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Bacterial Biofilm: Its Composition, Formation and Role in Human Infections

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Review Article

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ABSTRACT

Biofilm is an association of micro-organisms in which microbial cells adhere to each other on a living or non-living surfaces within a self-produced matrix of extracellular polymeric substance. Bacterial biofilm is infectious in nature and can results in nosocomial infections. According to National Institutes of Health (NIH) about about 65% of all microbial infections, and 80% of all chronic infections are associated with biofilms. Biofilm formation is a multi-step process starting with attachment to a surface then formation of micro-colony that leads to the formation of three dimensional structure and finally ending with maturation followed by detachment. During biofilm formation many species of bacteria are able to communicate with one another through specific mechanism called quorum sensing. It is a system of stimulus to co-ordinate different gene expression. Bacterial biofilm is less accessible to antibiotics and human immune system and thus poses a big threat to public health because of its involvement in variety of infectious diseases. A greater understanding of bacterial biofilm is required for the development of novel, effective control strategies thus resulting improvement in patient management.

HISTORICAL BACKGROUND OF BIOFILM

Bacterial cell exhibit two types of growth mode i.e. planktonic cell and sessile aggregate which is known as the biofilm. Biofilm is an association of micro-organisms in which cells stick to each other on a surface encased within matrix of extracellular polymeric substance produced by bacteria themselves [1]. A Dutch researcher, Antoni van Leeuwenhoek, for the first time observed 'animalcule' on surfaces of tooth by using a simple microscope and this was considered as the microbial biofilm discovery [2]. Later in 1973, Characklis indicated that biofilms are not only tenacious but show higher resistant nature to disinfectants e.g. chlorine. Costerton, in 1978, coined the term biofilm and alert the world about the importance of biofilm [2]. Biofilms are present everywhere in nature and can be found in industrial places, hotels, waste water channels, bathrooms, labs, hospital settings and commonly occur on hard surfaces submerged in or exposed to an aqueous solution. It can also be formed as floating mats on surface of liquid. Its formation can occur on both living and non-living surfaces [3].

COMPOSITION OF BIOFILMS

Biofilms are group or micro-organisms in which microbes produced an extracellular polymeric substances (EPS) such as proteins (<1-2%) including enzymes), DNA (<1%), polysaccharides (1-2%) and RNA (<1%), and in addition to these components, water (up to 97%) is the major part of biofilm which is responsible for the flow of nutrients inside biofilm matrix **(Table 1)**. The architecture of biofilm consists of two main components i.e. water channel for nutrients transport and a region of densely packed

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cells having no prominent pores in it [4]. The microbial cells with in biofilms are arranged in way with significant different physiology and physical properties. Bacterial biofilms are normally beyond the access of antibiotics and human immune system. Micro-organisms that produce biofilm have enhanced potential to bear and neutralize antimicrobial agents and result in prolonged treatment. Biofilm forming bacteria switch on some genes that activate the expression of stress genes which in turn switch to resistant phenotypes due certain changes e.g. cell density, nutritional or temperature, cell density, pH and osmolarity [5]. When the biofilm water channels are compared with system of circulations showed that biofilms are considered primitive multi-cellular organisms [6]. Various components of biofilms are shown in **Table1** signify the biofilm integrity and making it resistant against various environmental factors [7].

Table 1. Biofilm chemical composition [114].

S. No	Components	Percentage of matrix
1	Microbial cells	2-5%
2	DNA and RNA	<1-2%
3	Polysaccharides	1-2%
4	Proteins	<1-2% (including enzymes)
5	Water	Up to 97%

HOW BIOFILMS ARE FORMED?

Biofilm formation is a highly complex process, in which microorganism cells transform from planktonic to sessile mode of growth [8]. It has also been suggested that biofilm formation is dependent on the expression of specific genes that guide the establishment of biofilm [8,9]. The process of biofilm formation occurs through a series of events leading to adaptation under diverse nutritional and environmental conditions [10-12]. This is a multi-step process in which the microorganisms undergo certain changes after adhering to a surface **(Figure 1)**. Microorganisms which form biofilms are shown to elicit specific mechanisms. Biofilm formation has following important steps (a) attachment initially to a surface (b) formation of micro-colony (c) three dimensional structure formation (d) biofilm formation, maturation and detachment (dispersal) [2].

