cloned in pET23b as *Eco*RI and *Xho*I fragment to express Orf96 with C-terminal His-tag	This study
pT113W	Ap^r, *orf113* cloned in pET23b as *Eco*RI and *Xho*I fragment to express Orf113 with C-terminal His-tag	This study
pTRWG	Ap^r, *repA* cloned in pGEX-4T-1 as *Eco*RI and *Xho*I fragment to express RepA as GST-fusion protein	This study
pT96G	Ap^r, *orf96* cloned in pGEX-4T-1 as *Eco*RI and *Xho*I fragment to express RepA as GST-fusion protein	This study
pT113G	Ap^r, *orf113* cloned in pGEX-4T-1 as *Eco*RI and *Xho*I fragment to express RepA as GST-fusion protein	This study
pPAGT	Ap^r, *Abgst01* cloned in pET23b as *Nde*I and *Xho*I fragment to code AbGST01 without any tag	This study
pTAGTH	Ap^r, *Abgst01* cloned in pET23b as *Nde*I and *Xho*I fragment to code AbGST01 with C-terminal His-tag	This study
pPAOXR	Ap^r, *oxyR* cloned in pTZ vector	This study
pSUP*oxyR::kan*	Suicidal vector pSUP202 containing insertionally inactivated *oxyR* cloned as *Eco*RI fragment	This study
pTSR6K	Shuttle vector constructed using indigenous plasmid pTS4 of *A. baumannii* DS002	This study
pTSPH	A 1.5 Kb *opd* containing fragment cloned in pTSR6K as *Bam*HI fragment	This study
pTSTC	A *Ptac* promoter cloned immediately upstream of MCS of pTSR6K as *Bgl*II and *Bam*HI fragment	This study
pTSPM	A 2 Kb *PNP momooxygenase* gene cloned in pTSR6K as *Bam*HI and *Pst*I	This study

	fragment	
pTSPD	A 1.5 Kb *xylE* gene cloned in pTSR6K as *Bam*HI and *Xba*I fragment	This study

2.1.6 Primers

Primers	**Sequence [Forward Primer(F*)/ Reverse Primer (R*)]**	**Purpose**
DS00101 DS00102	GCAGAAAGTTTGTAATTCG (F*) GCTTCTGAAATATGGGCTT (R*)	Primers used for amplification of DSO
DS00103 DS00104	GTCTGTAAGTTTGCTTTGAAG (F*) CTTCAAAGCAAACTTACAGAC (R*)	Primers used to generate RepA Y265P
DS00105 DS00106	CATTCCTATATATGTAATGATCAGGTG (F*) CACCTGATCATTACATATATAGGAATG (R*)	Primers used to introduce stop codon in *orf113* coding region
DS00107 DS00108	GAGCTTAGACAATGTGATAATTACAAGG(F*) CCTTGTAATTATCACATTGTCTAAGCTC (F*)	Primers used to introduce stop codon in *orf96* coding region
DS00111 DS00110	CCGAGAATTCTATGAAGTCCTTTAG (F*) CCCTCGAGCTGTGCGGTGCGTACGG (R*)	Primers used to amplify *repA* from first ATG
DS00109 DS00110	GTGGGGGAATTCGATGCTTAAAAAAGAC(F*) CCCTCGAGCTGTGCGGTGCGTACGG (R*)	Primers used to amplify *repA* from predicted alternate start codon TTG
DS00112 DS00113	CCCTCATATGATTAGTGTACACCACCTGG (F*) CCCGTTCCTCTCGAGTTATATATCAGCAC (R*)	Primers used to amplify *Abgst01* gene
DS00114 DS00115 DS00116	GGTGTACACTGCAGATGGTATTGCCTA (R*) CCAGCGGCCGAATTCAATAC (F*) CTGAGTTTAGGAATTCATACTGTGC (F*)	Primers used to amplify *Abgst01* promoter region
DS00117 DS00118	GAACCATATGGCTGCATTACCC (R*) AGACTCCTTTGAATTCGGTTCATGTAAGTTTG TGG (F*)	Primers used to amplify *oxyR* gene
DS00119 DS00120	CCATC ACGCGTGAATTGCTTCG (F*) ACCCTGACGCGTGCATGCCTGC (R*)	Primers used to amplify Kan cassette and MCS having R6K*ori*
DS00121 DS00122	CTGGGAGCGAATTCAATGATTAAT (F*) CACCTCGAGCAAACTTTCTGCAAG (R*)	Primers used to amplify *orf96* from pTS4
DS00123 DS00124	GTGAATCACATTGAATTCTATGAA (F*) CCTTGTCTCGAGATCATTGTCTAA (R*)	Primers used to amplify *orf113* from pTS4
DS00125 DS00126	CGNTTYGGNTTYTAYGGNYTNTAGAAGAG (F*) CCTTGAARACKNGAYTGTARNGCYTCNAGNG G (R*)	Primers used to amplify partial *gst* gene
DS00127 DS00128	GGGGAGAATTCATGCTTAAAAAAGACA (F*) GGGCTCGAGTTACTGTGCGGTGCGTACGG (R*)	Primers used for cloning *repA* in pGEX-4T-1
DS00129	CTGGGAGCTGAATTCATGATTAAT (F*)	Primers used for cloning

DS00130	CCCCTCGAGTTACAAACTTTCTGCAAG (R*)	*orf96* in pGEX-4T-1
DS00131 DS00132	CCCGAATTCATGAAAAAATCAGGTGAG (F*) CCTCTCGAGTTAATCATTGTCTAA (R*)	Primers used for cloning *orf113* in pGEX-4T-1
DS00133 DS00134	TCATCCGCTTGGCAAGGCACC (F*) ACGGCATAAGTGAGCCTTCGGC (R*)	qPCR primers for amplification of *Abgst01*
DS00135 DS00136	TGGCGGGTCCTTGGGAAGCT (F*) CCAACACTTACATCTGCGGCGGT (R*)	qPCR primers for amplification of *Abgst02*
DS00137 DS00138	TGGTCCACGTGGTGAGCCAA (F*) GGACCTAAGCCACCCATTTGCCA (R*)	qPCR primers for amplification of *Abgst03*
DS00139 DS00140	GGTTGATCATAGCCAAAACCCGCCA (F*) AGGTGCACTGCCCATTTGCCA (R*)	qPCR primers for amplification of *Abgst04*
DS00141 DS00142	TCAGCCCACCTCCACTCCACT (F*) AGGCGTTGCAACATCAGGGCA (R*)	qPCR primers for amplification of *Abgst05*
DS00143 DS00144	ACCATCGCTGGATTGCTGAGTCG (F*) TGAGCAAGTGCCCAACGACGT (R*)	qPCR primers for amplification of *Abgst06*
DS00145 DS00146	CCCGTATCGCTCGCTACCTAGACG (F*) TGCAGGACGGCCAATACTGCT (R*)	qPCR primers for amplification of *Abgst07*
DS00147 DS00148	GCAACCATTGCCGACTGCTGC (F*) TGGTTGTTGTTCTGGCGCCG (R*)	qPCR primers for amplification of *Abgst08*
DS00149 DS00150	GGCTACTTGGCTGAGGGAACGG (F*) AACGCTTTATGGCCTTTTGGTTGT (R*)	qPCR primers for amplification of *Abgst09*
DS00151 DS00152	CCCCTCGAGTATATCAGCACAATTTCTTTCG (F*) GTTCCTTTTCTCGAGTATATCAGCAC (R*)	Primers used to cloned *Abgst01* gene with His-tag

2.2 Preparation of stocks

2.2.1 Ampicillin

Ampicillin stock solution was prepared by dissolving 500 mg of ampicillin in 5 ml of milli-Q water and sterilized by filtration. The sterilized solution was stored in 1 ml aliquots at -20 ^{0}C. When required 100 µl of ampicillin stock solution is added to 100 ml of medium after cooling it to 45 ^{0}C to get a final concentration of 100 µg/ml.

2.2.2 Chloramphenicol

Chloramphenicol stock solution was prepared by dissolving 300 mg of chloramphenicol in 10 ml of 100 % ethanol. The stock solution was stored in 1 ml aliquots at -20 ^{0}C. When required 100 µl of chloramphenicol stock solution was added to 100 ml of medium after cooling it to 45 ^{0}C to get a final concentration of 30 µg/ml.

2.2.3 Kanamycin

Stock solution of kanamycin was prepared by dissolving 250 mg of kanamycin sulfate in 10 ml of milli-Q water and sterilized by filtration. The stock solution was stored in 1 ml aliquots at -20 ^{0}C. When required 100 µl of kanamycin stock solution is added to 100 ml of medium after cooling it to 45 ^{0}C to get the working concentration of 25 µg/ml.

2.2.4 Streptomycin

Stock solution of streptomycin was prepared by dissolving 200 mg of streptomycin in 10 ml of milli-Q water. The stock solution was stored in 1 ml aliquots at -20 ^{0}C after filter sterilization. When required 100 µl of streptomycin stock solution was added to 100 ml of medium after cooling it to 45 ^{0}C to get the working concentration of 20 µg/ml.

2.2.5 Tetracycline

Tetracycline stock solution was prepared by dissolving 100 mg of tetracycline hydrochloride in 10 ml of 70 % (v/v) ethanol/water and sterilized by filtration. The stock solution was stored in 1 ml aliquots at -20 ^{0}C. When required 100 µl of tetracycline stock solution was added to 100 ml of medium after cooling it to 45 ^{0}C to get the working concentration of 10 µg/ml.

2.2.6 Isopropyl-β-D-thio-galactoside (IPTG)

IPTG stock (1 M) solution was prepared by dissolving 236.8 mg of IPTG in 1 ml of autoclaved milli-Q water and stored at -20 ^{0}C. When required the stock solution was thawed on ice bath and 100 µl of stock solution was added to the cooled medium (45 ^{0}C) to get 1 mM working concentrations of IPTG.

2.2.7 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal)

X-gal stock (4 %) solution was prepared by dissolving 40 mg of X-gal in 1 ml of N, N' -dimethylformamide. When required, 100 µl of stock solution of 4 % (w/v) X-gal was

added to 100 ml of medium after cooling it to 45 ^{0}C.

2.2.8 Benzoate

Benzoate stock (1 M) was prepared by dissolving 12.1 g of benzoate in 100 ml of double distilled water and the solution was filter sterilized by passing through a 0.2 µm filter (Pall Corporation, USA) and stored at 4 ^{0}C until further use. Whenever needed adequate amount of stock was added to minimal media as source of carbon.

2.2.9 Sodium succinate

Sodium succinate stock (1 M) solution was prepared by dissolving 27 g of sodium succinate in 100 ml of distilled water and the solution was filter sterilized by passing through 0.2 µm filter (Pall Corporation, USA) and the contents were stored at 4 ^{0}C until further use. Whenever needed adequate amount of stock was added to minimal media to get a final concentration of 10 mM.

2.2.10 Cumene hydroperoxide (CHP)

Cumene hydroperoxide (100 mM) stock was prepared by diluting 172 µL (from 88 %, 5.789 M) of commercially procured CHP to 10 ml with DMSO. Whenever needed adequate amount of stock was taken to get a final concentration of 100 µM.

2.2.11 Ethacrynic acid (EA)

Ethacrynic acid (50 mM) stock was prepared by dissolving 151 mg of EA in few ml of ethanol before adjusting the volume to 10 ml. Whenever needed adequate amount of stock was added to reaction mixture to get concentrations of 100 µM.

2.2.12 Hydrogen peroxide (H_2O_2)

H_2O_2 stock (100 mM) was prepared by diluting 51 µL of 30 % H_2O_2 (9.8 M) to 5 ml with sterile milli Q water. Whenever necessary 10 µl of stock solution of H_2O_2 was added to 10 ml of culture to obtain a final concentration of 100 µM.

2.2.13 Methylene blue (MB)

Methylene blue stock (10 mM) was prepared by dissolving 128 mg of MB in 5 ml of sterile milli Q water. Whenever needed adequate amount of stock was added to the culture medium to get concentrations of 5 µM.

2.2.14 Methyl parathion (MeP) (*O,O*-dimethyl-*O*-paranitrophenyl phosphorothioate)

Stock solution (0.2 M) of methyl parathion was prepared by dissolving 263.2 mg of methyl parathion in 5 ml methanol. The contents were then stored at -20 ^{0}C until further use. When used as assay substrate to measure enzyme activity, 1 µl of stock solution of methyl parathion was added to 1 ml of reaction mixture to obtain a final concentration of 200 µM.

2.2.15 *p*-Nitrophenol (PNP)

p-Nitrophenol (50 mM) stock was prepared by dissolving 347.7 mg of PNP in 50 ml of double distilled water and the solution was filter sterilized by passing through 0.2 µm filter (Pall Corporation, USA) and the sterile contents were stored at 4 ^{0}C until further use. PNP was used at a final concentration of 50 µM.

2.2.16 Paraquat (PQ**)**

Paraquat (100 mM) was prepared by dissolving 128 mg of paraquat in 5 ml of H_2O. The stock solution (10 µl) of PQ was added to 10 ml of culture medium to obtain a final concentration of 100 µM.

2.2.17 *tert*-Butyl hydroperoxide (tBHP)

t-Butyl hydroperoxide stock (100 mM) was prepared by dissolving 128 µL of 70 % (7.7778 M) tBHP to 10 ml with sterile milli-Q H_2O. Whenever needed adequate amount of stock was taken to get a final concentration of 100 µM.

2.3 Media

The following media were used to propagate bacteria. All media were sterilized by autoclaving for 20 min at 15 lb/Sq. When required appropriate amounts of antibiotics of Ampicillin (100 µg/ml), Tetracycline (25 µg/ ml), Chloramphenicol (30 µg/ ml), Streptomycin (20 µg/ml) and Kanamycin (25 µg/ml) were added after cooling the media to 45 ^{0}C.

2.3.1 Luria Bertani (LB) broth

The LB medium was prepared by dissolving 10 g of peptone, 5 g of yeast extract and 10 g of NaCl in 500 ml of distilled water. The contents were stirred and finally made up to 1 liter with milli-Q water. The pH of the medium was adjusted to 7.0 with 1 N NaOH and then sterilized it by autoclaving. The LB agar plates were prepared by adding 2 % agar to LB broth.

2.3.2 Minimal Medium

The minimal medium was prepared by dissolving 4.8 g of K_2HPO_4, 1.2 g of KH_2PO_4 and 1 g of NH_4NO_3 in a few ml of milli-Q water and finally the volume was made up to 978.5 ml. Then the salt solution was sterilized by autoclaving for 15 minutes at 15 lb pressure. $MgSO_4.7H_2O$ (1 g/10 ml), $Fe_2(SO_4)_3$ (1 g/100 ml) and $Ca(NO_3)_2.4H_2O$ (4 g/20 ml) were prepared separately and sterilized by autoclaving as described above. About 2 ml of $MgSO_4.7H_2O$, 100 µl of $Ca(NO_3)_2.4H_2O$ and 50 µl of $Fe_2(SO_4)_3$ were added to sterile 1 litre of minimal medium under aseptic conditions. When required filter sterilized benzoate (5 mM or 50 mM) or succinate (10 mM) were added to minimal medium as carbon source.

2.4 Preparation of solutions and buffers for DNA manipulations

2.4.1 Tris Acetate EDTA (TAE) buffer

A stock solution of 50 X TAE buffer was prepared by adding 121 g of Tris, 28.6 ml of acetic acid and 50 ml of 0.5 M EDTA (pH 8.0) to 1000 ml of distilled water. The contents were thoroughly dissolved before adjusting the volume of the contents to 400 ml with distilled water. The buffer was then stored in a container fixed with a tap. When necessary adequate volume of the stock buffer was diluted to get 1 X TAE with distilled water and used for preparing agarose gels.

2.4.2 Tris Borate EDTA (TBE) buffer

A stock solution of 10 X TBE buffer was prepared by dissolving 108 g of Tris, 55 g of boric acid in 900 ml of distilled water. Once the contents were dissolved, 40 ml of 0.5 M EDTA (pH 8.0) was added and finally the volume of the contents was adjusted to 1000 ml with distilled water. The buffer was then stored in a container fixed with a tap. When necessary adequate volume of the stock buffer was diluted to get 0.5 X TBE with distilled water and used for preparing agarose gels.

2.4.3 TE buffer

TE buffer was prepared by dissolving 121 mg of Tris and 37.2 mg of EDTA (pH 8.0) in 80 ml of distilled water. The pH of the buffer was adjusted to 8.0. Finally the volume was made up to 100 ml with distilled water to get TE buffer having 10 mM Tris and 1 mM EDTA.

2.4.4 Sample Loading buffer (6X) for agarose gel electrophoresis

Bromophenol blue (5 mg), 25 mg of xylene cyanol and 4 g of sucrose were dissolved in few ml of distilled water and finally volume was made up to 10 ml of distilled water. The buffer was stored at 4 ^{0}C until further use.

2.5 Buffers for southern hybridization

2.5.1 20 X SSC

Sodium citrate (0.3 M) and sodium chloride (3 M) buffer was prepared by dissolving 88.2 g of sodium citrate and 175.3 g of sodium chloride in 500 ml of distilled water and the solution was made up to 1000 ml with double distilled water.

2.5.2 Denaturation solution

Denaturation solution (1.5 M NaCl and 0.5 M NaOH) was prepared by dissolving 87.65 g of sodium chloride and 20 g of sodium hydroxide pellets in few ml of distilled water and finally the solution was made up to 1000 ml with double distilled water.

2.5.3 Neutralization buffer

Tris (121.14 g) and 87.6 g of sodium chloride were dissolved in 500 ml of distilled water and the pH of the solution was adjusted to 7.4 with 1 N HCl. The final volume of the buffer was made up to 1000 ml with double distilled water to get neutralization buffer containing 1 M Tris (pH 7.4) and 1.5 M NaCl.

2.6 DNA manipulations

2.6.1 Genomic DNA isolation

Genomic DNA was isolated by following method described elsewhere (Christopher et. al., 2002). About 5 ml cells were harvested from culture (O.D.= 0.8) and washed with 0.75 ml of 1 % NaCl before resuspending in 0.75 ml of TES buffer (10mM Tris-HCl, 10mM EDTA pH 8.0, 2% SDS). The cell lysate was then extracted with phenol:chloroform (3:1 ratio) mixture. The clear aqueous layer collected from the phenol:chloroform extract was then added to equal volumes of chloroform and re-extracted to remove traces of phenol. The phenol free aqueous layer obtained was used to precipitate DNA by adding 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol. The contents were then incubated at room temperature for 15 minutes and the precipitated DNA was

centrifuged at 12,000 rpm for 20 min. The DNA pellet was then washed with 70 % ethanol before resuspending it in TE (10 mM Tris-HCl, 2 mM EDTA pH 8.0 and 50 ug/ml of RNase).

2.6.2 Purification of plasmids

Plasmids were purified using QIAgen mini preparation kit especially when used for performing cloning and sequencing. An overnight culture (3 ml) of LB was developed by inoculating plasmid carrying colony in LB broth containing appropriate antibiotics. The cells were harvested by centrifuging the contents at 13,000 rpm. The bacterial cell pellet was resuspended in 250 µl of buffer P1. The cells were then lysed by adding 250 µl of buffer P2 followed by mild shaking through gentle inversion for 4-6 times. After lysis of the cells 350 µl of buffer N3 was added and the tube was inverted immediately for 4-6 times. Then tube was centrifuged at 13,000 rpm for 10 minutes to pellet down the cell debris. After centrifugation, the supernatant was directly transferred to a QIAprep column placed in the collecting tube and centrifuged for 1 minute at 13,000 rpm. The column was then washed with 0.75 ml of buffer PE. The residual wash buffer PE was removed from column through a brief centrifugation at 13,000 rpm for 1 minute. Finally, plasmid DNA was eluted from the column by adding 50 µl buffer EB (10 mM Tris-HCl, pH 8.5) or H_2O to the centre of QIA preparation column followed by brief centrifugation at 13,000 rpm. The plasmid DNA was stored at -20 ^{0}C until further use.

2.6.3 Isolation of Plasmid by Alkaline Lysis method

Mini preparations of plasmid DNA was carried out by following the procedures of Birnboim and Doly (1979) and Ish-Horowicz and Buker (1981) with slight modifications. A single bacterial colony carrying plasmid to be isolated was inoculated into 3 ml of LB medium containing appropriate antibiotic and was incubated overnight at 37 ^{0}C with

vigorous shaking. Bacterial cells were collected from 1 ml of overnight culture by centrifugation at 13,400 rpm for 1 minute. The cell pellet was resuspended in 100 µl of ice-cold solution I (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0) by vigorous vortexing. The above bacterial suspension was mixed with 200 µl of freshly prepared solution II (0.2 N NaOH, 1 % SDS) before adding 150 µl of ice-cold solution III (3M potassium acetate, pH 4.8). The contents were then thoroughly mixed by inverting the tube 4-5 times. Then tube was kept on ice for 3-5 minutes. Precipitate formed in the above mixture was centrifuged by spinning the contents at 13,400 rpm for 10 minutes. The supernatant was then transferred into a fresh tube and extracted with equal volumes of phenol: chloroform. The clear aqueous phase was transferred to a fresh tube and the plasmid DNA was precipitated by adding 1/10th volumes of 3 M sodium acetate and 2 volumes of ethanol and tubes was kept at -20 ^{0}C for 30 minutes. Then the tubes were centrifuged at 13,400 rpm for 20 minutes at 4 ^{0}C in a microfuge to pellet down the plasmid DNA. The DNA pellet was further washed with 70 % ethanol to remove traces of salts associated with plasmid. Subsequently the plasmid DNA was dried before redissolving it in 50 µl of TE (pH 8.0) and stored at -20 ^{0}C until further use.

2.6.4 Isolation of indigenous plasmids

Large indigenous native plasmids were isolated essentially by following the Currier-Nester protocol with the following modifications (Currier and Nester, 1976). A single colony of *A. baumannii* DS002 was streaked on succinate containing minimal medium plate supplementing with 30 µg of Cm. The plate was subsequently incubated at 30 ^{0}C for 48 hrs to obtain a lawn of cells. These cells were then taken with the help of a sterile tooth pick and suspended in 10 mM Tris buffer pH 8.0 to get a bacterial cell suspension with a cell density of OD 0.75-0.8 (425 nm). The cells were harvested from the

suspension by centrifuging at 10,000 rpm for 10 min at 4 ^{0}C and the pellet obtained was resuspended in a 5.4 ml of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) containing 0.21 ml of lysozyme (10 mg/ml). The contents were then incubated at 37 ^{0}C for 15 min to facilitate cell wall lysis due to lysozyme activity. After incubation, 0.3 ml of freshly prepared 20 % (w/v) SDS (in TE buffer) was added and the contents were mixed by inversion to ensure uniform distribution of the SDS. The lysed mixture was vortexed at maximum speed for 2 min to shear chromosomal DNA and the sheared DNA was denatured by addition of 0.2 ml of 3 N NaOH. Then the contents were incubated at room temperature for 15 min with occasional shaking to ensure complete denaturation of DNA. After denaturation, the contents were neutralized by adding 1 ml of 2 M Tris pH 7.0. Subsequently, 1.5 ml of 5 M NaCl was added before extracting the contents with salt-saturated phenol. The clear aqueous phase was collected in a fresh tube and the plasmids present in the aqueous phase were precipitated with ethanol. The plasmid DNA pellet was washed with 70 % of ice cold ethanol to remove traces of salts and the salt-free plasmid DNA was stored at -20 ^{0}C until further use after dissolving it in 50 µl of TE buffer (pH 8.0).

2.6.5 Isolation of total RNA

Single colony of *A. baumannii* DS002 was inoculated in 3 ml of LB broth having 30 µg of Cm and incubated at 30 ^{0}C to obtain overnight culture. About 1 % of this overnight culture was used as inoculum in 10 ml LB broth to prepare mid log phase cultures. The total RNA was isolated from these cells by using TRI REAGENT (Sigma) following manufacturer's protocol. Cells from 1 ml culture were harvested by centrifugation at 6000 rpm for 5 minutes at 4 ^{0}C. The pellet was then resuspended in 1 ml of TRI REAGENT followed by addition of 0.2 ml of chloroform. The content was then vigorously shaken for

15 sec and allowed to stand for 2-15 minutes at room temperature. The resulting mixture was then centrifuged at 12,000 g for 15 minutes at 4 ^{0}C to separate the mixture into three layers; a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase with RNA. The aqueous phase was transferred to a fresh tube, to which 0.5 ml of isopropanol was added before incubating at room temperature for 5-10 minutes. The contents were then centrifuged at 12,000 g for 10 minutes at 4 ^{0}C to pellet down precipitated RNA. The RNA pellet was then washed with 1 ml of 75 % ethanol before allowing the RNA pellet to dry for 5-10 minutes at room temperature. The RNA pellet was finally dissolved in 20 µL of DEPC treated water. The purity of RNA was determined by taking ratio of spectrophometric readings taken at 260 and 280 nm.

2.6.6 cDNA synthesis

The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit supplied by Thermo scientific (Germany) following the manufacture's protocol. About 2 µg of total RNA was incubated with 1 µl of random hexamer primer (0.2 µg/µl) in DEPC-treated water (12 µl reaction mixture) at 70 ^{0}C for 5 minutes. The reaction mixture was then kept on ice and the following components were added with the following order: 4 µl of 5 X reaction buffer, 1 µl of Ribolock Ribonuclease Inhibitor (20 U/ µl) and 2 µl of 10 mM dNTP mix. The contents were gently mixed and incubated at 25 ^{0}C for 5 minutes before adding 200 unit of Revert Aid H Minus M-MuLV Reverse Transcriptase. The contents were further incubated for an additional 10 minutes at 25 ^{0}C and shifted to 42 ^{0}C water bath for 1 hr. Finally the reaction was stopped by heating at 70 ^{0}C for 10 minutes and stored at -20 ^{0}C until further use.

2.6.7 Preparation of DNA standards for Real-time PCR

For calculating copy number of DNA the following formula was used:

1 µg of 1 Kb DNA = 9.1 X 10^{11} molecules

Molecular weight of clone (with insert) is X Kb

Concentration of clone is Y ng/µl

Therefore, 1 µg is present in 1000/Y = Z µl

To know how many copy number is present in 1 µg of clone:

9.1 X 10^{11}/Mol. Wt. of clone (in Kb) = N X 10^{11} molecules

Take Z µl and make upto N ml,

This gives 1 X 10^{11} copies/ml or 1 X 10^{8} copies/ µl

2.6.8 Real-time PCR

The mRNAs of the *Abgsts* were quantified by performing Real Time PCR. The RNA samples isolated from *A. baumannii* DS002 grown under different stress conditions were used as templates. The 16S rRNA was used as an internal control. Total RNA (2 µg) was reverse transcribed using random hexamers and Reverse transcriptase (Fermentas) and the corresponding cDNAs (2 µl) were used as templates in a 25 µl PCR reaction mixture containing SYBRGreen assay mix (Biorad). In the SYBR green assay, the concentrations of forward and reverse primers were kept at 250 nM. Primers designed using Primer-BLAST program that gave an amplicon size of less than 250 bp with annealing temperature of 60 ^{0}C were selected for qPCR. The thermal cycling was carried out with a gradient mastercycler (eppendorf, Germany) under manufacturer's universal thermal cycling conditions; the denaturation program (94 ^{0}C for 2 minutes), amplification and quantification program repeated 40 times (94 ^{0}C for 15 seconds and 60 ^{0}C for 1 minutes),

melting curve program (60 ^{0}C to 95 ^{0}C with the heating rate of 1.75 ^{0}C per min) and finally cooling to 40 ^{0}C.

2.6.9 PCR amplification

PCR amplification reactions were performed in a 25 µl volume containing 2.5 mM $MgCl_2$, dNTP mix containing 200 µM each of dATP, dCTP, dGTP and dTTP mix (Fermentas), 10 pico mol of each forward and reverse primers, 1.0 Unit of *Taq* polymerase or *pfu* DNA polymerase, 10-20 ng of plasmid / genomic DNA used as a template. Amplifications were carried out in the thermal cycler (Biorad) by suitably adjusting the PCR programme depending on the amplicon size and Tm of the primers. Amplification products were analyzed on 0.8 % agarose gel electrophoresis.

2.6.10 Purification of PCR products

After performing the polymerase chain reaction (PCR), the total PCR reaction mixture (25 µl) was taken into an eppendorf tube and the PCR product was purified using QIAgen PCR purification kit by following the manufactures protocol. The 25 µl PCR reaction mixture was mixed with 125 µl of buffer PB (binding buffer) and passed through Qia quick spin column fitted in a collecting tube. The flow through was discarded and the QIA quick spin column was washed with 0.75 ml of PE (wash buffer). The column was then placed in a new eppendorf tube and subjected for brief centrifugation to remove the traces of ethanol. The column was then placed in a 1.5 ml eppendorf tube and the DNA was eluted by adding 50 µl of buffer EB (10 mM Tris- Cl, pH 8.5).

2.6.11 Agarose gel electrophoresis

Agarose gel electrophoresis was performed by the following standard procedures described elsewhere (Sambrook et al., 1989). Required amount of agarose was dissolved in TBE or TAE by heating in micro oven. The solution was then cooled to 50 - 55 ^{0}C

before adding ethidium bromide (0.5 µg/ml) and poured on a clean sterile gel tray fitted with combs. The gel was allowed to solidify at room temperature. After solidification the gel along with the gel tray was immersed in the TBE or TAE buffer poured in the electrophoretic tank. Buffer level was adjusted to cover the gel to a depth of about 1 mm. Appropriate amount of DNA samples were mixed with 6 X loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol, 40 % sucrose) and loaded into the wells of submerged gel. Electrophoresis was carried out at 100 volts till the bromophenol blue reaches the end of the gel. The gel was then visualized under UV transilluminator and the electrophoretic mobility of DNA was recorded by taking the photograph of the gel using gel documentation system.

2.6.12 Purification of DNA fragments from agarose gel

The DNA fragments were extracted from agarose gel by using QIAgen gel extraction kit. After performing agarose gel electrophoresis, appropriate portion of the gel containing desired DNA fragment was sliced, weighed and carefully taken into a microcentrifuge tube. Then 3 volumes (W/V) of buffer QG was added and incubated at 50 ^{0}C for 10 min to dissolve the gel. After the gel slice was completely dissolved, 1 volume (w/v) of isopropanol was added. Meanwhile the QIA quick column was placed in a collection tube and the contents were carefully transferred into the column. Immediately the column fitted in collection tube was placed in a microfuge and the contents were centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the column was again washed with 0.75 ml of buffer PE (70 % alcohol). Finally the column was subjected to a brief spin to remove residual amount of ethanol found in buffer PE. The QIA quick column was then placed in a 1.5 ml eppendorf tube and the DNA was eluted in 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) or Milli-Q water.

2.6.13 DNA ligation

Concentration of vector and insert were estimated and were taken in a ratio of 1:3 (vector : insert) in a sterile eppendorf tube. One unit of T_4 DNA ligase and 2 µl of 10 X ligation buffer were added and the reaction volume was adjusted to 20 µl with milli-Q water. Ligation reaction was incubated at 16 ^{0}C for 16 hours. A portion of ligation mixture was taken to transform *E. coli* competent cells.

2.6.14 Preparation of Ultra-Competent cells

Ultra Competent cells were prepared following the procedures of Inoue et al., (1990). *E. coli* cells required to make competent were grown at 18 ^{0}C in 250 ml of LB broth taken in 1 L flask by inoculating with an initial inoculum of 1 % overnight culture. The cultures were allowed to grow (overnight) till the cell density reached to 0.4-0.5 OD at 600 nm. The culture was then chilled on ice for 10 minutes and centrifuged at 2500 g for 10 minutes at 4 ^{0}C to harvest the cells. The cell pellet was then suspended in 80 ml of ice cold Inoue transformation buffer [55 mM $MnCl_2.4H_2O$, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 10 mM PIPES (0.5 M, pH 6.7)] and centrifuged at 2500 g for 10 minutes at 4 ^{0}C. The pellet was then resuspended into 20 ml of ice cold Inoue buffer and incubated on ice for 10 minutes after adding 1.5 ml of DMSO. Working quickly, the cells were dispensed into sterile microfuge tubes in 100 µl aliquots and the tightly closed tubes were then snap freezed by placing them in a bath of liquid nitrogen. The tubes were stored at -70 ^{0}C until further use.

2.6.15 Transformation

The frozen competent cells were thawed by placing them on ice bath. The ligation mixture/plasmid of interest were added and incubated on ice for 30 minutes. After 30 minutes, the cells were subjected to heat shock at 42 ^{0}C for exactly 90 sec and

immediately chilled on ice for 2 min. Further, 800 µl of LB broth was added and incubated at 37 ^{0}C for 45 min. The cells were collected by centrifugation and resuspended in 200 µl of LB broth before plating on LB agar plates containing appropriate antibiotic. The plates were then incubated at 37 ^{0}C for 12 hr to observe transformed colonies.

2.6.16 Preparation of electro-competent cells

Electro-competent cells were prepared following the procedures described elswhere (Sharma and Schimke, 1996). Overnight culture (1 %) of *E. coli* or *A. baumannii* DS002 were grown in 100 ml of LB broth at 37 ^{0}C and 30 ^{0}C respectively with an initial inocolum of 1 %. The cultures were allowed to grow till the cell density reached to 0.3-0.4 OD at 600 nm. The culture was chilled on ice for 30 minutes and centrifuged at 4000 rpm for 10 minutes to harvest the cells. The cell pellet was then suspended in 50 ml of ice cold 10 % glycerol. The process was repeated thrice to remove traces of salts. Finally, the cells were resuspended in sterile 10 % glycerol (10 ml) and dispensed in 100 µl aliquots before flash freezing by dipping them in liquid nitrogen. The frozen competent cells were stored at -80 ^{0}C until further use.

2.6.17 Electroporation

About 1 to 2 µg of DNA was added to 100 µl of electro-competent cells thawed on ice. The suspension was mixed vigorously by flicking the tube. The cell/DNA mixture was placed in a pre-chilled cuvette, between the electrodes and a pulse of 2.5 kV was applied for 4.5 sec using Genepulser (BioRad, USA). Following the pulse, immediately 1 ml of SOC medium was added to the cells (SOC: 2% Bacto tryptone, 0.5% Bacto yeast extract, 1 mM NaCl, 2.5mM KCl, 1 mMMgCl2, 10 mM MgSO4, 20 mM glucose). The cells mixed in the broth were taken in a 1.5 ml tube and incubated at 37 ^{0}C for 1 hour with constant

shaking. After the incubation period, the cells were diluted appropriately in SOC and plated on LB agar plates containing appropriate antibiotics.

2.6.18 Conjugation

Biparental matings were performed as follows: The cells of the donor strain *E. coli* S17-1 having plasmid of interest and the *A. baumannii* DS002, the recipient strain were mixed in 1:1 ratio before spreading uniformly on the surface of an LB plate. After 24 h incubation at 30 ^{0}C, the cells were scrapped using sterile tooth pick and resuspended in 0.9 % NaCl solution. Serial dilutions made for the cell suspenssion was plated on LB plate containing appropriate antibiotics.

2.6.19 Genomic DNA library construction

Genomic DNA library of *Acinetobacter baumannii* DS002 was constructed using CopyControl Fosmid Library Production Kit (EPICENTRE Biotechnologies, USA). The genomic DNA was sheared and the DNA fragments of 40 Kb were selected by performing gel electrophoresis. The sheared DNA was then end-repaired to get blunt ended DNA. It was then size resolved by running in 1% agarose gel electrophoresis along with control DNA. The blunt-end DNA was then ligated to the 8.1 Kb linear pCC1FOS vector. The ligated DNA was then mixed with MaxPlaxTH Lambda Packaging Extracts and incubated at

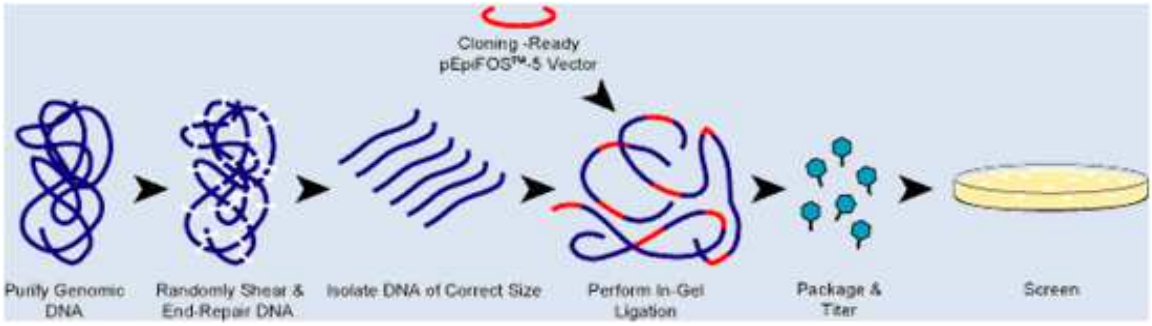

Figure 1. Overview of strategy used for construction of fosmid library for the genomic DNA of *Acinetobacter baumannii* DS002.

