



Convergence of Biofilm Formation and Antibiotic Resistance in *Acinetobacter baumannii* Infection

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Acinetobacter baumannii (*A. baumannii*) is a leading cause of nosocomial infections as this pathogen has certain attributes that facilitate the subversion of natural defenses of the human body. *A. baumannii* acquires antibiotic resistance determinants easily and can thrive on both biotic and abiotic surfaces. Different resistance mechanisms or determinants, both transmissible and non-transmissible, have aided in this victory over antibiotics. In addition, the propensity to form biofilms (communities of organism attached to a surface) allows the organism to persist in hospitals on various medical surfaces (cardiac valves, artificial joints, catheters, endotracheal tubes, and ventilators) and also evade antibiotics simply by shielding the bacteria and increasing its ability to acquire foreign genetic material through lateral gene transfer. The biofilm formation rate in *A. baumannii* is higher than in other species. Recent research has shown how *A. baumannii* biofilm-forming capacity exerts its effect on resistance phenotypes, development of resistome, and dissemination of resistance genes within biofilms by conjugation or transformation, thereby making biofilm a hotspot for genetic exchange. Various genes control the formation of *A. baumannii* biofilms and a beneficial relationship between biofilm formation and “antimicrobial resistance” (AMR) exists in the organism. This review discusses these various attributes of the organism that act independently or synergistically to cause hospital infections. Evolution of AMR in *A. baumannii*, resistance mechanisms including both transmissible (hydrolyzing enzymes) and non-transmissible (efflux pumps and chromosomal mutations) are presented. Intrinsic factors [biofilm-associated protein, outer membrane protein A, chaperon-usher pilus, iron uptake mechanism, poly- β -(1, 6)-N-acetyl glucosamine, BfmS/BfmR two-component system, PER-1, quorum sensing] involved in biofilm production, extrinsic factors (surface property, growth temperature, growth medium) associated with the process, the impact of biofilms on high antimicrobial tolerance and regulation of the process, gene transfer within the biofilm, are elaborated. The infections associated with colonization of *A. baumannii* on medical devices are discussed. Each important device-related infection is dealt

with and both adult and pediatric studies are separately mentioned. Furthermore, the strategies of preventing *A. baumannii* biofilms with antibiotic combinations, quorum sensing quenchers, natural products, efflux pump inhibitors, antimicrobial peptides, nanoparticles, and phage therapy are enumerated.

Keywords: *Acinetobacter baumannii*, antimicrobial resistance, biofilm regulation, biofilm-associated infections, adult, paediatric, biofilm prevention

INTRODUCTION

Acinetobacter baumannii occupies the upper echelon of the WHO priority pathogen list- “critical,” a position indicative of how important this nosocomial pathogen is, particularly when they are resistant to the “last resort” antibiotic, carbapenem (1–3). *Acinetobacter* causes a wide range of infections mostly acquired in clinical settings and is frequently associated with high morbidity and mortality rates (26–60%) (4, 5). The rate of mortality due to multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *A. baumannii* infections is high and several outbreaks have been reported worldwide (5, 6). *A. baumannii* infections frequently occur in patients in ICUs on life-support systems prolonging their stay in hospitals and treatment failures are frequently encountered (7). Outside the hospital environment, *A. baumannii* has been isolated from a wide range of environmental samples including soil, aquatic environment, animals, humans, food items including raw vegetables, gardens, inanimate objects and even from body lice samples of homeless people, which serve as the reservoirs for this bacterium (8, 9). This species has now gone beyond hospitals and is being reported to cause community-acquired infections (10). Infections in both paediatric and adult populations indicate that as with other pathogens, the vulnerable are targeted (11). All these factors clearly indicate why *A. baumannii* is considered a critical pathogen.

Pathogens can grow and replicate even under unfavorable conditions. The tools that they use are diverse and many, sometimes as simple as the ability to persist in an environment. This ability to persist, particularly in adverse conditions such as hospitals where the use of antibiotics and disinfectants are high, gives *Acinetobacter* a clear advantage. *Acinetobacter* can survive in hostile environments (desiccation, antimicrobial therapies, nutrient unavailability) and can colonize biotic and abiotic surfaces for prolonged periods of time due to their ability to form complex structures called biofilms (12, 13). Biofilm formation is an important virulence mechanism and a hallmark characteristic of *A. baumannii*. Numerous microbial features (e.g., adhesins, capsular polysaccharides, surface appendages, virulence genes, resistance determinants), physicochemical factors (temperature, growth media, surface hydrophobicity, pH, oxygen concentration), and various other factors [biofilm-associated protein (Bap), the outer membrane protein A (OmpA), chaperon-usher pilus (Csu), iron uptake mechanism, poly- β -(1,6)-N-acetyl glucosamine (PNAG), two-component system (BfmS/BfmR), PER-1], facilitate the formation and maintenance of the *A. baumannii* biofilms (13).

Acinetobacter spp. can form biofilm at both air-liquid and solid-liquid interface. The biofilm formation rate in *A. baumannii* at the solid-liquid interface is 80–91%, which is 3 times higher than other *Acinetobacter* species (5–24%) (14–16). In addition, these isolates are able to form biofilm at the air-liquid interface, known as pellicle, which increases the surface-associated motility of the bacterium. However, pellicle formation is a rare trait in *A. baumannii* and a limited number of genes are essential for the expression of this phenotype, but within the ACB-complex, pellicle formation was almost four times higher for *A. baumannii* than other *Acinetobacter* genospecies (15, 17). *Acinetobacter* infections may be more difficult to treat when forming a biofilm and may be readily transmissible from patient to patient, making outbreaks that are difficult to control. Hospital surfaces and surfaces of medical devices such as cardiac valves, artificial joints, ventilators, urinary or intravascular catheters, endotracheal tubes made of polystyrene, polypropylene, polytetrafluoroethylene, and glass are excellent for biofilm formation (18). Indwelling devices provide pathogens a mode of entry into the body, therefore patients admitted to the hospitals are at high risk of *Acinetobacter* infection as *Acinetobacter* can colonize on abiotic surfaces efficiently. A recent study suggested that the clinical isolates of *A. baumannii* have better ability to form biofilm on abiotic surfaces than non-clinical isolates. Therefore, the high capability of *A. baumannii* to colonize and form biofilm on abiotic surfaces is considered an important factor contributing to chronic and persistent infections in hospital settings (19). This subsequently enhances the risk of infectious diseases such as cystic fibrosis, periodontitis, bloodstream infections, urinary & respiratory tract infections, burn-wound infections, chronic non-healing injury, endocarditis, necrotizing fasciitis, etc. (18, 20, 21). Moreover, *A. baumannii* is able to maintain its virulence even after long periods of survival in the hospital environment, which could facilitate infections (22).

In addition to the ability to form biofilms, the deft with which *Acinetobacter* acquires antibiotic resistance genes and also transmits them provides the species with an additional advantage in hospitals where the use of antibiotics is always higher than in other environments. Studies have been showing horizontal gene transfer (HGT) of antibiotic resistance genes (carbapenemases, oxacillinases, metallo-beta-lactamases, or metal resistance genes) *via* conjugation, transformation, bacteria phage-mediated, nanotube-mediated, or *via* outer membrane vesicles (23–29). The mechanism of resistance is similar to other Gram-negative bacteria (GNB) which employ hydrolyzing enzymes or modifying enzymes, pumps to efflux antibiotics,

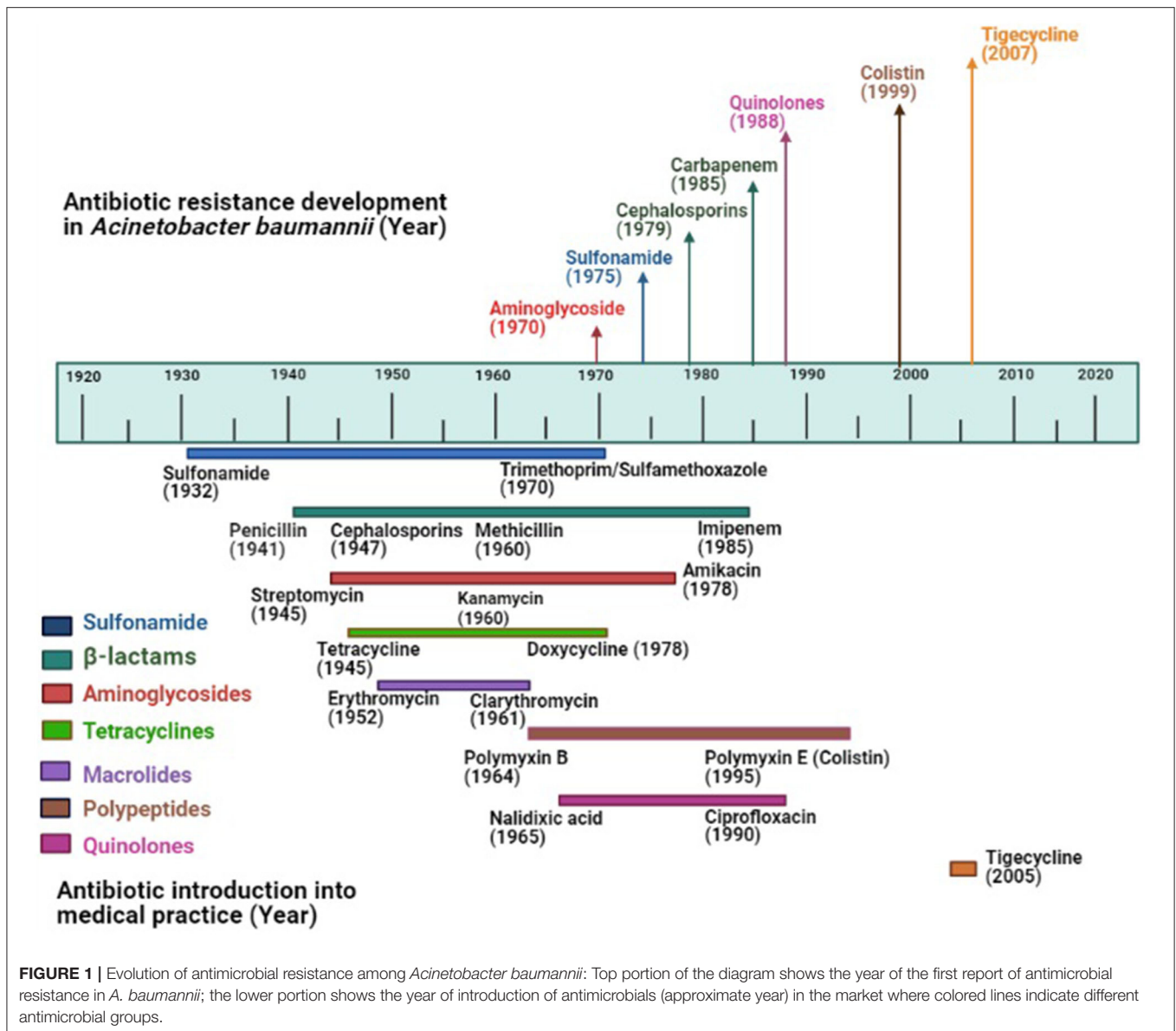


FIGURE 1 | Evolution of antimicrobial resistance among *Acinetobacter baumannii*: Top portion of the diagram shows the year of the first report of antimicrobial resistance in *A. baumannii*; the lower portion shows the year of introduction of antimicrobials (approximate year) in the market where colored lines indicate different antimicrobial groups.

possess carbapenemase activity. OXA-23, OXA-24/40, OXA-58, OXA-143, and OXA-235 are examples of oxacillinases that are detected in *A. baumannii* and are able to hydrolyze carbapenems (10, 30, 62–64). Insertion of IS_{Aba1} in the *bla*_{OXA-23} promoter sequence has been reported to be associated with overexpression of *bla*_{OXA-23}, *bla*_{OXA-51}, or *bla*_{OXA-58} in *A. baumannii* (65, 66). Class B β -lactamases which are very different from the other classes and known as MBLs (metallo- β -lactamases), can be inhibited by EDTA as they possess Zn at their active site (30). Several MBLs have been reported in *A. baumannii* such as IMP (imipenemases), VIM (Verona integron-encoded MBL), SPM (Sao Paulo MBL), SIM (Seoul imipenemase), GIM (imipenemase from Germany), and NDM (New Delhi MBL) (30, 67–71). These MBLs are the primary reason for carbapenem resistance in *A. baumannii* along with oxacillinases.

Apart from hydrolysis of antimicrobials by β -lactamases, enzymatic modification of the antibiotics is another mechanism of enzymatic resistance in *A. baumannii*. One of the best examples is the presence of three different aminoglycoside-modifying enzymes (acetyl transferases, nucleotidyl transferases, and phosphotransferases) which modify amino or hydroxyl-groups of the aminoglycosides (67).

Non-enzymatic Mechanisms of Resistance: The Active Pumps and More

Most GNB including *A. baumannii* also possess several non-enzymatic mechanisms of resistance which include efflux pumps, modifications of drug binding sites, and permeability defects.

To date, different categories of efflux pumps have been identified in *A. baumannii*: RND-family (resistance-nodulation-division), MFS-family (major facilitator superfamily), MATE-family (multidrug and toxic compound extrusion), and SMR-family (small multidrug resistance). The RND system more actively participates in antimicrobial resistance in *A. baumannii* and this family includes the AdeABC, AdeIJK, and AdeFGH efflux pumps (72). These efflux pumps are controlled by certain regulators such as AdeRS (two-component system), AdeL, and AdeN (72). Some other efflux pumps detected in *A. baumannii* include MATE-family (AbeM and CraA), MFS-family (AmvA, AbaF, and AbaQ), and SMR-family (AbeS) (72). tet(A), tet(B), and tet(G) are specific transposon-mediated efflux pumps also detected in *A. baumannii* (73).

