slimy material that can anchor them to a variety of materials including metals, plastics, soil particles, medical implant materials, and tissue. Microbial biofilm formation is known to be a sequential bacterial development process (O'Toole et al., 2000; Stoodley et al., 2002) and is regulated by a series of genetic and phenotypic determinants (O'Toole et al., 2000; Stoodley et al., 2002; Sauer et al., 2002; Davey et al., 2003). Simple screening methods such as isolation of biofilm defective mutants have contributed greatly to understanding the genetics of biofilm developmental process and added significant information into the genetic basis of biofilm development.

II. Definition of Biofilm

A number of working definition has come over the year. The structure and composition of the biofilm being different from one environmental niche to another, the definition of biofilm is ambiguous. Costerton et al. (1987) described biofilm as a complex matrix containing nutrient channels analogous to the tissues of higher organisms. Carpentier and Cerf (1993) described it as "a community of microbes embedded in an organic polymer matrix, adhering to a surface'. Percival et al. (2000) recognized biofilm as "microbial cells immobilized in a matrix of extracellular polymers acting as an independent functioning ecosystem, homeostatistically regulated". Microbially derived biofilm community is now currently defined and characterized by Donlan and Costerton (2002) as follows:

-Cells that are irreversibly attached to the substratum or interfaces or to each other.

-Cells that are embedded in a matrix of extracellular polymeric substances that they have produced, and

-Exhibit an altered phenotype with respect to growth rate and gene transcription.

III. Biofilm mode of growth

The development of biofilm is a complex and multistage process which is dependent on a variety of variables including the type of microorganism, the surface of attachment, environmental factors and expression of biofilm specific genes (Carpentier and Cerf, 1993; Dunne, 2002). Initial adhesion of bacteria to the abiotic surfaces is primarily non-specific (e.g. involving hydrophobic) interaction, whereas adhesion to living tissue involves molecular interaction with ligand, lectin or adhesin (Carpentier and Cerf, 1993; Dunne, 2002). The initial adhesion between bacteria and abiotic surface is generally said to be reversible and occurs between conditioned surface and planktonic cell. After the successful attachment to the surface, the bacteria aggregate together and produce an extracellular

polysaccharide matrix (Costerton et al., 1999), which encases the cells together in a biofilm. A mature biofilm consists of heterogeneous microbial community, interstitial voids and water-filled channels within the matrix (Sutherland 2001). These channels are known to promote the influx of nutrients and the efflux of waste products. Occasionally some individual cells from the mature biofilm are released; these then disperse and multiply rapidly, in so doing they colonize other places. The formation of mature biofilm is influenced by a number of biological factors and hydrodynamics features (Stoodley et al., 2002). The biological factors include cell-to-cell signaling between the biofilm bacteria, the growth rate of the bacteria, extent of EPS production, motility of the biofilm bacteria as well as possible competition and cooperation between the bacteria (Kolari, 2003).

IV. Developmental stages in biofilm formation

The process of development of bacterial biofilm has been reviewed by many authors (Costerton et al., 1995; O'Toole et al., 2000; Reisner et al., 2003; Hall-Stoodley et al., 2004; Watnick and Kolter, 2000; Parsek and Fuqua, 2004; Stoodley et al., 2002; Donlan and Costerton, 2002; Davey and O'Toole, 2000) and found to be similar in most of the organisms including *Escherichia coli* , *Pseudomonas fluorescence* (Pratt and Kolter, 1998; Reisner et al., 2003; Tremoulet et al., 2002), *Pseudomonas aeruginosa* (Stoodley et al., 2002; Sauer et al., 2002), *Vibrio cholerae* (Watnick and Kolter, 1999), *Staphylococcus aureus* (Fux et al., 2003). The development process consists of some basic steps including initiation, microcolony formation and maturation along with EPS production and finally dispersal of individual cells so as to continue the process at some other locations. Palmer and White (1997) described biofilm development as a five stage process including (i) Development of surface conditioning film (ii) Movement of organism into closer proximity with the surface (iii) Adhesion (reversible and irreversible adhesion of the microbe to the conditioned surface) (iv) Growth and division of the organisms with the colonization of the surface, microcolony formation and biofilm formation; phenotype and genotype changes (v) Biofilm cell detachment/ dispersal. According to Sauer et al. (2002) the five stages of biofilm development includes i) reversible attachment ii) irreversible attachment iii) maturation-1 iv) maturation-2 and v) dispersal. Biofilm formation *in vitro* involves the following five stages (Stoodley et al., 2002; Annous et al., 2009):

