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Quorum sensing and swarming migration in bacteria

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Abstract

Bacterial cells can produce and sense signal molecules, allowing the whole population to initiate a concerted action once a critical concentration (corresponding to a particular population density) of the signal has been reached, a phenomenon known as quorum sensing. One of the possible quorum sensing-regulated phenotypes is swarming, a flagella-driven movement of differentiated swarmer cells (hyperflagellated, elongated, multinucleated) by which bacteria can spread as a biofilm over a surface. The glycolipid or lipopeptide biosurfactants thereby produced function as wetting agent by reducing the surface tension. Quorum sensing systems are almost always integrated into other regulatory circuits. This effectively expands the range of environmental signals that influence target gene expression beyond population density. In this review, we first discuss the regulation of AHL-mediated surface migration and the involvement of other low-molecular-mass signal molecules (such as the furanosyl borate diester AI-2) in biosurfactant production of different bacteria. In addition, population density-dependent regulation of swarmer cell differentiation is reviewed. Also, several examples of interspecies signalling are reported. Different signal molecules either produced by bacteria (such as other AHLs and diketopiperazines) or excreted by plants (such as furanones, plant signal mimics) might influence the quorum sensing-regulated swarming behaviour in bacteria different from the producer. On the other hand, specific bacteria can reduce the local available concentration of signal molecules produced by others. In the last part, the role and regulation of a surface-associated movement in biofilm formation is discussed. Here we also describe how quorum sensing may disperse existing biofilms and control the interaction between bacteria and higher organisms (such as the *Rhizobium*-bean symbiosis).

Keywords: Quorum sensing; Swarming; Surface translocation; Population density-dependent; *N*-acyl-homoserine lactone; AI-2

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1. Introduction

1.1. Short overview of quorum sensing

Although bacteria are unicellular organisms, they often show group behaviour: e.g. in living biofilms individual cells at different locations in the biofilm may have different activities. This led to the proposal that biofilm communities may represent an evolutionary step between unicellular non-specialized organisms and multicellular organisms that possess specialized cells [1]. For this, bacteria have to monitor their own population density. This can be achieved by quorum sensing. This process relies on the production of a low-molecular-mass signal molecule (often called 'autoinducer' or recently quormon), the extracellular concentration of which is related to the population density of the producing organism. Cells can sense the signal molecule allowing the whole population to initiate a concerted action once a critical concentration (corresponding to a particular population density) has been reached. Gram-negative and gram-positive bacteria use different signal molecules to measure their population density (Fig. 1).

Cell–cell communication using *N*-acyl-homoserine lactone (AHL) signals is one of the known mechanisms by which bacteria can communicate with each other and is a widespread phenomenon in gram-negative bacteria [2]. The first example and the paradigm of gram-negative quorum signalling is the *luxI–luxR* quorum sensing system of *Vibrio fischeri*, involved in population density-dependent regulation of bioluminescence. The AHL signalling system of *V. fischeri* involves two major components: *luxI* is the AHL synthase gene that is part of the bioluminescence operon *luxICDABEG* and *luxR* codes for the transcriptional activator. At low population density the transcription of *luxICDABEG* is weak. The AHL quorum sensing signal molecule produced by LuxI at a basal level, 3O,C_6-HSL (see below), diffuses through the membrane. The LuxR transcriptional activator is inactive at this moment. With increasing population density, the AHL concentration increases. When a threshold concentration is reached, the signal molecule binds to the LuxR transcriptional activator. This complex is active and binds to the promoter region of the bioluminescence operon *luxICDABEG*. This leads to a rapid amplification of the AHL signal 3O,C_6-HSL and consequently induces bioluminescence. *V. fischeri* is a free-living marine bacterium that also occupies the light organ of the squid *Euprymna scolopes*. The high population density required for bioluminescence is only reached in the microenvironment of the light organ.

AHL-mediated gene regulation was originally termed 'autoinduction' for two reasons. First, the *lux* operon of *V. fischeri*, which includes *luxI*, is itself positively regulated by the AHL whose synthesis is directed by the LuxI synthase, and second, each bacterial species was originally believed to produce a unique AHL signal. The intensive study in the field of quorum sensing during the past ten years indicates that the system is far more complex than initially thought. AHL quorum sensing appears to be widespread among the Proteobacteria [3], and the AHL signalling systems all have two major components: an AHL synthase enzyme (mostly LuxI homologues) catalyzes the formation of AHLs, whereas the receptor protein (mostly LuxR homologues) binds the AHL signal molecule and functions as transcriptional regulator. Synthases without similarity to LuxI-type proteins, such as AinS in *V. fischeri* [4,5], can also catalyze the formation of AHLs but they will not be discussed here. In general, LuxI-type proteins direct the formation of an amide linkage between SAM and the acyl moiety of the appropriately charged acyl-ACP. The LuxI-type synthase in *Pantoea stewartii*, EsaI, catalyzes the formation of 3O,C_6-HSL. According to the three-dimensional structure of EsaI, the 3O,C_6-phosphopantetheine of acyl-ACP fits neatly into the V-shaped hydrophobic cavity of the enzyme [6]. During this interaction, a predicted hydrogen bond between the C3 carbonyl in 3O,C_6-ACP and the Thr140 hydroxyl of EsaI is formed [6]. In the following steps, a conformational rearrangement of the N-terminal domain, SAM binding, and finally *N*-acylation of SAM occurs [6,7]. Subsequent lactonization of the ligated intermediate with the release of methylthioadenosine follows. This step results in the formation of the AHL

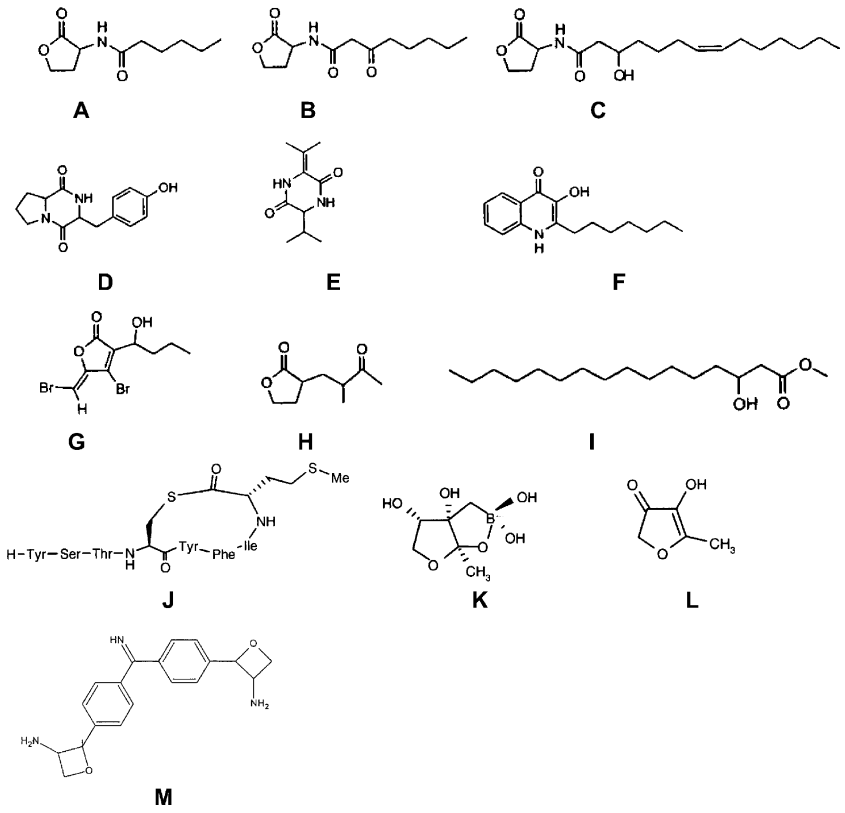

Fig. 1. Different quorum sensing signal molecules mentioned in the text (adapted from [29]). (A–C) Examples of microbial AHLs without substitution on the C3, or with an oxy or hydroxyl group. (A) *N*-hexanoyl-L-homoserine lactone or C_6-HSL. (B) *N*-(3-oxooctanoyl)-L-homoserine lactone or 3O,C_8-HSL. (C) *N*-(3R-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone or 3OH,$C_{14:1}$-HSL. (D, E) Microbial diketopiperazines: (D) cyclo(L-Pro-L-Tyr). (E) cyclo(Δ Ala-L-Val). (F) 2-Heptyl-3-hydroxy-4-quinolone (PQS) produced by *P. aeruginosa*. (G) 4-Bromo-5-(bromomethylene)-3-(1′-hydroxybutyl)-2(5*H*)-furanone of *D. pulchra*. (H) γ-butyrolactone produced by *X. campestris*. (I) 3-Hydroxypalmitic acid methyl ester of *R. solanacearum*. (J) Group IV cyclic thiolactone from *S. aureus*. (K) Putative structure for *Vibrio harveyi* AI-2. It is also possible that this compound and 4-hydroxy-5-methyl-3(2H)furanone (MHF) are interconvertable (L). (M) bradyoxetin, a four-membered oxetane ring, from *B. japonicum*.

(Fig. 2) [8–10]. At low population density, a basal level of *luxI*-type gene expression can be observed. Results obtained with an in vitro fatty acid-3-oxo-AHL synthesis system (known as the Fab–Las system) with purified *Pseudomonas aeruginosa* Fab proteins, ACP and *P. aeruginosa* LasI 3-oxo-AHL synthase, demonstrate that FabG activity (β-ketoacyl acyl carrier protein reductase) in the biosynthetic pathway is a determining factor of 3-oxo-HSL acyl chain lengths [11]. It was observed that when the FabG activity is high, turnover of the short chain 3-oxo-acyl-ACP substrates is rapid and LasI cannot compete for these, presumably because its affinity for these substrates is lower than that of FabG. Once the acyl chain length reaches 12 carbons, LasI competes for the appropriately charged ACP, resulting in synthesis of 3O,C_{12}-HSL. When the FabG catalyzed step becomes rate limiting, short chain 3-oxo-acyl-ACPs accumulate. This enables LasI to compete for these short-chain ACPs and use them for synthesis of the corresponding short chain 3-oxo-AHLs. Some of the observations made with the in vitro system were supported by preliminary in vivo data [11].