Random point mutations, which are an important mechanism of bacterial resistance, alter the target site of antibiotic binding. The examples of such mechanisms among *A. baumannii* are (i) fluoroquinolone resistance due to spontaneous mutations in gyrase and topoisomerase IV encoding genes, i.e., *gyrA*, *gyrB*, and *parC*, *parE*; (ii) rifampin resistance due to point mutations in the RNA polymerase encoding gene *rpoB*; (iii) colistin resistance due to mutations in PmrAB two-component system and *lpxA*, *lpxC*, *lpxD* genes; and (iv) aminoglycoside resistance due to mutations in 16S ribosomal RNA gene *armA* (10, 30, 67, 74). Moreover, carbapenem resistance in *A. baumannii* is also associated with mutations in Penicillin Binding Protein PBP-2 (30, 67, 75).

Porins also play a significant role in antimicrobial resistance among *A. baumannii*. Decreased expression of several porins (Omp22–23, Omp33–36, Omp37, Omp43, Omp44, Omp47, OmpA, and CarO) has been noted in carbapenem-resistant *A. baumannii* (30, 76). A schematic diagram of the several antimicrobial resistance mechanisms has been depicted in **Figure 2**.

A. baumannii AND BIOFILM: INVOLVEMENT OF INTRINSIC AND EXTRINSIC FACTORS

Biofilm is a complex multicellular three-dimensional structure of the assembled population of bacterial cells which is enclosed within an exopolymer matrix called extracellular polymeric substance (EPS) (77). The EPS comprises nucleic acids, carbohydrates, proteins, and other macromolecules. In the EPS matrix, the most abundant carbohydrates are glucose, galactose, and mannose followed by fucose, arabinose, xylose, rhamnose, galacturonic acid, and *N*-acetyl-glucosamine. The complexity of the biofilm is enhanced by the presence of extracellular proteins which stabilize the biofilms and extracellular DNA (eDNA) secreted by the cells (77).

Formation of the biofilms, also known as the biofilm cycle, involves six major stages: (i) reversible attachment of planktonic bacteria with surfaces followed by settlement; (ii) proliferation and aggregation of the adherent bacteria (irreversible attachment); (iii) formation of microcolonies which either look like mushrooms or towered structures; (iv) initiation of quorum sensing (communication pathway between cells) at a

critical cell density facilitating biofilm formation, positioning of cells in the microcolonies, formation of water channels within the biofilm and detachment of cells; (v) biofilm maturation (maximum antibiotic tolerance can be observed at this stage due to the presence of thick polysaccharide matrix surrounding them); and (vi) detachment and dispersion of cells to colonize in another location (78).

INTRINSIC FACTORS ASSOCIATED WITH A. baumannii BIOFILM FORMATION: THE TOOLS WITHIN

Biofilm formation in *A. baumannii* on biotic and abiotic surfaces is regulated and influenced by several intrinsic factors such as virulence genes or proteins, cellular structures, and phenotypic or genotypic features. The factors associated with biofilm formation and regulation in *A. baumannii* are summarized in **Table 1** and shown in **Figure 3**.

Biofilm Associated Protein (Bap)

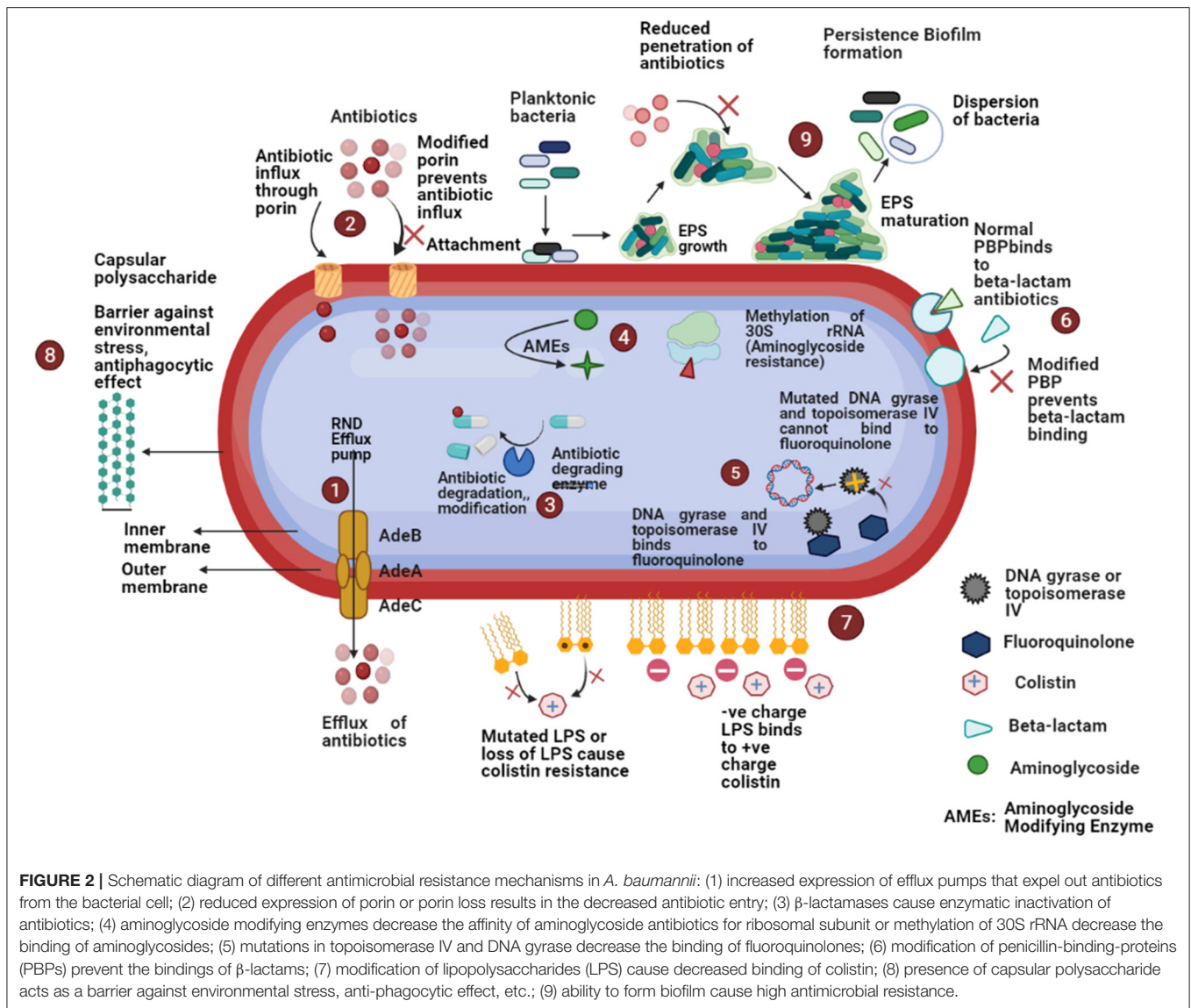
Biofilm-associated protein (Bap) is a high-molecular-weight protein (8,620 amino acids), essential for cell-cell interactions, biofilm formation, and maturation on various surfaces including polypropylene, polystyrene, and titanium (89). BAP was first identified in *A. baumannii* by Loehfelm et al. and is identical to the *Staphylococcus aureus* Bap protein (79). *A. baumannii* Bap mainly targets carbohydrates in the host cells and is secreted via a type I secretion system. Several studies have documented the presence of Bap in *A. baumannii* strains and its association with strong biofilm formation (80, 90). Loehfelm et al. showed that Bap protein increases adherence to both normal human neonatal keratinocytes and bronchial epithelial cells (79). Many Bap-like proteins, BLP1 and BLP2 are also harbored by *A. baumannii* which coordinately contribute toward mature biofilm formation and adhesiveness to epithelial cells in a similar fashion as Bap (80).

Poly-β-(1, 6)-N-Acetylglucosamine (PNAG)

Poly-β-(1, 6)-N-acetylglucosamine (PNAG) is one of the major components of polysaccharides and is important for the development of biofilm in both GPB and GNB (81). PNAG is encoded by a cluster of four genes (*pgaA*, *pgaB*, *pgaC*, and *pgaD*) (82) which share similarities with *E. coli* and *Y. pestis* (91). *pgaA* plays an important role in protein-protein interaction and contains a porin domain which facilitates PNAG translocation through the outer membrane, *pgaB* involves in PNAG exportation via the outer membrane lipoprotein, *pgaC* helps in the synthesis of PNAG and *pgaD* restricts the cytoplasm and promotes *pgaC* in the synthesis of PNAG (91). Numerous studies showed PNAG is essential for maintaining the integrity of *A. baumannii* biofilms in a more dynamic and stressful environment (81, 91).

PER-1 β-lactamase

Acinetobacter baumannii adhesion and biofilm formation in both biotic and abiotic surfaces are enhanced by the presence and the



expression of the *bla_{PER-1}* gene which is a class A extended-spectrum β -lactamase (92). Several studies found that increased cell adhesiveness and biofilm formation was higher in strains harboring the *bla_{PER-1}* gene than in those that do not harbor this genetic trait (83, 92, 93). However, Bardbari et al. reported no relationship between biofilm formation and production of *PER-1* β -lactamase (94). Therefore, the presence of *bla_{PER-1}* probably increases the adhesion property of cells carrying this gene but does not necessarily contribute to biofilm formation.

Csuab-A-B-C-D-E Chaperone-Usher Pili Assembly System

Chaperon-Usher secretion (CUS) system is required for bacterial attachment on abiotic surfaces that results in microcolony formation and development of biofilm (95). *Csu* pili are polycistronic in nature, adhesive surface organelles that consist of a tip fibrillum and adhesion protein (84). *A. baumannii* strains

produce type I *Csu* pili that is encoded by an operon: *Csuab-A-B-C-D-E*. Furthermore, the expression of the *Csuab-A-B-C-D-E* operon in *A. baumannii* is regulated by a two-component system *BfmRS* where *BfmR* acts as a response regulator and *BfmS* acts as a sensor kinase (85, 96). Moreover, a second two-component system *GacSA* controls the *Csu* pili gene expression and is indirectly involved in biofilm formation in *A. baumannii* (97). Other putative chaperone usher pili systems and *Pap* pili systems, which are homologous to the *P* pili of *E. coli*, have also been implicated in *A. baumannii* biofilm formation and maintenance (98).

Outer Membrane Proteins

Porins are the outer membrane proteins (Omps) that modulate cellular permeability, have an essential role in adaptation, environmental communication, and also play an important role in microbial virulence through drug exclusion mechanisms

TABLE 1 | Factors implicated in *Acinetobacter baumannii* biofilm formation and regulation.

Effectors	Gene determinants	Functions	References
<i>A. baumannii</i> Biofilm-associated protein (BAP)	<i>bap-Ab</i>	Bap is a surface exposed protein, plays an important role in cell-cell adhesion, interactions, biofilm formation, and maturation.	(79, 80)
Poly- β -(1, 6)-N-acetylglucosamine (PNAG)	<i>pgaA, pgaB, pgaC, and pgaD</i>	PNAG is a polymeric exopolysaccharide essential for cell-cell adherence, biofilm formation, and thickness of biofilm.	(81, 82)
Beta-lactamase PER1	<i>bla_{PER-1}</i>	<i>bla_{PER-1}</i> is a broad-spectrum of β -lactamase gene responsible for adhesion and biofilm formation to both biotic and abiotic surfaces.	(83)
Csuab-A-B-C-D-E. chaperone-usher pili assembly system	<i>csuA, csuB, csuC, csuD and csuE, bfmRS (bfmR and bfmS), gacSA</i>	Cus pili are surface homo or heteropolymer protein structures, play a key role in the adhesion, pili production and assembly, biofilm formation, and maintenance on abiotic surfaces.	(84, 85)
Outer membrane protein A	<i>ompA</i>	The <i>OmpA</i> are key virulence factors in adhesion, invasion, biofilm formation, and cytotoxicity in biotic surfaces.	(86)
Quorum Sensing (QS)	<i>abaR, abaM, and abaI</i>	QS produces the signaling molecules, autoinducers to maintain bacterial cell-to-cell communication, population density, synchronized behavior, and interaction. QS is also responsible for activation and regulation of gene expression of virulence factors, motility, plasmid transfer, drug resistance, and biofilm formation.	(87, 88)

across the outer membrane channels (86). The outer membrane protein A (OmpA) is a well-recognized and well-characterized virulence factor of *A. baumannii* and is necessary for the development of robust biofilms on abiotic surfaces (86). The two-component system BfmS/R regulates biofilm formation, pilus, and OmpA expression, along with serum sensitivity (99). Another outer membrane protein of *A. baumannii* is the Omp 33–36 kDa protein which acts as a channel for water and is associated with resistance to carbapenem antibiotics. Moreover, knockout of *omp 33–36* gene in *A. baumannii* strains had defective growth rate and significantly reduced capability of adherence, invasion, biofilm formation, and cytotoxicity in biotic surfaces (100).