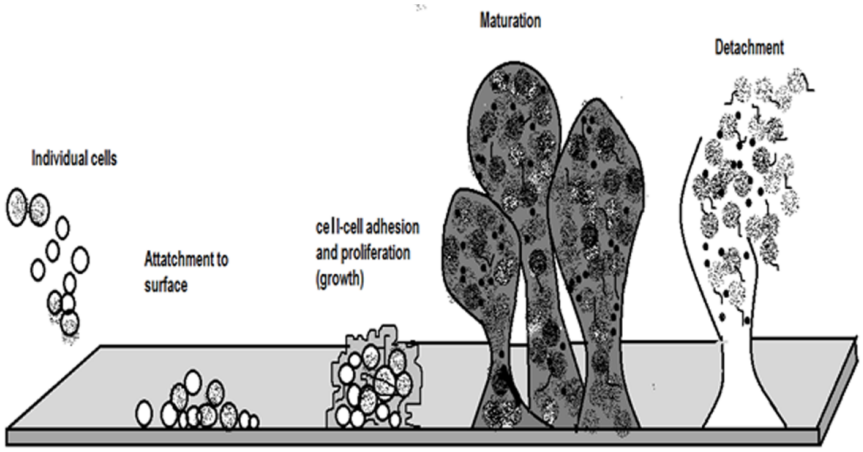

Figure 1. The biofilm life cycle in three steps: attachment, growth of colonies (micro-colony formation and formation of three dimensional structures) and detachment in clumps.

ATTACHMENT

When a bacterium cell reaches to near some surface/support so close that its motion is very slow down, it make a reversible connection with the surface and /or already adhered other microbe to the surface. For biofilm formation, a system of solid–liquid interface can provide an ideal environment for micro-organism to attach and grow (e.g. blood, water) [2]. For most frequent attachment and biofilm formation rough, hydrophilic and coated surfaces will provide better environment. Increased attachment may also occur due to increase but not exceeding critical level in flow velocity, temperature of water or nutrients concentrations. Presence of locomotor structures on cell surfaces such as flagella, pili, fimbriae, proteins or polysaccharides are also important and may possibly provide an advantage in biofilm formation when there are mixed community [13].

MICRO-COLONY FORMATION

Micro-colony formation takes place after bacteria adhered to the physical surface/biological tissue and this binding then becomes stable which results in formation of micro-colony. Multiplication of bacteria in the biofilm starts as a result of chemical signals. The genetic mechanism of exopolysaccharide production is activated when intensity of the signal cross certain threshold [2]. So by this way using such chemical signal, the bacterial cell divisions take place within the embedded exopolysaccharide matrix, which finally result in micro-colony formation [14].

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THREE-DIMENSIONAL STRUCTURE FORMATION AND MATURATION

After micro-colony formation stage of biofilm, expression of certain biofilm related genes take place. These gene products are needed for the EPS which is the main structure material of biofilm. It is reported that bacterial attachment by itself can trigger formation of extracellular matrix. Matrix formation is followed by water-filled channels formation for transport of nutrients within the biofilm. Researcher have proposed that these water channels are like a circulatory systems, distributing different nutrients to and removing waste materials from the communities in the micro-colonies of the biofilm [15].

DETACHMENT

After biofilm formation, the researchers have often noticed that bacteria leave the biofilms itself on regular basis. By doing this the bacteria can undergo rapid multiplication and dispersal. Detachment of planktonic bacterial cells from the biofilm is a programmed detachment, having a natural pattern [2]. Sometime occasionally due to some mechanical stress bacteria are detached from the colony into the surrounding. But in most cases some bacteria stop EPS production and are detached into environment. Dispersing of biofilm cells occur either by detachment of new formed cells from growing cells or dispersion of biofilm aggregates due to flowing effects or due to quorum- sensing [16]. In biofilm of cells are removed due to an enzyme action that causes alginate digestion [2]. Phenotypic characters of organisms are apparently affected by the mode of biofilm dispersion. Dispersed cells from the biofilm have the ability to retain certain properties of biofilm, such as antibiotic in-sensitivity. The cells which are dispersed form biofilm as result of growth may return quickly to their normal planktonic phenotype [16]. The different steps in biofilm life cycle are shown in **Figure 2**.

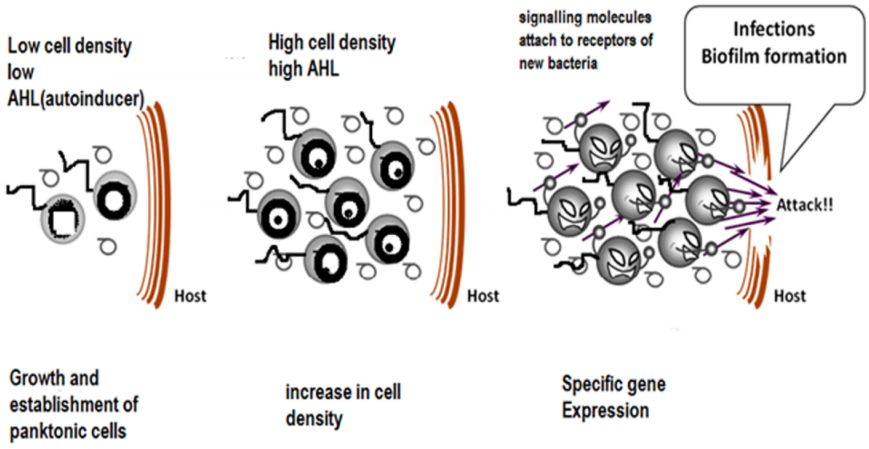

Figure 2. Cell density dependent gene expression in quorem sensing.