The LuxR-type proteins contain two conserved domains, an amino-terminal domain for AHL-binding and dimerization and a carboxy-terminal helix-turn-helix DNA-binding domain. Based on the *Agrobacterium tumefaciens* TraR crystal structure, 3O,C_8-HSL produced by the corresponding synthase TraI, binds to the alpha/beta/alpha sandwich in the N-terminal domain of this LuxR-protein. During this interaction, the AHL lies fully embedded within the protein with virtually no solvent contact [12,13]. Specific interactions are made between the conserved lactone ring and the binding pocket, and the pocket's shape suggests how specificity may be mediated by the differences found in the alkyl chain [12,13]. Recently, studies with new synthetic agonists of the *P. aeruginosa* LasR system with the alkyl chain kept constant but containing various amines and alcohol substitutions instead of the homoserine lactone ring suggest that the HSL ring binding pockets in the regulatory proteins are not absolutely conserved and

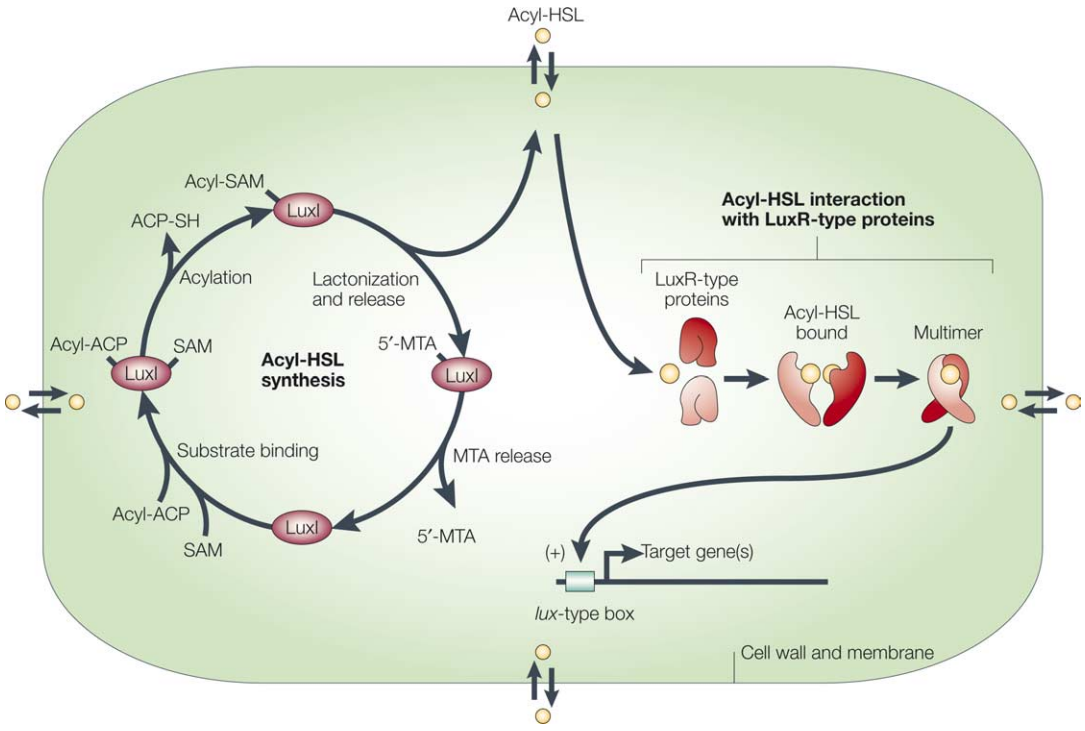

Fig. 2. General model of AHL signal transduction (taken from [3], with permission from *Nature Reviews*). A single quorum-sensing cell is shown. Tentative models for AHL synthesis cycle and AHL interaction with LuxR-type proteins are depicted. Double arrows with filled circles at the cell envelope indicate the potential two-way traffic of AHLs into and out of the cell. The LuxR-type protein is shown as dimerizing, although higher-order multimers may be important in other systems. Although the act of binding to the AHL and multimerization are represented as different events, these may occur simultaneously. ACP, acyl carrier protein; MTA, methylthioadenosine; SAM, *S*-adenosylmethionine; HSL, homoserine lactone; AHL, *N*-acyl-homoserine lactone.

interact differently with the ring analogues found in synthetic ligands [14]. In general, binding of an AHL to its receptor requires a threshold signal concentration (depending on both the numbers of receptors and cells and the diffusibility of the local environment [15]) and presumably unmasks the carboxy-terminal domain of the LuxR-type protein relieving inhibition. This enables the receptor-AHL complex to bind to specific promoter sequences in the genome and to induce gene expression. Often, the target genes include the *luxI* homologue, creating a positive feedback circuit, although systems exist in which the AHL synthase gene is not autoregulated [16]. A microarray analysis of the *P. aeruginosa* quorum-controlled genes revealed that the strings of adjacent quorum-controlled genes are in operons, and that the genes in a given string show similar quorum responses [17]. The transcriptome analysis also suggests that the timing of quorum-controlled gene induction is on a continuum (induction from early in growth until induction during the stationary phase) and timing is not related to signal concentration. The level of LasR was hypothesized to be a critical trigger for quorum-activated gene expression. In fact, the *lasR* and *rhlR* transcript levels increase during the late logarithmic and early stationary phases, which coincides with the induction of most quorum-activated genes. More evidence is required to determine the validity of this hypothesis. The binding site for the transcriptional activator is often a sequence with a dyad symmetry, called the *lux* box, although other essential but non-palindromic *cis*-elements exist [18,19]. Furthermore, *lux* boxes are not apparent in promoter regions of all quorum-regulated genes [20]. In this regard, it is interesting that a number of quorum sensing-regulated genes are transcriptional regulators or members of two-component systems. As a consequence, the target genes of these two-component systems may be regulated indirectly via quorum sensing [21]. Although no membrane-spanning sequences are present in *V. fischeri* LuxR, it has been proposed that LuxR contacts the interior leaflet of the cytoplasmic membrane bilayer through amphipathic interactions [22]. In line with this and as reported for *A. tumefaciens*, in the absence of AHLs, monomeric TraR cofractionates with cytoplasmic membranes, whereas in the presence of ligand, TraR appears to be largely cytoplasmic [23].

The *V. fischeri* AHL, known to regulate bioluminescence as a function of population density, was the first identified AHL and shown to be 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl) hexanamide, more commonly known as *N*-3-(oxohexanoyl) homoserine lactone or 3O,C_6-HSL [24]. AHLs may be saturated or unsaturated and

mainly vary with respect to the length (4–14 carbons) and the substituent (H, O or OH) at the third carbon of the acyl-side chain (Figs. 1A–C). Recently, AHLs with acyl-chains up to 16 and 18 carbons were isolated from *Rhodobacter capsulatus* and *Sinorhizobium meliloti* [25,26] by using a radiotracer technique.

The AHL signal is released into the environment, either by passive diffusion, as observed for 3O,C_6-HSL in *V. fischeri* and *Escherichia coli* cells [27], or by a combination of diffusion and active efflux of AHLs with longer acyl-side chains as in *P. aeruginosa* [28], and accumulates with growth of the bacterial population. At least in *V. fischeri*, the signal freely diffuses back into the cells such that its intracellular concentration also rises as a function of the increase in bacterial population. Transduction of this information to response regulators of gene expression leads to the elaboration of an appropriate phenotype when a quorum is reached (Fig. 2). The quorum sensing process is summarized in Fig. 2.

The physiological processes regulated by AHLs in different bacterial species, including pathogens from humans, animals, and plants may vary from conjugal plasmid transfer to bioluminescence, exopolysaccharide synthesis, biofilm formation, antibiotic synthesis, or virulence (reviewed in [29]). Often, the regulated genes are crucial to the colonization or infection of eukaryotic hosts [20]. Quorum sensing was thought to provide many plant and animal pathogens with a mechanism by which they delay the production of tissue-damaging virulence factors until sufficient bacteria have been amassed, required to produce sufficient quantities to influence the surrounding environment, and as a consequence circumvent the host defence response. According to Redfield [30], the postulated benefits of quorum sensing are accepted uncritically, as neither the need for group action nor the selective conditions required for its evolution, have been demonstrated. The author argued for a more direct function of signal molecule secretion and response: the ability to determine whether secreted molecules rapidly move away from the cell [30]. Diffusion sensing allows cells to regulate secretion of degradative enzymes and other effectors to minimize losses owing to extracellular diffusion and mixing and as such could also be designated as 'compartment sensing'. Both quorum sensing and diffusion sensing should be treated sceptically until each has been rigorously tested. One needs to ask whether the regulation acts under natural conditions where quorum sensing is possible. Some signal molecule-regulated processes are true quorum sensing systems. Others might be found to be more dependent on diffusion.

Since different species of bacteria often produce the same, or very similar AHLs, there is opportunity for interspecies communication and trickery. Bacteria in natural environments may be able to use AHL signalling to monitor proximity of other bacterial species as well as their own (e.g. *Salmonella*) [31,32]. Microbial consortia now appear to play a role in quorum sensing signal turnover and mineralization [33]. Six strains with the capacity to degrade AHLs were obtained from the tobacco rhizosphere following an enrichment procedure based on the utilization of C_6-HSL as the sole carbon source [34]. They fall within the genera *Comamonas* (about 16% of the isolates), *Pseudomonas* (64%), *Rhodococcus* (8%) and *Variovorax* (12%). One of the strains was identified as *V. paradoxus*, a species that has been already described as capable of AHL degradation [35]. *Arthrobacter* strain VAI-A can grow synergistically with *V. paradoxus*, which exhibits an HSL-releasing, AHL-acylase activity. Besides growth on the generated HSL, VAI-A grows on the nitrogenous AHL inactivation product, acyl-homoserine, generated chemically or by a lactonase in diverse bacteria [33].

