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Review

Beneficial biofilms in marine aquaculture? Linking points of biofilm formation mechanisms in *Pseudomonas aeruginosa* and *Pseudoalteromonas* species

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Abstract: For marine aquaculture it is suggested that a specific substrate coated with a beneficial biofilm could prevent fish egg clutches from pathogenic infestations and improve the water quality and health of adult fish while, at the same time, minimising the need for the application of antibiotics. In marine biotopes, the habitat of *Pseudoalteromonas* species (a strain with suggested beneficial properties), biofilms are mostly discussed in the context of fouling processes. Hence research focuses on unravelling the mechanisms of biofilm formation aiming to prevent formation or to destroy existing biofilms. Initially in this review, particular components of biofilm formation in *Pseudomonas aeruginosa*, a gram-negative model organism that is responsible for nosocomial infections and considered as a food spoiling agent, are described (extracellular appendages, role of matrix components, cell-cell signalling) to get an advanced understanding of biofilm formation. The aim of this treatise is to seek linking points for biofilm formation of *P. aeruginosa* and *Pseudoalteromonas* sp., respectively. Furthermore, approaches are discussed for how biofilm formation can be realized to improve fish (larvae) rearing by species of the genus *Pseudoalteromonas*.

Keywords: bacterial appendages; biofilm formation steps; extracellular polymeric substances; *Pseudomonas* sp.; *Pseudoalteromonas* sp.; quorum sensing; two component systems

Abbreviations

AHLs—acylated homoserine lactones; AI—autoinducers; Ca^{2+}—calcium; c-di-GMP—cyclic diguanylate monophospahte; Cup fimbriae—chaperone usher pathway; DGC—diguanylate cyclise;

eDNA—extracellular DNA; EPS—extracellular polymeric substances; HHQ—4-hydroxy-2-heptylquinolone; LPS—lipopolysaccharides; MSHA-pili—mannose-sensitive haemagglutinin; *P. aeruginosa—Pseudomonas aeruginosa; P. tunicata—Pseudoalteromonas tunicate;* Pel—referring to pellicle; Phd—prevent-host-death; PQS—*Pseudomonas* quinolone signal; Psl—polysaccharide synthesis locus; Roc system—regulator of *cup*; TBDR—TonB-dependent receptor; TCS—Two-component systems.

1. Introduction

Some *Pseudoalteromonas* sp. from marine habitats are able to inhibit *Vibrio anguillarum* (*V. anguillarum*) in a live cell assay [1]; they produce bioactive secondary metabolites [2] and this production of secondary metabolites, which comprises small molecules, antibiotics, and pigments, takes place during the stationary phase when bacterial physiology changes. Furthermore, secondary metabolites may act as signalling molecules to protect biofilms from occupation by other organisms [3]. In natural environments, biofilm formation represents the stationary phase of bacterial growth [4]. It is assumed that biofilm formation of *Pseudoalteromonas* sp. on specific abiotic surfaces protects fish spawn (deposits on this special prepared abiotic surface) against fish pathogenic bacterial and/or fungal infestation. *Pseudoalteromonas* sp. has potential to improve larvae rearing in aquaculture and ornamental fish farming.

Initially, in this review particular components of biofilm formation in *Pseudomonas aeruginosa* (*P. aeruginosa)* are described (extracellular appendages, role of matrix components, cell-cell signalling) to get an advanced understanding of biofilm formation. The aim of this review is to compare biofilm formation mechanisms of *P. aeruginosa*, a thoroughly examined biofilm forming bacterium, with the mechanisms of *Pseudoalteromonas* species. *P. aeruginosa* serves as a reference bacterium, since it is a model for gram-negative bacteria forming biofilms, and, as for *Pseudoalteromonas* sp., it belongs to the phylum of gamma-proteobacteria. Most of the investigations dealing with biofilms are connected with pathogenicity of *P. aeruginosa*, such as in the cases of cystic fibrosis patients. However, there are beneficial applications of biofilms in wastewater treatment [5].

In natural habitats bacterial cells are capable of sensing changes in their environment such as physiological and chemical signals, thus, allowing for production of intercellular signalling molecules and adherence to biotic or abiotic surfaces by cell appendages and/or biopolymers [6,7,8]. As a result a bacterial biofilm is formed: a biofilm is a cohabitation of multicellular complexes surrounded by an extracellular matrix sticking cells together and preventing hostile attacks. In this process, the resulting extracellular matrix is generated by the microbes themselves [9]; it consists of bacteria in stationary phase, polysaccharides, proteins, and extracellular DNA [10]. In prevalent models four steps of biofilm formation are described (Figure 1): (i) flagella and type IV pili are used for the initial surface-attachment [11], (ii) type IV pili are responsible for moving on surfaces, leading to microcolonization, (iii) reduction of flagella, (iv) a self-produced matrix stabilizes the 3D-structure of the biofilm [12]. Biofilm formation and structuring depends on a number of

environmental factors such as nutrient availability, physical impacts, intercellular signal molecules, extracellular polymeric substances (EPS), cell migration and cell proliferation [6]. For example, citrate medium in flow chambers induces flat biofilms [13; Figure 1], while glucose medium induces "mushroom"-shaped biofilms [8; Figure 1]. One advantage of the biofilm community is the protection against hostile offense by other organisms or antimicrobial agents because of their compact structure [3,14]. In addition, biofilms promote genetic exchange [15].