1. Reversible adhesion of bacterial cells to a surface.
2. Irreversible adhesion involving the formation of exoploymeric material.
3. Formation of microcolonies and the initiation of biofilm formation.

4. Maturation of biofilm with a three-dimensional structure consisting of cells packed in clusters with channels between the clusters that allow transport of water and nutrients and waste removal.
5. Detachment and dispersion of cells from the biofilm and initiation of new biofilm.

Fig. 1: Developmental stages of biofilm.(1) Initial attachment; (2) Irreversible attachment with EPS formation; (3) Formation of microcolonies; (4) Maturation with the formation of voids and water channels; (5) Dispersal

Initial adherence to the surface (reversible and irreversible adhesion to the surface)

The initial adhesion of bacterial cells to the surface consists of two-steps, reversible and irreversible (Zobell, 1943). The reversible attachment is a temporary process and involves weak interactions such as Lifshitz-van der Waals and Lewis acid-base which are considered important for the initial attachment of bacteria (Israelachvili, 1992.),while the irreversible permanent attachment involves the expression of specific bacterial adhesins (Whittaker et al., 1996). The bacterium possesses many surface appendages which aid in the initial adhesion to the solid surface; these include pili, flagella, fimbriae and EPS. In *Pseudomonas aeruginosa,* flagella and type IV pili facilitates the early attachment (O'Toole and Kolter, 1998). *Staphylococcus epidermidis* and *S. aureus* produces a polysaccharide intercellular adhesion, which is related with cell-to-cell adhesion and biofilm formation (Crampton et al., 1999; Heilmann et al., 1996). In *Acinetobacter baumanii,* pilli plays a critical role in the initial adhesion to a surface (Gaddy and Actis, 2009). Fimbriae and antigen 43 are found to be important adhesins in *Escherichia coli* (Houdt and Michiels, 2005). In *Vibrio cholerae* flagella and fimbriae are necessary for initial adherences to solid surfaces (Heithoff and Mahan, 2004).

Another important phenomenon in the initial adhesion of bacteria to abiotic surfaces is the surface conditioning (Carpentier and Cerf, 1993; Korber et al., 1995). Surface conditioning is the deposition of organic and inorganic matter onto the surfaces immersed in aquatic environment (Loeb and Neihof, 1977; Characklis and Escher, 1988; Taylor et al., 1997). In aquatic or terrestrial environment the conditioning layer is made up of complex polysaccharides, glycoproteins and humic compounds (Chamberlain, 1992; Marshall et al., 1971; Baier, 1980; Rittle et al., 1990). Generally most bacteria moving from the aqueous phase towards a surface have their initial contact with conditioned surfaces. The formation of conditioned film may alter the physico-chemical properties of the surface such as surface free energy, hydrophobicity and electrostatic charges (Dickson and Koohmarare, 1989) and thus affect the microbial attachment process (Characklis et al., 1990; Vaudaux et al., 1994).

