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DEDICATED TO
OUR LOVING PARENTS
whose blessings & constant
encouragement have always been with us
throughout our endeavour

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PREFACE

Pseudomonas aeruginosa: The superbug is primarily a nosocomial pathogen and accounts for 10% of all healthcare-associated infections. It causes high morbidity and mortality in hospitalised patients worldwide, especially in developing countries. This book has been written to present essential and updated information about *P. aeruginosa* in general, our experimental study regarding antibiotic resistance β-lactamases production, and biofilm formation by *P. aeruginosa*. The implication of this study is immense as antibiotic sensitivity profile and detection of β-lactamases production by phenotypic methods can help the medical fraternity to treat patients infected with *P. aeruginosa* strains effectively. It can also help implement Infection Control Programme in a healthcare setup. No corrective measure can be taken unless the Superbug is detected.

We hope that this book will be helpful for Postgraduate M.D. and PhD students of Medical Microbiology, Research workers, Clinicians, Health Care Workers (HCWs) and Officers of Hospital Infection Control Committees.

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Dr. Silpi Basak

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INTRODUCTION

The most important advancement in the history of modern medicine is the discovery of antimicrobials in the twentieth century. The discovery of Penicillin in 1928 and its first clinical use on an Oxfordshire constable, Albert Alexander, led people to think that the war against microorganisms causing infectious diseases has been won. With clinical usage of Penicillin, the suffering of especially the war victims in World War II was reduced to a great extent and millions of lives were saved. It was only a year after Albert Alexander became the first recipient of penicillin that Remmel and Kemp reported identification of isolates of *Staphylococcus aureus* resistant to the miracle drug. Antimicrobial resistance and its worldwide spread not only question the effectiveness of antimicrobials but also jeopardise the global health security. Within a short span of seventy years from the discovery of miracle drug Penicillin to Tigecycline, mankind is facing problems with some bacterial strains that are resistant to almost all antimicrobials and busy in writing the obituary for antimicrobials. Currently, antimicrobial resistance is a major threat to patient care in any healthcare set-up worldwide because of the following reasons:

i. it increases morbidity and mortality
ii. it increases the cost of healthcare
iii. it challenges infection control program in healthcare set-up
iv. it threatens a return to pre-antibiotic era

In 2011, the WHO declared ‘combat drug resistance - No action today, No cure tomorrow’. The fact is that the pace at which bacteria develop resistance is much higher than that of development of newer antimicrobials.

P. aeruginosa is one of the most commonly isolated bacterial species in Microbiology laboratories, and causes infections in hospitalised patients with strains resistant to many antimicrobials. In addition, it can form biofilm especially in cystic fibrosis patients and patients with medical devices. Extracellular polymeric substances (EPS) or extracellular matrixes in biofilm prevent access of antimicrobial agents, disinfectants and host-produced immune responses. Antimicrobial resistance in *Pseudomonas*

aeruginosa is multifactorial and can be due to mutation, transfer of genes by plasmids, or transposons, among other causes. Production of β-lactamases causes resistance to β-lactam drugs including Carbapenems.

Against this background of emergence of antibiotic resistance, this book studies different types of β-lactamases produced by *Pseudomonas aeruginosa* strains routinely in clinical microbiology laboratories. It shows that, although molecular methods like Polymerase Chain Reaction (PCR) are the gold standard for detection of different types of β-lactamases, they are very costly and require expertise.