2. Biofilm Formation by Extracellular Appendages

Biofilm formation starts with the interplay of planktonic cells with an abiotic or biotic surface mediated by suitable environmental signals [11]. In general, biofilm formation is a process of replaying steps: the initial bacterial attachment will be preceded by a transport of bacteria from the environment to a surface and subsequently, the bacteria form microcolonies and the biofilm matures [16,17].

The movement and attachment of *P. aeruginosa* in fluid environment to a surface is mediated by flagella, which have a sensory role in terms of temperature and chemicals [18,19]. The typical flagellum consists of the protein flagellin arranged in helical filaments with the shape of a hollow tube of 20 nm in diameter [20]. More than 40 genes are involved in flagellar motility (e.g. *fleQ, fleS, fleR, fliA, flgM, fleN*) [21], whereby two master genes are responsible for flagella synthesis in *P. aeruginosa*: *fleQ* and *fliA* [22,23], (Table 1). As the expression products of these genes exceed a threshold, other genes at a higher level are transcribed [24].

In total, the three surface motilities; swarming, twitching, and sliding have been described in connection with biofilm formation by *P. aeruginosa*. These different types of motility are suggested to be responsible for the activity of bacteria to pass long distances against repulsive forces and, thus, enhancing the probability of a close approach [25]. Swarming is mediated by flagella, twitching is mediated by type IV pili, and sliding appears in the absence of type IV pili and flagella [26,27]. Initially, the adherence phenotype of *P. aeruginosa* is powered by flagella and type IV pili [6] (treated in the next section). Flagella and type IV pili are responsible for the motility of *P. aeruginosa* subpopulations, existing along with non-motile subpopulations after surface binding [3,8,28,29]. Although, not much is known about the association of swarming motility and biofilm formation, O´May et al. (2012) [30] have shown that the blocking of swarming motility by tannin derivations led to an increase in biofilm formation of *P. aeruginosa*. Thus, it is suggested that the non-swarming cells of *P. aeruginosa* are involved in the development of biofilms.

Following the movement in fluid environment, the initial attachment to surfaces is mediated by type IV pili [3]. According to the model of Klausen et al. (2003a) [3] type IV pili also mediate movements along the surface (called twitching) ending up in cellular aggregation and microcolony formation [31]. Structurally, *P. aeruginosa* flagellin resembles the flagellin of archaeal flagella and assembling involves the *pilGHIJKL* cluster [32,33] (Table 1) and more than 50 proteins [26], while the motility is enabled by rhamnolipid [34,35,36]. Type IV pili, numerously formed at the cell poles, were discovered to sense extracellular DNA of dead cells, which were initially involved in biofilm formation [37,8]. Furthermore, studies by Wang et al. (2013) [38] prove the formation of a fibre-like Psl matrix (a biofilm matrix component discussed below) when bacterial surface-adhesion is

mediated by type IV pili. Thus, the involvement of type IV pili in biofilm formation is beyond dispute.

In addition to type IV pili there exist so called Cup fimbriae (chaperone usher pathway, Table 1), known for targeting biotic and abiotic surfaces, though, their specific function is obscure. The fimbrial gene clusters are conserved in gram-negative bacteria [39], and Filloux et al. (2004) [40] identified five gene clusters for *P. aeruginosa*, named *cupA-E*. The chaperone-usher pathway is responsible for assembling and secreting these adhesive surface structures. It follows three steps: (i) production of fimbrial subunits in the cytoplasm and transport to periplasm, (ii) binding to chaperones in the periplasm leads to a correct folding of the subunits, (iii) subsequent transport to the usher (forming pores in the outer membrane) and assembling and exposure of pilus fibres at the cell surface [40]. In *P. aeruginosa* CupE was detected as the first non-archetypal system assembling fimbriae under biofilm conditions. Fimbriae formed in such way were proven to play a role in early biofilm formation during micro and macrocolonization and in 3D shaping [41]. Previous studies by Vallet et al. (2004) [42] proved a role of the *cupA* gene cluster from *P. aerugniosa* in biofilm formation on abiotic surfaces. In the course of the latter study a gene was detected (*mvaT*) that encodes a negative regulator of *cupA* transcription. Interestingly, *mvaT* mutants displayed an increase in biofilm formation.

While flagella and pili belong to the class of formed adhesins, SadB belongs to the class of conditionally synthesised adhesins [43]. The latter is known to be involved the coordination of biofilm formation and swarming motility, but the mechanism has yet to be elucidated. Thus, SadB is another component in the transition from planktonic to sessile lifestyle [44,45].

On the other hand LecA (or PA-IL) and LecB (or PA-IIL) are lectins of *P. aeruginosa*, belonging to a group of proteins, capable of recognizing carbohydrates (Table 1). Both of the lectins play a role in biofilm formation. LecA mediates specific binding with D-galactose and its derivatives [46], while LecB, associated with the outer membrane, specifically bind to L-fucose and its derivatives [47]. As a result, polysaccharides of the surrounding cell matrix and sugar components on cell surfaces can be detected leading, eventually, to the interaction with other cells and surfaces.

