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Antibiotic Resistance, Biofilms and Quorum Sensing in *Acinetobacter* Species

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1. Introduction

Acinetobacter is a Gram-negative coccobacillus that is strictly aerobic, nonmotile, catalase positive and oxidase negative. It is ubiquitous in nature, being found in soil and water. Members of the genus *Acinetobacter* have now clearly emerged as opportunistic nosocomial pathogens (Forster et al., 1998). Bacteremia, pneumonia, meningitis, urinary tract and surgical wound infections are the most common infections caused by this organism (Cisneros et al., 2002; Dijkshoorn et al., 2007). The taxonomy of the genus *Acinetobacter* has undergone extensive revision during the last two decades, and at least 31 named and unnamed species have now been described (Dijkshoorn et al., 2007). Of these, *Acinetobacter baumannii* and the closely related unnamed genomic species 3 and 13 sensu Tjernberg and Ursing (13TU) species were the most clinically relevant. In recent years, multidrug-resistant (MDR) *A. baumannii* are increasingly held responsible for nosocomial infections and MDR *A. baumannii* clones are spreading into new geographic areas with increasing number of strains acquiring many resistance genes (Navon venezia et al., 2005). Unfortunately, newer extended-spectrum β-lactamases and different carbapenemases are emerging fast, leading to pan-resistant strains of *A. baumannii*.

A. baumannii appears to have the propensity for developing multiple antimicrobial resistances extremely rapidly. This bacterium has shown a remarkable tendency to develop resistance to virtually every antibiotic class (Henwood et al., 2002). The emergence and quick dissemination of multiple drug resistant (MDR) *A. baumannii* and its genetic potential to carry and transfer diverse antibiotic resistance determinants pose a major threat in hospitals world-wide. The complex interplay of MDR clones, its rapid spread, their persistence through biofilm formation, their regulation by quorum sensing (QS), transfer of resistance elements and other interactions are contributing to the increasing woes and creating additional difficulties in treating infections caused by these organisms. This review article mainly focus on antibiotic resistance in *Acinetobacter*, the current understanding of biofilm production and its correlation with antibiotic resistance as well the quorum sensing mechanisms in *Acinetobacter* species.

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2. Antibiotic resistance in *Acinetobacter* spp.

A. baumannii is considered the paradigm of multi-resistant bacteria as the organism has an ever-increasing list of resistance determinants that can rapidly nullify most of the therapeutic armamentarium. Both acquired and intrinsic resistance mechanisms can contribute this multi-resistance. The ability to acquire such resistance for multiple drugs may be due to either the acquisition of genetic elements carrying multiple resistant determinants or mutations affecting the expression of porins and/or efflux pump(s), which can minimize the activity of unrelated antimicrobial agents (Vila, 2007). It is also indicated that the outer membrane of *Acinetobacter* spp. acts as a substantial barrier against the penetration of these antibiotics. The results of one of the earliest studies suggest that one of the causes for the high antibiotic resistance of *Acinetobacter* is attributable to the presence of a small number of small-sized porins (Sato et al., 1991). Apart from this, it was also shown earlier that the amount of *Acinetobacter* porin was less than 5% of the Total outer membrane proteins (OMP), while that of *E. coli* it was reported to be about 60% (Rosenbusch, 1974) that contributes to reduced permeability. The most widespread β-lactamases with carbapenemase activity in *A. baumannii* are carbapenem hydrolysing class D β-lactamases mediated by OXA genes that are most specific for this species. In addition, metallo-β-lactamases have now been reported worldwide that confer resistance to all β-lactams except aztreonam (Dijkshoorn et al., 2007). Resistance to aminoglycosides in *A. baumannii* is mediated principally by aminoglycoside-modifying enzymes (AME's). Further, multidrug efflux pump such as AdeABC may have a role in aminoglycoside resistance (Wieczorek et al., 2008). Quinolone resistance is often caused by modifications in the structure of DNA gyrase secondary to mutations in the quinolone resistance determining regions of the *gyrA* and *parC* genes.

The main underlying resistance mechanisms to multiple antibiotics in *Acinetobacter* sp. can be summarily outlined as follows (i) production of hydrolysing enzymes for e.g. β-lactam hydrolysis by different kinds of β-lactamases (Class A to D β-lactamases), (ii) changes in penicillin-binding proteins (PBPs) that prevent action of β-lactams, (iii) alterations in the structure and number of porin proteins that result in decreased permeability to antibiotics through the outer membrane of the bacterial cell and (iv) the activity of efflux pumps that further decrease the concentration of antibiotics within the bacterial cell. But, among these β-lactamases, OXA- and metallo-carbapenemases seem to be more significant with their increasing incidence when compared to other β-lactamases (Livermore et al., 2006).

2.1 Resistance to β-lactam antibiotics

Resistance for β-lactams in *Acinetobacter* is been associated with the production of β-lactamases.

2.1.1 β-lactamases

Resistance due to the expression of hydrolysing enzymes such as cephalophorinases and amber class A–D β-lactamases remains as one of the extensively studied and skilful resistance mechanism among the species of *Acinetobacter*. These enzymes to some extent hydrolyze carbapenems along with other β-lactams. The most common carbapenemases detected in *A. baumannii* were either Class B β- lactamases such as metallo β- lactamases

(MBL) or class D β- lactamases (also referred as carbapenem hydrolyzing oxicillinases (CHDLs)) (Livermore, 2007). While class A carbapenemases have been frequently detected in bacteria belonging to *Enterobacteraceae* family, they were not usually found in *Acinetobacter* spp. However, *A. baumannii* producing extended-spectrum β-lactamases (ESBLs) have been reported, though it is not a common phenomenon (Livermore & Woodford, 2006). As there are emerging reports of arrival of newer broad spectrum β- lactamases such as New Delhi metallo-beta lactamase -1 (NDM-1) (Karthikeyan et al., 2010) among Gram-negative pathogens including *Acinetobacter* and their progressing hydrolysing abilities makes this group of Gram-negative bacterial pathogens as superbugs by assisting them to survive in extreme conditions. The genes that code for multiple resistances are reported to be plasmid as well as chromosomally encoded.

2.1.2 A- Class

A. baumannii, like *Pseudomonas aeruginosa*, produces a naturally occurring AmpC β-lactamase, together with a naturally occurring oxacillinase with carbapenemase properties. ESBLs are plasmid-mediated β-lactamases of predominant class A. ESBLs are capable of efficiently hydrolyzing penicillin, cephalosporin, the oxyimino group containing cephalosporins (cefotaxime, ceftazidime) and monobactams (aztreonam). β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) generally inhibit ESBL producing strains. A wide range of class A ESBLs have been reported in *Acinetobacter* sp. such as TEM, SHV, CTX-M, GES, SCO, PER and VEB. However, these resistant determinants are not universally present in *Acinetobacter*, as there are only isolated reports of them. Some of the documented ESBLs world-wide are PER-1 from Turkey, Korea, Russia, Romania, Belgium and France; VEB-1 from France and Belgium; TEM-116 and SHV-12 from China and The Netherlands; CTX-M-2 from Korea; *bla* Shv-5 –EBSL, TEM -92 from Italy and VEB -1 from Northern France and Belgium (Naiemi et al., 2005; Nass et al., 2006, 2007; Endimiani et al., 2007). Nevertheless, they were not as common as MBL and CHDL in *Acinetobacter* species.

2.1.3 B- Class

Carbapenemases are the most versatile of all β-lactamases and many of them recognize almost all hydrolysable β-lactams. The most common hydrolyzing enzyme carbapenemases found in *A. baumannii* belong to either the class B family of beta-lactamases such as MBLs (IMP/VIM) or the OXA class D family of serine β-lactamases (Poirel, 2006). Class B β-lactamases are also referred as MBLs has the highest level of carbapenem-hydrolyzing activity among the three classes of carbapenemases. MBLs have been identified in many Gram-negative bacteria including *Acinetobacter* genomic species 13 TU and *A. baumannii* and are resistant to the commercially available β-lactamase inhibitors but susceptible to inhibition by metal ion chelators. Potent class B metallo-carbapenemases of the IMP, VIM, SIM and NDM type have been found in *A. baumannii*.

There are numerous existing reports on IMP type of MBL in *Acinetobacter* spp (Lee et al., 2003; Livermore, 2007). In *A. baumannii,* six IMP variants belonging to three different phylogroups have been identified and reported namely IMP-1 in Italy, Japan and South Korea; IMP-2 in Italy and Japan; IMP-4 in Hong Kong; IMP-5 in Portugal; IMP-6 in Brazil and IMP-11 in Japan (Poirel and Nordmann, 2006). In addition, IMP-4 has been identified in clinical isolates of *Acinetobacter junii* in Australia (Peleg et al., 2006). On the contrary, there

are only few studies that have documented MBL VIM type in *Acinetobacter*. In fact more than 100 clinical isolates screened by our group showed non-existence of VIM in this part of world. Surprisingly, *P. aeruginosa* isolates collected from the same hospital in our region showed the presence of VIM type of MBL and there was no cross transmission observed (Unpublished data). VIM-2-producing *Acinetobacter* spp. have been isolated in the Far East (Lee, et al., 2003) and in Germany (Toleman, 2004), while the VIM-1 determinant has been reported only in Greece (Tsakris 2006). One study recently identified VIM-4, which is nothing but a point mutant of VIM-1 and that has been previously identified only in *Enterobacteriaceae* (Luzzaro et al., 2008) and *Pseudomonas* spp. (Pournaras et al., 2003). This report on MBL VIM-4 determinant in *Acinetobacter* spp., emphasizes the fact that CHDLs are not the solitary factor for emergence of resistance to carbapenems in this genus. Interestingly, *bla*VIM-4 was identified in a non-*A. baumannii* isolate, thereby indicating that clinically insignificant Gram-negative bacterial species may also be reservoirs for MBL-encoding genes. It is also noteworthy that the occurrence of VIM-4 in *Acinetobacter* in a country that has reported VIM-4 in *P. aeruginosa* previously (Pournaras et al., 2003). Concurrently, it was also observed in Greece that *bla*VIM-1 which is widespread in *P. aeruginosa* had apparently crossed the species barrier to reach *Acinetobacter* spp. Such examples might be yet another example of resistance genes crossing genus barrier (Tsakris et al., 2006).