SECTION I

REVIEW OF LITERATURE

CHAPTER 1

GENERAL CHARACTERISTICS

History & Taxonomy

Pseudomonas aeruginosa belongs to the family Pseudomonadaceae and is also included in the genus Pseudomonas-rRNA group I (Washington et al. 2006, 319). The genera Pseudomonas lived hundreds of millions of years ago. It was described for the first time by Walter Migula. In Greek, 'pseudes' means false and 'monad' means a single-celled organism. These terms were used in the *Pseudomonas aeruginosa's* early history of Microbiology to denote single-celled organisms that meant false unit. (Palleroni 2010, 1377) But it was described as a distinct species at the end of the Nineteenth century. Carl Gessard, a chemist and bacteriologist, discovered the bacilli in 1882 as the cause of blue pus in the wounds and presented his experimental findings in the paper, 'On the Blue and green coloration that Appears on Bandages.' (Gessard 1984, S775) It was named *Bacillus pyocyaneous*. In 1850, Sedillot changed the name to *Pseudomonas pyocyanea.* Schroeter, in 1872, renamed it to *Pseudomonas aeruginosa* as he observed that the designation 'aeruginosa' has priority over *pyocyanea.* (Rachhpal 2016, 3) Later, many species including a few environmental isolates were assigned to the genus *Pseudomonas*. But many other *Pseudomonas* species have now been classified in a different genus *Burkholderia and Ralstonia*. (Cornelis 2008, 20)

The RNA/DNA hybridisation studies demonstrated that Pseudomonas could be subdivided into rRNA homology groups. Thereafter, *Pseudomonas aeruginosa* has been included in rRNA group. The genus Pseudomonas includes two groups – fluorescent and nonfluorescent species. The fluorescent species was named according to their phenotypic characters e.g. *P. aeruginosa, P. fluorescence, P. putida, P. aureofaciens,* etc. (Palleroni 2005, 1591)

Genomic Structure

The genome size of *P. aeruginosa* is about 5.2 to 7 million base pairs (Mbp) with a Guanine plus Cytosine (G+C) content of 65%. The genome is a combination of variable accessory segment and a conserved core. The accessory segment contains a set of genomic islands and islets from a tRNA-integrated island type. The core segment consists of a low level of nucleotide divergence of 0.5% and a conserved synteny of genes, meaning two or more genes, whether linked or not, are on the same chromosome. (Wiehlmann et al. 2007, 8101) The genome of *P. aeruginosa* is quite large compared to other sequenced bacteria such as *E. Coli* (4.6Mbp), *Bacillus subtilis* (4.2Mbp), etc. Therefore, *P. aeruginosa* can encode a large number of regulatory proteins including enzymes needed for metabolism, transportation, and efflux of different compounds leading to high adaptability to a wide range of environments. (Silby et al. 2011, 652; Klockgether et al. 2011, 150; Stover et al. 2000, 959) *P. aeruginosa* has a single and supercoiled circular chromosome in the cytoplasm. (Fick 1993, 1)

P. aeruginosa carries a lot of chromosome-mobilising plasmids that are important for its pathogenicity. The plasmids like TEM, OXA, and PSE are encoded for β-lactamase production which is required for resistance to antibiotics. The two strains having the complete genome sequence are *P. aeruginosa* PA01 and *P. aeruginosa* PA14. (Craig and Ebert 1994, 478) These strains have been compared with one another and approximately 96.3% of DNA sequence of PAO1 is in PA14 and 92.4% of PA14 DNA sequence is in PAO1. (Fredman and Kolter 2004, 4457)

Morphology

P. aeruginosa is Gram-negative rod and its size is about 1.5-3 μm by 0.5 μm with rounded ends and parallel sides. It is arranged singly or in pairs and has a single flagellum at the tip of the cell.

Cultural Characteristics

In 1966, it was reported that *P. aeruginosa* could survive between 4^0C to 42^0C and the optimum temperature for its growth is 37^0C. The optimum pH is 7.4 to 7.6. (Stainer, Palleroni and Doudoroff 1966, 159) The ability to grow at 42^0C differentiates *P. aeruginosa* from other species. (Brooks et al. 2010, 230) *P. aeruginosa* is an obligate aerobe and grows on any

routine culture media with a characteristic sweet or grape-like odour. In 1965, Jessen described five colony characteristics of *P. aeruginosa* which may be observed on solid media – circular smooth colonies, irregular contoured colonies, dry flat colonies, mucoid colonies, and rugose colonies. (Jessen 1965) The different types of colonies are described as follows**:**

*Circular smooth colonies***:** The circumference of these colonies is mostly circular with an entire edge. The colonies are convex and dome-shaped with a smooth shining surface. They are translucent, homogeneous, and whitish grey in colour. The consistency of these colonies is soft.