30 ^{0}C for 90 min. The packaged phages were then serially diluted before infecting *E. coli* EP1300 cells by incubating together at 37 ^{0}C for 25 min. The infected cells were then plated on chloroamphinicol plates and incubated at 37 ^{0}C for overnight for colonies to appear. The strategy used to construct genomic library is shown in Fig. 1.

2.6.20 Southern hybridization

2.6.20.1 Preparation of DNA probes for hybridization

About 25 to 50 ng of probe DNA amplified using target gene specific primers were taken independently in a clean eppendorf tube and denatured it by incubating for 5 minutes on a boiling water bath. The denatured DNA probe was then added to the reaction mixture containing 2.5 µl of 10 X assay buffer, 1 µl of random primer (100 ng/µl), 2.5 µl of 20 mM DTT, 2 µl of dATP, dGTP and dTTP mixture. Immediately 30 µCi of ^{32}P labelled dCTP was added and the labelling reaction was initiated by adding 1 µl of Klenow enzyme (3 units/µl). The labelling reaction was allowed to proceed by incubating at room temperature for 2 hours. After the completion of labelling reaction, the DNA was ethanol precipitated to remove free nucleotides. The ^{32}P labelled double stranded DNA was denatured by placing the tube at 100 ^{0}C for 5 minutes followed by immediately chilling on ice before adding to the membrane submerged in the hybridization solution.

2.6.20.2 Southern transfer

The DNA gel was depurinated by submerging it for 10 min at room temperature in 250 mM HCl. The gel was rinsed with double distilled water and subjected to denaturation at room temperature by submerging it in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 25 minutes. After denaturation, the gel was neutralized by placing it in a neutralization buffer (0.5 M NaCl, 0.5 M Tris-HCl pH 7.5) for 30 min. The blotting apparatus (Biorad Trans-Blot SD) was assembled, according to the manufacture's

protocol (Biorad), using 0.5 X TBE as the transfer buffer and the DNA was transferred onto Hybond N+ at a constant current of 3.55 mA/cm^2 for 1 hour. After transfer, the membrane was UV cross-linked and the membrane was inserted into hybridization tube containing prehybridization buffer (0.5 M Phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 7 % SDS). The tube was then placed in hybridization oven set at 65 ^{0}C for two hours. After two hours of incubation, the prehybridization buffer was replaced with similar amount of the hybridization buffer (Prehybridization buffer with 1 % BSA W/V) which contained denatured labelled probe. The hybridization reaction was performed for 16 hours at 65 ^{0}C.

2.6.20.3 Washing and detection

After completion of hybridization, the membrane was removed from hybridization solution and immediately immersed in a large volume of (300 to 500 ml) wash solution 1 (2 X SSC + 0.1 % SDS). The washing process was continued at room temperature for 5 minutes with gentle agitation. After preliminary wash, the membrane was transferred into hybridization tube containing 300 to 500 ml of wash solution 2 (1 X SSC + 0.1 % SDS) and the process of washing was continued for 1.30 hrs at 68 ^{0}C. The process is repeated twice and the washed membrane was air dried before exposing to X-ray film after wrapping it in a clean saran wrap paper. The X-ray film was then developed to capture the image of DNA hybridized to the probe.

2.6.21 Colony Hybridization

Colony hybridization was performed by following the procedure described elsewhere (Sambrook et al., 1989). Master plates containing clones to be screened were taken and a dry, sterile detergent-free nitrocellulose filter was placed on the surface of the master plate so that it gains contact with the bacterial colonies. The filter was then carefully

lifted out of the plate and the colonies stuck on the surface of the filter were lysed by placing the filter for 5 minutes in denaturing (1.5 M NaCl, 0.5 M NaOH) solution. The filter was then neutralized by submerging in neutralizing buffer (1 M Tris-Cl pH 7.4, 1.5 M NaCl) followed by rinsing it in 2 X SSPE (0.3 M NaCl, 0.02 M NaH_2PO_4, 0.002 M EDTA) buffer. The DNA was then fixed onto the membrane by exposing it to UV light keeping DNA side down for 5 minutes. After cross-linking, the process of hybridization was done following protocol described elsewhere.

2.7 Rescue-cloning of indigenous plasmids

2.7.1 *In vitro* transposition

Rescue-cloning was done by tagging the mini-transposon having R6Kγori replicative origin. The isolated plasmids were treated with plasmid safe (Epicenter Biotechnologies, USA) to remove linear DNA and were used to tag with EZ-Tn5™ <R6Kγori/KAN-2> using the Transposon Insertion Kit (Epicenter Biotechnologies, USA). A 10 µl reaction mixture contained 1 µl of 10 X buffer, 1 µg of plasmid DNA, equimolar concentration of transposon and 1 U of transposase. The reaction mixture was incubated for 2 hours at 37 ^{0}C and the reaction was stopped by adding 1 µl of stop solution followed by incubation of the reaction mixture at 70 ^{0}C for 10 min. A 1 µl aliquot of the transposition mixture was then electroporated into *E. coli* EC100D *pir-116* cells at 2.5 kV, 200 Ω for 4.5 sec using a GenePulser (Bio-Rad Laboratories, USA). After electroporation 1 ml of SOC medium was added to the electroporated cells and incubated for 1 hour at 37 ^{0}C with vigorous shaking. After incubation, the cells were plated on LB plates supplemented with kanamycin and incubated for 12 hours at 37 ^{0}C. The Kan^R colonies were independently subcultured in LB medium supplemented with arabinose (1 mM) and the rescued plasmids were isolated following a conventional plasmid isolation technique (Birnboim and Doly, 1979). The plasmids were then digested with different restriction

endonucleases and those which gave a unique restriction profile were isolated and analyzed further.

2.7.2 DNA sequencing and analysis

DNA sequencing was done following Sanger method (Sanger et. al*.,* 1975). The sequence was analyzed using online bioinformatic tools. The NCBI GLIMMER programme (www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi, Delcher et al., 1999) was used to predict open reading frames (ORFs) and for promoter prediction, BPROM software (www.softberry.com/berry.phtml?topic=bprom) was used. NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST, Altschul et al., 1990), EBI ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/, Thompson et al., 1994) and REPFIND software (http://cagt.bu.edu/page/REPFIND_submit, Betley et al., 2002) were used for sequence similarity search, multiple sequence alignment and for finding repeat sequences, respectively. The double-stranded origin (DSO) of replication was identified by aligning the pTS4 DNA sequence with known sequences of other rolling-circle (RC) plasmids available in the database (Khan, 1997). DNA secondary structure was predicted using the Mfold programme (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form, Zuker, 2003), and antigenic determinants in PrP and pTS4 encoding proteins were predicted using online bioinformatics tools (Kolaskar and Tongaonkar, 1990).

2.7.3 Site-directed mutagenesis

Site-directed mutagenesis was performed following the procedure described elsewhere (Fisher and Pei, 1997). Initially the region to be mutated was selected and overlapping primers were generated by introducing desired mutations in the primer sequence. The plasmid containing the gene of interest was used as template and the PCR was performed using appropriate primers having mutation at required place. The PCR

was performed for 20 cycles using *pfu* polymerase. The PCR product was then digested with *Dpn*I to eliminate template plasmid and to enrich the amplified DNA. After *Dpn*I treatment the template was used to transform into *E. coli* DH5α cells. The colonies appeared on selection plates were randomly selected to isolate plasmid and to confirm sequence by performing sequencing.

2.7.4 Assay of nicking activity

The nicking activity was assayed by assessing conversion of super-coiled (SC) DNA to open-circle (OC) form. The plasmid DNA to be used for nicking activity was isolated and analyzed in a preparative agarose gel (0.8 %). The band corresponding to the SC DNA was gel extracted and used as substrate for enzyme creating nick at specified site. While performing nicking assay the SC form of DNA was used as a substrate. About 0.8 µg of SC form of plasmid was incubated with either increased protein concentration or with increased incubation time with fixed protein (RepA) concentration. The amount of converting SC form of DNA to OC form is assayed by analyzing on agarose gel (0.8 %). Decrease in concentration of SC form DNA and a proportionate increase in concentration of OC form is taken as indication of nicking activity.

2.7.5 Electro-mobility shift assay (EMSA)

EMSA was performed as described previously (Hellman and Fried, 2007). A 100 bp DNA fragment containing the double stranded origin of replication (DSO) region was amplified using the primer pair DS00101/DS00102 designed taking sequence flanking to DSO of pTS4. The PCR amplicon containing pTS4 DSO was end-labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase following the standard protocols with the following modification (Sambrook, 1989). The PCR product was gel-extracted using a Qiagen kit, 40 picomoles of DNA were incubated with 10 µCi [γ-^{32}P] ATP and 10 units of T4

polynucleotide kinase in 50 µl of 1 X kinase buffer at 37 ^{0}C for 20 min. The labeled DNA was purified by using a PCR purification kit (Qiagen, Germany). The following concentration (0 ng, 100 ng, 250 ng, 500 ng and 1000 ng) of purified $RepA_{6His}$ were incubated with 2 picomoles of the labeled probe in 20 µl of binding buffer [20 mM Tris-HCl pH 8.0, 1.0 mM EDTA, 6 mM $MgCl_2$, 50 mM KCl, 50 µg/ml bovine serum albumin (BSA), and 5 % (w/v) glycerol] containing 5 µg/ml of herring sperm DNA for 20 min at 25 ^{0}C. The DNA-protein mixture was resolved on a 6 % (w/v) native polyacrylamide gel and was analyzed by autoradiography.

2.8 Isolation of Virus

2.8.1 PEG precipitation

Culture supernatant of *A. baumannii* DS002 (1 L) was prepared form an overnight culture by centrifuging at 8000 rpm for 10 min. The clean supernatant was then used to precipitate viral particles. Initially, the culture supernatant was brought to 4 ^{0}C by placing it in a cold room. After cooling, the culture was placed on a magnetic stirrer and salt was added to bring the final concentration of NaCl to 1 M. After adding salt, PEG 8000 (Himedia) was slowly added to the salted culture supernatant to reach PEG 8000 to a final concentration of 10 % (W/V). The contents were then left overnight in cold room (4 ^{0}C) with constant stirring. The PEG 8000 precipitated viral particle were then collected by centrifuging at 15,000 rpm for 20 min. The obtained pellet was then resuspended in 2 ml of 20 mM phosphate buffer (pH 7.0) and left at 4 ^{0}C until further use.

2.8.2 Density gradient ultracentrifugation

The sucrose solutions with 10, 20, 30 and 40 % were prepared by dissolving appropriate amount of sucrose in 20 mM phosphate buffer pH 7.0. While preparing sucrose gradient, initially 10 ml of 40 % sucrose was taken in a transparent centrifuge

tube fixed to a stand. Subsequently similar amount of 30 % and 20 % sucrose solution were slowly layered without disturbing the gradient. Finally 5 ml of 10 % sucrose solution was layered before adding 2 ml of viral suspension. The tubes were then placed in swinging-bucket rotor (SW 28) and centrifuged at 25,000 rpm for 3 hrs. After centrifugation, the band containing virus particles were seen by passing visual light through centrifuge tube. The band with viral particles was collected with the help of a long needle fitted to a syringe. Sucrose from viral preparation was removed by exclusive dialysis.

2.9 Preparation of buffers and solutions for SDS-PAGE

2.9.1 Acrylamide solution

Acrylamide solution (30 %) was prepared by dissolving 30 g of acrylamide and 0.8 g of N, N- methylene-bis-acrylamide in 70 ml of distilled H_2O. The contents were then filtered and the solution was finally adjusted to 100 ml. The stock solution prepared in this manner is stored at 4 ^{0}C until further use.

2.9.2 Staking gel buffer for SDS-PAGE

Tris (3.03 g) was dissolved in 50 ml of double distilled H_2O and the pH of the solution was adjusted to 6.8 using 1 N HCl before adjusting the volume to 100 ml with distilled H_2O.

2.9.3 Running gel buffer for SDS-PAGE

About 59.93 g of Tris was dissolved in 400 ml of double distilled H_2O and pH of the solution was adjusted to 8.8 using 1 N HCl. Finally the volume of the buffer solution was made up to 500 ml using distilled H_2O.

2.9.4 Tank buffer (pH 8.5) for SDS-PAGE

Tank buffer (1X) concentration was made by dissolving 3.03 g of Tris, 14.4 g of

glycine and 1 g of SDS in 500 ml of double distilled H_2O. Finally, the volume of the buffer was made up to 1000 ml. The buffer at 1 X concentration contains 0.025 M Tris, 0.192 M glycine and 0.1 % SDS.

2.9.5 Sample loading buffer for SDS-PAGE

The following components (1.2 ml of 0.5 M Tris pH 8.0, 2 ml of 10 % SDS, 1 ml of 10% glycerol, 0.5 ml of *β*- mercaptoethanol and 0.001 g of bromophenol blue) were taken in a 10 ml reagent bottle. The contents were mixed well before adjusting the volume to 10 ml with double distilled H_2O. The solution was stored at 4 ^{0}C and when necessary, adequate amounts of loading buffer were used to prepare protein samples.

2.9.6 Staining solution

Coomassie brilliant blue (0.25 g) was dissolved in 50 ml of methanol. To this 10 ml of acetic acid was added and finally the volume was made up to 100 ml with double distilled H_2O. The contents were stored at room temperature in amber color bottle until further use.

2.9.7 Destaining solution

About 30 ml of methanol was mixed with 10 ml of glacial acetic acid before making up the volume to 100 ml using double distilled H_2O. The contents were freshly made and used once to destain the SDS-PAGE gels.

2.9.8 Protein Markers

Low molecular weight protein markers supplied by MBI Fermentas were used. Size of the protein markers include, 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa.

2.10 Preparation of buffers for Western blotting

2.10.1 Protein transfer buffer

Trizma-base (3.03 g) and 14.4 g of glycine were dissolved in 650 ml of double distilled

H_2O. To this 200 ml of methanol was added and final volume was made up to 1000 ml with distilled water. The buffer was stored at 4 ^{0}C until further use.

2.10.2 TBS-T Buffer

Tris (2.42 g) and 8 g of sodium chloride were dissolved in 500 ml of double distilled H_2O. The pH of the solution was then adjusted to 7.6 using 1 N HCl before adding 1 ml of 0.1 % Tween-20. Finally the volume of the buffer solution was made up to 1000 ml with distilled water.

2.10.3 Blocking Buffer

The blocking buffer was prepared just before use by dissolving 0.5 g of skimmed milk powder in 10 ml of TBS-T. The contents were stirred to ensure complete dissolution of milk powder before adding to the membrane.

2.10.4 Phosphate buffered saline (PBS) buffer

1 X PBS (pH 7.3) was prepared by dissolving the following compounds; 8.18 g of NaCl, 0.2012 g of KCl, 1.4196 g of Na_2HPO_4 and 0.2449 g of KH_2PO_4 in 800 ml of double distilled H_2O. The pH of the buffer was adjusted to 7.3 before adjusting the volume to 1000 ml. The buffer was sterilized by autoclaving and stored at room temperature.

2.10.5 Stripping solution

Stripping solution was prepared by dissolving 1.5 g of glycine, 0.1 g of SDS and 1 ml of tween 20 in 100 ml of double distilled water. The pH of the solution was adjusted to 2.2 with 6 N HCl.

2.11 Protein analysis

2.11.1 Protein estimation

An aliquot of sample was pipetted out into a 1.5 ml microfuge tube. The volume of the sample was adjusted to 0.1 ml with double distilled water. One ml of Bradford

reagent (100 mg/L Coomassie Brilliant Blue G-250, 50 ml/L 95% ethanol and 100 ml/L 85% (w/v) phosphoric acid) was added and the contents were mixed by gentle inversion. After 15 min, absorbance at 595 nm was measured spectrophotometrically against a reagent blank prepared from 0.1 ml of appropriate buffer and 1 ml of Bradford reagent. Protein concentration of the sample was calculated from a standard curve drawn using bovine serum albumin.

2.11.2 SDS-polyacrylamide gel electrophoresis

The protein samples were separated by SDS-Polyacrylamide gel electrophoresis following the procedures of Laemmli (1970). 12.5 % running acrylamide gel solution was prepared by mixing 3.6 ml 30 % acrylamide solution, 4.5 ml running buffer (0.98 M Tris-Cl, pH, 8.8), 0.9 ml distilled water, 90 µl of 10 % SDS, 4.5 µl of TEMED and 45 µl of freshly prepared 10 % ammonium persulphate. The solution was mixed well before adding ammonium persulphate. The contents were then poured in between two sealed glass plates containing 1 mm spacers to form a slab. These contents were over-layered with 0.1 ml water saturated n-butanol and allowed to polymerize for 15 minutes at room temperature. After polymerization of running gel, butanol was removed by repeated washing and the traces of water were removed by wiping with filter paper strips. The 7.5 % stacking acrylamide gel solution was prepared by taking 1.25 ml of 30 % acrylamide solution, 2.5 ml of stacking gel buffer (0.325 M Tris-Cl, pH, 6.8), 1.25 ml of water, 50 µl of 10 % SDS, 5 µl of TEMED and 50 µl ammonium persulphate. The stacking gel solution was carefully over layered on the running gel. Immediately a comb of required size was placed to form the wells. The stacking gel was allowed to polymerize for 15 minutes. After 15 minutes the comb was removed and the wells were washed with tank buffer (50 mM Tris-Cl, 0.384 M glycine, 0.1 % SDS, pH 8.5). Protein samples were mixed with equal

volumes of 2 X sample loading buffer (50 mM Tris HCl, pH 6.8, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol, 100 mM 2-mercaptoethanol) and kept in a boiling water bath for 5 minutes. The contents were then briefly centrifuged before loading the sample into the wells. The electrophoresis was carried at 100 volts till the tracking dye reached the anode end of the gel. The gel was removed from the glass plates and the protein bands were stained with staining solution containing 0.2 % W/V coomassie brilliant blue in methanol : water : acetic acid (50 : 40 : 10) for 6-8 h. The gel was then destained in the destaining solution containing methanol : water : acetic acid (30 : 60 : 10) for another 6-8 h.

2.11.3.1 Western blotting

Western blotting was performed using ECLPlus western blotting detection system supplied by Amersham Pharmacia Biotech, USA. Left-hand corner on the top of the gel was cut to mark the orientation of the gel. An extra thick sheet of Whatman filter paper soaked in towbin buffer (25 mM Tris, 192 mM glycine and 20 % methanol, pH 8.3) and placed on anode graphite plate. Polyvinylidene fluoride (PVDF) membrane (Highbond-P) was kept in methanol for 30 seconds to prewet the membrane and immediately rinsed with towbin buffer and placed on top of the filter papers. Then the SDS-PAGE gel submerged in towbin buffer was placed on top of the membrane. On top of the gel, an extra thick sheet of filter paper soaked in towbin buffer was placed. Transfer of proteins onto PVDF membrane was carried out at constant voltage of 18 V for 30 min.

Once the transfer process was finished, membrane was stained with ponceau reagent to check the transfer of proteins on to the PVDF membrane. The Ponceau was removed by rinsing the membrane with 1 X TBS-T buffer. Then the membrane was blocked with blocking reagent such as 10 % skimmed milk powder in 1 X TBS-T buffer (20 mM Tris-base; 137 mM NaCl; pH 7.6). Blocking was continued for 1 hr at room

temperature with constant shaking. Then the membrane was washed three times for 15 min in large volumes of 1X TBS-T buffer. Either the primary antibody or the commercially procured anti-His antibodies were diluted in a ratio of 1:5000 in 1 X TBS-T buffer containing 10 % blocking agent and the membrane was incubated at room temperature with constant shaking for 3 hrs. After incubation the membrane was washed with 1 X TBS-T three times for 15 min each wash to remove unbound primary antibody. After primary antibody incubation, the membrane was incubated for 45 min at room temperature with a secondary antibody (HRP conjugate anti-mouse IgG supplied by ECL+Plus kit, Amersham Pharmacia Biotech, UK) diluted in a ratio of 1:5000 in 1 X TBS-T buffer containing 10 % blocking reagent. Then the membrane was washed three times with 1 X TBS-T as mentioned above and protein signals were detected by following the manufacturer's instructions.

2.11.3.2 Detection

Detection was performed using ECL+Plus kit procured from Amersham Pharmacia Biotech, UK following manufacture's protocol. Solution A and B were taken in a ratio of 1:40 and mixed them gently in dark room. Membrane was then incubated in the reagent mix for exactly one min and dried with tissue papers to remove excess detection reagent before wrapping in a clean cling film. Then the membrane was exposed to Biomax X-ray film for 30 seconds to 2 min. The film was developed by immersing it in a Kodak developer solution and fixed by transferring it to Kodak - fixer solution for 2 min. The film was then washed in water and dried before analyzing the results.

2.12 Expression of recombinant proteins

The recombinant expression plasmid, coding proteins were transformed into *E. coli* BL21 (DE3) and were initially grown in LB with appropriate antibiotics at 37 ^{0}C in an orbital shaker. After reaching mid-log phase (0.4 O.D. at 600 nm), the cells were induced

with 0.5 mM IPTG and were grown for 16 hours at 18 ^{0}C. Cells from 1 ml culture were harvested and the cell pellet was dissolved in appropriate amount of sample buffer before boiling for 5 min in boiling water bath. The induction of recombinant protein was tested by analyzing the proteins on 12.5 % SDS-PAGE. The cell pellet collected from uninduced cells served as controls. If necessary the cell lysate was prepared from induced culture and separated it into particulate and soluble fractions. These two fragments were also analyzed on 12.5 % SDS-PAGE along with total proteins to gain information on formation of inclusion bodies from induced proteins.

2.13 Protein purification

2.13.1 Preparation of cell free extracts

The cell free extracts from *E. coli* cells expressing desired protein were harvested and cell pellet was washed twice in 20 mM TE buffer pH 8.0 and resuspended in the 7 volumes of 50 mM Tris buffer (pH 8.0) having 1 mM EDTA. After resuspension of the cells were disrupted by sonication for a period of 10 min with a pulse on and off cycle of 30 sec each at 4 ^{0}C. The resulting homogenate was centrifuged at 15,000 rpm for 30 min and the supernatant was again centrifuged at 45,000 rpm for 1 hour at 4 ^{0}C. The supernatant thus obtained was considered as the cytoplasmic fraction and was used for purification of desired protein.

2.13.2 Ammonium Sulphate Fractionation

To the cytoplasmic fraction, ammonium sulphate was added slowly with constant stirring at 4 ^{0}C in order to bring the solution to 20 % saturation. Later, the solution was centrifuged at 15,000 rpm for 30 min to pellet out the precipitated proteins. Similarly, the supernatant obtained was further saturated to 40 % and 60 % respectively by adding appropriate amounts of ammonium sulphate. The proteins precipitated in each fraction were used for identification of desired protein.

2.13.3 Anion Exchange Chromatography

Anion exchange chromatography was done on a manually packed DEAE Sepahrose column (XK 16/40) operated on an AKTA basic FPLC system (Amersham Biosciences, UK). The protein sample was loaded on to the column pre-equilibrated with 50 mM Tris-Cl (pH 8.0) having 1 mM EDTA. The protein was eluted at a flow rate of 1 ml/min with a linear gradient of 1 M NaCl in 50 mM Tris buffer.

2.13.4 Hydrophobic Interaction chromatography

Hydrophobic Interaction chromatography was performed on a manually packed Phenyl sepharose column operated under gravity flow. The proteins from the anion exchange fractions having high GST activity were pooled, and loaded on to the phenyl sepharose column pre-equilibrated with a 50 mM Tris-Cl buffer having 50 mM NaCl, 1 mM EDTA and 15 % $(NH_4)_2SO_4$. The proteins were eluted using a 50 mM Tris-Cl buffer having 50 mM NaCl, 1 mM ETDA with a negative stepwise gradient of $(NH_4)_2SO_4$.

2.13.5 Gel Permeation Chromatography

Gel permeation chromatography was performed on a manually packed Sephacryl 200 HR columns (XK 16/100) operated at a flow rate of 0.5 ml/min using an AKTA basic FPLC system (Amersham Biosciences, UK). The protein from the Phenyl sepharose fractions having high GST activity were pooled and concentrated by ammonium sulphate precipitation. Further, the protein was dialysed to remove ammonium salt and then loaded on to Sephacryl 200HR column pre-equilibrated with 50 mM Tris-Cl (pH 8.0) buffer having 0.18 M NaCl.

2.13.6 Affinity chromatography

2.13.6.1 Purification of His-tagged proteins

The cells expressing recombinant proteins with C-terminal His-tag were harvested by centrifugation at 6000 rpm for 10 minutes and the collected pellet was washed in binding

buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl). The cells pellet was then resuspended (1 g /10 ml) in lysis buffer (5 mM imidazole and 10 % glycerol in binding buffer) and disrupted by sonication by placing the cell suspension on ice. The lysate was centrifuged at 15,000 rpm for 30 minutes and the clear supernatant was applied to the Ni sepharose column, pre-equilibrated with binding buffer. The column was the extensively washed with wash buffer (binding buffer with 50 mM imidazole) and the bound protein was eluted with a gradient of 0 - 500 mM imidazole in binding buffer.

2.13.6.2 Purification of GST-tagged proteins

The proteins which were expressed as GST-fusions were purified using glutathione sepharose column (GE Healthcare, UK). The cell extracts were prepared in equilibration buffer (1 X PBS, pH 7.4) and applied to the column pre-equilibrated with the equilibration buffer. The column was then washed extensively (10 column volumes) with the same buffer before eluting the bound proteins with elution buffer (10 mM glutathione, 50 mM Tris-HCl pH 9.6).

2.14.1 Generation of antibodies for RepA, Orf96 and Orf113

Polyclonal antibodies against plasmid pTS4 coded RepA, Orf96 and Orf113 proteins were raised in rabbit by following standardized protocol described elsewhere (Cooper and Patterson, 2008). Rabbits were allowed to acclimatize in the animal house for a week prior to the immunization. Before giving the first dose, 10 ml of blood was collected and allowed to coagulate to separate about 5 ml of clear pre-immune serum. While obtaining serum, the blood collected in falcon tube was kept at room temperature for 1 hour and left in refrigerator (4 ^{0}C) for more than 12 hours. The sample was then centrifuge at 3000 rpm for 5 min at 4 ^{0}C to separate the serum from the coagulum. The obtained serum was stored at -20 ^{0}C until further use.

The three purified recombinant proteins were further separated independently on 12.5 % SDS-PAGE and the corresponding bands were excised before subjected them for electro-elution. The immunogen (250 µg each) is diluted in 0.5 ml of PBS buffer and mixed with 0.5 ml of complete Freund's adjuvant. The antigen and adjuvant were mixed thoroughly to form a stable emulsion. The emulsion form was then injected subcutaneously near the shoulder and intra-muscularly into the large muscle of the rear legs. Four weeks after the first immunization, three immune booster doses were administrated each with an interval of two weeks. In each immune booster 125 µg of the antigen was taken in a final volume of 0.5 ml (addition of PBS to the antigen to 0.25 ml + 0.25 ml of Incomplete Freund's Adjuvant) and administrated intramuscularly as described before. One week after administration of each immune booster, blood samples were collected and the serums prepared from these samples were used to test generation of antibodies against injected proteins. Finally, blood was collected from the central ear artery with a 19-gauge needle and nearly 15 and 30 ml of whole blood was collected from each animal. The collected blood was used to prepare serum.

2.14.2 Antibody purification

Affinity chromatography was performed to purify IgG's for the serum collected from immunized rabbits. Initially required amount (2 ml) of serum was taken and diluted it with equal volumes equilibration buffer (20 mM sodium phosphate buffer pH 7.0). The diluted serum was then applied on to a protein A column pre-equilibrated with the equilibration buffer at the flow rate of 1 ml/min. The column was then washed with 10 column volumes (CV) of equilibration buffer and finally the IgG's bound to the column were eluted with elution buffer (0.1 M glycine-HCl, pH 2.7). The eluted IgG's were then stored at -20^0 C in a 100 µl aliquots and used when necessary.

2.15 Enzyme assays

2.15.1 Glutathione S-transferase assay

Glutathione S-transferase assay was performed by using Chloro-Di-Nitro Benzene (CDNB) as a substrate essentially by following the procedures described elsewhere (Habig and Jakoby, 1974). Glutathione (GSH) conjugation with CDNB was monitored at 340 nm using spectrophotometer. Reactions were performed in 50 mM Sodium phosphate buffer (pH 6.5) containing 1 mM CDNB and 1 mM GSH, with appropriate amount of protein (50 µl) and the reaction was initiated by the addition of GSH. A unit of enzyme activity was expressed as the amount that catalyzes the formation of 1 µmol S-2, 4-dinitrophenyl-GSH adduct per minute, using a molar extinction coefficient of 9.6 mM-1 cm-1 for CDNB. Similar assay was performed for ethacrynic acid (EA) except that the CDNB was replaced with 0.2 mM EA and the conjugation with GSH was measured at 270 nm.

For GST kinetic studies, initial velocities were determined at pH 6.5 using the spectrophotometric assay described above. CDNB concentrations were varied from 0.2 mM to 5 mM while keeping the GSH concentration constant at 1 mM. Assay volume remained constant. Assuming Michaelis-Menten kinetics, apparent K_m and V_{max} values of the AbGST01 activities were calculated using a non-linear regression computer program generated by GraphPad Prism 5 (www.graphpad.com).

2.15.2 Peroxidase assay

Peroxidase activity towards cumenehydroperoxide (CHP), t-butyl hydroperoxide (TBHP) and 13-HPODE was determined by using a glutathione reductase (GR)-coupled assay (Lawrence et al., 1976). Briefly, 0.5 mL reaction mixture containing 1 mM GSH, 0.2 mM NADPH, 2.265 units glutathione reductase in 100 mM sodium phosphate buffer, pH 6.5, was incubated with 50 µg of AbGST01 at 30 ^{0}C for 5 min. The reaction was initiated

by the addition of CHP. The consumption of NADPH was monitored at 340 nm for 5 min at 30 ^{0}C. A no substrate blank and a no-enzyme blank were used to correct for glutathione reductase independent NADPH oxidation and non-enzymatic peroxidase reaction.

2.15.3.1 Dealkylation of methyl parathion

The assay for GST-mediated biotransformation of MeP was performed at 37 ^{0}C in a shaking water bath as previously described (Anderson et al., 1992). In a 1.7-ml plastic vial, 100 µg of purified GST was added to 145 µL incubation buffer (100 mM Tris, pH 7.4) with glutathione (5 mM). The mixture was gently vortexed and prewarmed for 5 min. Following this pre-incubation, 0.6 µl of MeP stock solution was added to get a final concentration of 300 µM MeP in the reaction mixture. The contents were thoroughly mixed and returned to 37 ^{0}C water bath for 30 min incubation period, when necessary the incubation period was prolonged to 60 minutes. Protein concentration and incubation times were modified to ensure that the reaction proceeded within the linear range for the entire incubation period. After the incubation period, the enzyme reaction was stopped by addition of 200 µL of ice cold methanol containing 250 µM *p*-phenylphenol. The samples were stored at -20°C until further use. Reaction replacing GSTs with similar amount of BSA serve as negative control. The degradation studies were performed by conducting three independent experiments. Each experiment contained reaction mixtures in triplicates. Samples were prepared for HPLC/UV analysis by centrifugation at 10,000 × *g* for 3 min. Subsequently 200 µl of the supernatant was transferred to an HPLC vial.

2.15.3.2 HPLC protocol

Reversed-phase HPLC with ion-pairing was used to detect the MeP metabolites and

internal standard. A 4.6 × 250 mm C18 RP column was used for metabolites separation

Time (min)	% A	% B
0	90	10
3	70	30
10	65	35
16	10	90
22	10	90

Table 2.11.1. HPLC mobile phase gradient.

using Waters HPLC instrumentation. The aqueous mobile phase (A) consisted of 0.25 mM tetrabutylammonium phosphate (TBAP, ion-pairing reagent) in water, while the organic phase (B) consisted of 0.25 mM TBAP in 80 % acetonitrile. Each phase was brought to pH 3.0 with 10 % phosphoric acid. A 22-min gradient (Table 2.11.1) was used for metabolite separation and began with 90 % A with a flow rate of 1 ml per minute with an injection of 20 µl sample. The detection of the metabolites was monitored at 280 nm wavelength.

2.15.4 (13-HPODE) assay

GST-peroxidase activity towards 13-HPODE was determined using high performance liquid chromatography (HPLC) as described below. The substrate 13-HPODE (50 µM) was incubated in 100 mM potassium phosphate buffer, pH 6.5 containing; 1.0 mM GSH, 5 % ethanol, and 10 µg of AbGST01 at 30 ^{0}C. All reactions were performed in triplicate. After 30 min, the reactions were placed on ice and quenched by adding concentrated HCl to lower the pH to 4.0. The reaction mixtures were then extracted three times with freshly distilled ether. All extractions were kept in cold to minimize peroxide decomposition. The ether extracts for each reaction were combined and the solvent removed in a vacuum

centrifuge (Speed-Vac). The residue was dissolved in HPLC-grade hexane containing 0.8 % isopropyl alcohol (IPA) prior to analysis by straight phase HPLC. A 25 cm Beckman Ultrasphere column was eluted at 1.0 mL/min with 0.8 % IPA and 0.1 % acetic acid in hexane. Product elution was determined by monitoring the absorbance at 235 nm. The substrate and product, 13-HPODE and 13-HODE were completely resolved and eluted at about 14.5 min and 12.3 min, respectively. Control incubations in the absence of enzyme were performed and subtracted from the data to correct for non-enzymatic reactions.

2.15.5 Dichloromethane assay

The enzyme reaction was carried out in 2 ml reaction mixture containing 100 mM Tris-Sulfate (pH 8.0), 5 mM GSH, 200 µg AbGST01 and 5 mM dichloromethane. The reaction mixture was incubated at 30 ^{0}C for 5-30 minutes before stopping the reaction by addition of 20 % TCA. The content was then centrifuged at 1000 g and the supernatant were analysed for presence of formaldehyde by method of Nash (Nash, 1953). The Nash reagent consists of reagent A (10 mM acetylacetone and 100 mM ammonium acetate) and reagent B (2 M ammonium acetate, 5 mM acetic acid and 2 mM acetylacetone), the reagents were mixed in equal volume. About 1 ml of the supernatant obtained was added to 1 ml of Nash's reagent, and the yield of formaldehyde was measured in a spectrophotometer at 412 nm.

2.15.6 Tetrachlorohydroquinone (TCHQ) assay

AbGST01 activity against tetrachlorohydroquinone was carried out as described elsewhere (McCarthy et al., 1996) by using a discontinuous HPLC assay. Reaction mixtures contained 100 µM TCHQ, 5 mM GSH, 5 mM ascorbate (to protect hydroquinone substrates and products from oxidation), 1 mM EDTA, 25 mM DTT and 500 µg AbGST01 in 25 mM potassium phosphate buffer, pH 7.2. At intervals after the addition of enzyme,

aliquots were removed and quenched by the addition of an equal volume of 1 N HCl. Reaction mixtures were analyzed by reverse-phase HPLC on a Agilent C18 column. For analysis of TCHQ disappearance, an isocratic elution with 25 % acetonitrile was performed keeping the detector was set at 210 nm. While analysing the product, a gradient of 0.1 % acetic acid and acetonitrile (0-50 % between 0 and 10 min and 50-55 % between 10 and 40 min) was employed. The detector was set at 285 nm.

2.15.7 *β*-Galactosidase assay

β-Galactos idase activity was performed according to protocol described elsewhere (Miller, 1972) with the following slight modification. Overnight cultures of *A. baumannii* DS002 were diluted 1/100 in fresh medium and were grown to mid-log phase. Once the culture density reached to mid log phase various oxidative stress inducers were added and grown for additional 4 hrs at 30 ^{0}C. The cultures were then kept on ice bath for 20 minutes to stop growth. Cell pellet obtained from at least 2 ml of culture was resuspended in the same volume of chilled Z buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol pH 7.0). The cells were lysed by adding 100 µl chloroform and 50 µL 0.1 % SDS (sodium dodecyl sulfate, sodium laurel sulfate). The contents were thoroughly mixed and incubated at 28 ^{0}C water bath for 5 minutes to bring the temperature of lysate to 28 ^{0}C. The reaction was started by adding 200 µL of *O*-nitrophenyl-β-D-galactoside (ONPG; 4mg/ml) and soon after observation of formation of yellow colour the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The debris and chloroform were removed by centrifugation at maximum speed for 5 minutes and the clear supernatant was used to measure the optical density at 420 nm and 550 nm respectively. The β -galactosidase activity was calculated by using the formula given below and expressed in miller units.