Concerning cell appendages and outer membrane proteins, being responsible for biofilm formation, there is still an open question: which factors cause the change from the planktonic to the sessile lifestyle in bacteria? It is noteworthy that biofilm formation depends on environmental conditions, such as temperature, pH, oxygen, toxic substances, and nutrition. Two-component systems (TCS) serve as the regulators as they control the production of appendages in response to environmental conditions, for details see [48]. A classical TCS consists of a sensor kinase, which can be cytosolic but is mostly membrane bound and monitors environmental conditions. The second part of a TCS is the response regulator, which is activated as a transcription factor by phosphorylation through the sensor kinase [49].

The above described *cup* gene expression is regulated by the Roc system (regulator of *cup*) among other things. This system contains a sensor kinase (RocS1), a DNA binding response regulator (RocA1, activating cup genes) and an antagonist of RocA1 activity (RocR), which regulate the transcription of cupB and cupC [50].

However, the expression of the *pil* genes, involved in the development of type IV pili goes along with a high complexity. Hobbs et al. (1993) [51] proved that the transcriptional regulator PilR

controls the expression of *pilA* gene, coding for the structural subunit PilA. PilR is a response regulator, belonging to the cognate histidine sensor kinase PilS.

Another regulation of twitching motility is realized by AlgR, which is the response regulator of the two component system formed with FimS (sensor kinase). Due to the phosphorylation of AlgR, genes within the *fimU-pilVWXY1Y2E* operon are activated. This cluster is essential for type IV pilin biogenesis [52].

Furthermore, a chemotactic system is active; measuring the direction and rate control of type IV pili mediated movement depending on surrounding conditions [53]. Swarming motility is regulated by the GacS/GacA-system [54]. As mentioned above more than 40 genes are responsible for flagella biogenesis. The complex regulation concerns four classes of genes (class I-IV). The two master genes *fleQ* (transcriptional regulator) and *fliA* (sigma factor σ^{28}), forming class I, are constitutively expressed. Again FleQ and RpoN (sigma factor σ^{54}) are responsible for the transcriptional activation of the TCS encoding genes *fleSR* (class II genes). Together with RpoN, FleR is responsible for the positive regulation of class III genes. FliA influences the transcription of class IV genes, ["following the export of the FliA specific antisigma factor FlgM through the basal body rod-hook structure (assembled from class II and III gene products)"] [21]. Collectively, these findings describe the sensing of environmental changes by TCSs, followed by the transcriptional regulation of genes required for the production of cell appendages.

In the final step, biofilm maturation, microcolony forming cells protect themselves by a matrix (next chapter). The formation of aqueous channels enables the supply of nutrients, oxygen, and signalling molecules as well as the removal of waste products [55]. Additionally, type IV pili are involved in biofilm maturation as they ensure the movement of motile bacteria to form the mushroom-like cap [8]. Living in a biofilm allows nutrient supply and protection against environmental attacks simultaneously.

3. The Role of Matrix Components in Biofilm Formation

As already mentioned, attached bacteria forming microcolonies are able to surround themselves with a self-produced matrix containing polysaccharides, nucleic acids, and proteins.This matrix is required for encasing the cells, and it forms a protective barrier.

An overview of the involved biofilm components, with their function and kind of regulation is given in Table 1.

The exopolysaccharides of *P. aeruginosa* consists of three components: alginate, Pel (referring to pellicle), and Psl, a polysaccharide resulting from the transcription and translation of the polysaccharide synthesis locus [56].

The first one, alginate, ["is a high molecular weight, acetylated polymer composed of non-repetitive monomers of ß-1,4 linked L-guluronic and D-mannuronic acids"] [56]. The genes, responsible for alginate biosynthesis, are clustered in the *algD* operon [57] and require the alternative sigma-factor σ22, which is regulated post-translationally by MucA [58]. In the context of biofilm formation, some studies show the effect of alginate on the biofilm architecture, while, at the same

Table 1. Overview of the involved biofilm components with their associated gene cluster, function and kind of regulation in *P. aeruginosa*.

Biofilm Involved Component	Type	Component	Gene Cluster	Function	Regulation
Extracellular Appendages	polar flagellum	protein flagellin arranged in helical filaments (diameter: 20 nm)	master genes: *fleQ, fliA*	swarming: movement in fluid environment and reach most favourable environment; sensors temperature and chemicals	flagellar biogenesis: i.a. FleS/FleR; swarming motility: GacS/GacA
	type IV pilus	flagellin of archaeal flagella; pilin subunits	*pilA, fimU-pilVWXY1Y2E, pilGHIJKL*	twitching: initial attachment in biofilm formation; senses eDNA	*pilA*: PilS/PilR ; *fimU-pilVWXY1Y2E*: FimS/AlgR; chemostatic system
	Cup fimbrium	fimbrial subunits	*cupABCDE*	targeting biotic and abiotic surfaces; involved in early biofilm formation during micro- and macrocolonisation; specific function is obscure	*cupA*: MvaT, a negative regulator of cupA transcription; *cupB, cupC*: RocS1/RocA1/RocR
	lectin (LecA and LecB)	outer membrane protein	*lecA, lecB*	recognize carbohydrates (LecA → D-galactose; LecB → L-fucose) → interaction with other cells and surfaces	quorum sensing: las and rhl; sigma-factor RpoS
Matrix	exopolysaccharide	alginate (in mucoid strains)	*algD* operon: *algD844KEGXLIJFA*	maintains biofilm architecture	*algD* operon: sigma-factor 22; alginate synthetase: c-di-GMP
		Pel (in non-mucoid strains)	*pelA - pelG*	conveys cell-cell interaction → structural support for community; increased resistance to aminoglycoside antibiotics	quorum sensing: *las* and *rhl;* c-di-GMP
		Psl (in non-mucoid strains)	*pslA - pslO*	supports biofilm structure	c-di-GMP
	proteins	pili, fimbriae, lectins	see above	see above	see above
	eDNA	-	-	biofilm formation and stability	release limitted by BfmR
Quorum Sensing	AHL	las system: N-3-oxododecanoyl homoserine	*lasI*	intercellular communication and coordination → activates transcription of certain genes	constitutively produced (autoinducer)
		rhl system: N-butyryl homoserine lactone	*rhlI*	intercellular communication and coordination → activates transcription of certain genes	constitutively produced (autoinducer)
	PQS	quinolone	*pqsA - pqsE*	supposed to be involved in LecA production; formation of eDNA; repress swarming motility	PqsE/PqsR