*Irregular contoured colonies***:** The circumference of these colonies is irregularly lobate with an entire or fimbriated edge. The colonies are convex and contoured with a beaten-copper appearance, and smooth shining surface. They are translucent, slightly granular, and have a whitish grey colour. The consistency of these colonies is soft.

*Dry flat colonies***:** The circumference of these colonies is mostly circular with lobular edge. The colonies are slightly raised with an irregular surface. They are opaque, granular, and have a whitish grey colour. The consistency of the colonies is easily friable.

*Mucoid colonies***:** The colonies are larger compared to other colony types. They are convex with entire edges. Their surface is smooth, moist, and shiny. The colonies are opaque, homogeneous, and are whitish or greyish green. Their consistency is soft.

*Rugose colonies***:** These colonies are smaller compared to other colony types. They are round with lobular margins. Their surface is dry and wrinkled with irregular or radiant crests. The colonies are opaque, and granular with greyish or whitish colour. The consistency of the colonies is membranaceous.

The first three types of the colonies are flat and have restricted swarming outgrowth. Mucoid colonies occur due to production of large amount of a polysaccharide called alginate that surrounds the cell. Mucoid colonies are observed in *P. aeruginosa* strains isolated from cystic fibrosis patients. Usually, a combination of characteristics is observed in majority of *P. aeruginosa* strains grown on solid culture media such as the green and fluorescing pigments, the fruity odour, the shining metallic patches on top of the culture, and a localised swarming from the edge of the colony.

Growth on nutrient agar: Bluish green pigmentation of *P. aeruginosa* can be observed and the pigmentation is diffusible in the medium. On nutrient agar, *P. aeruginosa* colonies form iridescent patches, with a metallic sheen. Sometimes, beneath the patches, crystals are formed which are salts of fatty acids liberated by autolysis. The organisms are lysed within the patches and the plaques like lesions in the growth are called autoplaques. The autoplaques are formed due to autolysis which is caused by proteolytic enzymes that digest cells. (Brooks et al. 2010, 227)

Growth on blood agar: *P. aeruginosa* colonies are often β-hemolytic, large, pigmented with metallic sheen, and sometimes spreading periphery can also be seen. In the heaviest area of growth, *P. aeruginosa* colonies may have a metallic sheen and a scaling appearance which is called alligator skin appearance. (Washington et al. 2006, 319)

Growth on MacConkey's agar: *P. aeruginosa* form large pale and nonlactose fermenting to red at acidic pH. Pyocyanin can be extracted by chloroform.

*Fluorescin (pyoverdin)***:** These pigments are water soluble, diffuse freely into the medium, and fluoresce brightly under long wavelength (400nm) ultraviolet light. It is insoluble in colonies. Sometimes, circular transparent nonlactose fermenting colonies are also formed. In broth culture, most of the *P. aeruginosa* strains form a pellicle at the top of the broth. Greenish pigmentation can be seen near the surface. (Pitt 1990, 256)

P. aeruginosa is resistant to quaternary ammonium compounds e.g. Cetyl trimethyl ammonium bromide (Cetrimide) and this characteristic has been used to prepare the selective media such as Cetrimide Agar for isolation of *P. aeruginosa* from heavily contaminated material.