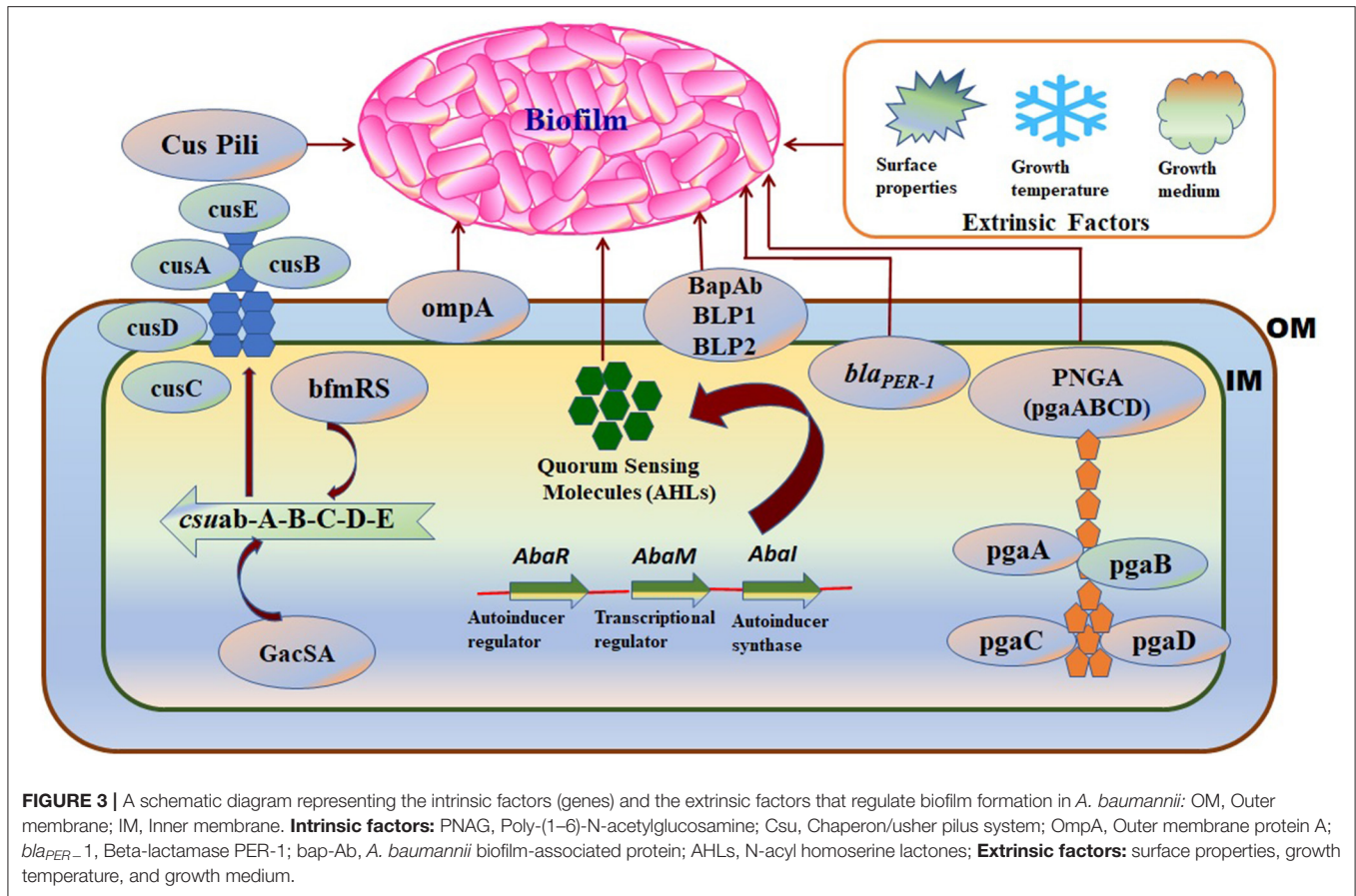
Quorum Sensing (QS)

Quorum sensing (QS) is a cell-to-cell communication process that depends on the bacterial population density. Several small diffusible signaling molecules are involved in QS which activate the expression of genes that control a variety of functions such as virulence, motility, biofilm formation, bioluminescence, and sporulation (87, 101). These diffusible signaling molecules termed autoinducers include oligopeptides in GPB and N acyl-homoserine lactones (AHLs) in GNB (102). The predominant AHL produced by *A. baumannii* is 3-hydroxy-C12-homoserine lactone. The QS system in *A. baumannii* is regulated by a two-component system, AbaI/AbaR which is homologous to the typical LuxI/LuxR system found in GNB. *abaI* encodes the autoinducer synthases which catalyze the synthesis of AHL and AbaR that functions as receptor proteins for AHLs (103). A

previous study had shown that mutation of the AHL synthase AbaI affects the surface-associated motility and biofilm formation in *A. baumannii*. A functional QS system is required for surface-associated motility and robust biofilm formation in *A. baumannii* ATCC® 17978 (104). In addition, another gene named *abaM* is an uncharacterized member of the RsaM protein family located between *abaR* and *abaI*, has been found to play a key role in regulating *A. baumannii* QS, virulence, surface motility, and biofilm formation (88). The expression and upregulation of another two-component system BfmS/R has also been linked to the QS molecules that enhance the ability of *A. baumannii* to form biofilm on abiotic surfaces (105). Quorum sensing deficiency causes thinner biofilm formation and lower EPS production, thereby increasing the susceptibility to antibiotics.

Efflux Pumps

Efflux pumps are membrane proteins, that can extrude a wide group of substrates, including antibiotics, detergents, dyes, toxins, and waste metabolites. Several studies suggest that efflux pumps play major roles in biofilm formation and maturation by different mechanisms: efflux of EPSs and quorum quenching (QQ) molecules to facilitate biofilm matrix formation; indirect regulation of genes involved in biofilm formation and efflux of antibiotics or metabolic intermediates (106, 107). There are three types of RND efflux pumps associated with *A. baumannii*: AdeABC, AdeFGH, and AdeIJK. Yoon et al. reported that mutant strains of AdeABC, AdeFGH, and AdeIJK efflux pumps have significantly reduced biofilm formation in comparison with the wild-type strain. Therefore, biofilm formation in *A. baumannii*



requires expression of efflux pump genes to initiate and maintain biofilm. Another study reported that the mutation of AdeABC and AdeIJK efflux genes were associated with lower expression of several pilus system-encoding proteins CsuA/B, CsuC, and FimA. These proteins play a key role in the initial stages of adhesion, surface colonization, and biofilm formation in *A. baumannii* (108). Richmond et al. presented that knockout of AdeAB efflux pumps in *A. baumannii* mutant strain caused significant reduction in biofilm formation on mucosal tissue compared with wild type strain. Therefore, the over-expression of AdeABC and AdeIJK efflux pump regulate the expression of pilus genes and biofilm production, and altered membrane composition in *A. baumannii* (108, 109). The third RND-type efflux pump AdeFGH is regulated by a LysR-type AdeL transcriptional regulator system. The over-expression of this efflux pump confers multidrug resistance and is linked to the synthesis and transport of autoinducer molecules during biofilm formation in *A. baumannii*.

EXTRINSIC FACTORS ASSOCIATED WITH *A. baumannii* BIOFILM FORMATION: THE EXTERNAL INFLUENCES

Certain environmental factors that affect *A. baumannii* biofilm formation are summarized in Table 1 and shown in Figure 3.

Surface Property

Several factors such as roughness, physicochemical properties of a surface, and the presence of biological materials influence the attachment of *A. baumannii* to abiotic surfaces and the formation of biofilm (110). The ability of *A. baumannii* to form mature biofilms on polypropylene, polystyrene, titanium, and other medical-associated devices has been associated with several factors including pH, ionic composition, and biomaterial of protein adsorption (111). The presence of biomaterial such as blood, tears, urine, saliva, interstitial fluid, wound cultures and respiratory secretions influence the attachment of bacteria to its surface and promote the formation of biofilm (112). Polycarbonate surfaces are known to develop statistically more biofilm mass than glass, rubber, porcelain, and polypropylene (110). Latex catheters are low-priced and have more elasticity but are prone to bacterial adhesion and biofilm formation. Hence, silicone catheters are preferred over latex catheters (113).

Growth Temperature

Temperature also has an effect on biofilm formation. *A. baumannii* successfully survived at -20 to 44°C (114). However, different studies have been reported different optimum temperatures for biofilm formation in *A. baumannii* including 30°C at pH 7 in a medium containing sodium chloride or 25°C (115, 116). Another study showed that biofilm formation in

A. baumannii on plastic surfaces was high at 28°C due to the upregulation of certain biofilm-associated proteins (BAPs), Csu pili, and iron-uptake proteins (111).

Growth Medium

The growth medium is also a factor that affects biofilm formation. It has been reported that a static environment with high nutrient containing medium (Tryptic Soy Broth or Brain Heart Infusion Broth) and supplemented with glucose, carbon, and cation sources (Na + sodium, Ca²⁺ calcium, Fe³⁺ ferric ion) influences the formation of *A. baumannii* biofilm than in hydrodynamic environment (117). These modulatory properties of medium and supplemented sources also influence the structural and mechanical properties of *A. baumannii* biofilms by lowering stiffness and increasing adhesiveness (117). However, clinical isolates of *A. baumannii* showed a significant reduction in adhesiveness and biofilm formation in the presence of an iron-chelating agent and ethanol on abiotic surfaces (83, 118).

A. baumannii BIOFILMS AND ANTIBIOTIC RESISTANCE: A DANGEROUS LIAISON

Antimicrobial Resistance by Biofilm Cells: Understanding the Mechanisms

The term “biofilm resistance” signifies the survival of cells embedded within biofilms for long periods of time in presence of antimicrobials. Biofilm resistance does not indicate that biofilm cells show increased MIC compared to antibiotic-resistant planktonic cells (119). Biofilms are better equipped to evade antimicrobials than planktonic cells because biofilms are not easily destroyed by antimicrobials. Factors that are responsible for biofilm resistance and explain better survival of biofilm cells compared to planktonic cells in presence of antimicrobials are described below (Figure 4).

Diffusion Inhibition of Antibiotics: Restricted Entry

Inhibition of antibiotic diffusion within biofilms can be attained via several mechanisms such as the presence of EPS matrix that serves as the diffusion barrier for antibiotic entry, antibiotic degradation with the help of production of exoenzymes (β -lactamases, β -galactosidase), metal immobilization or chelation, extracellular signaling, a mutation in the antibiotic target site and oxidation-mediated inactivation of antibiotics (120). These processes can lower antibiotic concentration to sublethal levels, leading to the selection of antibiotic-resistant cells within a biofilm. In addition, negatively charged eDNA facilitates the lowering of antibiotic concentrations within biofilms by binding to positively charged antibiotics such as aminoglycosides and gathers antibiotics up to 25% of its weight (121). eDNA can also enhance antibiotic resistance within biofilms by chelating cations such as Mg²⁺ and creating a cation-depleted environment leading to activation of two-component systems which are linked to AMR (121). It facilitates the transfer of antibiotic resistance genes within biofilms.

eDNA not only comes from lysed cells but is also actively secreted which indicates that eDNA has an important role in biofilm formation. Its negative charge works as a repulsive force in the initial attachment, but when the distance between cell and surface becomes a few nanometers, eDNA interacts with receptors on the substratum surface to facilitate adhesion (122). Sahu et al. characterized eDNA from a multidrug-resistant clinical strain of *A. baumannii* and demonstrated its role in *in vitro* biofilm formation on abiotic surfaces. They supplemented biofilms with eDNA in various preparations, for example, whole-cell lysate, cell-free supernatant, MV suspension, and purified eDNA to mimic its natural availability during growth. Their experiment showed that eDNA supplemented in any of the given forms was able to augment the biofilms on polystyrene microtiter surface significantly (224.64%), whereas biofilm inhibition was 59.41% after DNase I treatment: confirming that eDNA facilitates biofilm formation in *A. baumannii* (123).

Heterogeneity and Decreased Growth Rate: The Environment Within Matters

The growth rate of organisms embedded in biofilms varies due to oxygen and nutrient depletion especially inside the microcolonies and in the deep cell layer, leading to gradients of nutrients that result in different growth rates such as fast/slow-growing cells, dormant cells, persister cells. These differences in growth rates cause population heterogeneity within the biofilms (124). The slow growth of the bacteria has been observed in mature biofilms (125). Generally, antimicrobials kill rapidly growing cells. Therefore, slow growth undoubtedly contributes to biofilm resistance (126). The phase of the cell division cycle is also known to influence antibiotic resistance within biofilms (127). Apart from heterogeneity observed within biofilms, population heterogeneity has been noted in *A. baumannii* which exists as opaque-virulent (VIR-O) and translucent-avirulent (AV-T) colonies. The AV-T cells produce denser biofilms than VIR-O cells, thereby showing an antibiotic resistance phenotype (128).