A small number of reports are available on other MBL types such as SIM-1, NDM-1 encountered in *Acinetobacter* spp (Lee et al., 2005, Karthikeyan et al., 2010). Recently, a novel acquired MBL gene namely *bla*SIM-1 was detected in clinical isolates of *A. baumannii* from Korea (Lee et al., 2005). This SIM-1 is encoded by a class 1 integron-borne gene cassette and is more closely related to IMP-type enzymes than to other MBLs. Very recently new β–lactamase such as NDM-1 has been reported in *A. baumannii* (Karthikeyan et al., 2010). Interestingly, in this report NDM-1–positive isolate was also positive for both OXA-23 and IMP. The *bla*NDM-1– positive strain was more resistant to antibiotics than the strains that were harbouring both OXA-23 and IMP. Fortunately, it was found that this *bla*NDM-1–positive *A. baumannii* strain was susceptible to several fluoroquinolone antibiotics and to polymyxin B (Chen et al., 2011).

2.1.4 D- Class

The most common carbapenemases detected in *Acinetobacter* are CHDLs that are also referred as Class-D oxacillinases. Among the nine clusters of carbapenem hydrolysing oxacillinases, four have been identified to date in *A. baumannii*. These included members of OXA-23, -24, -51, and -58 families. In addition, recently a novel class D enzyme named OXA-143 has been reported from Germany. OXA-58 oxacillinase was the first enzyme to be identified in an *A. baumannii* isolate in France and subsequently this has been reported among *A. baumannii* isolates in several countries (Coelo et al., 2006). Contrary to many workers, one investigation opined that the carriage of OXA-58 but not of OXA-51 β-lactamase gene correlates with carbapenem resistance in *A. baumannii* (Tsakris, 2007). In one of the studies, epidemiologically unrelated *Acinetobacter* isolates that were positive for the presence *bla*OXA-51- and *bla*OXA-58-like carbapenemase genes was also shown to carry the *bla*VIM-1 in a class 1 integron, which is of much concern (Tsakris, 2008).

Recently, a new OXA class D β–lactamase Oxa-97 has been reported in Tunisia which belongs to Oxa58-like (subgroup) in Africa (Poirel et al., 2008). In one instance, a novel Oxa-

143- CHDL in *A. baumannii* (Higgins et al., 2009) which is not associated with insertion sequence (IS) elements or integron features has been reported, which is bracketed by 2 replicase genes and its incorporation was shown to be by homologous recombination. Oxa-143 is a class D carbapenemase is similar to OXA-66/OXA-51-like enzyme that contributes to imipenem resistance, which was first reported from Taiwan. Off late, OXA-72 oxacillinase has been also reported in several carbapenem resistant *A. baumannii* isolates in Taiwan (Lu et al., 2009).

It has also been discovered that *bla*OXA-51-like genes may be associated with carbapenem resistance in isolates with an adjacent copy of insertion sequence (IS) ISAbA1 (Turton et al., 2006). IS elements presumed to enhance β-lactamase gene expression by providing additional promoters. Repeated observations such as ISAba1, ISAba2, ISAba3, ISAba4 IS elements being often found upstream of the different β- lactamases genes in *A. baumannii* can be taken as evidence for such assumption (Chen et al., 2008; Poirel 2006a. 2006b & 2008). In addition, one recent work demonstrated that a plasmid-borne CHDL with appropriate upstream ISs was enough to confer a high level of carbapenem resistance in *A. baumannii*. Moreover, a *bla*OXA-58 gene with an upstream insertion of a truncated ISAba3 and IS1008 was detected on a plasmid obtained from a clinical carbapenem resistant isolate in one of the studies (Chen et al., 2008). Acquisition of a plasmid-borne *bla*OXA-58 gene with an upstream IS1008 insertion is also shown to confer a high level of carbapenem resistance to *A. baumannii* (Chen et al., 2008). Therefore, as observed for the natural *bla*AmpC gene of *A. baumannii*, ISAba1 might provide promoter sequences that enhance expression of associated genes. These promoter sequences are probably extremely efficient in *A. baumannii*, so that insertion of ISAba1 upstream of *bla*OXA-51-like genes might represent a true mechanism of carbapenem resistance, or at least decreased susceptibility. Hence, it is sensible to believe that the association of *bla*OXA -51 like genes with IS elements may have a role in increasing carbapenem resistance. At least in one instance, it was conclusively shown that the reduced susceptibility to carbapenems was related to selection of the ISAba1-related overexpression of *bla*OXA-66 that belongs to *bla*OXA-51 subgroup (Figueiredo et al., 2009).

2.2 Modifications in target proteins

2.2.1 Penicillin binding protein

Carbapenem resistance in *A. baumannii* may be because of penicillin binding proteins (PBP) or porin modifications. The penicillin-binding domains of PBPs are transpeptidases or carboxypeptidases involved in peptidoglycan metabolism. Reduced expression level of PBP was observed in multidrug resistant strains in order to resist the activity of antibiotics. Some of the strategies adopted by *A. baumannii*, which have been uncovered, are the acquisition of an additional low-affinity PBP, overexpression of an endogenous low-affinity PBP and alterations in endogenous PBPs by point mutations or homologous recombination.

One recent study strongly indicated an association between down-regulation of PBPs and/or alteration in PBPs for β-lactam resistance in *A. baumannii* (Vashist et al., 2011). In this study, it was shown that one of the PBP designated PBP-7/8 is critical for the survival of *A. baumannii* strain AB307–0294 in the rat soft tissue infection and pneumonia models. Furthermore, it was shown PBP-7/8 either directly or indirectly contributes to the resistance of this strain to complement-mediated bactericidal activity (Russo, 2009).

2.2.2 *gyr*A and *par*C

Quinolone resistance is often caused by modifications in the structure of DNA gyrase secondary to mutations in the quinolone resistance determining regions (QRDR) of the *gyr*A and *par*C genes. DNA gyrase and DNA topoisomerase IV encoded by *gyr*A and *par*C genes respectively, are among the housekeeping genes involved in DNA replication and processing are the targets for ciprofloxacin and other fluoroquinones. A point mutation on the *gyr*A gene (Ser-83 to leu) was observed in MDR strains of *A. baumannii* which is consistent with fluoroquinolone resistant phenotype. Sequencing of the *par*C gene also indicated mutations in the *par*C gene that caused an amino acid change at either Ser-80 or Glu-84 (Deccache et al., 2011)

2.3 Alternations in permeability

2.3.1 Changes in OMPs & porins

Reducing the transport of β-lactam into the periplasmic space via changes in porins or OMPs reduces the access to PBPs. The outer membrane in MDR *A. baumannii* is less permeable to antimicrobial agents than that in other susceptible ones. Alternations in permeability characteristics disturbs the β-lactam assimilation into the periplasmic space, resulting in the weak activity of antibiotics. Several porins, including the 33-kDa CarO protein, that constitute a pore channel for influx of carbapenems, might be involved in such resistance. Sometimes disruption of OMP genes by ISAba10 element may lead to the inactivation of the OMPs like CarO thereby reducing the extent of which the antibiotic enters the cell. When the chromosomal locus containing the *car*O gene was cloned from clinical isolates and characterised, it was shown that only a single copy of *car*O, present in a single transcriptional unit, was present in the *A. baumannii* genome. The *car*O gene encodes a polypeptide of 247 aminoacid residues, with a typical N-terminal signal sequence and a predicted trans-membrane β-barrel topology (Siroy et al., 2006). Remarkably, many recent studies have revealed that disruption of the *car*O gene by the IS elements such as ISAba1, ISAba125, or ISAba825 results in loss of activity of CarO OMP leading to carbapenem resistance in *A. baumannii* (Mussi et al., 2005; Poirel et al., 2006). Many recent reports of outbreaks caused by carbapenem resistant phenotypes and their characterization having revealed the loss or reduction of porins such as OMPs of 22-29 kDa, 47, 44, and 37kDa and one of 31 to 36 kDa substantiates the findings of many previous investigations on OMPs. Additional gene expression studies, along with phenotypic characterization, of these membrane proteins will conclusively clarify the role of membrane permeability in β-lactam resistance.

2.4 Efflux pumps

Efflux pumps are the ones among the well studied mechanisms of resistance in *A. baumannii*, by which the bacterial cells overcome the action of antibiotics by expelling them out. For example the 3.9-Mb genome of *A. baumannii* AYE is reported to harbour 46 open reading frames (ORFs) encoding putative efflux pumps of different families (Fournier et al., 2006). The over expression of efflux pump genes have been reported in the antibiotic resistant strains which provides the evidence for the role of efflux pumps in making the bacteria multi-drug resistant. To date, five classes of efflux pumps have been reported to be present

in *A. baumannii* such as ATP binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), resistance–nodulation–cell division (RND) and small multidrug resistance (SMR).

The efflux systems in *A. baumannii* that are completely characterized functionally so far include AdeABC, AdeFGH and AdeIJK (RND type), AbeM (MATE type), and CraA (MFS type) (Peleg, 2008; Roca, 2009; Damier-Piolle, 2009). We have only partial knowledge on the functionality of ABC and SMR efflux pumps (Iacono et al., 2008; Srinivasan et al., 2009)

2.4.1 RND type efflux pump

The RND class efflux pumps that are commonly found in Gram-negative bacteria are usually tripartite in nature, i. e. they comprise of three protein components such as cytoplasmic, inter-membrane or membrane fusion protein (MFP) and peripalsmic or outer membrane protein which are encoded by three different genes present in a single operon. The cytoplasmic protein is otherwise termed as transporter protein which is involved in the export of substrates such as drugs or antibiotics from the cell, MFP and OMP help in export mechanisms. Different classes of RND family efflux pumps have been reported till date in *Acinetobacter* sp. Among these *ade*ABC, *ade*FGH and *ade*IJK functions and specificities have been studied extensively and overexpression of all these efflux pumps is controlled by two-component regulatory systems such as sensor and regulator kinase cascade.

In *A. baumannii*, AdeABC is one of the common types of efflux pumps which are involved in posing resistance to antibiotics such as aminoglycosides, β-lactams, chloramphenicol, tetracyclin, trimethoprim, erythromycin and drugs such as ethidium bromide (Magnet et al., 2001; Peleg et al., 2008). However, many studies seemed to indicate that the presence of *ade*ABC and *ade*DE is species specific, wherein *ade*ABC is being restricted to *A. baumannii* and adeDE to *Acinetobacter* genomespecies 3 (Chau et al., 2004). Contrastingly, one recent study for the first time showed the involvement of AdeABC pump in a non-*A. baumannii* strain and this study also described it in detail and characterized this pump. This investigation had also revealed that all three types of RND pumps coexist in non-*A. baumannii* strains (Roca et al., 2011). In AdeABC pump, AdeB is the multidrug transporter protein, AdeA is the membrane fusion protein and AdeC is the OMP. The efflux transporter AdeB captures the substrates either from within the phospholipid bilayer or the cytoplasm and then transports them out via OMP (AdeC). The periplasmic protein AdeA acts as an intermediate component which acts as an overpass between AdeB and AdeC components. AdeR-S two-component system is likely to control the expression of AdeABC type pumps. Further, point mutations in components of AdeABC and its regulatory proteins have been associated with overexpression of AdeABC leading to multidrug resistance (Marchand et al., 2004).