Pigment production: Many strains of *Pseudomonas* produce pigments, some of which are water soluble. Pigments are very characteristic and help in detection of the species. Pigments are lost by mutation or several pigments can be produced by a single strain. *P. aeruginosa* can produce four different pigments such as pyocyanin, fluorescin, pyorubin, and pyomelanin. King et al. developed two media A and B for production of pyocyanin and fluorescin respectively. (King, Ward and Raney 1954, 303)

*Pyocyanin***:** It is a blue phenazine derivative, is characteristic of *P. aeruginosa* and is diffusible in the culture medium. This pigment with sheen on the colonies and a characteristic odour is useful criteria for detection of *P. aeruginosa.* Pyocyanin is soluble in chloroform and water.

Its colour depends on pH. It has been observed that magnesium, sulphates, potassium, phosphates, and iron are essential to produce pyocyanin. If the pH of aqueous solution is changed, the colour of the solution changes from colourless at alkaline pH to yellow, and then chloroform gives yellowish tinge to culture. It can also be produced by other Pseudomonas species and is observed in media having low iron content. King B media (King, Ward and Raney 1954, 301) and addition of 10% egg white to a common complex medium (Garibaldi 1967, 1296) can be used for production of fluorescin pigment. Production of fluorescin pigment can also be enhanced in media with high phosphate content. (King, Ward and Raney 1954, 301) This fluorescin pigment has a powerful siderophore activity. (Meyer et al. 2002, 2745)

*Pyorubin***:** Another water soluble bright red pigment, pyorubin can be produced by *P. aeruginosa* strains. It was described initially by Gessard. (Gessard 1984, S775) It is insoluble in chloroform. It is a phenazine compound and is produced by fresh isolates. It is detected by growing *P. aeruginosa* strains in a medium containing mannitol, glycine, leucine, mineral salts, and potassium nitrate as nitrogen source. In low oxygen concentration, pyorubin is irreversibly reduced to colourless form. Two other red pigments are produced by some *P. aeruginosa* strains which were described by Herbert and Holliman as 'aeruginocin A' and 'aeruginocin B'. (Herbert and Holliman 1964, 19; Holliman 1957, 1668)

*Pyomelanin***:** It is a brown to black pigment and is not related to animal melanin. It is not commonly produced by clinical isolates of *P. aeruginosa*. (Yabuuchi and Ohyama 1972, 53)

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CHAPTER 2

VIRULENCE FACTORS AND ANTIGENIC CHARACTERS

Virulence Factors

Pseudomonas aeruginosa are opportunistic pathogens. It can cause cystic fibrosis, Ventilator-associated pneumonia (VAP), burn and wound infections, and catheter-associated urinary tract infections (CAUTI), etc. It can produce a large number of cell-associated and extracellular virulence determinants such as capsule, adhesion factors, extracellular enzymes, biofilm production, etc. These virulence factors antagonise host defences and mainly cause impaired phagocytosis by polymorphonuclear leukocytes and the bactericidal effect of serum (Ben et al. 2011, 393). Three important virulence factors of *P. aeruginosa* which have been studied extensively are - i) Quorum sensing (qs), ii) Type III secretion (TTS) system, and iii) Lipopolysaccharide (LPS) O-antigen. (Le Berre et al. 2011, 2113)

Adhesion factors: The surface adhesion factors are multifactorial and include pili, flagella, and lipopolysaccharides (LPS). (Walker, Redman and Elimelch 2004, 7736) *P. aeruginosa* pili or fimbria are of many types and are known as CupA, CupB, and CupC fimbriae. (Vallet et al. 2011, 6911) The CupA fimbriae play an important role in adherence to abiotic surfaces and biofilm formation. The pili of *P. aeruginosa* adhere to the host epithelial cells of the upper respiratory tract. These adhesions bind to the specific galactose or mannose receptor present on the surface of epithelial cells. The LPS of *P. aeruginosa* is lethal against mice and has pyrogenic action. But the extent to which the endotoxin of *P. aeruginosa* contributes to the toxaemia is uncertain. (Pitt 1990, 256)

In *P. aeruginosa*, the type III secretion system requires contact of individual cells to host-cell membranes, (Cornelis 2006, 811) and this contact is mediated by several adhesions, particularly, the type IV pili (TFP). TFP are long, motorised fimbriae that also provide cells with surface-specific twitching motility and play an important role in virulence.