Development of Persisters Within Biofilm: Temporary Resistance

The capacity of microorganisms to grow and survive at higher antibiotic concentrations than their MIC is called antibiotic tolerance. In contrast to resistance, tolerance is basically a transient phase and after exposure to antibiotics for a long time, antibiotics kill the bacteria. Bacterial populations in biofilms that exhibit increased antimicrobial tolerance are called “persister cells” (126). Persister cells are actually responsible for biofilm-associated infections (126, 129). The formation of biofilms and antibiotic-tolerant persisters contributes to the heterogeneity of *A. baumannii* populations, facilitating their adaptation to fluctuating environments. It was proposed that environmental stress (such as desiccation) causes the death of the main stressed population within the biofilm, where few viable surviving bacteria (persister cells) can resume growth and restore the original population, once the environmental conditions are suitable. This strategy is called the “bust-and-boom” strategy

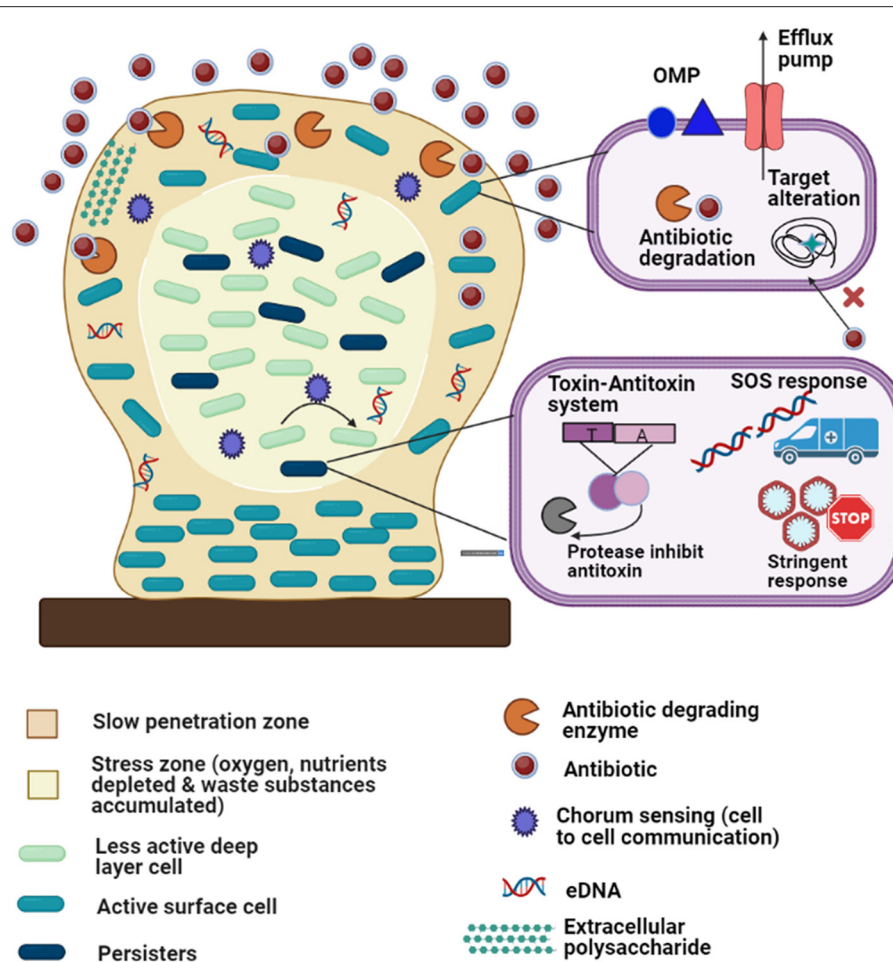


FIGURE 4 | Diagrammatic representation of the antibiotic resistance mechanisms of biofilm-embedded bacterial cells: The biofilm is attached to a biotic or abiotic surface (brown rectangle). Development of persister cells (dark green) and less active deep layer cells (light green) in the stress zone (the core of the biofilm, light cream color) where fewer nutrients are available. The various resistant mechanisms depicted in the figure are as follows: (1) matrix exopolysaccharides cause slow penetration of antibiotics; (2) extracellular DNA (eDNA); (3) multidrug efflux pumps; (4) outer membrane protein; (5) antibiotic degrading enzymes and target modifications (6) quorum sensing; (7) stress responses (oxidative stress response, stringent response, etc.); (8) toxin-antitoxin system and (9) SOS responses.

and *A. baumannii* follows this strategy (130, 131). Multiple mechanisms trigger antibiotic tolerance and development of persisters in biofilm such as stress triggered by antibiotics, the host immune responses, high osmolarity, ROS, changes in pH, efflux pumps, quorum signaling, oxidative stress responses, desiccation, or nutrient starvation. In addition, stringent response (SR), SOS response, and toxin-antitoxin (TA) modules can be activated during persister formation. These pathways result in decreased metabolism, protein aggregation, depletion of ATP, and inhibition of translation (132, 133). In the stringent response, the production of (p)ppGpp (also known as alarmone) is activated by the (p)ppGpp synthetases such as RelA and SpoT. (p)ppGpp regulates various transcriptional and metabolic pathways, such as phosphate, lipid, and amino-acid metabolism (134). Ultimately, the SR shuts down almost all metabolic processes leading to increase tolerance to antibiotics. The formation of *A. baumannii* persister cells which showed tolerance to rifampicin and colistin due to the deficiency of ppGpp has also

been reported (135). DNA damage leading to activation of the SOS response which is also associated with antibiotic tolerance has been noted (136). Together, SR and SOS response molecules activate the TA system in which one gene encodes toxin that shows activities against DNA, RNA, membrane, cell wall synthesis, ATP, and the other encodes antitoxin that binds and inhibits the toxin. Alkasir et al. (137) reported that up-regulation of two toxin-antitoxin systems HigB/HigA, and DUF1044/RelB resulted in high ceftazidime tolerance among *A. baumannii* persister cells.

Change in Bacterial Morphology due to Environmental Stress: Cause of Resistance

Stress responses result in physiological changes that control the composition and arrangement of the cellular envelope. Such changes in cell morphology can protect cells from nutrition deficiency, cold shock, heat shock, pH change, and also increase

their non-susceptibility to several antimicrobial agents (138). *A. baumannii* embedded within biofilms also face stress due to high osmolarity that causes induction of porins such as OMP33–36 and CarO, leading to carbapenem resistance (30).

IS THERE AN ASSOCIATION BETWEEN BIOFILM FORMATION AND ANTIMICROBIAL RESISTANCE IN *A. baumannii*?

The type of correlation that exists between antibiotic resistance phenotypes and biofilm formation among *A. baumannii* is controversial.

Several studies have shown that antibiotic-resistant *Acinetobacter* spp. form strong biofilms compared to susceptible bacteria, indicating a positive correlation between antibiotic resistance and biofilm formation (83, 92–94, 115, 139–143) (Table 2). In the above studies, a statistically significant difference was found between strains with the high biofilm-forming ability and those with low/no biofilm-forming ability with resistance to several categories of antimicrobial agents (β -lactam group, cephalosporin group, aminoglycosides, quinolones, tetracycline, oxytetracycline, aztreonam, etc.). A particular study revealed high biofilm-forming ability among MDR *Acinetobacter* strains isolated from ICU patients compared to *Acinetobacter* strains isolated from non-ICU patients which showed resistance to fewer antibiotics (144). Similarly, another study reported high biofilm production among *A. baumannii* isolated from burn units. These isolates showed high resistance to antibiotics including carbapenems and also showed co-production of AmpC and ESBLs (145). Bardbari et al. compared biofilm-production ability between clinical and environmental *A. baumannii*. Clinical strains showed strong biofilm production ability compared to environmental strains (58.7 vs. 31.2%). However, a significant correlation was observed between the MDR phenotype and biofilm formation ability in both groups ($P = 0.008$) (94). Few of the above-mentioned studies also showed a high prevalence of biofilm-related genes including *ompA*, *bfmS*, *bap*, *csuE*, *bla_{PER-1}*, and *epsA* in MDR *A. baumannii* with a high biofilm-forming ability (83, 92, 93, 139–141).

The diversity and abundance of antibiotic resistance genes (ARGs) in biofilms of *Acinetobacter* spp. have been investigated by several authors to highlight the fact that the probability of accumulation of ARGs (*bla_{OXA-51}*, *OXA-23*, *OXA-58*, *OXA-72*, *OXA-24/40*-like genes, *bla_{TEM}*, *bla_{VIM}*, *bla_{NDM}*, *bla_{SIM}*, *ompA*, *xerC*, and *gyrA*) or IS elements (*ISAb1*, *ISAb3*) in biofilms is higher rather than in the planktonic cells (93, 94, 141).

Though most studies have shown a positive association between biofilm formation and antibiotic resistance in *Acinetobacter* spp., some studies have documented an inverse relationship between the biofilm formation capacity of clinical strains of *A. baumannii* and MDR/XDR phenotype (146–152). These studies are comparatively fewer than the studies that show a positive correlation. These studies have been described in detail in Table 2.

HGT WITHIN BIOFILMS OF ACINETOBACTER SPP.: EXCHANGE MATTERS

Antibiotic resistance genes (ARGs) are often encoded in mobile genetic elements (MGEs) such as conjugative and non-conjugative plasmids, integrative and conjugative elements, transposons, and bacteriophages (153–156). Plasmids and MGEs can easily be transferred to closely related or distantly related bacteria *via* HGT. HGT also occurs in biofilms and certain factors that enhance the HGT within biofilms are the EPS matrices of biofilm that limit bacterial motility, increased cell-to-cell contact, quorum sensing, high cell density that helps in bacterial interactions. Apart from these, the presence of eDNA, oxygen availability, the SOS response, extracytoplasmic stress, and biomass surface increase the efficiency of plasmid transfer (157).

During conjugation, transfer of plasmid occurs through conjugation pilus which is proteinaceous in nature and serves as a link between donor and recipient cells (155). Transfer of ARGs within a biofilm is mainly associated with conjugation because biofilm cells are attached to a matrix, located close together that provides direct cell to cell contact. Cells remain metabolically active and are embedded within the EPS matrix in which the cells are protected against the harsh environment. These factors enhance HGT within biofilms *via* conjugation and thereby are considered as HGT hotspots (158–161). Apart from conjugation, transformation is another important mechanism that transfers naked genes within biofilms (162). Free DNA which is released by cell lysis can serve as the donor for transformation.

All AMR genes can be transferred *via* HGT within the biofilm. However, there are limited studies that show the transmission of specific genes/resistance plasmids within *A. baumannii* biofilm *via* conjugation or transformation. Willium et al. provided the first evidence of the natural transformation of *Acinetobacter* BD413 cells in river biofilms with a mercury resistance plasmid pQM17 (163). Hendrics et al. showed effective natural transformation in biofilms of *Acinetobacter* spp. strain BD413. The nature of transformants changed with the change in the amount of exogenous DNA. When the amount of DNA was low, transformants formed at the biofilm attachment surface while with an increasing amount of DNA, the accumulation of transformants was observed at the bottom of the biofilm (23). Another study showed both conjugal transfer and natural transformation of plasmids from *A. baumannii* to *E. coli* HB 101 and *A. baylyi*, respectively (115). The spread of a highly promiscuous carbapenem hydrolyzing gene *bla_{NDM-1}* was noted from *E. coli* J53-*bla_{NDM-1}* transconjugant to *A. baumannii* biofilms *via* conjugation (24).

Apart from conjugation and transformation, the transfer of plasmid DNA is also mediated *via* the formation of nanotubes (elongated extracellular structure employed cell to cell contact and composed of proteins), outer membrane vesicles, and bacteriophages in *Acinetobacter* spp. (25–29) but their role in the transfer of ARGs among biofilm-embedded *Acinetobacter* has not been elucidated yet. HGT also occurs in the microbial ecosystem in the human intestinal tract. HGT in the human

TABLE 2 | Association between biofilm formation and antimicrobial resistance in *Acinetobacter spp.*

Sr. no	Study	Country	Strain (numbers)	Sources of isolation	% of biofilm formers	Observations	References
Positive correlation between biofilm formation and antibiotic resistance							
1.	Rao et al., 2008	India	<i>A. baumannii</i> (50)	Endotracheal aspirates, cerebrospinal fluid, wound swabs, urine, blood	62%-high biofilm former	<ul style="list-style-type: none"> Resistance to four antibiotics such as amikacin (82 vs. 17.6%, $P < 0.001$), cephotoxime (88 vs. 11%, $P < 0.001$), ciprofloxacin (70 vs. 29%, $P = 0.005$), and aztreonam (38 vs. 11%, $P = 0.039$) was comparatively higher among biofilm producers than non-biofilm producers. <i>bla</i>_{PER-1}-horbouring <i>A. baumannii</i> was able to form strong biofilm in comparison to the isolates that did not possess the gene. 	(92)
2.	Lee et al., 2008	Korea	<i>A. baumannii</i> (23)	Blood, sputum, urine	100%- biofilms former	<ul style="list-style-type: none"> Cell adhesiveness and biofilm formation were significantly higher in isolates carrying the <i>bla</i>_{PER-1} as compared with isolates without this gene ($P < 0.005$ and $P < 0.001$, respectively). RT-PCR showed a positive correlation between the level of expression of the <i>bla</i>_{PER-1} and the level of biofilm formation ($P < 0.0001$). 	(83)
3.	Pour et al., 2011	India	<ul style="list-style-type: none"> <i>A. baumannii</i> (47); <i>A. lowffii</i> (3) 	Urine samples, urinary catheters	<ul style="list-style-type: none"> 12%- strong biofilm former 10%-low biofilms former 	<ul style="list-style-type: none"> High biofilm forming strains exhibited high resistance to 27 antibiotics from different groups including β-lactam group (83.3%), cephalosporin group (94.4%), aminoglycosides (97%), quinolones (75%), tetracycline (66.6%) and oxytetracycline, and imipenem (33.3%). 	(115)
4.	Nahar et al., 2013	Bangladesh	<ul style="list-style-type: none"> <i>A. baumannii</i> (32) from ICU patients <i>A. baumannii</i> (20) from non-ICU patients 	Tracheal aspirates, blood, central venous catheter, peripheral blood, urine, wound swab, pus, throat swab, endotracheal tubes, burn samples, ascitic fluid, sputum, aural swab, oral swab, cerebrospinal fluid, and catheter tip	<ul style="list-style-type: none"> 87.5%- biofilm former from ICU patients 55%- biofilm former from non-ICU patients 	<ul style="list-style-type: none"> Resistance to antibiotics such as gentamicin (100 vs. 88.9%), amikacin (85.7 vs. 55.6%), netilmicin (85.7 vs. 11.1%), ciprofloxacin (82.1 vs. 54.4%), imipenem (81.0 vs. 22.2%) and colistin (7.1 vs. 0%) was higher among biofilm forming <i>Acinetobacter spp.</i> isolated from ICU than non-ICU isolates. 	(144)
5.	Emami and Eftekhari, 2015	Iran	<ul style="list-style-type: none"> <i>A. baumannii</i> (30) from burn unit <i>A. baumannii</i> (30) from non-burn unit 	<ul style="list-style-type: none"> The burn isolates were mostly from wounds, blood, urine. Non-burn isolates were from sputum, wound specimens, catheters, blood, cerebral spinal fluid, trachea 	<ul style="list-style-type: none"> 55.5%- biofilm former in non-burn isolates 40.5%- biofilm former in burn isolates 	<ul style="list-style-type: none"> Non-burn strains significantly produced more biofilm compared to the burn strains ($P < 0.05$). Biofilm-producing non-burn isolates were significantly more resistant to amikacin, meropenem, and tobramycin compared to the biofilm negative strains within the same group ($P < 0.05$). AmpC and ESBL was much higher among the non-burn isolates compared to the burn samples (33.0 vs. 3.3%, $P < 0.05$). 	(145)