time, it is not necessary for the construction of biofilms [59,60,56,61]. Furthermore, it is an important component in mucoid *P. aeruginosa* strains, mainly present in the slime of cystic fibrosis patients [7].

In non-mucoid *P. aeruginosa* strains, such as PAO1 and PA14, two additional loci with polysaccharide encoding genes were discovered by Friedman and Kolter (2004) [62], evidencing that production of these exopolysaccharides is uncoupled from alginate production [61]: *pel* and *psl*. Transcription and translation of the *pel* locus (genes *pelA* to *pelG*) leads to the synthesis of a glucose-rich matrix component. Colvin et al. (2011) [63] revealed two roles of Pel in the *P. aeruginosa* matrix. On the one hand, it conveys cell-cell interactions in a PA14 biofilm, giving the community structural support, and on the other hand it is involved in the increased resistance to aminoglycoside antibiotics.

The second locus consists of *psl* genes -*A* to -*O* and is responsible for the production of mannose- and galactose-rich exopolysaccharides, which have accepted roles in the structure of biofilms [64].

It is conceivable, that Pel as well as Psl are synthesized simultaneously. In association with these findings, enhanced levels of c-di-GMP (cyclic diguanylate monophosphate) in cells were proposed to lead to the upregulation of *pel* and *psl* genes [65,66]. c-di-GMP is one of the factors which causes changes between planktonic and surface associated lifestyle in gram-negative bacteria [67], as in the case of *P. aeruginosa.* Thus, it is important for the biofilm development and it acts as a second messenger. The synthesis of c-di-GMP is realized from two GTP molecules by the enzyme diguanylate cyclase (DGC), which contains the conserved GGDEF domain, responsible for enzymatic activity. However, the degradation of the linear dinucleotide pGpG is performed by phosphodiesterase A, accommodating EAL and HD-GYP domains, which are responsible for enzymatic activity (in detail reviewed by [68]). A mutation of the genes encoding DGCs resulted in a decreased capability for biofilm formation and since this discovery c-di-GMP is considered to be a positive regulator for biofilm development [69]. Previous studies have shown that pelD, one of the genes of the *pel* operon, responsible for pellicle production as well as Pel exopolysaccharide synthesis, is a downstream target of c-di-GMP [70,71]. A recent study by Li et al. (2012) [72] presents the structural and biochemical features of PelD and describes it as a novel c-di-GMP effector.

Beside the upregulation of *pel* and *psl* genes, c-di-GMP serves as a secondary signalling nucleotide for motility in *P. aeruginosa*. Motility is suppressed by c-di-GMP and, thus, SadC, working as an integral membrane DGC influences biofilm formation directly by the junction of two GTPs to a c-di-GMP [73]. Currently, findings by Irie et al. (2012) [74] show an increase of SadC activity by the matrix polysaccharide Psl. Thus, a positive feedback regulatory loop is closed because Psl production is upregulated in the presence of c-di-GMP and Psl enhanced the activity of the DGC SadC, which mediates junctions of GTP molecules to c-di-GMP. Furthermore, there are different tertiary structures formed by c-di-GMP *in vitro*. For proteins from *P. aeruginosa* it is a fact that c-di-GMP interacts with them in both its monomeric and dimeric tertiary structure but not in the G-octaplex structure [75]. In addition, c-di-GMP is involved in the regulation of transcription factors. In the course of this regulation FleQ-binding to the *pel* promoter is prevented by high levels of c-di-GMP. Hence, FleQ is an activator of gene expression, responsible for flagella biosynthesis, and, thus, an activator for planktonic lifestyle [66]. Overall, there is a positive regulation by c-di-GMP in

matrix polysaccharide synthesis, acting as a repressor of motility, and promoting the surface-associated lifestyle.

In addition to the above regulatory system, regulation is influenced transcriptionally and post-transcriptionally by other bacterial proteins. The σ-factor RpoS, influences the transcription of *psl* expression positive, which was revealed by a mutation in *rpoS* leading to a decreased level of Psl. On the other hand, an increased level of Psl was observed when *rpoS* was overexpressed. Another regulator of Psl production is the post-transcriptionally acting RsmA, which is described along with RpoS. It binds to the 5′-untranslated region of the *psl* mRNA and, thus, represses *psl* translation [76]. Regulation systems of matrix polysaccharide biosynthesis were reviewed by Karatan and Watnick (2009) [43] in detail.