QUORUM SENSING

During biofilm formation many species of bacteria are able to communicate with one another through a mechanism called quoreum sensing [17]. It is a system of stimulus to co-ordinate gene expression with other cells and response related to the density of their local population. During quorum sensing signalling molecules attach to receptors of new bacteria and help in transcription of genes within a single species of bacteria as well as between different bacterial species [18] **(Figure 2)**. QS system enables communication between intraspecies and interspecies which involves in terms of biofilm formation, food shortages and environmental stress conditions, such as disinfectants, antibiotics, bacterial colonization, the identification of annoying species, the establishment of normal intestinal flora as well as the prevention of harmful intestinal flora. Many clinically-associated bacteria use QS for the regulation of the collective production of virulence factors. QS in Gram-positive bacteria occur through a series of events such as production, detection and response to AIs. The oligopeptide auto inducing peptides in many Gram positive bacteria are detected by membrane-bound two component signal transduction systems [19-21]. They are encoded as precursors (pro AIPs) and possess sequence diversity [8,18,22-25].

BIOFILM FORMING BACTERIA

Nearly all (99.9%) of micro-organisms have the ability to form biofilm on a wide range of surfaces i.e. biological and inert surfaces [26]. When micro-organisms bind to a surface, they produce extracellular polymeric substance (EPS) and form biofilm. Biofilm posing a great problem for public health due to its resistant nature to antibiotics and disease associated with indwelling medical devices [2,27]. It is found that *H. influenza* has the ability to form biofilm in human body and can escape from human immune system [26]. Biofilm forming capability has been reported in large number of bacterial species such as *P. aeruginosa*, *S. epidermidis*, *E. coli spp*, *S. aureus*, *E. cloacae*, *K. pneumoniae* [15,18,28,29] **(Table 2)**. A few biofilm forming bacterial species have described below.

Table 2. A list of common biofilm forming bacterial species [17].

S.No	Common biofilm forming bacterial species
1	*E. coli*
2	*P. aeruginosa,*
3	*S. epidermidis,*
4	*S. aureus,*
5	*Staphylococcus epidermidis*
6	*E. cloacae*
7	*K. pneumoniae*
8	*Actenomyces israelii*
9	*Haemophilus influenza*
10	*Burkholderia cepacia*

Escherichia coli

E. coli is a rod shaped Gram negative bacteria causing a large number of nosocomial and community infections such as urinary tract infections (UTIs) and prostatitis. It has the ability to secret toxins, polysaccharide and can form biofilm. It can also form biofilm *in-vitro* [17]. *E. coli* capsules are high molecular weight molecules and are attached to the cell surface. *E. coli* capsule play an indirect role in biofilm by protecting bacterial surface adhesion. Different environmental conditions affect *E. coli* capability to form biofilm [30]. Thickness of *E. coli* biofilm may be of hundreds of microns and posing a difficulty in treatment with antibiotics due to presence of exopolymers [30].

Pseudomonas aeruginosa

P. aeruginosa is a Gram negative notorious opportunistic pathogen found along with other Pseudomonas species as part of normal flora of human skin [30-32]. *P. aeruginosa* is a ubiquitous human pathogenic organism present everywhere, and can be isolated from different sources such as humans, plants and animals [33-37]. It has a strong tendency to form biofilm [38,39] and such biofilm has been found to be partially responsible for chronic infections. *P. aeruginosa* biofilm can be eradicated by using silver. Silver has antibacterial activity and bactericidal concentration of silver necessary to eradicate the bacterial biofilm was found to be 10-100 times higher as compared to that used to eradicate planktonic bacteria [10]. The biofilms of *P. aeruginosa* are developed communities of individual cells that are encased in an extracellular polysaccharide matrix. These are extremely resistant to antibiotics .Their biofilm formation involves an initial attachment to a solid surface which leads to the formation of micro-colonies. These micro colonies differentiate into exopolysaccharide-encased, mature biofilms [2]. *P. aeruginosa* is a multidrur resistantant bacteria even including ciprofloxacin, that are commonly used for the treatment of lung infections [40].

Staphylococcus aureus

S. aureus is a multi-drug resistant bacteria causing a number of nosocomial infections. It grows on catheters and chronic wounds as biofilm [41,42]. *S. aureus* recycles proteins for the formation of the extracellular matrix in the cytoplasm. The cytoplasmic proteins also working as matrix proteins allows enhanced flexibility and adaptation to *S. aureus* in forming biofilms in infectious conditions and could encourage the formation of mixed-species biofilms in chronic wounds [43].