(Comolli et al. 1999, 3625; Burrows 2012, 493) Because TFP dynamically interacts with the substrate, it mechanically attaches cells with surfaces. Consequently, although TFP has been viewed as adhesion and motility structures, it can also function as mechanical sensors to rapidly signal surface contact. This signal transduction mechanism requires attachment of type IV pili to a solid surface followed by pilus retraction and signal transduction through the Chp chemosensory system – a chemotaxis-like sensory system that regulates cAMP production and transcription of hundreds of genes, including key virulence factors.

*Motility***:** It is an important factor in colonisation of *P. aeruginosa*. Three different types of motilities can be observed in *P. aeruginosa*–swimming, swarming, and twitching. Swimming is due to flagellar movement and occurs due to rotation of single polar flagella. Swimming can activate neutrophil extracellular traps. (Floyd et al. 2016) Swimming also plays an important role in phagocytosis by neutrophils. (Lovewell, Patankar and Berwin 2014, L591) Twitching motility occurs due to extension and retraction of Type IV pili (TFP) and is important for bacterial attachment and colonisation on mucosal cell surface. (Hahn 1997, 99) TFP is also an important virulence factor. Swarming motility occurs over semisolid surfaces and requires involvement of multicellular factors. The role of motility in chronic lung infections by *P. aeruginosa* in cystic fibrosis patients has been extensively studied by several research workers. (Mahenthiralingam, Campbell and Speert 1994, 596)

Tamm-Horsfall protein (THP)**:** It is encoded by UMOD gene, (Pennica et al. 1987, 83) and is produced in ascending limb of loop of Henle in the kidneys and is most abundantly present in urine. THP is also known as Uromodulin. THP may help *P. aeruginosa* in evading host immune response and increase virulence. (Hawthorn, Bruce and Reid 1991, 301)

Leucocidin: The leucocidin produced by *P. aeruginosa* is a cytotoxic protein which acts on granulocytes and lymphocytes and causes morphological changes that ultimately leads to cell lysis. (Hirayama et al. 1983, 575) The leucocidin was isolated by Scharmann in 1976. (Scharmann 1976a, 836; Scharmann 1976b, 292) In 1984, Homma et al. reported that all clinical isolates of P. aeruginosa produced leucocidin and other exotoxins. (Homma et al. 1984, 855)

*Siderophores***:** These are iron-chelating compounds produced by the bacteria and help those bacteria survive in low iron-containing condition. *P. aeruginosa* produces pyoverdine and pyochelin which are siderophores,

and mainly act as virulence factors. Pyoverdine is required for virulence in models of lung infection and burn models. (Visca, Imperi and Lamont 2007, 22) Pyochelin is associated with inflammation occurring in chronic infections. (Cornelis and Dingemans 2013, 75) Pyoverdine has the highest affinity for iron. In the urinary bladder, there is low iron environment and siderophore may promote bacterial growth.

Toxins and enzymes: *P. aeruginosa* can produce many toxins which include four type III toxins – Exoenzymes named ExoS, ExoU, ExoT, and ExoY. In cases of acute infection, expression of these toxins has been observed in vitro. (Hauser et al. 2002, 521)

Exotoxins: The invasion of host tissue by *P. aeruginosa* depends upon many extracellular enzymes and toxins that cause damage to host cells. *P. aeruginosa* produces two extracellular ADP-ribosyltransferases-like exotoxins A and S. Both exotoxins are immunologically distinct. 90% of *P. aeruginosa* strains form exotoxin S in the human body which can be detected by presence of its antibody in the sera of patients recovering from *P. aeruginosa* bacteraemia. (Pitt 1990, 256) Exotoxin S is produced when *P. aeruginosa* grows in the burnt tissue and may be detected in the blood before the bacteria are present. (Deng et al. 2007, 255) Exotoxin A binds to a specific receptor on animal cells and causes inhibition of protein synthesis in the affected cell. (Wolf and Elsasser-Beile 2009, 161)