One study supports the hypothesis that the increased expression of *ade*B is associated with increased MICs of tigecycline. However, in the absence of an *ade*B gene knockout experiments, it is difficult to ascertain the overall contribution of the AdeABC efflux pump to tigecycline nonsusceptibility (Peleg et al., 2007; Hornsey et al., 2010). But, one recent study demonstrated that overexpression of the *ade*ABC efflux pump resulted in tigecycline nonsusceptibility by quantizing transcripts of the *ade*B gene and

demonstrating conversion of the tigecycline resistance pattern in the presence of an efflux pump inhibitor without any previously known mutation (Sun et al., 2010). When the isolates were analysed separately, there was an association between a higher MIC and elevated *ade*ABC expression, although more isolates would need to be investigated to confirm this observation.

AdeIJK is the second RND type efflux pump reported in *A. baumannii*, in which *ade*I, *ade*J, and *ade*K genes encode the MFP, transporter and outer membrane components of the pump, respectively. This type of pumps are found to be involved in exporting β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, safranin, pyronine and sodium dodecyl sulphate (Piolle et al., 2008).

The third RND type efflux pump is AdeFGH, which was found to be functional in the mutant in which AdeABC and AdeIJK were non-functional. In one clinically relevant study, it was shown that the increased expression of AdeFGH in *A. baumannii* is an additional mechanism for high-level resistance to fluoroquinolones and decreased susceptibility to tigecycline. The efficiency of AdeFGH pump is less when compared to the other type of efflux pumps because its overexpression was not reported during antibiotic stress and is found be constitutively expressed in the cells. AdeL, a LysR type regulator controls the expression of AdeFGH operon. The presence of the *ade*FGH operon in 90% of the strains was shown in one study (Coyne et al., 2010). This work also revealed that overexpression of *ade*FGH is likely due to point mutation in *ade*L, suggesting that this event may possibly occur in all clinical strains under selection pressure. More molecular and biochemical studies on the transcriptional regulator AdeL should allow better understanding of the mechanism of AdeFGH expression in *A. baumannii* (Coyne et al., 2010)

2.4.2 MFS type efflux pump

Major facilitator superfamily (MFS) acts as efflux pumps to decrease the intracellular concentrations of multiple toxic substrates and confer multidrug resistance. TetA and TetB efflux pumps from the MFS, involved in the tetracycline and minocycline resistance in *A. baumannii*. Many believe that MFS efflux pump is also responsible for the intrinsic chloramphenicol resistance described in *A. baumannii* strains, and therefore it was suggested that it can be named CraA, for **c**hloramphenicol **r**esistance ***A****cinetobacter* (Magnet et al., 2001; Peleg et al., 2008). Recently, a novel efflux pump AmvA (Methyl Viologen resistance) that mediates antimicrobial and disinfectant resistance in *A. baumannii* has been characterized (Rajamohan et al., 2010). AmvA is known to be responsible for the transport of toxic substances such as acridine orange, acriflavine, benzalkonium chloride, DAPI, deoxycholate, ethidium bromide, methyl viologen, SDS and tetraphenylphosphonium chloride(TPPCl). In yet another study, two different MFS type efflux pumps such as CmlA and CraA that are specific for chloramphenicol resistance have been reported (Roca et al., 2009).

2.4.3 MATE type efflux pump

The MATE (Multidrug and Toxic Compound Extrusion) family is the most recently categorized, one among the five multidrug efflux transporter families. There are almost twenty different types of MATE type transporters reported in bacteria. A proton driven

MATE family of efflux pump AbeM is reported in *Acinetobacter* which was characterized to be responsible for exerting resistance to kanamycin, erythromycin, chloramphenicol, tetraphenylphosphonium chloride (TPPCl), norfloxacin, ciprofloxacin and trimethoprim (Su et al., 2005)

2.4.4 SMR type efflux pump

One most recent study for the first time described the role of the SMR efflux pump in *Acinetobacter* spp (Srinivasan et al., 2009). The regulatory protein of this pump AbeS mediates resistance to various antibiotics, hydrophobic compounds, detergents, and disinfectants in *A. baumannii* strain AC0037 (Srinivasan et al., 2009). The SMR type pump is composed of four transmembrane α-helices of approximately 100–140 amino acids in length driven by H+ gradient. A related study concluded that the coupling ion in the AbeM pump is H+ and not Na+. It is worthwhile to note that some H^+ - norfloxacin antiport activity is seen earlier in vesicles of *E. coli* KAM32/pUC18 (Su et al., 2005).

2.4.5 ABC transporters

ATP Binding Cassette (ABC) transporters form a special family of membrane proteins, characterized by homologous ATP-binding and large, multispanning transmembrane domains. Several members of this family are primary active transporters. Whole cell proteome analysis of *Acinetobacter* has revealed the presence ABC transporters which are proposed to be responsible for the transport of ferric ion and drug resistance (Iacono et al., 2008).

2.5 Aminoglycoside-modifying enzymes (AMEs)

Resistance to aminoglycosides by AMEs is also a major threatening feature which leads to resistant phenotypes which shows resistance to aminoglycoside antibiotics such as gentamycin, kanamycin and streptomycin in *Acinetobacter* spp. All three classes of aminoglycoside-modifying enzymes reported have been found in *Acinetobacter*. These enzymes are the O-nucleotidyltransferases (ANT) and O-phosphotransferases (APH) that catalyse the nucleotidylation (adenylation) and phosphorylation of the hydroxyl groups and finally the N-acetyltransferases (AAC) that catalyse acetylation of amino groups thereby rendering the antibiotics inactive. Studies have shown that the genes encoding all these enzymes to be present on plasmids, transposons or within integron-type structures.

In summary, emergence of MDR *A. baumannii* isolates that are resistant to almost all available antibiotics are a serious problem in clinical settings. More ominously, pan drug-resistant (PDR) and extremely drug-resistant (XDR) *A. baumannii* isolates that have been recently emerged (Park et al., 2009). As a consequence, colistin is now considered as a therapy of last resort against MDR *Acinetobacter* infections (Nation & Li, 2009). Unfortunately, colistin resistance has also been reported now (Adams et al., 2009). The overexpression of components of PmrAB two-component system such as *pmr*B and/or *pmr*A appear to be only partially responsible for colistin resistance as shown by Park et al (Park et al., 2011). All kinds of mechanisms of antimicrobial resistance in *Acinetobacter* species have been clearly illustrated in Figure – 1.

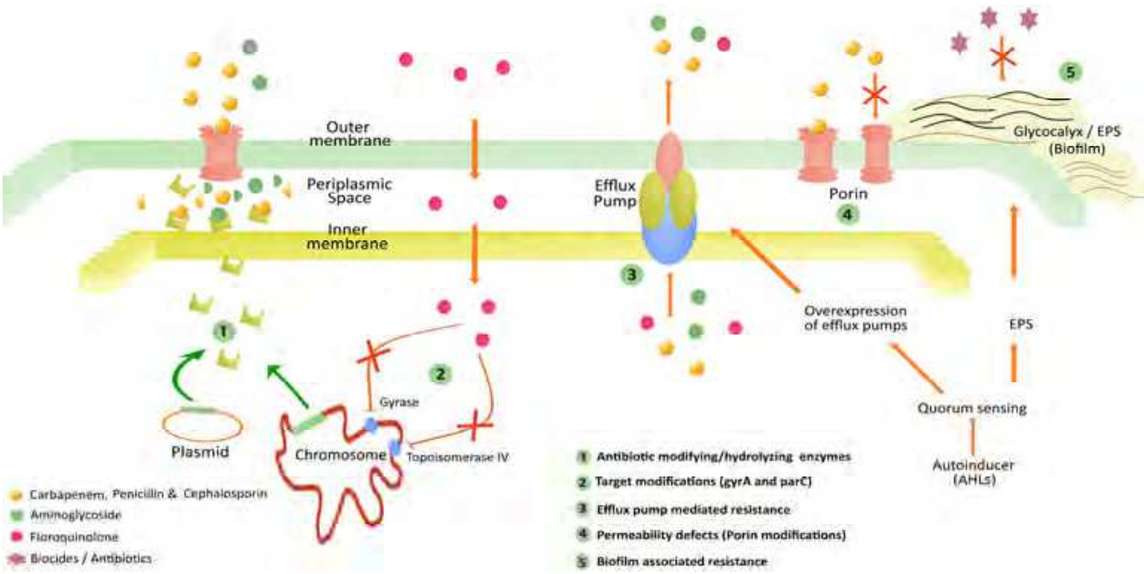


Fig. 1 Potential mechanisms of antimicrobial resistance in *Acinetobacter* species. General depiction of different kinds of antimicrobial resistance mechanisms operating in A*cinetobacter* spp. Five types resistance mechanisms are illustrated in the figure, which is of self explanatory

Finally, more experiments are in need that elucidates the performance of gene knockout studies particularly, knockout of the genes for β-lactamases and efflux systems and restoration of the genetic support for deficient mechanisms (e.g., porins) will further define their roles in *Acinetobacter* clinical isolates.

3. Biofilms and antibiotic resistance

The ability of *A. baumannii* to adhere to and form biofilms on biotic and abiotic surfaces (inanimate objects) may explain its success in the hospital environment. Biofilms might contribute to the environmental persistence of *Acinetobacter* leading to host infection and colonization

3.1 Bacterial biofilms

The bacterial biofilm have been in nature since very long but, it was not until 1970s that science could decipher and appreciate the biofilm lifestyle of bacteria. Biofilm is a complex aggregation of microorganisms, wherein the cells are embedded in a self-produced matrix of extracellular polymeric substance (EPS). The new definition of a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, 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 (Donlan & Costerton, 2002). It is now becoming clear that aggregation of bacterial cells are natural assemblages of bacteria within the biofilm matrix and it functions as a cooperative consortium, in a relatively complex and coordinated

manner. Biofilm phenotype of a pathogen promotes increased colonization and persistence and therefore is the leading cause for device-related infections. The ability of these pathogens to adhere to human tissues and medical devices and produce biofilms is a major virulence factor that correlates with increase in antibiotic resistance, reduced phagocytosis, and overall persistence of the bacterial population. Moreover, these biofilms are notoriously difficult to eradicate and are a source of many recalcitrant infections. The medical importance of the scientific studies of biofilms and its architecture resides more in our ability to explain the characteristics of device-related infections and other chronic infections and to design strategies to counter their refractory nature.