Besides the immunomodulatory activity of the *P. aeruginosa* 3O,C_{12}-HSL in immuno-compromised individuals [36,37], another eukaryotic response to bacterial AHLs was recently reported. The legume plant, *Medicago truncatula*, is able to detect bacterial AHLs from both symbiotic and pathogenic bacteria [38]. This eukaryotic host responds by significant changes in the accumulation of over 150 proteins and in the secretion of quorum sensing-mimicking signals. These results indicate that AHLs may also play important roles in the beneficial or pathogenic outcomes of eukaryotic–prokaryote interactions [38].

1.2. Principles of swarming

A large number of reviews describing AHL-mediated quorum sensing have appeared over the past 10 years [2,3,29,39–44]. However, because no reviews on quorum sensing-regulated swarming are available at present, a summary of quorum sensing-regulated swarming in a diverse range of bacteria is presented. For this, both AHL-mediated swarming regulation and quorum sensing signal molecules with a different structure are reported. Quorum sensing regulation of swarming presumably allows optimal dissemination of bacterial cells when a population is getting too large to inhabit a single given niche [29]. From the data obtained for *P. aeruginosa* where excess iron prevents swarming, it is suggested that less favourable nutritional conditions in general may elicit swarming motility and biosurfactant production, presumably as a means to find a new niche with more propitious nutrient supplies instead of settling and forming a biofilm [45]. Perhaps because we are social beings, we find the idea that bacteria have evolved communication and cooperation very appealing. Following Redfield [30], the regulation of motility by signal molecules could also reflect the benefits of sensing the physical structure of the environment rather than the presence of other bacteria. The presence of a solid

barrier will cause the signal to accumulate and might induce shifting to a mode of motility better suited to movement along surfaces rather than to movement in liquid [30].

In processes of surface colonization and biofilm formation, certain bacteria exhibit a primitive form of multicellularity that leads to co-ordinated behavioural patterns [46]. Henrichsen recognized six different types of translocation [47]. *Mycococcus xanthus* displays a mode of surface translocation, referred to as gliding social motility that depends on type-IV pili and is required for fruiting body formation [48]. This type of surface movement is not discussed here. Neither will the flagellum-independent surface translocation as observed for *Serratia marcescens* (also called sliding) [49,50], *Vibrio cholerae* and *E. coli* [51], be reviewed. Bacterial swarming, the type of translocation discussed in detail here, is a flagella-driven movement in the presence of extracellular slime (a mixture of carbohydrates, proteins, peptides, surfactants, etc.) by which bacteria can spread as a biofilm over a surface. This process was found in members of *Proteus*, *Vibrio*, *Bacillus*, *Clostridium*, *Chromobacterium*, *Escherichia*, *Salmonella*, *Azospirillum*, *Aeromonas*, *Yersinia*, *Serratia*, *Burkholderia*, *Pseudomonas*, and *Sinorhizobium* [52–57]. In contrast with swimming, where bacteria move through the water channels in the agar (0.2–0.4% agar), swarming is a social phenomenon across the agar (0.4–1.2% agar). The *flhDC* master operon is a key regulator in swarmer cell differentiation in several *Enterobacteriaceae* (such as *Proteus mirabilis*, *Serratia*, *E. coli*, *Salmonella* and *Yersinia*) and *Bacillus subtilis*: the increased viscosity (surface contact) and intracellular signals (nutritional state) are integrated, resulting in hyperflagellated, elongated and multinucleated swarmer cells. These motile cells move in groups or rafts, organized parallel to their long axis to maximize cell–cell contact, colonizing the entire surface available. The migration front is preceded by a visible layer of slime-like extracellular material, which gives the colony a glistening effect [54]. As a consequence of this embedding in a matrix of extracellular polymeric material, the population densities are obviously extremely high in these surface-attached communities [58]. Several lines of evidence support the fact that differentiation into the swarmer cell state is coupled to the expression of certain virulence factors [57,59].

The glycolipid or lipopeptide biosurfactants such as rhamnolipid (*Pseudomonas*), surfactin (*Bacillus*), and serrawettin (*Serratia*) (see Sections 2.1.1 and 2.1.2) function as wetting agents by reducing the surface tension, as illustrated in Fig. 3 [60]. Mutants deficient in biosurfactant production are unable to spread over the solid surface. Toguchhi et al. [61] suggested that the LPS O-antigen directly or indirectly improves the surface wettabilty required for swarm colony expansion in *Salmonella enterica* Serovar Typhimurium. The rescue of

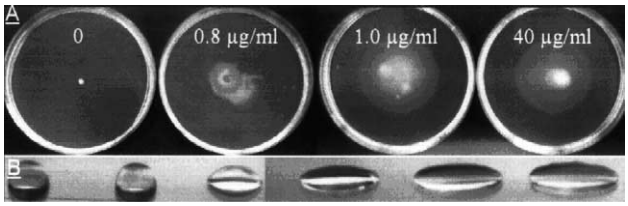

Fig. 3. (A) Swarming of the *S. liquefaciens swrI* mutant, deficient in serrawettin W2, on plates containing increasing amounts of serrawettin W2 (0, 0.8, 1 and 40 μg/ml). Production of serrawettin W2 by SwrA is regulated by the SwrIR quorum sensing system. (B) Effect of serrawettin W2 on the surface tension of water. The droplets contain 0, 0.5, 0.8, 1, 2 and 40 μg/ml serrawettin (taken from [72]).

LPS mutants with surfactin is consistent with this hypothesis. Furthermore, a role for the LPS O-antigen in *P. mirabilis* and *S. marcescens* swarming [62,63] and for a capsular polysaccharide (CPS) in enhancing medium surface fluidity during *P. mirabilis* population migration and in influencing cell–cell interactions, was previously reported [64]. Although mutagenesis of the core-LPS biosynthesis gene in *P. mirabilis* and *S. marcescens* showed a clear reduction in O-antigen LPS molecules, the investigators could not exclude that the inner-core change observed in the mutants could also play a role in swarming [63].

At least for *Proteus*, the swarmer cells, located at the front of the migrating colony, are separated from the vegetative cells in the centre, which are important for growth and cell division. The *Proteus* swarming colony shows terraces as a result of the differentiation to swarmer cells and dedifferentiation to vegetative cells (also known as consolidation). Within a *Serratia liquefaciens* colony, the bacteria at the perimeter differentiate into swarmer cells. Formation of a surface-conditioning film on media of intermediate hardness by bacteria in the region behind this swarm region, results in a circulation between subcultures of swarm and vegetative cells, continuously creating new zones of growth [65–67]. Consistent with this, vegetative cells, also called breeders, may play the dominant role in secreting serrawettin. Non-differentiating *flhDC* mutant strains have recently been found to express *swrA* mainly in the swarmer band [67]. Recently, Tolker-Nielsen et al. [66] showed that the *flhDC* expression and mRNA levels are not increased in *S. liquefaciens* swarmer cells in contrast to the increased level in *Proteus* (30-fold increase in mRNA level; 50 times more flagella). *Serratia* cells elongate and the average swarmer cell carries many more flagella but these cells are strictly speaking not hyperflagellated, probably due to a posttranscriptional regulation of *flhDC* [66].

2. Quorum sensing-regulated surface migration

Quorum sensing systems are almost always integrated into other regulatory circuits. This effectively expands the range of environmental signals that influence target

gene expression, such as those for biosurfactant production, beyond population density. In Sections 2.1 and 2.2 we discuss the regulation of AHL-mediated and other quorum sensing systems involved in biosurfactant production in different bacteria. Later on, regulation of swarmer cell differentiation will be described (Section 3). Because both interspecies signalling and interference with quorum sensing systems can affect swarming, this topic is summarized in Section 4. Although production of *B. subtilis* surfactin, a bacterial cyclic lipopeptide, is regulated as a function of population density by the ComX pheromone [54], quorum sensing in gram-positive bacteria will not be discussed here.

2.1. LuxI/LuxR-mediated swarming migration

2.1.1. Serrawettin production by the genus Serratia

Serratia species include strains which are opportunistic pathogens colonizing a wide variety of surfaces in water, soil, plants, insects, fishes, and humans [68]. *S. liquefaciens* is generally motile, by means of peritrichous flagella. The formation of a swarming colony in the case of the non-pigmented *S. liquefaciens* MG1, was shown to involve two genetic switches (Fig. 4). The first involves the *flhDC* master operon, which regulates the expression of the flagellar regulon and governs control over swarmer cell differentiation [69]. The second encodes a quorum sensing control mechanism and will be described here [70,71].

According to a two-dimensional PAGE analysis, at least 28 genes are under the control of the *swrIR* quorum sensing system in *S. liquefaciens* [71]. Production of serrawettin (Fig. 5), a lipodepsipentapeptide biosurfactant, by SwrA, a multidomain enzyme complex, is quorum sensing regulated. s*wrI*, a *luxI* homologous gene, encodes an AHL synthase and *swrR* encodes a LuxR-type transcriptional activator. In an *swrI* mutant, the formation of a swarming colony is abolished but can be restored by the addition of exogenous AHLs. As seen

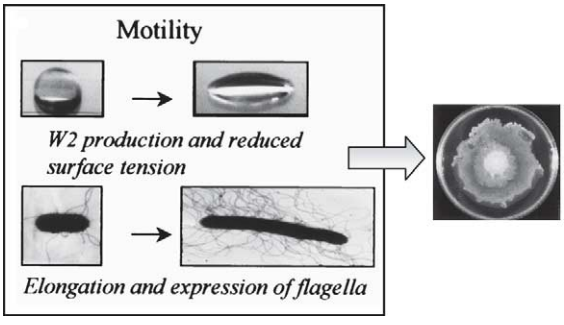

Fig. 4. Activation of the signalling system (quorum sensing) and the flagellar master operon (surface sensing) in *S. liquefaciens*, results in serrawettin W2 production and swarmer cell differentiation (elongated and hyperflagellated cells). These biological processes, combined with an active metabolism (bacterial growth) lead to colony expansion (taken from [75]).