Miller units = 1000 x [(OD_{420}-1.75 x OD_{550})] / (T x V x OD_{600})

T = time of the reaction in minutes, V = volume of culture used in the assay in mLs.

The units give the change in A_{420}/min/mL of cells/OD_{600}

2.15.8 Organophosphate hydrolase (OPH) assay

The cells to be used for measuring OPH activity were suspended in 5 ml of 10 mM Tris HCl (pH 8.0) buffer before lysing them by sonication at 4 ^{0}C with a pulse of 30 sec for 5 min. The lysate was centrifuged at 13,000 rpm for 10 minutes. The supernatant fraction was used to assess paraxonase activity. 1 ml of reaction mixture contained 790 µl of double distilled water and 100 µl of 200 mM CHES and 10 µl of enzyme source. Whenever needed 10 µM of $CoCl_2$ was added to the reaction mixture. The reaction was started by adding 100 µl of 10 mM methyl parathion with gentle mixing. An increase in the absorbance at 410 nm due to formation of *p*-nitrophenol was determined (Dumas et al, 1989). The concentration of *p*-nitrophenol formed in the reaction was determined using the extinction coefficient (16500 M-1 Cm -1). The specific activity of the enzyme was expressed as micromoles of PNP produced per mg of protein.

Plasmids provide great insight for understanding the molecular evolution of bacteria. Horizontal transfer of the genes through conjugation is believed to be the genuine method of transfer and most prevalent among the bacteria. This contributes to the evolution, diversification and adaptability of the bacterial population to the changing environments. There is lot to be unraveled in the field of plasmid biology to unfold lots of mechanisms of adaptability, survival and the contributing factors. This information in the prokaryotic systems could also through some light on the evolutionary aspects of archaea and eukaryotes. *A. baumannii* has been shown to have multiple indigenous plasmids (Vallenet et al., 2008). The strain's ability to take up linear and circular DNA molecules under natural conditions is believed to be a reason for the acquisition of multiple plasmids and resistance genes for multiple drugs from microbial communities (Woodford et al., 2011). Our lab has been studying regulation and horizontal mobility of organophosphate degrading (*opd*) genes among soil bacteria (Siddavattam et al., 2003; Pandeeti et al., 2011). As *A. baumannii* DS002 is isolated from OP-pesticide polluted agricultural soils, the strain was analyzed to detect indigenous plasmids, apparently to establish link, if any, between the plasmids and OP pesticide degradation.

3.1.1 Isolation of Indigenous plasmid from *A. baumannii* DS002

Plasmid typing is used in many cases for identification of *Acinetobacter* spp. during the out- breaks of infections (Gerner-Smidt, 1989). The plasmids have been previously isolated by the methods of Birnboim and Doly, 1979, Currier and Nester, 1976. It was observed that most of the protocols do not isolate all the plasmids present in the native organisms. In the present study we have shown presence of four indigenous plasmids in *A. baumannii* DS002 by using modified Currier and Nester method as described elsewhere (Materials and methods). Four indigenous plasmids were detected in this soil

isolate (Fig. 3.1) and were designated as as pTS1, pTS2, pTS3 and pTS4. Initial hybridization studies using a well-conserved organophosphate-degrading (*opd*) gene, gave no signal with any of the plasmids or with the chromosomal DNA suggesting that the OP degradation mechanism of *A. baumannii* DS002 might be novel.

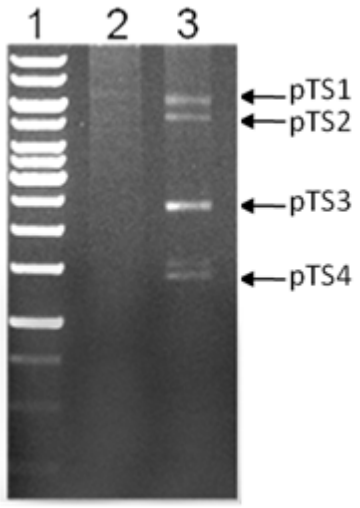

Fig. 3.1. Agarose gel electrophoresis showing indigenous plasmid of *A. baumannii* DS002: Lane 1 and 2 represent kilobase DNA ladder and plasmid preparations from plasmid less *E. coli* strain used as negative control. Four indigenous plasmids found in *A. baumannii* DS002 are shown in lane 3.

3.1.2 Rescue-cloning of Indigenous plasmid

In order to gain further insights on biology and genetics, these indigenous plasmids were isolated and rescue cloned into *E. coli pir116* cells after tagging with a mini-transposon (EZ-Tn5<R6Kγ*ori*/KAN-2>) having R6Kγ replicative origin. The plasmid preparation of *A. baumannii* DS002 was treated with plasmid safe (Epicentre, USA) to remove the genomic DNA contamination, if any. The pure plasmids present in the preparation are used to perform *in vitro* transposition using EZ-Tn5<R6Kγori/KAN-2> cassette (Materials and methods). Insertion of the Kanamycin cassette does not result in any scorable mutation other than giving Kanamycin resistance (Singer and Finnerty, 1984). These transposons tagged plasmids are electroporated into *E. coli pir116* cells. As

the transposon provides the R6Kγ origin of replication, the transposon tagged plasmids can replicate and maintain in the *E*. *coli* cells. The mini-transposon borne kanamycin gene permits selection of mini-transposon tagged *A. baumannii* DS002 plasmid in kanamycin plate. The R6Kγ origin of replication depends on *pir* protein for replication. In *E. coli pir116* cells, the *pir* coding gene is cloned under the control of *ara* promoter. The copy number of mini-transposon tagged plasmids can be enhanced by growing them on 1 mM arabinose. In *pir* induced cultures, the plasmid will be in high copy and hence were isolated using alkaline lysis method. The plasmids were screened by digestion with different restriction enzymes, to identify those with single transposon insertion. All four plasmids detected in *A. baumannii* DS002 were rescue cloned in *E. coli pir116* cells and the sizes of the plasmids pTS1, pTS2, pTS3 and pTS4 were found to be ~2kb, ~5kb, ~12kb, and ~14kb respectively (Fig.3.2). After rescue cloning, the indigenous plasmid containing *E. coli pir116* cells were tested to know if they can degrade either methyl parathion (OP-insecticide) or its catabolic intermediate *p*-nitrophenol. No degradation was observed,

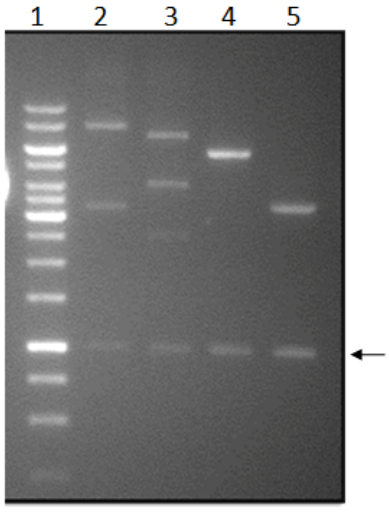

Fig. 3.2. Restriction profile of rescue cloned indigenous plasmids of *A. baumannii* DS002 isolated from *E. coli pir116* cells: Lane 1 represents 1 Kb DNA ladder. Lanes 2-4 represent pTS1, pTS2, pTS3 and pTS4 plasmids digested with *Xho*I- *Bam*HI. The 1 kb portion of mini-transposon will be released from rescue cloned plasmids upon digestion with *Xho*I and *Bam*HI.

suggesting there is no such genetic information on indigenous plasmids of *A. baumannii* DS002.

3.1.3 Sequence analysis of pTS4

Initially all the four indigenous plasmids of *A. baumannii* DS002 were partially sequenced, using the mini-transposon specific primers, to be able to gain partial information on their functions. Further, as plasmid pTS4 was the smallest, it was assumed that it can be an ideal candidate for constructing a shuttle vector to clone and express genes in *A. baumannii*. Therefore, complete sequence was determined by using initially the mini-transposon specific primers and subsequently by performing primer walking. The complete sequence and the predicted ORF's, promoter and terminator sequences are shown in figure 3.3.

The 2252 bp of pTS4 sequence contained three ORFs (Fig. 3.3), one of them has shown homology to *repA*, generally seen in rolling circle (RC) replicating plasmids. The other two ORFs code for proteins with a length of 113 and 96 amino acids. These two proteins showed no homology to any known protein sequences available in the databases and hence were designated as Orf113 and Orf96. These two ORFs appear to be translationally linked, as the predicted translational stop codon of *orf113* overlapped with the start codon of *orf96* (Fig. 3.3). Moreover, only in the upstream region of *orf113* a consensus σ^{70} dependent promoter was identified and no predicted promoter sequences were found upstream of *orf96* (Fig. 3.3). The absence of promoter elements upstream of *orf96* and the existence of translational coupling suggest possible organization of these two ORFs as one operon. Furthermore, a perfect inverted repeat that shows structural resemblance to *rho*-independent terminator was identified immediately downstream of *orf96* (Fig. 3.3), supporting the proposed arrangement of these two genes as an operon.

A 12 bp long sequence that matched perfectly with the double-stranded origin of replication (DSO) typically found in rolling-circle (RC) replicating plasmids was found in the intergenic region between *orf96* and *repA* (Fig. 3.3). Indeed, the -10 hexameric

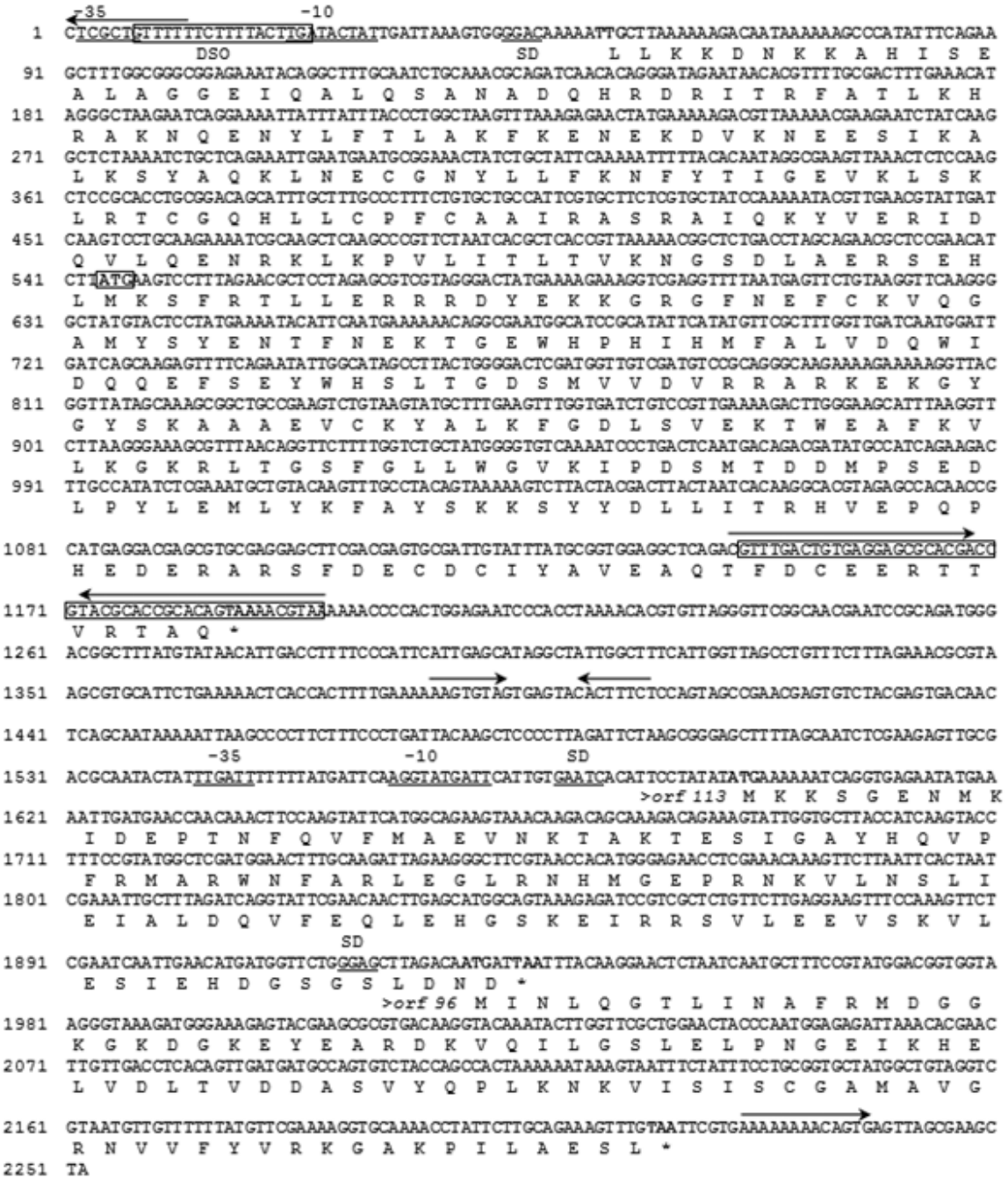

Fig. 3.3. Nucleotide and deduced amino acid sequence of plasmid pTS4. Putative DSO is shown with an open box. The consensus σ^{70} promoters and RBS sequences are underlined. The alternate start codon TTG is indicated in bold case. The codon ATG specifying 165 methionine (M165) is indicated with open box. The predicted *Rho* independent terminators found downstream of *repA* and *orf96* are shown with inverted arrows. The putative SSO is indicated with an open box.

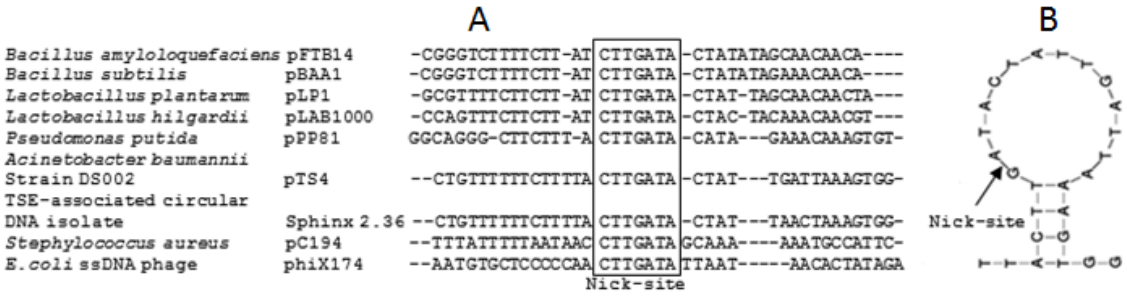

Fig. 3.4. Panel A: Alignment of Double Stranded Origin of replication (DSO) of plasmid pTS4 with the known rolling circle replicating plasmids. Panel (B) shows the secondary structure of DSO. The proposed site of nick is shown with an arrow.

```
2.pPP81         ------------------------------------MMLEH----------------- 5
1.pTS4          LLKKDNKKAHISEALAGGEIQALQSANADQHRDRITRFATLKHRAKNQENYLFTLAKFKE 60
3.pC194         --------------------VVFMCYNMEKYTEKKQRNQVFQK----------------- 23
4.pLAB1000      ------------------MSKKILKDVSRNRKERPWRERKLEN----------------- 25
                                                    :::

2.pPP81         -----LREAPSPASTKTAASLASCGNYLHFREYFTVGKVRLHNATFCKQHLVCPLCAIRR 60
1.pTS4          NYEKDVKNEESIKALKSAQKLNECGNYLLFKNFYTIGEVKLSKLRTCGQHLLCPFCAAIR 120
3.pC194         -------FIKRHIGENQMDLVEDCNTFLSFVADKTLEKQKLYKANSCKN-RFCPVCAWRK 75
4.pLAB1000      ---LQYAEYLRILNFKKANRVKECGEVLRFVAD-DEGRLRLYQTWFCKS-RLCPLCNWRR 80
                              :    : .*.  * *       . :* :   * .  .**.*   :

2.pPP81         GAK-ALGAYLARWEVLQEQHP-ELRPYLITLTVKNGDDLEERQAHLTRSLKRLTDRRRFF 118
1.pTS4          ASR-AIQKYVERIDQVLQENR-KLKPVLITLTVKNGSDLAERSEHLMKSFRTLLERRRDY 178
3.pC194         ARKDALGLSLMMQYIKQQEKK-EFIFLTLTTPNVMSDELENEIKRYNNSFRKLIKRKKVG 134
4.pLAB1000      SMGQSNQLMQVLDEAHKQRKTGRFLFLTLTAENASGENLKQEVRKMGRAISKLFQYKKPA 140
                .   :            :.:  .:    :*     ..:* :.  :  .::  * . ::

2.pPP81         NAGKRGSPWTELCKAQGAVYTLELTNKGKG--WHPHCHMIALAAS--------QPSQSDL 168
1.pTS4          EKKGRG--FNEFCKVQGAMYSYENTFNEKTGEWHPHIHMFALVDQ--------WIDQQEF 228
3.pC194         ------------SVIKGYVRKLEITYNKKRDDYNPHFHVLIAVNKSYFTDKRYYISQQEW 182
4.pLAB1000      KN------------LLGYVRSTEITINKNG-TYHQHMHVLLFVKPTYFKDSANYINQAEW 187
                              * : . * * : :   :: * *::  .            .* :

2.pPP81         SAEWH-----RITGDSMIVDVRPITGDP----SEGFMEVFKYAVKFSDLTLEDNWHAAQV 219
1.pTS4          SEYWH-----SLTGDSMVVDVRRARKEKGYGYSKAAAEVCKYALKFGDLSVEKTWEAFKV 283
3.pC194         LDLWR-----DVTGISEITQVQVQKIRQNN--NKELYEMAKYSGKDSDYLINKSKSL--- 232
4.pLAB1000      SKLWQRAMKLDYQPIVNVEAVRSNKAKGKNSLIASAQETAKYQVKSKDILTNDQERDLQV 247
                   *:            :  *:              *  **  *  *   :.

2.pPP81         LKGKRLLNSFGLFRGVDIPDSLLD-EPLDELPYWDRFYRYLGGEYQFTG--------EAP 270
1.pTS4          LKGKRLTGSFGLLWGVKIPDSMTDDMPSEDLPYLEMLYKFAYSKKSYYDLLITRHVEPQP 343
3.pC194         ------------------------------------------------------------
4.pLAB1000      VEDLEQG--LAGSRQISYGGLFKEIRKQLQLEDVDAHLINVDDDKVKIDEVVREVVAKWD 305


2.pPP81         KGRERG---------------------------- 276
1.pTS4          HEDERARSFDECDCIYAVEAQTFDCEERTTVRTAQ 378
3.pC194         ----------------------------------
4.pLAB1000      YNKQNYFIW--------------------------- 314
```

Fig. 3.5. Multiple sequence alignment of RepA sequence of pTS4 with RepA sequences of other rolling circle replicating plasmids, pPP81 (CAC01244.1), pC194 (NP_040435.3), pLAB1000 (AAA98163.1), and pE194 (AAA25603.1). The conserved tyrosine residue involved in initiation of replication is shown in open box.

sequence of a putative σ^{70}-dependent promoter of *repA* gene was located in the middle of the DSO sequence motif (Fig. 3.3). The predicted DSO of pTS4 aligned very well with DSOs of other RC plasmids showing high sequence identity (Fig. 3.4A). The sequence that contributes to form a loop structure was 100 % conserved (Fig. 3.4B), indicating that pTS4 may be RC plasmid. In consistent with the structural conservation of DSO, deduced amino acids sequence of RepA aligned well with replication protein (Rep) of other rolling circle plasmids such as pPP81, pC194, pLAB1000 and pT181 isolated from *Pseudomonas putida* (Holtwick et al., 2001), *Staphylococcus aureus* (Khan and Novick. 1983), *Lactobacillus hilgardii* (Josson et al., 1990) and *S. aureus* (Novick, 1987) respectively (Fig. 3.5). The RepA of pTS4 showed considerable homology to the Rep protein of other RC plasmids, mainly the conservation was seen only in the central region of the protein. The C-terminus and N-terminus region of RepA showed no significant similarity. The conserved tyrosine residue involved in initiation of replication was identified at 265 residue in RepA of pTS4 and this residue was found to be highly conserved in all the Rep proteins of RC plasmids (Fig. 3.5).

3.1.4 Detection of single stranded DNA of pTS4

During the RC mode of replication Rep protein will create a nick at DSO, the exposed hydroxyl group will be then used by the host replication machinery to replicate DNA. In the process the positive strand gets displaced and circularized to form a ssDNA intermediate. The ssDNA intermediate is a hallmark of the RC replication mechanism. Therefore, detection of ssDNA confirms if the plasmid in question replicates through RC-mode. Before designating pTS4 as RC plasmid with reasonable confidence, experiments were conducted to detect SS form of pTS4 in cultures of *A. baumannii* following

established procedures described elsewhere (te Riele et al., 1986). Initially indigenous plasmids of *A. baumannii* DS002 were isolated and a portion of it was treated with S1 nuclease while the other portion was left untreated. These samples were separated on a 0.8 % agarose gel and were then transferred to nylon membrane without denaturation step, which facilitates only the single stranded DNA to be available for annealing with the DNA probe. The membrane with the immobilized plasmid was then hybridized using αP^{32} labeled pTS4 plasmid as probe. The pTS4 specific signals obtained in the lanes loaded with S1-treated and untreated plasmid preparations were compared to identify single-stranded intermediate of pTS4. As seen in RC plasmids, a strong signal was obtained only in the plasmid samples that were not treated with S1 nuclease. Since S1 nuclease treatment preferentially eliminated SS form of pTS4 no such signal was seen in the treated sample (Fig. 3.6), giving clear indication that pTS4 is a RC plasmid and it replicates via ssDNA intermediate.

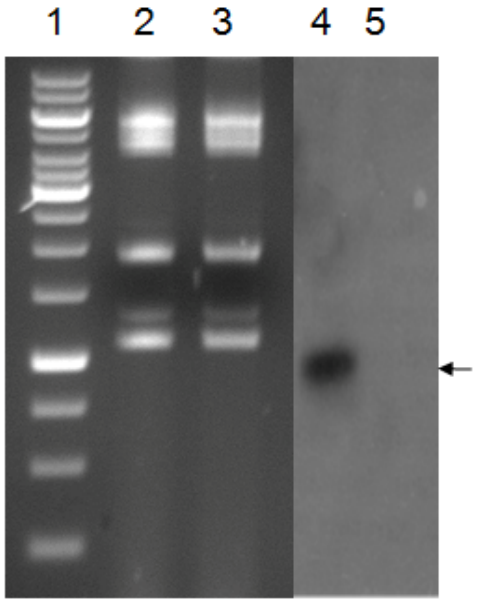

Fig. 3.6. Detection of single stranded intermediate of pTS4: Lane 1 represents 1 kb DNA ladder, lanes 2 and 3 represent total plasmid preparation treated with (2) and without (3) S1 nuclease. Lanes 5 and 6 represent corresponding autoradiogram developed using labelled pTS4 as probe. Single stranded pTS4 seen in S1 nuclease untreated sample is shown with an arrow mark.

3.2 Characterization of RepA protein

3.2.1 RepA has an alternate translational start codon

The bioinformatic tool GLIMMER, which is used to predict ORFs, has indicated that initiation of RepA translation occurs from an alternate start codon UUG, which is located 21 bp downstream of the putative promoter element of *repA* (Fig. 3.3). The first AUG codon was found 513 bp downstream of the predicted promoter element. The predicted amino acid sequence coded by the DNA region present between these two codons shows strong homology to the N-terminal portion of the RepA sequence coded by a well-characterized RC plasmid pPP81 (Holtwick et al., 2001). Such observation supports possible initiation of RepA synthesis from the alternate start codon UUG. As this is an unusual observation, further studies were conducted to determine if RepA is translated from an alternate start codon UUG.

3.2.2 Construction of RepA expression plasmids

For constructing RepA expression plasmid expressing RepA from alternate start codon UUG, the *repA* gene was amplified from plasmid pTS4 plasmid using the forward (DS00109) and reverse (DS00110) primers carrying *Eco*RI and *Xho*I restriction recognition sequences, respectively. The *repA* amplicon was then digested with *Eco*RI and *Xho*I and ligated into pET23b digested with the same enzymes (Fig. 3.7A). The cloning strategy places *repA* in-frame with the vector-encoded His-tag of pET23b and the recombinant plasmid was designated as pTRW1, which encodes RepA with a C-terminal His-tag. Similar strategy was followed while cloning of *repA* from its first ATG except that the primers DS00109 and DS00110 were used as forward and reverse primers and the resulting expression plasmid was named as pTRW2 (Fig. 3.7B). In both the cases, start and stop codons were modified to facilitate incorporation of *Eco*RI and *Xho*I sites at 5’ and 3’ ends

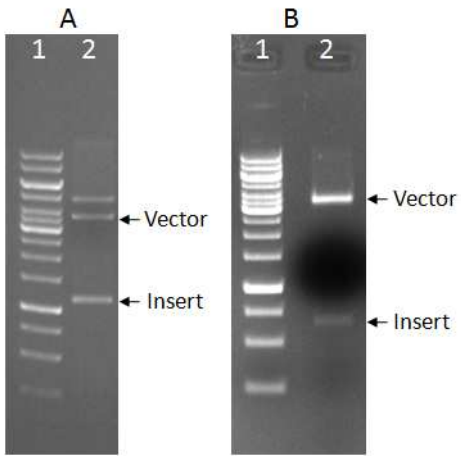

Fig. 3.7. Construction of RepA expression plasmids from alternate start codon and from first ATG: First lanes represent 1 Kb DNA ladder. Second lanes represent RepA expression plasmids digested with *Eco*RI and *Xho*I; pTRW1 (Panel A) and pTRW2 (Panel B). The vector and insert are shown with arrow marks.

of *repA* sequence.These expression plasmids were then transformed into *E. coli* BL21 cells and its expression were confirmed by performing western using anti-his antibody as described in materials and methods (Fig. 3.7).

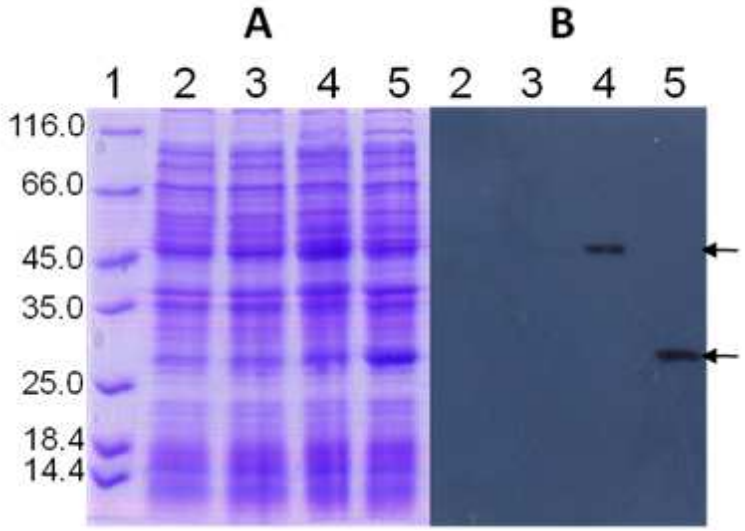

Fig. 3.7. Expression of RepA in *E. coli* BL21: SDS-PAGE (Panel A) and corresponding western blot (Panel B) probed using anti-His antibody. Lane 1 represents protein molecular weight marker. Lanes 2 and 3 represent protein extracts prepared from uninduced *E. coli* BL21 cells having either pTRW1(lane 2) or pTRW2 (lane 3). Lanes 4 and 5 represent similar extracts prepared from induced cultures. Lanes 2-5 in panel B indicate signals obtained with anti-his antibody.

3.2.3 Complementation assay

Initially, during rescue-cloning of *A. baumannii* DS002 plasmids, all the rescue-cloned pTS4 plasmids having a single mini-transposon insertion were collected and established their restriction profile to locate precisely the point of mini-transposon insertion. In one of the rescue-cloned pTS4 derivatives, the mini-transposon was inserted between the predicted alternate codon UUG and the conventional start codon AUG (Fig.

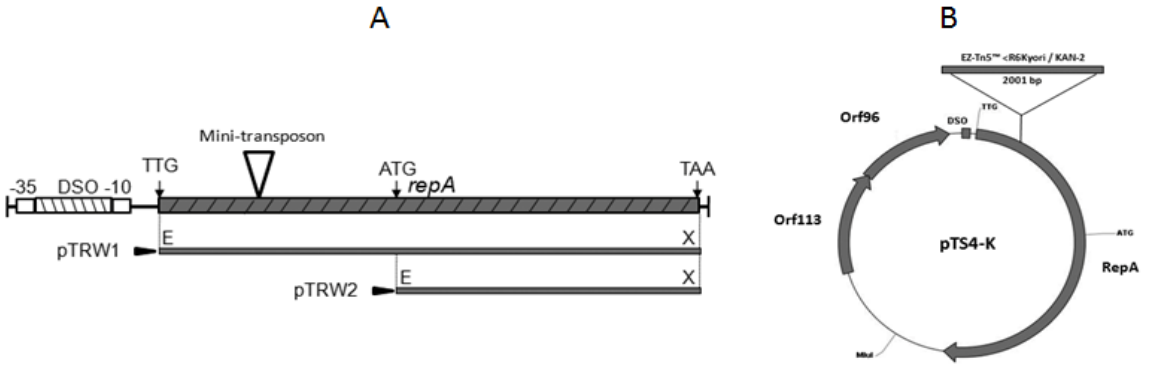

Fig. 3.8. Panel A shows the physical map of the DSO and *repA* regions of pTS4. DSO is shown as a hatched box. The extent of the *repA* sequence cloned to generate expression plasmids coding pTRW1 (translation from alternate start codon UUG) and pTRW2 (translation from AUG specifying 165 methionine) is shown. The triangle indicates the mini-transoson insertion site. Panel B: A schematic representation of pTS4-K with a mini-transposon insertion between the alternate start codon UUG and a AUG codon of *repA*. Arrows indicate transcriptional orientation of *repA*, *orf113* and *orf96.* The DSO found upstream of the *repA* start codon UUG is shown as a solid box.

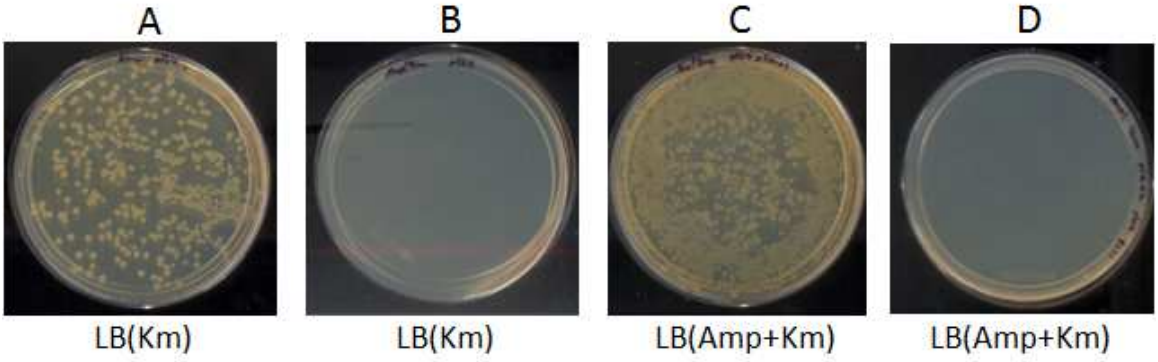

Fig. 3.9. Complementation assay: Panel A and B represent replication of pTS4-K in permissive (*E. coli pir116*) and non-permissive (*E. coli* BL21) hosts respectively. Panel C and D indicate growth of *E. coli* BL21 (pTRW1) and *E. coli* BL21 (pTRW2) respectively, after transforming with pTS4-K.

3.8). This plasmid, pTS4-K failed to replicate in *pir* negative *E. coli* cells (Fig. 3.9B). This derivative was therefore used to develop a complementation assay to assess the functional status of RepA expressed from these two putative translational start codons. For the complementation assay, *E. coli* BL21 cells harboring the two expression plasmids encoding RepA either from the alternate start codon (pTRW1) or from the conventional start codon ATG (pTRW2) were used as host to co-transform with plasmid pTS4-K. When selected on kanamycin plates, colonies were found only in *E. coli* BL21 (pTRW1) cells expressing RepA from alternate start codon UUG which indicates the replication ability of pTS4-K (Fig. 3.9C). No such growth was found in *E. coli* cells with RepA encoded from AUG found 513 bp downstream of promoter sequences (Fig. 3.9D). This observation clearly suggests that the N-terminal portion of RepA specified by the sequence found upstream of the AUG is essential for the RepA function.

3.2.4 RepA-DSO interactions

3.2.4.1 Nicking assay

The first step in RC replication is an interaction of the plasmid DSOs with their initiator protein RepA. The initiator-DNA interaction results in the extrusion of a cruciform structure with a nick site (Koepsel et al., 1985b; Noirot et al., 1990). The Rep proteins then nick at their specific cleavage sites and become covalently attached to the 5' phosphate end of the DNA through a phosphotyrosine linkage (Dempsey et al., 1992b; Thomas et al., 1988). In order to assess any interaction of RepA with the predicted DSO of pTS4, recombinant $RepA_{6His}$ was purified as described elsewhere (Materials and methods) and used to perform two independent *in vitro* studies. The RepA expression plasmid (pTRW1) which codes RepA from the alternate start codon was transformed in *E. coli* BL21 and its expression was achieved by inducing with IPTG. The soluble fraction was

then used to purify the recombinant RepA using Ni affinity column. A homogenous band of 46 kDa was observed in SDS-PAGE (Fig. 3.10A) which corresponds to the predicted size of recombinant RepA protein.

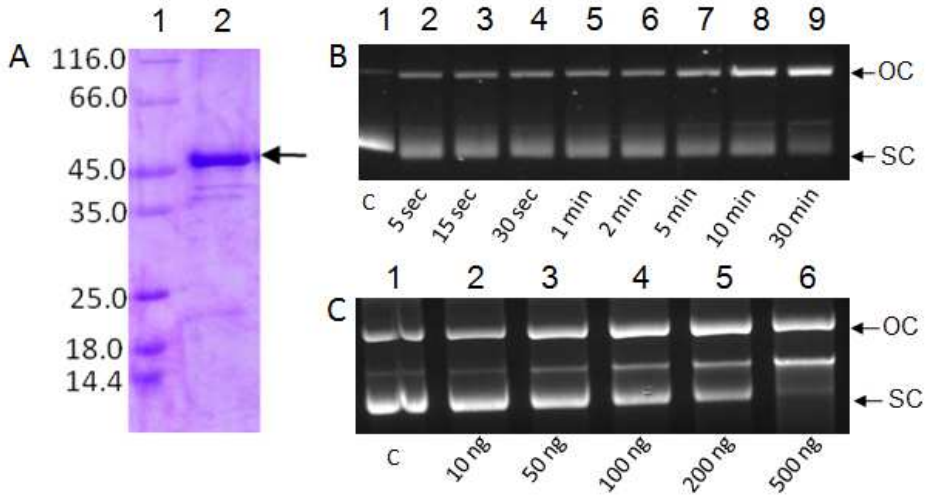

Fig. 3.10. Panel A: Purification of RepA by Ni-sepharose chromatography. Lane 1 represent protein molecular weight marker, lane 2 represent purified recombinant RepA protein indicated by an arrow mark. Panel B&C: RepA mediated generation of open-circular (OC) form of plasmid pTS4-K from a super-coiled (SC) form. Panel B indicates SC to OC conversion of pTS4-K due to increase of time. Panel C represents conversion due to increased in concentration of RepA. 'C' represents pTS4-K without RepA.

Initially SC form of pTS4 plasmid was prepared by extracting super-coiled band of pTS4 from 0.8 % agarose gel. The purified recombinant $RepA_{6His}$ was incubated with the super-coiled form of pTS4-K for various time intervals, keeping the DNA (0.8 µg) and protein (170 ng) at constant concentrations. The conversion of pTS4-K (SC) to pTS4-K (OC) was analyzed by running on 1 % agarose gel. Generation of OC form of pTS4-K increased with the increase in incubation time and most of the SC form was converted to OC form within 10 minutes (Fig. 3.10B). Similarly, with the increase in concentration of RepA protein, proportionate increase of pTS4-K (OC) was seen from pTS4-K (SC) (Fig. 3.10C). No conversion of SC form was seen in control samples prepared by omitting RepA (Fig. 3.10B&3.10C).

3.2.4.2 Electrophoretic Mobility Shift Assay (EMSA)

Though the nicking assay clearly suggests a possible RepA-dependent generation of the OC species from the super-coiled plasmid, it does not indicate whether the generated nick was at the DSO region. In order to identify a precise nick region in plasmid pTS4, Electrophoretic Mobility Shift Assay (EMSA) was performed. Initially, about 100 bp of sequence flanking the predicted DSO was amplified using primers DS00101 and DS00102. This DSO containing amplicon was then end-labeled with ^{32}P and was used to perform EMSA by incubating with pure recombinant $RepA_{6His}$ (Materials and methods). An apparent shift was observed in the mobility of the amplicon due to mobility retardation of the DSO-RepA complex (Fig. 3.11). The complex was not dissociated in the presence of BSA or herring sperm DNA suggesting that the interactions between DSO and RepA were specific. Thus, it is likely that generation of OC of pTS4-K was due to introduction of a nick in the predicted DSO.