Beside polysaccharides, nucleic acids are another important component of the biofilm matrix, known as extracellular DNA (eDNA). Bacteria can release or secrete DNA actively. Furthermore, bacterial lysis (realized by a mechanism involving genomic prophages, [77]) and formation of outer membrane vesicles are the source for eDNA. Cell death is important for biofilm differentiation and supply, as nutrients from lysed cells represent an energy source for the survivial of surrounding cells [77]. In surface-associated microcolonies, eDNA ensures attachment, aggregation, and stabilization of bacteria. Additionally, it plays a role in maturation [78]. However, Whitchurch et al. (2002) [53] proposed functionality of eDNA only in early biofilm formation, because *P. aeruginosa* biofilms in early development were disrupted by DNase treatment, while DNase treatment of matured biofilms had no such influence. A Psl matrix-free cavity was described [79], that contains dead cells and eDNA. This cavity is formed during biofilm maturation in the centre of microcolonies leading to a distribution of cells. According to the finding of eDNA localization to the stalk structure in mature microcolonies [80] it was proved that ["dead cells were concentrated in a region close to the substratum and in the stalk portions of mushroom-like colonies, which was also the location of the matrix cavity"] [79]. A main function of eDNA is to stabilize the matrix indirectly, by exhibiting antimicrobial activity. This activity is realized because of cell lysis of invasive cells by chelating cations. Again, these cations, which are no longer available after chelating, are responsible for stabilization of lipopolysaccharides (LPS) and the outer membrane. Furthermore, a decrease of cations, especially Mg^{2+} cations activates a two-component system, called PhoPQ/PmrAB system [81]. Among other things this system is relevant for the control of *pmr* genes, and responsible for spermidine production on the outer membrane. Spermidines are able to fend off attacks by antimicrobial peptides, known to be toxic for bacterial cells (for detail see [82]). Furthermore, Chiang et al. (2013) [83] proved protection by eDNA against aminoglycoside antibiotics. Another distinct feature of eDNA is its role as a source of phosphate, nitrogen, and carbon [84]. The release of eDNA is proposed to be regulated by BfmR, a two-component regulator. BfmR limits bacteriophage-mediated lysis as its target is the promoter of genes encoding proteins of the prevent-host-death (Phd) family [85]. Hence, eDNA causes a development of cation gradients, induces genomic DNA release, and protects the bacterial cells against antibiotic treatment ensuring a protected, surface-associated community.

A third component within the biofilm matrix are proteins. Proteins occurring in the biofilm matrix as fimbriae, pili, lectins and sugar binding proteins, were all described and dealt with in the second paragraph (Biofilm formation by extracellular appendages).

In summary, these findings reveal the biofilm matrix structure built up on polysaccharides, eDNA, and proteins. Thereby, the matrix acts as a barrier to fend off hostile agents adjusted at different levels. Thus, a biofilm is not just a collection of cells randomly held together, but it can be considered as a self- and well-organized cohabitation.

4. Cell-cell Signalling Effects Biofilm Formation

Bacterial cells are able to communicate and coordinate with each other by small extracellular molecules, called autoinducers (AI). This intercellular communication is called quorum sensing. Quorum sensing is launched when AIs, which are produced and secreted, reach and exceed a threshold level [86–89].

As with most of the gram-negative bacteria, *P. aeruginosa* uses a quorum sensing system based on acylated homoserine lactones (AHLs) acting as AIs. Upon reaching the threshold level, AHL synthases modulate the activity of an AHL-responsive, transcriptional regulator. Subsequently, the transcription of AHL synthesis genes is activated, when the AHL binds the regulator. There are two main AHL-based systems known for *P. aeruginosa*: *las* and *rhl*. The AHL synthases called LasI and RhlI, the transcriptional regulators called LasR and RhlR, and the AHL signals consisting of N-3-oxododecanoyl homoserine lactone and N-butyryl homoserine lactone [90]. To effect communication the AHL signals have to leave the cells. This is realized either by direct membrane diffusion, as in the case of N-butyryl homoserine lactone, or by secretion through an efflux pump, as in the case of N-3-oxododecanoyl homoserine lactone [91]. Furthermore, a non-AHL extracellular signal exists, which is involved in the AHL signalling pathway: the *Pseudomonas* quinolone signal (PQS; reviewed in detail by [92]). The biosynthesis of this 2-heptyl-3-hydroxy-4-quinolone is encoded by several genes [93,94] together with the transcriptional regulator (pqsR) and the response effector (pqsE, [95]). The substance belongs to the family of 2-alkyl-4-quinolones. It is supposed to be involved in LecA production, responsible for biofilm maturation [94] and in formation of eDNA [80]. In contrast to AHL signals, PQS and its precursor 4-hydroxy-2-heptylquinolone (HHQ) are highly hydrophobic and are transported through the membrane by vesicles [96,97]. Furthermore, HHQ probably induces the production of phenazine-1-carboxylic acid, again suggested to repress swarming motility [98]. Thus, in quorum sensing self-generated signalling molecules lead to coordination of gene expression depending on the cell density. Different linking points of quorum sensing and biofilm formation have been discussed in previous studies. Davies et al. (1998) [99] reveal the involvement of the Las quorum sensing system, in contrast to Rhl quorum sensing, for differentiation and maturation of biofilms. Lectins, which are responsible for the detection and the binding of sugar components on cell surfaces, are also under the control of quorum sensing [100]. It is suggested that the *pel* operon dependency of quorum sensing is connected with the biosynthesis of matrix components [101]. The biosynthesis genes of rhamnolipids are also under the control of quorum sensing. In context with biofilm formation rhamnolipids are thought to influence microcolony formation, preservation of aqueous channels, mushroom-like-cap formation, and release of cells from the community (detailed Review [90]). With respect to motility, quorum sensing is involved indirectly by the control of swarming through rhamnolipid production [102]. However, direct interaction in motility was not shown for *P. aeruginosa* but was suggested for a variety of