3.1.1 Biofilms and resistance

The mechanisms by which biofilms contribute to reduced susceptibility still remain unclear, but a number of different explanations have been proposed. Biofilms are inherently resistant to the antimicrobial agents, reasons being failure of an agent to penetrate full depth to cells of biofilm or cells slow growing state due to the organism's slow metabolism. For chemically reactive disinfectants such as chlorine, iodine and peroxygens, and for highly charged antibiotics, such as the glycopeptides, the glycocalyx does indeed greatly affect the ability of the antimicrobial agent to reach those cells that are deep within the biofilm. On the other hand, for relatively unreactive, uncharged agents, such as the β-lactams, such reaction-diffusion limitation is unlikely to occur. The glycocalyx may however, contribute to reduced susceptibility to β-lactams, if the antibiotic is susceptible to inactivation by β–lactamases and if the β-lactamase is derepressed while the bacterium is in the biofilm mode of growth. In such cases, the enzyme is concentrated within the extracellular polymer matrix and hydrolyses the drug as it penetrates (Gilbert & Brown, 1998). Reduced susceptibility to β-lactams amongst biofilm bacteria is more likely to be a function of a diminished growth rate within the deeper recesses of the biofilm which causes the expression of penicillin-binding proteins that are unrepresentative of those normally targeted by these antibiotics (Gilbert & Brown, 1998). Retarded growth also affects the bactericidal action of the β-lactams because transpeptidase inhibition, which induces cellular injury, is directly related to growth rate. One investigation revealed that gene transfer in biofilms occurs far more frequently than previously noticed (Hausner & Wuertz, 1999) and horizontal gene transfer inside a biofilm matrix offers a great advantage in terms of both frequency and stability (Hausner & Wuertz, 1999). The knowledge that attached cells of the same species differ in their ability to maintain incoming plasmids hints at specific physiological conditions in biofilms which lead to individual cells experiencing different environmental pressures.

3.1.2 Biofilm cycle – a multistep process

Attachment to abiotic surface is mainly dependent on cell surface hydrophobicity, whereas surface proteins mediate adhesion to host matrix-covered implants. After adhesion to the surface, exopolysaccharide, specific proteins and accessory macromolecules aid in intercellular aggregation (Otto, 2009). At critical cell density, cells co-ordinate through a communication pathway involving signalling molecules, termed quorum sensing (QS), resulting in biofilm formation (Costerton et al., 1999). Further, at a later stage, due to physical forces or intercellular signalling, the cells detach and disperse to colonize new areas (Costerton et al., 1999).

3.1.3 Biofilm and disease

While many biofilm infections are "stealthy," in that they develop slowly and initially produce few symptoms, they may be very damaging because they promote immune complex sequelae and act as reservoirs for acute exacerbations in hosts. Many a times host immune response products of oxidative bursts rarely penetrate the biofilm matrix accounting for the inability of phagocytes to destroy the pathogen (Costerton et al., 1999). The exact processes by which biofilm associated organisms elicit disease in the human host are poorly understood. However, suggested mechanisms include: (i) detachment of cells or cell aggregates from indwelling medical device biofilms, resulting in bloodstream or urinary tract infections, (ii) production of endotoxins and (iii) resistance to the host immune system (Peleg et al., 2008). One should begin to examine any infection that is refractory to antibiotic therapy and to host defences in terms of the genes that are expressed to produce the refractory bacterial phenotype. Furthermore, one must begin to use the biofilm phenotype of each chronic pathogen in the development of new vaccines and antibiotics aimed at biofilm-specific targets that can be the means of controlling burgeoning group of diseases caused by biofilm phenotype.

3.2 Biofilm development mechanisms in *A. baumannii*

There are three important factors which contribute to the persistence of *A. baumannii* in the hospital environment, namely: resistance to major antimicrobial drugs, resistance to desiccation and resistance to disinfectants. This survival property is most likely to play a significant role in the outbreaks caused by this pathogen (Tomaras et al., 2003). The potential ability of *Acinetobacter* to form biofilms may explain its outstanding antibiotic resistance against a wide range of antibiotics (Rao et al., 2008; Dijkshoorn et al., 2007; Donlan & Costerton, 2002). *A. baumannii* has the ability to colonize both abiotic and medical devices (Tomoras et al., 2003) and form biofilms that display decreased susceptibility to multiple antibiotics (Uma Karthika et al., 2008). Adherence of *A. baumannii* to human bronchial epithelial cells and erythrocytes has already been demonstrated, with pilus like structures appear to be important for adherence (Gospodarek et al., 1998, Lee et al., 2006). This process is considered to be a first step in the colonization process of *A. baumannii*. Survival and growth on host skin and mucosal surfaces requires the clones that can resist inhibitory agents and the conditions that are exerted by these surfaces. Outgrowth on mucosal surfaces and medical devices, such as intravascular catheters and endotracheal tubes can result in *A. baumannii* biofilm formation, which enhances the risk of infection of the bloodstream and airways (Tomoras et al., 2003). One of the studies had showed that the common source of *Acinetobacter* bacteremia is intravascular catheters and the colonization of respiratory tract (Cisneros et al., 2002). Interestingly, it has also been demonstrated that biofilm formation in *Acinetobacter* is phenotypically associated with exopolysaccharide (EPS) production and pilus formation (Tomoras et al., 2004). The protein equivalent to CsuE of *Vibrio parahaemolyticus*, a chaperone has been identified as a key factor in pilus and biofilm formation in a pioneer study (Tomoras et al., 2004). Surprisingly, considerable variation in quantitative adherence was observed among different strains of *A. baumannii* isolated from the same geographical region (Lee et al., 2006). This observation of varying degree of adherence among the strains is in concordance with our studies (unpublished data). Our earlier investigation also demonstrated a high propensity among the clinical isolates of *A.*

baumannii to form biofilm and a significant association of biofilms with multiple drug resistance (Rao et al., 2008). Thus, biofilm production by *A. baumannii* promotes increased colonization and persistence leading to higher rates of device related infections. Identification of new genes involved in biofilm formation is required for better understanding of molecular basis of strain variation and various pathogenic mechanisms implicated in chronic *Acinetobacter* infections.

3.2.1 Factors associated with *A. baumannii* biofilm formation

3.2.2 Poly-β-(1, 6)-N-acetlyglucosamine (PNAG)

One of the important polysaccharides is poly-β-(1, 6)-N-acetlyglucosamine (PNAG), which has been now portrayed as a major component of biofilms of bacteria was first described in the genus *Staphylococci* (Maira-Litran et al., 2002). PNAG seems to be having profound effects on host-microbe interactions. PNAG affects colonization, virulence, and immune evasion in infections caused by both Gram-positive and Gram-negative species (Itoh et al., 2008). Apart from its role in surface and cell-to-cell adherence (Cramton et al., 1999), PNAG is described as an important virulence factor (Kropec et al., 2005) and provides protection against the antibiotics and shown to protect *Staphylococci* against innate host defences (Lewis 2001 and Voung et al., 2004). Pga locus encodes for the proteins involved in the synthesis and translocation of PNAG on to the bacterial surface (Kropec et al., 2005; Shiro et al., 1995; Vuong et al., 2004). In *S. aureus*, PNAG confers resistance to killing mediated by innate host immune mediators. Overall, PNAG production by *S. aureus* appears to be a critical virulence factor as assessed in murine models of systemic infection (Kropec et al., 2005). PgaB and IcaB (from *Staphylococci*) contain polysaccharide N-deacetylase domains belonging to carbohydrate esterase family 4. PNAG is also shown to be essential for the formation of the nonrandom or periodic cellular architecture in *E. coli* biofilm microstructure and for conversion from temporary polar cell surface attachment to permanent lateral attachment during the initial stages of biofilm development (Itoh et al., 2008). PgaB of *pga*ABCD peron of *E. coli* is predicted to be an outer membrane lipoprotein. The *hms* locus in *Yersinia pestis*, which is equivalent to PNAG operon apparently promotes the transmission of the plague bacillus *Y. pestis* from the flea vector to the mammalian host (Jarrett et al., 2004). PgaB ortholog in *Y. pestis* designated as HmsF, co-purifies with the outer membrane fraction in this bacterium.

PNAG, the most important EPS secreted by the bacterial population also forms the major component of the biofilms in *Acinetobacter* spp. (Choi et al., 2009). Recent study on *pga*ABCD of Gram-negative bacteria with the typical reference strain of *A. baumannii* showed that the four gene loci share a high degree of similarity with *E. coli* and *Y. pestis* (Choi et al., 2009). *A. baumannii pga*A encodes for a predicted 812-amino-acid OMP and it contains a porin domain suggesting that it facilitates PNAG translocation across the outer membrane and a superhelical periplasmic domain that is thought to play a role in protein-protein interaction (Itoh et al., 2008). PgaB is made up of 510 amino acids with a putative polysaccharide deacetylase domain. PgaB is an outer membrane lipoprotein that along with PgaA, is necessary for PNAG export (Itoh et al., 2008). *pga*C encodes for a 392-amino-acid N-glycosyltransferase that belongs to the glycosyltransferase 2 family. Gene *pga*D encodes for a 150 amino acid protein which localizes in the cytoplasm and assists PgaC in the synthesis of PNAG (Itoh et al., 2008). One recent investigation speculated that in a more dynamic

environment with higher shear forces, PNAG is more essential for maintaining the integrity of *A. baumannii* biofilms (Choi et al., 2009).

3.2.3 Biofilm-associated protein (Bap)

Biofilm-associated proteins (Bap) were first characterized in *S. aureus* (Cucarella et al 2001) and recent research findings indicated that *Acinetobacter* has a homologue of Bap protein of *Staphylococcus*. Bap family members are high-molecular weight proteins present on the bacterial surface, contain a core domain of tandem repeats, and play a critical role in cell-cell interactions and biofilm maturation (Loehfelm et al., 2008; Lasa & Penades, 2006). Bap is made up of 8620 amino acids, arranged in tandemly repeated modules A-E (Rahbar et al 2010). It has a higher proportion of negatively charged amino acids in the tandem repeats compared to non-tandem repeat parts (Loehfelm et al., 2008). As it has no transmembrane anchoring domain, its interaction with the cell wall is unclear and yet to be investigated.