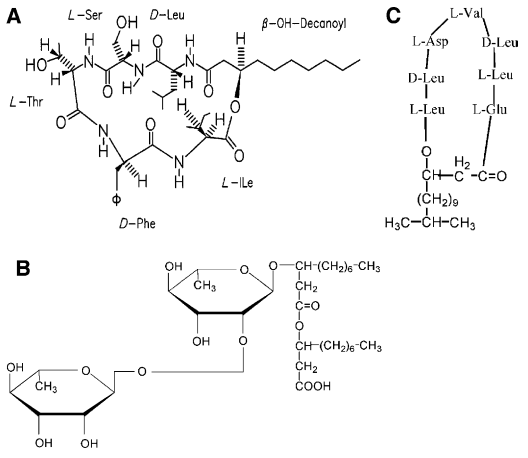

Fig. 5. Molecular structure of serrawettin W2 produced by *S. liquefaciens* (taken from [72]) (A), rhamnolipid of *P. aeruginosa* (B), and surfactin of *B. subtilis* (C) (taken from [213]).

in Fig. 3, media supplemented with purified serrawettin W2, allows the *swrI* mutant to travel across the agar surface [72]. One has to realize that inactivation of *swrI* neither affects growth rate or swimming motility, nor the development of hyperflagellation and cell elongation [73,74]. SwrI produces C_4-HSL and a lesser amount of C_6-HSL (10:1) [73]. At high population density, the AHL concentration reaches a threshold above which the active SwrR/C_4-HSL complex activates transcription of target genes such as *swrA*, which results in biosurfactant production essential for swarming [75]. Recently, it was observed that a *S. liquefaciens estA* esterase mutant produces greatly reduced amounts of AHLs when Tween was used as a carbon source [76]. When cells are grown on lipidic substrates such as Tween, the enzymatic action of the outer membrane esterase EstA will provide the cell with fatty acids. As a consequence, the cellular pool of charged acyl-ACPs may be replenished, which otherwise may be the bottleneck for AHL synthesis under these conditions. Unexpectedly, the *estA* mutant was still able to develop a swarming colony on medium containing Tween 20 as the carbon source. These results can be explained by the fact that Tween 20 is a detergent that lowers the surface tension of the medium [76]. Whether EstA is also involved in AHL biosynthesis in *S. liquefaciens* under more natural life conditions, e.g. during colonization of plant roots, has yet to be investigated.

Bacterial swarming was also described in *S. marcescens* [77] and a similar requirement of biosurfactants for this surface motility has been demonstrated [49]. Synthesis of AHLs, presumably by the product of *smaI* [78], a LuxI homologue in *S. marcescens*, is inhibited by multiple copies of the RNA binding protein *rsmA* (repressor of secondary metabolites; see Section 2.1.2.3.4). Furthermore, overexpression reduces biosurfactant production [79]. Overexpression of *rsmA* in

S. marcescens inhibits swarming without influencing swimming or swarmer cell differentiation. SpnR, the LuxR-type protein in the pigmented isolate of *S. marcescens* SS-1 is a negative regulator of a biosurfactant, which facilitates surface translocation. SpnI directs the synthesis of two major (3O,C_6-HSL and C_6-HSL) and two minor AHLs [50]. SpnR is de-repressed by 3-oxo-C_6-HSL and the non-cognate 3O,C_8-HSL. In addition, long chain AHLs antagonize the biosurfactant-mediated surface translocation of this bacterium as does a protein SpnT. Further analysis revealed that *S. marcescens* SS-1 is unable to produce flagella and as a consequence does not swim or swarm [50]. This type of quorum sensing-regulated flagella-independent surface translocation corresponds to sliding motility.

2.1.2. Rhamnolipid synthesis by the genus Pseudomonas

2.1.2.1. Introduction: the las *and* rhl *systems.* *P. aeruginosa* is a gram-negative bacterium that contains a single polar flagellum and several type IV pili [80], living in soil and aqueous environments. Furthermore, it is a typical opportunistic pathogen that colonizes the lungs of cystic fibrosis patients and causes infections in immunocompromised hosts (reviewed in [81,82]). This bacterium is discussed here because it regulates rhamno-lipid biosurfactant production essential for swarming via the quorum sensing network.

P. aeruginosa possesses two well-characterized cell-to-cell signalling systems, *las* and *rhl*, which contain the LasR [83] and RhlR [84] transcriptional regulators, and their cognate AHL synthases, LasI and RhlI. LasI synthesizes 3O,C_{12}-HSL together with small amounts of 3O,C_8-HSL [85–87] and RhlI synthesizes C_4-HSL and C_6-HSL (15:1) [88,89]. Using a novel detection method, also 3O,C_{14}-HSL previously unreported for *P. aeruginosa* and 3O,C_{10}-HSL were detected in a biofilm [90]. The two quorum sensing systems are hierarchically arranged (Fig. 6), with the *las* system being on top of the signalling cascade [91]. LasR positively regulates genes controlled by the *las* quorum sensing system, including *rhlR* and *rhlI*. Recently, a regulatory region was identified upstream of *rhlI*. Expression studies revealed that this regulatory region is important for *rhlI* expression and although the *rhl* quorum sensing system will induce *rhlI*, the *las* system is the dominant one [92]. Quorum sensing in *P. aeruginosa* controls the expression of a number of extracellular virulence factors (e.g. toxins, elastases, proteases), and secondary metabolites such as rhamnolipids (Figs. 5 and 7).

Although both *rhl* and *las* regulatory systems are required for the production of elastase, the RhlI-dependent

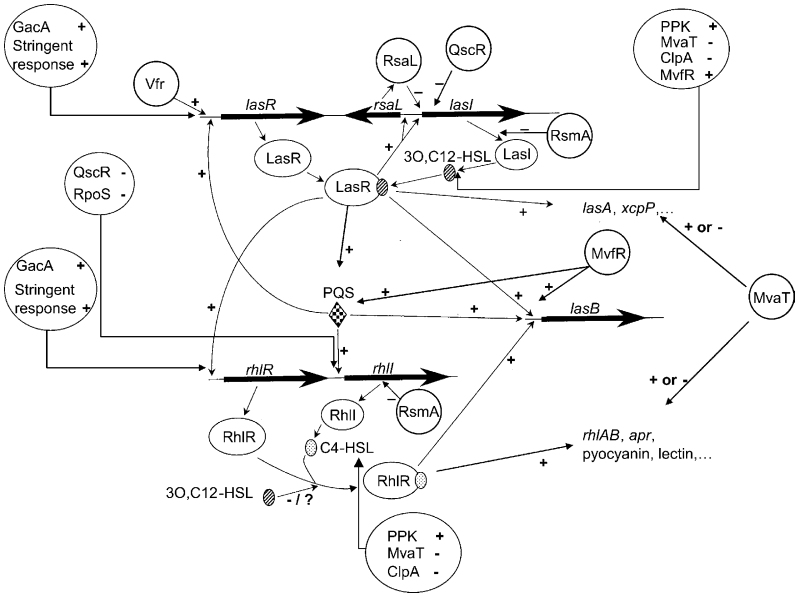

Fig. 6. Hierarchical quorum sensing control in *P. aeruginosa* (adapted scheme [29,107]). The quorum sensing cascade begins with the induction of the *las* quorum sensing system when cells reach a threshold density. Vfr induces *lasR*, and the concentration of 3O,C_{12}-HSL, synthesized by LasI, increases to the point where it binds to and activates LasR. The LasR/3O,C_{12}-HSL complex induces genes controlled by the *las* system, including a negative regulator gene *rsaL*, *rhlR*, and unidentified genes required for PQS (2-heptyl-3-hydroxy-4-quinolone) production. PQS induces *rhlI*, leading to the production of C_4-HSL that binds to and activates RhlR. The RhlR/C_4-HSL complex can then induce genes controlled by the *rhl* quorum sensing system. PQS induces *lasB*. Other regulators such as GacA (+), QscR (−), MvfR (+), RpoS (−), PPK (+), MvaT (−), ClpA (−) regulate quorum sensing-dependent components. RsmA posttranscriptionally regulates *lasI* and *rhlI*. The stringent response induces quorum sensing prematurely, independent of population density. Furthermore, MvaT controls target gene expression both positively and negatively. Genes and proteins are indicated by thick arrows and unfilled circles, respectively. Plus and minus symbols (at the end of the arrow) indicate transcriptional activation or repression of the gene(s), respectively. Blocking of the association between RhlR and C_4-HSL by 3O,C_{12}-HSL observed in *Escherichia coli* but not in *P. aeruginosa* is indicated by a minus/question mark symbol next to the arrow between 3O,C_{12}-HSL and C_4-HSL at the bottom of the figure.