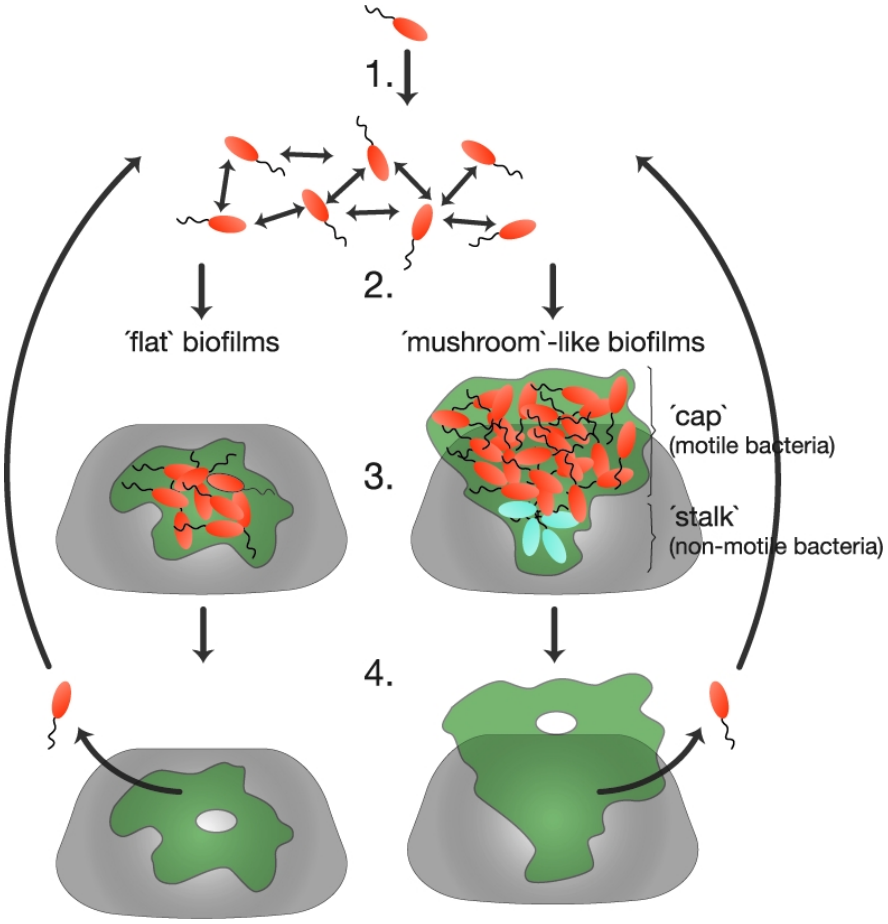

Figure 1. Model for biofilm formation by *P. aeruginosa*. 1. Cell division leads to an increase of bacteria number and depending on a designated cell density quorum sensing (⟷) is activated. 2. Subsequently, swarming (by flagella), lectins (controlled by quorum sensing indirectly and directly, respectively), and type IV pili mediated twitching effect binding to (cell-) surfaces. 3. Bacteria surround themselves by self-produced EPS (protection against extraneous attacks) and the formed 3D-structure (´flat´ or ´mushroom´-like) depends on nutrient source. In flow chambers citrate medium leads to ´flat´ biofilms and glucose leads to ´mushroom´-like biofilms. 4. The maturation is accompanied by differentiation and dispersal of a biofilm. Thereby, unfavourable conditions are responsible for cell death and cell release from the centre of the microcolonies. If conditions in the biofilm are favourable again, bacteria are able to enter the surface-associated community again. As bacteria in biofilms represent the stationary phase, they are capable of producing bioactive compounds. The grey area represents a solid surface, while the green area depicted the biofilm matrix. Modified according to [132] and [90].

bacterial species. High cell density can lead to surface motility because nutrition supply is not any longer favourable. It is believed that bacteria leave the community to face more favourable conditions than available in the environment (for a detailed Review see [103]).

5. Biofilm Formation in *Pseudoalteromonas* Species

A comparison of biofilm formation processes in *P. aeruginosa* and *Pseudoalteromonas* sp. May be helpful as the understanding could help to create a beneficial *Pseudoalteromonas* sp. biofilm on surfaces with usage in marine aquaculture.

Aside from the well-studied biofilm mechanisms in *P. aeruginosa*, information from marine bacteria, such as *Pseudoaltermonas* sp., is rare. Studying mechanisms of artificial biofilm formation in *Pseudoalteromonas* sp. has a great potential as these marine bacteria produce bioactive compounds and display probiotic activity [104,105].

