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Review Article

Revamping the role of biofilm regulating operons in device-associated *Staphylococci* and *Pseudomonas aeruginosa*

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Abstract

Extensive use of indwelling devices in modern medicine has revoked higher incidence of device associated infections and most of these devices provide an ideal surface for microbial attachment to form strong biofilms. These obnoxious biofilms are responsible for persistent infections, longer hospitalization and high mortality rate. Gene regulations in bacteria play a significant role in survival, colonization and pathogenesis. Operons being a part of gene regulatory network favour cell colonization and biofilm formation in various pathogens. This review explains the functional role of various operons in biofilm expression and regulation observed in device-associated pathogens such as *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*.

Key words: *Biofilm, operon, implant, Pseudomonas, Staphylococcus*

Introduction

Biofilm is a conglomerate aggregation of microorganisms which are irreversibly associated with a surface and enclosed in a hydrated matrix.[1] They are made up of single or mixed species population and differ from planktonic forms by showing reduced growth, increased drug resistance and adaptability. The fundamental unit of a biofilm is a microcolony, wherein close contact of cells provides a perfect environment for the creation of nutrient gradients, genetic exchange and signalling.[2] Channels present in biofilm favour exchange of water, bacterial waste, nutrients, enzymes, metabolites and oxygen. Biofilm-related infections may be caused by a single monopolized species or a mixture of species. The seeding of biofilm on any device is triggered by microorganisms present on the skin of the host, cross-contamination of healthcare workers, tap water to which entry ports are exposed, or other sources within the local environment.[3] The occurrence of a biofilm on a medical device may also hamper the function of the device itself.[4] This, in turn, can cause weakening of both health and quality of life of an individual. Increasing use of medical devices in healthcare systems is always associated with a definitive risk of bacterial infections which is commonly termed as "foreign body-related bacterial infections" (FBRI).[5] FBRIs comprise bloodstream infections, orthopaedic implant-associated infections and catheter-associated infections. Organisms observed on these devices are either Gram-positive or Gram-negative bacteria or yeasts or a mixture of all. These pathogens are either commensals on skin or are nosocomial in origin. Various studies on the incidence of device associated pathogens have revealed that biofilms primarily consist of *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*.[5] Voluminous work has been carried out in understanding the molecular mechanisms involved in biofilm formation. Operons, being one of the major molecular circuits in biofilm expression, have been well studied in these pathogens. Operons are the clusters of coregulated genes with related functions found commonly in bacterial genomes. The series of genes in an operon are transcribed as a single mRNA and consists of an upstream promoter and a downstream terminator.[6] Genes transcribed in an operon are shown to be functionally related and are often engaged in regulating the same metabolic pathway.[7] Extensive research on biofilm forming pathogens has opened new avenues in exploring the role of these operons in biofilm expression and regulation. The current review is focused on various operons and their functions which are reported in device-associated pathogens such as *S. aureus, S. epidermidis* and *P. aeruginosa*. These operons are also regulated by various transcription and global regulators. A short description on well-reported transcriptional and global regulators of *Staphylococcus* and *Pseudomonas aeruginosa* biofilms have been explained in this review.

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Operons of Staphylococcal biofilms

Staphylococci are commonly found on indwelling medical devices such as central venous catheters, needleless connectors, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, tympanostomy tubes, urinary catheters and voice prostheses.[8] After inserting indwelling devices into the patients, they are rapidly coated with host-derived extracellular matrix components and plasma proteins which can function as adhesion molecules for *Staphylococcal* attachment.[8] One of the reasons for catheter and implant infections to occur frequently is the poor vascularization at implantation site. The inability of the innate and adaptive defence mechanisms to reach these sites makes it difficult for the host to fight infection.[8] In regions devoid of circulation, *Staphylococci* are free to grow, spread and form a resistant biofilm structure.[8] Among the *Staphylococcal* species that appear in the list of the leading aetiological agents, *Staphylococcus aureus* and *S. epidermidis* are, respectively, at the first and the second positions. There are a certain number of Coagulase-negative *Staphylococci* (CoNS) that are emerging as new pathogens, such as *S. hominis*, *S. haemolyticus*, *S. capitis* and *S. warneri*.[8] With reference to *S. aureus* and *S. epidermidis,* the regulation of biofilms by operons and their known functions are explained below. Table 1 gives an overview of biofilm regulating operons, encoded proteins and their functions.

Intercellular adhesin operon (icaADBC)

Production of the extracellular polysaccharide, termed 'poly-N-acetylglucosamine (PNAG)' in *S. aureus* and 'polysaccharide intercellular adhesin (PIA)' in *S. epidermidis* is the well-known mechanism observed in *Staphylococcal* biofilms.[9] PNAG/PIA is produced by enzymes regulated by the *ica* operon[9] and this operon is frequently observed in isolates obtained from indwelling devices rather than the carriage strains.[10] High incidence (nearly 50%) of *ica* positive *S. epidermidis* isolates has been reported in intensive care unit isolates.[11] In contrast, the observed tendency of *ica* operon in *S. aureus* clinical isolates was 100%.[12] These data suggest that the presence of *ica* operon in clinical and device-associated *Staphylococci* can trigger biofilm formation and thus it has become an essential biofilm regulatory component.