Stereptococcus epidermidis

S. epidermidis is well known as an opportunistic pathogen that has greater potential to cause infections in patients with immune-compromised state, intravenous drug abusers, AIDS patients, immuno-suppressive therapy patients and premature new born [24,44]. During surgical implantation of polymeric devices the *S.epidermidis* contamination chances increases because of its biofilm forming capability. Biofilm formation is responsible for device related infections of *S. epidermidis* and this consequently leads to the pathogenesis [45]. In *staphylococci*, the main factor which is responsible for adhesion is called the polysaccharide intercellular adhesion (PIA). In *Staphylococcus* cells are covers by PIA which hold them together as the most important component of the extracellular matrix [46]. In a recent study Rohde et al. [47] have reported PIA-independent biofilm formation in about 27% of biofilm-forming *S. epidermidis* strains isolated from prosthetic joint infections.

Enterobacter cloacae

E. cloacae is a Gram positive bacteria causing a range of nosocomial infections in human i.e. lower respiratory tract infection, bacteraemia, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, skin and soft tissue infections, osteomyelitis and ophthalmic infections. *Enterobacter* causing nasocomial infections is most frequently isolated species and in recent years has emerged as important pathogenic bacteria [29]. Bloodstream infections which are responsible for morbidity and mortality in both developing and developed countries are caused by *E. cloacae* [27]. It also causes biofilm associated infections such as UTIs and biliary tract infections. *Enterobector* also has property of intrinsic resistance to certain antibiotics such as ampicillin and narrow-spectrum cephalosporins. It is also showing high frequency of mutations to expanded-spectrum and broad-spectrum cephalosporin. It also possesses ß-lactamase and showing resistance to third generation cephalosporins [48].

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Klebsiella pneumoniae

K. pneumoniae is a Gram negative bacterium, frequently causing nosocomial infections, belongs to the genus *Klebsiella*. *K. pneumonie* is very important species among genus *Klebsiella* and causing a considerable proportion of nosocomial infections such as urinary tract infections (UTI), pneumonia, septicemias and soft tissue infections [49]. Field emission scanning electron microscopy (F-ESEM) and confocal laser scanning microscopy were used to investigate the biofilm of *K. pneumoniae*, isolated from clinical strains. In a study on different strains of *K. pneumonia* isolated from various human samples such as urine, blood sputum and wound swabs, it was reported that about 40% strains had the ability to form biofilms. However, increasing temperature from 35°C to 40°C resulted in consistent growth of biofilm on abiotic surfaces. Also the ability of *Klebsiella pneumonia* to form biofilm takes place more successfully in a mix strains than individual strain [50,51].

BIOFILMS AND ANTIBIOTIC RESISTANCE

Mechanisms of antibiotics and biocides resistance of biofilms are categorized into four classes which include (a) active molecule inactivation directly (b) altering body's sensitivity to target of action, (c) reduction of the drug concentration before reaching to the target site and (d) efflux systems **(Figure 3)**. Biofilm antibiotic resistance level may vary among different sittings and the key factors responsible for this resistance may also differ. Regarding resistance, the primary evidence shows that conventional mechanisms are unable to explain the high resistance to antibacterial agents associated with biofilms, although this evidence cannot be ignored in resistance in the growth of adherent cells. So it is suggested that the resistance posed by the adhered bacteria or biofilms may have some intrinsic mechanisms and are responsible for conventional antibiotic resistance [52]. Several mechanisms have been explored that are considered to be key factors in high resistance nature of biofilms. These mechanisms are (a) limited diffusion, (b) enzyme causing neutralizations, (c) heterogeneous functions, (d) slow growth rate, (e) presence of persistent (non-dividing) cells and (f) biofilm phenotype such adaptive mechanisms e.g. efflux pump and membrane alteration [53,54].

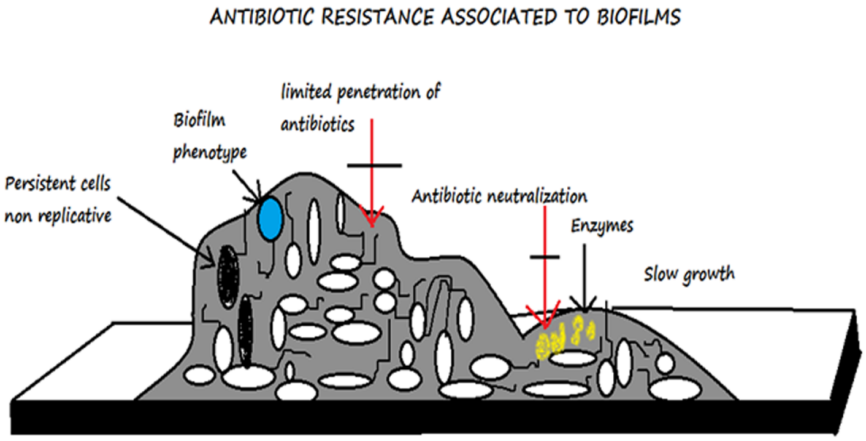

Figure 3. Antibiotic resistance associate to biofilm. Description of the key mechanisms involved in antibiotic resistance such as enzyme causing neutralizations, presence of persistent (non- dividing) cells and biofilm phenotype.