(Continued)

TABLE 2 | Continued

Sr. no	Study	Country	Strain (numbers)	Sources of isolation	% of biofilm formers	Observations	References
6.	Thummeepak et al., 2016	Thiland	<i>A. baumannii</i> (221)	Sputum, urine, pus, blood, pleural fluid, ascetic fluid, and wound	76.9%- biofilm former	<ul style="list-style-type: none"> The association between biofilm forming ability and gentamicin resistance was found to be significant ($P = 0.017$). Antibiotic-resistant isolates possessed <i>ompA</i> (84.4%), <i>bfmS</i> (84%), <i>bap</i> (48%), <i>bla_{PER-1}</i> (30.2%) and <i>epsA</i> genes (30.2%). However, biofilm formation related genes <i>ompA</i> and <i>bap</i> were associated with multidrug-resistant <i>A. baumannii</i> strains. 	(139)
7.	Bardbari et al., 2017	Iran	<ul style="list-style-type: none"> <i>A. baumannii</i> (75) from clinical samples <i>A. baumannii</i> (32) from environmental samples 	<ul style="list-style-type: none"> Sputum, bronchoalveolar lavage, endotracheal aspirates, ventilators, sink Area, floor, hand staff, trolleys and bedside table, pillow and linens, and other fomites 	<ul style="list-style-type: none"> 31.2%- strong biofilm forming clinical isolates 58.7%- strong biofilm forming environmental isolates 	<ul style="list-style-type: none"> Clinical strains showed strong biofilm production ability compared to environmental strains (58.7 vs. 31.2%). Significant correlation was observed between the frequency of multidrug-resistant isolates and biofilm formation ability in both clinical and environmental strains ($P = 0.008$). The study revealed the presence of <i>bla_{OXA-51}</i>, <i>bla_{OXA-23}</i>, <i>bla_{OXA-24}</i>, <i>bla_{OXA-58}</i>, and <i>bla_{PER-1}</i> among biofilm forming <i>A. baumannii</i>. 	(94)
8.	Khamari et al., 2019	India	<i>A. baumannii</i> (14)	Blood, pus, urine, pleural fluid, endotracheal tube	<ul style="list-style-type: none"> 100%- biofilm former 71.4%-strong biofilm former 	<ul style="list-style-type: none"> <i>bla_{TEM}</i>, <i>bla_{OXA}</i>, <i>bla_{NDM}</i>, <i>bla_{VIM}</i>, <i>bla_{SIM}</i>, and <i>bla_{PER-1}</i>; class 1 integron were detected among the isolates. 	(93)
9.	Yang et al., 2019	Taiwan	<i>A. baumannii</i> (152)	No data available	<ul style="list-style-type: none"> 45.4%- strong biofilm former 32.5%- moderate biofilm former 15.6%- weak biofilm former 	<ul style="list-style-type: none"> A positive correlation was observed between biofilm forming capacity and resistance to ticarcillin, amikacin, gentamicin, ceftazidime, piperacillin, imipenem, and sulfamethoxazole-trimethoprim antibiotics ($P = 0.018$, 0.004, 0.003, 0.003, 0.033, 0.017, 0.007, respectively). The study also revealed that biofilms-related genes such as <i>bap</i>, <i>bla_{PER}</i>, <i>ompA</i>, and <i>csuE</i> genes were found in 81, 39, 91, and 69% of the biofilm producers, respectively. The strains carrying these genes formed stronger biofilm than the isolates without these genes. 	(140)
10.	Ranjbar et al., 2019	Iran	<i>A. baumannii</i> (161)	Burn wood infections	<ul style="list-style-type: none"> 70.6%- strong biofilm former 12.2%- moderate biofilm former 17.2%- weak biofilm former 	<ul style="list-style-type: none"> A significant association was observed between biofilm-forming ability and XDR phenotype ($P = 0.001$). Multiple genes (<i>bla_{OXA-23-like}</i>/<i>bla_{OXA-40-like}</i>/<i>bla_{OXA-51}</i>, <i>bla_{PER-1}</i>/<i>bla_{VEB-1}</i>, <i>bla_{IMP}</i>, and <i>bla_{VIM}</i> and <i>tetB</i>) were found to be responsible for detection of drug-resistance in burn patients. 	(141)

(Continued)

TABLE 2 | Continued

Sr. no	Study	Country	Strain (numbers)	Sources of isolation	% of biofilm formers	Observations	References
11.	Celik et al., 2020	Turkey	<i>A. baumannii</i> (60)	Tracheal aspirates, blood, urine, wound, sputum, CSF, abscess, bronchoalveolar lavage fluid	90%- biofilm former	<ul style="list-style-type: none"> In biofilm-positive strains, antibiotic resistance was significantly higher against ampicillin/sulbactam, cefoperazone-sulbactam, chloramphenicol, piperacillin/tazobactam, and ciprofloxacin ($P = 0.008, 0.038, 0.017, 0.027, 0.005$, respectively). 	(142)
12.	Asaad et al., 2021	Egypt	<i>A. baumannii</i> (161)	Sputum, endotracheal aspirate, wound swab	<ul style="list-style-type: none"> 20.2%- strong biofilm former 34%- moderate biofilm former 16%- weak biofilm former 	<ul style="list-style-type: none"> Biofilm-producing isolates showed statistically significant higher resistance rate to ceftazidime, ampicillin/sulbactam, piperacillin/tazobactam, piperacillin, gentamycin, trimethoprim/sulfamethoxazole, tigecycline, and imipenem ($P = 0.041, <0.001, 0.006, 0.034, 0.028, 0.002, 0.002$, and 0.02, respectively). Presence of <i>ompA</i> gene ($P = 0.002$), <i>bap</i> gene ($P = 0.012$), MDR ($P = 0.017$), and XDR ($P = 0.002$) was significantly associated with biofilm-producing capability of the isolates, compared to non-biofilm producing capabilities. 	(143)
Negative correlation between biofilm formation and antibiotic resistance							
1.	Rodríguez-Baño et al., 2008	Spain	<i>A. baumannii</i> (92)	No data available	63%- biofilm former	<ul style="list-style-type: none"> In comparison to non-biofilm forming <i>A. baumannii</i>, biofilm forming isolates were less frequently resistant to ciprofloxacin and imipenem (47 vs. 25%, $P = 0.04$; and 94 vs. 66%, $P = 0.004$, respectively). 	(146)
2.	Han et al., 2014	China	<i>A. baumannii</i> (70)	No data available	<ul style="list-style-type: none"> 50%- strong biofilm former 29%- moderate biofilm former 21%- weak biofilm former 	<ul style="list-style-type: none"> Resistance to levofloxacin (85.71%, 45.00%, 38.24%, $P = 0.010$), cefepime (71.43%, 45.00%, 29.41%, $P = 0.027$), and gentamicin (78.57%, 55.00%, 38.24%, $P = 0.037$) significantly decreased when biofilm-forming ability was strong. 	(147)
3.	Zhang et al., 2016	China	<i>A. baumannii</i> (120)	Sputum	<ul style="list-style-type: none"> 27.3%- strong biofilm former 54.5%- moderate biofilm former 18.2%- weak biofilm former 	<ul style="list-style-type: none"> Isolates which produced strong biofilm exhibited low-level resistance to gentamicin, minocycline, and ceftazidime ($P < 0.05$). 	(148)
4.	Qi et al., 2016	China	<i>A. baumannii</i> (268)	No data available	<ul style="list-style-type: none"> 23%- strong biofilm former 74.7%- weak biofilm former 	<ul style="list-style-type: none"> Among the strong biofilm-formers, 79.4% were non-MDR isolates and, 20.6% were MDR/XDR ones. Among the weak biofilm-formers, 12.4% non-MDR and 87.6% MDR/XDR isolates. Strains that were negative for biofilm formation consisted of 8.7% non-MDR and 91.3% MDR/XDR isolates. 	(31)

(Continued)

TABLE 2 | Continued

Sr. no	Study	Country	Strain (numbers)	Sources of isolation	% of biofilm formers	Observations	References
5.	Krzyżosiak et al., 2017	Poland	<i>A. baumannii</i> (15)	Blood, central nervous system, pulmonary	80–90%- biofilm former	<ul style="list-style-type: none"> • Strains showing sensitivity to amikacin, tobramycin, trimethoprim/sulfamethoxazole and ciprofloxacin from ICU patients produced more biofilm than strains showing resistance to these antibiotics. 	(149)
6.	Wang et al., 2018	Taiwan	<i>A. baumannii</i> (269)	Blood	26%- biofilm former	<ul style="list-style-type: none"> • MDR isolates was significantly lower ($P = 0.006$) in the biofilm-forming group. • Biofilm-forming isolates were significantly more susceptible to most commonly used antibiotics including amikacin, gentamicin, ceftazidime, cefepime, ciprofloxacin, imipenem, and meropenem ($P = 0.040, 0.043, 0.003, 0.009, 0.001, 0.035, 0.018$, respectively). 	(150)
7.	Shenkutie et al., 2020	China	<i>A. baumannii</i> (104)	Sputum, blood, urine, soft tissue, hospital environments	<ul style="list-style-type: none"> • 25%- strong biofilm former • 14.4%- moderate biofilm former • 20.2%- weak biofilm former 	<ul style="list-style-type: none"> • Non-MDR strains (66.1%) showed strong biofilm formation. 	(151)

Studies have been arranged in chronological order.

gut microbiome can occur *via* different mechanisms like transduction or conjugation. Different bioinformatics tools and experimental approaches have been developed to determine the association and transfer of antibiotic resistance genes in the gut microbiome (164). The HGT in the gut microbiome may lead to the development and spread of antibiotic resistance genes among commensals and opportunistic pathogens (165).

RESISTANCE OF *A. baumannii* BIOFILM-ASSOCIATED CELLS TOWARD DISINFECTANTS

Disinfectants are chemical agents used to play a key role in the prevention of nosocomial transmission of infectious pathogens (166). The commonly used disinfectants against infectious pathogens in the hospital or industrial environments are 70% ethanol, chlorhexidine, sodium hypochlorite, quaternary ammonium compounds, benzalkonium chloride (BZK), benzethonium chloride (BZT), phenolic disinfectants, hydrogen peroxide, and silver ions (167). Several factors lead to the enhanced resistance toward disinfectants in *A. baumannii*, e.g., reduced diffusion or reaction limitations of disinfectants in biofilms, overexpression of the EPS matrix, biofilm-specific efflux pumps, phenotypic adaptations of biofilm cells to sublethal concentrations of disinfectants, alterations in genotypic features like gene transfers and mutations due to stress responses, and specific microenvironment conditions that inactivate biocides (168–170). A study has reported that resistance to antiseptics and disinfectants in *A. baumannii* is largely mediated by efflux proteins encoded by *qac* genes. The *qacA/B* genes encode proteins of the MFS-family whereas *qacC*, *qacE*, and *qacF* encode efflux proteins of the SMR-family which are located on mobile elements, thus facilitating their spread and resistance to disinfectants (171). Indeed, inappropriate use of disinfectant solutions with an adequate concentration leads to the selection and emergence of microorganisms resistant to disinfectant in the hospital setting (172).