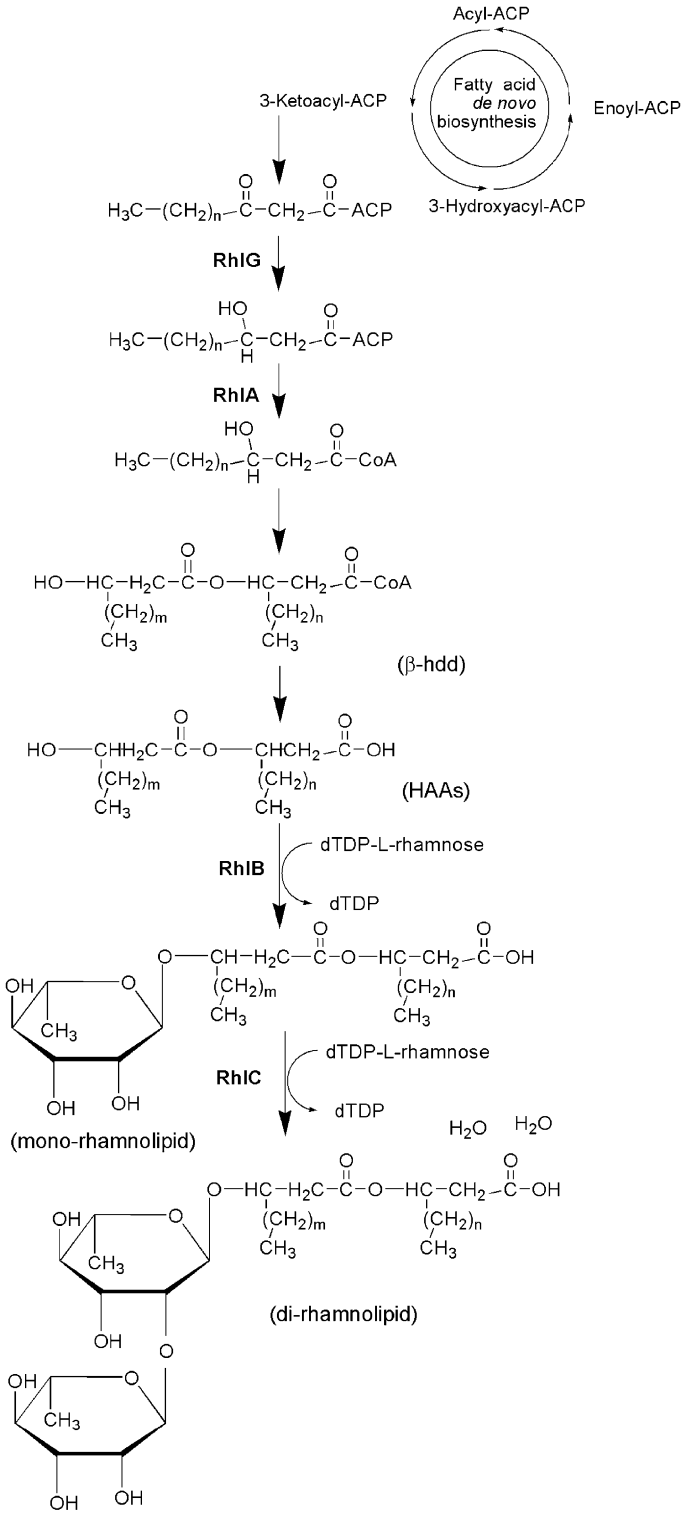

Fig. 7. Schematic representation of the fatty acid biosynthetic pathway showing the predicted roles for the RhlG protein, RhlA and the RhlB and RhlC rhamnosyltransferases in the production of HAA and rhamnolipids in *P. aeruginosa* (adapted from [45,100]). The rhamnolipid production starts with a specific ketoacyl reduction step catalyzed by RhlG. dTDP-L-rhamnose, thymidine-diphospho-L-rhamnose; β-hdd, β-hydroxydecanoyl-β-hydroxydecanoate; CoA, coenzyme A; ACP, acyl carrier protein; HAA, 3-(3-hydroxyalkanoyloxy)alkanoic acid; n, m = 4, 6 and 8.

C_4-HSL does not bind to LasR to form an active complex [93,94]. Cross-regulation between RhlR and LasR regulators, present in multiple copies, was obtained together with their cognate autoinducers. These effects were much less than the activation of *rhlA* by the *rhl* system and *lasB* by the *las* system [94]. Using a heterologous

host, a posttranslational regulation of the *rhl* via the *las* quorum sensing system was demonstrated [95]. C_4-HSL and 3O,C_{12}-HSL compete for the RhlR binding in *E. coli*. However, the increased expression of some target genes [19,96] in the presence of both AHLs in a homologous genetic background, supported the idea that 3O,C_{12}-HSL does not function as a posttranslational regulator of the RhlR/C_4-HSL system [97]. Transcriptome analysis performed by two different research groups suggested that the final set of quorum-regulated genes represents about 6% of the genome. Schuster et al. [17] found 315 induced and 38 repressed overlapping genes in 2 independent types of analysis and Wagner et al. [21] found 394 and 222 such genes, respectively. The most overrepresented categories consist of genes involved in the production of secreted products, in the adaptation and protection categories and in the central intermediary metabolism categories. Also quorum-repressed genes were identified such as those involved in carbohydrate utilization or nutrient transport. These genes are activated only in the mutants during the late logarithmic and stationary phases [17].

2.1.2.2. Regulation and role of rhlAB *and* rhlC. Swarming in *P. aeruginosa* is induced on semisolid surfaces (0.5–0.7% agar). Cells isolated from the swarm edge as well as from the centre possess two polar flagella. Evidence for rhamnolipids being a biosurfactant involved in swarming motility was given [98]. Rhamnolipids are produced as a complex mixture of congeners containing one or two 3-hydroxy fatty acids of various length, linked to a mono- or dirhamnose moiety. In general, the two more abundant rhamnolipids are L-rhamnosyl-beta-hydroxydecanoyl-beta-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-beta-hydroxydecanoyl-beta-hydroxydecanoate [45]. The *rhlAB* operon, an *rhlIR* -mediated target gene, catalyzes the synthesis of mono-rhamnolipid (L-rhamnosyl beta-hydroxydecanoyl-beta-hydroxydecanoate) from dTDP-L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) moieties of various lengths (Fig. 7) [93]. Recently, evidence was presented indicating that *rhlA* is required for production of HAAs, the actual precursors of rhamnolipid biosynthesis and that these HAAs also display potent surface-active properties [45]. Based on its homology, RhlA could potentially be an acyltransferase catalyzing the transfer of the 3-hydroxyacyl moiety from the ACP thioester to CoA. HAAs would result from the condensation of two of these 3-hydroxy-CoA residues. RhlB was hypothesized to be the catalytic subunit of the rhamnosyltransferase 1 that is anchored in the inner membrane and to have a preference for longer chain and saturated HAAs [45,93]. It was observed that swarming requires the expression of *rhlA* but does not necessitate rhamnolipid production, as HAAs act as surfactants [45]. A recent study suggested that RhlC is an inner membrane-bound rhamnosyltransferase that produces di-rhamnolipid from mono-rhamnolipid and dTDP-L-rhamnose (Fig. 7) [99]. Some of the mono-rhamnolipid is secreted directly, whereas a portion is transformed by RhlC and then secreted into the extracellular environment. The synthetic pathway for the fatty acid moiety of HAAs and rhamnolipids is not linked with the general fatty acid synthetic pathway, starting with a specific ketoacyl reduction step catalyzed by the RhlG protein, a FabG homologue. Production of C_4-HSL is not affected by *rhlG*, encoding an NADPH-dependent beta-ketoacyl-ACP reductase (Fig. 7) [100]. The *rhlR* mutant does not swarm and compared with PAO1 wild type, the *lasR* mutant exhibits a reduced swarming behaviour due to the hierarchical organization of two quorum sensing circuits in *P. aeruginosa* [97,98]. The production of wetting agents involved in *P. aeruginosa* swarming is mainly controlled by the *rhl* quorum sensing system, which activates the transcription of both *rhlAB* and *rhlC* when the C_4-HSL concentration reaches a threshold [93,99]. This implies that reducing the C_4-HSL concentration affects swarming. The presence of a *lux* box in the *rhlG* promoter region and the fact that direct involvement of LasR in the regulation was ruled out, suggest that *rhlG* is transcriptionally regulated by RhlR [100]. The *las* system is capable of mildly activating *rhlA*, and similarly, the *rhl* system partly activates elastolysis through *lasB*, a virulence gene mainly induced by the LasR/3O,C_{12}-HSL complex [94]. The strongly reduced biosurfactant production in a specific genetic background (PAO-B1) [94] is mainly due to the previously unknown NfxC phenotype, characterized by overexpression of the MexEF–OprN efflux system (see Section 2.1.2.3) [101]. Overproduction of this MexEF–OprN multidrug resistance efflux pump is correlated with a decrease in C_4-HSL concentration. The *nfxC* mutants produce lower levels of extracellular virulence factors, controlled by the *las* and *rhl* quorum sensing systems in *P. aeruginosa*.

Unlike all other swarming bacteria, *P. aeruginosa* was initially thought to require type IV pili for this type of motility in addition to flagella [98]. It seemed likely that the type IV pili assist the flagella in surface propagation. Alternatively, the pili may be involved in sensing the viscosity of the surface and sensing a signal for initiation of swarming. The inability of the *rhl* mutant to swarm was initially ascribed to both a reduced rhamnolipid production and a decreased surface piliation while the synthesis of the pili per se is not affected. This observation appeared to have been a consequence of a secondary mutation in a key regulator affecting a variety of phenotypes (discussed below). In line with this, Rashid and Kornberg [102] reported that a *pilA* mutant is not affected in swarming.

2.1.2.3. Superregulation of P. aeruginosa *quorum sensing*. Although more regulators such as Vfr (required for a

basal level of *lasR* expression) [103], and RsaL (repression of *lasI*) [104] have been described in literature (Fig. 6), only those affecting the *rhl* quorum sensing system and C_4-HSL production and/or rhamnolipids or HAA will be discussed in this section as putative regulators of the *P. aeruginosa* swarming behaviour. An overview of these regulators is given in Fig. 6.