The mechanism by which the Bap contributes to biofilm development is unknown, though their large size and the presence of a high number of repeats suggest that these proteins could mediate homophilic or heterophilic intercellular interactions (Lasa & Penades, 2006). Structural studies suggest that the main target for Bap is carbohydrates, for maintenance of biofilm complex (Rahbar et al., 2010). Time course confocal laser scanning microscopy and three-dimensional image analysis of actively growing biofilms demonstrate that Bap mutant is unable to sustain biofilm thickness and volume, suggesting a role for Bap in supporting the development of the mature biofilm structure. In *A. baumannii,* Bap is identified as a specific cell surface protein and is involved in intercellular adhesion within the mature biofilm. Future studies in *A. baumannii* must explore Bap-mediated interactions like direct mediation of intercellular adhesion from one bacterium to a surface receptor on a neighboring bacterium, autoadhesion between Bap molecules on adjacent bacteria and/or whether cells may be linked indirectly via shared interactions with some extracellular biofilm matrix component. However, one can hope that Bap can be a potential target to develop a novel vaccine that can abolish biofilm development (Rahbar et al., 2010).

3.2.4 Chaperone-usher secretion system

A. baumannii require chaperone-usher pili assembly for the production of biofilm on inanimate surfaces as revealed from the study of Tomaras et al (2003). This secretion system encodes for a putative pili-like structure/adhesion protein essential for the initiation of biofilm formation. The *csu* operon expressing chaperone-usher pili assembly comprised of a gene cluster that encompasses six ORFs: *csu*AB-A-B-C-D-E and is polycistronic in nature (Tomaras et al., 2003). The translational products of the *csu*D and *csu*E are highly related to chaperone and usher bacterial proteins, respectively, the four remaining ORFs encode hypothetical proteins potentially involved in pili assembly (Tomaras et al., 2003). The *csu* operon is regulated by a two-component system, *bfm*RS. BfmS is a sensor kinase, which senses environmental conditions and activates a response regulator encoded by *bfm*R. Over-expression of the *csu*AB operon is caused by higher BfmR intracellular concentration (Tomaras et al., 2003). Current models on biofilm formation clearly implicate the participation of bacterial surface related flagella and pili (O' Toole & Kolter, 1998) and cellular appendages (Tolker-Nielson et al., 2000). Bacterial cells in the biofilm community are linked to each other through extracellular appendages that resemble pili structures (Tomaras et al., 2003).

All the above data suggest that there may be an overlap in factors required for the initiation and maturation of biofilms on abiotic and biotic surfaces, bacterial attachment and pathogenesis *in vivo*. Though one can articulate that quorum sensing may be a central mechanism for autoinduction of multiple virulence factors such as genes those involved in the cell envelope, EPS production, pilus biogenesis, iron uptake and metabolism (Smith, 2007) and type IV virulence/secretion systems.

4. Quorum sensing in bacteria

Many bacteria use cell to cell communication to monitor their population density, synchronize their behavior and socially interact. Such communication used by the bacteria is chemical in nature and generally designated as quorum sensing (QS) which is nothing but a coordinated gene regulation and is generally termed as QS. Small diffusible molecules produced by bacteria are 'signals' which can reach other cells and elicit 'answers'. This phenomenon relies mainly on cell density and with the increase in cell density, a critical concentration of signaling molecule will be reached that allows sensing of the signalling molecule and enables the other bacteria to respond. QS is a type of community behaviour prevalent among a diverse group of bacteria to switch between planktonic phenotype to high cell density biofilm phenotype. Irrespective of either Gram-negative or Gram-positive bacteria, the process of QS is analogous in both the groups. The stepwise process involving intracellular synthesis of low molecular weight molecules and secrete them to the extracellular milieu. When the number of cells in a population increases, the concentration of QS molecules also increases and once the minimal threshold level crosses, the molecules are recognised by the receptors that trigger signal transduction cascades that result in a population wide change in gene expression. Such molecular cascades enable the population to function in harmony to survive and proliferate. Depending upon the bacterial species, the physiological processes regulated by QS are extremely diverse, ranging from maintaining the biofilms to regulating the antibiotic resistance. A flurry of research over the past decade has led to significant understanding of many aspects of QS molecules including their synthesis, the receptors that recognize the signal and transduce this information to the level of gene expression and the interaction of these receptors with the transcriptional machinery. Recent studies have begun to integrate QS into global regulatory networks and establish its role in developing and maintaining the structure of bacterial communities.

QS network in Gram-negative bacteria regulate the expression of specific sets of genes in a cell density-dependent fashion (Ng & Bassler, 2009). Pathogenic bacteria typically use QS in the regulation of genes encoding extracellular virulence factors. Gram-positive bacteria like *S. aureus* secrete small peptides for cell to cell communication. On the other hand Gram-negatives like *A. baumannii* predominantly produce small molecules like acylated homoserine lactones (Acyl-HSL) as QS entities. Sometimes other signalling molecules such as 2-heptyl-3-hydroxy-4-quinolone and diketopiperazines are also produced by Gram-negative bacteria (Holden et al., 2000)

4.1 Quorum sensing molecules

Acyl homoserine lactones (AHLs) are a major class of autoinducer signals used by Gram-negative proteobacteria for intraspecies communication that are best characterised till date. AHLs of QS signalling system seem to control diverse physiological functions such as

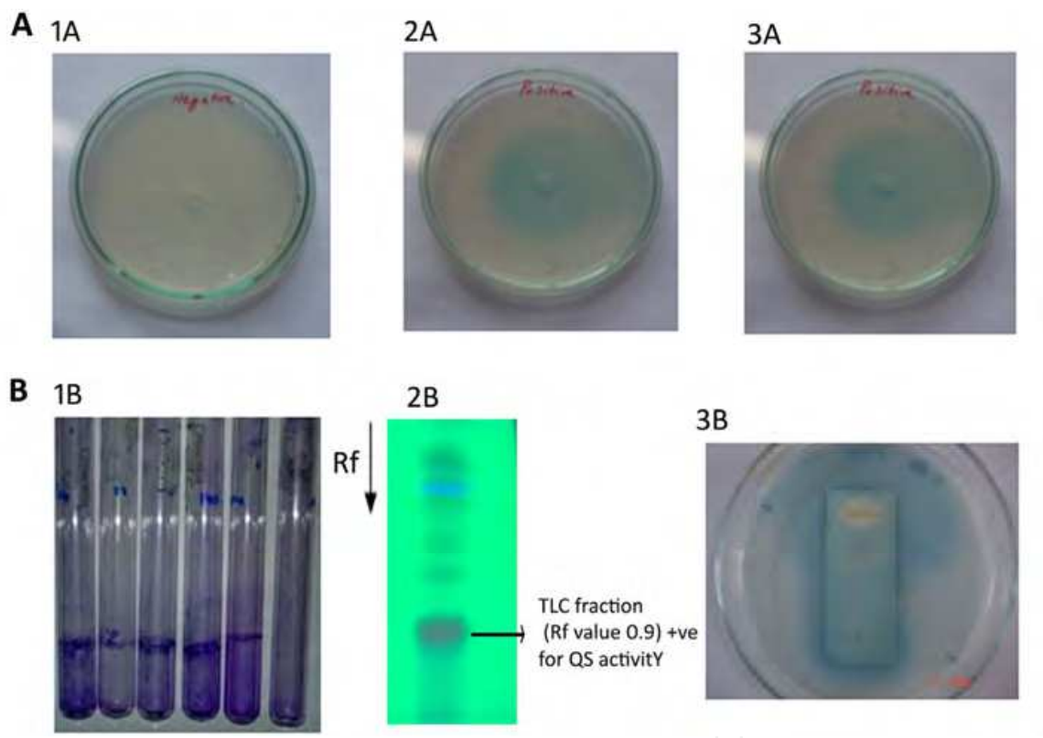
biofilm formation, Ti plasmid conjugation, production of antibiotics, and competence in certain bacteria (Fuqua et al., 2001; Antunes et al., 2010). AHLs are composed of HSL rings carrying acyl chains of C4 to C18 in length. These side chains harbour occasional modification, notably at the C3 position or unsaturated double bonds. The first AHL autoinducer and its cognate regulatory circuit has been first discovered in the bioluminescent marine bacterium *Vibrio fischeri*. Two proteins, LuxI and LuxR, are essential for QS control of bioluminescence in *V. fischeri*. The LuxI/LuxR regulatory system of *V. fischeri* is considered the paradigm for the control of gene expression by QS in Gram-negative bacteria. Homologs of *lux*I and *lux*R have been identified in a large number of bacterial genomes and these other LuxIR-type QS systems control global cell density dependent gene expression. In *V. fischeri*, LuxI is the synthase of the QS autoinducer N-3-(oxo-hexanoyl)-homoserine lactone (3OC6HSL). LuxI catalyzes acylation and lactonization reactions between the substrates S-adenosylmethionine (SAM) and hexanoyl-ACP. Following synthesis, 3OC6HSL diffuses freely in and out of the cell and its concentration increases as the cell density of the population increases (Stevens et al., 1994). LuxR is the cytoplasmic receptor for 3OC6HSL as well as the transcriptional activator of the luciferase operon. Without the 3OC6HSL ligand, the LuxR protein is unstable and is rapidly degraded. When 3OC6HSL accumulates, it is bound by LuxR and the LuxR-AHL complex recognizes a consensus binding sequence (*lux* box) upstream of the luciferase operon and activates its expression. Because expression of luxI is also activated by 3OC6HSL-bound LuxR, when the QS circuit engages, autoinducer production is induced, and the surrounding environment is flooded with the signal molecule. This autoinduction positive feedback loop is presumed to enforce synchrony as the population of cells switches from low cell density mode to high cell density QS mode (Stevens et al., 1994; Schaefer et al., 1996).

The QS networks are increasingly gaining importance in clinical isolates as they function as global regulators. One of the well studied organisms in clinical context is *P. aeruginosa*, which uses AHL as a QS signaling molecule. In *P. aeruginosa*, the QS network is found to play a major role in maintaining biofilm, this biofilm matrix in turn helps the bacteria to survive hostile conditions by becoming resistance to bactericidal agents, resisting nutrition depleted conditions and thereby helps them to remain persistent in hospital environment that makes complete eradication of this organism a challenging quest.