Marine biofilms, which consist of organic (e.g. protein and glycosidic fragments) and inorganic compounds, such as salts [106], and of microalgae [107] in addition to the bacteria, are often associated with fouling processes. Most of the biofilm literature in aquaculture systems deals with the development of surfaces that are able to cope with surface-fouling organisms. As early as 2009, Iijima et al. [108] suggested biofilms as beneficial partners in fish breeding. The counterpart of *P. aeruginosa* in marine biotopes, in terms of a model organism, is *Pseudoalteromonas tunicata* (*P. tunicata*). However, studies have been carried out on the genus of *Pseudoalteromonas* not identified at the species level.

In *P. tunicata* genome analysis revealed several structures required for sessile life: curli, type IV pili, MSHA-pili (mannose-sensitive hemagglutinin) and capsular polysaccharides (O-antigen; [109]; Table 2). The gene locus for the flexible, peritrichous MSHA-pili was identified as *mshI1I2JKLMNEGFBACDOPQ* with homology to *Vibrio cholerae*. Furthermore, the production of these pili is upregulated depending on the cellulose concentration [110]. In turn, curli are constructed from proteinaceous fibres by many Enterobacteriaceae and are important components of the formed matrix [111]. No genes for alginate biosynthesis were detected in *P. tunicata*, but gene clusters with homology to algZ/algR (a TCS involved in regulation of motility in *P. aeruginosa*, [112]), this system may be involved in the expression of pili components [109]. Another factor regulating the production machinery of type IV pili is the ToxR-like transcriptional regulator WmpR [113]. Furthermore, WmpR is involved in the regulation of iron uptake, thus ["important for survival under low-iron conditions"] [114].

Previous studies by Ritter et al. (2012) [115] identified four proteins, bound to the outer membrane of *Pseudoalteromonas* sp. D41, which are involved in biofilm construction, membrane transport, and main cellular metabolic processes. Biofilm formation was tested on hydrophobic surfaces as well as on hydrophilic surfaces. One of the detected proteins was PilF, which is involved in biogenesis of type IV pili. As mentioned for *P. aeruginosa*, type IV pili are responsible for twitching motility and, thus, for mushroom-like structures. The deletion of PilF in *P. aeruginosa* resulted in the loss of this structure [3,37]. In context with genes required for type IV pilus production, *Pseudoalteromonas haloplanktis* and *P. tunicata* also have homologous gene sequences [116,109]. Hence, in strains of *Pseudoalteromonas* sp. there are proteins responsible for construction of type IV pili, involved in twitching ability. For the second protein discovered, OmpA, a role as an adhesion factor was supposed. OmpA belongs to the group of porins and its homologue in *P. aeruginosa* is OprF. Lower et al. (2005) [117] demonstrated a role for OmpA in biofilm formation on abiotic surface structures and a mutation in *oprF* in *P. aeruginosa*, which leads to

changed biofilm structures and decreased biomass compared to the wild type biofilm. Another protein belonging to the group of porins and initially described in conjunction with biofilm formation is OmpW. Its homologue in *P. aeruginosa* is OprG and Ritter et al. (2012) [115] proved a change in biofilm formation and a decrease in biofilm biomass formed by a respective mutant. The fourth protein highlighted by MS/MS *de novo* sequencing analysis was a TonB-dependent receptor (TBDR). As it is reported for other gram-negative bacteria, TBDRs take up large molecules and subsequently transport them across the outer membrane [118,119]. They are upregulated by the above mentioned WmpR [114]. For *Pseudoalteromonas* sp. D41 a function in ["adhesion, sugar assimilation, and/or remodelling of the extracellular polysaccharide matrix"] is suspected [115]. This reveals, that *Pseudoalteromonas* sp. D41 harbours a specific biofilm mechanism as in the case of *P. aeruginosa* (type IV pili, OmpA and OmpW porins) and mechanisms specific for marine habitats (TBDR, [115]).

Moreover, calcium [Ca^{2+}] is a factor participating in biofilm formation in *Pseudoalteromonas* sp. 1398. In marine habitats [Ca^{2+}] occurs on surfaces and in association with other organisms in the marine environment. For instance, the concentration of flagellin, an important swarming component, is decreased at higher [Ca^{2+}] concentration, resulting in sessile lifestyle. Furthermore, higher [Ca^{2+}] concentrations are connected with an increase of biofilm-associated polysaccharides leading to a matrix construction, surrounding, stabilizing, and protecting the cells [120]. In *P. aeruginosa* the control of pilus extension and retraction, realized by PilY1, a pilus biogenesis factor, depends on [Ca^{2+}] concentration [121].

As it is described for *Pseudoalteromonas* sp. D41, the biofilm matrix mainly consists of exopolysaccharides, proteins and glycoproteins, similar to *P. aeruginosa*. Studies by Leroy et al. (2008) [122] show that subtilisin, a protease, prevents biofilm formation by *Pseudoalteromonas* sp. D41 in polystyrene microtitreplates, but it cannot destroy already formed biofilms. Thus, proteins seem to be an important factor for initial *Pseudoalteromonas* sp. D41 adherence. Genomic analysis of *P. tunicata* revealed the existence of several enzymes necessary for extracellular matrix construction. It includes a gene cluster for capsular polysaccharides (*cspA-D*; [109]).