2.1.2.3.1. The Pseudomonas *quinolone signal (PQS).* *P. aeruginosa* produces another signal molecule, 2-heptyl-3-hydroxy-4-quinolone, which is designated as the PQS derived from anthranilate, an intermediate in the tryptophan biosynthetic pathway [105,106]. This molecule belongs to the 4-quinolone family, which is best known for antibiotic activity. It was reported that PQS is produced maximally when cultures reach the late stationary phase of growth, long after the *las* and *rhl* systems have been activated [107]. Recently, the direct analysis of culture supernatants with LC/MS revealed that PQS is produced essentially during the early stationary phase of growth [108]. The assay used by the former researchers is not directly reflecting PQS concentration and possibly, the ethyl acetate extract to be tested for PQS contained additional compounds. Finally, strain differences and different growth media might also contribute to explain the contradictory conclusions about the timing of PQS production. Moreover, the bulk of the PQS produced is mostly associated with the surface of the cells [108]. The genes required for PQS synthesis include a cluster in the *phnAB* region: PhnA and PhnB (previously associated with phenazine biosynthesis) presumably synthesize the anthranilate precursor from chorismate while PqsA may be involved in activating anthranilate for PQS synthesis. Furthermore, *pqsB*, *pqsC*, *pqsD*, and *pqsH* (final step addition of hydroxyl group) additionally play a role in PQS synthesis. Another gene, *pqsE*, may participate in the cellular response to PQS [109,110]. Although the *pqsH* homologous *pqsL* gene could encode an enzyme that also acts on PQS, its exact function is not yet clear [110]. *pqsR* encodes a member of the LysR family of transcriptional regulators. Furthermore, PqsR corresponds to MvfR of strain PA14 [109] and plays an essential role in PQS biosynthesis and perhaps signalling [109,110]. PqsR is required for the expression of *phnAB* [111] and also regulates the *pqsABCDE* genes [109]. In a previous study, MvfR (multiple virulence factor regulator) was identified as a novel LysR-type membrane-associated quorum sensing transcriptional factor that positively regulates 3O,C_{12}-HSL and/or PQS synthesis (the assay did not distinguish between these two substances) [111]. In the stationary phase, a unique negative feedback mechanism is activated to signal the downregulation of the MvfR protein. The signal for cleavage of MvfR is secreted and the production is controlled by MvfR itself [111].

Neither *lasI* nor *rhlI* synthase genes are responsible for synthesis of PQS, which depends on LasR [105]. Transcription of *pqsH*, a gene required for PQS synthesis, was severely reduced in the *lasR* mutant background [109,112]. Furthermore, it was shown that the *phnAB* operon is subject to quorum sensing regulation [17,21,112]. In addition, the microarray data obtained by Hentzer et al. [112] showed that the entire *pqs* operon is controlled by the *las* system. Interestingly, a *las*-dependent upregulation of *mvfR* expression precedes AHL-induced expression of the *pqs* operon [112]. PQS controls expression of *lasB* [105] and causes a major induction of an *rhlI lacZ* fusion. Increased expression of *rhlI* leads to the production of C_4-HSL [107]. PQS acts as a link between the *las* and *rhl* quorum sensing systems by transcriptionally regulating *rhlI* and is probably not involved in sensing population density (Fig. 6) [107]. A different study indicated that loss of PQS biosynthesis and signalling does not prevent *rhlI* transcription [109]. In the areas of highest cell density in PQS-overproducing strains autolysis occurs during surface growth. As the band of peripheral cells spread outward form the central lysed area, the centre of the band itself developed plaque-like holes, which coalesced, forming concentric zones of lysis as this process repeated [110]. Moreover, autolysis is completely suppressed in the PQS biosynthesis mutants (*pqsABCD* and *pqsR*). Although a *pqsL* mutant, deficient in the *P. aeruginosa* monooxygenase, showed a pronounced lysis due to overexpression of PQS, the link between PQS and the monooxygenase is not yet clear (e.g. PQS degradation or modification) [110].

Recently, the MexEF–OprN efflux system was proposed to affect intracellular PQS levels through the transport of PQS by this pump or through the efflux of a precursor required for PQS biosynthesis [101]. Overexpression of the MexEF–OprN efflux system decreases the transcription of *rhlI* and as a consequence, C_4-HSL production decreases. Furthermore, overexpression of the pump negatively regulates the transcription of *rhlAB* resulting in lower levels of wetting agents. In this case, expression of *lasR* and *rhlR* is not affected. A study by Hentzer et al. [112] revealed upregulation of the *mexEF* genes by a synthetic furanone, known as an antagonist of bacterial quorum sensing (see below). The *nfxC* mutant, overproducing the MexEF–OprN efflux system, is unable to swarm [101]. Furthermore, the MexEF–OprN efflux pump may contribute to the secretion of the hydrophobic 3O,C_{12}-HSL [101] as was previously shown for the MexAB–OprM efflux system [28]. *P. aeruginosa* is known for its ability to develop resistance to a number of structurally unrelated antibiotics. This phenomenon can be attributed predominantly to chromosomal mutations leading to overexpression of multidrug efflux systems. These strains are likely to be less virulent because the reduced levels of the quorum sensing signal molecules (PQS, 3O,C_{12}-HSL and C_4-HSL) decrease the transcription of quorum sensing-regulated virulence genes.

2.1.2.3.2. The GacS/GacA and AlgR2 global regulators. The global regulator, GacA, was shown to activate, directly or indirectly, the expression of *rhlR*, and hence modulates *rhlI* expression and production of C_4-HSL and the *rhl* controlled phenotypes [113,114]. The environmental signals for the GacS/GacA two-component system are at present unknown [20]. However, in the same organism, the post-transcriptional control by GacA of the genes involved in the production of extracellular products, such as hydrogen cyanide, also follows an AHL-independent signal transduction pathway involving the ribosome-binding site (see CsrA-type RNA binding proteins) [114–116].

Pseudomonas syringae, a causal agent of bacterial brown spot on beans, swarms with a characteristic dendritic pattern on semisolid (0.4%) agar plates. In this bacterium, a direct link between this global regulator and swarming was observed. Mutations in either *gacS* or *gacA* eliminate swarming without obvious effects on motility [117]. Although a *P. syringae* AHL synthase mutant, *ahlI*, still swarms, the ethyl acetate extract of the wild-type strain appeared to weakly restore the initiation of swarming in *gacS* and *gacA* mutants, known to be deficient in AHL production [117].

The *P. aeruginosa* global regulator AlgR2 (AlgQ) was originally identified as a regulatory protein in alginate production. Recently, Ledgham et al. [118] demonstrated for the first time that AlgR2 (AlgQ) negatively modulates the expression of the two QS regulatory genes *lasR* and *rhlR* by directly binding to the respective promoters in the mucoid strain. This observation is consistent with the observed downregulation of rhamnolipid biosurfactant synthesis. Apart from the effect on both quorum sensing systems, the global regulator AlgR2 (AlgQ) in the mucoid *P. aeruginosa* strain modulates the level of ppGpp and polyphosphate (see stringent response) [118]. The global effect of the AlgR2 mutation on rhamnolipid synthesis might thus represent actions at more than one level.

2.1.2.3.3. Growth phase-dependent superregulation (via RpoS, MvaT, ClpA). In *V. fischeri* and *Erwinia carotovora*, quorum sensing controlled phenotypes can be induced prematurely by addition of their cognate AHL signal molecule. In *P. aeruginosa*, a number of genes (so called class II and class IV genes) were identified whose expression is enhanced but not advanced by addition of AHLs [96]. The same was found for the expression of the RhlR/C_4-HSL-dependent lectin gene, *lecA*, and *rhlR* in *P. aeruginosa* [19,97]. Recently, a number of genes were identified that modulate the timing of quorum sensing controlled processes in *P. aeruginosa*. In most cases, these gene products serve to prevent the early activation of quorum sensing [119]. Quorum sensing regulation of virulence gene expression is linked with the growth phase and the metabolic state of the cell. A high AHL concentration on its own is insufficient to advance gene expression [97].

A study by Latifi et al. [91] indicated that transcription of the stationary phase sigma factor (*rpoS*) is controlled by RhlR/C_4-HSL. The microarray analysis of *P. aeruginosa* quorum sensing regulons agreed with a quorum sensing promotion of this gene [21]. Quorum sensing regulation of RpoS was recently questioned after transcriptional analysis of a chromosomally *rpoS* promoter fusion [120]. In this latter study, this sigma factor was reported to negatively regulate *rhlI* transcription, and C_4-HSL synthesis in early logarithmic phase. RpoS was suggested to repress all early C_4-HSL-regulated genes [120]. This observation is in line with the observed stimulation by PQS in the late stationary phase of growth (see the PQS). In addition, a more detailed analysis revealed that the stationary phase sigma factor is required for swarming in *P. aeruginosa* [97].

The first systematic screening for quorum sensing superregulators in *P. aeruginosa* revealed that like the *rpoS* mutant, the *mvaT* and *clpA* mutants, all produce high levels of both C_4-HSL and 3O,C_{12}-HSL compared with the wild-type PAO1 and are affected in multiple quorum sensing phenotypes, suggesting that these genes influence the quorum sensing circuit to some extent [97]. MvaT is a novel global regulator of the expression of some virulence genes as a mutation in *mvaT* results in an enhanced *lecA* expression (a lectin structural gene) and pyocyanin production. Addition of exogenously added AHLs to the mutant, in contrast to the wild type, significantly advances expression, suggesting that MvaT is involved in growth phase-dependent regulation [97]. MvaT is homologous to the heterodimeric transcriptional regulator of the initial reactions of the mevalonate catabolism in *Pseudomonas mevalonii*. ClpA forms, together with ClpP, a protease involved in the degradation of misfolded proteins in *E. coli*. How inactivation of *clpA* influences quorum sensing-regulated phenotypes in *P. aeruginosa*, needs to be established. Such a growth phase-dependent superregulation occurs at least at two levels: control of the quorum sensing cascade itself and control of the target gene expression [97]. It is important to note that although AHL levels are increased in an *mvaT* mutant, some quorum sensing-dependent phenotypes are downregulated. Compared with PAO1 wild type, the *mvaT* mutant, and the *clpA* mutant exhibit reduced swarming behaviour as observed for the *lasR* mutant [97]. As indicated before, the *rhlR* mutant does not swarm at all.

2.1.2.3.4. Regulation and role of the RNA binding protein RsmA. The global RNA binding protein RsmA (repressor of secondary metabolites) exerts a negative effect on the production of AHLs controlled by *las* and *rhl* in *P. aeruginosa*. This was confirmed by translational fusions of both synthase genes. The data highlighted the temporal expression control of *lasI*, and *rhlI* but to a

lesser extent [121]. From the regulation of both LasI- and RhlI-mediated AHL production one may suggest a hypothetical role for RsmA on quorum sensing-regulated phenotypes such as swarming. Unfortunately, the possible regulatory effect of this RNA binding protein on swarming was not yet studied.