4.2 Quorum sensing in *A. baumannii*

Quorum sensing (QS) in *A. baumannii* appears to have a regulatory role in biofilm formation (Smith et al., 2007). Environmental survival and growth require attributes such as resistance to desiccation and antibiotics, versatility in growth requirements, biofilm forming capacity and possibly, QS activity (Dijkshoorn et al., 2007; Smith, 2007). QS has been shown to regulate a wide array of virulence mechanisms in many Gram-negative organisms (Antunes et al., 2010) and *Acinetobacter* is no different. The presence of QS has been inferred from the detection of a gene that is involved in autoinducer production (Gaddy et al., 2009) that could control the various metabolic processes, production of virulence factors, including biofilm formation. QS network in *Acinetobacter* is mediated by acyl homoserine lactones (AHL). Up to five different QS signal molecules that are more detectable (produced abundantly) during the stationary phase have been identified in *Acinetobacter*, indicating that this may be a

central mechanism for autoinduction of multiple virulence factors (Gonzalez et al., 2001;Joly-Guillou et al., 2005; Niu et al., 2008; Gonzalez et al., 2009). In one most recent study, different species of *Acinetobacter* were analyzed for the production of AHL and it was shown that QS sensors were not homogenously distributed among species, though one particular AHL was specifically present in most of the strains belonging to *A. calcoaceticus-A. baumannii* complex (Gonzalez et al., 2009). Furthermore, it was revealed that no distinction could be made between the QS signals secreted by typical opportunistic strains of the *A. calcoaceticus-A. baumannii* complex isolated from patients and strains belonging to other species of the genus (Gonzalez et al., 2009). In our investigation, we have also identified more than six different QS signal molecules in majority of the *A. baumannii* clinical isolates wherein chromatographic separation (Thin Layer Chromatography) of ethyl acetate extracts followed by β-galactosidase assay for determining QS activity using *A. tumefaciens* reporter strain NT1, containing plasmid pZLR4 carrying traR and a traG::lacZ reporter fusion was used. However, among these only one kind of QS molecule was produced abundantly (Figure – 2).



A:- Biosensor overlay test using reporter strain (*Agrobacterium tumefaciens* pZLR4) for detection of quorum sensing (QS) molecules; 1A – Negative control; 2A *A. tumefaciens* Positive control NTL4(pTiC58ΔaccR); 3A - QS activity positive reaction produced ethyl acetate extract of *A. baumannii* clinical isolate confirming the production Acyl Homoserine lactone
B:- 1B- Biofilm production in *A. baumannii* isolates detected through Tube method using 1% crystal violet stain. Thick violet ring was witnessed between liquid air interfaces; 2B - Thin layer chromatography (TLC) of crude ethyl acetate extract of *A. baumannii* culture supernatant; 3B - Ethyl acetate extracts obtained from *A. baumannii* culture supernatants were separated by TLC and over laid with *A. tumefaciens* (pZLR4).

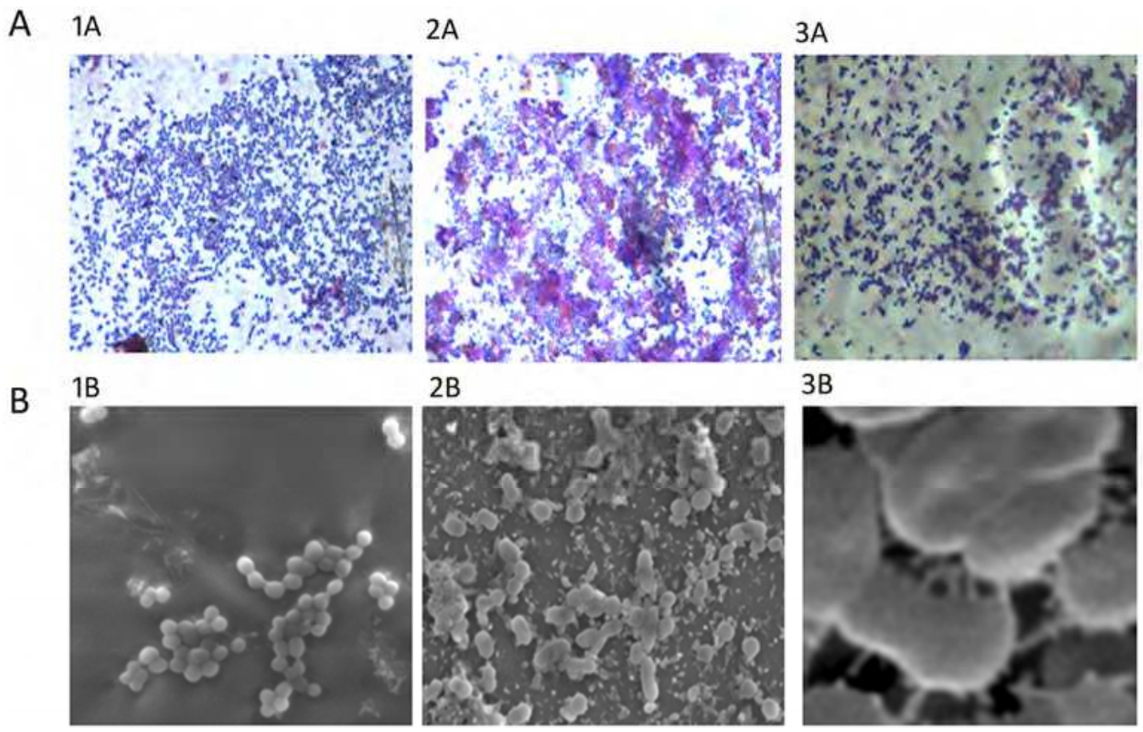
Fig. 2. Quorum sensing activity in *A. baumannii*

Another recent investigation on *A. baumannii* M2 strain characterized an AHL and one AHL synthase gene was identified, which was held responsible for predominant of kind QS molecule produced (Niu et al., 2008). Although additional AHLs were detected in this study, they were not able detect any other gene related to them. Hence, it appears that the auto inducer synthase that was discovered has low specificity and may be capable of synthesizing other QS signals as well. Some interesting questions arising out of above study are: do the diversity of QS signals observed respond only to particular synthase? or is there any existence of more than one AHL synthase?

However, since AHL signals produced by acyltransferases do not have similarity to LuxI or LuxM/AinS, it cannot be ruled out that additional AHL signals are present in *A. baumannii* M2. The AbaI protein was similar to members of the LuxI family of autoinducer synthases and was predicted to be the only autoinducer synthase encoded by *A. baumannii*. The expression of *aba*I at the transcriptional level was activated by ethyl acetate extracts of *A. baumannii* culture supernatants or by synthetic 3-hydroxy-C12-HSL. Further an *aba*I mutant failed to produce any detectable AHL signals and was impaired in biofilm development indicting that there is direct role QS molecules in biofilm development (Niu et al., 2008). QS machinery in *A. baumannii* appear to be mediated by a two component system AbaIR (Niu et al., 2008). This two-component system is homologous to a typical LuxIR family of proteins found in Gram- negative bacteria. This system includes a sensor protein AbaI that functions as an enzyme synthesizing AHLs and AbaR that functions as receptor by recognizing the AHL and induces a cascade of signaling pathway(s). QS was found to play a major role in biofilm maintenance and maturation in *Acinetobacter*. Niu et al (2008) have revealed that in abaI null mutants, there is about 40% reduction of biofilm and this was restored when AHL supplied externally. Consistent with this study, scanning electron microscopy (SEM) analysis data from our laboratory has shown biofilm formation in biofilm-negative clinical isolates when AHL was provided exogenously, as well there was enhanced biofilm formation in weakly adherent clinical isolates after such supplementation (Figure– 3). *A. baumannii* was found to produce more than 5 types of AHLs with varying fatty acid chains (Gonzalez et al., 2001 & 2009; Niu et al., 2008). The *aba*I autoinducer synthase was found to produce N-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C12-HSL) when cloned and expressed in *E. coli*. Genomic sequence analysis of *A. baumannii* ATCC 17978 has revealed that *aba*I and acyltransferase may be the core mediator for synthesis of AHLs with varying chemical nature. Such observations as whole underpin a positive correlation between QS and biofilm formation

A comparative study using *in silico* tools have shown that the autoinducer synthase gene *aba*I is more than 45% identical to autoinducer synthase gene from environmental non-pathogenic organisms like *Halothiobacillus neapolitanus*, *Acidithiobacillus ferrooxidans* ATCC23270 and less identical to RhlI and LasI system of pathogenic *P. aeruginosa* but about 47.3% identical to autoinducer synthase genes of an environmental strain *Pseudomonas* sp RW10S (Bhargava et al., 2010). These similarities and dissimilarities between environmental and clinical isolates clearly demonstrates how *Acinetobacter* as evolved from an environmental form to a pathogenic individual. Further, in-depth analysis has revealed that *A. baumannii* has more similarity with *Burkholderia ambifaria* at organism level and in stark contrast, its *aba*I gene shares similarity with *H. neapolitanus*. Similarly, *aba*R was found to

share more similarity to *H. neapolitanus* but it is unrelated to *B. ambifaria*. This can raise another question that *aba*I and *aba*R are two different genes yet they share similarity with homologs of another organism and it is because *aba*R is present just 63 base pairs upstream of *aba*I and it can be easily transferred as a single unit from one organism to the other.



1A- Growth of *A. baumannii* (biofilm-negative) on glass cover slip stained with 0.1% of crystal violet; 2A- Effect of N-AHL (200µM) extracted from *A. baumannii* biofilm phenotype on growth of biofilm non-producer; biofilm development can be observed. 3A - Effect of garlic extract on growth and biofilm formation; inhibition of biofilm was observed.
1B & 2B - SEM images of preparations similar to 1A & 2A respectively (X 5000 magnification); 3B magnified images showing cell to cell adherence through pili-like appendages (X 15000 magnifiction).

Fig. 3. A & B – Microscopic and scanning electron microscopic (SEM) analysis of effect of N-Acyl homoserine lactone (AHL) and garlic extract (Quorum quenching agent) on biofilm-negative strains of *A. baumannii* of clinical origin.

The likely lateral gene transfer between two distinct bacteria can be attributed to the natural competence of *A. baumannii* that has made them to acquire genetic information from other organisms. Interestingly, QS sensing is well known to increase competence in bacteria which further illuminates the importance of these chemical mediators.

A. baumannii being found to be a major threat in many hospitals, recent studies have clearly demonstrated the alarming need for an intense research on QS in *A. baumannii*. Pandrug resistance of *A. baumannii* is attributed to a number of antibiotic resistance mechanisms and biofilm formation. This biofilm formation is in turn regulated by QS networks, which make them to be considered as an important drug target to combat these multidrug resistant superbugs.

4.3 Quorum sensing and Antibiotic resistance

Multiple drug resistance can be attributed to a number of mechanisms, which includes synthesis of enzymes that degrade the drugs, modified targets that does not respond to the drug and presence of efflux pumps that pumps out the bactericidal drugs from the bacterial system to the extracellular milieu. QS was found to be regulating multidrug resistance in two ways, one involves up regulation of biofilm associated EPS matrix and other by up regulation of efflux pump genes.