Extracellular enzymes: *P. aeruginosa* produces several proteolytic enzymes which act on casein, gelatin, collagen, fibrin, and other substrates. Three different proteases produced by *P. aeruginosa* are general protease, alkaline protease, and elastase. Proteases and elastase produced by *P. aeruginosa* are associated with virulence that exerts their activity at the invasive stage. (Pitt 1990, 256) The elastase cleaves collagen, IgG, IgA, and complement. Both elastase and protease destroy the ground substance of cornea. (Jorgensen et al. 2008, 802) *P. aeruginosa* produces two distinct haemolysins – a heat labile enzyme Phospholipase C and a heat stable rhamnolipid. Increased level of haemolytic version of Phospholipase C (PlcH) was detected in 100% of cystic fibrosis patients with chronic *P. aeruginosa* infections. (Hollsing et al. 1987, 1868) In urinary tract infection, the Phospholipase A might be involved in apoptosis of host cells. (Steinbrueckner et al. 1995, 54) The haemolytic activity of Phospholipase C might increase iron concentration in iron-limited condition of urinary tract. (Ostroff, Vasil and Vasil 1990, 5915) Phospholipid D could cause persistence of urinary tract infection and also invasion of urinary tract. (Russell et al. 2013, 508; Jiang et al. 2014, 600)

Quorum Sensing (QS)

Quorum sensing is a cell to cell communication by producing diffusible signal molecules called autoinducers that coordinate production of virulence factors, motility, and biofilm formation. (Karatan and Watnick 2009, 310) Quorum sensing regulates expression of genes within the DNA of the bacterium which is dependent on cell-population density and extracellular environment, etc. Gram-positive bacteria use processed oligopeptides and Gram-negative bacteria use N-acylhomoserine lactones as autoinducers. (Miller and Bassler 2001, 165) Autoinducers increase in concentration as a function of cell density. Cell signalling molecules called 'autoinducers' turn gene expression on and off depending on the number of bacteria in the population. Hence, quorum sensing allows a large population of bacteria to work in a unit like a 'bacterial hive'.

*Quorum sensing in P. aeruginosa***:** The first component is called the "Las system" which contains the autoinducer 'LasI'. It causes expression of the gene encoding the protein LasB elastase. The second component is called the "rhl system" which controls the gene expression for producing rhamnolipid. The autoinducer found inside this system is called rhlI, which also plays a role in producing LasB in an optimal manner. Many virulence factors and other genes of *P. aeruginosa* are regulated by these two acyl-homoserine lactone (AHL) quorum-sensing (QS) systems - LasI-LasR (LasIR) and RhlI-RhlR (RhlIR). LasI catalyses production of N-3-oxododecanoyl homoserine lactone (3OC12-HSL) which binds itself to transcription factor LasR and then activates expression of multiple genes including lasI, rhlI, and rhlR. RhlI catalyses production of butyryl-HSL (C4-HSL) which binds to the transcription factor RhlR. In *P. aeruginosa* strains such as PAO1, the RhlIR QS system requires induction by LasR. As a result, deletion of either lasR or lasI in strain PAO1 diminishes expression of QS-activated genes. LasR-null mutants of strain PAO1 have impaired virulence in acute infection of animal models. These two AHL QS systems interact with a third system which is mediated by the signal 2-heptyl-3-hydroxy-4-quinolone [Pseudomonas quinolone signal (PQS)] and its biosynthetic precursor 2-heptyl-4-quinolone (HHQ) (Déziel et al. 2004,1339; Gallagher et al. 2002, 6472; Pesci et al. 1999, 11229; Diggle et al. 2006, 83) Two main QS systems (las & rhl) in *P. aeruginosa* drive production of autoinducers N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-c12-HSL) and N-butanoyl-L-homoserine lactone respectively.