The *ica* gene locus consists of *ica*ADBC genes.[1,13] [Figure 1a] and all these genes are regulated by *icaR*[9,14] and the teicoplanin-associated locus regulator, *tcaR*.[14] PIA is composed of β-1,6-linked N-acetylglucosamine residues (80-85%) and an anionic fraction with a lower content of non-N-acetylated D-glucosaminyl residues that contains phosphate and ester-linked succinate (15-20%).[15] Upregulation of *ica* gene locus is influenced by environmental conditions, such as glucose, ethanol, high osmolarity, high temperature, anaerobiosis, sub-inhibitory concentrations of tetracycline or quinupristin–dalfopristin.[16] *tagO* gene involved in the production of wall teichoic acids has also been reported to regulate the expression of *ica* loci.[17] Besides, transcriptional regulators such as alternative sigma factor B (SigB),[16] *Staphylococcal* accessory regulator A (SarA)[16] and *Staphylococcal* accessory regulator X (SarX)[18] have been reported to influence biofilm production by controlling the expression of *ica*ADBC operon. An outlook on the function of these transcriptional and global regulators in *ica*ADBC expression is explained in Table 2. The occurrence of phase variation due to alternating insertion and excision of insertion sequence 256 (IS256) to *ica* operon has shown to elicit PIA-independent proteinaceous biofilms.[10,19] Thus, a thorough understanding of these regulators, IS256 elements and environmental factors involved in *ica* operon expression and regulation is very much essential to know more about *Staphylococcal* biofilms.

D- alanine poly (phosphoribitol) ligase operon (dltABCD)

The role of teichoic acids, highly charged cell wall polymers in biofilm formation have been reported in *Staphylococci*.[20] Teichoic acids being a major component of bacterial cell wall help in the attachment of cell surface proteins and favour biofilm development.[20] In *Staphylococci*, two classes of teichoic acids have been reported; peptidoglycan-bound wall teichoic acids and membrane-anchored lipoteichoic acids.[17] The operon *dltABCD* programs the synthesis of peptidoglycan-bound wall teichoic acids with the release of four functional proteins responsible for the esterification of teichoic acids with D-alanine [Figure 1b]. Isolates with mutations in the *dltABCD* operon specifically in *dltA* has shown to increase net negative charge on the cell surface and unable to colonize on glass or polystyrene surface.[20] They also failed to produce D-alanine esters in its teichoic acids and did not show confluent growth on U-bottom plates when stained with safranin.[20] The expression of *dltABCD* operon is repressed by high concentration of Na^+ and moderate concentrations of Mg^{2+} and Ca^{2+}.[21] Moreover, the ArlRS two component system negatively regulates the transcription of *dlt* operon in presence of high concentration of Mg^{2+} ions.[21] These facts strongly support the functionality of teichoic acid's negative charge in bacterial non-adherence and reduced biofilm formation. Thus, *dltABCD* operon regulates the expression of biofilms in *Staphylococci* by generating a net positive charge on the surface. Any mutation in *dltABCD* operon will lead to reduced biofilm production due to the generation of net negative charge on bacterial cell wall.

Holin-like protein (cidABC) operon

The role of bacterial cell death with the release of extracellular DNA has been implicated in the establishment

Table 1: Genes of *Staphylococcal* biofilm operons, encoding proteins and their functions

Gene	Encoding proteins and their functions
icaADBC operon	
icaA	N-acetylglucosaminetransferase
icaD	N-acetylglucosaminetransferase
icaB	PIA deacetylase
icaC	Putative PIA exporter
icaR	Negative transcriptional regulator for the expression of *ica* operon
dltABCD operon	
dltA	D-alanine-D-alanyl carrier protein ligase which activates D-alanine by hydrolysis of ATP
dltB	Encodes a vital protein DltB which facilitates the incorporation of D-alanine into teichoic acids and transfers the activated D-alanine across the cytoplasmic membrane
dltC	Encodes a D-alanine carrier protein (Dcp) and relocates activated D-alanine to phosphopantetheine, a cofactor of Dcp
dltD	Produces DltD protein and transfers D-alanine from the membrane carrier protein to teichoic acids
cidABC operon	
cidA	Encodes hydrophobic protein supposed to function as holins
cidB	Encodes a hydrophobic protein but function of this protein is yet to be identified
cidC	Encodes pyruvate oxidase which shows increased accumulation of acetic acid when grown in excess glucose
cidR	Encodes a positive transcription regulator known to increase the expression of *cidABC* operon
psmβ operon	
psmβ 1	Synthesizes peptides which disrupt non-covalent molecular interactions
psmβ 2	Synthesizes peptides which disrupt non-covalent molecular interactions
agrACDB operon	
agrA	Synthesis a response regulator AgrA and upregulates promoters P2 and P3
agrC	Activates auto-phosphorylation of response regulator
agrD	Synthesizes pro auto-inducing peptide
agrB	Processes pro-auto inducing peptide released from *agrD*

