Discussion

5. DISCUSSION

5.1 β-lactam resistance determinants of *Z. mobilis* and their homologues

Multiple β-lactam resistance determinants such as beta-lactamase (BLA), penicillin binding proteins (PBP) and beta-lactamase domain containing proteins (BDP) are present in *Z. mobilis.* They seem to be homologues of the antibiotic resistance genes in *Sphingomonas wittichii* RW1, *Sphingobium chlorophenolicum* L-1, *Sphingomonas* sp. SKA58, *Mesorhizobium opportunistum* WSM2075, *Caulobacter* sp. K31 and *Gluconobacter hansenii* ATCC23769. *S. wittichii* RW1 and *S. chlorophenolicum* degrade xenobiotic compounds (Takeuchi *et al*., 2001, Yabuuchi *et al*., 2001). *S. wittichii* RW1, *Sphingomonas* sp. and *S. japonicum* are reported to have antibiotic resistance (Basta *et al*., 2004, Pal *et al*., 2005, Yabuuchi *et al*., 2001). *S. wittichii* RW1 is an effective metabolizer of dibenzo-p-dioxin and has been reported to show resistance to beta-lactam antibiotics like aztreonam and moxalactam (Yabuuchi *et al*., 2001). *S. chlorophenolicum* has been reported to completely mineralize pentachlorophenol (Takeuchi *et al*., 2001). The existence of insertion sequence IS6100 has been reported in hexachloro cyclohexane-degrading *S. paucimobilis* that has the ability to spread antibiotic resistance genes across diverse group of bacteria by horizontal gene transfer (Dogra *et al*., 2004). This particular IS element is reported to be linked with antibiotic-resistance genes (Mahillon and Chandler, 1998). These genes are involved in antibiotic resistance in *Sphingomonas* sp. (Basta *et al*., 2004). The *S. japonicum* sp. shows resistance to ampicillin (Pal *et al*., 2005).

The β-lactamase BLA2 of *Z. mobilis* is comparable to the β-lactamase of *Citrobacter freundii* GN7391 (Tajima *et al*., 1980). The molecular weight (42 kDa) and pI (5.96) of BLA3 of *Z. mobilis* are close to that of β-lactamase of *Psychrobacter immobilis* A8 (Feller *et al*., 1995). Similarly the molecular weight and pI of BLA3 are similar to those of class A beta-lactamases (Arakawa *et al*., 1989). In addition, the BDP1 has predicted molecular weight and pI matched to the VIM-2 metallo–β-lactamase characterized previously (Poirel *et al*., 2000). The substitution of amino-acids in the active-site of the ß-lactamases could be responsible for the broad range of theoretical iso-electric point and wide-range of pH stability. This wide range of pI could be due to the variation in SXXK motif, in the active site of these proteins. Such amino-acid substitutions have been reported to change the pIs of the ß-lactamases (Ambler, 1980). The predicted aliphatic index value range of BLAs, BDPs and PBPs suggested that they are highly thermo-stable as reported earlier (Arakawa *et al*., 1989, Feller *et al*., 1995, Poirel *et al*., 2000, Tajima *et al*., 1980).

The predicted half-life of the ß-lactam resistance determinants indicated their stability and possible transfer to potential pathogenic *E. coli* strains by horizontal gene transfer. Such horizontal gene transfer has been reported in pathogenic and non-pathogenic strains conferring antibiotic-resistance (Couvalin, 1996). The horizontal transfer of antibiotic resistance genes between *Sphingomonas* sp and *S. aromaticivorans* F199 has been reported (Basta *et al*., 2004). *S. japonicum* sp. is resistant to ampicillin (Pal *et al*., 2005). Recently, the similarity between beta-lactamase II of *E. litoralis* and New Delhi metallo-beta-lactamase having carbapenemase activity has been reported (Zheng *et al*., 2011). *S. wittichii* RW1 is

resistant to wide-range of antibiotics (Yabuuchi *et al*., 2001). All these reports suggested the close resemblance of *Z. mobilis* with these ß-lactamase producers.