The maturation (differentiation and dispersal) of biofilms formed by *P. tunicata* is considered to be connected to cell death brought about by hydrogen peroxide. Hydrogen peroxide is produced from the oxidation of L-lysine by the lysine oxidase, called AlpP [123]. Recent studies revealed the regulation of AlpP (190 kDa) by WmpR, mentioned above in context with type IV pili production [124]. In contrast, cell death in *P. aeruginosa* is triggered by a prophage [77], pointing at different mechanisms in *P. aeruginosa* and *P. tunicata*. In both, cell death occurs inside the microcolonies leading to the formation of cell free cavities [77,124]. As previously mentioned, cell death is an important mechanism in releasing eDNA. Again, eDNA causes a development of cation gradients, induces genomic DNA release, and protects the bacterial cells against antibiotic treatment ensuring a protected, surface-associated community.

To avoid predation by protozoans *P. tunicata* has different defence strategies: (i) a small polar heat-stable anti-larval molecule [125], (ii) a large antibacterial protein [126], (iii) a putative antialgal peptide [127], (iv) an antifungal tambjamine alkaloid (yellow pigmented; [128,129]), (v) and violacein (a purple pigment; [130]).

Table 2. Overview of the involved biofilm components with their associated gene cluster, function and kind of regulation in *Pseudoalteromonas* species.

Biofilm Involved Component	Type	Component	Gene Cluster	Function	Regulation
Extracellular Appendages	curli	proteinaceous fibres	*csgABEFG*	suggestion: attachment to algal host surfaces	CsgD: regulator protein of the *csg* operon
	MSHA-pilus (belongs to the family of type IV pili)	pilin subunits	*mshI112JKLMNEGF BACDOPQ*	Twitching	algZ/algR: may be involved in pili component expression; WmpR: type IV pilus production and iron uptake regulation
Matrix	exopolysaccharides	capsular polysaccharides	*cspA - cspD*	biofilm matrix formation	unknown (e.g. described for *E. coli* K12)
	outer membrane protein involved in the biofilm construction in *Pseudoalteromonas* sp. D41	PilF (lipoprotein)	*pilF*	involved in biogenesis of type IV pili → twitching	unknown
		OmpA (porin)	Gene cluster is unknown for *Pseudoalteromonas* sp.	adhesion factor	unknown
		OmpW (porin)	Gene cluster is unknown for *Pseudoalteromonas* sp.	adhesion factor	unknown
		TBDR	Gene cluster is unknown for *Pseudoalteromonas* sp.	adhesion, sugar assimilation, and/or remodelling of the extra-cellular polysaccharide matrix	WmpR: upregulation of TBDR

In conjunction with beneficial effects of *Pseudoalteromonas* sp., is the appearance of extracellular proteases during biofilm formation [108]. Such proteases play an important role in eliminating increased protein load of fish farm sediments. Thus, biofilms are not only damaging in terms of fouling processes, but also ["can benefit to keep the health status of breeding fishes"] [108].

6. Conclusion

This review presents the latest findings of biofilm research, covering extracellular appendages, matrix components, and cell-cell signalling in *P. aeruginosa* and *Pseudoalteromonas* sp. One aim of this review was to work out similarities of biofilm formation in *P. aeruginosa* and *Pseudoalteromonas* sp. As shown for *P. aeruginosa*, biofilms are complex structures depending on the environmental conditions and the bacterial strain. Due to this complexity, not all processes of biofilm formation and regulation have been unravelled so far. As it is demonstrated,

Pseudoalteromonas sp. biofilm formation has traits similar to the biofilm formation mechanisms of *P. aeruginosa*. Thus, it is conceivable to match gene sequences, which are integrated in the biosynthesis of biofilm formation components in *P. aeruginosa*, with those in *P. tunicata* to elucidate further mechanisms of biofilm formation in *P. tunicata*. Furthermore, genetic patterns involved in factors influencing biofilm formation in other subspecies of *Pseudoalteromonas* could be identified by comparison with the known genetic patterns in *P. tunicata*.

Another starting point could be the development of surfaces or objects with attractive properties for biofilm formation. Thus, artificial biofilms with *Pseudoalteromonas* sp. could be created. The biofilms formed in this way are suggested to protect fish egg clutches from fish-pathogenic infestation. This protection is probably achieved by sequestered bioactive compounds from *Pseudoalteromonas* sp. These beneficial biofilms can be useful in aquaculture, due to the improvement of fish (larvae) rearing [131].

Additionally, bacteria of *Pseudoalteromonas* sp. could be applied to aquacultures coupled to fish food to improve fish health as probiotics. *Pseudoalteromonas* sp. may have two effects: (i) they may protect egg clutches from fish-pathogenic infestations, (ii) and as probiotic bacteria, *Pseudoalteromonas* sp. stabilize fish health and thus avoid the inset of antibiotics. Antibiotics should be a last resort, as pathogenic microorganisms are able to develop resistance against them and thus the antibiotic becomes ineffective, which requires increased research for and an inset of other antibiotics for this pathogenic microbe.

This review illustrates the importance of studies dealing with biofilm formation and regulation mechanisms on the genus of *Pseudoalteromonas*.

Thus, for future research and investigations it is unavoidable to connect disciplines of biology (such as microbiology, molecular biology, and biophysics) and other scientific disciplines.

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Conflict of Interest

The author declares no conflicts of interest in this paper.

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