of biofilms in various organisms.[22] Regulated bacterial cell death and lysis in *S. aureus* is favoured by an enzyme murein hydrolase.[22] This enzyme targets cleavage of cell wall peptidoglycans and has shown to take part in cell growth, cell division, separation of daughter cells, peptidoglycan recycling and regulated cell lysis.[22] The regulation of murein hydrolase activity is favoured by *cid* operon which acts as an effector by encoding holin-like proteins.[23] Advanced research on *cid* operon has shown their importance in biofilm development.[23] *cid* operon consists of three genes *cidA, cidB, cidC* and a regulator *cidR* [Figure 1c]. This operon is positively regulated by the transcriptional factor *cidR* which is a part of this operon. A study on *cidA* mutant (lysis-defective mutant) showed loosely compacted cells with decreased adherence in both static and flow-cell biofilm systems.[23] This study further helped scientists to look into the role of extracellular DNA released during cell lysis for the development of biofilms. Treatment of wild type *S. aureus* with DNAse showed increased destabilization.[24] But a similar treatment on *cidA* mutants had a minimal effect.[23] *cidA* mutants have also shown the reduced biofilm formation even in animal models.[22] The expression of *cid* operon is regulated by LytSR two component regulatory system.[24] Its role on *cid* operon expression has been explained in Table 2. These data opine that the *cid* operon-mediated cell lysis coupled with the release of extracellular DNA has an essential role in maintaining the structural integrity of *Staphylococcal* biofilms.

Phenol-soluble Modulin β operon

PSM belongs to a class of short peptides which function as surfactants and pro-inflammatory molecules in *Staphylococci*.[25] They are subdivided into three classes namely PSM-α consisting of ~ 20 amino acids and PSM-*β* consisting of ~ 40-45 amino acids and PSM-γ (encoded by *hld* gene) is a delta toxin.[1,26] During the maturation of biofilm, *β*-type PSM peptides are encoded by *psmβ* operon[27] [Figure 1d]. The role of PSM-*β* in biofilm maturation was first observed in *S. epidermidis* using an isogenic *psmβ* mutant.[27] This mutant expressed a dense and extended biofilm in flow cell systems when compared to its wild type.[27] This study showed that PSM-*β* can favour the detachment of biofilms by disrupting the non-covalent molecular interactions within the biofilm.

Accessory Gene Regulator (agrACDB) operon

Agr is considered as a global regulator in *Staphylococcal* virulence and quorum sensing mechanism

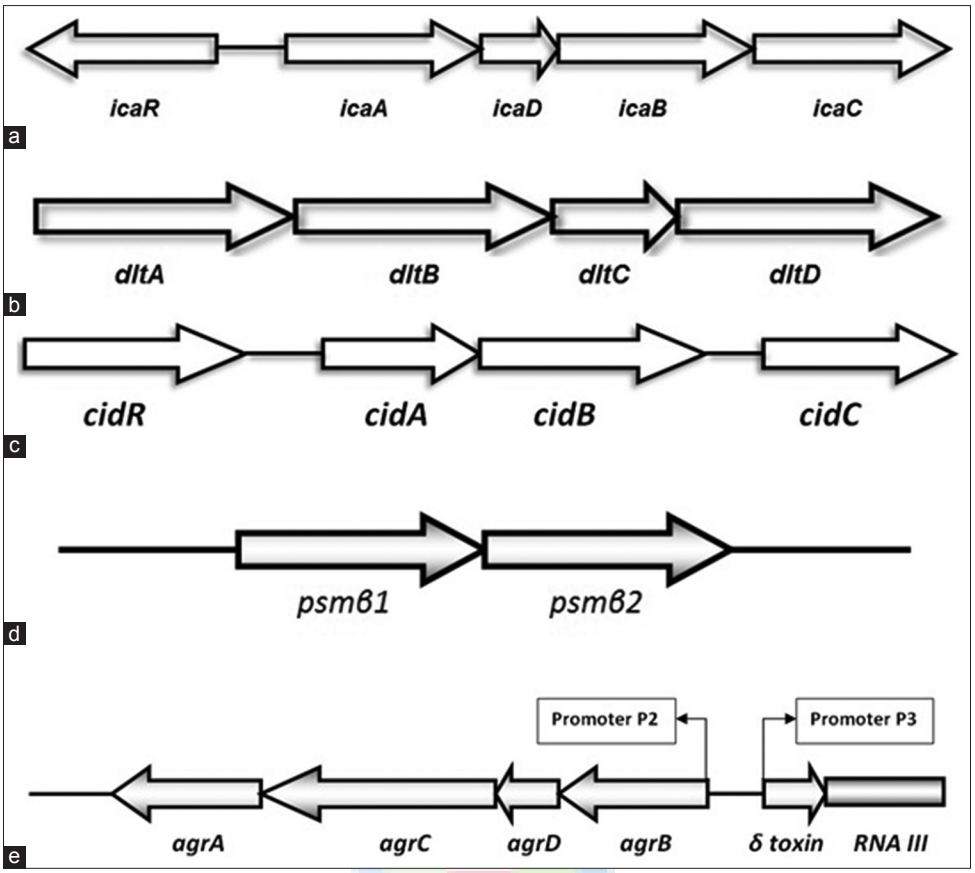

Figure 1: Biofilm-regulating operons in *Staphylococci* (a) *icaADBC* operon (b) *dltABCD* operon (c) *cidABC* operon (d) *psmβ* operon (e) *agrACDB* operon

in *Staphylococcus aureus*.[28,29] The *agr* genetic locus is nearly 3 kb in size and comprises of diverse transcription units.[30] This locus is activated by two promoters P2 and P3 [Figure 1e]. The P2 programs the expression of *agrACDB* operon and an autoinducing ligand.[30] Whereas, P3 promoter directs the expression of RNAIII molecules, which, in turn, regulate the expression of virulence and quorum-sensing genes.[30]