RsmA's mode of action and its complex regulation were comprehensively studied in bacteria different from *P. aeruginosa*. The RsmA homologous protein in *E. coli*, CsrA (carbon storage regulator), binds to target mRNA in a region surrounding the ribosome binding site, controls access to this site and alters mRNA stability [121]. In *E. coli*, the regulatory activity of CsrA is modulated by an untranslated RNA *csrB*, which binds to about 20 CsrA molecules, titrating the available concentration of free CsrA and preventing mRNA decay [122]. Whereas the RsmA/CsrA proteins are well conserved in different bacteria, such sequence conservation is not observed for the antagonistic regulatory RNAs [115]. An additional level of control on the CsrA-type RNA binding protein is exerted by the GacS/GacA superregulatory system in *Pseudomonas fluorescens* CHAO. In this strain, which does not produce AHLs, a CsrA homologue, RsmZ, was identified. GacA up-regulates the expression of regulatory RNAs such as RsmZ, in response to a non-AHL bacterial signal in *P. fluorescens*. These regulators may relieve translational repression of target mRNAs by RsmA towards the end of exponential growth [116]. The non-AHL signal is produced under GacS/GacA control and requires a functional GacS/GacA system to exert its positive effect on the secondary metabolism [115]. Clearly, a number of regulatory elements are still missing. Presently, the possible regulation of the *P. aeruginosa* RsmA, a quorum superregulator in this bacterium, has not yet been unraveled.

2.1.2.3.5. The third LuxR-type protein in P. aeruginosa. The completed *P. aeruginosa* genome-sequencing project revealed a gene encoding for a homologue of the signal transducers, LasR and RhlR, that was called quorum sensing-control repressor *qscR* [123]. The authors suggested that QscR negatively regulates all quorum sensing controlled genes by repressing transcription of *lasI* in the early logarithmic phase of growth although direct expression of swarming-related genes such as *rhlAB* was not tested. The *qscR* mutant produces the 3O,C_{12}-HSL and C_4-HSL signal molecules prematurely when compared with the wild-type strain. Furthermore, the LasI-generated signal is synthesized earlier than the RhlI-generated AHL. In addition, the *qscR* mutant advances transcription of quorum sensing-regulated genes such as *rhlI*, *hcnA* (hydrogen cyanide structural gene) and *phzA* (phenazine structural gene) [123].

2.1.2.3.6. The stringent response. One important phenomenon during nutrient starvation is the stringent response, which results in inhibition of stable RNA synthesis. The effector of the stringent response is ppGpp, synthesized by RelA after ribosome binding of uncharged tRNA. Furthermore, during the stringent response, the cellular levels of inorganic polyphosphate increase (see below) [124].

Overexpression of *relA* elicits the stringent response under constant nutritional abundance, thereby minimally disturbing the cellular physiology. The global effect on quorum sensing is positive: both AHL production and *lasR* and *rhlR* expression are prematurely activated [124]. The stringent response might be able to activate quorum sensing independently of population density. Furthermore, overexpression of *relA* activates the expression of the stationary phase sigma factor *rpoS* in *P. aeruginosa* [124]. This is not in accordance with the discussed negative effect of RpoS on the *rhl* quorum sensing system (see above) [97]. *rpoS* mutant analysis demonstrated that the sigma factor is not required for the premature stimulation of quorum sensing during *relA* overexpression [124].

During the stringent response also the level of inorganic polyphosphate increases. The polyphosphate kinase (*ppk*) gene, encoding PPK, is responsible for the synthesis of inorganic polyphosphate (poly P), a linear polymer of hundreds of orthophosphates, from ATP. The most significant function in *E. coli* is its regulatory role in adapting to nutritional stringencies and environmental stresses, and for survival in the stationary phase of growth [102]. Both 3O,C_{12}-HSL and C_4-HSL levels are reduced in the *ppk* mutant. Furthermore, production of quorum sensing controlled virulence factors, such as rhamnolipids, is severely reduced and *rhlA lacZ* expression is decreased in the *ppk* mutant [125]. These data suggest that PPK and/or poly P affects the synthesis of AHLs and probably also the formation of AHL complexes with cognate regulatory proteins. Alternatively, the *ppk* mutation may affect the transcriptional activation of downstream target genes. In addition to this AHL-mediated effect on swarming, the flagella-driven surface movement of the *ppk* mutant is determined by its ability to swim. The *ppk* mutant is moderately defective in flagella-mediated swimming, despite possessing an apparently normal flagellum and, in addition, is defective in flagella-dependent swarming [102]. The *ppk* mutant swarmer cells are neither elongated, nor hyperflagellated when compared with the wild type [102]. In *E. coli*, the chemotaxis signal transduction system is essential for swarming. Poly P might substitute for ATP in CheY phosphorylation or phospho-PPK might directly transfer phosphate to some CheY-like proteins [126]. Poly P might also interfere with the cellular Ca^{2+} level to affect the activity of CheY-like proteins or might act directly on the flagellar motor [127].

2.1.3. Biosurfactant production in Burkholderia cepacia

B. cepacia has been recognized as an important pathogen in patients with cystic fibrosis. Infection often occurs in patients, already colonized with *P. aeruginosa*. It was demonstrated that CepR and CepI, homologues of LuxR and LuxI, respectively, mediate C_8-HSL and as a minor product C_6-HSL synthesis in *B. cepacia* [128,129]. Three higher-level regulators of the *cep* quorum sensing system, *yciR*, *suhB* and *yciL* are postulated to influence the level of AHLs via post-transcriptional control of *cepR* expression, or by affecting the activity status of the receptor protein [130]. YciR contains a GGDEF motif and was therefore thought to be a member of a signal transduction system [131]. The SuhB protein in *E. coli* possesses inositol monophosphatase activity and may have a role in mRNA decay [132,133]. The third higher-level regulator YciL is homologous to pseudouridine synthases [134]. The corresponding mutant in *P. aeruginosa* is defective in several parameters related to osmotic stress [135]. Fifty-five proteins out of 985 detected spots were differentially expressed in the *cepI* mutant (5% of the proteome was downregulated and 1% upregulated) [136]. It was shown that *B. cepacia* displays swarming motility and biofilm formation. Evidence was provided that swarming motility is quorum sensing-regulated via *cep* in *B. cepacia*, possibly through the control of biosurfactant production [58]. Swimming behaviour of the *cep* mutants and the wild type is indistinguishable. Biofilm maturation by *B. cepacia* requires a functional *cep* quorum sensing system [130]. Complementation of the *cepIR* quorum sensing mutants with surfactin and serrawettin W2 biosurfactants restores swarming. Moreover, the exogenous supply of these biosurfactants does not significantly increase biofilm formation. This result suggested that swarming motility per se is not essential for the later steps in biofilm formation required developing a typical three-dimensional biofilm structure in this bacterium (see Section 5.1) [58].

2.2. Other types of quorum sensing-regulated movement over a solid surface

The most intensive studied group of quorum sensing molecules in gram-negative bacteria is the class of *luxIR*-mediated AHLs. Recently, other quorum sensing signal molecules such as AI-2, produced by *Vibrio harveyi*, have also been isolated from a number of other bacteria. In literature, a link between the AI-2-mediated quorum sensing system and swarming was found for *P. mirabilis* and *Vibrio parahaemolyticus*.

2.2.1. Capsular polysaccharide synthesis in Proteus mirabilis

P. mirabilis is a pathogenic gram-negative bacterium that frequently causes kidney infections, established by ascending colonization of the urinary tract. Swarmer cell formation and movement are stimulated by peptides and amino acids resulting from extensive proteolysis with broad spectrum proteases [54,137].

The acidic capsular polysaccharide produced by *P. mirabilis* plays a key role in swarming motility by enhancing medium surface fluidity (Fig. 8). Mutants lacking this polysaccharide are inhibited in their migration [138]. Extracellular signals might be sensed by two-component regulators such as RcsC–RcsB [54]. In *E. coli*, YojN, carrying a histidine-containing phosphotransmitter domain (Hpt), serves as a link between RcsC and RcsB that is involved in acid capsular polysaccharide synthesis and swarming [139]. In *P. mirabilis*, a *yojN* homologous gene, named *rsbA*, is involved in swarming [140,141]. Consistent with the effect on mRNA stability in *E. carotovora* and *S. marcescens*, a plasmid copy of *rsmA* caused suppression of *P. mirabilis* swarming motility and differentiation probably by promoting mRNA degradation. In contrast, the swimming ability was not affected [142]. It is possible that RsbA may regulate swarming by modulating the expression of the *rsmA*/*rsmB* system. Takeda et al. [139] reported a previously unrecognized Hpt domain in RsbA, in contrast to the study of Belas [140] (see below), and suggested an analogous RcsC–RsbA (YojN)-RcsB phosphorelay in *P. mirabilis* swarming. The lag period prior to *P. mirabilis* swarming depends on the density of cells in the preswarming colony. Mutations in *rsbA* result in a reduced lag period. Belas et al. [140] characterized RsbA as a membrane sensor protein with homology to *V. harveyi* LuxQ. In *V. harveyi*, LuxQ acts as a sensor for AI-2 in conjunction with the activated (via AI-2) AI-2 receptor protein LuxP [143]. To illustrate the AI-2-mediated quorum sensing, the regulation of bioluminescence in *V. harveyi* is described shortly. Besides the production of an AHL, *V. harveyi* produces also AI-2 via LuxS, an enzyme involved in the methyl cycle of some gram-positive and gram-negative bacteria (Fig. 9) [144]. In SAM-dependent methyltransferase reactions, *S*-adenosylhomocysteine (SAH) is formed. AI-2 producing bacteria convert SAH in two steps, catalyzed by methylthioadenosine/SAH nucleosidase (MTA/SAHase, also known as Pfs) and *S*-ribosylhomo-cysteine (RH) cleavage enzyme, also known as LuxS [144]. Recently, the structure for AI-2 was predicted to be a cyclic borate

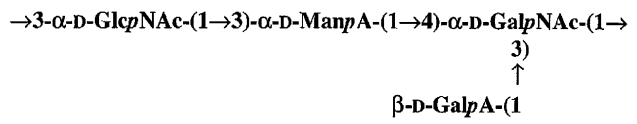

Fig. 8. The structure of a colony migration factor from *P. mirabilis*, composed of a tetrasaccharide repeating unit (taken from [64]). GlcNAc, *N* -acetylglucose; Man, mannose; Gal, galactose; GalNAc, *N*-acetylgalactose.