LOW PENETRATION OF ANTIBIOTICS

Diffusion of antibiotics can take place through the matrix of the biofilm. Diffusion or penetration of antibiotics to deeper layers of biofilm is affected by exopolysaccharide acting as a physical barrier. When molecules direct interact with this matrix, their movement to the interior of the biofilm is slow down, resulting antibiotic resistance. This may also acts as a hindrance for high molecular weight molecules such as complement system proteins and lysozyme, and in liquid culture bacterial cells are readily exposed to antibiotics as compare to compact structure biofilm. Bacteria escape from biofilm that do not produce polysaccharide and are easily attack by immune system cells. Inactivation of antibiotic takes place when bind to biofilm matrix. *P. aeruginosa* have alginate exopolysaccharide, which is anionic in nature. Presence of this matrix explains slow penetration of fluoroquinolones and aminoglycosides [39,55,56]. Low penetration of antibiotic is not sufficient to explain the biofilm resistance, other mechanisms have been assumed that must be involved. This is also suggested recently that slow diffusion of antibiotics permit plenty of time to establish a protective response to stress [39].

NEUTRALIZATION BY ENZYMES

Antibiotics resistance in biofilm may be due to the presence of neutralizing enzymes which degrade or inactivate antibiotics. These enzymes are proteins which confer resistance by mechanisms such as hydrolysis, modification of antimicrobials by different biochemical reactions. Accumulations of these enzymes occur in the glycocalyx from the biofilm surface by the action of antibiotics. Neutralization by enzymes is enhanced by slow penetration of antibiotics and also antibiotics degradation in the biofilm. In cystic fibrosis which is caused by *P. aeruginosa*, overproduction of *cephalosporinase* AmpC enzymes is responsible for

resistance to different antibiotics. This enzyme confers resistance to β-lactam in the presence of even much more concentration of carbapenems [57]. During a study when filters impregnated with antibiotics was applied on *K. pneumoniae* biofilm (mutant cells β-lactamases), in spite of good diffusion of antibiotic, growth was observed, suggesting that there would be another mechanism of resistance which need to be explored [58].

HETEROGENEOUS NATURE

Studies performed on determination of microbial growth in biofilms by using a microelectrode with probes to direct measure oxygen concentration in different areas of the biofilms [39]. The biofilms are heterogeneous nature both metabolically and structurally and both processes such as aerobic and anaerobic occur at the same time. So response against antibiotics may be different in different areas of the biofilms. On surface of biofilm there is a high level of activity of antibiotics while inside the biofilms, slow or absent growth reduces the sensitivity of the cells to antimicrobials [59]. In the various sub layers of biofilm, aerobic or facultative anaerobic microbial populations help us to know the differential susceptibility to various antibiotic therapies. Antibiotics response to the planktonic forms is different from the adhered cells. Action of aminoglycosides is affected by limitation of oxygen and anaerobic growth of microorganisms, which is affected by the presence of oxygen and pH gradients.

CELLS SLOW GROWTH RATE

Slow growth of microorganisms occurs due to limited availability of nutrients which confer resistance to antibiotics. In case of biofilm a gradient of nutrients resulting in metabolically active cell (periphery or surface layer) and inactive cells (within its interior) [60]. Bacterial cells are attack by both penicillin and ampicillin only when they are growing. Some other antibiotics that attack cells in stationary phase are β-lactams, aminoglycosides, cephalosporin and fluoroquinolones [2]. So due to slow growth resistance has been determined in different bacterial strains such as resistance to cetrimide on *E. coli*, piperacillin and tobramycin in *P. aeruginosa* and ciprofloxacin on *S. epidermidis*. It has been shown that this resistance was due to slow growth [13,61]. There are some natural peptides produce during host innate immune response act as antibacterial providing protection to the body [62]. A peptide named polymyxin E is effective in treating cancer patient's biofilms and cystic fibrosis, caused by *P. aeruginosa* [63,64]. In Cystic fibrosis patients, using ciprofloxacin and tetracycline can clear active growing cells and it is suggested that a combination of antibiotic colistin with other two antibiotics (ciprofloxacin and tetracycline) will be very effective in clearing *P. aeruginosa* [65].