Agr system has been reported to negatively regulate the expression of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which are involved in biofilm initiation.[1,30] These molecules are highly expressed during colonization and once it is achieved, *agr* down-regulates MSCRAMMs expression.[30] Furthermore, the dependence of *agr* operon in biofilm expression is mainly observed by tight regulation of PSM expression.[31] With the aid of green fluorescent protein expression, a study showed that *agr*-favoured biofilm detachment by upregulating the expression of *psmβ* operon.[29] In the same study, *agr* mutants formed compact biofilms in *in vitro* conditions when compared to its wild type.[29] Thus, *agr* system negatively regulates the expression of biofilm by positively regulating the expression of *psmβ* operon.

Operons of Pseudomonas aeruginosa biofilm

Pseudomonas aeruginosa is a major nosocomial pathogen which causes life-threatening infections in immunocompromised individuals and patients suffering from respiratory illness. *P. aeruginosa* is responsible for ventilator-associated pneumonia with high mortality when compared to other pathogens.[32] It is also a dominating pathogen in cystic fibrosis patients leading to chronic infections followed by death. The formation of biofilm by *P. aeruginosa* can prolong the disease status in cystic fibrosis patients and favours the growth of diverse microorganisms leading to secondary infections.[33] *P. aeruginosa* is also involved in urinary tract infections with severe complications by forming irreversible biofilms on urinary catheters.[34] It is frequently reported as a major pathogen in ventilator-assisted devices coronary stents and endotracheal tubes.[35] These reports infer that the pathogen *P. aeruginosa* has a notable impact in device associated infections through biofilm formation. The production of extracellular polymeric substance (EPS) serves as a backbone for biofilm establishment and plays a vital role in initial attachment, cell-cell interactions, tolerance and exchange of genetic materials.[33] *P. aeruginosa* EPS matrix primarily consists of alginate, polysaccharides (*psl and pel*), proteins, cyclic-di-GMP-regulated adhesin A protein (*cdrA*),

cup fimbria, type IV pili, lectins and extracellular DNA (eDNA). Different operons have been discovered for the expression and regulation of these EPS components.[33] Here we describe few of the well-known operons involved in EPS matrix and biofilm production in *P. aeruginosa*. Biofilm regulating operons in *P. aeruginosa,* its encoded proteins and their functions are explained in Table 3.

Alginate operon

Alginate is an acetylated polymer made up of high molecular weight non-repetitive monomers of β-1,4 linked L-Glucuronic and D-Mannuronic acids.[36] The alginate operon is made up of twelve genes [Figure 2a]. Momentous work has been carried out in understanding

Table 2: Regulators of *Staphylococcal* and *Pseudomonas aeruginosa* biofilm operons

Operon	Regulators	Functions	References
Regulators of biofilm operons in *Staphylococci*			
ica ADBC	σ^B factor	Repress *icaR* expression and enhances biofilm production	[16]
	Staphylococcal accessory regulator A (SarA)	Down-regulates *icaA* expression in *S. aureus*	[16]
	Staphylococcal accessory regulator X (SarX)	Down-regulates *icaA* expression in *S. epidermidis*	[18]
	Wall techoic acid regulator (TagO)	Repress *icaR* expression and enhances biofilm production	[17]
*dlt*ABCD	Two component regulatory system ArlRS	In presence of high concentration of Mg^{2+} ions ArlRS repress *dlt* operon	[21]
cidABC	Two component regulatory system LytSR	Upregulates *cid* operon	[24]
Psmβ	Agr system	AgrA binds to the promoter of *psmβ* operon and downregulates its expression	[29, 31]
agrACDB	SarA	In association with SarA, AgrA (response regulator) activates promoter P2 and P3	[30]
Regulators of *P. aeruginosa* biofilm operons			
alg	AlgT	Alg T is the primary transcriptional regulator of *alg* operon	[41, 46]
	MucA	Binds to AlgT and Negatively regulates its expression	[46]
	MucB	Favours binding of MucA to AlgT and negatively regulates AlgT expression	[46]
	MucD	Negative regulator of AlgT	[46]
	AlgB, AlgR, AmrZ	AlgB, AlgR and AmrZ positively regulates *alg* operon by binding *algD* promoter	[46]
pel	Quorum sensing regulators (LasR, RhlI)	Repress *pel* expression	[48]
	Two component regulatory systems (RetS, GacS/GacA)	Repress *pel* expression	[48]
	Post-transcriptional regulator RsmA	Repress *pel* expression	[61]
	c-di-GMP responsive transcriptional regulator FleQ	Functions as both repressor and activator of *pel* operon in presence of c-di-GMP	[43]
psl	Alternative sigma factor RpoS	Positively regulates *psl* expression	[61]
	Post-transcriptional regulator RsmA	Repress *psl* translation	[61]
	Two sensor kinase LadS and RetS	Negatively regulates *psl* expression	[61]
cdrAB	c-di-GMP responsive transcriptional regulator FleQ	Functions as both repressor and activator of *cdrAB* operon	[43]
cupA	Transcriptional regulator MvaT	Downregulates *cupA* operon	[55]
	Global regulator of anaerobic respiration Anr	Upregulates *cupA* operon	[56]