A. baumannii SNEAKS INTO HOSPITALS: ADULT AND PAEDIATRIC PATIENTS

Modern medicine and improved healthcare systems save numerous lives. However, these medical interventions have also provided conditions for microbial growth, entry into human bodies, and infections. Several indwelling devices in the hospital create breaches in the body’s defense mechanisms allowing easy access for microorganisms to enter the body. The patients in ICUs already have underlying conditions and are vulnerable to infections; indwelling devices offer more opportunities for such infections. *A. baumannii* can survive for long periods in the hospital environment, particularly on inanimate surfaces, which may act as a reservoir for cross-colonization and infection outbreaks. Moreover, a previous study showed that *A. baumannii* can retain its virulence under stress (desiccation and/or starvation in hospital settings) which could facilitate infections (22). In healthcare setups, biofilms pose a serious problem due

to the increased antimicrobial tolerance and the potential of biofilm-associated organisms to cause infections in patients with indwelling medical devices (173). *A. baumannii* easily acquires resistance and the biofilm formation rate in *A. baumannii* is higher than other species, making this organism a major cause of concern in ICUs. In most cases, indwelling medical device-related infections including bloodstream infections and urinary tract infections are biofilm-associated infections (173). Central venous catheters (CVCs) are the most common medical devices, followed by endotracheal tubes (ETT), ventilators, medical implants that pose a risk of device-related infection. Several *in vitro* and *in vivo* studies reported biofilm-formation of *A. baumannii* on several abiotic surfaces including hospital equipment and indwelling medical devices, such as catheters, endotracheal tubes etc (174–176). Carbapenem-resistant *A. baumannii* were also found to form biofilm on extracorporeal membrane oxygenation catheters (177). Several reports have also indicated the presence of *A. baumannii* biofilm on different hospital material, such as latex, anodized aluminum, stainless steel, and polycarbonate surfaces (86, 110, 174). Development of biofilm on medical devices depends on several factors including adherence of microorganisms for prolonged periods of time that results in irreversible attachment of organisms, physicochemical characteristics of the surface, cell density and types of the adherent cells, nutrient composition of the medium, flow rate of liquid through the device, drug concentration, ambient temperature and most importantly hydrophilicity and surface charge of the material of the medical devices (178). The surface proteins of microorganisms that act as virulence factors can specifically bind to host extracellular matrix proteins, such as fibrinogen, fibronectin, and collagen by van der Waals forces and H-bonds (179). These proteins have a high affinity for implants and become easily attached to the implant surface and develop as microcolonies over the entire surface of the host (179). The infections associated with colonization of *A. baumannii* on medical devices are discussed below. Each important device-related infection is dealt with separately and both adult and pediatric studies have been categorized (178).

Ventilator-Associated Pneumonia (VAP)

Ventilators are devices that support breathing in seriously ill patients by forcing oxygen into the lungs. Some ventilators have tubes that are inserted into the bronchus bypassing an important defense of the lungs, the ciliated cells, introducing microbes easily into the lungs. This entry is facilitated in organisms that form biofilms. Biofilm formation in the oropharynx, tracheostomy, and endotracheal tubes of ventilated patients has been suggested to play a role in the development of ventilator-associated pneumonia (VAP) (180). Sometimes, microorganisms may directly reach the lower airways by inhalation as a result of contamination of medical equipment, and they may reach the lungs. These tubes are indwelling prostheses and are typically made from polyvinyl chloride, latex rubber, or silicone materials, providing a potential surface for the growth of bacteria, especially *A. baumannii*. A very recent study detected *A. baumannii* as one of the most common organisms capable of forming a biofilm on tracheostomy tubes among critically ill patients (181). A

large surveillance study from the United States showed the association of *A. baumannii* with ~ 5 and 10% of ICU-acquired pneumonia (182). Malacarne et al. (183) reported 28.6% of the cases of late-onset VAP due to *A. baumannii* which were preceded by tracheobronchial colonization with *A. baumannii*. The formation of biofilm on ETT and its association with VAP was analyzed in several studies where the most frequent bacteria were *A. baumannii* which lead to high mortality of patients (175, 184–189).

Ventilator-associated pneumonia (VAP) is the second most frequent cause of nosocomial infection in children in ICUs in developing countries (190). Children who develop VAP also have an increased risk of mortality and morbidity (191, 192). *A. baumannii* is increasingly recognized as an important pathogen causing VAP in neonatal and children ICUs with a trend of high resistance to broad-spectrum antibiotics including carbapenems and colistin (176, 193–196). These studies clearly indicate the association of *A. baumannii* and VAP.

Bloodstream Infections (BSIs)

Bloodstream infections (BSIs) are primarily associated with the presence of the CVC or as a consequence of extensive hospital-acquired pneumonia (197). Catheters introduce microorganisms into the body as they are directly inserted in the vein to inject antibiotics or other medicines in hospitalized patients. The CVC-related infections are dependent on the patients' age and insertion procedure of CVC. The degree of severity of catheter-related bloodstream infections (CRBSI) is increased when microorganisms form biofilms. The best way to avoid CRBSI is to reduce the unnecessary catheterization, reduce the indwelling duration of CVC, use antibiotic-impregnated (like minocycline/rifampin) catheters, and use preventive locks (197). CRBSI among ICU patients due to *A. baumannii* biofilm formation on CVC has been noted (198, 199). Recent studies also showed BSI with highly virulent *A. baumannii* ST2 and ST191 belonging to International Clonal Lineage II that showed strong biofilm-forming ability (200, 201).

CVCs are also used in modern pediatric medication for various purposes including hemodynamic monitoring, infusion of vasoactive medication, hemodialysis, long-term use for chemotherapy, antibiotic treatment, or immunological diseases. CRBSI and central line-associated bloodstream infection (CLABSI) are also reported among children (202–204).

Urinary Tract Infection (UTI)

Among adults, urinary tract infection (UTI) is mostly associated with urinary catheters. Catheter-associated UTIs (CAUTIs) represent about 40% of all nosocomial infections as most of the hospitalized patients need an indwelling urinary catheter throughout their hospital stay (205). The placement of the catheter leaves the sphincter open allowing unbridled access to pathogens present in the hospital environment, the washing action of the urine is also absent in catheter-fitted patients. The catheter also presents a perfect surface for micro-organisms to adhere to and start biofilm formation (206). Given these multiple factors, UTIs are common. Biofilm formation depends on the duration of catheterization as 10–50% of short-term catheterized

patients (≤ 7 days) experience biofilm formation meanwhile biofilm is formed inevitably in all long-term catheterized patients (> 28 days) (173, 178). *A. baumannii*-associated UTI causes serious medical problems because of treatment difficulties due to their resistance to carbapenems and third-generation cephalosporins. Various studies showed *Acinetobacter* as one of the biofilm-producing organisms associated with CAUTI (5, 207–212).

Urinary tract infections (UTIs) are common infections among children in the first 2 years of life and are considered a common disease in school and pre-school children. Most *A. baumannii* strains are capable of producing biofilm in percutaneous nephrostomy tubes or urinary catheters, therefore *A. baumannii* may contribute significantly to UTIs in hospital admitted children and also cause community-acquired UTIs (213, 214).

Traumatic Battlefield, Wound, Burn, Skin, and Soft Tissue Infections

Biofilm formation on medical devices draw significant attention in healthcare settings, but the biofilm formation ability of *A. baumannii* on biotic surfaces such as on wound, burn, skin and soft tissue have also been noted. Such tissue-related infections in immunocompromised, cancer, and diabetic patients have raised serious concerns (215). Bacterial colonization in open wounds damages the healing process. It has been reported that biofilms are causative factors for many chronic non-healing ulcers (216). Nosocomial *A. baumannii* deep wound and burn wound infections have been reported in natural or man-made disasters (earthquakes, bombing, military operations) (217–219). Evidence of *A. baumannii* biofilm in wounds has also been provided by several other studies (220–224). Skin and Soft Tissue Infections (SSTIs) are often accompanied by *A. baumannii* bacteremia (225–228). The spectrum of infection can extend from cellulitis to necrotizing fasciitis.

Orthopedic Implant-Related Infections

Orthopedic devices are commonly used worldwide for a wide number of procedures including hip or knee replacements, fracture treatment, joint, ligament, and tendon replacements, and other surgical processes. These procedures have become extremely common to restore the function of affected joints, bones, or limbs. Implant-associated infections remain a major problem in orthopedic procedures and is caused by surface-adhering bacteria that form biofilm. The reported rate of implant-related infection is about 5% (229). Implant-associated infection can either be early (within the first 2 months of surgery) or delayed (between the third and the 24th month). Among different microorganisms that cause implant-associated infections, *A. baumannii* has been diagnosed in the case of periprosthetic joint infections (230, 231).

Neonatal Intensive Care Units

Acinetobacter baumannii has been a major cause of neonatal sepsis and several studies have reported outbreaks in neonatal units (232–234). Neonatal sepsis with drug-resistant and even carbapenem-resistant *A. baumannii* has also been reported (235,

236). Sepsis in neonates can also lead to meningitis causing high mortality rates. Several neonatal meningitis cases have also been reported due to MDR *A. baumannii* (237–241).

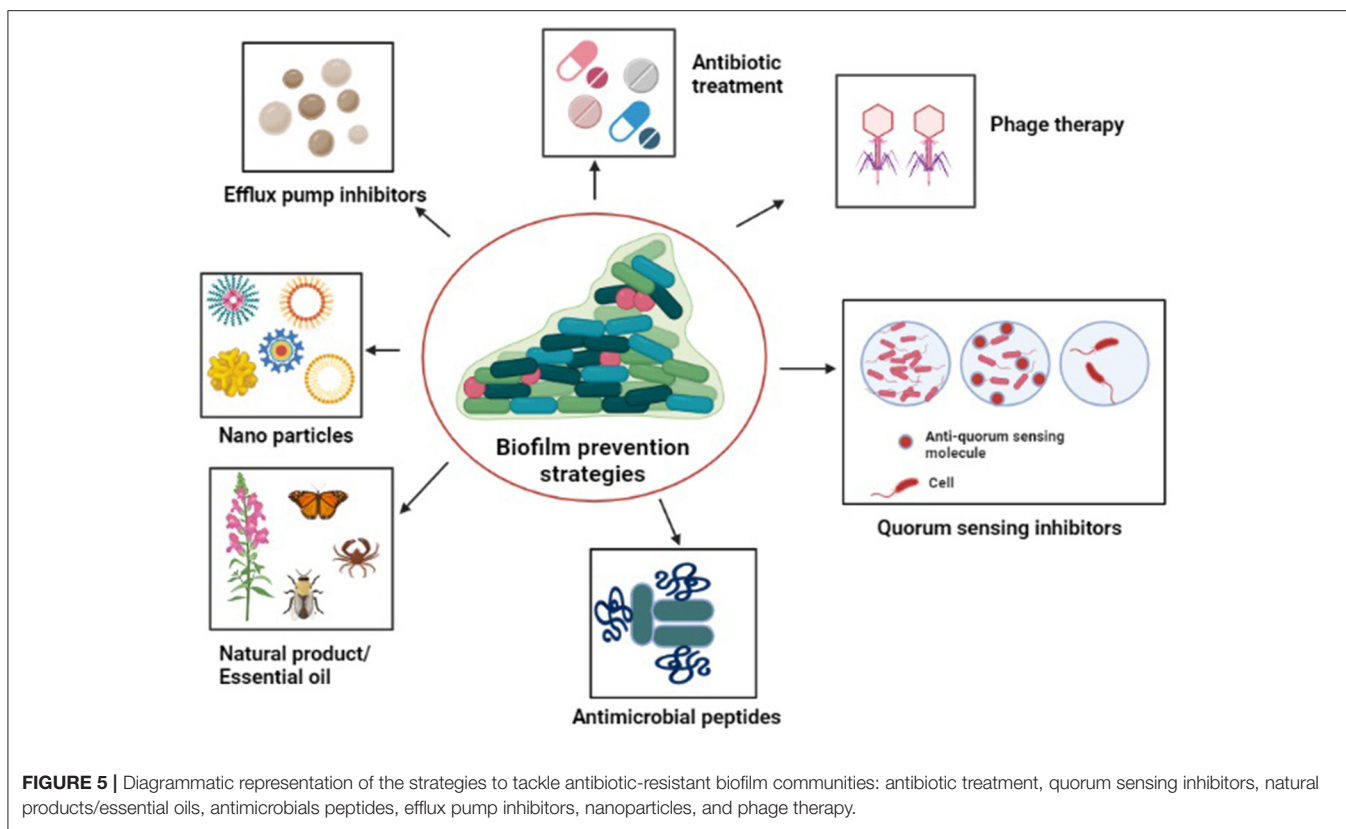
As with adult ICUs, neonatal ICUs also provide the same set of conditions and devices for *A. baumannii* to flourish. Most neonates in the ICUs are premature or of low birth weight and require prolonged hospitalization. As they are vulnerable and already fighting for life, they also require life-support systems such as ventilators. Prolonged stay at the hospital on life-support systems always increases the chances of infection particularly in neonates who are on antibiotics and have an immature immune system (242). Apart from infection, colonization of the gut with *A. baumannii* has also been reported in hospitalized neonates, increasing the possibility of subsequent sepsis due to translocation of the gut bacteria (243). A comparison of bacterial etiology of neonatal sepsis reveals that higher the level of care (Level III against Level II/I) greater the rate of *A. baumannii* sepsis. A recent study noted *A. baumannii* as the predominant cause of neonatal sepsis; this study was carried out in 3 tertiary care hospitals in New Delhi, all with level III care (244). Level III units are equipped with devices, which facilitate the formation of biofilms clearly indicating the link. Sources of infection could be varied and unexpected, the devices, the hospital staff, or even mothers who handle the neonate for breastfeeding or kangaroo care (193, 194, 245). Vigilance and infection control are of utmost importance particularly in units that care for neonates.

STRATEGIES OF PREVENTING *A. baumannii* BIOFILMS: FIGHTING BACK

With the aim to limit *Acinetobacter* adhesion to biotic or abiotic surfaces and to inhibit biofilm growth, numerous effective novel anti-biofilm remedies have been developed, few of which have been discussed below (Figure 5).