The production of an EPS matrix is one of the distinguishing characteristics of biofilm and it has been suggested that EPS prevents the access of antibiotics into the bacterial community. Our investigations reveal that there is a strong association between multidrug resistance and biofilms wherein majority of our clinical isolates, which were strong biofilm producers were also exhibiting multidrug resistance (Rao et al., 2008). Compared to non-biofilm producers, biofilm producers showed a significantly higher resistance to cephotaxime, amikacin, ciprofloxacin and aztreonam. Thus, it is clear that clinical isolates of *A. baumannii* have a high propensity to form biofilm and there is a significant association of biofilms with multiple drug resistance. Further investigation showed that presence of antibiotic resistant determinant *bla*PER-1 is more critical for cell adherence, which is the first step in biofilm formation cycle. One of the success stories of *Acinetobacter* is its ability to withstand stress conditions like exposure to high dose of antibiotics. Previous studies on *Pseudomonas* have shown that exposure to the macrolide antibiotics found to enhance biofilm formation. Such responses suggest biofilm as a potential defence mechanism against antibiotics. Similar mechanism is seen in *A. baumannii* in which strong biofilm producers are commonly multidrug resistant. The role of QS molecule as a key player in antibiotic resistance can be understood from their mechanism of enhancing replication and transfer of plasmids, which are the major carriers of antibiotic resistant genes. Thus in a biofilm microstructure there is an increased possibility of gene transfer including genes for antibiotic resistance. Consequently, the biofilm forming capacity of *A. baumannii* combined with its multidrug resistance contributes to the organism's survival and further dissemination in the hospital settings.

Evidences for the role of QS in upregulating efflux pumps arise from studies in *E. coli* in which over expression of *E. coli* luxR homologue SdiA lead to the overexpression of AcrAB efflux pumps and its knockout lead to decrease in AcrAB efflux gene expression. This study clearly demonstrates how QS directly play an indispensable role in regulating efflux pump gene expression. SdiA as well regulates cell division in a cell density-dependent manner. It was also shown that SdiA controls multidrug resistance by positively regulating the MDR pump AcrAB and overproduction of SdiA confers multidrug resistance and increased levels of AcrAB. Conversely, *sdi*A null mutants are hypersensitive to drugs and have decreased levels of AcrB protein. These observations provide a direct link between QS and MDR achieved through efflux pump. Combined with earlier reports, this data support a model in which a role of drug efflux pumps is to mediate cell–cell communication in response to cell density (Rahmati et al., 2002). Now, it is clear that *sdi*A positively regulates the AcrAB efflux pump to mediate multiple drug resistance in *E. coli.*

In *P. aeruginosa*, when the cells are in the logarithmic growth phase, the MexR repressor negatively regulates *mex*AB-*opr*M efflux pump expression by binding at the MexR-MexAB-OprM operator-promoter region. As the cells enter the stationary growth phase, they sense a high population density and turn on a QS switch producing an autoinducer, C4-HSL, which

independently induces the expression of *mex*AB-*opr*M operon directly or it inactivates the MexR repressor, as a consequence it enhances the transcription of MexAB–OprM efflux pump (Maseda et al., 2004). This study also revealed that MexAB mutants accumulate 3O-C12-HSL intracellularly, which shows how QS signals form a part of efflux pump networks. In *A. baumannii*, antibiotic resistance is also brought about by a number efflux pump genes and the RND efflux genes which are found to share about 47% similarity with MexAB pumps are the major efflux pumps in *A. baumannii*. *A. baumannii* also produces C12-HSL compounds as QS molecules, which shows that there may be an interconnecting role between efflux pumps and QS that imparts multiple drug resistance.

To overcome stress, cells express various factors and one of them is RpoS which is regulated by the global regulator Hfq. In one of the studies conducted in *P. aeruginosa*, *las*R knockout mutants showed decreased resistance to ofloxacin, whereas the resistance was restored when RpoS was over expressed in *las*R knockouts. This finding suggests the strong role of stress regulators in multiple-drug resistance. As Hfq was found to regulate RpoS which in turn involved in orchestrating QS controlling antibiotic resistance, one can understand the pivotal role of stress regulators in QS and multidrug resistance. *Acinetobacter* genome analyses provide evidences for the presence of both Hfq and RpoS in *A. baumannii* though their interconnecting role is not yet elucidated.

4.4 Biofilm associated gene expression and virulence factors

Many recent investigations have revealed differential gene expression of genes during biofilm formation. Since biofilm helps in persistence of the organism in various stressful environments including survival in human hosts, many stress tolerating factors (can also termed as virulence factors) are produced to overcome a range of stress conditions. In this regard, our investigations have shown a positive correlation between biofilm and virulence factors. Our study which included majority of clinical isolates of *A. baumannii* that are biofilm producers were also found to be positive for production of virulence factors like protease, gelatinase, phospholipase, serum resistance and haemolysis (unpublished data). These factors are highly helpful for the pathogens survival in human hosts. Thus our observation sturdily supports a positive correlation between biofilm and virulence factors. Some cells in biofilm have slow growth rate, which is related to general stress response rather than nutrient limitations. To overcome stress, cells express various factors and one of them is RpoS which is regulated by the global regulator Hfq. As Hfq was found to regulate RpoS which in turn leads to QS controlled expression of virulence factors, one can understand the pivotal role of Hfq during harsh conditions. A general model of QS network with overall role of AHL in signal transduction regulated by AbaR, possible role of AbaI, Hfq, RpoS in *Acinetobacter* spp. is depicted in Figure – 4.

In conclusion, QS sensing works as a global regulator in regulating a diversified network of signalling cascades which helps the organism to resist infinite hostile conditions that are yet to be unveiled. Bacterial virulence being shown as one of the functions regulated by QS may therefore be a right target for designing newer therapeutics. Consequently, interference with QS-based inter-cellular communication might become the basis of new therapeutic schemes. Moreover, understanding QS cascades apart from revealing the communal relationships between the cells may help in designing potential drugs which can tackle multidrug resistant superbug *A. baumannii.*

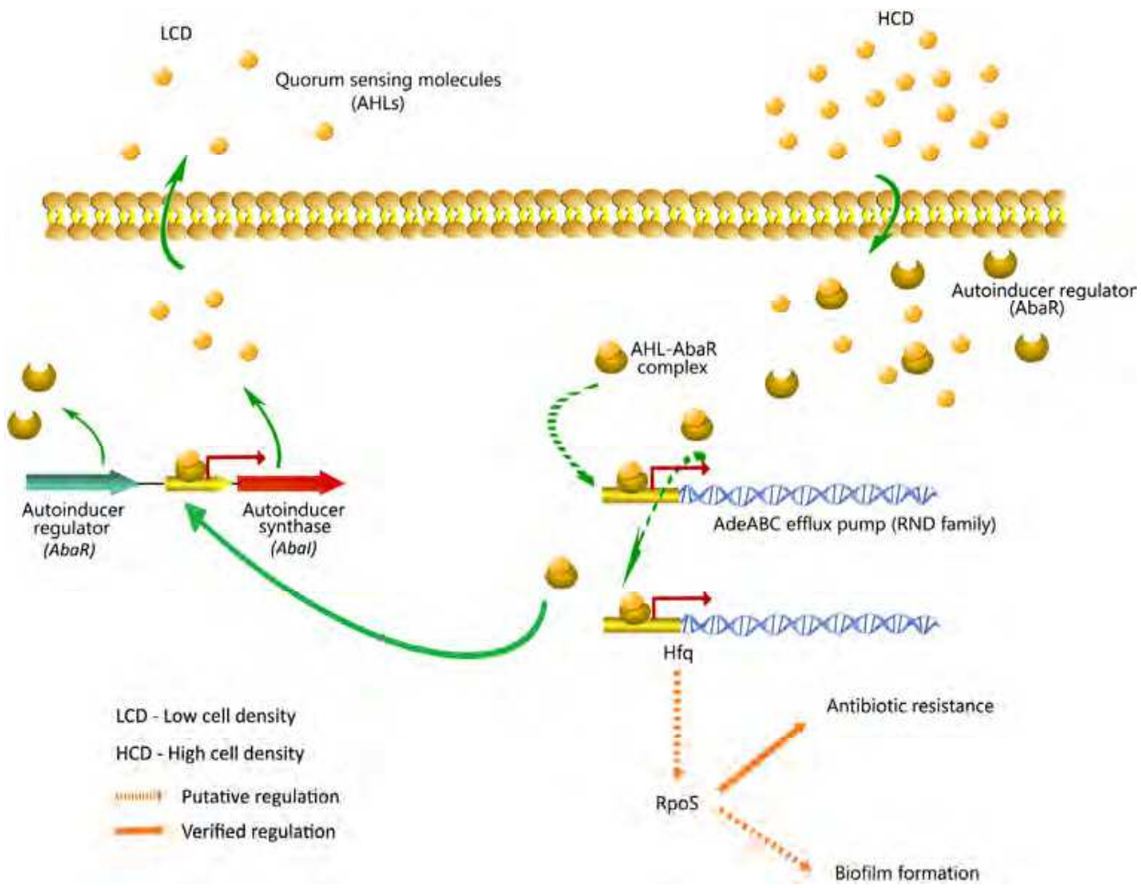


Fig. 4. A General model of acyl-homoserine lactone (AHL) signal transduction in *Acinetobacter* spp. by quorum sensing is shown. Tentative model for AHL synthesis (*left side*) and AHL interaction with AbaR-type regulatory proteins (*right side*) are depicted. Green solid arrows on the outer membrane indicate the potential two-way traffic of AHLs into and out of the cell. Putative regulatory role of AbaR and its interaction with AbaI or AbaI-type protein is shown at left side. Putative activation and overexpression of AdeABC efflux pump by AHL- AbaR complex is depicted by green dotted arrows at the right side. The presumed role of AHL- AbaR complex in up regulation of Hfq expression and putative regulation of RpoS by Hfq are shown. Finally, role of RpoS in antibiotic resistance and its probable role in biofilm development are illustrated.

5. Future prospective

Ironically, many have started believing that we are nearing the post antibiotic era as no new groups of antibiotics have been discovered after 1980s. As such, we are in a desperate need for searching new therapeutic solutions for infections caused pan-drug resistant bacteria. We might achieve this with respect to nosocomial pathogen *A. baumannii* after some careful studies of the genomics and proteome of *Acinetobacter* species looking for possible promising targets. In the following paragraphs, we describe one of the potential targets as an example, where we have tried to relate iron metabolism to biofilm production, which is based entirely on indirect evidences but strong correlation of *A. baumannii* with its other close relatives in a genomic perspective.