Table 3: Genes of *P. aeruginosa* biofilm operons, encoding proteins and their functions

Gene	Encoding proteins and their functions
Alginate operon	
algD	Synthesizes an enzyme GDP-Mannose dehydrogenase which converts mannose to GDP-Mannuronate and acts as a precursor for the formation of alginate polymer
alg8	Encodes Alg8 protein which binds bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) for alginate polymerization
alg44	Synthesizes Alg44 protein; in association with Alg8, it binds to c-di-GMP
algK	Expresses a scaffold protein AlgK, which plays a vital role in the assembly of periplasmic proteins
algE	Synthesizes an outer membrane protein AlgE which shows an instantaneous assembly in planar lipid bilayer to form a highly anionic selective channel
algG	Programs the synthesis of AlgG protein involved in the epimerization of the D-mannuronate to L-guluronate. This epimerization alters the structural properties of alginate such as the ability to form gel and the binding of divalent ions like calcium
algX	Synthesizes AlgX protein which combines with multi-protein complex. Deletion mutagenesis in *algX* results in the release of depolymerized alginate
algL	Encodes AglL protein, involved in the lysis of alginate polymer
algI, algJ, algF	Synthesizes a complex enzyme which converts polymannuronic acid to mature alginate by O-acetylation
algA	Synthesizes a bifunctional enzyme which shows phosphomannose isomerase and GDP-mannose pyrophosphorylase activity. These enzymes favor the formation of alginate precursor ManUA
pel operon	
pelA	Encodes a large 105kDa protein with unknown function
pelB	Synthesis a scaffold protein PelB for the assembly of secretion complex and protects the polysaccharide from degradation
pelC	Programs an outer membrane lipoprotein PelC which plays an essential role for polysaccharide production
pelD	Programs an inner membrane protein PelD which favors polysaccharide production by binding to c-di-GMP. PelD is functionally similar to Alg44
pelE	Enhances the assembly of secretion complex by interacting with PelA and PelB
pelF	Encodes a protein that localizes to the cytoplasm and predicted to have glycosyltransferase activity
pelG	Encodes a protein which may favor the export of Pel polysaccharide across the inner membrane
psl operon	
pslA	Encodes a putative glucose glycosyltransferase enzyme which favors assembly of repeating oligosaccharide units onto the isoprenoid lipid at the cytoplasmic face of the inner membrane
pslB	Synthesizes a bifunctional enzyme with phosphomannose isomerase and GDP Mannose Phosphorylase activity and plays a vital role in sugar nucleotide precursor production
pslC	Encodes a rhamnose glycosyltransferase enzyme
pslD	Programs a secretery protein PslD which acts as a UDP-glucose lipid exporter and favors Psl formation
pslE	Functions as a transporter and help in polymerization of Psl
pslF	Encodes an enzyme with glycosyltransferase activity
pslG	Encodes a protein with glycosyl hydrolase activity. Believed to be responsible for the processing of growing polysaccharide
pslH	Encodes an enzyme with glycosyltransferase activity.
pslI	Encodes an enzyme with glycosyltransferase activity
pslJ	Encodes an enzyme with Polymerase activity
pslK	Encodes an enzyme with glycosyltransferase activity
pslL	Encodes an enzyme with acetylase activity
pslM	Encodes an enzyme with dehydrogenase activity
pslN	Encodes an enzyme with topoisomerase activity
pslO	Encodes a Protein with unknown function
cdrAB operon	
cdrA	Encodes cyclic diguanylate regulated TPS partner A (CdrA) protein which functions by cross-linking with mannose part of Psl and amplifies EPS matrix and biofilm formation by auto-aggregation

(*Contd*...)

Table 3: (Continued)

Gene	Encoding proteins and their functions
cdrB	Encodes cyclic diguanylate regulated TPS partner B (CdrB) protein. This functions as a transporter of CdrA
cupA operon	
cupA1	Encodes precursors for fimbrial protein
cupA2	Encodes periplasmic chaperon
cupA3	Encodes fimbrial usher proteins. This protein is essential for biofilm formation
cupA4	Encodes precursors for fimbrial protein
cupA5	Encodes periplasmic chaperon