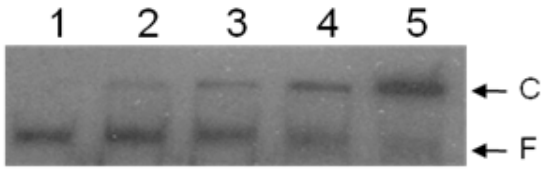

Fig. 3.11. Electrophoretic Mobility Shift Assay (EMSA) for DSO and RepA. Lane 1 represents P^{32} labelled DSO without RepA. Lanes 2-5 represent labelled DSO incubated with 0.1 µg, 0.25 µg, 0.5 µg and 1 µg of RepA respectively. A shift in DSO mobility due to formation of DSO- RepA complex is shown with an arrow mark (C) while F represents the free probe.

3.2.5 Site directed mutagenesis of RepA

Plasmids that replicate using the rolling-circle mechanism have a functionally conserved tyrosine residue near the C-terminus of RepA protein (Fig. 3.5). This invariant tyrosine residue has been implicated in initiation of the replication process by generating a nick at DSO (Chang et al., 2000). The RepA of pTS4, when aligned with the well

characterized protein of RC plasmids, the region containing invariant tyrosine is conserved (Fig. 3.5). In order to assess if the conserved tyrosine of RepA plays a role in replication of pTS4, site-directed mutagenesis was performed to substitute tyrosine 265 of RepA with phenylalanine. *Dpn*I mediated site-directed mutagenesis was carried out as describe elsewhere (Materials and methods), to replace the tyrosine 265 to phenylalanine RepA of pTS4. The expression plasmid coding RepAY265F is designated as pTRM. The site directed mutagenesis was further confirmed by sequencing the pTRM plasmid (Fig. 3.12).

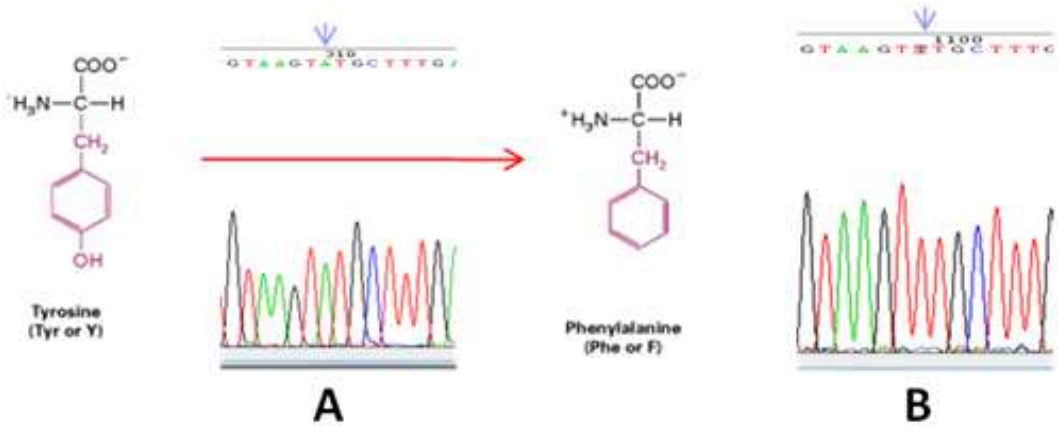

Fig. 3.12. Sequence indicating substitution of phenylalanine to tyrosine: Panel A indicates wildtype RepA sequence. Panel B shows sequence of similar region indicating change in sequence to facilitate incorporation of phenylalanine in RepA.

3.2.6 Tyrosine 265 is essential for repA function

Once the tyrosine residue has been replaced by phenylalanine, a complementation assay was performed by transforming plasmid pTS4-K into *E. coli* BL21 cells having expression plasmids coding for either RepA (pTRW1) or RepAY265F (pTRM) proteins (Fig. 3.13B). Only BL21 with the plasmid pTRW1 exhibited kanamycin resistance suggesting that replication of plasmid pTS4-K was dependent on the invariant tyrosine residue. In order to further substantiate the complementation assay, an *in vitro* nicking assay was carried out using purified RepA mutant protein. RepA Y265F was purified using Ni-

sepharose affinity chromatography and was analyzed on SDS-PAGE (Fig. 3.13A). The pure RepA Y265F was incubated with the super-coiled form of pTS4-K and ability of it to form the OC species of the plasmid was analyzed (Fig. 3.13C), no conversion was seen in all the samples indicating that tyrosine residue is required for creating a nick at DSO.

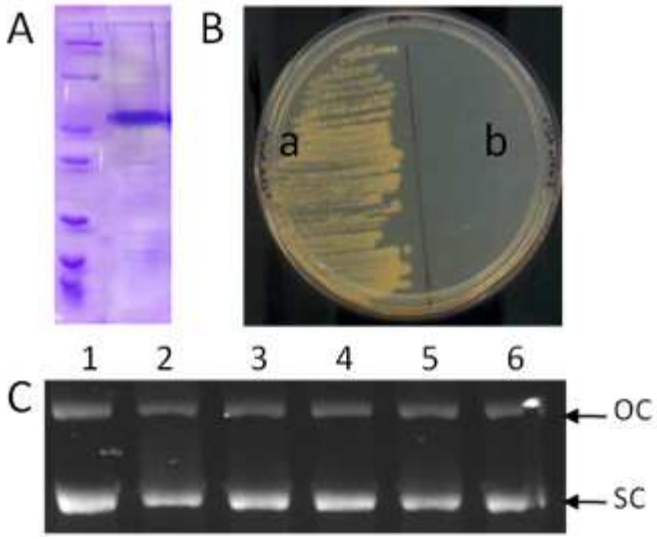

Fig. 3.13. Panel A; RepA Y265F purification by Ni affinity chromatography. Panel B; Complementation assay with RepA Y265F expression plasmid in *E.coli* BL21. Sector (a) and (b) represent replication of pTS4-K in *E. coli* BL21 expressing wildtype RepA and RepA Y265F respectively. Panel C; *Initro* nicking assay. pTS4-K incubated with increased concentration of RepA Y265F: Lane 1; Control without protein, lanes 2-6; 10ng , 50ng, 100ng, 200ng and 500ng RepA Y265F protein.

3.3 Functional analysis of Orf 113 and Orf 96

As mentioned before, the two ORF's present on the pTS4 plasmid did not show homology to any known protein sequence in the database. Therefore, a preliminary study was conducted in order to assess whether they have any role in replication of pTS4 plasmid or not. A complementation assay, similar to the described above, was performed to determine whether the ORF's *orf113* and *orf96* have any role in the replication process. Initially, two variants of pTS4-K were generated by introducing translational termination codons immediately downstream of the predicted start codons of either *orf113* or *orf96.* Site-directed mutagenesis was performed using primers having

the stop codon immediately downstream of start sites. Plasmid pTS4-K was used as template and primer sets (DS00105/DS00106) and (DS00107/DS00108) were used for incorporating termination codon in *orf113* and *orf96* respectively. The pTS4-K variants obtained are designated as pT113M and pT96M respectively. These pTS4-K variants pT113M and pT96M were then independently transformed into *E. coli* BL21 cells having RepA expression plasmid (pTRW1). Colonies were observed in the both the plates (Fig. 3.14), indicating that neither of these ORF are required for replication of pTS4.

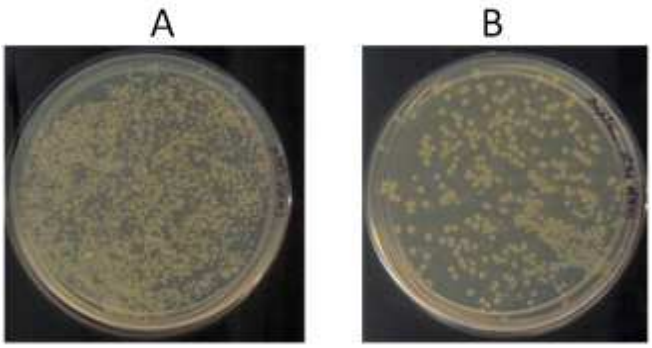

Fig. 3.14. Complementation assay: Panel A and B represent replication of pTS4-K variants pT113M and pT96M in *E. coli* BL21 (pTRW1).

3.4.1 pTS4 is homologous to Sphinx 2.36

Although database search to find homologues of pTS4 identified *repA* genes from similar RC plasmids such as pPP81 (Khan and Novick, 1983), a clear overall homology was only seen with the recently reported circular DNA Sphinx 2.36 and an uncharacterized *Acinetobacter baumannii* plasmid p4ABAYE. The circular DNA of Sphinx 2.36 was co-purified with Bovine Spongiform Encephalopathy (BSE) infected brain samples collected from different geographical regions (Manuelidis, 2011). The SPHINX, which stands for Slow Progressive Hidden INfections of variable (X) latency, were found to be enriched in infectious Transmissible Spongiform Encephalopathy (TSE) preparations. About 67%

identity was found between pTS4 and Sphinx 2.36 DNA sequences. The DSO sequences of these two circular DNA molecules were found to be highly similar to the DSOs of other RC

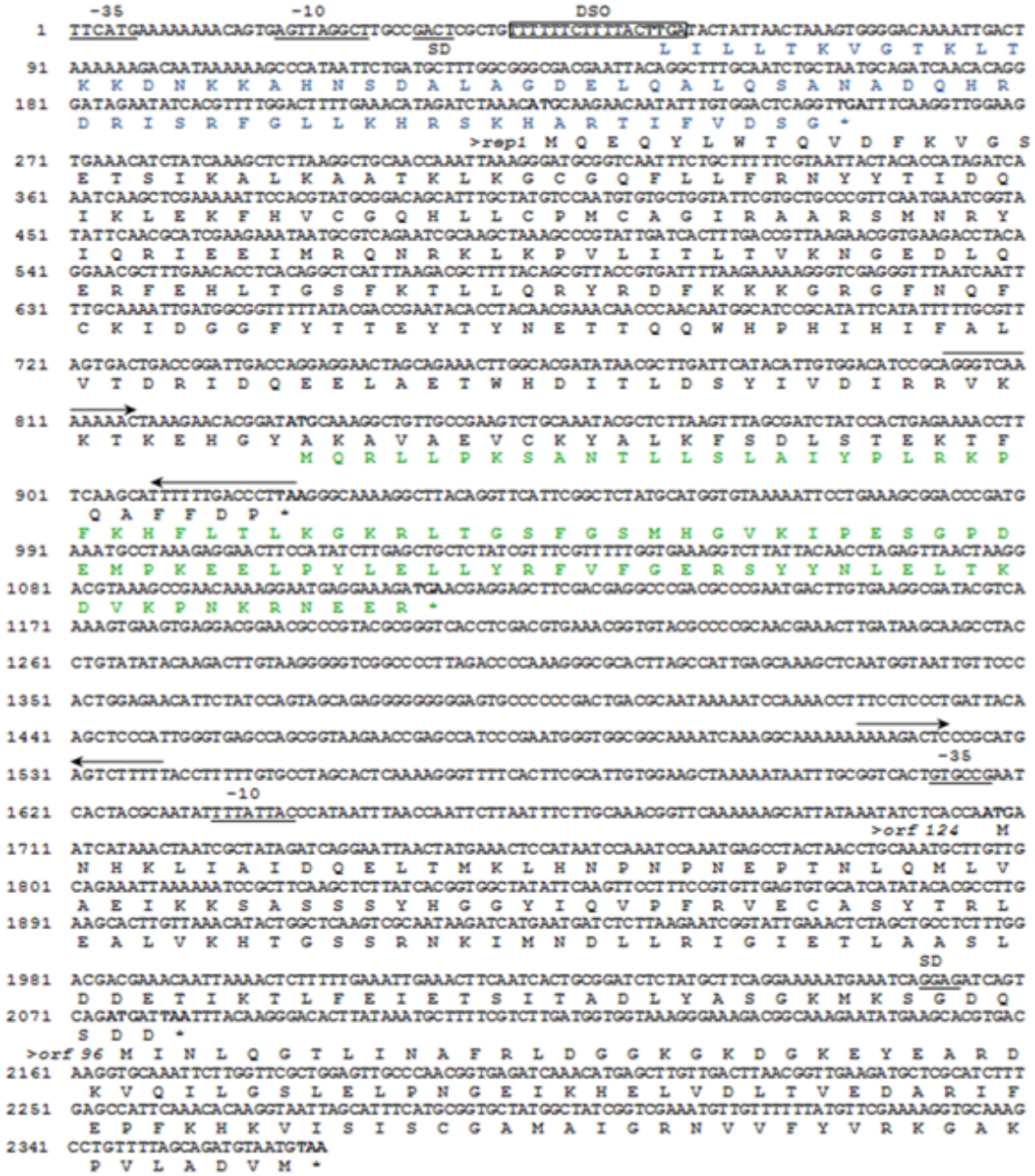

Fig. 3.15. Nucleotide and deduced amino acid sequence of Sphinx 2.36. The protein sequences coded by different frames of *repA* are shown. The protein showing homology to N-terminous of RepA of pTS4 is represented with blue colour. The central portion of RepA found to be similar both in Sphinx 2. 36 and RepA of pTS4 is shown in black letters. The protein predicted from +1 frame and show homology to the C-terminous of RepA of pTS4 is indicated with green colour. DSO, transcriptional terminators are shown with an open box and inverted arrows respectively. Putative promoter and SD sequences are underlined.

plasmids. Interestingly, the Sphinx 2.36 also codes for Orf113 and Orf96 homologues. As seen in pTS4, the two ORFs in Sphinx 2.36 appear to be translationally coupled, suggesting functional conservation of the gene products (Fig. 3.15).

Despite of sharing identical DSO sequences, the RepA sequences coded by these two circular DNA molecules were not highly conserved. This unusual observation has prompted to reanalyze the *repA* sequence of Sphinx 2.36 to identify possible frame-shifts in the sequence. Initially, the proteins coded in all six frames of the *repA* sequence were aligned with the RepA sequence of pTS4. The protein encoded by frame +2 was 47 % similar to the N-terminal part of pTS4 RepA. Similarly, the protein encoded by frame +1 was again similar (43 %) but with the C-terminal portion of pTS4 RepA. The RepA sequence reported by Manuelidis was encoded by frame +3 and it matched the RepA of pTS4 from amino acids 48 to 281 (Fig. 3.15). Reconstruction of the RepA-coding sequences of Sphinx 2.36 into a single frame by arbitrarily changing the sequence revealed a gene product with 62 % identity (92 % sequence coverage) and 79 % similarity to the RepA of pTS4. Interestingly, the Sphinx 2.36 *repA* also has a predicted UUG translation initiation codon, along with a putative promoter overlapping DSO. Taken together, these data suggest possible frame-shifts in the coding region, which could result either from sequencing errors or mis-incorporation of bases while amplifying the Sphinx 2.36 sequence by using ø29-polymerase from infected brain samples (Manuelidis, 2011). Although no data are available which proves the predicted RepA of Sphinx 2.36 is functional, nevertheless, if the experimental evidence shown in this study is taken into consideration, it suggests that RepA initiated from the alternative UUG codon might be needed for replication of Sphinx 2.36 in neuronal cells. A better understanding is only possible through thorough analysis of *repA* sequence of Sphinx 2.36.

Conclusions

1. Four indigenous plasmids were detected in *A. baumannii* DS002.
2. All four detected plasmids were rescue-cloned in *E. coli pir116*.
3. These indigenous plasmids were named as pTS1, pTS2, pTS3 and pTS4.
4. The 2.2 Kb pTS4 plasmid was completely sequenced. It contains three open reading frames designated as *repA*, *orf113* and *orf96*.
5. In plasmid pTS4 a putative double stranded and a single stranded origin of replication were identified.
6. The plasmid pTS4 is a rolling circle replication plasmid and replicates by generating single stranded intermediate.
7. The RepA encoded by *repA* gene of pTS4 is involved in initiation of replication of plasmid pTS4.
8. The translation of RepA of pTS4 is initiated from alternate start codon.
9. It creates nick at DSO of pTS4.
10. The conserved tyrosine (RepAY265) residue of RepA is required for initiating replication of pTS4.
11. The proteins coded by *orf113* and *orf96* genes do not participate in replication of pTS4.
12. The plasmid pTS4 sequence showed 67 % sequence identity to Sphinx 2.36, a circular DNA found associated with TSE infected brain samples.
13. The homologues of *repA*, *orf113* and *orf96* are found in Sphinx 2.36. The *repA* is monocistronic both in pTS4 and Sphinx 2.36.
14. Most probably the *orf113* and *orf96* are organized as one operon.

4.1 Link between pTS4 and Sphinx 2.36

4.1.1 pTS4 is a unique plasmid

The aim of the present study was to gain clearer insights into the plasmid profile of *A. baumannii* DS002 isolated from agricultural soils polluted with OP insecticides. Plasmid pTS4 was smallest of the rescued plasmids of *A. baumannii* DS002. Analysis of its sequence led to the discovery of a link between pTS4 and Sphinx 2.36. Homology between Sphinx 2.36 and pTS4 is more than 67 % and it is not confined to the conserved region but covers through the entire length of the sequence. Recently, Fondi et al., have generated an evolutionary relationship for 29 Acinetobacter plasmids available in database (Fondi et al., 2010). They have achieved this by retrieving all the 493 sequences of Acinetobacter plasmid-encoded proteins and used as input for the B2N software (NCBI), generating a set of network showing sequence identities existing among all the proteins. It can be assumed that the higher the degree of amino acid identity between two proteins, the more recent would be the event (recombination/transposition/duplication/vertical transmission) responsible for the presence of the two orthologous/paralogous coding genes in different plasmids. In this network, most of the plasmids showed some interconnections, even from the plasmids isolated from different *Acinetobacter* strains, indicating their evolutionary relationship. However, four plasmids (pRAY, pAC450, pYA1, and p4ABAYE) did not show any link to any of the proteins in the network. Interestingly the p4ABAYE plasmid is a pTS4 homologue, which suggests that pTS4 is a unique plasmid found in *A. baumannii* cells. As a homologue of Sphinx 2.36, it might lead to have new evolutionary origin.

4.2 Transmissible Spongiform Encephalopathies (TSE)

Prion diseases, also known as the transmissible spongiform encephalopathies

(TSEs), are a group of fatal neurodegenerative disorders that affect humans and animals (Prusiner and McKinley, 1987; Kimberlin and Walker, 1988; Weissmann, 1991a,b; Prusiner, 1993, 1998, 2001; Collinge and Palmer, 1997; Collinge, 2001). Transmissible Spongiform Encephalopathies (TSEs) include endemic sheep scrapie and epidemic BSE, human kuru, human Creutzfeldt–Jakob disease (CJD), and rapidly spreading cervid TSEs. Few theories have been proposed regarding the propagation of this endemic and epidemic desease. According to the protein-only theory, otherwise also called as prion hypothesis, a misfolded form of host prion protein (PrP) is infectious agent of BSE (Prusiner, 1998). The normal cellular prion protein (PrP^{C}) is found in most cell types within the body. Whereas in disease state, this protein undergoes a structural transition to its disease-causing scrapie form (PrP^{SC}) with profoundly altered physicochemical properties (Prusiner, 1998). The accumulation of PrP^{SC} is toxic to cells and may eventually lead to widespread cell death that is characteristically accompanied by a spongiform degeneration of the brains of afflicted individuals. Despite a wealth of data on the evolutionary conservation, cellular localization, structure, molecular environment and metal-binding properties of PrP^{C}, its precise cellular functions are still debated (Ehsani et al., 2011).

Though a 'protein only' theory has gained wider acceptance, detection of nucleic acids in TSE preparations supported the hypothesis that contradicts this otherwise established notion (Akowitz et al., 1994; Manuelidis, 2003). One school of thought which argues against the protein-only theory suggest involvement of a nucleic acid-based 'cofactor' during conversion of the cellular form of the prion, PrP^{C} into the disease-causing, protease-resistant PrP^{SC} (Geoghegan et al., 2007; Manuelidis, 2007). In support of this proposition, a vertebrate single-stranded RNA was shown to act as a 'cofactor' for

the *in vitro* amplification of PrP^{SC} (Deleault et al., 2003). Viral particles and RC-replicating plasmid-like elements designated as Sphinx were also seen associating with BSE-infected samples (Manuelidis, 2007). Identical Sphinx sequences were isolated from the infected brain samples collected from diverse geographical regions (Manuelidis, 2011). Though no direct involvement of Sphinx has been demonstrated in the generation of PrP^{SC}, their amplification, particularly in infected brain samples is of potential significance. Even in the absence of a causal link between Sphinx and TSE, the existence of a circular DNA in mammalian brain, which has a resemblance to a plasmid from *Acinetobacter baumannii* DS002 is an important observation. The present study has shown plasmid pTS4 has a rolling-circle mechanism of replication. Considering the overall structural similarity with pTS4 and their identical origins of replication, in all probability Sphinx 2.36 might be replicating through a single-stranded intermediate. However, whether a single-stranded form of Sphinx has any role in formation of PrP^{SC} from PrP^{C} remains to be established.

4.3 The pTS4 coded proteins and PrP have similar epitopes

Acinetobacter sp. are the most robust microbes that can adapt to a variety of habitats, including soil. They can easily gain entry into animals while grazing or drinking water. In fact, they were implicated in TSE due to existence of molecular mimicry between myelin basic protein (MBP) and 4-carboxymuconolactone decarboxylase (CMLD) (Hughes et al., 2003; Zhu, 2005). Supporting this proposition all infected animals tested BSE positive had highest levels of Acinetobacter specific antibodies (Wilson et al., 2003). Taking this information into account we have predicted antigenic determinants of PrP and the three predicted ORFs of pTS4 using the web based software (http://bio.dfci.harvard.edu/Tools /antigenic.html). Some of the epitopes mapped in PrP (155 to 165 residues) and RepA (210 to 230 residues) and Orf96 (45 to 53 redidues) of

pTS4 have shown the kind of molecular mimicry seen between MBP and CMLD (Fig. 4.1) and suggested the possibility of RepA/Orf96 antibodies cross reacting with PrP. In the light of these observations experiments were conducted to establish the cross reactivity of PrP antibodies with pTS4 coded RepA, Orf113 and Orf96 and vice versa. While gaining such experimental evidence all three pTS4 coded proteins; RepA, Orf113 and Orf96 were expressed in *E. coli* and the pure proteins were used to raise antibodies.

```
prion_protein      -GSKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGWGQPHGGGWGQPHGGGWGQPHG 59
pTS4_RepA          LLKKDNKKAHISEALAGGEIQALQSANADQHRDRITRFATLKHRAKNQENYLFTLAKFKE 60
                     .*.. *.  .   .*..  .  *...:::  :        * .   : :    .: :
prion_protein      GGWGQPHGG-GWGQPHGGGGWGQGGTHGQWN--------KPSKPKTNMKHVAGAAAAGAV 110
pTS4_RepA          NYEKDVKNEESIKALKSAQKLNECGNYLLFKNFYTIGEVKLSKLRTCGQHLLCPFCAAIR 120
                   .   : :.  .    :..   .: *.:  ::        * ** :*  :*:  . .*.
prion_protein      VG-GLGGYML----------------------GSAMS----------RPLIHFGSDYED 136
pTS4_RepA          ASRAIQKYVERIDQVLQENRKLKPVLITLTVKNGSDLAERSEHLMKSFRTLLERRRDYEK 180
                   .. .:  *:                        ** ::          *.*:.   ***.
prion_protein      R--------------------YYRENMHRYPNQVYYRPVDQYSNQNNFVHDCVNIT---- 172
pTS4_RepA          KGRGFNEFCKVQGAMYSYENTFNEKTGEWHPHIHMFALVDQWIDQQEFSEYWHSLTGDSM 240
                   :                    : .:. . :*:   :  ***: :*::* .   .:*
prion_protein      ---------------------------------VKEHTVTTTTKGENFTETD-----IK 193
pTS4_RepA          VVDVRRARKEKGYGYSKAAAEVCKYALKFGDLSVEKTWEAFKVLKGKRLTGSFGLLWGVK 300
                                                     *   . .. **:.:* :      :*
prion_protein      MMERVVEQM---------CITQYQRESQAYYQRGA------------------------ 219
pTS4_RepA          IPDSMTDDMPSEDLPYLEMLYKFAYSKKSYYDLLITRHVEPQPHEDERARSFDECDCIYA 360
                   : : :.::*          : ::  ..::**:
prion_protein      ------------------
pTS4_RepA          VEAQTFDCEERTTVRTAQ 378
```

Fig. 4.1. Sequence alignment of Prion Protein (PrP) with pTS4 RepA. Predicted antigenic determinants are shown in open box.

4.4.1 Cloning, expression and purification of Orf96 and Orf113

In order to generate antibodies against Orf96 and Orf113, expression plasmids were constructed by cloning *orf113* and *orf96*. The Orf96 coding gene was amplified from plasmid pTS4 using a forward primer (DS00121) having *Eco*RI site and a reverse primer (DS00122) having *Xho*I site. Similarly *orf113* gene was amplified with a pair of primers (DS00123/DS00124) having *Eco*RI and *Xho*I sites. The respective amplicons were then digested with *Eco*RI and *Xho*I and ligated independently into pET23b digested with the same enzymes (Fig. 4.2A). The cloning strategy places both *orf96* and *orf113* in-frame with the vector-encoded His-tag of pET23b and the corresponding recombinant plasmids

were designated pT96W and pT113W respectively. These two proteins were expressed and purified following the protocol described for RepA protein expression and purification (Fig. 4.2B&C).

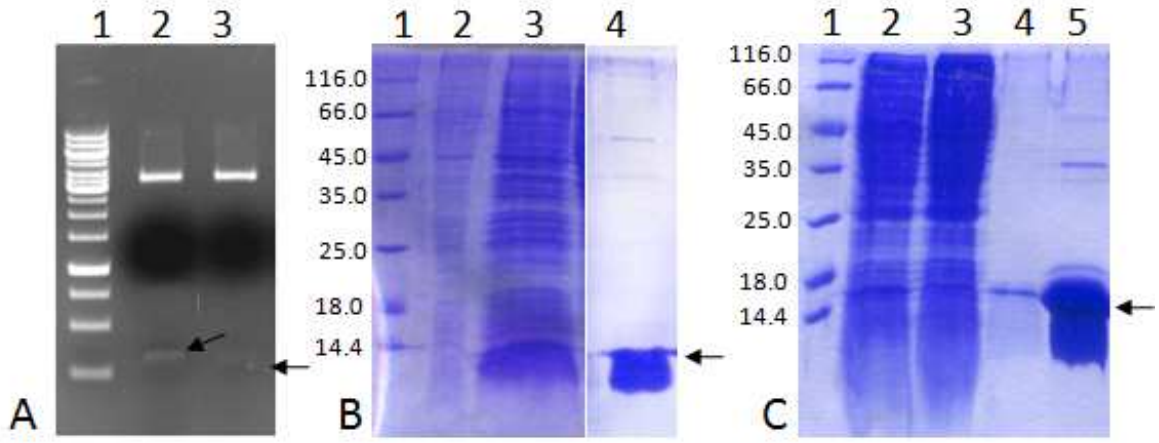

Fig. 4.2. Panel A: Cloning of *orf96* and *orf113* in pET23b. Lane 1 represents 1 Kb DNA ladder, lanes 2 and 3 represent *Eco*RI and *Xho*I digestion of expression plasmids pT96W and pT113W. Release of 0.34 Kb and 0.29 Kb of *orf113* and *orf96* inserts are shown with arrow marks. Panel B: 15 % SDS-PAGE. Lane 1 represents protein molecular weight marker, lanes 2 & 3 represent uninduced and induced culture of *E. coli* BL21 (pT96W) cells and lane 4 represent affinity purified Orf96. Panel C: 15 % SDS-PAGE showing purification profile of Orf113. Lane 1 represents protein molecular weight marker, lane 2 & 3 indicate protein expressed from *E. coli* BL21 (pT113W) induced and uninduced cells. Affinity purified of Orf113 is shown with an arrow mark (lane 5).

4.4.2 Cloning and expression of RepA, Orf96 and Orf113 as GST fusion proteins

The commercially procured PrP antibody was raised using His-tagged recombinant bovine prion protein as primary antigen. Therefore, there is a possibility of having antibodies produced against hexa histidine epitope. In such a scenario, PrP-6His polyclonal antibodies can cross react with plasmid pTS4 proteins expressed with C-terminal His-tag. That kind of false reactivity can be avoided by producing pTS4 coded proteins either with GST-tag or without having any affinity tag. The His-tag was removed from the C-terminus of RepA by digesting the plasmid pTRW1 coding RepA with *Xho*I. The digested plasmid was end-filled and religated to create frame shift between *repA* and vector coded his sequence. The corresponding plasmid was designated as pTRW1X. The

E. coli BL21 cells expressing RepA without his-tag was confirmed by probing the cells lysate with anti-his antibody.

The RepA, Orf96 and Orf113 were further expressed as GST fusion protein by cloning in pGEX-4T-1 vector. Initially three pairs of primer were designed, to amplify *repA* (DS00127/DS00128), *orf96* (DS00129/DS00130) and *orf113* (DS00131/DS00132) genes, having *Eco*RI and *Xho*I sites. After the PCR amplification of all the genes using pTS4 as template, the amplicons were digested with *Eco*RI and *Xho*I and ligated into pGEX-4T-1 digested with the same enzymes. The clone was confirmed by digesting with *Eco*RI and *Xho*I enzymes (Fig. 4.3A) and the resulting plasmids encode the respective proteins with N-terminal GST fusion protein. Theses expression plasmids designated as pTRWG, pT96G and pT113G were then transformed in *E. coli* BL21 cells and their expression were analysed on SDS-PAGE. A clear overexpressed band was seen in the gel corresponding to the size of each protein (Fig. 4.3B) and these cultures were used for performing western with various antibodies.

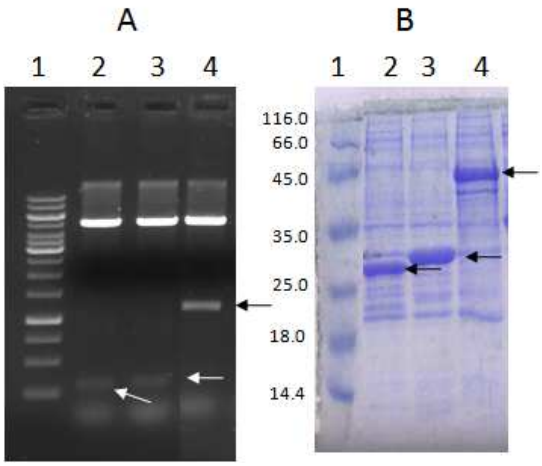

Fig. 4.3. Panel A: Confirmation of *repA*, *orf96* and *orf113* cloning in pGEX-4T-1. Lane 1 represents 1 Kb DNA ladder, lanes 2 to 4 represent *Eco*RI and *Xho*I digestion of expression plasmids pT96G, pT113G and pTRWG. Release of *orf96*, *orf113* and *repA* inserts are shown with arrows. Panel B: 12.5 % SDS-PAGE. Lane 1 represents protein molecular weight marker, lanes 2 to 4 indicate cell lysates of *E. coli* BL21 cultures coding GST-Orf96, GST-Orf113 and GST-RepA respectively. The overexpressed fusion proteins are indicated with arrows.

4.5 Generation of Antibodies RepA, Orf96 and Orf113

Antibodies against RepA, Orf96 and Orf113 were generated in Rabbit as described in materials and methods. The serum obtained were then used to purify IgG using Protein A column following the procedure described elsewhere (Materials and methods). The purified IgG's were used for western analysis.

4.5.1 PrP antibody interacts with RepA

Initially, in order to gain preliminary evidence on interaction between RepA and PrP antibody, western blotting was carried using commercially procured PrP antibody (Abcam, USA). The lysates of *E. coli* BL21 cells expressing recombinant RepA and purified mouse PrP protein were used as positive controls. Immuno blotting was done to gain information on the reactivity of prion antibody with plasmid pTS4 coded proteins. As shown in figure 4.4A&B, a positive signal was observed in the lane corresponding to *E. coli* BL21 cells containing RepA expression plasmid, whereas no such signal was seen in *E. coli* BL21 cells alone. Since the commercially procured PrP antibody was generated from

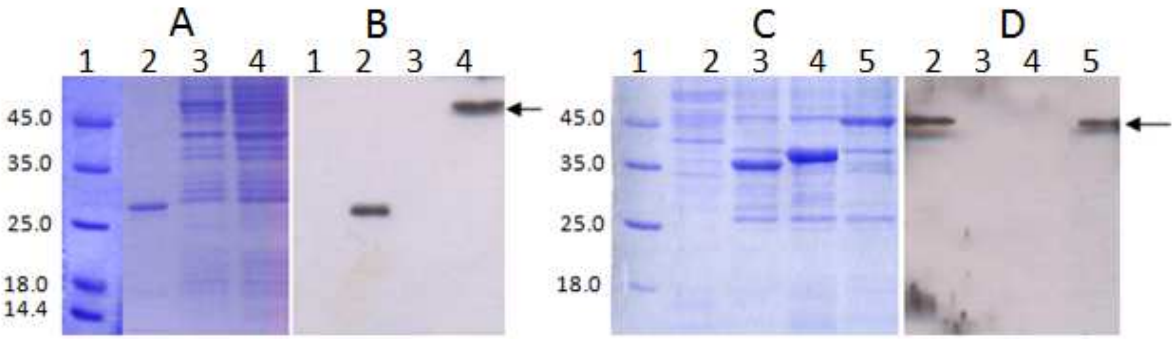

Fig. 4.4. SDS-PAGE (A) and corresponding western blot (B) probe using anti-PrP antibody. Lane 1 represents protein molecular weight marker, lane 2 represents recombinant mouse PrP, lanes 3 and 4 represent lysate prepared from *E.coli* BL21 cells without and with RepA expression plasmid (pTRW1) respectively. The positive signal corresponding to RepA is shown with an arrow. Panel C: SDS-PAGE and corresponding western blot (D) probe using anti-PrP antibody. Lane 1 represents protein molecular weight marker, lane 2 represents cell lysate prepared from *A. baumannii* DS002, lanes 3 & 4 indicate *E. coli* BL21 cells expressing GST-fusion protein of Orf96 and Orf113 respectively, and lane 5 represents recombinant RepA protein without any tag. The RepA specific signal is shown with arrows.

recombinant PrP_{6His} protein, the cross-reactivity shown might be due to antibodies raised against "His" epitope. In order to avoid such situation, the experiment was executed using *E. coli* BL21 cells expressing RepA without His-tag. Similar hybridization pattern was observed (Fig. 4.4C&D) suggesting that Prp antibodies cross react with RepA protein of plasmid pTS4.

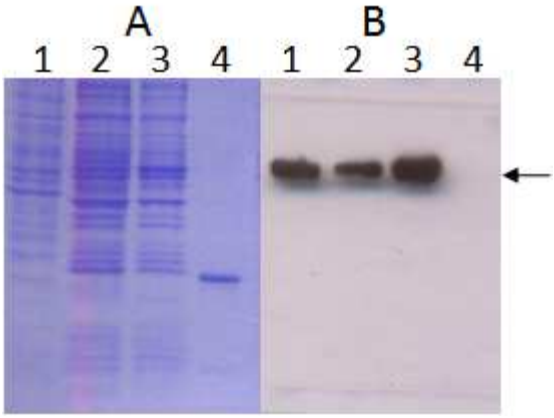

Fig. 4.5. Western analysis using RepA antibody: Panel A; 12.5 % SDS-PAGE and its corresponding western blot (Panel B). Lane 1 represents cell lysate prepared from *A. baumannii* DS002, lanes 2 & 3 represent cell lysate of *E. coli* BL21 culture harbouring RepA expression plasmid without (2) or with His-tag (3), and lane 4 represent recombinant PrP protein. The RepA specific signal is shown with an arrow. No cross reactivity was seen between antibodies raised against RepA and PrP protein.

The purified antibodies from the serum of immunized rabbits showed specific cross reactivity. Initially the RepA antibodies were used to probe protein extracts prepared from *A. baumannii* DS002 and *E. coli* BL21 expressing with $RepA_{6His}$ and RepA. In all these extracts a clear signal corresponding to RepA was alone noticed indicating the antibodies produced were giving specific signals against RepA (Fig. 4.5A&B). Interestingly, these RepA polyclonal antibodies showed no cross reactivity with PrP protein. If these results are any indication, the proposed molecular mimicry between RepA and PrP is not a viable/valid hypothesis. However, the PrP used in this study is not purified from bovine brain. It is a recombinant PrP expressed in *E. coli*. There could be a possibility of having

differences in the structure features of native and recombinant PrP. If native PrP cross reacts with RepA antibodies remains to be established.