Biofilm production

Recently, biofilm is defined as sessile communities of microbial cells irreversibly attached to a surface or interface with each other and are embedded in a self-produced matrix of extracellular polymeric biomolecules (such as DNA, proteins, and polysaccharides) and are physiologically different from planktonic cells with respect to growth rate and gene transcription. (Doulam and Costerton 2002, 167) Biofilms are responsible for 65-80% of all microbial infections. Biofilms are called a 'city of microbes' that involves multiple bacterial machineries including Quorum Sensing (QS) systems and the two component regulatory systems. The rhamnolipids produced by the rhl system control and play an important role in biofilm formation and are involved in forming microcolonies that maintain open channel structures, facilitate three-dimensional mushroom-shaped structures in *P. aeruginosa* biofilms, and also facilitate cell dispersion from *P. aeruginosa* biofilm. (Jimenez et al. 2012, 46)

Role of biofilm (Cokare, Chakraborty and Khopade 2009, 159)**:** The exopolysaccharide (EPS) matrix of *P. aeruginosa* consists of biomolecules such as polysaccharides (alginate, psl and pel) lipopolysaccharides, proteins (protease, elastase), extracellular DNA (eDNA), metabolites, exotoxins, and siderophores. The EPS matrix is important for overall structural scaffold and architecture of the biofilm. Biofilm plays a significant role in pathogenesis of *P. aeruginosa* infections. Alginates are overproduced by *P. aeruginosa* after infection of cystic fibrosis patients. Psl is associated with the caps of mushroom like microcolonies. Pel plays a role in cell to cell interactions in *P. aeruginosa* PA14 biofilms. The same function was found in eDNA also. The EPS provides protection from environmental changes like pH shift, UV radiation, osmotic shock, and desiccation to the bacteria residing in a biofilm. EPS can prevent the access of antimicrobial agents in biofilm by acting as anion exchanger. It restricts the diffusion of compounds into the biofilm. The water channels provide effective nutrients in the major aqueous phase by changing the nutrient availability and removal of toxic metabolites. Horizontal gene transfer and acquisition of new genes is important for the microbial communities in a biofilm. *P. aeruginosa* can form biofilm remarkably and cause antimicrobial treatment ineffective leading to chronic infection. Usually biofilm production can be detected by Congo red agar method, tube method, and tissue culture plate method. (Mathur et al. 2006, 25) It is important to note that antibiotics are less effective for bacteria growing in biofilm. Besides production of many virulence factors, *P. aeruginosa* can remarkably form biofilms in different environmental conditions leading to

failure of antibiotic therapy. Three global non-microbicidal strategies have been proposed to combat infections caused by biofilm-forming pathogens. These are - i) avoiding microbial attachment to a surface, ii) disrupting biofilm development and or affecting biofilm architecture, and iii) affecting biofilm maturation and or inducing its disruption and degradation. (Bjarnsholt 2013, 1)

Antigenic characteristics

Nineteen distinct group-specific heat stable O antigens and two heat labile flagellar H antigens have been detected in *P. aeruginosa*. LPS O-antigen is a variable polysaccharide and is present on the outer surfaces of Gram-negative bacteria. It protects against complement mediated lysis. (Pier 2007, 277) Serogroup O6 and O1 are commonly present in clinical material and serogroup O11 has been associated with outbreaks of *P. aeruginosa* in hospitals in USA and Britain. (Farmer et al. 1982, 266; Noone et al. 1983, 341) The outer membrane of *P. aeruginosa* contains many antigenic proteins and six major proteins have been identified. *P. aeruginosa* has common fimbrial antigens which are serologically heterogeneous.

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