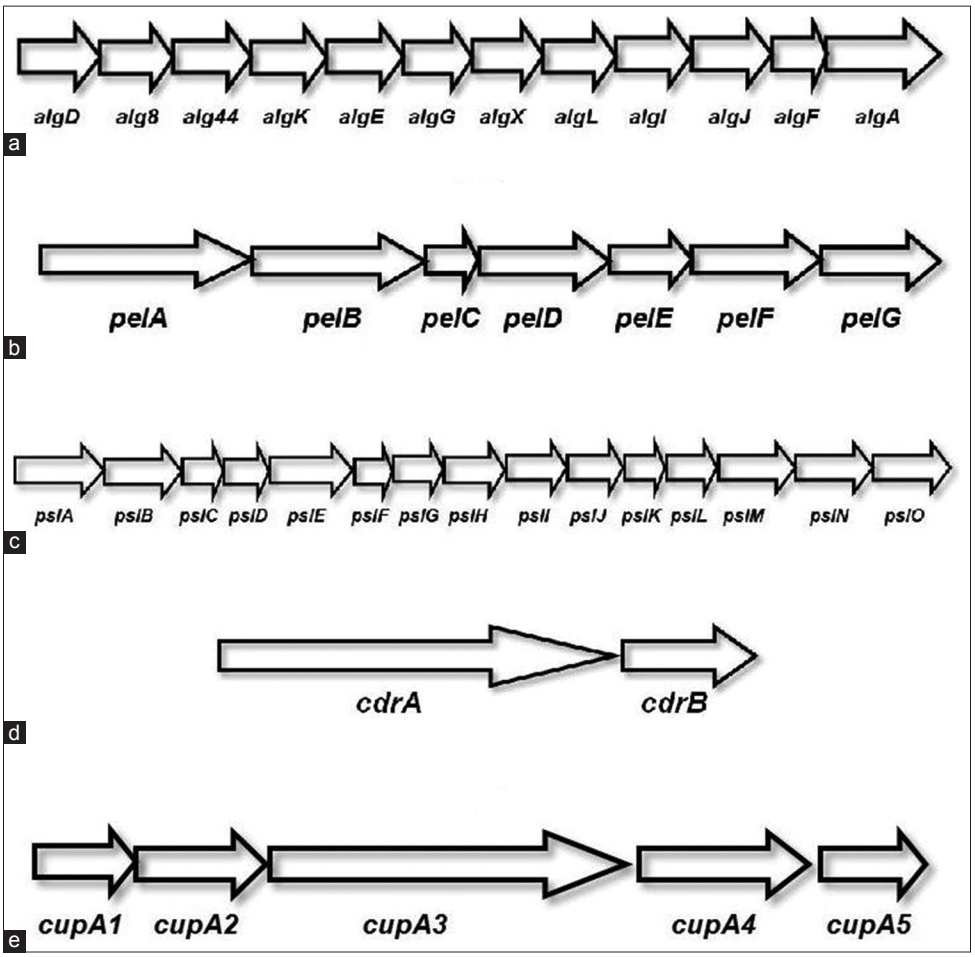

Figure 2: Biofilm-regulating operons in *Pseudomonas aeruginosa* (a) Alginate operon (b) *pel* operon (c) *psl* operon (d) *cdrAB* operon (e) *cupA* operon

the role of this exopolysaccharide alginate in *P. aeruginosa* biofilm formation. A defined architecture and resistance to tobramycin was reported in alginate over producing strain.[37] Another report has shown that super mucoid *P. aeruginosa* strain failed to attach properly and formed thicker biofilm with an outsized unmitigated mushroom like microcolonies.[38] These reports have shown that, the regulation of various genes in alginate operon has a considerable role in alginate production and biofilm regulation. Post-transcriptional regulation of membrane anchoring protein Alg44 and the glycosyl transferase Alg8 together plays a vital role in polymerization leading to alginate production.[39] Attenuated total reflection/Fourier transform-infrared spectrometry (ATR/FT-IR) and scanning confocal laser microscopy (SCLM) have revealed that alginate is not a major component of *P. aeruginosa* biofilm and also for the interfacial adhesion or growth.[40] In the same study, *in vitro* mutagenesis of *algD* and *algJ,* expressed an improper alginate and an alginate without O-acetylation respectively. These mutants failed to form

three-dimensional structures but showed flat biofilms similar to non-mucoid strains. These observations proved that alginate with O-acetylation can promote the structural stability of biofilms. However, it also suggests that the alginate may not be an essential structural component of *P. aeruginosa* biofilm.[40] Apart from alginate, polysaccharides were also observed in biofilms of non-mucoid strains.[41] These polysaccharides were later identified as pellicle polysaccharide and a polysaccharide synthesized at the polysaccharide synthesis loci.

Pellicle polysaccharide operon

The ability of *P. aeruginosa* to form biofilm at the air-liquid interface in static cultures suggested the role of a special pellicle polysaccharide in biofilm formation. It was first reported in PA14 strain by screening its transposon library for pellicle-deficient mutants.[42] This study disclosed the existence and functioning of a seven gene operon *pel* in PA14 strain[42] [Figure 2b]. To understand the role of pel operon in biofilm production, mutants lacking few *pel* genes were evaluated for biofilm initiation and integrity. This study revealed that Pel did not significantly affect biofilm initiation in PA14 *pel* mutants. Instead it disturbed the colony morphology and the ability of these cells to bind congo red.[42] A similar study comprehended the role of Pel in PAK strain which is known to show biofilm initiation using type IV pili. Mutational analysis of *pel* genes as well as pili associated genes in this strain showed failure to initiate biofilm.[42] Thus, *pel* locus is considered as an essential component of biofilm regulation in PA14 and PAK strains. The expression of *pel* loci is regulated by a secondary messenger molecule c-di-GMP and a transcription factor FleQ.[43] Another study has shown that the allosteric binding of c-di-GMP to *pelD* activates Pel synthesis.[44] These data suggest that *pel* operon is regulated by multiple factors and the expression of Pel is essential for biofilm formation.