4.5.2 Antibodies of Orf36 and Orf96 do not react with PrP

After obtaining antibodies for $Orf113_{6His}$ and $Orf96_{6His}$, similar studies were conducted to ascertain if these antibodies cross react with PrP protein. Initially, the antibodies were probed with total cellular protein extracts prepared from *A. baumannii* DS002 and *E. coli* cells expressing Orf113 and Orf96. When western blotting was done using antibodies raised against Orf113, interesting results were noticed. These anti-Orf113 antibodies gave no signal, neither with PrP nor with *A. baumannii* DS002 protein lysate. Signal was seen only with pure Orf113 (Fig. 4.6A&B). The signal obtained with Orf96 specific antibodies gave interesting results. It gave no signal with PrP. Further the signal obtained with *A. baumannii* DS002 protein extracts resembled a ladder of molecular weight marker (Fig. 4.7A&B). The experiment was repeated several times to know if it is an artefact. In all repetitions similar results were obtained. If the results

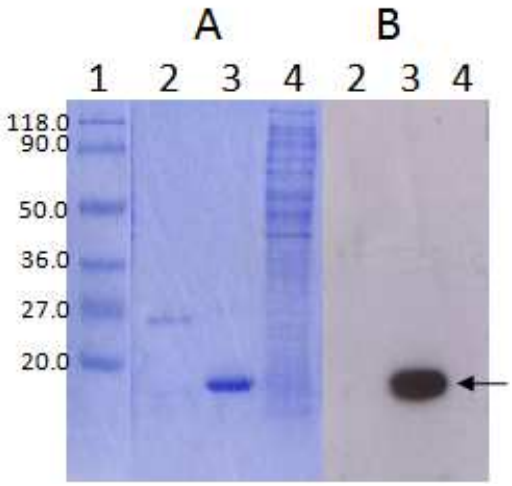

Fig. 4.6. Panel A: Western analysis using Orf113 antibody. Lane 1 represent protein molecular weight marker, lane 2 indicates PrP protein, lane 3 represent Orf113 protein, lane 4 indicates cell lysates of *A. baumannii* DS002. Panel B represents corresponding western blot. The positive signal is obtained only with pure Orf113 (Panel B; lane 3). No signal is seen either with PrP or with *A. baumannii* cell lysate.

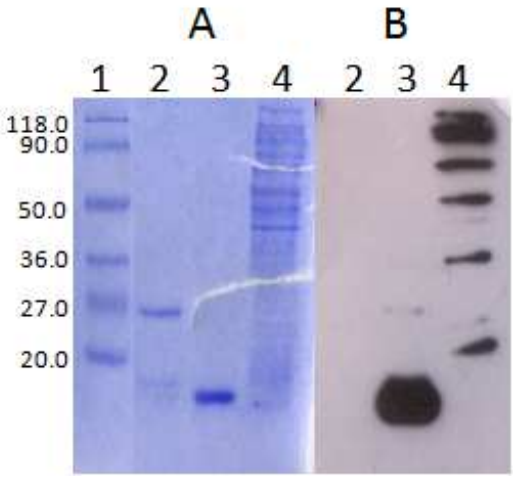

Fig. 4.7. Panel A: Western analysis using Orf96 antibody. Lane 1 represent protein molecular weight marker, lane 2 indicates PrP protein, lane 3 represent Orf96 protein, lane 4 indicates cell lysates of *A. baumannii* DS002. Panel B represents corresponding western blot. No reactivity is seen with PrP protein (Panel B; lane 2). The pure Orf96 has shown clear reactivity. Interestingly with total cell lysate, the obtained signal showed an image of ladder (Panel B; lane 4).

obtained are true it looks thatOrf96 is getting oligomerized in *A. baumannii* DS002. Why this kind of oligomerization?. Is pTS4 a replicative form of viral DNA having ssDNA as its genome? Is Orf96 is viral coat protein?

4.6.1 Is pTS4 is genome of virus?

After analysis of western blot results further experiments were carefully designed to assess if there exists a phage in *A. baumannii* DS002. The culture supernatant of *A. baumannii* DS002 was used to identify existence of phage. The polyethylene glycol 8000 (PEG) precipitate generated from 1 L culture of supernatant was subjected to sucrose density gradient ultracentrifugation. The PEG precipitate was loaded on a step gradient having 2 cm height of 10 %, 20 %, 30 % and 40 % sucrose solution. After placing the gradient tube in swing out rotor, the PEG precipitate was subjected to ultracentrifugation for 3 hours at 25,000 rpm. The tubes were then carefully removed and visualized in a dark room by passing visual light with the help of torch light. As shown in figure 4.8, two clear bands were seen at 20 % and 10 % sucrose solution. The first band from the bottom

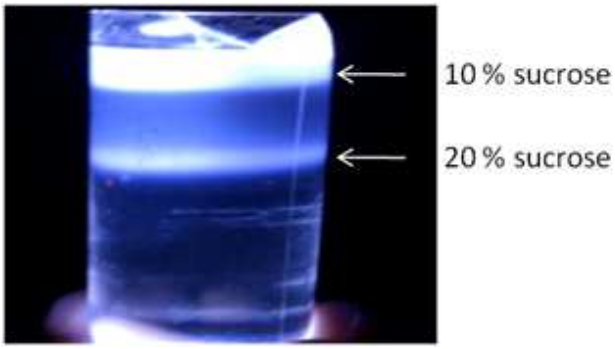

Fig. 4.8. Visualization of virus particles: The bands appeared after passing visual light are shown with arrow marks.

of the tube (BL) is a high density particle. It is banding out at a density of 1.2 g/ml. It is assumed as viral particle with nucleic acid. These bands were then carefully collected with the help of a syringe fitted to a long needle. The particles present in these bands were further precipitated by subjecting them for one more round of ultracentrifugation. The particles pelleted from these fractions were independently subjected for SDS-PAGE and subsequent western blotting using antibodies against Orf113 and Orf96. A clear signal was obtained when probed with Orf96 antibody with these two fractions collected from the tube. The size of the obtained signal was little bigger than the recombinant

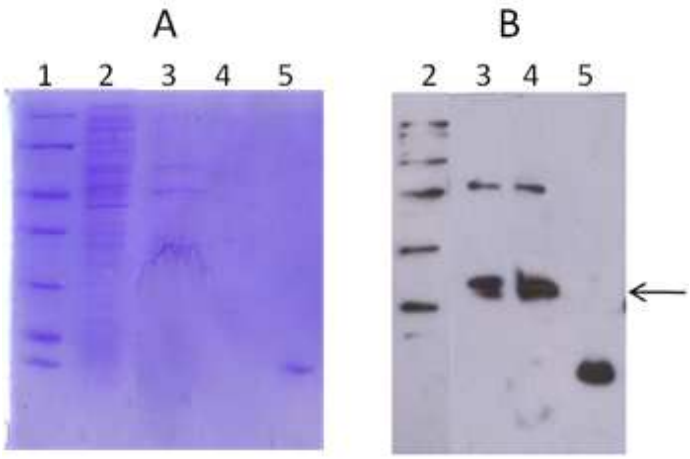

Fig. 4.9. Analysis of proteins found in 10 and 20 % sucrose gradient fractions (Panel A) and corresponding western blot using Orf96 antibody (Panel B). Lane 1 represents protein molecular weight marker, lane 2 represents cell lysate of *A. baumannii* DS002, lanes 3 & 4 represent particles obtained at 10 and 20 % sucrose gradient and lane 5 indicates Orf96 pure protein. Arrow mark indicates signals obtained from the two fractions.

Orf96. The viral coat proteins frequently undergo posttranslational modifications (Fang et al., 1999). These modifications, especially if were through glycosylation, the size of the protein is increased (Vigerust & Shepherd, 2007). Though the size of the signal is not coinciding with the size of recombinant Orf96, existence of a protein in the particles obtained in 20 % sucrose gradient points towards existence of its homologues.

4.6.2 Visualization of viral particles

In order to have further insights into nature of the particles found in 20 % sucrose gradient, the samples were visualized under transmission electron microscope (TEM). A drop of solution containing particles was placed on carbon coated copper grid (Icon analytical system, USA). The particle was then stained with uranyl acetate and observed under TEM. A clear uniformly sized spherical particles were observed in the fraction obtained from 20 % sucrose gradient (Fig. 4.10). This clearly indicates existence of 30-40 nm sized particles and in all possibility appears to be viral particles. The protein found in these particles appears to be Orf96. Formation of SDS resistant Orf96 multimers and interaction of the protein found in the particles with the antibodies of Orf96 point towards existence of a phage in *A. baumannii* DS002, probably with the ssDNA of pTS4 as

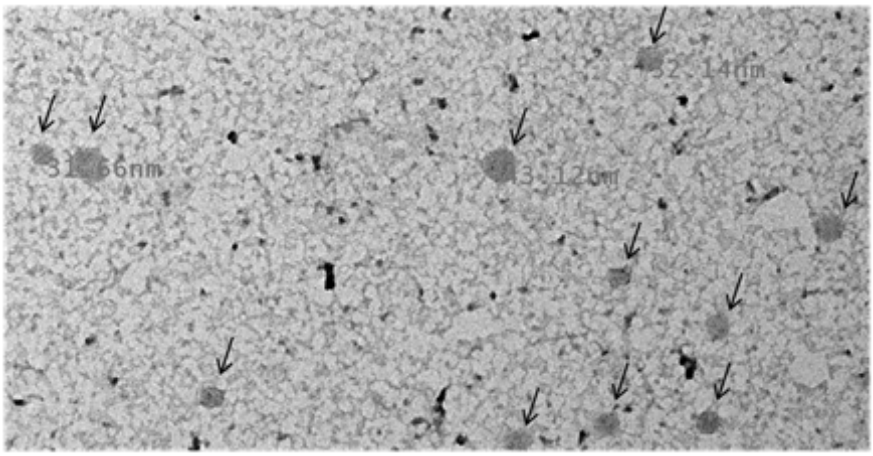

Fig. 4.10. TEM image of particles obtained at 20 % sucrose gradient. The virus particles are indicated with arrow marks.

its genome. Further work to analyze nucleic acid found in the phage like particle is in good progress in our laboratory.

Prion disease is one of the most complex of all animal diseases. Despite of best brains working on the subject, the topic is still controversial. Though a number of theories were proposed, none of them have given any convincing evidence on the cause of the disease. The prion only theory, proposed by Stanley Prusiner, suggests that the disease is due to misfolding of cellular prion (PrP^{C}) to PrP^{SC} (Scrapie form). It doesn't explain the reasons for such misfolding (Prusiner, 1998). In the absence of any change in sequence information of PrP^{C} and PrP^{SC}, the force that disrupts folding of a protein, the 'factors' that that contribute for its misfolding needs to be identified. There are reports of existing single stranded nucleic acid acting as cofactors for converting PrP^{C} to PrP^{SC} (Deleault et al, 2003). In contradiction of this hypothesis, a group of scientists believe that BSE a viral disease. They have shown existence of a circular DNA in all BSE infected samples collected from diverse geographical regions (Manuelidis, 2007; Manuelidis, 2011). The pTS4 is homologue of one of the circular DNA separated in BSE infected brain samples. The present work provides reasonable evidence to show that pTS4 as replicative form of DNA of a phage using *A. baumannii* DS002 as a host. *A. baumannii* DS002 is an environmental isolate and there is ample scope for reaching *A. baumannii* DS002 to animals. In the light of these preliminary findings, further investigations are warranted to established link, if any, between pTS4 and BSE.

Conclusions

1. Antigenic determinant predicted both on Prion protein of Bovine and RepA of pTS4 showed close amino acids identity, indicating the possibility of interaction between RepA antibodies with prion protein.

2. Antibodies against RepA, Orf96 and Orf113 were generated in Rabbit.
3. PrP antibody cross-reacted with RepA of pTS4, however the RepA antibody did not react with recombinant PrP protein.
4. Orf113 and Orf96 antibodies did not interact with PrP protein.
5. Phage like particles were detected in the culture supernatant of *A. baumannii* DS002.
6. The antibodies of Orf96 cross react with protein found in these particles.

Acinetobacter baumannii DS002, as mentioned before, is isolated from the OP insecticide polluted agricultural soils. Along with this strain we have also obtained a *Flavobacterium balustinum* that used OP insecticide methyl parathion as a source of carbon and nitrogen (Somara and Siddavattam, 1995). Further studies have shown existence of a plasmid borne organophosphate degrading (*opd*) gene in this isolate (Somara and Siddavattam, 1995). Although *A*. *baumannii* was enriched along with *Flavobacterium balustinum*, it has never grown using OP compound methyl parathion as carbon source. However, it has dealkylated OP insecticide methyl parathion. In support of this observation, a glutathione S-transferase (GST) like protein got induced only when the culture was grown in presence of methyl parathion (unpublished data). The 2-D profile of the proteome extracted from the culture grown in presence of methyl parathion has shown upregulation of GST-like protein (unpublished data). Absence of a conserved organophosphorous degrading (*opd*) gene and induction of GST suggested existence of an alternate pathway to metabolize methyl parathion in *A. baumannii* DS002. Therefore, in the present study an attempt was made to understand the role of GST in biotransformation of methyl parathion a prominent OP-insecticide used to control insect pests.

4.1 Identification of *gst* gene in *A. baumannii* DS002

4.1.1 Construction of genomic DNA library

In order to obtain *gst* gene from *A. baumannii* DS002, a genomic DNA library was constructed using Copy Control Fosmid Library Production Kit (EPICENTRE Biotechnologies, USA). The Copy Control Cloning System, as reported elsewhere, provides the user with total control over the clone copy number (David et al., 2006). The system ensures the clone stability due to its existence as a single copy in absence of copy

number induction. However, when needed the copy number of the recombinant fosmids can be enhanced by inducing the *pir* protein by growing the culture in presence of arabinose. The details of the genomic DNA construction are giving in materials and methods section. The recombinant fosmid DNA was isolated as described in materials and methods and the existence of 40 Kb insert was confirmed by digesting the recombinant fosmid with *EcoR*I (Fig. 4.1).

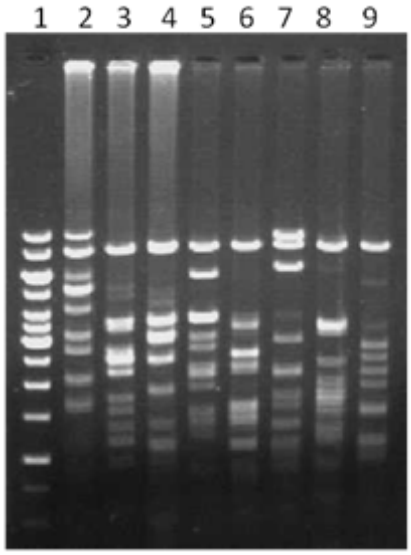

Fig. 4.1. Digestion of randomly selected recombinant fosmids with *Eco*RI: Lane 1 represents 1 Kb DNA ladder, Lanes 2 to 9 indicate DNA fragments generated upon *Eco*RI digestion. The genomic fragment profile indicates existence of different regions of *A. baumannii* genome in recombinant fosmid.

The completeness of library was calculated using standard formula

$$N = \ln (1-P) / \ln (1-f)$$

Where P is the desired probability (expressed as a fraction); f is the proportion of genome contained in a single clone; and N is the required number of clones with recombinant fosmid. The size of *Acinetobacter* sp. genome is approximately 4 Mb. As shown in figure 4.1 each recombinant fosmid is having an insert of approximately 40 Kb

and hence the number of clones required to ensure a 99 % probability of a given DNA sequence of each recombinant having 40 Kb insert is:

N = ln (1-0.99) / ln (1 – [4 X 10^4 bases / 4 X 10^6 bases]) = - 4.61 / -0.01 = 461 clones.

If calculation is made incorporating this data in the formula shown above, it requires to have 461 clones to claim it as complete library. The present study has generated about 1500clones and hence it can be claimed as complete library. These colonies were designated as FSTS giving numbers ranging from 0001 to 1500. The library is maintained in the laboratory.

4.1.2 Screening of Genomic DNA library to identify clones containing *gst* gene

In our previous studies our laboratory has identified GST as one of upregulated proteins in protein extracts isolated from methyl parathion grown *A. baumannii* DS002 cultures. Based on the MALDI/TOF/TOF data *de novo* sequence was generated for randomly selected fragments. The sequence information was used to generate degenerate primers (DS00125/DS00126) to amplify partial *gst* sequence (Fig. 4.2). After establishing that the amplicon is partial *gst*, through sequencing, it was used as probe for screening *A. baumannii* DS002 genomic library.

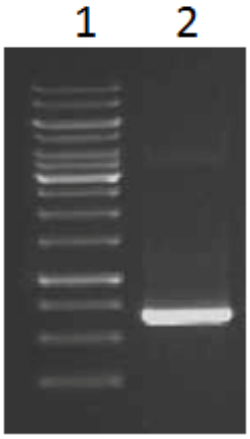

Fig. 4.2. Lane 1 represents 1 Kb DNA ladder and lane 2 indicates amplification of partial *gst* gene.

4.1.3 Identification of recombinant fosmids with *gst* genes

Colony hybridization was employed (materials and methods) to screen the genomic DNA library. Initially all the clones from the genomic DNA library were transferred to nitrocellulose membrane and the presence of *gst* genes were determined by using radiolabelled partial *gst* gene as probe. In total 9 clones have given positive signal, and they were designated as FSTS0009, FSTS0021, FSTS0023, FSTS0051, FSTS0437,

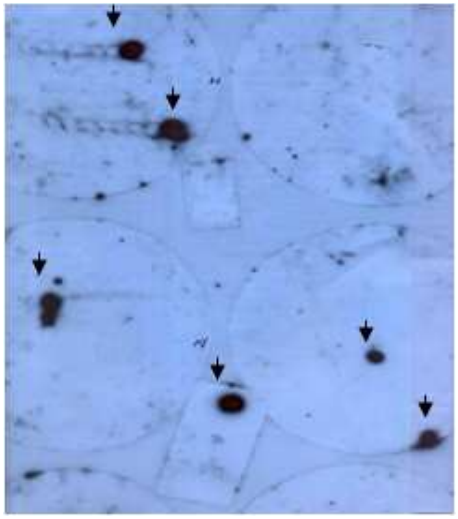

Fig. 4.3. Colony hybridization: The colonies that gave signal when hybridized with radiolabelled partial *gst* (PCR amplicon) are indicated with arrow marks.

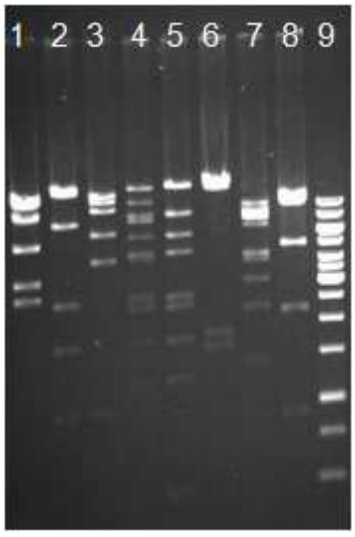

Fig. 4.4. *Eco*RI fragment profile of recombinant fosmids containing *gst* gene. Lane 9 represent Kilobase ladder. Lanes 1 to 8 indicate fragment profile generated from recombinant fosmids FSTS0009, FSTS0021, FSTS0051, FSTS0437, FSTS0438, FSTS0526, FSTS0585 and FSTS 1016.

FSTS0438, FSTS0526, FSTS0585 and FSTS 1016 (Fig. 4.3). The recombinant fosmids isolated from these clones were digested with *Eco*RI to establishe the similarity/dissimilarity of the inserts (Fig. 4.4). Interestingly, all of the 9 colonies that gave positive signals have shown unique restriction pattern, indicating existence of multiple *gst*s in *A. baumannii* DS002. The fragments that gave positive signals were subcloned and the sequence information was obtained for all 9 *gsts* identified in *A. baumannii* DS002. As convention these *gsts* were designated as *Abgst01, Abgst02, Abgst03, Abgst04, Abgst05, Abgst06, Abgst07, Abgst08,* and *Abgst09*.

4.2 Sequence analysis of *Abgsts*

A. baumannii DS002 as indicated in the introduction section is a robust organism. It has ability to withstand stress conditions and to use a variety of aromatic compounds as source of carbon (Schirmer et al., 1997). Such metabolic diversity and capability to withstand stress conditions indicate existence of novel genetic information. GSTs are known to contribute for degradation of a variety of xenobiotics. Their involvement in stress resistance is a well known phenomenon. In the light of such information, existence of multiple *gsts* in *A. baumannii* acquires significance and naturally generates inquisitive to know information pertaining to their expression. Therefore, the sequence information obtained for all nine *gsts* were analyzed to predict promoter elements using online bioinformatics tools (www.softberry.com/berry.phtml?topic=bprom). Interestingly, each of the cloned *gst*s has shown to contain unique promoter structure and appears to depend on novel transcription factors for expression (Fig. 4.5-4.12).

As in the case of *Abgst01* (Fig. 4.5), a putative OxyR binding site was predicted upstream of σ^{70} dependent promoter along with an RpoS and ArgR binding sites. OxyR is

a member of LTTR family (LysR Type Transcriptional Regulator) and it induces expression of several defensive genes against oxidative stresses (Zheng et al., 1998). The members of LTTR family have also been shown to repress expression of their own gene (Schell,1993). The stationary phase sigma factor RpoS (σ^S) is a stress regulator that plays a critical role in survival under stress conditions in *Escherichia coli* and other γ-proteobacteria (Dong et al., 2008). Under condition of cellular starvation, σ^S accumulates, binds and directs the RNA polymerase holoenzyme to over 100 genes constituting RpoS regulaon (Ishihama, 2000). Whereas the ArgR belongs to the ArgR/AhrC family, whose members are widely distributed in bacteria (Makarova, 2001). The ArgR is involved in repression of several genes involved in biosynthesis of arginine, transport of histidine, and its own synthesis (Lim et al., 1987; Charlier et al., 1992; Caldara et al., 2006) and activates genes involved in arginine catabolism (Kiupakis and Reitzer, 2002). Considering the transcriptional requirement, the *Abgst01* appears to play a critical role in protecting *A. baumannii* DS002 from oxidative stress.

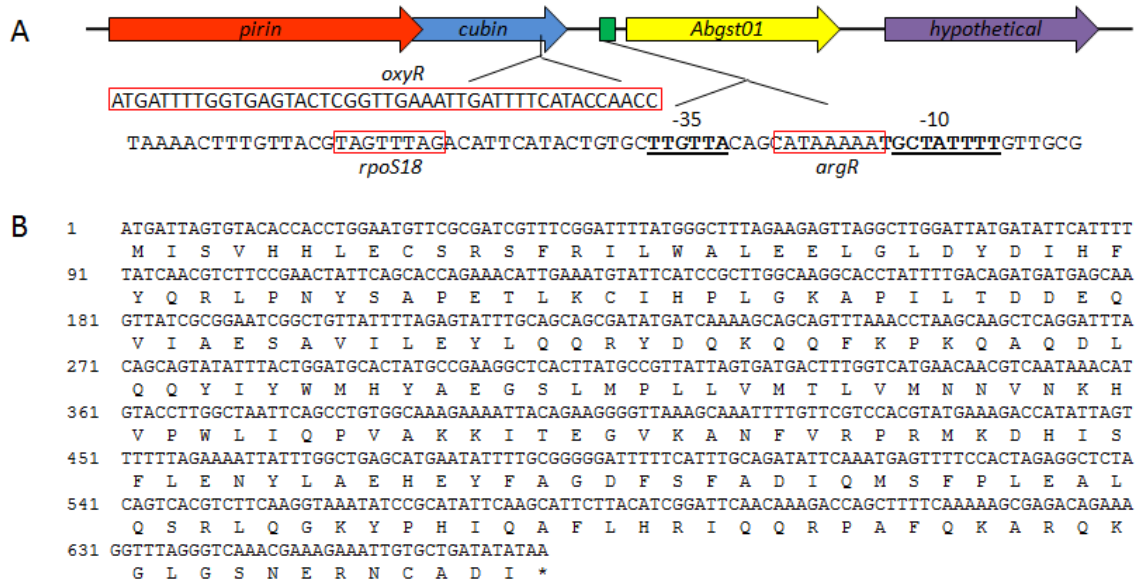

Fig. 4.5. Panel A: Schematic representation of *Abgst01* and its flanking regions. Putative transcription factor binding sites are shown with an open box (Red).The underlined sequence indicates putative promoter element. Panel B represents nucleotide and deduced amino acid sequence of *Abgst01*.

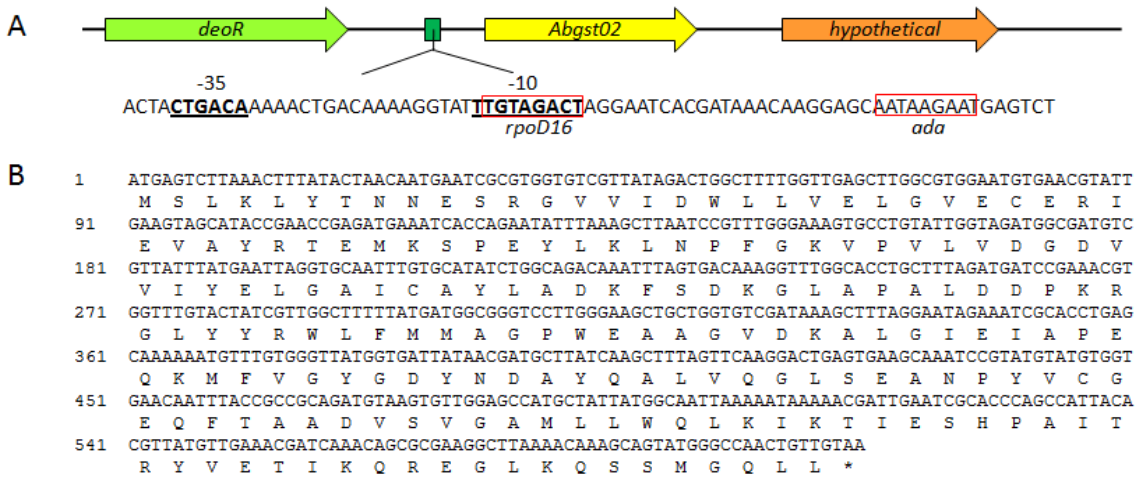

Fig. 4.6. Panel A: Schematic representation of *Abgst02* and its flanking regions. Putative transcription factor binding sites are shown with an open box (Red). The underlined sequence indicates putative promoter element. Panel B represents nucleotide and deduced amino acid sequence of *Abgst02*.

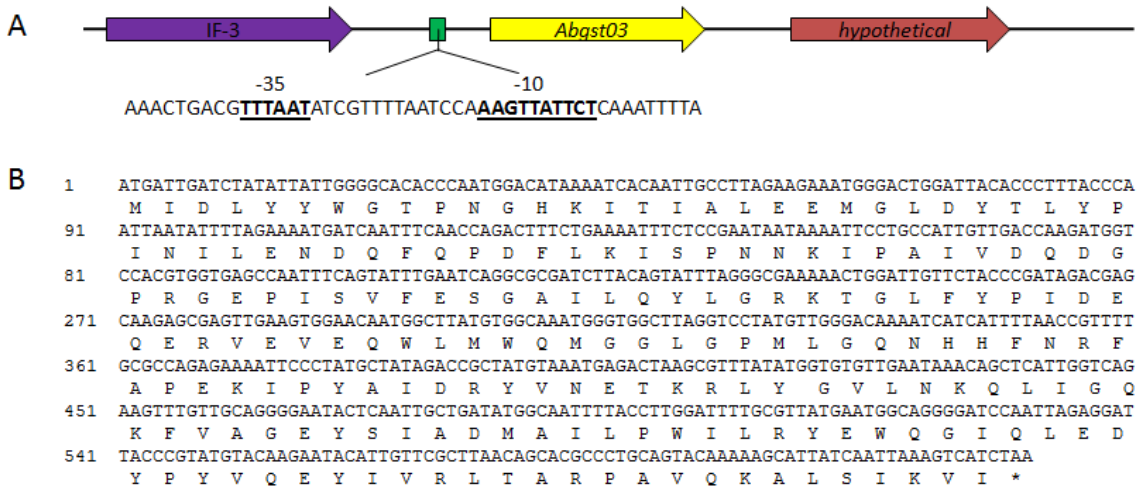

Fig. 4.7. Panel A: Schematic representation of *Abgst03* and its flanking regions. Putative promoter sequence is underlined. Panel B represents nucleotide and deduced amino acid sequence of *Abgst03*.

The *Abgst02* as shown in figure 4.6 is flanked by a transcription repressor DeoR and a hypothetical protein. When the promoter region of *Abgst02* was analysed, a typical RpoD16 dependent promoter motif was identified. Downstream of this putative promoter a consensus sequence to which transcription activator Ada binds was identified. Interestingly, there is no consensus DeoR binding site upstream of *Abgst02*. Therefore it is not clearly known if DeoR is involved in repression of *Abgst02*. The Ada

type of transcription regulators are involved in regulation of genes that contribute for protection of DNA damage in presence of alkylating agents (Lindahl et al., 1988; Sedgwick and Lindahl, 2002). Existence of such motif immediately downstream of *Abgst02* promoter, if seen together with the role of GSTs in dealkylating xenobiotics, indicates its involvement in biotransformation of alkylating agents that contribute for the DNA damage.

The *Abgst03* (Fig. 4.7) and *Abgst08* (Fig. 4.11) contain a typical σ^{70} dependent promoter without having any putative transcription binding site. If promoter sequence is taken into consideration, these two GSTs appear to express constitutively in *A. baumannii* DS002 and probably must be playing a critical role in housekeeping activities.

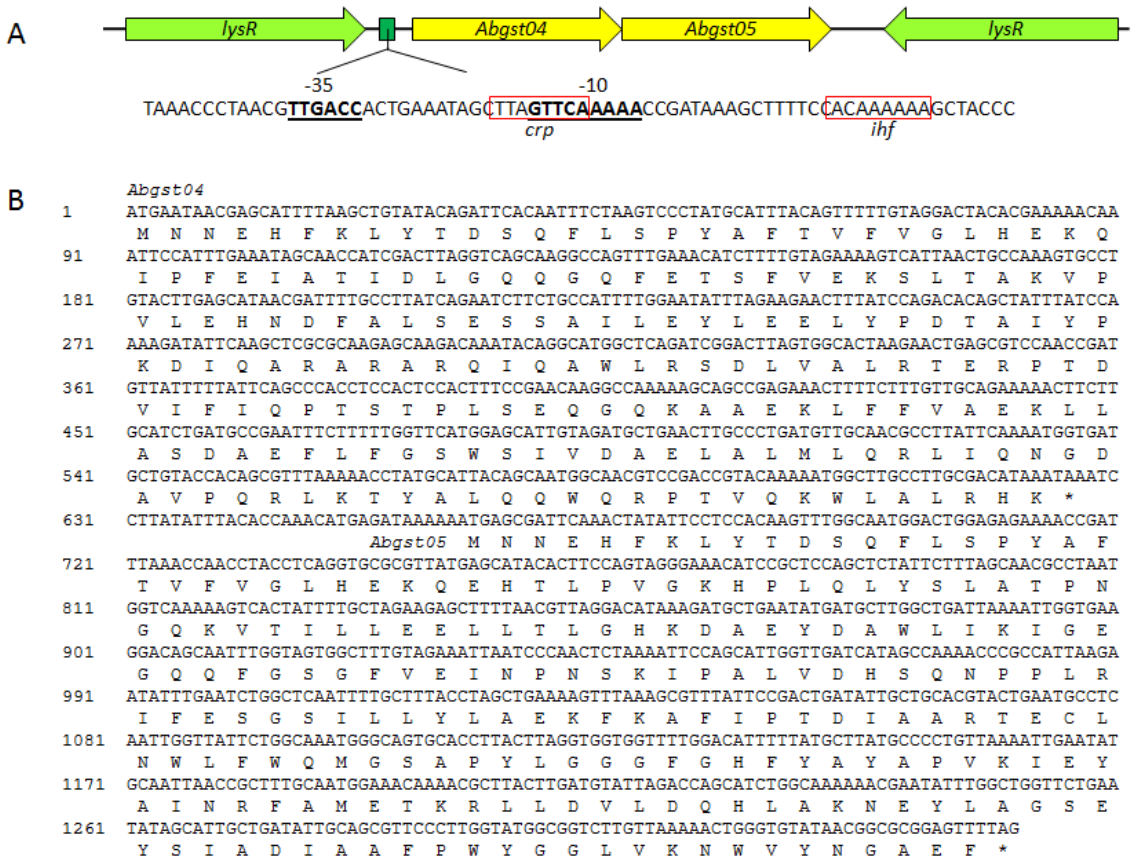

Fig. 4.8. Panel A: Schematic representation of *Abgst04* and *Abgst05* with its flanking regions. Putative transcription factor binding sites are shown with open box (Red). The underlined sequence indicates putative promoter element. Panel B represents nucleotide and deduced amino acid sequences of *Abgst04* and *Abgst05*.

Abgst04 and *Abgst05* (Fig. 4.8) have unique organizational features. These two GSTs appear to have organised as one operon. Both upstream and downstream of this operon *lysR* genes are identified. The identified *lysR* genes have opposite transcription orientation. As shown in figure 4.8, the *Abgst04*/*05* operon appears flanked between oppositely transcribed *lysR* genes. Analysis of the promoter sequence of *Abgst04* revealed interesting features. It contains a typical σ^{70} dependent promoter. Almost overlapping the -10 sequence a consensus Crp binding site is identified. It also contains consensus sequence that serves as binding sites for integration host factor. These consensus motifs and existence of *lysR* gene, which codes for a transcription factor that regulates the expression of number of genes, points towards induction of *Abgst04/05* operon under a defined physiological condition. Considering the organization of two *Abgsts* as one operon, an attempt was made to investigate if the *Abgst04*/05 operon is generated due to an event of gene duplication. Initially, AbGST04 and AbGST05 protein sequences were aligned to know if there is high degree of conservation between these two proteins. As shown in figure 4.14 these two GSTs have minimum sequence similarity, in fact there is less than 22 % sequence similarity among nine GSTs identified in *A. baumannii* DS002. Existence of structurally dissimilar GSTs under the control of a common promoter and terminator speaks functional significance and certainly warrants further investigation.