Microcolony formation, EPS production and maturation of biofilm

After the initial attachment to the surface, irreversible adhesion is continued through the secretion of extracellular cementing substance known as extracellular polymeric substances (EPS). This polymeric substance also provides the structural framework for the biofilm. The association of EPS in bacterial attachment has been well documented by many authors (Corpe, 1970; Marshall et al., 1971; Czaczyk and Myszka, 2007). The EPS may account for 50% to 90% of the total organic carbon of biofilm (Flemming et al., 2000) and is primarily composed of polysaccharide. In a number of gram negative bacteria the polysaccharide is neutral or polyanionic. The anionic nature is thought to be due to the presence of uronic acids or ketal-linked pyruvate which is thought to enhance the binding of divilant cations such as calcium and magnesium, thus allowing greater strength to the biofilm (Davey and O'Toole, 2000). Apart from polysaccharides the EPS may consist of different classes of macromolecules such as glycoprotein, protein, cellulose, lipid, glycolipid and nucleic acid (Czaczyk & Myszka, 2007; Sutherland, 2001; Branda et al., 2005).

Further the surface attached bacterial cells multiply resulting in microcolony formation and maturation of the biofilm. The formation of micrcolonies has been well studied in *P. aeruginosa* (Harmsen et al., 2010). The cellular multiplication and aggregation in *P. aeruginosa* results in the formation of mushroom-shaped microcolnies with interconnecting void channels that are thought to be involved in nutrient transport and waste efflux within the microcolonies. These mushroom-shaped multicellular structures form due to a maturation process that requires cell-to-cell signaling (Costerton et al., 1999; O'Toole et al., 2000; Stoodley et al., 2002). During the maturation process, the gene expression pattern of attached cell undergoes significant changes from the free floating (planktonic) cell.

More than 300 proteins have been detected in biofilm cells of *P. aeruginosa*, that were absent in the planktonic cell (Sauer et al., 2002; Whiteley et al., 2001).

Bacterial cell-to-cell communication: Quorum sensing

The role of quorum sensing in the regulation of biofilm has been first reported by Davies et al. (1998) which initiated a period of active research in the cell-to-cell signaling in biofilms. He showed that *lasI*-mutant cells of *P. aeruginosa* that were unable to synthesize the QS signaling molecule [3OC12-HSL (3-oxododecanoylhomoserine lactone)] produced undifferentiated biofilm architecture and are also sensitive to biocide SDS. Supplementation of *lasI*- mutant cells with 3OC12-HSL resulted in architecture similar to the wild type biofilm. The process of cell-to-cell communication in bacterial population is known to occur via small diffusible signaling molecules recognized as autoinducer. These signal molecules are produced by the bacterial cells and their concentration in the environment depends on the population density. When a threshold concentration is reached, the signal can induce other bacteria leading to the induction or repression of certain target genes.

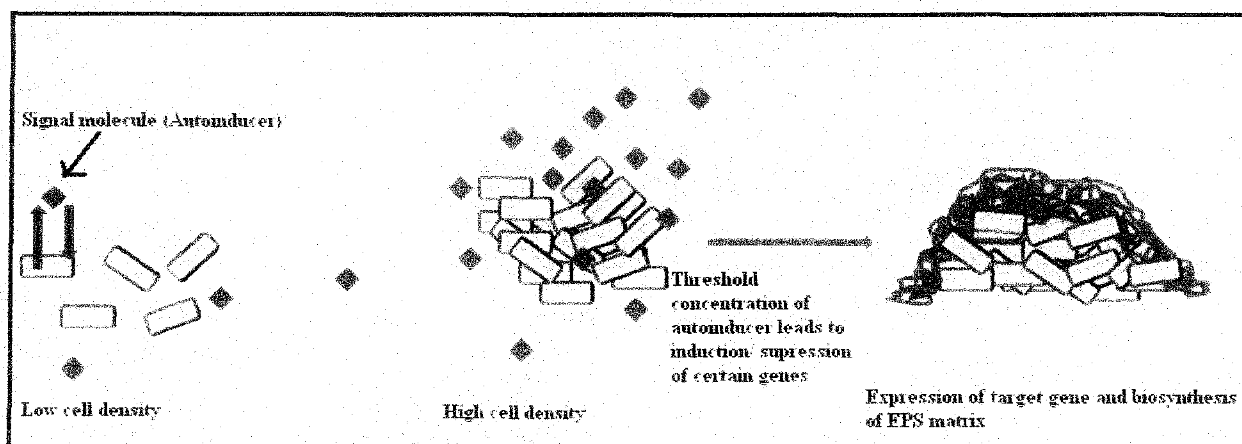

Fig. 2: Bacterial quorum sensing and EPS synthesis.