Polysaccharide Synthesis Locus Operon

The role of *psl* operon in *P. aeruginosa* biofilm formation was discovered by conducting mutagenesis studies on PAO1 and PA14 strains. The disruption of alginate operon in these strains, were earlier thought to reduce biofilm formation. But interestingly biofilms were observed in these strains with rich polysaccharide matrix even in the absence of alginate. Identification of *psl* operon by three independent research groups confirmed the presence of 15 co-transcribing genes (*pslA* to *pslO*) [Figure 2c] and displayed an essential role in biofilm regulation.[45] The mannose-rich polysaccharide Psl expressed by *psl* operon is reported to be a highly conserved in numerous *P. aeruginosa* strains.[46] Cell-to-cell interactions and cell-to-surface interactions mediated by this polysaccharide is very much essential for the formation of biofilm matrix and its maintenance.[47] Helical symmetry of Psl has been explored and this has shown to favour cell-cell and cell-surface interactions.[47] Lectin staining to localize Psl polysaccharide in mushroom-shaped biofilms showed its localisation at the periphery.[47] This confirmed that Psl accumulates at the periphery of microcolonies in mushroom shaped biofilms.[47] The expression of Psl polysaccharide is regulated by two sensor kinases: Lost adherence Sensor (LadS) and Regulator of Exopolysaccharide and type III Secretion (RetS).[48] Besides, it is also regulated by c-di-GMP.[43] A novel protein cyclic di-guanylate monophosphate regulator A (CdrA) expressed by *cdrAB* gene complex is reported to crosslink with Psl and this displayed elevated biofilm matrix production[49] With all these findings we can infer that in *P. aeruginosa* Psl polysaccharide is an essential component of EPS matrix and its expression can favour biofilm formation in non-mucoid strains.

Cyclic di-guanylate monophosphate regulator operon

Research on extracellular matrix expansion has revealed that cyclic diguanylate monophosphate (c-di-GMP) positively regulates production of matrix components at the transcriptional and allosteric level.[50] It is now known that higher concentrations of intracellular c-di-GMP triggers expression of biofilms in liquid medium and at lower levels they promote motility and planktonic lifestyle.[50] The sum total of genes induced in response to elevated levels of c-di-GMP was comparatively low in *P. aeruginosa* and most of these genes belonged to the *pel* and *psl* operons.[51] However, further investigation revealed that two genes termed as PA4625 and PA4624 encoded two unique proteins which enhanced EPS matrix expansion and biofilm formation.[50] These genes exhibited a two-partner secretion system (TPS) and expressed a large concealed adhesin protein with transporter function.[50] They were termed as cyclic diguanylate-regulated TPS partner A (*cdrA*) and cyclic di-guanylate-regulated TPS partner B (*cdrB*), respectively and both were contiguous to form a single operon[50] [Figure 2d]. CdrA is dependent on the presence of Psl polysaccharide and aggregation can be disrupted by adding mannose.[50] CdrB functions as a transporter of CdrA and this was confirmed by mutational analysis, wherein CdrB mutants failed to show auto-aggregation of cells and biofilm formation.[50] Thus *cdrAB* operon with two important genes is very much essential for proper functioning and expansion of EPS matrix,

Chaperone Usher Pathway A operon

Extracellular appendages such as pili or fimbriae in Gram-negative bacteria can play a pivotal role in attachment, cell mobilization, protein transport, genetic exchange and invasion.[51] Based on the biosynthetic pathways these pili are classified into five types such as chaperone–usher pili (CU pili), type IV pili, type III secretion needle pili