EXISTENCE OF PERSISTENT CELLS

After a purling antibiotics treatment of biofilm, a very small number of bacterial cells remain viable, called persistent cells. These cells may or may not give this resistance to their progeny and return to their normal state after the release of the applied stress or pressure. The persistent cells stop their replication for small duration for the survival of the community. Their adaptive mechanism is not related to the mechanism followed by the cells during stress (environmental damage). Persistent cells can bear multiple antibiotic doses and work for survival. When density of bacterial cells number in stationary phase raised to maximum, persistent cells increase in number indicting their main role in survival [66]. There are certain evidences for the presence of persistent cells in biofilm: a) there is existence of a biphasic dimension in biofilms which means that large number of cells population is attacked while the rest of the population is not attacked (resistant) even with an extensive antibiotics treatment, b) persistence gene description function as a circuits of regulation, c) bacteriostatic antibiotics contribute to the growth of persistent cell and biofilm preservation by inhibiting growth of sensitive cells and d) reshaping of biofilm into original form when the antibiotics therapy is withdrawn [67].

BIOFILM PHENOTYPE

During biofilm formation, bacteria produce some products called secondary metabolites. These products are not required by the cell for their growth. These metabolites function as signaling molecules thus enhancing formation process of biofilms [68]. Biofilm phenotype is regarded as community cells that confer no response to antibiotics treatment. These characteristics have proposed the presence of specific genes. In *B. subtilis* only 6% difference in gene expression was observed for biofilm by DNA microarrays as compared to their planktonic culture cells, while for P. aeruginosa this difference was only 1%. However at present time, this differential gene expression has not been proven fruitful for describing this mechanism [28].

EFFLUX PUMPS

Efflux pumps are protein structures, either express constitutively or intermittently. These pumps may have substrate specificity. Similar compounds can be transported by these pumps that may be involved in multidrug resistance [69,70]. Efflux pumps, inside the bacteria in the periplasmic area, are involved in antagonized accumulation of antibiotics. The show resistant to multiple antibiotics such as tetracycline, macrolides, fluoroquinolones, β-lactam and thus reducing these antibiotics concentration at low toxic level. Five families of efflux transporters have been identified in prokaryotes. Over expression of the efflux pumps have been considered to be responsible for antibiotic resistance in *P. aeruginosa* biofilms [71,72].

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MEMBRANE PROTEINS ALTERATIONS

Permeability of outer membrane is very important for the antibiotic diffusion through different routes. A key role is played in transportation of hydrophilic molecules by outer membrane channel proteins (porins) present in Gram-negative bacteria from the outer environment to the periplasmic space. Mutation in porins encoding genes can result in production of non-functional or altered proteins. These mutant porins have low permeability for the passage of hydrophobic molecules [73]. OprD is a specific porin present in *P. aeruginosa* which enhances the absorbance of basic amino acids and imipenem. Loss of OprD in *P. aeruginosa* is responsible for resistance to imipenem, resulting in three-dimensional disturbance of imipenem molecule. The differential expression of porins coding genes, occur in biofilm, leading to antibiotic resistance. When the expression of *ompC* and three other genes (osmotically regulated) is increased then the bacterial cells grow as biofilms [39].

PHASE VARIATION

Diverse phenotype within biofilms plays a significant role and is responsible for the resistant infections. Authors have reported this phenomenon for many genera and species which are exemplified by *Pseudomonas* and *Staphylococcus* genus, and also some species of *Enterobacteriaceae* [2]. Biofilms have the capability to develop bacterial subpopulation to switch to the quiescent state as small-colony known as small colony variants (SCVs). These variants cells have very less susceptibility to growth phase dependent killing of antibiotics. Also have a defective catalase activity which interferes with oxidative metabolism [74,75]. Detectable colonial morphological changes caused by SCVs in biofilms, leads to increased adherence, auto-aggregation, increased hydrophobicity and low level motility. It can also withstand wide range of harsh environmental stress conditions so this is considered as survival mechanism for biofilms. Phase variation was considered as the process of cellular internal rearrangement but recently it is consider as phase variation that occur due to genetic elements interaction [76,77]. Various mechanisms involved in antibiotic resistance due to biofilm are shown in **Figure 3**.

BIOFILM ROLE IN INFECTIONS

Microscopic evaluation of specimens from chronic wounds often indicates the presence of biofilms. Traditionally, three phases are use to describe wound microbiology. These three phases described as: contamination, colonization, and infection. Contamination refers to the presence of bacteria in the wound, whereas the term colonization is used for bacterial community which is multiflying within the wound but not causing tissue damage. The “critical colonization” is used to describe bacteria that are growing inside the wound but do not possess classical symptoms of infection. However, they adversely affect wound healing [78]. Some researchers have suggested that bacteria may play a vital role in normal wound healing [78]. However, the specific role of bacterial community in wound healing is still under debate. The wound communities have polymicrobial nature. Bacteria can find its way to a wound from exogenous (soil and water) and endogenous (skin, saliva, urine, and faeces) sources. Bacteria that do multiply are not considered “infective” unless and until they pose detrimental effects. This is particularly the case for *Corynebacteria* and *coagulase-negative Staphylococcus* which are considered as commensal skin organisms. However, the multiple microbial population interactions in chronic infections are not completely understood. Trengove and his co-workers studied 52 patients and concluded that bacterial diversity and wound chronicity is correlated [79,80].