Abgst06 gene is located in between Acyl-CoA dehydrogenase and Gln/Asp transporter coding sequences (Fig. 4.9). The promoter of *Absgt06* is unique in number of ways, it contain RpoD dependent promoter. Overlapping with -10 hexameric sequence ArgR binding site was predicted. About 7 bp upstream of -35 consensus hexameric sequence, two transcription factor binding sites are identified. One of them is LexA

binding site whereas the other one is PhoB binding site. Downstream of the promoter element, an Lrp binding site was also identified. In *E. coli*, the Lrp otherwise known as leucine responsive regulatory protein regulate transcription of at least 10 genes (Tani et al., 2002). Some of these genes are involved in carbon metabolism and also the genes

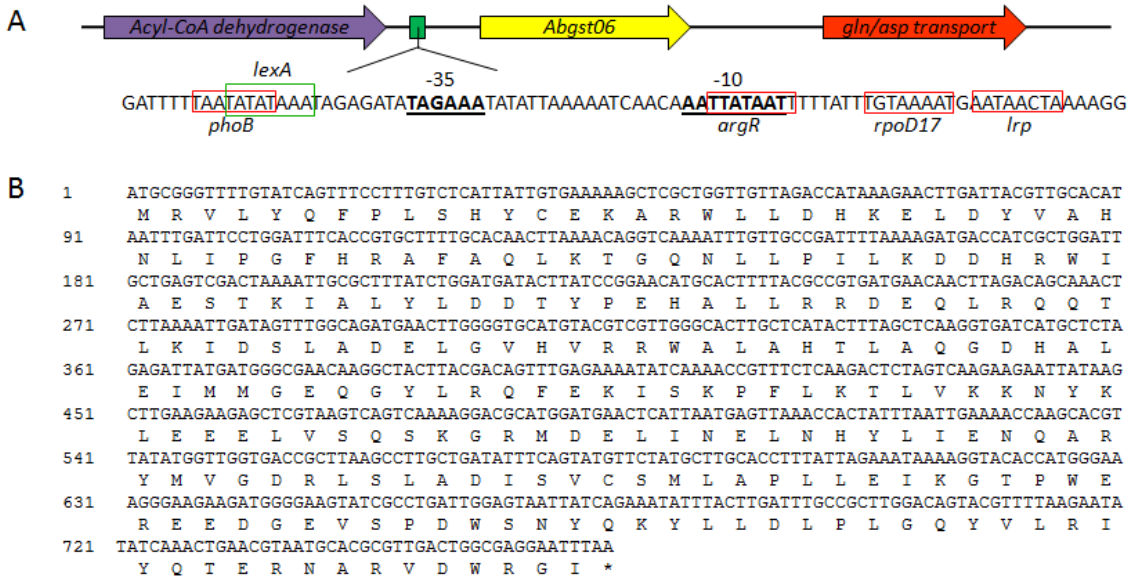

Fig. 4.9. Panel A: Schematic representation of *Abgst06* with its flanking regions. Putative transcription factor binding sites are shown with open box (Red). The underlined sequence indicates putative promoter element. Panel B represents nucleotide and deduced amino acid sequence of *Abgst06.*

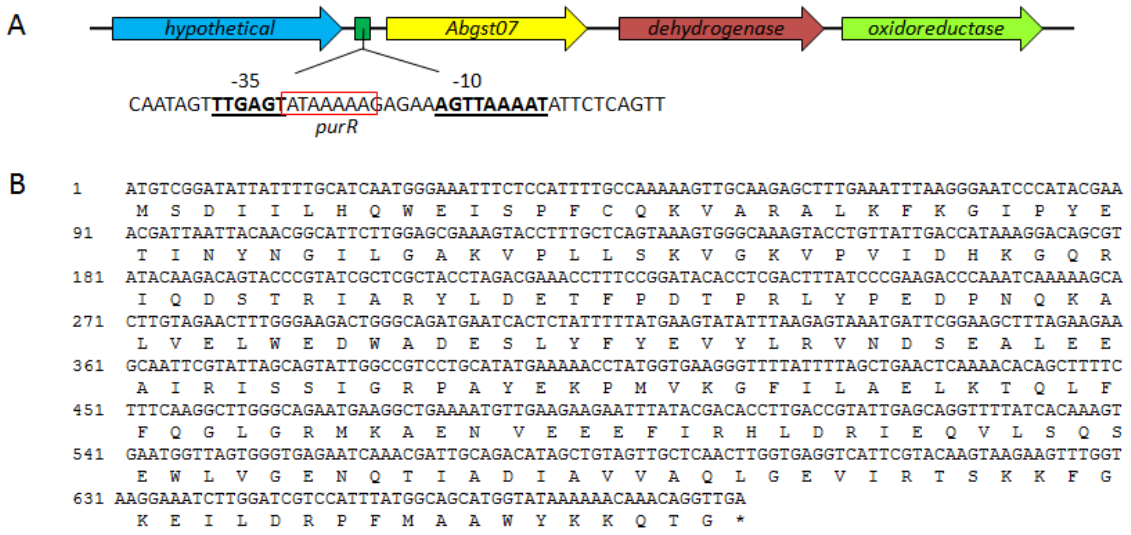

Fig. 4.10. Panel A: Schematic representation of *Abgst07* with its flanking regions. Putative transcription factor binding sites are shown with open box (Red). The underlined sequence indicates putative promoter element. Panel B represents nucleotide and deduced amino acid sequence of *Abgst07.*

that are induced entering into stationary phase (Brinkman et al., 2003; Ernsting et al., 1992; Cho et al., 2008). Interestingly, the Lrp can act both as repressor and activator of its target genes (Platko and Calvo, 1993). Similarly, LexA, PhoB and ArgR are transcription regulators. LexA represses the transcription of several genes involved in cellular response to DNA damage. Its role in inhibition of DNA damage as well as its own synthesis is well documented (Fernandez et al., 2000). PhoB is the transcriptional factor that belongs to two component regulatory system. Its role in expression of Pho regulon is well documented (Makino et al., 1989). PhoB has recently been shown to contribute for expression of virulence factor in pathogenic *E. coli* (Crepin et al., 2011). In view of existence of multiple transcription factor binding sites, expression of *Abgst06* is predicted under multiple stress conditions. Further studies are warranted to elucidate its role in growth and physiology of *A. baumannii* DS002.

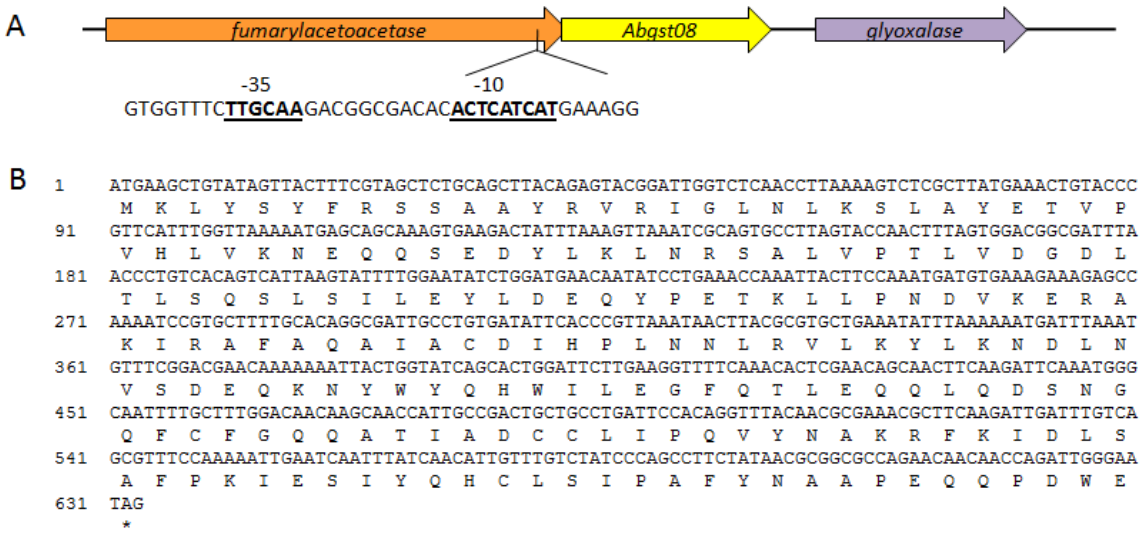

Fig. 4.11. Panel A: Schematic representation of *Abgst08* with its flanking regions. Putative promoter element is underlined. Panel B represents nucleotide and deduced amino acid sequence of *Abgst08.*

The Lrp binding site was also seen immediately downstream of RpoD dependent promoter of *Abgst09* (4.12). Existence of transcription factor binding site that specifically

contribute for amino acid metabolism is indeed an interesting observation. It is not clearly known the linked between *Abgst06* and amino acid metabolism, however existence of Lrp binding site immediately downstream of *Abgst09* and *Abgst06* promoter points towards their role in amino acids metabolism. Likewise in *Abgst07* (Fig. 4.10) the transcription factor PurR binding site was identified. The PurR belongs to GalR/LacI family of transcriptional regulator and it controls several genes involved in purine nucleotide biosynthesis (Fukami-kobayashi et al., 2003). Its involvement in regulation of its own expression is also well documented (He et al., 1990; Choi and Zalkin, 1992). Existence of PurR binding site overlapping the promoter motif predicted upstream of *Abgst07* creates interest on its physiological role and points towards its involvement in purine nucleotide metabolism.

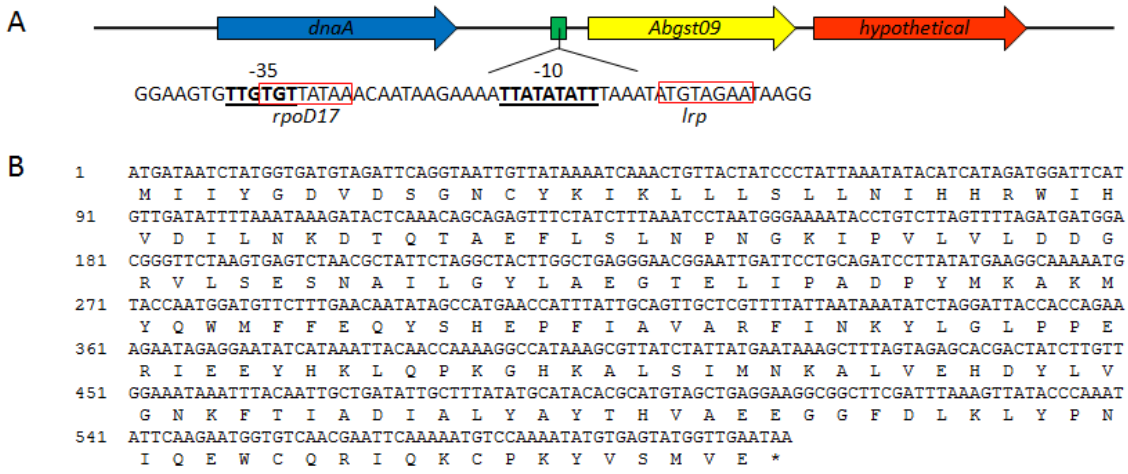

Fig. 4.12. Panel A: Schematic representation of *Abgst09* with its flanking regions. Putative transcription factor binding sites are shown with open box (Red). The underlined sequence indicates putative promoter element. Panel B represents nucleotide and deduced amino acid sequence of *Abgst09.*

4.3 Phylogenetic analysis

Since clones that gave positive signals gave different restriction profile, apparently it has generated lots of curiosity to know the relatedness among various AbGST sequences. Therefore, the predicted amino acid sequences of AbGSTs were aligned using

clustalW (www.ebi.ac.uk/Tools/msa/clustalw2/). Interestingly, apart from few conserved amino acid residues at the C-terminal domain, the sequence identity was found to be less than 22 % among the AbGSTs (Fig. 4.13) indicating their diversity due to primary sequence of AbGSTs found in *A. baumannii* SD002 (Fig. 4.13 & 4.14).

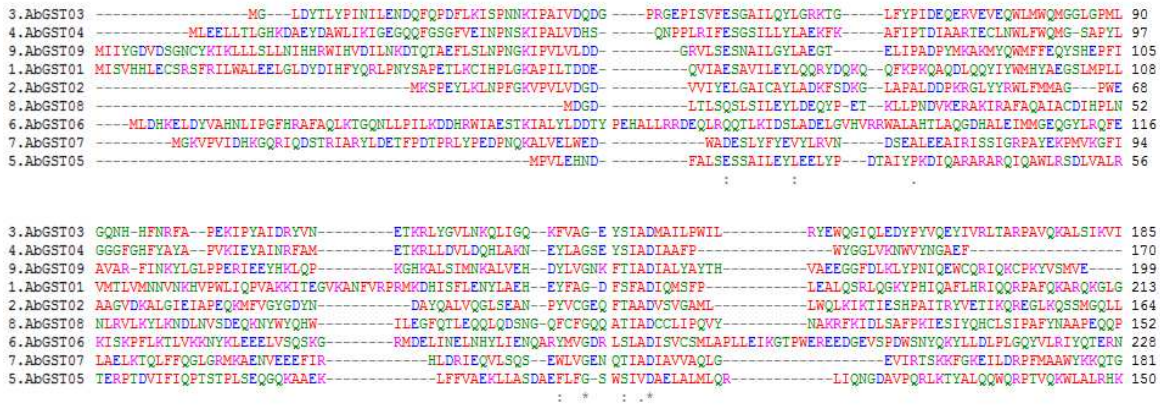

Fig. 4.13. Multiple sequence alignment of AbGSTs.

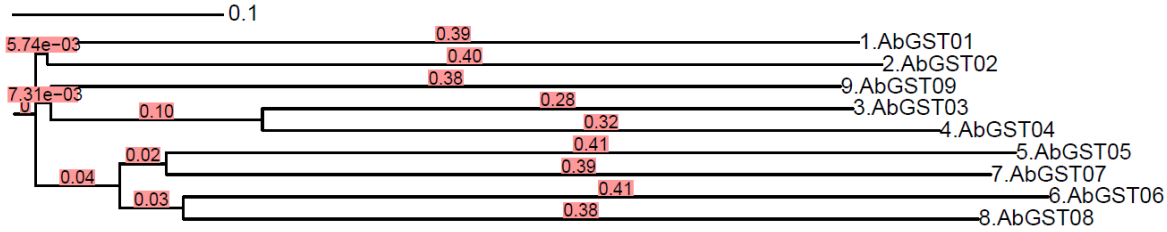

Fig. 4.14. Phylogram of AbGSTs.

Considering the diversity and AbGST sequences, an attempt was also made to know the relationship of AbGSTs with the known GST classes. A pylogenic tree was constructed to evaluate relationship of AbGSTs with known GST classes (Fig. 4.15). The AbGST01 and AbGST06 have shown close similarity with *Agrobacterium tumefaciens* GST which belongs to Tau GST class. Similarly, the AbGST02 and AbGST08 clustered with mammalian Zeta class. The AbGST03 and AbGST04 have aligned well with GST of *Rhodococcus* AD45, which belongs to Pi class. While the AbGST05 showed close relationship with *Sphingomonas paucimobilis* LigB that falls under Beta class of GST. AbGST07 has shown distant relationship to non-mammalian Pi and Chi classes of GSTs. Finally,

AbGST09 has grouped together with GSTs of *Thermosynechococcus elogatus* BP-1 and *Synechococcus elongates* PCC 6301, belonging to Chi class GSTs. Presence of such diversified GSTs in *A. baumannii* DS002 generates another argument on their acquisition. However, the generated phylogenetic tree further clarifies existence of minimum relationship among the AbGSTs of *A. baumannii* DS002 and shows presence of GSTs belonging to various groups in *A. baumannii* SD002.

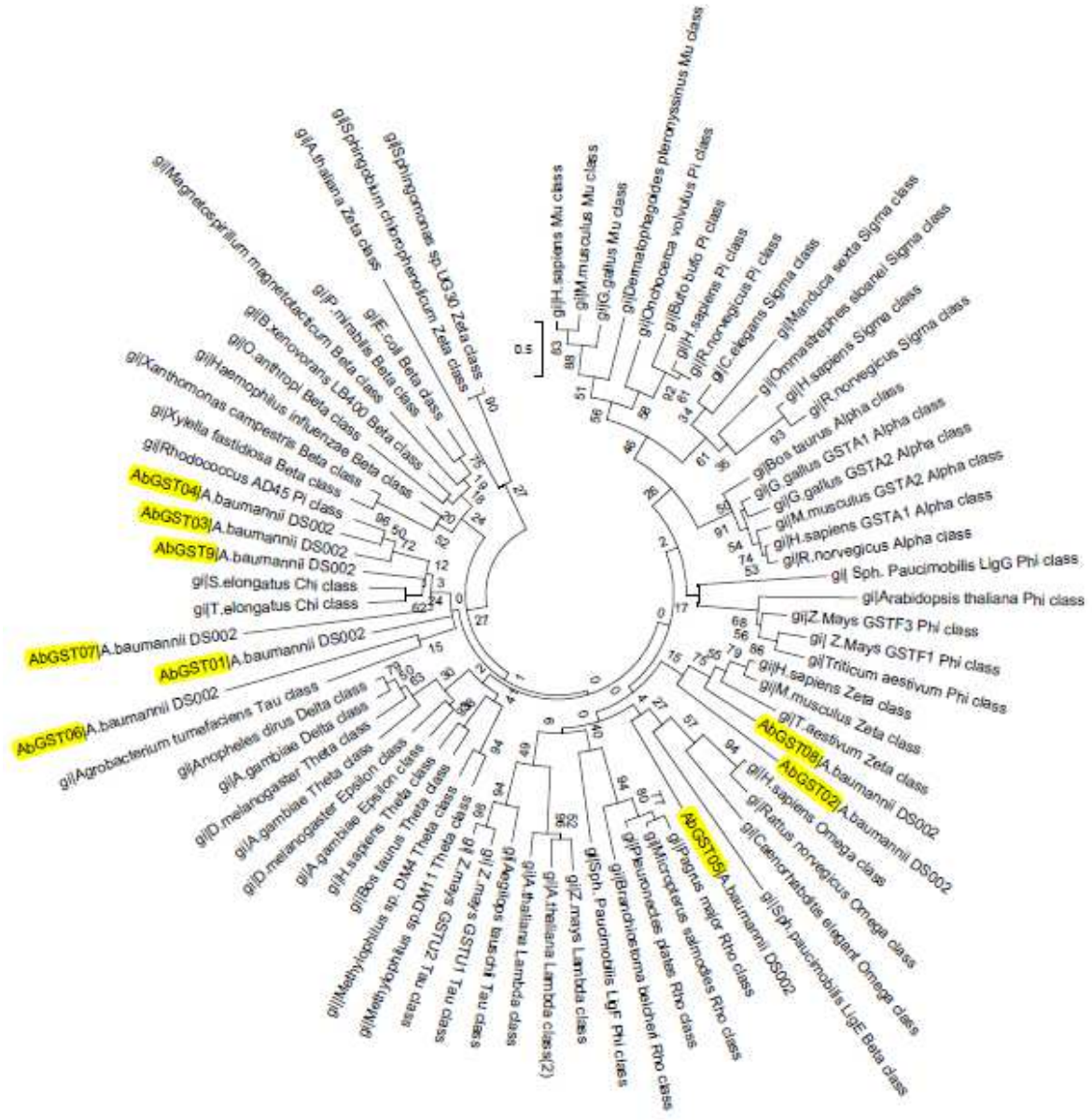

Fig. 4.15. Phylogenetic tree indicating relationship of nine AbGSTs with representative bacterial and eukaryotic GSTs. AbGSTs are indicated with yellow background.

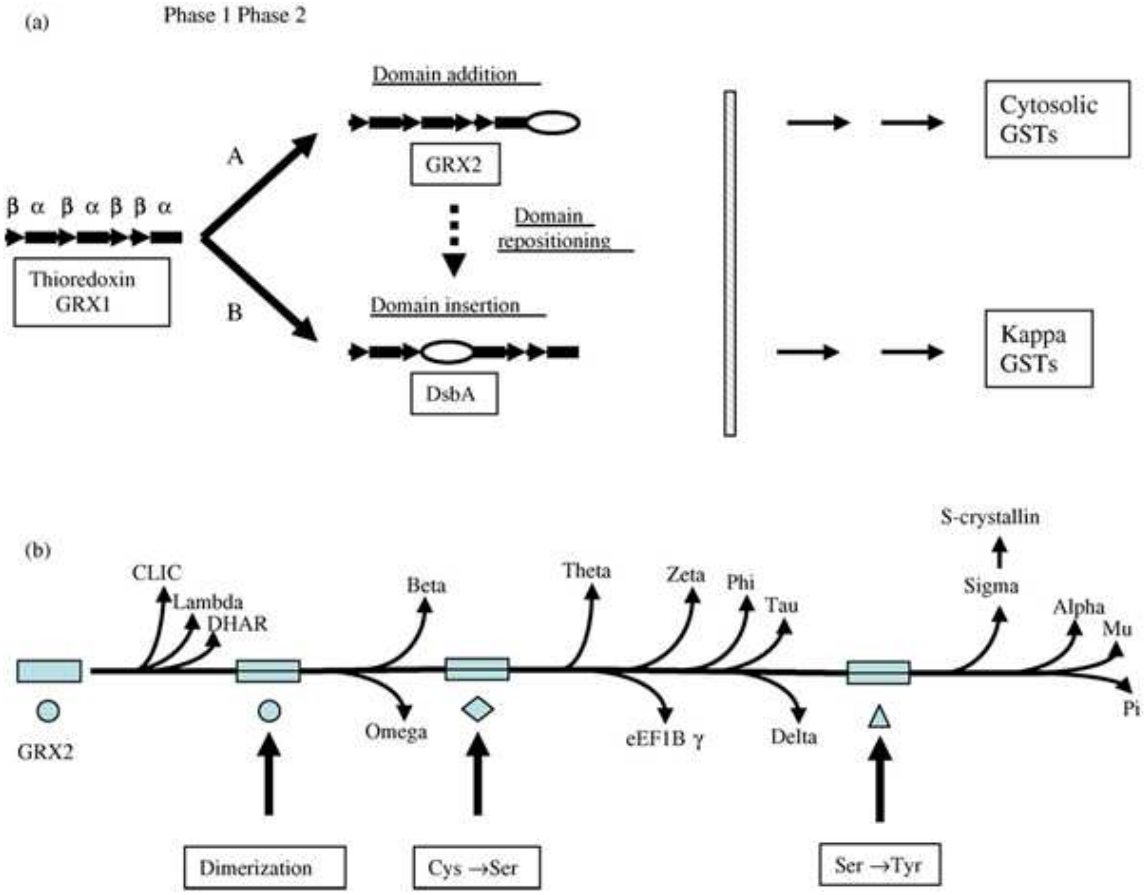

Fig. 4.16. Model of TRX proteins evolution (Frova 2006); (a) represents the most ancient steps. (b) represents phase 2 differentiation of cytosolic GSTs. Thick arrows in (b) indicate the likely sequence of critical evolutionary steps, dimerization and changes in active site residues. Geometric symbols; Circle = Cys, diamond = Ser and triangle = Tyr.

According to the evolutionary model proposed by Frova, thioredoxin is the ancestor form of GSTs (Frova, 2006). As shown in figure 4.16a, during the first phase of evolution, two all-helical domain got inserted in thioredoxin. One of them, the bacterial glutaredoxin 2 (GRX2), got inserted at the C-terminal of thioredoxin, resulting in generation of cytosolic GSTs (cGST). The second domain belongs to bacterial disulfide-bond-forming oxidoreductase A (DsbA). When DsbA domain is inserted in between thioredoxin fold, it has generated Kappa GSTs. In the second phase of evolution, cGSTsfurther diversified into different classes (Fig. 4.16). The Beta class enzymes, which include most of bacterial GSTs, have evolved after the dimerization of GRX2. The other

GST classes were believed to be generated due to shift from cysteine to serine chemistry (Fig. 4.16b).

The existence of multiple GSTs in *A. baumannii* DS002 could be a good example for gene duplication events, resulting in generation of groups of enzymes with a myriad of functions. As discussed before, GST family appears to have expanded through multiple duplications (Frova 2006; Hayes et al, 2005). The fact that GST enzymes perform multiple roles, both enzymatic and non-enzymatic, and facilitates the process of neofunctionalization through optimization of a secondary function (O'Brien and Herschlag, 1999). Since *A. baumannii* is an opportunistic pathogen, they are exposed to all sorts of oxidative stress elements inside the host. The need for protection against the products of oxidative damage must have undoubtedly driven the expansion of GST family via positive selection (da Fonseca et al, 2010). In *A. baumannii* strain isolated from organophosphate insecticides polluted soils. GSTs play a key role in metabolism of xenobiotics, including OP insecticides (Fukami, 1980). Existence of OP insecticide residues might have driven the expansion of the family through substrate assisted gain of function (Afriat, 2006).

4.4 Regulation of *Abgst* expression

Existence of multiple *gst* genes with a variety of transcription factor binding sites naturally caused interest to know appropriate physiological conditions for their expression. Therefore, to gain insights into response of *Abgsts* for various stress inducers, the transcript level of all the *Abgsts* were quantified by qPCR in presence and absence of them. Initially the cells were grown in presence of organic and inorganic peroxides such as cumene hydroperoxide (CHP, 100 µM), t-butyl hydroperoxide (TBHP, 100 µM) and hydrogen peroxide (H_2O_2, 100 µM) to induced oxidative stress. After inducing oxidative

stress for two hours, total RNA was extracted to perform qPCR. The qPCR data is presented in figure 4.17. If qPCR is taken into consideration, all *Abgsts* got induced in presence of organic hydroperoxides except *Abgst04*, *Abgst05* and *Abgst08*. Interestingly, none of them got induced in presence inorganic hydroperoxide H_2O_2. However, *Abgst01* expression was found moderately increased in presence of methyl parathion and CDNB. Since the *A. baumannii* DS002 is an environmental isolate and was isolated from OP insecticides polluted environment, further studies were conducted to understand its regulation.

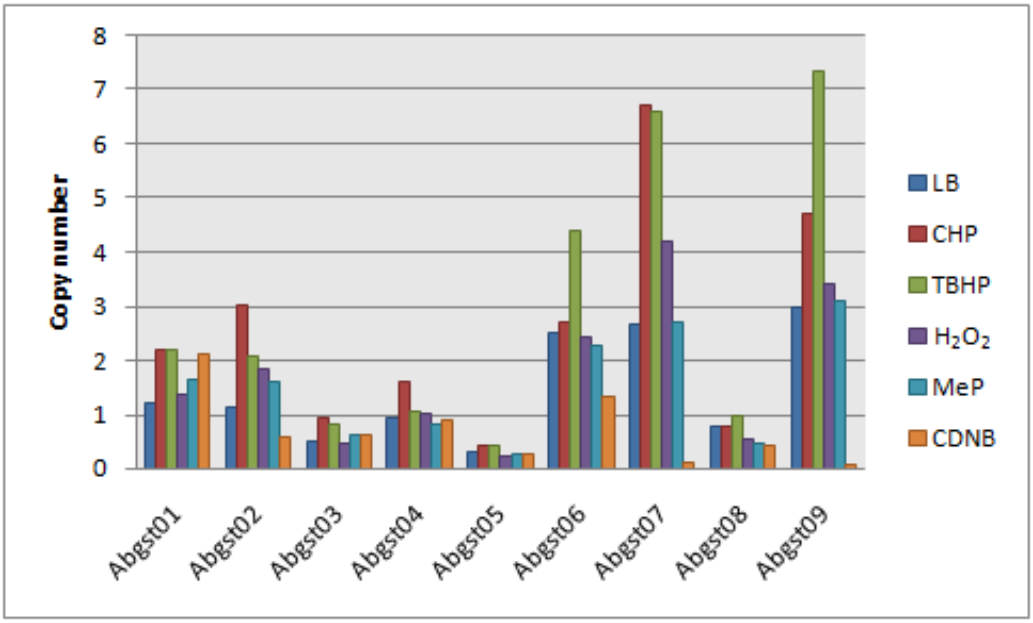

Fig. 4.17. Real time PCR analysis of *Abgsts* induced with various oxidative stress inducers.

4.5.1 Sequence analysis of *Abgst01* gene

The *Abgst01* was cloned with considerable length of upstream region from fosmid clone FSTS0438. A 2.7 Kb fragment have *Abgst01* was used for generating sequence. The sequence of the 2698 bp DNA fragment having *Abgst01* is shown in Figure 4.18. Three open reading frames were identified in the sequence and were designated as *orf316*, *orf115* and *orf222*.

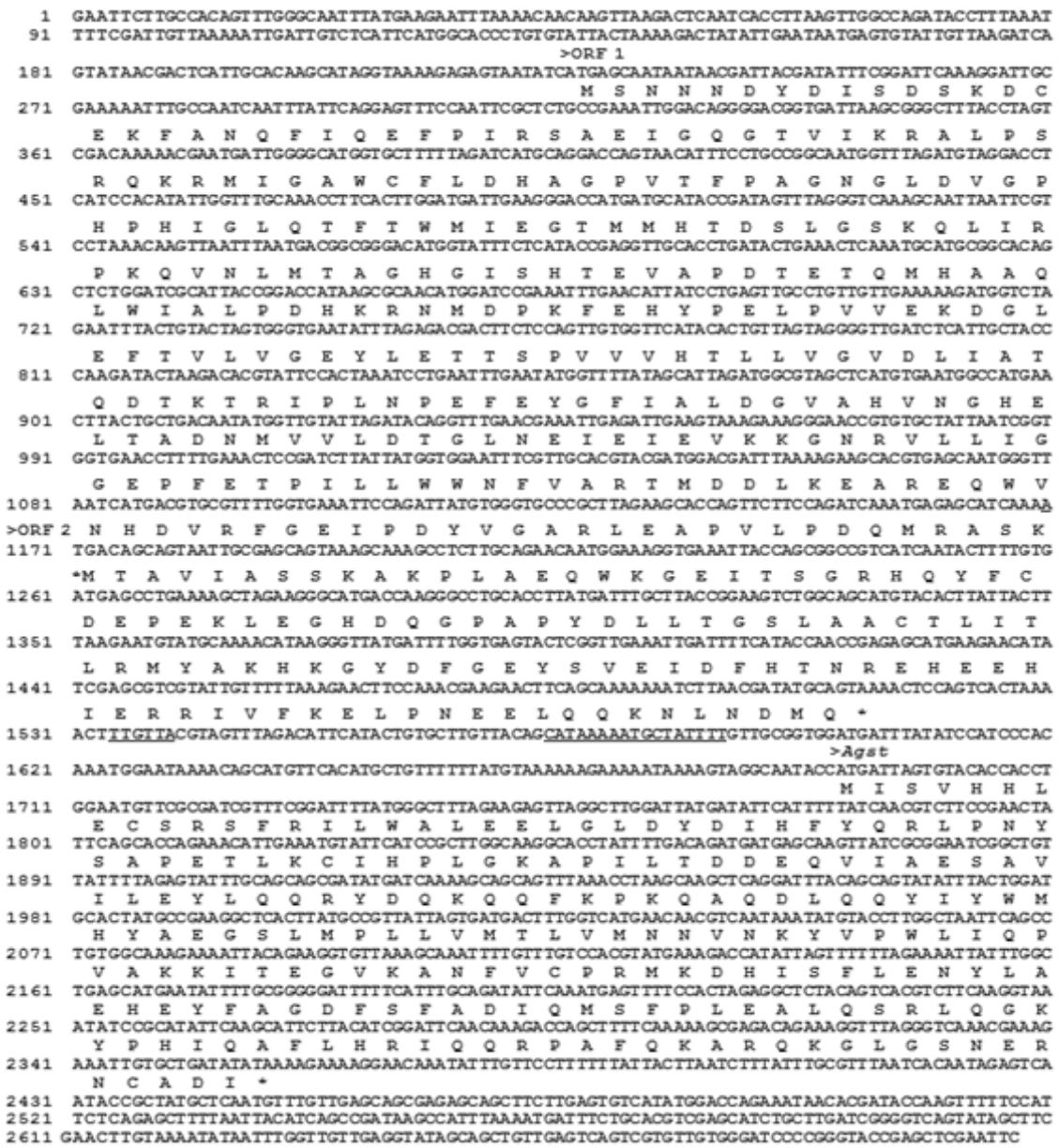

Fig. 4.18. Nucleotide sequence of 2.7 Kb *sal*I fragment containing *Abgst* gene. The amino acid sequence of predicted ORFs are shown with bold case.

The *orf316* has shown 99% identity with Pirin-related protein of *Acinetobacter baumannii* ATCC 17978. Pirins are highly conserved among mammals, plants, fungi, and prokaryotes and have been classified as a subfamily of the cupin superfamily on the basis of both sequence and structural similarity (Wendler et. al., 1997, Dunwell, J. M. 1998). The cupin superfamily proteins are among the most functionally diverse proteins described to date. It was named on the basis of the conserved β-barrel fold ('*cupa*' is the

Latin term for a small barrel), and comprises both enzymatic and non-enzymatic members, which have either one or two cupin domains (Jim M. Dunwell et. al., 2001).

The *orf115*, which starts at position 1,170, showed 98% identity to predicted redox protein that regulates disulfide bond formation in *Acinetobacter baumannii* ACICU. It also shows 58% identity with OsmC-like protein of *Acinetobacter* sp. ATCC 27244. OsmC is a stress -induced protein found in *Escherichia coli* and is induced in response to osmotic stress.

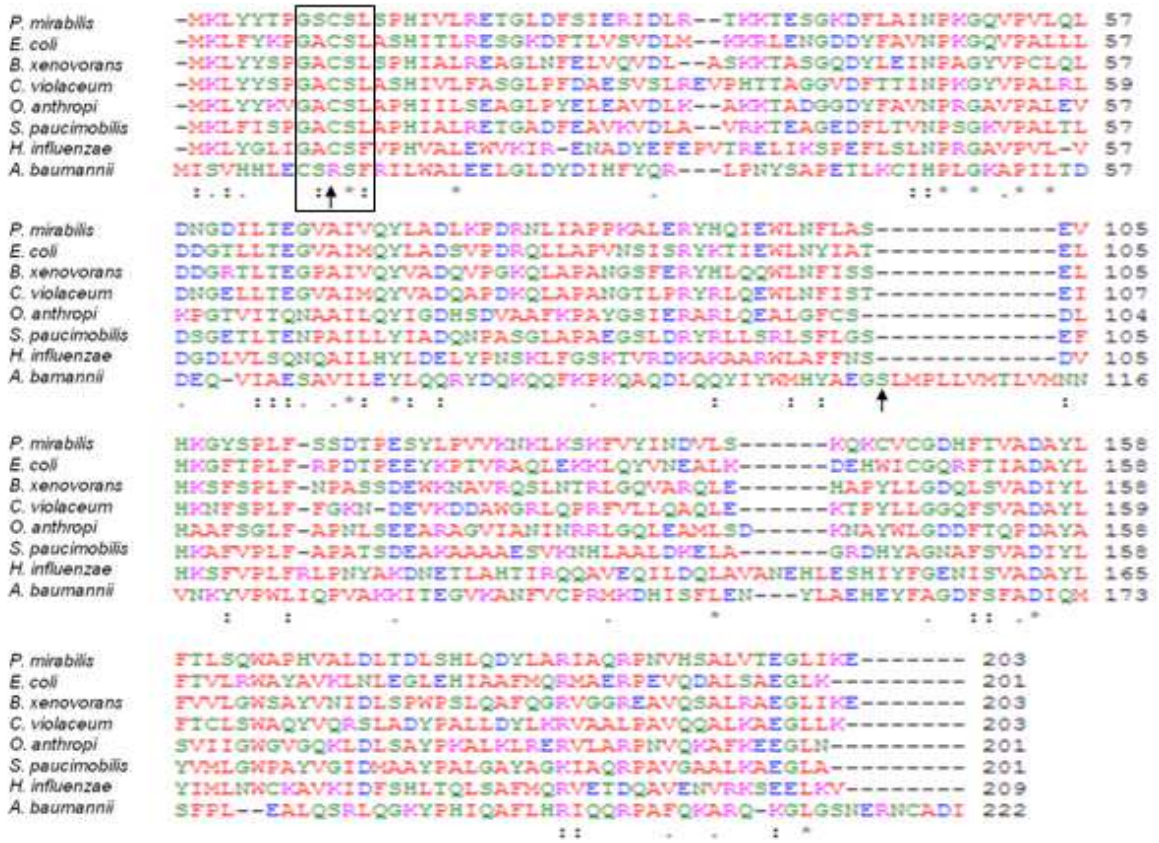

Fig. 4.19. Alignment of *Acinetobacter baumannii* DS002 with other bacterial GSTs. Variations found in AbGST01 at Cys10 and His 106 is shown with arrow marks. The conserved catalytic domain is shown in open box.

The predicted translation product of *orf222* showed 54% identity to the GST of *Yersinia mollaretii* and 66% identity (30% sequence coverage) to the GST identified in *Salmonella enterica*. When the deduced amino acid sequence of AbGST01 was aligned with other bacterial GSTs, the amino acid residues that contribute to catalytic domain

were found to be highly conserved (Fig. 4.19). However, there are significant differences with respect to the amino acid residues that contribute for catalytic activity. In GSTs of *E. coli* and *B. xenovorans*, Cys 10 is involved in hydrogen bonding with thiol group of GSH and is found closely associated with histidine residue located at position 106. These two residues are almost conserved in GST sequences known to date (Motohiko et. al., 1998, Elitza et. al., 2006). Interestingly, these two residues are not found in AbGST01. In place of Cys 10, Arg is found and His 106 is replaced with Serine (Fig. 4.19). No change is seen in the position of other conserved residues, such as Ser 11, Gly 50 and Glu 58. Such change in the active site residues acquires significance, might contribute for a novel catalytic properties for AbGST01.

The sequence found 500 bp upstream of *Abgst01* start codon was used to predict a putative transcription factor binding sites using Virtual Footprint software (Münch et. al., 2003). The predictions have shown 40 bp sequence motif having similarity to the OxyR binding region of *E.coli* (Zheng et. al*.,* 2001). The transcription factor OxyR is found in many prokaryotic organisms (Kazuyuki Tao et. al*.,* 1991). This LysR-type regulator activates the expression of numerous genes in response to oxidative stress, in particular upon exposure to hydrogen peroxide, and has served as a paradigm for understanding cellular sensing of oxidative stress (Aslund et. al., 1999). Hydrogen peroxide directly activates OxyR through the formation of an intramolecular disulfide bond. Oxidized OxyR then activates transcription of antioxidant genes, including *katG* (encoding hydroperoxidase I), *ahpCF* (encoding an alkyl hydroperoxide reductase), *dps* (encoding a nonspecific DNA binding protein), *gorA* (encoding glutathione reductase), *grxA* (encoding glutaredoxin I), and *oxyS* (encoding a small regulatory RNA) (Storz et. al., 2000).

Identification of putative OxyR binding site indicates its involvement in AbGST01 induction under oxidative stress.