and type IV secretion pili.[51] Among these, CU pili have gained much attention due to their complex assembly mechanisms.[52] The assemblage of CU pili is favoured by a chaperone synthesized at the periplasmic region and by a porous outer-membrane protein termed as usher.[52] Chaperons prevent polymerization of pilus in the periplasm, favour its folding and direct them towards the usher.[52] Usher as a molecular motor facilitates the assembly of chaperons to pilus subunits and secretes the pili through the usher pore.[52] Mutational studies have shown that the initiation of biofilm and its maturation in *P. aeruginosa* requires the expression of type IV pili and flagella on its cell surface.[53] These factors thwart the repulsive forces exerted on a surface, favouring the formation of biofilms.[53] However, Tn5 mutational analysis in *P. aeruginosa* strain revealed the presence of new adhesion factors other than pili and flagella.[54] Among these mutants few of them showed reduced expression of proteins similar to periplasmic chaperons exclusively observed in chaperon/usher pathways expressed by *cupA, cupB, cupC* and *cupD* gene clusters.[55] *cupA* mutant displayed reduced biofilm formation in the absence of type IV pili and further investigation disclosed the presence of *cupA1, cupA2, cupA3, cupA4* and *cupA5* genes[55] [Figure 2e]. In order to identify whether all the genes of *cupA* operon are involved in biofilm formation, loss of function mutations in *cupA2* and *cupA3* has been carried out. This study validated the essential role of *cupA3* in biofilm expression.[55] A transcriptional regulator termed as MvaT encoded by *mvaT* gene which was first reported in *P. mevalonii,* downregulates the expression of *cupA* operon and blocks the expression of biofilm.[55] This regulator also displayed flaunted virulence gene expression in *P. aeruginosa.*[55] Besides, upregulation of *cupA* operon in a phase variable manner is carried out by *cupA* gene regulator (*cgr)* comprising four genes *cgrA*, *cgrB*, *cgrC* and a global regulator of anaerobic gene expression (*Anr*) which is present upstream to *cupA* operon.[56] The presence of anaerobic environment in *P. aeruginosa* triggers higher expression of biofilm aided by *Anr* gene regulation.[56] These data suggest that the expression of *cupA* operon requires multiple factors and have a tremendous impact on biofilm expression when compared to other *cup* operons.

Biofilms and gene expression-message from transcriptome analysis

Transcriptome analysis plays a key role in understanding the expression of various genes at different stages of biofilm formation. It also helps us in identifying novel genes involved in biofilm regulation. Currently transcriptome analysis is being carried out either by microarray or by RNA sequencing technology.[57] Whole genome transcriptome profiling of *S. aureus* UAMS -1 strain showed altered expression of 580 genes during biofilm maturation.[58] When expression was compared in biofilm and planktonic state, nearly 48 genes showed enhanced expression and 84 genes showed reduced expression in biofilm than their planktonic counterpart.[58] Moreover, the expression of *icaD* showed consistent upregulation and *Spa* was extremely downregulated in *S. aureus* UAMS -1 biofilms.[58] Similar studies on *S. epidermidis* 1457 (non-producer of biofilm) and *S. epidermidis* RP62A (strong producer of biofilm) have shown that nearly 12% of genes expressed in varied fashion and 6% of genes showed equal up and downregulation during biofilm formation.[27] *agr* expression was downregulated and *SarA* expression was consistent with biofilm formation in *S. epidermidis* RP62A.[27] These data suggest that *S. aureus* and *S. epidermidis* show differential expression of genes during biofilm formation.

In *P. aeruginosa* transcriptome analysis has been carried out in different growth phases. Transcriptome of planktonic cultures obtained at logarithmic and stationary phase have been compared with the developing and confluent biofilm transcriptome.[59] Nearly 19.4% of the PAO1 genome showed differential expression of genes in planktonic cultures (10.5% genes were up regulated and 8.9% were down-regulated).[59] When the logarithmic phase transcriptome of planktonic culture was compared with 8 h developing biofilm transcriptome, only 3.1% of the genome showed differential expression. (0.8% of genes were up-regulated and 2.3% of genes were down-regulated).[59] However, when the transcriptome of stationary phase planktonic culture was compared with the transcriptome of confluent biofilms ~ 14.3% of the genes were differentially expressed.[59] Comparative transcriptome analysis between developing and confluent biofilms also revealed considerable variation in gene expression with 15.5% differential expression.[59] Furthermore, transcriptome of developing and confluent biofilm were found to be related to the transcriptome of logarithmic and stationary phase cultures. Genes encoding transport proteins and transcriptional regulators were also found to be upregulated in developing and confluent biofilms of *P. aeruginosa* PAO1 strain.[59]

Quantitative and qualitative RNA sequence data analysis also revealed the existence of more than 3000 transcriptional start sites and the expression of small RNAs in *P. aeruginosa.*[60] Thus, transcriptome analysis of *P. aeruginosa* biofilms has helped us in understanding the rate of genes and small RNA expression at different phases of biofilm growth. RNA sequence-based transcriptome analysis has also improved our understanding of gene expression in biofilms.

Conclusions

Global incidences of biofilm-associated infections are increasing at an alarming rate due to the excessive usage of medical implants. In association with host derived conditioning factors and hydrophobic, hydrophilic and electrostatic interactions, these implants enhance the growth of pathogens in patients leading to persistent infections with increased drug resistance. Operons provide an

idealistic model to understand various environmental and nutritional factors impeding or promoting the expression of biofilms. Pathogens such as *S. aureus, S. epidermidis* and *P. aeruginosa* are repeatedly shown to form biofilms on indwelling medical devices and caused dreadful long-term infections with increased drug resistance. Operons in these organisms demonstrate diverse function with differential expression. However, all these operons are either positively or negatively regulated by various genes, two component regulators, transcriptional factors and global regulators. More importantly, transcriptome and RNA sequence analysis of biofilms disclosed differential gene expression, existence of novel genes and transcription start sites. Thus, operons and its associated regulators depict an intricate molecular regulation with a profound impact on biofilm production and gene expression. With all these understandings and vast knowledge in the genetic regulation of biofilms, it is still unclear how these pathogens have evolved to exhibit enhanced drug tolerance and resistance. Spurred research in this context is the need of the hour to improve health status in our society.

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