This phenomenon of cell-density-dependent gene regulation is most commonly known as quorum sensing (QS). The chemical nature of signaling molecules involved in the quorum sensing is diverse, however gram negative bacteria most commonly employs *N*-acylhomoserine lactones (AHLs). For example species of *Acidithiobacillus, Acinetobacter, Aeromonas, Agrobacterium, Brucella, Burkholderia, Erwinia, Enterobacter, Chromobacterium, Hafnia, Mesorhizobium, Methylobacter, Paracoccus, Pseudomonas, Ralstonia, Rhodobacter, Rhizobium, Rhanella, Serratia, Sinorhizobium, Vibrio* and *Yersinia* (Williams et al., 2007) are known to use AHLs as their major signaling molecule. Not only in the biofilm formation but also in the dispersal, quorum sensing plays a significant role. In

Rhodobacter sphaeroides (mutant cells) the addition of 7,8-cis-tetradecenoyl-HSL to the cell aggregate resulted in cell dispersion leading to the growth of free individual cells in suspension (Greenberg, 1999). Similarly, O'Toole et al. (2000) reported the decrease of *P. fluorescens* biofilm and failure of EPS synthesis in presence of AHLs and/or another factor in stationary-phase culture supernatants. These studies suggest the possible role of AHL signals in biofilm dispersal.

Dispersal

Like all other organisms, surface attached biofilm must release and disperse cells into the surroundings in order to colonize a new site. Dispersal of cells from the biofilm is an essential step to continue the biofilm mode of the life cycle. The molecular mechanism of bacterial biofilm dispersal is another fascinating area of research that may result in the development of novel agents that inhibit biofilm formation or promote cell detachment. A general mechanism of cell dispersal in many bacterial biofilm involves a spontaneous process, such as erosion or sloughing of cells caused by the hydrodynamic shear force of aqueous medium (Stoodley et al., 2001a; Stoodley et al., 2001b). Bacterial cells are also routinely dispersed from the biofilms via an active dispersal event where sessile, matrix-encased biofilm cells are converted into free-swimming, planktonic form. Mechanisms involved in the active dispersal of biofilm cells are yet to be completely explored and little is known about the regulatory pathways involved in the release of bacterial cells from the biofilm. Processes now known to play a role in biofilm dispersal include the release of hydrolytic enzymes by the biofilm growing cell and subsequent breakdown of biofilm matrix (Boyd and Chakrabarty, 1994; Xun et al.,1990; Allison et al.,1998), and the production of surfactants which loosen cells promotes detachment from the biofilm (Davey et al., 2003). Dispersal can also be under certain regulatory process involving cyclic di-GMP levels inside the cells (Simm et al., 2004), that are central regulators between the transition from sessile (biofilm) to motile (planktonic) phenotype (or vice-versa), and signal molecules produced by the bacteria (quorum sensing) such acyl homoserine lactones (Rice et al., 2005). Other physico-chemical parameters like oxygen or carbon substrate concentration, and pH has also been reported to trigger dispersion of mature biofilms in various organisms, including *P. aeruginosa*, *P. putida*, and *Shewanella oneidensis* (Applegate and Bryers, 1991; Gjermansen et al., 2005; Thormann et al., 2005; Tolker-Nielsen et al., 2000). Recently the production of free radicals such as nitric oxide (NO) by *P. aeruginosa* has been implicated with the dispersal process (Barraud et al., 2006). In a microarray study, Firoved et al. (2004) has revealed that certain genes involved in the adherence of *P. aeruginosa* were down regulated upon exposure to NO.