4.6 Primer extension analysis

In order to have firm prediction on promoter and transcription factor binding sites, primer extension was performed. This technique enables the determination of precise start point of transcription. Initially, a primer was designed immediately downstream of the start codon of *Abgst01* and was labelled with fluorescent nucleotide. The total RNA isolated from cumene hydroperoxide induced culture of *A. baumannii* DS002 was used to perform primer extension (Fekete et al, 2003). The size of the extended product was then determined by running in sequencing gel along with standard. The 165 nt long primer extended product when aligned correspond to A residue located 121 bp upstream to the translational start site ATG (Fig. 4.20). The OxyR binding site was found at 124 bp upstream of σ^{70} dependent promoter.

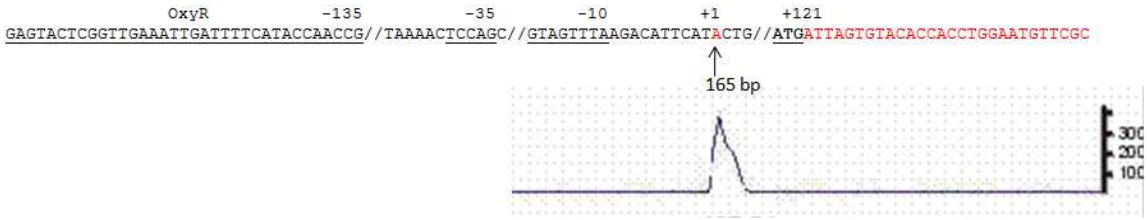

Fig. 4.20. Promoter mapping of *Abgst01*. The peak corresponds to primer extended product.

4.7 Promoter fusion analysis

In order to gain experimental evidence on involvement of OxyR in regulation of *Abgst01,* further studies were conducted. Initially, *Abgst-lacZ* fusions were constructed using a broad host range promoter probe vector pMP220 (Spaink et al., 1987). The strategy used for generation of *gst-lacZ* fusions was shown in Fig. 4.21. The upstream region of *Abgst01* gene was amplified using a constant reverse primer

appended with *pst*I site (DS00114) and two forward primers. One of them (DS00115) was designed just upstream of putative OxyR binding site of *Abgst01* gene and the second one (DS00116) was designed downstream of OxyR binding site (4.21). Both the primers were appended with *Eco*RI. The PCR products generate *Abgst01* upstream region with and without OxyR binding site. These amplicons were then independently cloned in pMMP220 to generate pST1 and pST2 respectively (Fig. 4.22).

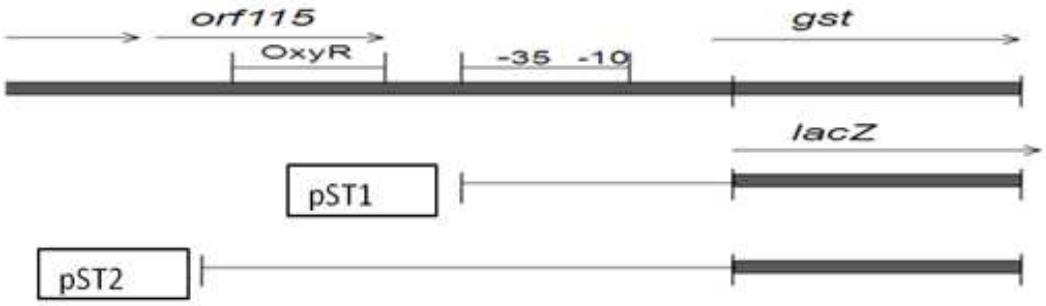

Fig. 4.21. Schematic diagram showing the construction of *Abgst-lacZ* fusions.

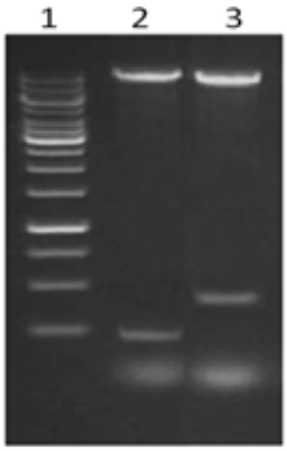

Fig. 4.22. Construction of *gst-lacZ* fusions: Lane 1 represents Kilobase ladder, lane 2 and 3 represent pST1 and pST2 plasmids upon digestion with *Eco*RI and *Pst*I.

In order to gain insights about the involvement of OxyR in regulation of *Abgst01* both pST1 and pST2 were mobilized into *A. baumannii* DS002 by performing typical conjugation experiment as described in materials and methods (Simon et al., 1983). Initially, *A. baumannii* DS002 carrying *Abgst-lacZ* fusions were independently grown to

assess promoter activity by monitoring β-galactosidase activity. Interestingly, deletion of OxyR binding site reduced promoter activity by three fold (Fig. 4.23). However, no promoter activity was seen in cell containing only the vector pMP220 (Fig. 4.23) clearly indicating existence of functional promoter upstream of *Abgst01.* Reduction of β-galactosidase activity due to removal of OxyR binding site indicates possible positive role of OxyR protein in expression of *Abgst01* in *A. baumannii* DS002.

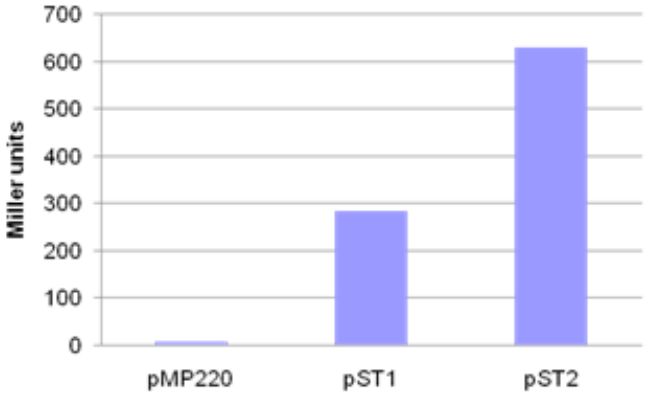

Fig. 4.23. β-galactosidase of *gst-lacZ* fusions in *A. baumannii* DS002.

The *A. baumannii* DS002 (pST2) were further used to know if oxidative stress inducers like cumene hydroperoxide (100 µM), *tert*-butyl hydroperoxide (100 µM), hydrogen peroxide (100 µM), paraquot (100 µM), methylene blue (10 µM), methyl parathion (100 µM), *p*-nitrophenol (50 µM) and CDNB (100 µM) have any influence on *Abgst01* promoter activity. As indicated in figure 4.24 only organic hydroperoxides have shown positive influence on *Abgst01* expression. The inorganic hydroperoxide like hydrogen peroxide and paraquot, methylene blue showed no influence on its expression. Only moderate influence was seen in presence of CDNB and methyl parathion. This is in clear agreement with qPCR data where enhancement of *Abgst01* was only seen in presence of organic hydroperoxides. Since CHP and tBHP are shown to be better inducers of *Abgst01* genes expression, the *A. baumannii* DS002 (pST1) and *A. baumannii* DS002

(pST2) were grown in presence of these two oxidative stress inducers. If CHP and tBHP are inducing *Abgst01* through OxyR *A. baumannii* DS002 (pST1) should not give more β-galactosidase activity as it does not contain putative OxyR binding motif. As, expected, these two organic hydroperoxides showed no influence on expression of *Abgst01* in absence of putative OxyR binding site. This is rather preliminary evidence to show influence of OxyR in regulation of *Abgst01* expression.

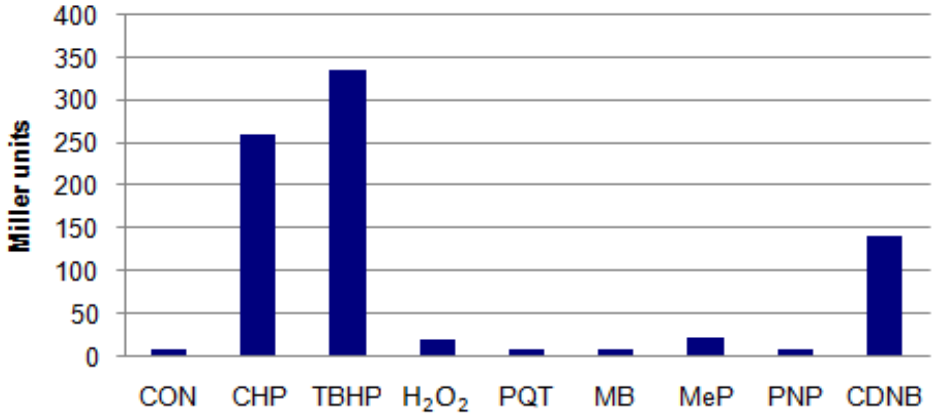

Fig. 4.24. β-galactosidase activity in *A. baumannii* (pST2) in presence of various oxidative stress inducers. CON indicates β-galactosidase activity in *A. baumannii* DS002 (untreated). CHP (cumene hydroperoxide), TBHP (*tert*-butyl hydroperoxide), H_2O_2 (hydrogen peroxide), PQT (paraquat), MB (methylene blue), MeP (methyl parathion), PNP (*p*-nitrophenol), CDNB (1-chloro 2, 4-dinitrobenzene) indicate β-galactosidase activity in presence of various stress inducers.

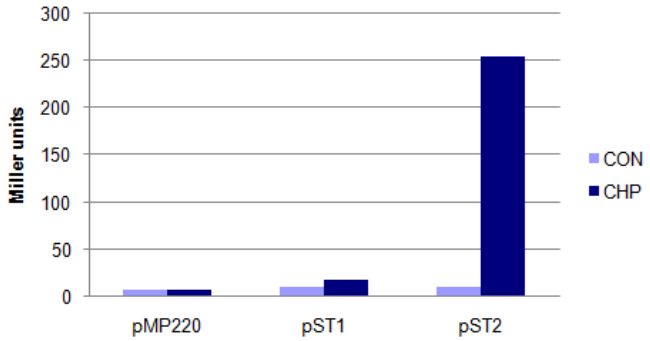

Fig.4.25. β-galactosidase activity of *gst-lacZ* fusions: Effect of cumene hydroperoxide induction. CON represent uninduced, whereas CHP represent cumene hydroperoxide induction.

4.6 Generation of *oxyR* knockout

In order to gain further insights into OxyR involvement in regulation of *Abgst01* gene expression, an *oxyR* knockout of *A. baumannii* DS002 was generated. Initially, the

insertionally inactivated *oxyR* was generated by inserting a kanamycin resistance gene in the coding region of *oxyR.* The *oxyR* was amplified using a primer set (DS00117)/(DS00118) as forward and reverse primer. The *oxyR* gene cloned in pTZ vector has a unique *Xho*I site in the coding region. The kanamycin gene taken as *Xho*I fragment was then cloned in the *Xho*I site of *oxyR* gene. The generated insertionally inactivated *oxyR::kan* was then taken as *Eco*RI fragment and cloned in suicidal vector pSUP202 (Simon et al., 1983). The strategy used for the generation of *oxyR* knockout in *A. baumannii* DS002 is shown in figure 4.26.

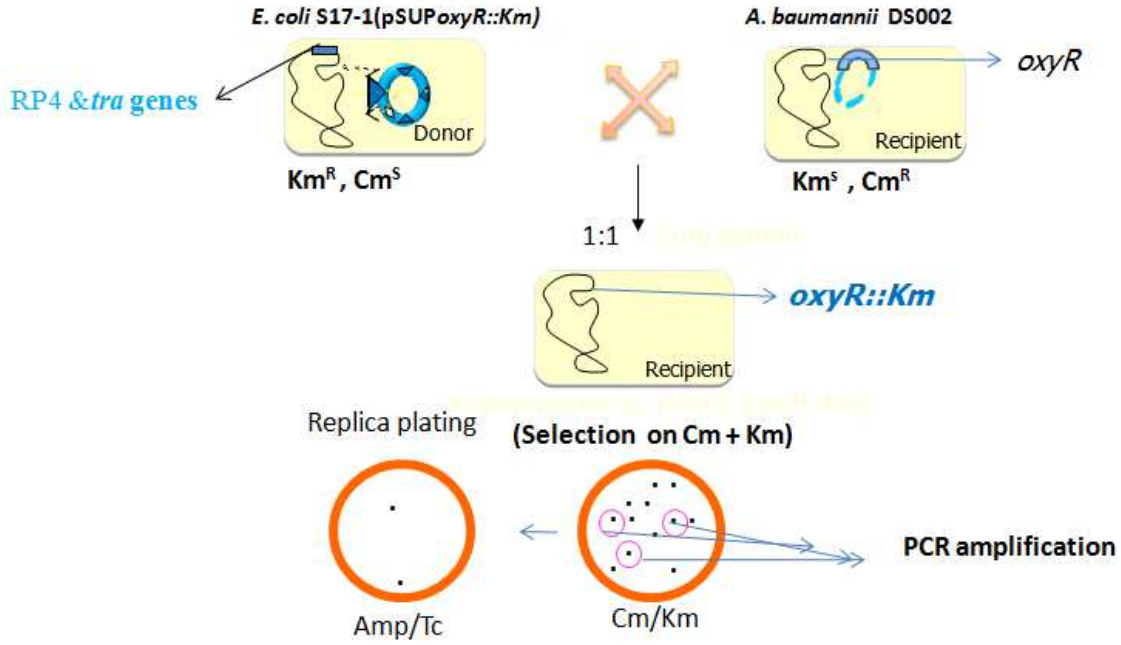

Fig.4.26. Strategy for generation of *oxyR* gene knockout in *A. baumannii* DS002.

The resulting plasmid was designated as pSUP*oxyR::kan* and was then mobilized into *A. baumannii* DS002 as described in materials and methods section (Fig. 4.26). The ex-conjugants that showed resistance to chloroamphinicol and kanamycin were selected and replica plated on LB agar plates containing tetracycline and ampicillin. Since tetracycline and ampicillin resistance gene are found on suicidal vector, the colonies showing sensitivity to these two antibiotics indicate elimination of vector DNA from ex-

conjugant. At the same time ex-conjugants showing resistance to kanamycin are indicative of *oxyR* knockout due to replacement of *oxyR* with *oxyR::kan* through homologous recombination. Therefore, DNA isolated from such ex-conjugant was used as template for amplifying *oxyR* using *oxyR* specific primers. The wildtype *oxyR* gene is 0.9 Kb, integration of Km cassette in *oxyR* gene elevates its sized by 2.6 Kb. As shown in figure 4.27, a clean amplicon equivalent to the size of 2.6 Kb was seen as opposed to 0.9 Kb *oxyR* amplicon in the wildtype strain DS002. This data confirms replacement of *oxyR* with *oxyR::kan* in *A. baumannii* DS002 and were treated as *oxyR* knockout.

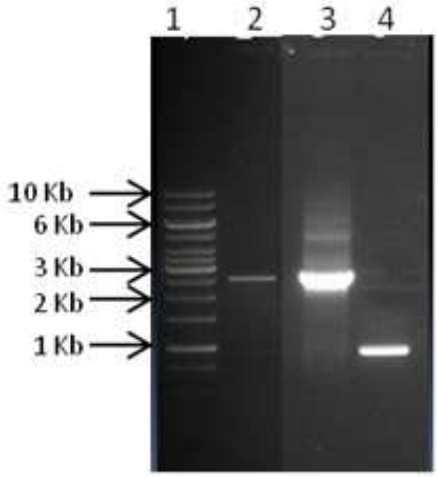

Fig. 4.27. Generation of *oxyR* knockout: Lane 1 represents 1 Kb DNA ladder, Lanes 2 represents PCR amplicon from ex-conjugant. The elevated size of *oxyR* specific amplicon indicates insertion of Kannamycin cassette in *oxyR* gene. Lanes 3 and 4 represent amplicon of *oxyR::Kan* and *oxyR* from suicidal plasmid pSUP*oxyR::Kan* and wildtype strain *A. baumannii* DS002 respectively.

4.7 Elucidating OxyR role in regulation of *Abgst01*

After constructing *oxyR* knockout, *Abgst-lacZ* fusions pST1 and pST2 were mobilized into *oxyR* negative mutant strains of *A. baumannii* DS002. These strains were then used for measuring β-galactosidase activity after inducing with cumene hydroperoxide. As expected, in the *oxyR* null mutant of *A. baumannii* DS002 (pST2) no induction of promoter activity was seen (Fig. 4.28). The activity in these cultures was similar to the *oxyR* mutant cultures having *Abgst-lacZ* fusion pST1, where OxyR binding site is removed.

This is rather direct evidence to show that OxyR is involved in expression of *Abgst01* in *A. baumannii* DS002.

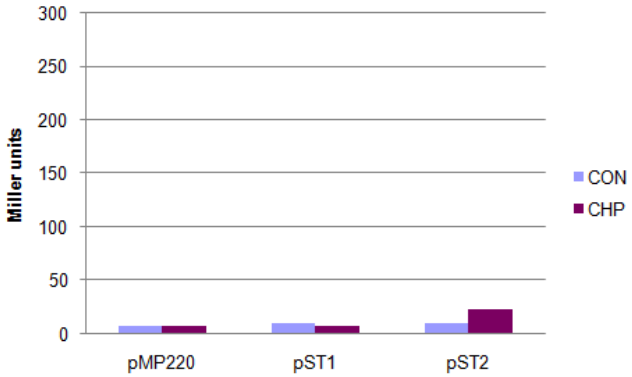

Fig. 4.28. *β*-galactosidase activity of *gst::lacZ* fusions in *oxyR* mutant strain of *A. baumannii* DS002. CON represents uninduced and CHP represents cumene hydroperoxide induced cultures.

One of the major challenges for living organisms is the oxidative stress derived from reactive oxygen species (ROS) produced via normal aerobic metabolism or by exposure to redox-cycling drugs, ionizing radiation or by stimulated human phagocytic cells during infections of multiple phyla (Wei et al., 2012). ROS in relatively low concentrations are capable of causing damage to virtually all biomolecules including DNA, RNA, lipid and protein. As a primary defense against ROS, both prokaryotes and eukaryotes have developed antioxidant defense systems to protect cells from the aforementioned lesions (Zheng et al., 1998). In bacteria, antioxidant defense systems have been extensively studied in *Escherichia coli* (Pomposiello and Demple, 2001; Storz and Imlay, 1999; Imlay, 2008). Key regulators modulating the oxidative stress response in bacteria are SoxR and OxyR, both of which are activated at the posttranslational level. O_2^- activates SoxR through oxidation of its [2Fe-2S] cluster (Ding and Demple, 1998; Gaudu and Weiss, 1996), and oxidized SoxR induces the expression of the second transcription factor SoxS, which directly activates transcription of several genes, including *sodA* in *Escherichia coli* (Jair et al., 1996; Li and Demple, 1994; Wu and Weiss, 1991). H_2O_2 induces at least 30

genes in *E. coli*, and the response of a subset of these genes depends on OxyR, a 34-kDa LysR-type transcriptional activator (Christman et al., 1989; Storz et al., 1990). In the presence of H_2O_2, OxyR forms an intramolecular disulfide bond which can be deactivated by enzymatic reduction upon relief of oxidative stress (Aslund et al., 1999; Zheng et al., 1998). Both the oxidized and the reduced forms of the *E. coli* OxyR protein have been shown to possess DNA binding activity (Tartaglia et al, 1992). OxyR homologs have also been identified in various bacteria, including other Proteobacteria such as *Neisseria gonorrhoeae*, *Legionella pneumophila*, *Xanthomonas campestris* and *Haemophilus influenza*. These proteins showed properties significantly different from those *of E. coli* OxyR with respect to the transcriptional regulation pattern, hydroperoxide-sensing behavior and conserved cysteine composition (Wei et al., 2012).

As an opportunistic pathogen, *Acinetobacter baumannii* causes severe acute nosocomial infections in ill patients in intensive care units (ICUs) (Towner, 2009). Intrinsic antibiotic resistance and versatile ecological adaptability, contribute to making the eradication of this bacterium from the infection sites highly problematic (Woodford et al., 2011). During both the infectious process in human disease as well as its free-living lifestyle, endogenous and exogenous ROS post a challenge to the organism. So far, little is known about the defence system against oxidative stress in *A. baumannii* and no study has been conducted on its regulator protein OxyR. In *P. aeruginosa* OxyR-regulated genes are upregulated in the presence of organic hydroperoxides (Ochsner et al., 2000). In their study, Ochsner at al., have characterized three OxyR-regulated genes such as *katB* (encoding catalase), *ahpB* and *ahpB* (encoding alkyl hydroperoxide reductase) involved in the defences against oxidative stress (Ochsner et al., 2000). They have shown that expression of *katB-lacZ* , *ahpB-lacZ* and *ahpC-lacZ* were induced up to 250-, 90- and 3-

fold higher respectively after exposure to oxidative stress-generating compounds (H_2O_2, paraquat, or organic peroxides) in *P. aeruginosa* PAO1. Further, expression of these genes was not observed in an *oxyR* mutant of *P. aeruginosa* PAO1. These findings support the present study which has clearly indicated activation of *Abgst01* by organic hydroperoxide through the involvement of OxyR in *A. baumannii* DS002.

4.8 Characterization of AbGST01

4.8.1 Cloning and expression of *Abgst01*

In order to characterize AbGST01, recombinant AbGST01 was generated by using *E. coli* expression system. Initially, *Abgst01* gene was amplified from the fosmid clone (FSTS0438), using a forward primer DS00112 having *Nde*I restriction site designed overlapping to the start codon ATG and a reverse primer DS00113 having *Xho*I restriction site. The PCR product was digested with *Nde*I and *Xho*I restriction enzymes and directionally cloned into pET-23b digested with same enzymes (Fig. 4.29A). The

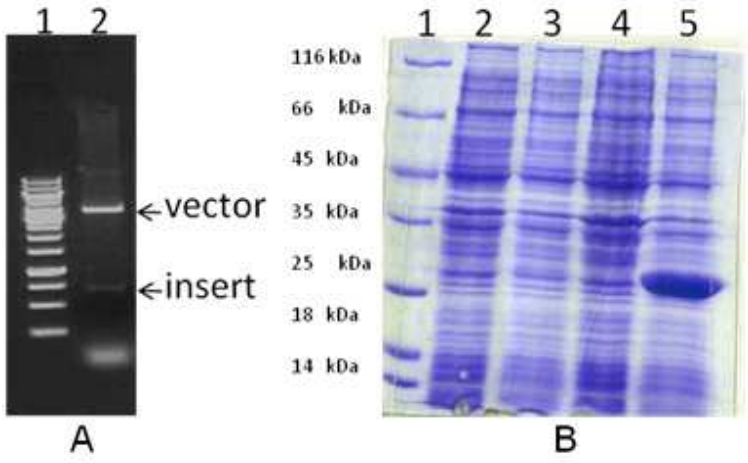

Fig. 4.29. Panel A: Cloning of *Abgst01* in pET23b; Lane 1 represents Kilobase ladder and lane 2 represents pPAGT plasmid upon digestion with *Nde*I and *Xho*I enzymes. The digested vector and insert are shown with arrow marks. Panel B: Expression of recombinant AbGST01; Lane 1 represents protein molecular weight marker, lanes 2 and 3 represent uninduced and induced cell lysate of *E.coli* BL21 respectively, and lane 4 and 5 represent uninduced and induced cell lysate of *E.coli* BL21 (pPAGT) respectively.

recombinant plasmid obtained was designated as pPAGT and its expression was achieved after transforming in *E.coli* BL21 (DE3) cells. The SDS-PAGE analysis of proteins from both induced and uninduced cultures revealed expression of 26 kDa AGST in SDS-PAGE (Fig. 4.29B). No such band is seen in uninduced cultures.

4.8.2 Purification of recombinant AbGST01

Initial attempt to purify the recombinant AbGST01 using glutathione matrix failed, as the protein did not bind to the column, which could be due to the replacement of catalytic Cys 10 by the Arg residue in AbGST01 (Fig. 4.13). Therefore, conventional protein purification techniques such as ammonium sulfate precipitation, anion-exchange chromatography, hydrophobic interaction chromatography and gel exclusion chromatography were applied to achieve recombinant AbGST01 purification. The expression vector containing the AbGST01 encoding DNA was used to transform *E.coli* BL21 (DE3) cells, which over-expressed AbGST01 after IPTG induction as mentioned above. An over-expressed band on SDS-PAGE corresponding to a protein of approximately 26 kDa was observed in the crude cell lysate which is in agreement with the expected size of the recombinant protein (Fig. 4.30, lane 2).

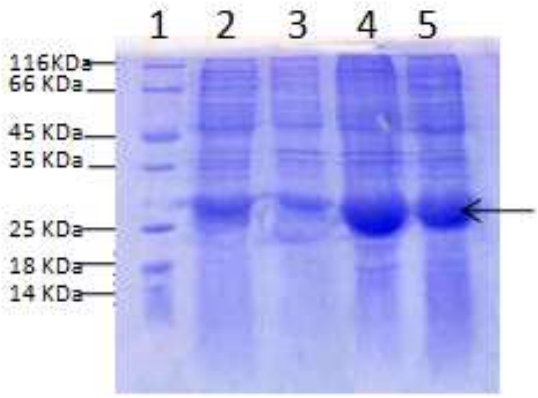

Fig. 4.30. Ammonium sulfate fractionation: Lane 1 represents protein molecular weight marker, Lane 2 represents crude cell lysate, Lanes 3-5 indicate proteins precipitated at 20 %, 40 % and 60 % ammonium sulfate respectively. Presence of AbGST01 in 40 and 60 % fractions are shown with arrow.

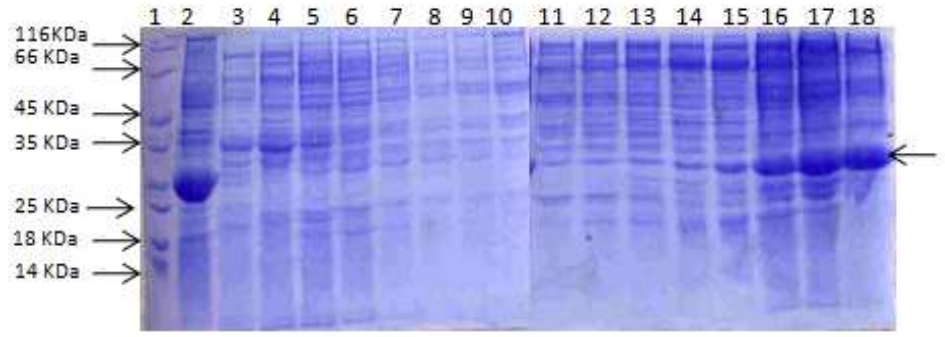

Fig. 4.31. DEAE sepharose chromatography: Lane 1 represents protein molecular weight marker, Lane 2 indicates 40 % ammonium sulfate used as input. Lanes 3-18 represent different elution fractions collected by using 0 to 0.5 M NaCl linear gradient. Presence of AbGST01 is shown with an arrow.

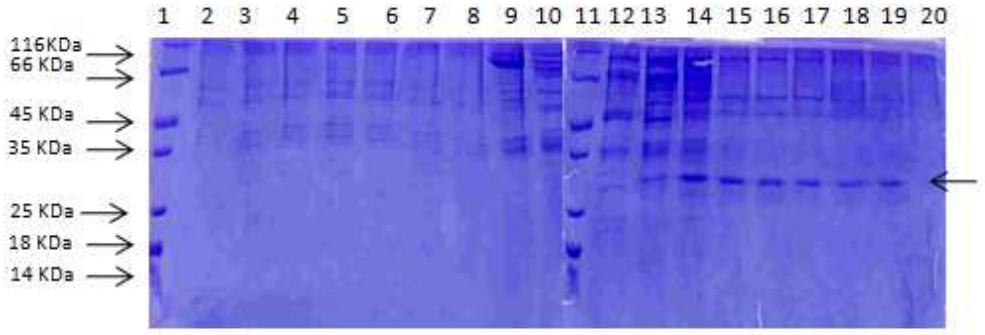

Fig. 4.32. Phenyl sepharose chromatography: Lane 1 & 11 represent protein molecular weight marker, Lanes 2-20 indicate protein obtained in various fractions. The partially pure AbGST01 is indicated with an arrow mark.

As most of the expressed AbGST01 was found in supernatant it was used for further purification following techniques described in materials and methods section. The 40 % ammonium sulfate fraction has showed highest activity when compared to the other fractions and hence was used for subsequent purification steps. Initially, the 40 % fraction was passed through DEAE column and the bound AbGST01 was eluted using salt gradient (0-0.5 M) (Fig. 4.31). As there were still impurities, the active fractions were pooled and applied on to phenyl sepharose column. The bound protein was eluted using ammonium sulfate gradient in the range of 50 mM to 5 mM (Fig. 4.32). This step has removed most of the contaminating proteins. The remaining impurities were eliminated by passing the active fractions through sephacryl S-200 column (Fig. 4.33).

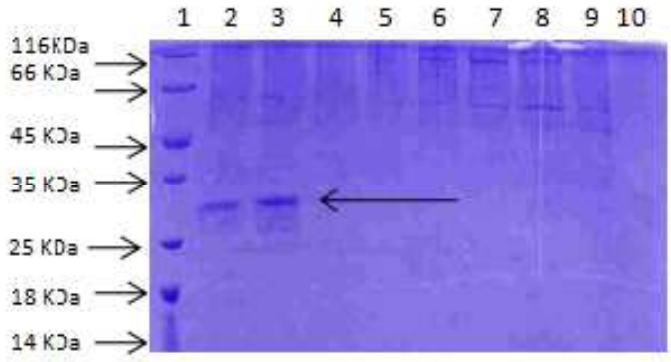

Fig. 4.33. Gel exclusion chromatography; Lane 1 represents protein molecular weight marker, Lane 2-10 indicate different fractions obtained from Sephacryl S-200 column. Pure AbGST01 is shown with an arrow mark.

4.8.3 Expression and purification of AbGST01$_{6His}$

Since the final yield from the gel permeation chromatography was low, AbGST01 was further expressed with C-terminal His-tag by cloning it in pET23b as described before using DS00151/DS00152 as forward and reverse primers. The corresponding plasmid was designated as pPATGH and was used for expression in *E. coli* BL21. The AbGST01$_{6His}$ was then purified using Ni affinity column as described in materials and methods. A homogenous band corresponding 26 kDa was observed in SDS-PAGE (Fig. 4.34), and hence this protein was used for further analysis.

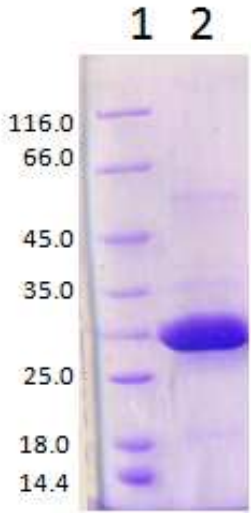

Fig. 4.34. Ni affinity chromatography: lane 1 represents protein molecular weight marker and lane 2 represents the purified recombinant AbGST01$_{6His}$ using Ni column.

4.8.4 Kinetics properties

Glutathione S-transferase activity with CDNB and other substrates were determined at 30 ^{0}C according the standard protocol (materials and methods), similarly glutathione peroxidase activity was determined at 30 ^{0}C with organic hydroperoxides as described in materials and methods. While determining kinetics parameters, only CDNB concentration is varied and GSH concentration was kept constant. Ethanol concentration was maintained such that it did not exceed 5 % of the reaction mixture. Using CDNB, fifteen concentrations of this substrate were assayed, ranging from 0.2 to 3 mM. Apparent K_m of AbGST01 activity towards CDNB (2.488 ± 0.4204 mM) was found to be higher than the other well characterized bacterial GST of *Proteus mirabilis* (0.73 mM). The specific activity was shown to be 3.26 µmol min^{-1} mg^{-1}.

The purified AbGST01$_{6His}$ also exhibited GSH-dependent peroxidase activity towards cumene hydroperoxide, t-butyl hydroperoxide and 13-hydroperoxyoctadecaienoic acid (13-HOPDE) and the specific activities were found to be 1.33, 0.669 and 2.4 µmol min^{-1} mg^{-1}, respectively. AbGST01$_{6His}$ also showed activity against ethacrynic acid (0.03 µmol min^{-1} mg^{-1}) however, the recombinant AbGST01$_{6His}$ did not show any activity against other bacterial GST substrates such as H_2O_2, dichloromethane, tetrachloro hydroquinone and antibiotics fosmomycin.

AbGST-peroxidase activity towards 13-HPODE was further determined using high performance liquid chromatography (HPLC) as described in materials and methods. The chromatogram obtained from HPLC analysis is shown in figure 4.22. The AbGST01 catalyzed conversion of 13-HPODE to 13-hydroxyoctadecaienoic acid (13-HODE) was observed in the test sample (Fig. 4.22B) where as no such activity was seen in the control sample (Fig. 4.22A).

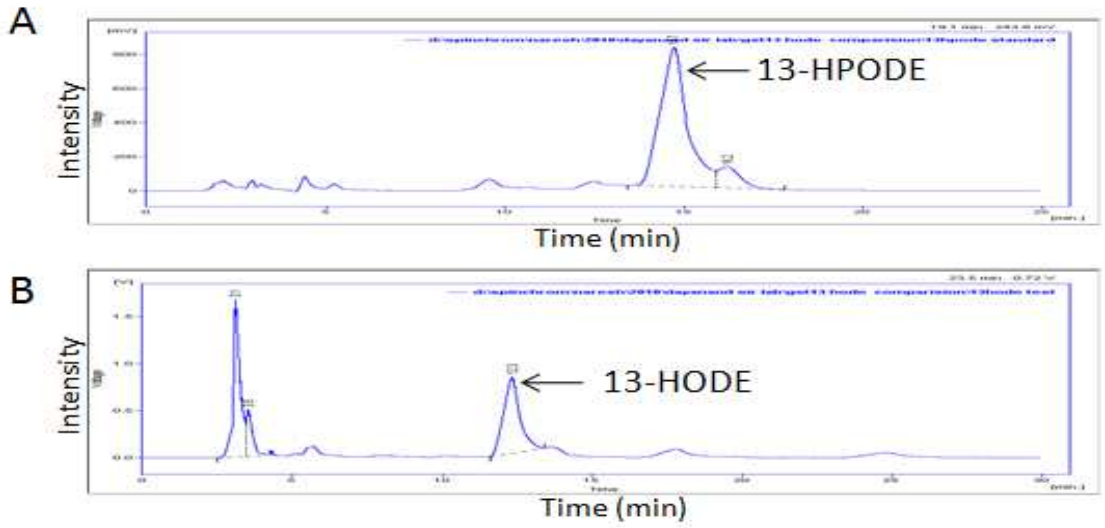

Fig. 4.35. HPLC analysis of AbGST01 activity towards 13-HPODE: Panel A represents control where AbGST01 was omitted whereas panel B represents the test.

In mammals, the oxidative metabolism of linoleic acid produces a number of bioactive products including 13-hydroperoxyoctadeca-9,11- dienoic acid (13-HPODE), 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE), and the 2,4-dienone 13-oxooctadeca-9,11- dienoic acid (13-OXO) (Podgorski and Bull, 2001; Bull et al., 2002). The GSTs were shown to be involved in the removal of these harmful by products (Seeley et al., 2006). Since AbGST01 catalyzed the conversion to 13-HPODE to 13-HODE *in vitro*, possibly such kind of protection is possible, especially when *A. baumannii'*s are inside the host.

4.8.5 Biotransformation of Methyl parathion by AbGST01

The purified recombinant AbGST01 were incubated with methyl parathion in presence of glutathione in tris buffer. After incubation, the product generated were analysed by HPLC and GC/MS (Materials and methods). Interestingly, both the methods have shown reduction in concentration of methyl parathion (MeP) in presence of AbGST01 (Fig. 4.36 and 4.37A). As expected the decrease of product concentration resulted in generation of a new peak only in the test sample (Fig. 36B). As the peak was eluted early indicating presence of a more hydrophilic compound and this could be a

glutathione conjugated with the methyl group of methyl parathion after dealkylation. Further a peak found only in the test sample was identified as desmethyl parathion by GC-MS analysis (Fig. 4.37B).

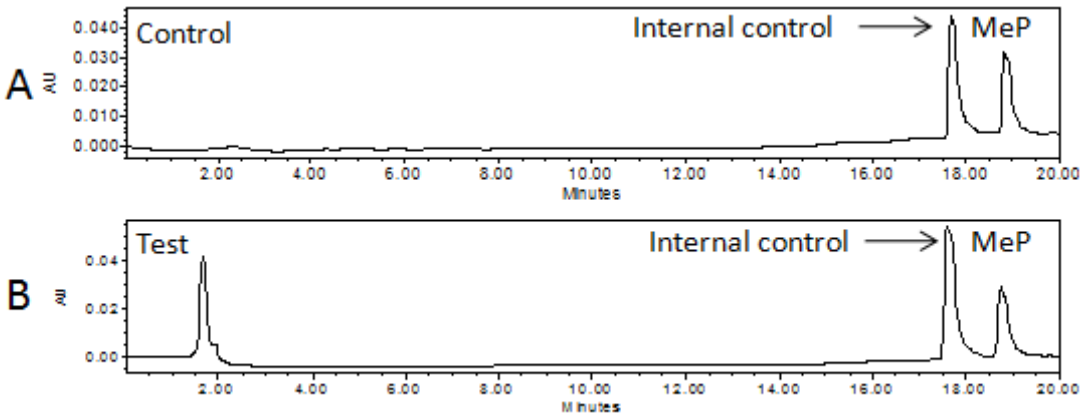

Fig. 4.36. HPLC chromatogram: Panel A represents control sample in which AbGST01 is replaced with water, whereas panel B represents test sample. Decreased in methyl parathion peak is clearly seen in the test sample.