Antibiotic Treatment: Active Combinations

Due to the high antibiotic tolerance of biofilm cells and restricted penetration of antibiotics through the matrix, it is difficult to treat biofilm-associated infections. Few antibiotics including quinolones, rifampicin, tetracycline, and macrolides show higher penetration ability. Monotherapy is generally inappropriate because of the high antibiotic tolerance of biofilm-embedded cells. With no new antibiotics in the pipeline, antibiotic combinations are the most logical option for the treatment of biofilm infections (246). Combination therapies such as imipenem-rifampicin, colistin-rifampicin, imipenem-colistin-rifampicin, meropenem-sulbactam, and tigecycline-sulbactam have shown significant inhibition of *A. baumannii* biofilms (247–249). Rifampicin generates hydroxyl radicals, which are highly reactive oxygen forms and the major components of bactericidal agents. A particular study investigated the biofilm inhibitory effect of five biofilm inhibitors (zinc lactate, stannous fluoride, furanone, azithromycin, and rifampicin) either alone or in combination with one of the four antibiotics (imipenem, meropenem, tigecycline, and polymyxin B). These biofilm inhibitors in combination with antibiotics showed



different degrees of *in vitro* synergistic and additive antibacterial effects against XDR *A. baumannii* under sub-minimal inhibitory concentrations (250). Different studies showed that antimicrobial combinations such as colistin-levofloxacin, colistin-tigecycline and tigecycline-levofloxacin or these combinations with clarithromycin were used as catheter lock solutions, therefore, effective in the treatment of *A. baumannii* catheter-related infections (251). Use of minocycline-rifampicin impregnated bladder catheters, gentamicin-releasing urethral catheters, and norfloxacin along with blends of a copolymer of ethylene-vinyl acetate and polyethylene oxide as catheter coatings showed a reduction of CAUTIs (252–254). Clinical trials also showed that the use of medicated CVCs that contain antimicrobial solutions (minocycline-rifampicin, chlorhexidine/silver sulphadiazine, rifampicin-miconazole) on both the external and the luminal surfaces of catheters, exhibited significant inhibition of bacterial attachment but only the minocycline-rifampicin impregnated CVCs were able to reduce CRBSIs (255–257). TRL1068 (a human monoclonal antibody active against an epitope of DNABII proteins that stabilizes biofilm eDNA in both GPB and GNB) in combination with imipenem showed a significant reduction of *A. baumannii* adherence to catheters (258).

Quorum Sensing Quenchers: Stopping the Communication

Since QS contributes to biofilm production, inhibition of QS signaling pathways result in a reduction of biofilm formation and can be used as a novel therapeutic strategy (259). Biofilm

formation in *A. baumannii* is dependent on the activation of a LuxI/LuxR-type QS network involving AbaI synthase, AbaR receptor, and various AHLs. QS inhibition could be achieved by targeting the synthesis of AHLs. For example, mutation of the AHL synthase AbaI affects the surface-associated motility and robust biofilm formation in *A. baumannii* ATCC[®]17978 (104). A low concentration of triclosan (chemical with antibacterial properties) could inhibit the enoyl-acyl carrier protein reductase (FabI), which is important for AHL acyl chain formation (260). The use of natural compounds such as allin, patulin/clavacin, and vanillin were found to interfere with AHL receptors, leading to the prevention of QS signals (261–263). Moreover, AHL analogs, AbaR antagonists (such as streptomycin), anOR antagonist (virstain), and antagonists for di-guanylate cyclase enzyme that synthesize cyclic di-GMP were found to inhibit QS and subsequently biofilm formation in *A. baumannii* and *A. noscomialis* (264–268). A marine steroid (Siphonocholin) which has anti-QS activity was found to inhibit the EPS production, swarming motility, and biofilm formation in *A. baumannii* (269). Certain genetically engineered quorum quenching enzymes such as AHL lactonase and MomL could also successfully inhibit QS signal, leading to disruption of biofilm structure (270–272).

Natural Products/Essential Oils: Nature to the Rescue

Natural products such as microbial, plant, and animal derivatives can effectively reduce *A. baumannii* infections. Metabolites produced by bacteria have been proved to be effective against *A.*

baumannii biofilm (273). A study showed that *A. calcoaceticus* could be strongly inhibited by other bacterial (*Burkholderia cepacia*, *Methylo bacterium* spp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata*, and *Staphylococcus* spp.) crude cell-free supernatants (274). Maipomycin A which is isolated from the metabolites of the marine actinomycete and acts as an iron chelator inhibits *A. baumannii* biofilm formation on medical materials including silicone catheters and endotracheal tubes (polyvinyl chloride) (275). Several sets of compounds derived from marine sponges showed inhibition of different bacterial biofilm including *A. baumannii* specifically through non-microbicidal mechanisms (276). 5-episinuleptolide isolated from *Sinularia leptoclados* (a genus of soft coral in the family Alcyoniidae), showed anti-biofilm activity against *A. baumannii* ATCC[®]19606TM and MDR *A. baumannii* strains by decreasing the expression of the *pgaABCD* locus, which encodes the extracellular polysaccharide (poly-PNAG) of the biofilm structure (277). A secreted serine protease, termed “PKF” was identified in *A. baumannii* that suppressed *A. baumannii* biofilm formation (278). Myrtenol which is a bicyclic monoterpene isolated from various plants showed strong anti-biofilm properties against clinical strains of *A. baumannii* at a concentration of 200 µg/ml. Myrtenol specifically inhibited the mature biofilm, reduced the biofilm thickness, inhibited the biofilm-associated virulence factors, and improved the susceptibility of strains toward conventional antibiotics. Upon myrtenol treatment, suppression of the biofilm-associated genes such as *bfmR*, *csuA/B*, *bap*, *ompA*, *pgaA*, *pgaC*, and *katE* was found (279). Natural products such as essential oils (EO), plant secondary metabolites could exert broad-spectrum antibacterial activity by disrupting bacterial membrane integrity and also by inhibiting ATP synthesis that result in leakage of metabolites/ions (280). Different EOs with MBIC between 0.3–1.25 µl/ml derived from flowery plants such as *Mentha pulegium* L. and *Ziziphora tenuior* L., could damage the *A. baumannii* biofilm structure to a great extent. D-isomenthone, pulegone, isopulegone, menthol, and piperitenone were found to be the major components of the plant extract (281, 282). Anti-biofilm properties of four essential oil components (carvacrol, eugenol, thymol, and vanillin) were assessed against the organisms adhering to food surfaces in the meat industry. Carvacrol and thymol showed the most remarkable antimicrobial effect against *A. baumannii* strains (283). Similarly, other studies also showed that different EOs derived from plants and rich with these essential oil components (Oregano oil rich in carvacrol and thymol contents; cinnamon oil rich in eugenol; Eucalyptus camaldulens oil rich in thymol content) were active against *A. baumannii* wound infections (284, 285). EO-based nanoemulsions, prepared from *Thymus daenensis* (plant), showed potent antibiofilm activity against *A. baumannii* on sublethal dose (56.43% inhibition in 1/2 MIC concentration) after 24 h of incubation (286). Shivaprasad et al. (287) reported the activity of different antibiotics such as imipenem, cefipime, cotrimoxazole, amikacin, ciprofloxacin, piperacillin/tazobactam, cefoperazone, and gentamicin was enhanced against MDR/XDR *A. baumannii* complex when used in combination with lemongrass EO which showed 65–79% of anti-biofilm activity at a dilution between 0.625–0.156 µl/ml.

Most of the essential oils have been tested for *in vitro* activity against bacteria, only few were subsequently tested in cellular or animal models. Ismail et al. (288) reported that anti-biofilm activity of *Pimenta dioica* leaf EOs (85% inhibit) was higher than *Pimenta racemosa* leaf EOs (34%) against *A. baumannii* and both *P. dioica* and *P. racemosa* leaf EOs showed a bactericidal action against *A. baumannii* within 6 h at 2.08 µg·ml⁻¹. In addition, a significant reduction of *A. baumannii* microbial load in the mice wound infection model was also found (288). Similarly, oregano oil showed significant antibacterial activity against 11 MDR clinical isolates including *A. baumannii* with a MIC ranging from 0.08 mg/ml to 0.64 mg/ml. An *in vivo* study demonstrated that oregano oil topically applied 24 h after bacterial inoculation, sufficiently reduced the bacterial load in the wounds by 3 log₁₀ in 1 h (289).

Antimicrobials Peptides: An Alternative to Antibiotics

Antimicrobial peptides (AMPs) are cationic peptides (15–30 amino acids long) produced by the innate immune response and target the negatively charged cell membrane of bacteria (290). Different AMPs of biological origins have been reported to inhibit *A. baumannii* biofilm. Human AMP LL37 is one such AMP that inhibits *A. baumannii* biofilm (291). Human milk lactoferrin which is an iron-chelating AMP also showed slightly more potent antibacterial activity than bovine milk lactoferrin against *A. baumannii* biofilm (292). Derivative (D-RR4) of a small synthetic peptide, RR (12 amino acids) exhibited potent antibacterial and anti-biofilm activity against *P. aeruginosa* and *A. baumannii* in macrophage cells as well as in a *Caenorhabditis elegans* model (293). Magainin 2 (an antimicrobial peptide consisting of 23 amino acids isolated from the skin of the African clawed frog *Xenopus laevis*), showed the ability to inhibit *A. baumannii* biofilm at a very low concentration (294). Potent activity against *A. baumannii* biofilm has also been observed with several AMPs derived from flies, such as cecropin AMP identified from *Musca domestica* and another complex of AMPs (Fly larvae immune peptides) from *Calliphora vicina* (295, 296). Jakiewicz et al. (297) investigated the antimicrobial activity of eight peptides (aurein 1.2, CAMEL, citropin 1.1., LL-37, omiganan, r-omiganan, pexiganan, and temporin A) of different biological origins against *A. baumannii* biofilm on tracheal tube fragments. Among these peptides, strong anti-biofilm activity was shown by CAMEL and pexiganan with MIC values of 2 µg/ml (297). More recently, four synthetically produced chimeric AMPs have been proven to show anti-biofilm activities against MDR *A. baumannii*. These AMPs worked synergistically with ciprofloxacin, cefotaxime, or erythromycin to inhibit *A. baumannii* biofilm (298). The activity of the cationic antimicrobial peptide WLBU2 (24-residue engineered cationic amphipathic peptide) showed promising activity in combination with imipenem and tobramycin against planktonic cells and biofilm of MDR *A. baumannii* (299). A synthetically produced cyclic peptide ZY4 (17 amino acid long) exhibited biofilm eradication activity by killing the persister cells of *A. baumannii* and *P. aeruginosa* within the biofilm. Moreover, the therapeutic potential of ZY4 was also determined

in an *A. baumannii*-induced bacteremia mice model (300). Some AMPs are used as ointments for medical device surfaces or for the reduction of superficial tissue infections (301). In an *in vitro* experiment, Temporin-L showed an anti-biofilm effect without cell damage, representing the great potential for clinical application (302). AMPs have great potential in clinical treatment by effective removal of biofilms.

Efflux Pump Inhibitors: Blocking an Important Machinery

Studies had shown that efflux pumps play various roles in biofilm formation in ESKAPE pathogens; hence, inhibiting their function by efflux pump inhibitors (EPI) could also inhibit biofilm formation. A compound can be considered as a potent EPI when it has a broad substrate specificity and low off-target toxicity (303). One of the most common EPIs that is usually used in *A. baumannii* is phenylalanine-arginine β -naphthylamide (PA β N) which was reported to inhibit *A. baumannii* biofilm formation (304). Two novel serum-associated EPIs known as ABEPI1 and ABEPI2 were identified that potentiated the activities of antibiotics against *A. baumannii* grown in human serum. Both compounds exhibited similar antibiotic potentiation profiles toward minocycline and ciprofloxacin and inhibited pump activity (305). Another study also generated a set of compounds (pharmacophores) consisting of 2-substituted benzothiazoles which showed significant inhibition of AdeABC efflux pumps in combination with ciprofloxacin (306). Krishnamoorthy et al. assessed the efficacy of two microbicides such as cetrimide and chlorhexidine which adversely modified the expression and function of AdeABC efflux pump in biofilm-associated *A. baumannii*. Furthermore, they established that these microbicides decreased the negative charges on *A. baumannii* cell membranes, causing dysregulation of the efflux pump, leading to cell death (307).

Nanoparticles: Small Is Beautiful

Nanoparticles (NPs) are very small in size (<100 nm) with a large surface area and extremely reactive nature. They show broad-spectrum activities against both GPB and GNB and sometimes they have been preferred over antibacterial agents. NP can disrupt biofilm integrity by penetrating bacterial cell membrane, generating ROS, causing ATP depletion, and interacting with polysaccharides, eDNA, proteins, and lipids (308). Different studies have been carried out to understand the role of NPs to inhibit *A. baumannii* biofilm. A study showed the use of nitric oxide (NO) releasing NPs to treat *A. baumannii* biofilm-related wound infections *in vivo* in murine models (309). Another study showed the disruption of *A. baumannii* biofilm on exposure to nanoemulsion of cetylpyridinium chloride, a quaternary ammonium salt (310). NPs coupled with metals or natural product extracts have been shown to possess inhibitory activity against both planktonic and biofilm-associated cells. A particular study showed the action of silver NPs, gold NPs and silver-gold bimetallic NPs against *A. baumannii* biofilms. These NPs showed 88% of *A. baumannii* biofilm inhibition (311). Several other studies also showed the efficiency of silver NPs (AgNPs) in inhibition of *Acinetobacter* biofilms as these NPs easily penetrate

the thick EPS in biofilms. Positively charged AgNPs interact with negatively charged eDNA that plays a major role in the inhibition of biofilms (312, 313). In addition, *A. baumannii* biofilm inhibition was also observed by selenium NPs, curcumin NPs, aluminum oxide NPs, etc. (314–316). NPs in combination with antibiotics have also been reported to possess substantial antibiofilm activity. AgNPs were found to act synergistically with imipenem, as imipenem lyses the cell wall of bacteria leading to increased penetration of AgNPs into the cells (317). NPs can also be used as drug delivery carriers or as catalysts to promote the penetration of drugs into biofilms; improve the solubility, stability, and biocompatibility of drugs (318).