5.2 Multiple β-lactam resistance characteristic motifs in *Z. mobilis*

Z. mobilis has multiple β-lactam-resistance characteristic motifs. The STFK motif is present in PBP3 which is the most frequently found motif in class D ß-lactamases (Couture *et al*., 1992, Donald *et al*., 2000). The STFK motif along with KTG motif is well-known to be involved in the serine active site function and regulation (Bush *et al*., 1995, Couture *et al*., 1992, Massova and Mobashery, 1998). KXG, SXXK and SXN motifs are the three catalytic centers of the transpeptidase activity in all PBPs (Ghuysen, 1991). KTG motif is known to be involved in the serine active site cleft formation, functional regulation and stability (Bush *et al*., 1995, Massova and Mobashery, 1998) and it is part of ß-sheet (Mottl *et al*., 1991). The conservative amino acid substitution with T (threonine) to S (Serine) in KXG motif is likely to maintain the structure and function of the ß-lactamases. However, this mutation could further lead to increased ß-lactam resistance (Harris *et al*., 2000). The absence of SXXK motif in BDP2, BDP3 and BLA4 indicates that they are metallo ß-lactamase proteins (Couture *et al*., 1992). YXN motif is reported to be present in a loop that connects two α-helices of the PBPs (Ambler, 1980).

5.3 ß-lactamase and PBPs in beta-lactam resistance in *Z. mobilis*

The occurrence of PBPs in *Z. mobilis* ZM4 have been reported earlier (Karibian and Starka, 1987) was confirmed in our study and suggested that two of them could be murein polymerase. Mutations in PBPs could have increased the levels of beta-lactam resistance in *Z. mobilis* through reduced affinity of PBPs to penicillin. *N. gonorrhoeae* is known to have three PBPs, similar to that of *Z. mobilis* (Dougherty

et al., 1980). The PBP1 and PBP3 contain transglycosylase and transpeptidase domains which is a characteristic of PBPs. These results are in accordance with the previous report (Di Gulmi *et al*., 2003). The N-terminal transglycosylation domain of PBP1 is considered to catalyse the elongation of the glycan chains while the transpeptidase domain is considered to catalyse the cross linking of the peptide and glycan chains during peptidoglycan biosynthesis (Di Gulmi *et al*., 2003).

5.4 Antibiotic resistance in *Z. mobilis* is similar to that of non-pathogenic bacteria

In this study, a detailed analysis of the various β-lactamases (BLA), β-lactam domain-containing proteins (BDP) and penicillin-binding proteins (PBP) in *Z. mobilis* revealed that they are evolutionally related to β-lactamases of other bacteria. *Saccharopolyspora erythraea* NRRL23338 contained antibiotic-resistance coding region in 1% of its genome (Oliynyk *et al*., 2007). The complete genome sequence of *Chromobacterium violaceum* ATCC 12472, non-pathogenic bacterium (Vasconcelos *et al*. 2003) reported to have virulence and antibiotic-resistance genes (penicillinase and cephalosporinase) and multidrug efflux pumps (Fantinatti-Garboggini *et al*., 2004).

Z. mobilis causes cider sickness in fruits and was isolated from samples from fruit spoilage. This bacterium shows resistance to several antibiotics including β-lactam antibiotics (Rajnish *et al*., 2011). The present study suggests that ß-lactamases of *Z. mobilis* may have significant role in the transfer of resistance genes to pathogenic bacteria in its habitat. *Z. mobilis* showed high level of resistance to cephalexin, cephalosporin C, cephalothin, benzylpenicillin and penicillin V.

Several pathogenic bacterial strains of *E. coli*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida* and *Salmonella* spp. also exhibit antibacterial resistance (Ninane *et al*., 1978, Ramos *et al*., 1995). These antibiotic-resistance genes could spread to pathogenic bacteria (Baquero *et al*., 2008). The presence of antibiotic resistance in *Branhamella catarrhalis*, a non-pathogenic bacterium with beta-lactamase has lead to bronchopulmonary infection (Ninane *et al*., 1978). Similarly, *L. enzymogenes*, *Saccharopolyspora erythraea* NRRL23338 and *Branhamella catarrhalis*, non-pathogenic bacteria posses genes encoding antibiotic resistance even though they were not exposed to ß-lactam antibiotics habitat (Ninane *et al*. 1978, Oliynyk *et al*., 2007, Richmond and Sykes, 1973). Resistance to penicillin by beta-lactamase produced by non-pathogenic *Staphylococci* has been reported (Ako-Nai *et al*., 2005).