Cell lysis within the biofilm is yet another process of dispersion which is best understood in *P. aeruginosa* and *Pseudoaltermonas tunicate* (Webb et al., 2003; Mai-Prochnow et al., 2006). In many biofilms, cell death regularly occurs with spatial organization inside mature microcolonies, and kills only a fraction of cells within the biofilm. It is proposed that the autolysis leads to destabilization and disruption of biofilm architecture and thus promote conversion of surviving cells to the motile dispersal phenotype (Webb, 2007). In *P. aeruginosa*, inside microcolonies, prophage-mediated cell death has been reported as an important mechanism of differentiation that assists dispersal of a subpopulation of surviving cells (Webb et al., 2003).

V. Protection from the Environment

The biofilm provide a shelter and homeostasis to the organisms living inside it and the important component of this shelter is the matrix made up of extracellular polymeric substance. This matrix has the potential to prevent the influx of certain antimicrobial agents thereby restricting the diffusion of these compounds from the environment into the biofilm (Gilbert et al., 1997). EPS has shown to have metal binding property and thus can sequester toxic metal ions and provide protective functions (Geddie and Sutherland, 1993; Wolfaardt et al., 1999). In addition to metal binding ability, the EPS can also sequester nutrients and minerals from the environment. This binding property of EPS is primarily due to the presence of ionizable functional groups such as carboxyl, phosphoric, amine, and hydroxyl groups in the EPS (Rudd et al., 1984). Mittleman and Geesey (1985) found that the purified EPS from the capsule of a freshwater sediment bacterium is capable of binding copper. Farag et al. (1998) reported the concentration of metals (Ar, Cd, Pb, Hg, and Zn) in different food web components including bacterial biofilms of the Coeur d'Alene River Basin in Idaho. In addition, various other authors have reported the stimulatory effect of metal ions on the biofilm formation. Lapaglia and Hartzell (1997) observed an induction of biofilm in the growing culture of *Archaeoglobus fulgidus* when exposed to high concentration of copper and nickel. Bereswill et al. (1998) reported an increased in the production of amylovoran; the main polysaccharide of EPS in *Erwinia amylovora*, in the presence of copper. Ordax et al. (2010) showed that the EPS extract, from *E.amylovora* can bind copper ions and thus concluded that the EPS favours the survival of *E. amylovora* under copper stress. Similar observations of increase in EPS production in the presence of metal stress have been reported for other bacterial species (Morita, 1997; Wolfaardt et al., 1999; Kidambi et al., 1995; Kazy et al., 2002; Iyer et al., 2004). EPS is also known to provide certain degree of protection to the biofilm cells from various environmental stresses,

such as UV radiation, pH shifts, osmotic shock, and desiccation (Flemming, 1993; Davey and O'Toole, 2000; Lewis, 2005; Allison and Mathew, 1992; Boke et al., 2010).

VI. Nutrient capture and metabolic co-operativity in a biofilm

The mature biofilm often contains voids and water channels that provide an increased surface area for nutrient exchange. As the water-channels are interconnected and goes deep into the biofilm, it ensures nutrient availability to microbial communities residing deep inside the biofilm. The biofilm traps the trace element and nutrient from external environment through physical trapping or electrostatic interaction (Stoodley et al., 1998).

The complex biofilm architecture also provides the opportunity for metabolic cooperation, and niches are formed within these structures. The microcolonies developed in these niches, that differ in their composition and metabolism. As these micrcolonies are arranged side by side, it provides an excellent opportunity for the exchange of substrate, removal and redistribution of metabolic end product. The cooperation and mutual dependence are well reviewed by Davey and O'Toole (2000).

VII. Horizontal gene transfer within biofilm

Biofilm provide a suitable niche in which bacteria of different microbial community can grow in close proximity to each other. This provides an area of hot spot for the exchange of extrachromosomal genetic elements like plasmid. Indeed, the transfer of plasmid DNA via conjugation occurs at higher frequency in the biofilm cells as compared to their planktonic counterparts (Ehlers and Bouwer, 1999; Roberts et al., 1999; Hausner and Wuertz, 1999). The horizontal transfer of conjugative plasmid adds to the development and stabilization of biofilm (Hausner and Wuertz, 1999; Ghigo, 2001; Molin and Tolker-Nielsen, 2003; Reisner et al., 2006; Burmølle et al., 2008). Since plasmid may have genes that provide resistance against many antimicrobial agents, biofilm formation also provides a mechanism for the spread of bacterial resistance to antimicrobial agents.