Phage Therapy: Cocktails That Work

The use of bacteriophages is another approach to control and remove biofilms. Different lytic bacteriophages such as AB7-IBB2 (family of Podoviridae), AB7-IBB1 (Siphoviridae), and vB_AbaMIME-AB2 were found to inhibit *A. baumannii* biofilms (10^8 CFU/well) on abiotic and/or biotic surfaces (60–>80%) (319–321). Lood et al. identified 21 distinct lysins (prophages) induced from 13 diverse *A. baumannii*. Among these lysins, PlyF307 showed the greatest activity, and treatment with PlyF307 was able to significantly reduce planktonic and *A. baumannii* biofilm both *in vitro* and *in vivo* (322). Thandar et al. (323) showed that the C-terminal amino acids (15, 108–115, 117–138) of a phage lysin named P307, alone could efficiently kill *A. baumannii* (>3 logs) while its engineered derivative (P307SQ-8C) showed improved activity (>5-log kill) along with polymyxin B. Two different phages (B_AbaM_ISTD, and vB_AbaM_NOVI), isolated from Belgrade wastewaters, were found to inhibit *A. baumannii* biofilms (324). *A. baumannii* biofilm biomass was inhibited when an environmental phage cocktail was used in combination with antibiotics such as ciprofloxacin, trimethoprim/sulfamethoxazole, gentamicin, tobramycin, imipenem, and meropenem which are generally used in the treatment of UTI. While phage cocktail combined with levofloxacin and amikacin, did not act synergistically (325). An excellent method of inhibiting *A. baumannii* biofilm was devised by Ran et al. (326) by combining photodynamic bacteriophages (ABP) and Nile blue photosensitizers (NB). NB photosensitizer possessing sulfur atom displayed ROS production ability. Both *in vitro* and *in vivo* experiments proved that NB-phage bioconjugate (APNB) could bind to the main components of biofilms and reduce drug resistance caused by biofilms (326).

Other Biofilm Inhibitors

Some other chemicals or compounds that do not belong specifically to the above-mentioned groups have also been reported to inhibit *A. baumannii* biofilms. In order to determine the effectiveness of biocides for the eradication of MDR *A. baumannii* biofilms, hydrogen peroxide, and hydrogen peroxide-based formulations were tested. Mixed-culture biofilm cells were found to be more resistant to some biocides, such as hydrogen peroxide and sulfathiazole, than the single-species *Acinetobacter* biofilm cells. Higher potential for biofilm removal and killing was found among oxidizing biocides, such as sodium hypochlorite

and hydrogen peroxide, compared to non-oxidizing biocides (sulfathiazole and glutaraldehyde) (327, 328). Reverse-amide class of 2-aminoimidazole compounds were also found to inhibit *A. baumannii* biofilms by >95% at 100 μ M and had the potential to readily increase the permeation of many conventional antibiotics into the bacterial cell membrane, therefore can be used as a new “drug delivery” mechanism in a variety of systems (329). The efficacy of octenidine dihydrochloride (OH) (disinfectant) was tested to reduce *A. baumannii* biofilms on polystyrene, stainless steel, catheters and was found to be effective in inhibiting *A. baumannii* biofilms at a concentration of 5, 10, and 15 mM (330). Biocidal activity of commonly used antiseptics and disinfectants [sodiumhypochlorite, chlorhexidine, orthophthalaldehyde (OPA), peracetic acid (PA), and peracetic acid] were tested against *A. baumannii*. About 78% of biofilm-producing *A. baumannii* became susceptible to all disinfectants and antiseptics tested (331). *A. baumannii* growth and biofilm formation in human serum was reduced by 16 μ M of gallium nitrate (hydrated nitrate salt), whereas a higher concentration (64 μ M) caused huge disruption of the preformed *A. baumannii* biofilms (332). N^2 , N^4 -disubstituted quinazoline-2,4-diamines which are dihydrofolate reductase inhibitors exhibited anti-biofilm activity (90%) against *A. baumannii* when the 6-position is replaced with a halide or an alkyl substituent (333). A clinical trial showed that the use of silver alloy on catheter material reduced bacterial colonization, thereby reducing CAUTIs (334). Different clinical trials showed that in addition to silver alloy-coated latex catheters, nitrofurazone-coated silicon catheters were also found to reduce CAUTI during short-term use (<30 days) (335).

SURFACE MODIFICATION AND PHYSICAL THERAPY

Surface modification and physical therapy are the most successful treatment options for eradication or prevention of microbial adhesion on medical devices when antibiotic treatment fails to effectively eliminate medical device-related biofilm infections. An effective physical method is photodynamic therapy (PDT) for removing microbial adhesion and biofilm formation. PDT inhibits biofilm formation and also fights against biofilm infections by producing Reactive Oxygen Species (ROS) and inhibiting the production of some toxic factors that affect bacterial adhesion and biofilm matrix formation (336, 337). PDT technique is also used to combat other implant-related biofilm infections, such as prosthetic joint infections and infections caused by ventilator-associated pneumonia biofilms (338). Low-intensity ultrasound at the physiotherapy level showed effective removal of biofilm by enhancing the activity of antibacterial agents (339). Another effective physical method is the use of water jets for removing biofilms on the surface of implants through the mechanical action of pulse and pressure (340).

Advances in surface engineering have led to the development of antibacterial agents or antiadhesion agents to coat the surface of medical devices that can effectively inhibit the growth of microorganisms. The use of Sharklet™ surface made by shark

skin and microscale ribs of various lengths are combined into a repeating diamond micropattern, preventing bacterial colonization and biofilm formation when incorporated into the surfaces of medical devices (341). Several antibacterial coatings, such as chlorhexidine, rifampicin, gentamicin, minocycline, silver sulfadiazine, amikacin, and vancomycin, have been widely used in clinical practice, showing the efficacy of preventing catheter-related and other implant-related infections (342). Hydrophilic polymers such as hyaluronic acid have been used to coat different medically relevant materials like polyurethane catheters and silicon shunts and have been shown to be effective in preventing biofilm formation on polystyrene surfaces. An isoeugenol coating has been shown to prevent the adhesion and formation of biofilms on stainless steel and polyethylene surfaces due to its good antibacterial activity (343). Various hydrogel coatings, heparin coatings, or bindings have been used in medical devices to reduce fibronectin deposition on vascular catheter surfaces due to high antiadhesive activity (344). In addition, inhibition of microbial growth on catheters also depends on the catheter constitutive polymers which are able to absorb large amounts of antibiotics. These polymers can be designed by introducing acidic or basic groups into the polymer side chains that are able to interact with different classes of drugs (345). Polyethylene glycol and bovine albumin could be incorporated into the polymer bulk together with antibiotic or antifungal molecules to increase and control drug release from the polymer matrices.

DISCUSSION

The genus *Acinetobacter* is not easy to deal with, both for researchers and clinicians alike, starting with the identification of the multiple species to its propensity to form biofilms and acquire antibiotic resistance genes readily (13, 30). *A. baumannii* has been particularly difficult in ICUs and notorious global clones ST1 and ST2 have also been reported from cases of neonatal sepsis (346, 347). Of the different species “baumannii” is most frequently reported; however, other species of *Acinetobacter* are also being reported to cause infections (348). The role of “non-baumannii” in human infection has been perceived recently because of the technological advances that allow correct identification of the bacteria at the species level. Identification of the bacteria at the species level heavily depends on the molecular methods or MALDI-TOF. The identification of the species is particularly challenging in low middle-income countries which lack the requisite infrastructure to differentiate the several species. Furthermore, *A. baumannii* and non-baumannii have distinct resistance mechanisms for antimicrobial agents though phenotypically they seem to be undifferentiated. Though this review primarily focused on baumannii, non-baumannii isolates could be lurching behind and due attention should be paid to them.

Despite all the effort that mankind has made to improve and save lives, it has been seen that microbes have found new strategies every time to evade technological or medical advancements. With the additional challenge of antibiotic

resistance, new approaches need to be utilized to restrict the usage of antibiotics. Some remedies (such as antibiotics, nano antimicrobial compounds, natural products, bacteriophages) that directly act on pathogens have already been discussed in this review but antimicrobial remedies that act on the host (several innate immune-enhancing peptides) to potentiate antibiotic action should be explored (349). Similarly, probiotics also confer a health benefit on the host against MDR *A. baumannii* when administered in adequate amounts combined with immunomodulators, such as lysophosphatidylcholine and antibiotics such as clarithromycin, colistin, tigecycline, or imipenem by stimulating the immune response (350–352). Though advances in medical science are appreciated, there is no underestimating the power of prevention. Hospital infection control is as relevant as in earlier times and can be important in reducing nosocomial infections and interrupting the transmission chain of pathogens such as *Acinetobacter*.

The ability of *Acinetobacter* to attach to biotic and abiotic surfaces is useful for the organism, particularly in hospitals. It can colonize the patients (sometimes without any symptoms), caregivers, and hospital surfaces or devices that increase the chances of infection. This realization has prompted several studies on approaches to prevent the formation of biofilms in hospital devices. The approaches are diverse ranging from antibiotic-coated devices, quorum sensing quenchers, natural products, phage cocktails and nanoparticles. However, very few of these approaches have been tested in *in vivo* models (288, 289) and reached the patient; most are *in vitro* studies, which have not been carried forward. *In vivo* studies address many of the shortcomings of *in vitro* studies and one can better evaluate the safety, toxicity, and efficacy of a drug candidate in *in vivo* models. Thereby, emphasis needs to be on *in vivo* models and further clinical trials.

Development of persister cells, antibiotic tolerance, population heterogeneity, and biofilm-related infections should be considered as significant risk factors in the course of choosing an appropriate therapy particularly in the case of *A. baumannii*. The possible role of the diversity of its reservoirs, its resistance to drying, its capacity to accumulate resistance genes especially plasmid-mediated resistance genes are challenges associated with this organism. Plasmids carrying carbapenem-resistant genes such as *bla*_{NDM} also carry other resistant genes, which make a panel of antibiotics ineffective. The evolution of new variants of antibiotic-hydrolyzing enzymes, which have better efficiency and better stability, also has made it difficult to treat MDR strains, especially *A. baumannii*.

As the authors reviewed the aspect of biofilm formation alongside antibiotic resistance in *Acinetobacter*, it was realized that there is still a lack of clarity regarding the association. It is presumed that this controversy prevails due to the methodology used to test the association. Most studies have used clinical *Acinetobacter* isolates, carried out *in vitro* biofilm assays, and tested the antibiotic susceptibility separately. As most clinical isolates are also antibiotic-resistant (independent of their biofilm-forming capability) such studies may not be appropriate in understanding the association. The difference in

the susceptibility of the isolates to antibiotics, when studied as planktonic growth as well as biofilms, will probably give a better understanding. As the entry of antibiotics is restricted within biofilms due to several mechanisms such as the presence of EPS matrix, antibiotic degradation, *Acinetobacter* as biofilms is resistant to the action of antibiotics but this phenomenon is irrespective of the presence of resistance determinants present.

Whole-genome sequencing has generated a vast amount of data that has enriched our understanding of pathogens. Analysis of such data can build an understanding of the epidemic clones, the ARGs or MGEs that are prevalent, their associations with particular clones, etc. The genome data can also be utilized to understand the strengths and weaknesses of antimicrobial agents. However, sequences are mere blueprints and different *in vivo* and *in vitro* laboratory assays need to be carried out along with genome sequences to understand potential therapeutic options. The focus should be on robust assays from the leads that are already identified.

CONCLUSION

Most GNB have an underlying similarity, yet there are several features unique to each, making some of these bacteria such as *A. baumannii* more challenging than others. The determinants that drive any organism to be a successful pathogen are a consequence of several diverse factors. These factors include antibiotic use, infection control practices, climate change, human behavior, deforestation, availability of resources, and several others, that can over the years determine how these pathogens evolve. Future research will increase our understanding of this pathogen. The clinicians' experience of treating patients with *Acinetobacter* infection can further strengthen our future approaches to treatment. Harnessing the "clinical eye" with the "scientific fervor" may help devise new strategies to deal with a pathogen that has various tools at its disposal. The use of antimicrobials in hospitals dealing with COVID-19 patients has increased. However, the attention that antibiotic resistance has been drawing for the past few years has waned off, at least temporarily. The strides that we have taken in controlling resistance need to be fostered to effectively control *A. baumannii* infections.

AUTHOR CONTRIBUTIONS

SR and GC performed literature search, wrote the manuscript, and prepared all the figures and tables. SB conception, design, wrote the introduction, and discussion along with a critical revision of the manuscript. AM and SD critically revised the manuscript. All authors read and approved the final manuscript.

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