Recently, among the *E. coli* strains isolated from diarrheic faeces, 62.8% of the non-pathogenic *E. coli* was found to be resistant to more than one antibiotic (Garcia *et al*., 2011). Among the bacterial strains isolated from non-diarrheic samples, 50% of the non-pathogenic *E. coli* showed antibiotic resistance. Recently, antibiotic resistance in diarrheagenic and non-pathogenic *E. coli* strains from Pigeons (*Columba livia*) in Brazil (Silva *et al*., 2009) has been reported. Similarly, the wide-range of antibiotic resistance in the human intestinal *E. coli* isolates has been reported. Multiple antibiotic resistances were due to Class-A beta-lactamases produced by *E. coli* (Amaya *et al*., 2011). Recently, there has been report of extended-spectrum beta-lactamase in *E. coli* and *K. pneumoniae* isolates and concluded that the transfer of antibiotic-resistance genes in the hospital and environmental settings thereby leading to continuous presence of these multi-drug resistance organisms (Vaidya, 2011).

Thus, it is evident that the non-pathogenic bacteria could be responsible for spread of antibiotic-resistance genes to pathogenic bacteria.

5.5 Regulatory elements of beta-lactamases

The expression of beta-lactamase gene is regulated by AmpR and AmpG present upstream to the gene (Poirel *et al*., 1999; Lauro *et al*., 2009). The helix turn helix motif of LysR is thought to be involved in binding upstream region at the *ampC* and thereby the regulating the expression of beta-lactamase (Poirel *et al*., 1999). All the beta-lactamases in *Z. mobilis* are chromosomally encoded. None of the sequenced plasmids (NC_013784, NC_013785, NC_013786, NC_013787, NC_013788, NC_004457) showed the presence of these beta-lactamase genes. These chromosomally encoded antibiotic resistance genes in *Z. mobilis* ZM4 is essential for their survival in antibiotic exposed environment. A chromosomally-encoded CTX-M, a class-A ß-lactamase from *Kluyvera* sp. has been reported already. This ß-lactamase is present in non-pathogenic bacteria (Decousser *et al*., 2001, Humeniuk *et al*., 2002, Poirel *et al*., 2002, Rodriguez *et al*., 2004).

5.6 Cellulose production has been reported in flocculating bacteria

Z. mobilis, a floc-forming bacterium, is capable of synthesizing extracellular cellulosic fibrils that help in cell adhesion to surfaces. Likewise, cellulose production has been reported in floc-forming bacteria such as *Agrobacterium tumefaciens*, *Escherichia coli*, *Gluconobacter xylinus*, *Klebsiella pneumoniae, Rhizobium leguminosarum* bv. *trifolii*, *Salmonella* spp., *Sarcina ventriculi*, and several species of cyanobacteria (Ausmees *et al*., 1999, Deinema and Zevenhuizen, 1971, Matthysse *et al*., 1995, Napoli *et al*., 1975 and Nobles *et al*., 2001). *Z. mobilis* has the capability to bind to red and calcofluor. Likewise, binding behavior of Congo red and calcofluor to

the cellulose molecule in cellulose-producing organisms has been reported (Ausmees *et al*., 1999, Deinema and Zevenhuizen, 1971, Matthysse *et al*., 1981). Like, *Z. mobilis, E. coli* and *Salmonella* strains have been reported to display red, dry and rough colony morphology on Congo red containing agar plates (Römling *et al*., 1998).