Conjugal transfer of DNA (plasmid) is not the only mechanism of gene transfer in a microbial biofilm, another mechanism such as transformation can also be expected, as a quantity of DNA is also found in biofilm structure. This DNA is thought to be released in the biofilm matrix by the lysis of bacterial cells as found in the case of *Streptococcus pneumoniae* and *Acinetobacter calcoaceticus* (Steinmoen et al., 2002; Palmen and Hellingwerf, 1995). The dense population inside the microcolonies of biofilm also provides an excellent opportunity for the uptake of this extracellular matrix DNA. Hendrickx et al. (2003) observed a high frequency transformation in the young and actively growing biofilm of

Acinetobacter sp. BD413 and correlated the increased transformation frequency with the DNA concentration and found no saturation.

VIII. The bacterium *Acinetobacter junii*

Acinetobacter junii belongs to the genus *Acinetobacter*. The genus *Acinetobacter* is composed of diverse aerobic, gram-negative, non-fermenting bacteria belonging to the Gammaproteobacteria. *Acinetobacter* species are commonly found in the environment and have been isolated from water, soil, human skin, vegetables and fruits (Baumann, 1968; Fournier and Richet, 2006).

Clinical significance

Acinetobacter species are generally considered nonpathogenic to healthy individuals. They have low virulence but are capable of causing infection in individuals with preexisting conditions or patients in high risk hospital. The species of Acinetobacter that have been associated with human infection and disease include *A. baumannii* and *A. calcoaceticus*, *A. lwoffi* , *A. johnsonii*, *A. ursingii*, *A. schindleri*. *A. junii* (Bouvet and Grimont, 1986) is a rare cause of disease in humans and was associated mainly with bacteremia in preterm infants and pediatric oncologic patients (Linde et al., 2002).

Biofilm formation is a common characteristic of many species of *Acinetobacter*. In *A. buamannii* biofilm formation is associated with environmental survival and infections associated with medical devices.

Biotechnological applications

Species of *Acinetobacter* have been attracting increasing attention in both environmental and biotechnological applications. Their biotechnological application has been well reviewed by Abdel-El-Haleem (2003). Some strains of this genus are known to be involved in biodegradation of a number of different pollutants such as biphenyl and chlorinated biphenyl, amino acids (analine), phenol, benzoate, crude oil, acetonitrile, and in the removal of phosphate or heavy metals. *Acinetobacter* strains are also well studied for the production of a number of extra-and-intracellular economic products such as lipases, proteases, cyanophycine, bioemulsifiers and several kinds of biopolymers.

The strain used in the present study *Acinetobacter junii* BB1A (LMG22734) is a metal-tolerant bacterium isolated from Torsa River of Northern West Bengal, India. The isolate BB1A was identified as a strain of *Acinetobacter junii* by Bhadra et al. (2006), following detailed analysis of morphological,

physio-biochemical and 16S rRNA gene sequence. The strain was found to be tolerant to Ni^{2+}, AsO^{2-}, Cd^{2+} and Hg^{2+} and form biofilm (Sarkar and Chakraborty, 2008).

The present study dealt with the following objectives:

IX. Objectives of the study

1. Standardization of current basic protocol for studying biofilm.
2. Effect of substrata and different stress components on biofilm formation.
3. Chemical characterization of extracellular polysaccharide involved in biofilm formation.
4. Relate the role of metal stress in inducing biofilm formation.
5. Review the role of quorum sensing in biofilm formation.
6. Isolation of mutant defective in biofilm formation.
7. Identification of genetic loci responsible for biofilm formation.

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