BIOFILM OCCURRENCE IN HUMAN WOUNDS

In 2001, it has been hypothesized that bacteria which colonize human chronic wounds may exist as biofilm communities. Later in 2003, Akiyama and his co-workers collected patients specimen who were suffering from the skin diseases bullous impetigo, atopic dermatitis, and pemphigus foliaceus and used safranin, ConA, and immunofluorescent staining with CSLM to study and demonstrate the presence of *S. aureus* biofilms in them [81]. Likewise Kirketerp-Moller *et al*. [82] assessed specimen wounds of 22 patients suspected of P. aeruginosa colonization. They used PNA FISH technique and anti-alginate antibodies and found that *P. aeruginosa* existed as biofilms rather than single cells in these wounds. Microscopic evaluation of specimens from 50 chronic wounds and 16 acute wounds was done by James et al. [83] and found the presence of biofilms in 60% of the chronic wounds and only 6% of the acute wounds.

BIOFILM FORMATION IN ACUTE WOUND

Biofilm formation in wounds has been investigated *in-vivo* using model organisms (murine and porcine). Akiyama and co-workers investigated *S. aureus* and *Streptococcus* biofilm formation in mice wounds [81-85]. These mice were treated with cyclophosphamide to inhibit leukocytes. Normal mice rapidly clear the inoculated bacteria because of a strong PMN response. *P. aeruginosa* biofilm, after examining burn wounds of murine model, shows that *P. aeruginosa* rapidly colonized burn wounds and formed biofilms around blood vessels [52].

BIOFILM FORMATION IN CHRONIC WOUND

Bacterial biofilm interrupts the human immune system in several ways. In initial stages of chronic wound, antibiotic treatment is considered to be the immediate step. But in case of mature and established biofilm, antibiotic therapy is least effective and

has only short term effects on both inflammation and healing. Clinicians have to depend on the results from a swab or biopsy, which barely represent all microorganisms present in the wound. The bacterial community residing in biofilm can be up to 1000 times resistant to antimicrobials [33]. Even silver treatment which is considered to be quite effective now a days and incorporated in wound dressings, is least effective on biofilm [86,87].

BIOFILM IN INFECTIOUS KIDNEY STONES

About 15-20% kidney stones are responsible for urinary tract infection and these stones are formed as a result of interaction between bacteria and mineral substances derived from urine. This result in the formation of complex biofilm composed of infecting bacteria and their exoproducts, and mineralized stone material. Hellstrom in 1938 for the first time examined the stones that were collected from his patients and discovered the occurrence of bacteria deep inside them [88]. Microscopic analysis of stones, which were removed from infected patients, have revealed that bacteria present in the stones are organized to form micro-colonies surrounded by an anionic matrix composed of complex polysaccharides and minerals [89]. Urine flow is obstructed by these infectious stones and thus causes severe inflammation and infection that can lead to kidney failure [90].

BACTERIAL ENDOCARDITIS

Bacteria and host components form complex biofilm that cause infection lesion in endocarditis. This biofilm is known as vegetation and can cause disease by three main mechanisms [61]. First, the vegetation disrupts the function of valve by creating leakage, turbulence and flow of blood. Secondly, the vegetation causes bloodstream infections, and may lead to recurrent fever, chronic systemic inflammation, and other severe complications. Thirdly, sometimes vegetation breaks off into pieces and these pieces are then carried to extremities in the circulation system (embolization). Brain and kidneys are particularly vulnerable areas of the body. Vegetation is usually treated by prolonged administration of intravenous antibiotics or surgical excision of the infected valve [91].

CYSTIC FIBROSIS AIRWAY INFECTIONS

Cystic Fibrosis patients most commonly afflicted with *P. aeruginosa* airway infections [92-94]. These infections are generally divided into two steps. First, intermittent respiratory infections are developed in CF patients [11]. In second stage, permanent infections with *P. aeruginosa* take place which last for the rest of the patient's life, as confirmed by genetic fingerprinting [18,93,95]. This persistant infection is clinically very important as it causes permanent failure of lungs [96,97].

OTHER BIOFILM DISEASES

Biofilms cause several diverse kinds of human infections. Biofilm is involved in otitis media with effusion. Fluid accumulates in the middle ear cavity of the patient, thus affecting speech development and learning capability of the patient. However, the complete etiology of the problem is still not clealy understood [97]. In acute osteomyelitis, certain areas of bone necrose and produce favourable conditions for biofilm development [98]. Biofilms have also been identified in most indwelling medical device infections and also in biliary tract infections, periodontitis, ophthalmic infections [99].