5.7 Structural genes of Cellulose synthase operon in *Z. mobilis*

In *Z. mobilis*, cellulose synthase catalytic subunit (*bcsA*), cellulose synthase regulator protein (*bcsB*), cellulose synthase operon C domain-containing protein (*bcsC*) form the cellulose synthase operon. Likewise, in *G. xylinus* strains, there has been report of cellulose biosynthesis operon comprising of above genes (Saxena and Brown, 1995). In *G. xylinus*, the *bcsA* and *bcsB* genes occur as single ORF as cellulose synthase (Kimura *et al*., 2001). *bcsC* has been reported in cellulose-producing bacterium such as *A. tumefaciens, G. xylinus* and *R. leguminosarum* bv. *trifolii* strains (Standal *et al*., 1994).

Genome sequencing of cellulose producing bacterium such as *Pseudomonas* spp. also revealed the presence of cellulose biosynthesis operon consisting of cellulose synthase catalytic subunit (*bcsA*), cellulose synthase regulator protein (*bcsB*), cellulose synthase operon C domain-containing protein (*bcsC*) encoding genes on their chromosome (Amikam and Benziman, 1989).

In *Z. mobilis*, cellulose synthase catalytic subunit shows the presence of characteristic motifs namely D, D, D, QRXRW motif, XAKAGNLN motif, QTP motif and FFCGS motif. These motifs have been reported in prominent cellulose producers. In *Agrobacterium* spp. Stasinopoulos *et al*., (1999) reported the presence of characteristic D, D, D35Q (R,Q) XRW motif, an indistinguishable HAKAGN(L,I)N motif, and QTP motifs.

5.8 The cellulose synthase regulator protein has a transmembrane domain

In *Z. mobilis*, cellulose synthase regulator protein (YP_162819) possess transmembrane domain at the C-terminus, and is inferred to bind c-di-GMP, the regulator of cellulose biosynthesis. One transmembrane domain located at the C-terminus has been determined by diverse algorithms for every BcsB protein reported so far (Mayer *et al*., 1991).

Cellulose synthase catalytic subunit and cellulose synthase regulator protein were predicted to be localized in cytoplasmic membrane. In *Z. mobilis*, cellulose synthase catalytic subunit and cellulose synthase regulator protein were predicted to be localized in cytoplasmic membrane. In *G. xylinus,* the cellulose synthase (BcsA) and the c-di-GMP binding protein (BcsB) are localized in the cytoplasmic membrane (Kimura *et al*., 2001).

5.9 ZMO0904 codes for a beta-galactosidase

The deduced amino acid sequence from the ORF ZMO0904 indicated high percentage identity to the beta-galactosidase of *Caulobacter segnis.* The ORF encodes a protein with predicted molecular weight of 114 kDa. The molecular weight of *Z. mobilis* Bgal is comparable with that of the *L. bulgaricus* beta-galactosidase characterized by Schmidt *et al*., (1989). Moreover, this protein had the signature sequence of GH family 35. The conserved domain database analysis of *Z. mobilis* Bga revealed the presence of the domains for glycosyl hydrolase superfamily 42 (Henrissat and Bairoch, 1993) and beta-galactosidase domain 2. Multiple sequence alignment of Bgal indicated the presence of conserved glutamate amino acid which is considered as the active site (Fowler *et al*., 1983). The three dimensional structure of *Z. mobilis* Bgal generated using SWISSMODEL indicated the presence of five

distinct domains similar to the structure of the *Penicillium* sp. beta-galactosidase characterized by Rojas *et al*., (2004).

Beta-galactosidase genes have been cloned using genomic library construction and PCR based methods (Schmidt *et al*., 1989). Genomic library construction was used for isolation and cloning of the beta-galactosidase gene of *Lactobacillus bulgaricus* (Schmidt *et al*., 1989), *Streptococcus thermophilus* (Herman and McKay, 1986) and *Pseudomonas haloplanktis* (Hoyoux *et al*. 2000). Recently Wang *et al*. (2010) used a metagenomic approach to isolate beta-galactosidase gene. In the present study, the ORF ZMO0904 was PCR amplified and cloned in the expression vector. Similarly, Vian *et al*., (1998) used PCR based cloning to express and purify the *Thermus* sp beta-galactosidase. Using a novel approach, chimeric beta-galactosidase was constructed by fusing the BgaA protein to the choline-binding domain of the major pneumococcal autolysin. The hybrid enzyme was purified in a single step by affinity chromatography on DEAE-cellulose.