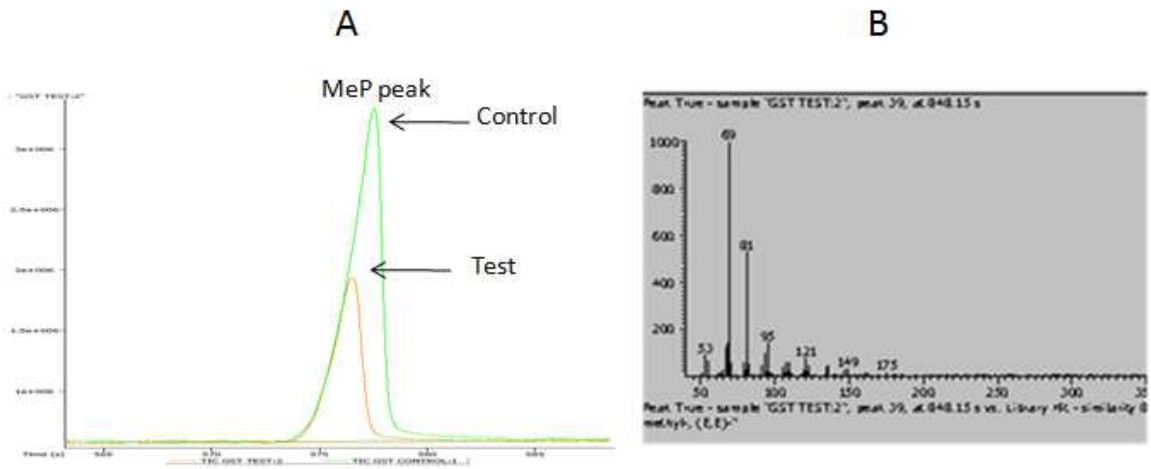

Fig. 4.37. Panel A: GC/MS chromatogram; The green colour peak represents MeP concentration in control sample, whereas the red colour peak represents similar parameter in the test sample. Decreased in MeP peak is clearly seen in the test sample. Panel B: Mass fragmentation pattern of a peak from GC/MS analysis.

Involvement of GSTs in transformation of xenobiotics is well documented both in prokaryotes (Hayes and Pulford 1995, Hayes and McLellan 1999 and Armstrong, 1997) and eukaryotes (Benke et al., 1974; Benke and Murphy, 1975; Clark et al., 1973). GSTs isolated from mammals especially from humans (Radulovic et al., 1986, Radulovic et al.,

1987) and rats (Benke and Murphy, 1975) have dealkylated methyl parathion generating desmethyl parathion. Prokaryoitic GSTs have been discovered very recently (Vuilleumier, 1997). Their role in transformation of structurally diverse group of xenobiotic is well documented (Armstrong, 1997), however, no reports are available to show their role in transformation of organophosphates. The experimental evidence shown in this report provides unequivocal support to implicate GSTs of *A. baumannii* DS002 in the transformation of organophosphate methyl parathion by dealkylation.

Conclusions

1. Genomic DNA library was constructed for *A*. *baumannii* DS002. Atleast 9 different gst-like genes were identified from the library and were designated as *Abgst01, Abgst02, Abgst03, Abgst04, Abgst05, Abgst06, Abgst07, Abgst08 and Abgst09.*
2. Sequence analysis has revealed little identity among these AbGSTs and most of them clustered with different GST classes.
3. Most of the *Abgsts* got upregulated when treated with organic hydroperoxides. Inorganic hydroperoxides like H_2O_2 showed no influence on *Abgsts* expression.
4. *Abgst01* was found to be upregulated in presence of CDNB and Methyl parathion.
5. Putative OxyR binding site was found upstream of *Abgst01*. In *oxyR* null mutant of *A. baumannii* DS002, no *Abgst01* expression was noticed. Organic hydroperoxide CHP failed to induce *Abgst01*. Indicating involvement of OxyR in regulation of *Abgst01*.
6. *Abgst01* was cloned and expressed in *E. coli* BL21. The purified recombinant AbGST01 showed activity against CDNB, CHP, t-BHP, 13-HOPE and EA but no activity was seen with H2O2, TCHQ, DCM and fosfomycin.
7. AbGST01 was able to dealkylate Methyl parathion. Generation of desmethyl parathion is only seen in presence of purified $AbGST01_{6His}$.

5.1 Development of genetic tools for manipulation of *A. baumannii* DS002

Acinetobacter baumannii is an important opportunistic pathogen that causes a variety of human infections. Due to existence of metabolic diversity and ability to grow in a variety of habitats, it has also been considered as one of the industrially important organisms having potential industrial applications. Despite of having clinical and industrial significance, no dedicated genetic tools are available for cloning and expression of genes in *Acinetobacter* strains. In the present study, as described in chapter one, a detailed investigation was undertaken on plasmidome of *A. baumannii* DS002. In total we have detected and rescue cloned four indigenous plasmids. One of these four indigenous plasmids, pTS4 is a smallest plasmid. Its complete sequence is determined in this study. The experiment conducted in this study further revealed that it is a rolling circle plasmid capable of generating a single stranded intermediate during the process of replication. All these properties made plasmid pTS4 as an ideal molecule for using it as a backbone for constructing a cloning and expression vector. This chapter describes construction of a pTS4 derived shuttle vector for cloning and expression genes in *A. baumannii* DS002.

5.1.1 Construction of shuttle vector

Before proceeding for construction of vector, a detailed examination was made to identify presence of unique restriction sites, especially in the intergenic regions. In the sequence of plasmid pTS4 a unique *Mlu*I site was noticed in the intergenic region of *repA* and *orf113.* Therefore, this unique site was exploited to insert multiple cloning site along with a kanamycin resistance gene and R6Kγ*ori* origin of replication. In plasmid EZ-Tn5™<R6Kγ*ori*/ KAN-2> (EPICENTRE Biotechnologies, USA) the kanamycin resistance gene, R6K$_{\gamma}$*ori* origin of replication and multiple cloning site are closely located (Fig. 6.1).

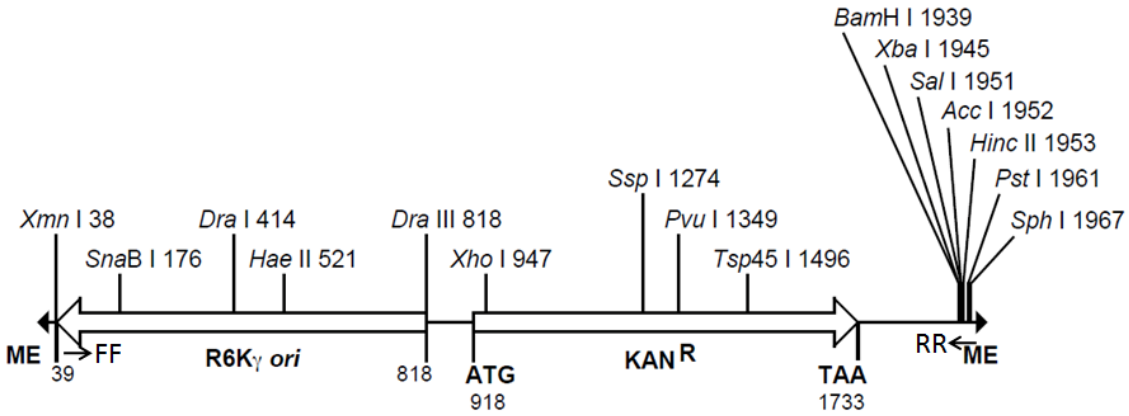

Fig. 6.1. EZ-Tn5™<R6Kγ*ori*/ KAN-2> map; Primers used for amplification of DNA sequences containing R6Kγ*ori,* kanamycin resistance gene and MCS are shown with arrows.

They can be amplified as a single 2 Kb fragment by designing primers. Therefore, a pair of primers (DS00119/DS00120) having *Mlu*I site were designed and used to amplify the region of mini-transposon containing MCS, R6Kγ*ori* and Km cassette. The PCR primers were designed by avoiding DNA region containing terminal repeats (ME). Therefore the PCR amplicon contains only the replicative origin, kanamycin resistance gene and MCS.

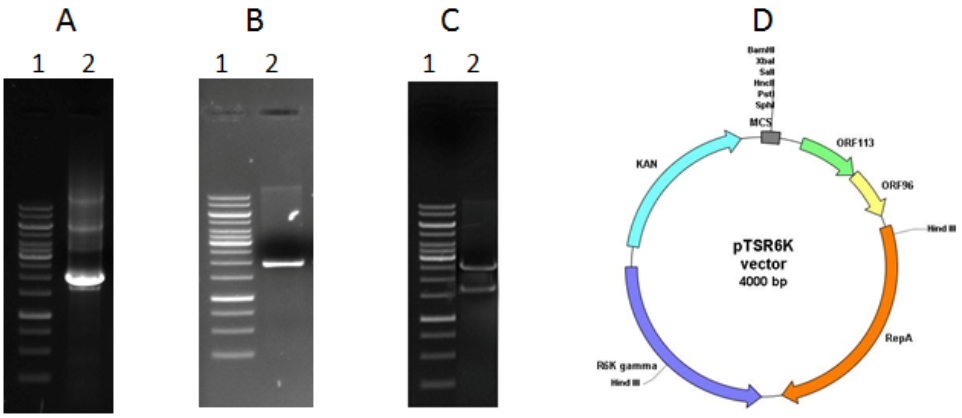

Fig. 6.2. Panel A: PCR amplification of MCS sites from EZ-Tn5™<R6Kγ*ori*/ KAN-2>.Panel B: pTS4 plasmid cut with *Mlu*I. Panel C: pTSR6K shuttle vector digested with *Hind*III. Panel D: Physical map of pTSR6K.

The PCR amplicon was digested with *Mlu*I and then ligated to pTS4 plasmid digested with same enzyme. The resulting plasmid designated as pTSR6K (Fig. 6.2) can replicate in *E.coli pir116,* due to existence of R6Kγ*ori* origin of replication. It can also replicate in *A. baumannii* as it has DSO and SSO replicative origin derived from pTS4.

Further if pTSR6K is transferred into *E. coli pir* negative background, it replicates in presence of an expression plasmid coding RepA (See chapter 1). Therefore, in case of necessity, it is possible to isolate single stranded version on pTSR6K. After construction, the pTSR6K was transformed into *A. baumannii* DS002 and selected on Km plate. A number of colonies were obtained indicating successful replication of the plasmid pTSR6K. One of the transformants was then selected and grown in LB medium for several generations in the absence of kanamycin. When such cultures were transferred into kanamycin containing LB medium, they have successfully grown showing stable maintenance of shuttle vector.

5.2 Expression of *opd* gene in *A. baumannii* DS002

As shown in the earlier chapter, *A. baumannii* DS002 dealkylates organophosphate (MeP) generating desmethyl parathion. Further degradation of desmethyl parathion is not seen. In order to achieve complete mineralization of OP compounds, a set of known genes have to be cloned into *A. baumannii* DS002. As shown in the figure 6.3, the first step in microbial degradation of methyl parathion (MeP), a model OP compound, is hydrolysis of triester linkage by organophosphate hydrolase (OPH), the product of organophosphate degrading (*opd*) gene (Brown, 1980; Serdar and Gibson, 1985). Therefore initially, the *opd* gene was cloned in pSTR6K to express OPH in *A. baumannii* DS002. The *opd* (1.5 Kb) gene was taken as *Bam*HI fragment from plasmid pPDL2 of *Sphingobium fuligensis* (Siddavattam et al., 2003) and ligated in pTSR6K digested with the same enzyme, the resulting plasmid was designated as pTSPH (Fig. 6.4A). This plasmid was then electroporated into *A. baumannii* DS002 (Materials and methods) and the degradation of methyl parathion was monitored.

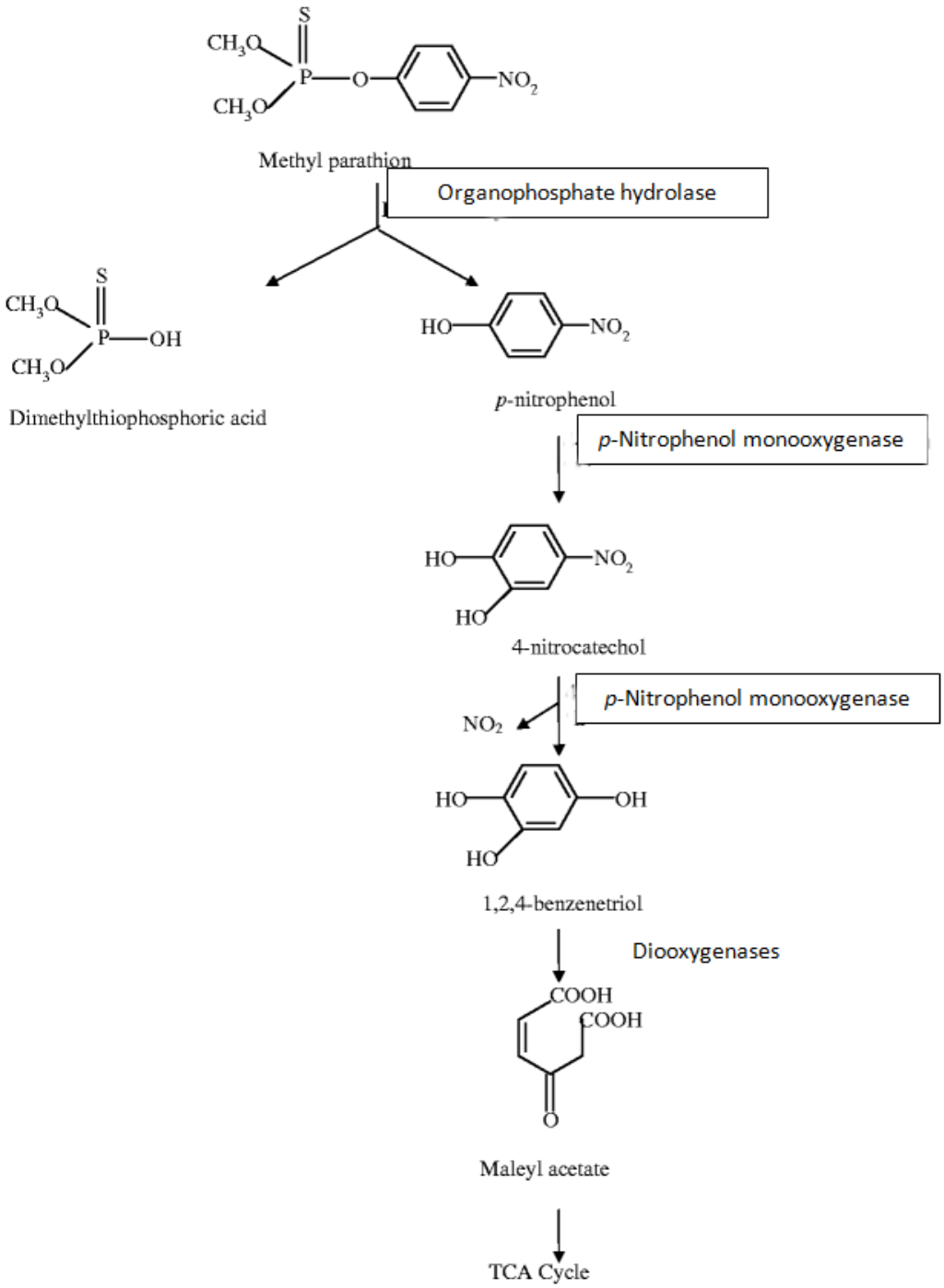

Fig. 6.3. Schematic representation of methyl parathion degradation pathway. Genes to be cloned in A. baumannii DS002 to achieved complete mineralization of methyl parathion are shown in open boxes.

The *A. baumannii* DS002 (pTSPH) cells having cloned *opd* gene were then tested to know if it is expressing OPH. Substantial amount of OPH activity was found in cells having recombinant plasmid pTSPH. However, no such activity was seen in wildtype *A. baumannii* DS002 (Fig. 6.4B). This is a clear indication to show that the cloned *opd* gene

of *Sphingobium fuligenesis* has expressed in *A. baumannii* DS002 using its indigenous promoter. Further, when methyl parathion was added to the culture medium, the culture quickly turned yellow indicating degradation of methyl parathion to produce *p*-nitrophenol (Fig. 6.4C). Such conversion of colour was not seen when methyl parathion was added to the culture medium having wildtype *A. baumannii* DS002 cells (Fig. 6.4C).

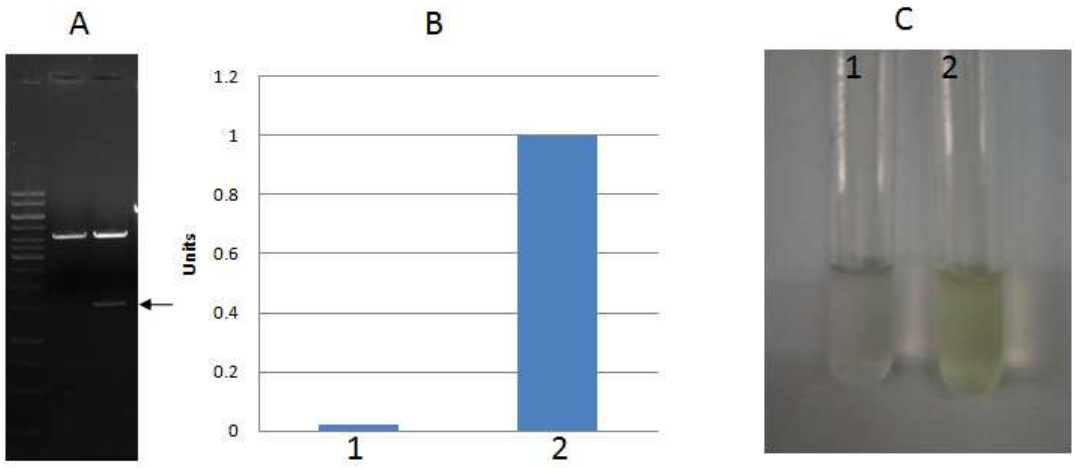

Fig. 6.4. Panel A: Lane 1; DNA ladder, Lanes 2 and 3 represent pTSR6K and pTSPH plasmids digested with *Bam*HI. Release of 1.5 Kb *opd* fragment is seen in lane 3. Panel B: OPH activity in *A. baumannii* DS002 (1) and *A. baumannii* (pTSPH) (2). Panel C: Degradation of methyl parathion in cultures containing vector pTSR6K (1) and pTSPH (2). Formation of *p*-nitrophenol due to OPH mediated hydrolysis is only seen in *A. baumannii* DS002 (pTSPH).

5.3 Expression of *PNP-monooxygenase* gene in *A. baumannii* DS002

The *A. baumannii* DS002 (pTSPH) cultures convert methyl parathion into of *p*-nitrophenol (PNP) and dimethylthiophosphoric acid (DMTPA) (Fig. 6.3). Though the DMTPA is used as source of carbon and phosphate, PNP accumulates in the environment. Since, PNP is toxic to the microflora it affects soil ecosystem and fertility (Anonymous, 1992). Elimination of PNP is therefore major concerns of environmental biologist. *A. baumannii* DS002 is a soil isolate and as stated in the introduction section, it gets enriched in presence of methyl parathion. However, it failed to use either methyl parathion or PNP as source of carbon. If *A. baumannii* DS002 has to be manipulated to achieve complete mineralization of OP compounds, it has to be manipulated by

mobilizing appropriate genes. In our unpublished studies we have noticed growth of *A. baumannii* DS002 on benzoate. Our laboratory has done further investigations to understand and elucidate benzoate degradation pathway. These studies have revealed existence of *ortho* pathway in benzoate degradation (Fig. 6.5). If benzoate degradation pathway is carefully examined, catechol is the key catabolic intermediate. After conversion of benzoate to catechol, the dioxygenase found in *A. baumannii* DS002 convert it into TCA cycle intermediates as shown in figure 6.5.

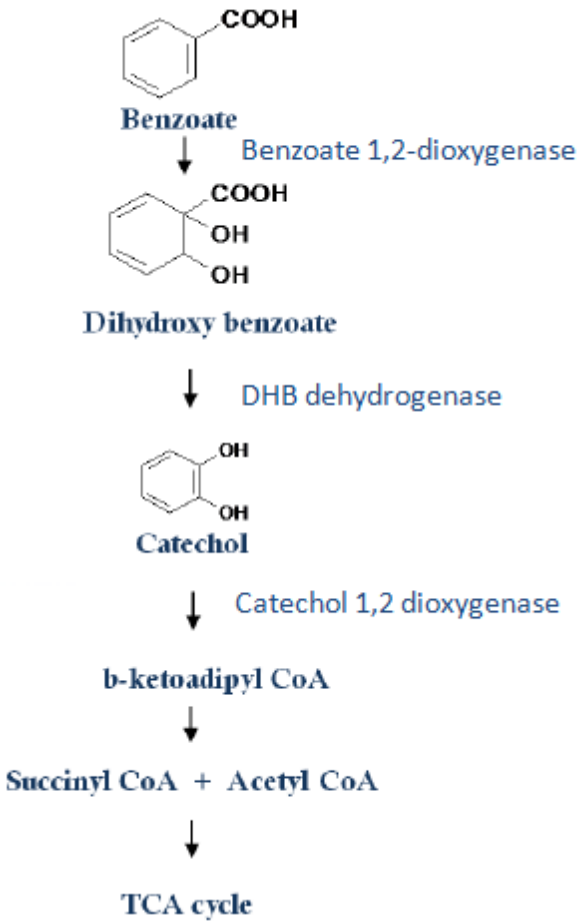

Fig. 6.5. Schematic representation of Benzoate degradation pathway in *A. baumannii* DS002.

Microbial degradation of *p*-nitrophenol is well known phenomenon (Ju and Parales, 2010). A wealth of information is available on PNP degradation pathways and genes involved in its catabolism. Novel monooxygenases convert *p*-nitrophenol to

benzenetriol (BT) via 4-nitrocatechol. The generated benzenetriol gets finally channelled to TCA cycle due to existence of ring cleavage dioxygenase. Since *A. baumannii* DS002 has dioxygenase, expression of PNP monoxygenase is expected to convert PNP to BT. Therefore a systematic approach is needed to express *PNP monoxygenase* of *Rodococcus* sp. in *A. baumannii* DS002. The constructed shuttle vector is useful only for cloning of the genes in *A. baumannii* DS002. It can't be used as expression vector due to lack of suitable promoter element. In order to generate an expression vector, initially the *Ptac* promoter was cloned in shuttle vector pTSR6K. In our previous study our lab has taken *Ptac* promoter as 100 bp *Bgl*II and *Bam*HI fragment. This *Ptac* promoter was then taken and cloned in *Bam*HI site of shuttle vector pTSR6K. Promoter found in proper orientation was then selected and the resulting expression vector was named as pTSTC (Fig.6.6).

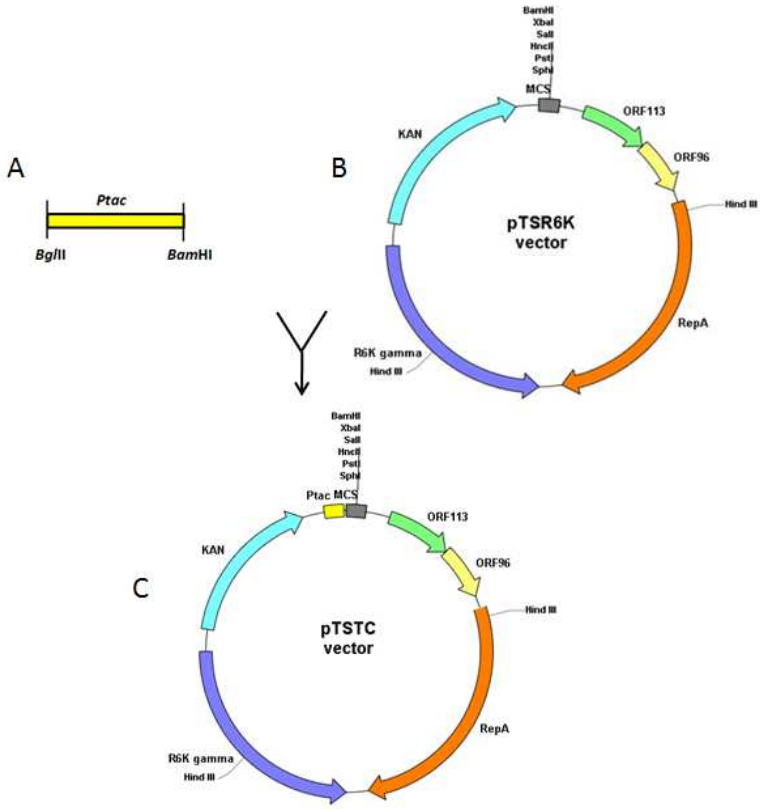

Fig. 6.6. Strategy for construction of an expression vector pTSTC: Panel A represents *Ptac* promoter. Panel B indicates shuttle vector pTSR6K and Panel c represents physical map of pTSTC.

After generation of expression vector pTSTC, the *p-nitrophenol monoxygenase* gene was cloned under the control of *Ptac* promoter. Initially a 2.2 Kb *PNP-monooxygenase* (*nphA1A2*) gene from *Rodococcus* sp. (Takeo et al., 2008) was excised from pUNPH-A12 plasmid (Takeo et al., 2003) as *Bam*HI and *Pst*I fragment. The fragment was then cloned in pTSTC vector (Fig. 6.7A) and the recombinant plasmid was designate as pTSPM. The pTSPM was then electroporated into *A. baumannii* DS002 as described elsewhere (Materials and methods) and the degradation of PNP was observed by plating the culture on PNP containing minimal medium plate. After overnight incubation, the otherwise yellow plate turned into brownish yellow suggesting conversion of PNP to 4-nitrocatechol and 1,2,4-benzenetriol. Such change in colour is seen only in *A. baumannii* DS002 (pTSPM) plate (Fig. 6.7C). No change in the colour of the plate was seen in wildtype *A. baumannii* DS002 plate (Fig. 6.7B). Further the *A. baumannii* DS002 (pTSPM) was grown in minimal medium supplemented with 50 µM PNP and the clean supernatant were scanned to identify formation of degradation products of PNP at various time intervals.

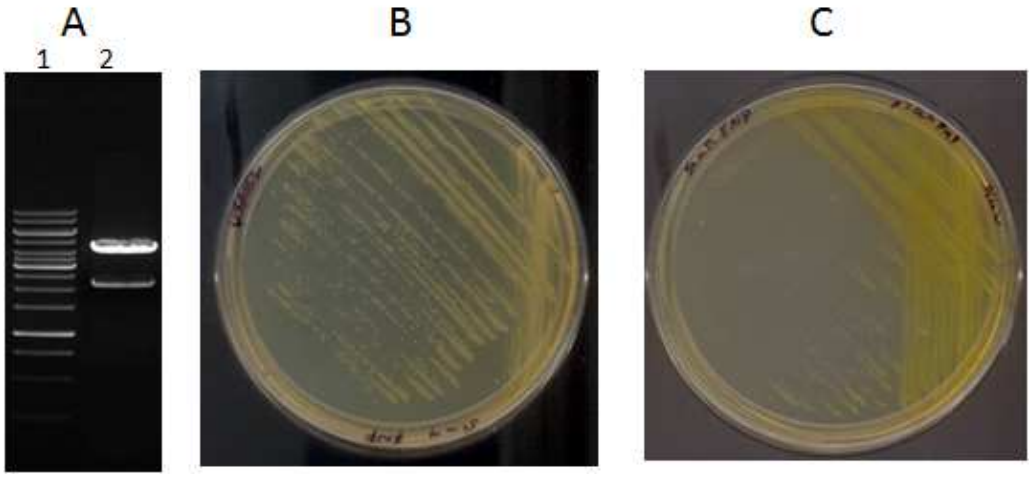

Fig. 6.7. Panel A: Lane 1 represents 1 Kb ladder and lane 2 represents pTSPM plasmid digested with *Bam*HI and *Pst*I. Release of 2.2 Kb *nphA1A2* fragment from pTSR6K plasmid is shown with an arrow. Panel B&C indicate degradation of PNP in minimal media plates. *A. baumannii* DS002 and *A. baumannii* DS002 (pTSM) expressing PNP-monooxygenase are shown in panel B and C respectively.

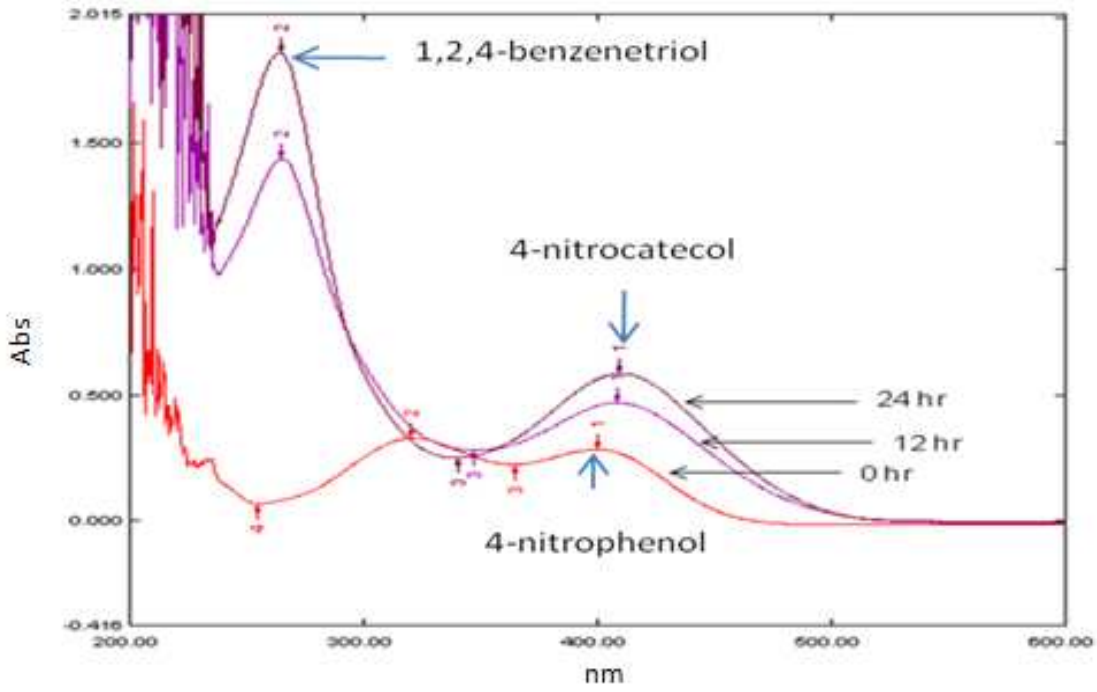

Fig. 6.8. Spectral scan of spent medium collected at various time intervals from *A. baumannii* DS002 (pTSM) culture. Shift in peak was observed at 12 hours and reached to maximum height at 24 hours of growth. Similarly peak of various metabolites are shown with arrow marks.

Appearance of a new peaks at 280 nm were observed to indicate generation of 1,2,4-benzenetriol and 4-nitrocatechol from PNP (Fig. 6.8). Accumulation of these PNP metabolites showed an interesting trend with increase of time.

5.4 Complete mineralization of organophosphates

The mixed cultures of *A. baumannii* DS002 expressing parathion hydrolase and PNP monooxygenase were then tested if they can eliminate methyl parathion supplemented to the culture medium. As expected the culture media, soon after addition of methyl parathion, turned yellow due to generation of PNP. The generated PNP then got slowly disappeared indicating complete elimination of PNP from the culture medium. This study clearly demonstrates mineralization of methyl parathion residues using manipulated strains of *A. baumannii* DS002. As *A. baumannii* DS002 doesn't have *opd* and *PNP-monooxygenase* genes, expression of these genes enhanced its ability to metabolize organophosphates and the recalcitrant metabolized like *p*-nitrophenol. Expressions of

these two genes generate metabolic intermediates of benzoate and methyl parathion. Such conversion channels the toxic OP compounds and their recalcitrant degradation products into benzoate degradation pathway leading to complete mineralization of OP compounds (Fig. 6.9).

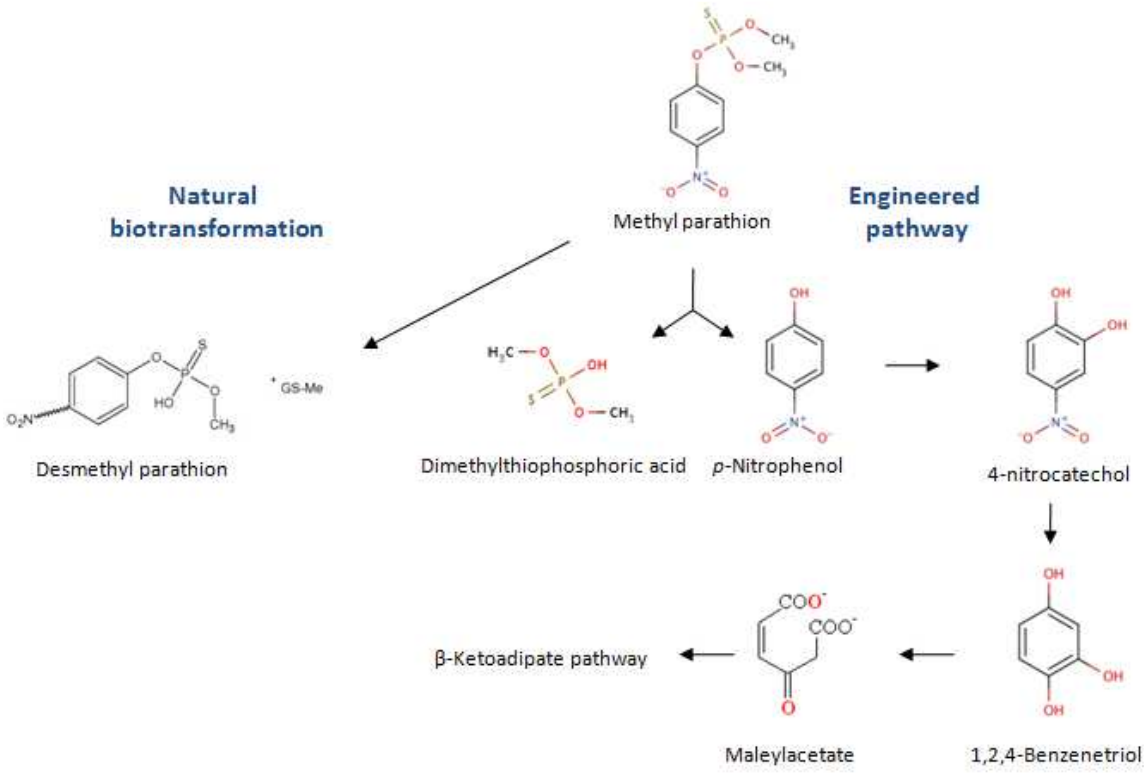

Fig. 6.9. Schematic representation of methyl parathion degradation pathways in engineered strains of *A. baumannii* DS002.

This study clearly demonstrated utility of manipulated strains of *A. baumannii* DS002 for elimination of OP compound methyl parathion. Expression of *opd* gene empowers *A. baumannii* DS002 with the ability of hydrolysing most of the OP insecticides used in agriculture. The aromatic compounds generated due to OPH mediated hydrolysis of OP insecticides are then channelled into TCA cycle due to expression of *p*-nitrophenol monooxygenase. These genetically manipulated microbe have restricted use, this can be used to clean the waste generated in pesticides manufacturing industries. Further, expression of *p*-nitrophenol monooxygenase clearly shows ability of pTSR6K as an

expression vector. It can be successfully used for expression of other gene in *A. baumannii* DS002.

Conclusions

1. Shuttle vectorpTSR6K was constructed using pTS4 of *A. baumannii* DS002.
2. The organophosphate degrading (*opd*) gene and *p*-nitrophenol monooxygenase genes were expressed in *A. baumannii* DS002 using shuttle vector pTSR6K.
3. The *A. baumannii* DS002 strains expressing *opd* and *PNP-momooxygenase* mineralized methyl parathion and *p*-nitrophenol, indicating suitability of shuttle vector for cloning and expression of genes in *A. baumannii* DS002.

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