The remarkable similarities between the prokaryote and eukaryote iron transport systems underscore the importance of our analysis with respect to the host-bacteria interactions leading to disease. An increased knowledge of the molecular mechanisms of microbial pathogenicity mediated by iron and host resistance will undoubtedly help in finding potential drug targets. The iron-scarce environment of a vertebrate host generates a non-specific defence mechanism as most iron is bound with host proteins such as haemoglobin, or complexed with high affinity ligands such as transferrin and lactoferrin (Neilands et al., 1995). To overcome this, *A. baumannii* and other Gram-negatives secrete high affinity iron chelators, called siderophores that gather this micro- but essential nutrient (Neilands et al., 1995; Crosa, 1989). Siderophores (from the Greek: "iron carriers") are defined as relatively low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress environment. The role of these compounds is to scavenge iron from the environment and to make the mineral and make it available to the cell. The ability to extract iron from these iron-scarce environments of the host often contributes to the virulence of a successful pathogen.

5.1 Iron metabolism in *Y. pestis* and *A. baumannii*

Indeed, there have been reports that iron deficient media suppress biofilm formation and hence decrease virulence (Weinberg, 2004; Yang et al., 2007). But, this would do nothing to hinder the growth of the pathogen as the siderophores perform superbly, the task of iron acquisition with their extremely high affinity for ferric ion (Neilands et al., 1995; Braun and Hantke, 2011). We certainly have a choice of targeting the iron acquisition system so as to abolish the virulence. Recent studies on human Gram-negative pathogen *Y. pestis* suggest that the HmsHFRS and HmsT operons regulating hemin-binding and storing system are also involved in biofilm formation (Perry et al., 1990; Kirillina et al., 2004). In fact, *Y. pestis* Hms+ phenotype, described by enormous adsorption of hemin or congo red to become red coloured, is a manifestation of biofilm formation during growth at 26–34 ^{o}C (Perry et al., 2004). *A. baumannii* has genes homologous to HmsH, HmsF and HmsR that occur end-to-end and (may) constitute an operon having a pair of hypothetical genes and spanning about a 4.7 kb region along the complementary strand of the genome (Figure-5).

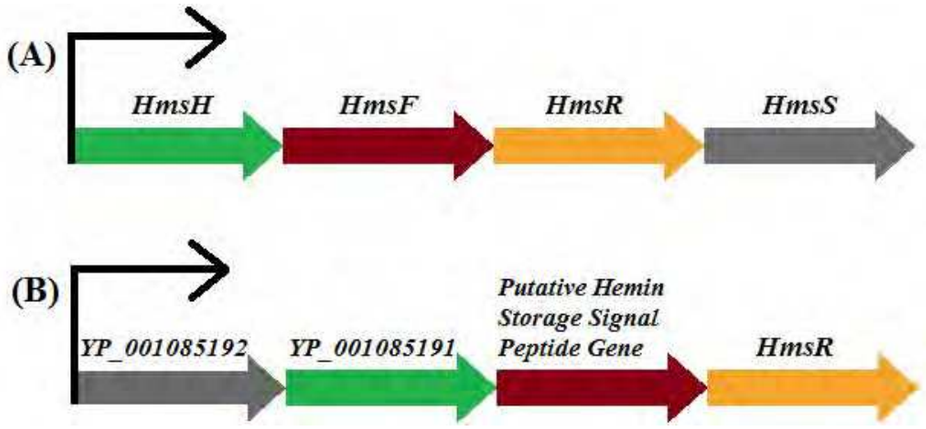


Fig. 5. A comparison between (A) *Y. pestis* Hms operon and (B) a 4.7 kb region of *A. baumannii* genome (see text) having four genes. Matching colours except dark grey colour showing homologues. Dark grey segments do not match. Promotor and intergenic sequences are ignored for the sake of simplicity.

Y. pestis HmsH is an outer membrane protein with a predicted β-barrel domain (Wortham et al., 2010) and has a weak homology to *A. baumannii* poly-beta-1, 6-N-acetyl-D-glucosamine (PGA) synthesis protein. HmsF is also an outer membrane protein with a predicted deacetylase domain. HmsR and HmsS are inner membrane proteins (Wortham et al., 2010). HmsR has a putative glycosyl-transferase domain where as HmsS homologue Ica is linked to the *Y. pestis* biofilm PGA synthesis protein PgaD. *Y. pestis* HmsH, HmsF, HmsR and HmsS have 58.2%, 60.8%, 83% and 50% sequence similarities to *E. coli* PgaA, PgaB, PgaC, PgaD respectively (Forman et al., 2006). But, they have very weak similarities with their (predicted) *A. baumannii* counterparts. Yet, from some recent investigations, it is now becoming obvious that genes from Hms operon have corresponding counterparts in *A. baumannii* (Zhou and Yang, 2001).

The above mentioned 4.7 kb region in genome of *A. baumannii* contains 4 genes in tandem, which consists of a pair of hypothetical proteins, bearing IDs YP_001085192 and YP_001085192 followed by a putative hemin storage signal peptide protein and hemin storage system protein HmsR. Neither the sequence nor the structural topology of YP_001085192 fits into any of the genes of *Y. pestis* HmsHFRS operon. Rather, according to UniProtKB annotations, it is a putative phosphotransferase, containing a nucleotide (possibly ATP) binding motif. There have been some evidences of phosphoenolpyruvate phosphotransferase (PTS) systems being involved in biofilm formation in *Vibrio cholerae*, *E. coli* and *Streptococcus gordonii* (Houot and Watnick, 2008; Lazazzera, 2010; Houot et al., 2010). YP_001085191, when searched against RefSeq (Pruitt et al., 2000) database, comes to be *A. baumannii* poly-beta-1, 6 N-acetyl-D-glucosamine export porin PgaA, which could be involved in the export of PGA to the cell exterior. The putative hemin storage signal peptide gene, as the name suggests, is involved in hemin storage. Searching results in the Conserved Domain Database (CDD) (Marchler- Bauer et al., 2011) suggested further that it has one each of polysaccharide deacetylase and poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase PgaB domains. The polysaccharide deacetylase domain is found in polysaccharide deacetylase. This family of polysaccharide deacetylases includes NodB (nodulation protein B from *Rhizobium*), which is a chito-oligosaccharide deacetylase. It also includes chitin deacetylase from yeast and endoxylanases which hydrolyses glucosidic bonds in xylan (Fukushima et al., 2004). Poly-beta-1, 6-N-acetyl-D-glucosamine N-deacetylase PgaB produces polysaccharides based on N-acetyl-D-glucosamine in straight chains with beta-1, 6 linkages. Deacetylation by this protein appears necessary to allow export through the porin PgaA (Itoh et al., 2008). The last one in the order, HmsR, as resulted in the CDD search, belongs to the cellulose synthase superfamily (Roberts and Bushoven, 2007) and also contains a DXD motif which binds to a metal ion that is used to coordinate the phosphates a nucleotide-sugar at the active site. These features suggest that *A. baumannii*, like its near relatives, depends on hemin-adsorption and storage for biofilm formation.

Neither the hemin acquisition (Zimbler et al., 2009) nor the biofilm function has remained uncharacterized in *A. baumannii*. But the above discussion correlates these two and suggests that they are not independent of each other. Even *A. baumannii* is able to survive without the help of iron chelators, if its Hms system is functional (Zimbler et al., 2009). On the contrary, an Hms negative almost does not develop biofilms (Figure-6) (Jarrett et al., 2004). One previous work (James et al., 2006) had revealed that genes coding for hemin and iron acquisition systems in *Porphyromonas gingivalis* are regulated by QS protein LuxS. Again QS is well known for inducing biofilm formation.

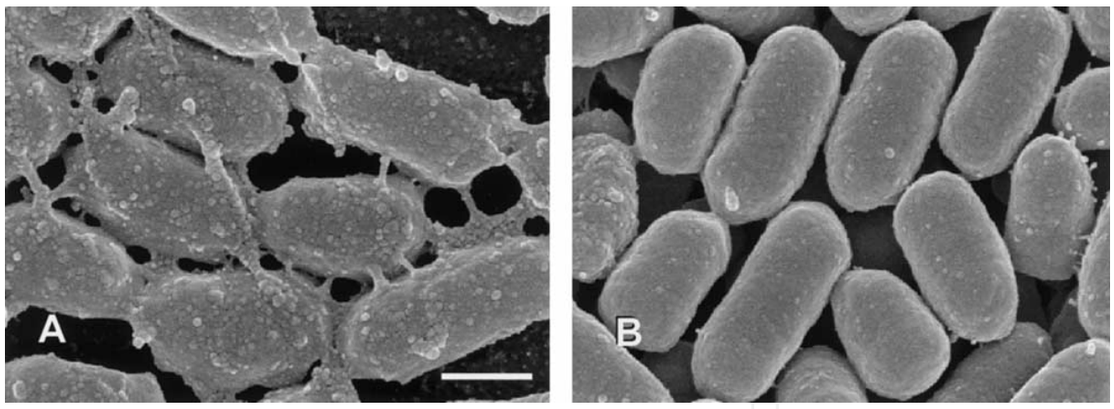


Fig. 6. Scanning electron microscopy of Hms-positive (A) and Hms-negative (B) *Yersinia pestis* grown on agar plates at 21˚C. Bar, 0.5 µm. Reproduced with the permission from Jarret et al., 2004.

6. Conclusion

This review attempted to give a glimpses of multiple mechanisms of antimicrobial resistance adopted by various species of *Acinetobacter*, described the current understanding of biofilm development and various factors regulating the biofilm formation in *Acinetobacter*. This write up also explained about the biofilm development and different virulence factors elaborated by *Acinetobacter* and its correlation with antibiotic resistance. Finally, quorum sensing has been elucidated in detail, which works as a global regulator in controlling and regulating diverse physiological functions such as biofilm formation, pilus biogenesis, production of multiple virulence factors, development of antibiotic resistance and increasing the competence of cells that helps in gene transfer. All the information discussed here will definitely help the future research in this area.

In conclusion, all the available evidence implies that *A. baumannii* is very important human pathogen that is gradually gaining more attention as a major global public health problem. It is responsible for a significant proportion of nosocomial infections among patients who are critically-ill receiving intensive care in the ICUs. With this situation together with the fact that certain biofilm phenotypes of *A. baumannii* being highly refractile and recalcitrant that are highly resistant to multiple drugs due to intrinsic resistance properties and those that can acquire resistant determinants with increasing propensity, makes this pathogen one of the most difficult challenges of the present days.

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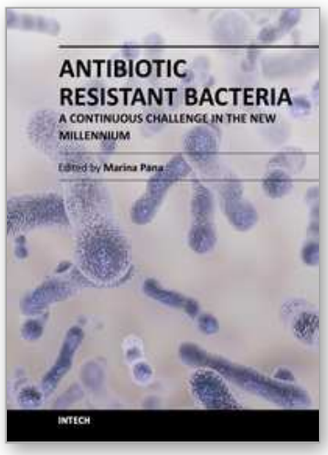

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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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