IMMUNE RESPONSE TO BIOFILMS

In-vitro studies have indicated that human leukocytes have the ability to penetrate biofilms of *S. aureus* [59]. Akiyama *et al*. [81] reported that antimicrobial efficacy against *S. aureus* biofilms was comparatively more effective in studying acute wound infection in normal mice than those who had less leucocytes count. They investigated the primary mechanism of antimicrobials in normal mice, and found that it was due to the penetration of PMNs into the biofilm. In *P. aeruginosa* biofilms, production of the extracellular polysaccharide, alginate, protected them from IFN-γ mediated phagocytosis by human leukocytes, primarily known as monocytes [100,101]. Similarly, polysaccharide intercellular adhesin (PIA) protected *S. epidermidis* against killing by polymorphonuclear leukocytes (PMN) and phagocytosis [101,102]. Thus, extracellular polysaccharides play a significant role in biofilm resistance to phagocytosis. In addition, *P. aeruginosa* biofilms cause killing of PMN through the production of rhamnolipids. Thus limits the effectiveness of innate immune factors. Severe cutaneous infections are caused by *S. aureus* which is capable of producing leukotoxins, including the Panton-Valentine leukocidin [102].

CONTROL OF BIOFILMS

literature, different methods have been described to control biofilms, some of them are described here.

PILICIDES

Pili are extracellular fibers of bacterial cells which allow binding and colonization on epithelial cells [103,104]. These pili are assembled via a specific mechanism called chaperone-usher pathway [116]. Interupting this mechanism of pili assembly is a new and feasible approach due to its potential application in biofilm eradication. Researchers have also designed small synthetic compounds known as pilicides which inhibit the synthesis of pili [105,106].

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ENZYMES

Another effective way to degrade biofilm is the application of enzymes. Biofilm consists of extracellular polymeric substances (EPS, therefore these enzymes have the potential to degrade EPS. Biofilm is mainly composed of bacteria and EPS. When the biofilm is degraded by enzymes which results release of components and plaktonic cells which is easily clear by immune systems [107].

INHIBITION OF QUORUM-SENSING

Using inhibitors of quorum sensing is the most studied novel way in control of biofilm. In last decade, many researchers have searched for compounds that could block the QS system [108].

ELECTRICAL CURRENTS

It has been shown that electrical current has antibacterial effect for several bacterial species [109]. In a study by the application of low intensity current, a substantial reduction was observed in number of both viable bacteria of *staphylococcus* and *pseudomonas* biofilms. Electrical currents in combination with electromagnetic fields and ultrasound have given enhanced results on biofilm eradication in studies conducted *in-vitro* as well as *in-vivo* [110,111].

SURFACE COATINGS

One of the most effective ways for eradication or blocking bacterial biofilm on surfaces of endotracheal tubes (ETTs) and catheters is coating the devices with metals, antiseptics or antimicrobials [112].

BACTERIOPHAGES

Use of bacteriophages for eradication/removal of biofilm is an efficient and novel strategy [113]. Bacteriophages ability to inhibit or reduce formation of biofilm *in-vivo* has also been proven [7]. During a study, when a genetically engineered lytic phage having a biofilm degrading enzyme, was used showed more efficient eradication of biofilm than wild type phages [114]. Similarly a phage cocktail (combination of multiple phages) can also be used for efficient and complete eradication of bacterial biofilm [115].

FUTURE ASPECTS OF BIOFILM TREATMENT

Treatment for chronic wounds can be made possible by understanding the bacterial biofilm communities [62]. Some of the treatment procedures enhance the host defense system to eradicate pathogens in the wounds. Biofilm production can be reduced or completely eliminated by surgically removing infected tissue. Removal may be surgical, chemical, mechanical or by maggot therapy. Moreover, manipulations have been done in quorum sensing molecules (autoinducer-2 (AI-2)) to increase the population of beneficial bacterial communities in gut thus promoting healthy gut microbiota [116]. Enhancements in pharmokinetics can improve the targeted drug delivery by encapsulating lead compounds in liposomes [117-120].

In conclusions, a thorough study of bacterial biofilm is required to better understand bacterial cells behaviour in biofilm, and also to understand bacterial resistance to multiple drugs. An elaborate molecular study is further needed to understand various gene expression/repression during biofilm formation [121-124]. These various molecular mechinisms can be targetd to control or inhibit biofilm formation. As biofilms show multiple drug resistance due to less penetration of antibiotics, therefore new therapeutic strategies are mandatory such as new agents are needed that can disrupt the biofilms to reach bacteria residing deep down in the biofilms. Main approaches for controlling these infections include the use of inhibitors of quorum sensing to prevent bacterial biofilm formation and disintegrate synthesis of polysaccharides [125-130].

Several authors have reported bacteriophages as possible potential therapeutic option for biofilm disruption [131-133]. In short a greater understanding of bacterial biofilm is required for the development of novel, effective control strategies which will subsequently result in health care improvement [134-136].

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