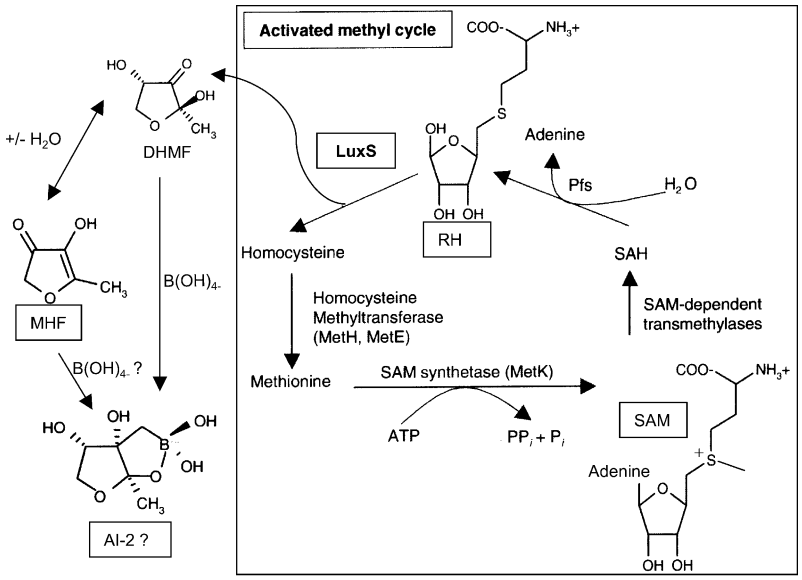

Fig. 9. AI-2, LuxS and the activated methyl cycle (adapted from [146,151]). All eukaryotes and Archaea, as well as some eubacteria, hydrolyze *S*-adenosylhomocysteine (SAH) to homocysteine and adenosine, using the enzyme SAH hydrolase (not shown). Other eubacteria, such as *E. coli*, convert SAH in two steps catalyzed by methylthioadenosine/SAH nucleosidase (MTA/SAHase, also known as Pfs) and *S*-ribosylhomocysteine (RH) cleavage enzyme. First SAH is hydrolyzed to RH and adenine. RH is then converted by the RH cleavage enzyme (LuxS) to homocysteine and 4,5-dihydroxy-2,3-pentanedione. The 4,5-dihydroxy-2,3-pentanedione formed by the action of LuxS on RH is considered to cyclize spontaneously to give a furanone (probably DHMF; 2,4-dihydroxy-2-methyl-3(2H)furanone). The formation of AI-2 and 4-hydroxy-5-methyl-3(2*H*)furanone (MHF) from DHMF is indicated. SAM, *S*-adenosylmethionine.

diester [145]. It is also possible that the borate compound and 4-hydroxy-5-methyl-3(2*H*)furanone (MHF) are interconvertible [146]. Quorum sensing signal transduction in *V. harveyi* of both AHL (AI-1) and AI-2 occurs via two parallel two-component proteins of the hybrid-sensor class. Both quorum sensing circuits channel phosphate to a shared signal integrator protein (LuxU), which transfers the signal to LuxO [147]. LuxO acts negatively to control the *lux* operon [148,149]. At high population densities, LuxO is unphosphorylated and inactive. This then allows the LuxR transcriptional activator (not homologous to *V. fischeri* LuxR) to bind the *lux* promoter and activate transcription of the bioluminescence genes [149].

Recent analysis showed that AI-2 activity in *P. mirabilis* is expressed during and correlates with the initiation of swarming migration on agar surfaces. The peak in AI-2 activity corresponds to the time at which the cells start swarming migration. This observation suggested that AI-2 plays a role in orchestrating this behaviour [150]. However, a mutation in *luxS* does not affect swimming or swarming motility, or swarmer cell differentiation [150]. This discrepancy in results is linked with the view of some researchers that AI-2 is a toxic metabolic compound rather than a quorum sensing signal molecule per se. Many of the quorum sensing systems described so far may turn out to be nonspecific. The signals may be common metabolites or even toxic metabolic end products. The question arises as to whether they are really communication systems in the strict sense [39]. Winzer et al. [151] proposed an alternative explanation for the extracellular accumulation of AI-2. The possibility arose that AI-2 has toxic properties, and is therefore excreted. Cells may minimize this loss of a four-carbon unit through controlled uptake and degradation of AI-2 at a later stage of growth [151]. Temporarily released metabolites and toxic compounds are often mistaken for cell-to-cell signal molecules.

2.2.2. *OpaR, the* V. harveyi *LuxR-homologue, negatively regulates swarming in* Vibrio parahaemolyticus

The nonluminescent *V. parahaemolyticus* BB22 produces two quorum sensing signal molecules, an AHL-like molecule and AI-2, which are capable of stimulating the dual system found in *V. harveyi* inducing luminescence [152–154]. *V. parahaemolyticus* swarms over the agar surface, concomitant with the production of lateral flagella, when compared with the typical single, sheated, polar flagellum during growth in liquid medium [155]. In addition to the swimmer-swarmer cell dimorphism, *V. parahaemolyticus* exhibits another kind of phenotypic switching, described as the opaque-translucent variation in colony morphology. It was postulated that differences in colony structure or packing result in differential light transmission [156]. The *opaR* gene, encoding a transcriptional regulatory protein homologous to LuxR of *V. harveyi*, controls opacity [154] and is involved in capsular polysaccharide production [157]. The *opaR*

expression is regulated by the particular state of the DNA: the gene is expressed in opaque strains but not in translucent strains [154]. An opaque colony of cells expressing *opaR* exhibits little or no movement across the surface on swarm plates. An *opaR* mutation in an opaque strain converts it into a translucent colony type, coinciding with the gain of swarming ability [154]. Furthermore, a translucent colony of cells without *opaR* expression was able to swarm over a surface. A similar *opaR* mutation in this translucent strain does not affect its swarming ability. Although no swarming-regulating signal molecules have been identified yet, it seems clear that the *V. harveyi* LuxR homologue OpaR negatively regulates swarming in *V. parahaemolyticus* [154].

3. Quorum sensing regulation of swarmer cell differentiation

Often, the same flagellar apparatus is utilized by the *Enterobacteriaceae* and *Bacillus* species for both swimming and swarming motilities [158]. Although *V. parahaemolyticus* senses viscosity with his polar flagellum, it still requires flagella assembly for the production of lateral flagella, which are needed for swarming of this bacterium [155]. The process of flagella-driven surface colonization requires that the bacteria sense the increased surface viscosity and as a consequence start swarmer cell differentiation. The *flhDC* operon encodes a regulator whose concentration or activity status determines whether cells swim or swarm [69]. The differentiated hyperflagellated and elongated swarmer cells can only migrate across the solid surface when also a surface wetting agent is produced. Quorum sensing regulation of this latter part of the process was discussed in part 2. The *flhDC* operon itself is subject to control by several regulatory circuits that are responsive to changes in environmental and nutritional conditions. The complexity of the assembly of the flagellar apparatus is well known but is not fully covered in this review. One particular form of superregulation, the quorum sensing-dependent regulation of the flagellar master operon, will be discussed in this part.

Harshey and Matsuyama [159] described swarming in *E. coli*: a few hours after inoculation of the bacterium onto the centre of an agar plate, streams of cells move outward, colonizing the entire surface within the next few hours. Enterohaemorrhagic *E. coli* (EHEC) colonizes the large intestine, where the bacterial flora is present in high population density, and produces a bacterial quorum signalling molecule, originally supposed to be AI-2, via the product of the *luxS* gene. It was demonstrated by Sperandio et al. [160] that the LuxS enzyme is involved in the synthesis of yet another autoinducer (AI-3) in this bacterium. Firstly, a 'quorum sensing *E. coli* regulator' of the LysR family, QseA, involved in the activation of the LEE genes (type III secretion system) was identified [161]. Recently, the 'quorum sensing *E. coli* regulators' *qseBC*, encoding a response regulator and a sensor kinase, respectively, were characterized. Sperandio et al. [162] showed that this two-component system is a positive regulator of the master regulatory operon *flhDC* in *E. coli* thereby regulating flagella expression (Fig. 10). Study of a *qseB* gene fusion supposed a quorum regulation and activation via the *luxS*/AI-2 quorum sensing system after addition of preconditioned medium (culture supernatants from the *luxS* mutant which does not contain AI-2 failed to activate transcription) [162]. However, the fraction containing AI-2 activity does not activate the quorum sensing regulated genes in EHEC. In contrast, another autoinducer in this extract, AI-3, was not able to induce luminescence in *V. harveyi* but activates transcription of *qseBC* [160]. It was demonstrated that AI-3 is the actual signal activating transcription of both the LEE and flagella genes. Furthermore, the mammalian endocrine hormone Epi (epinephrine) can substitute for AI-3 [160]. Transcription of *flhD*, *fliA*, *motA*, and *fliC* fusions is decreased in the *qseC* sensor kinase mutant [162]. In addition, a *qseC* mutant is unable to respond to both AI-3 and Epi to restore motility [160]. Given these data, it was hypothesized that both AI-3 and Epi are recognized by the same receptor, which is probably in the outer membrane of the bacteria because of the nonpolar nature of both signals. These signals might be imported to the periplasmic space where they interact most probably with QseC and other sensor kinases [160].

However, Winzer et al. [151] highlighted the metabolic function for the LuxS protein: LuxS fulfils a function in the methyl cycle (Fig. 9). Furthermore, conditioned medium prepared from the wild type and the *luxS* mutant, is very likely to differ not only with

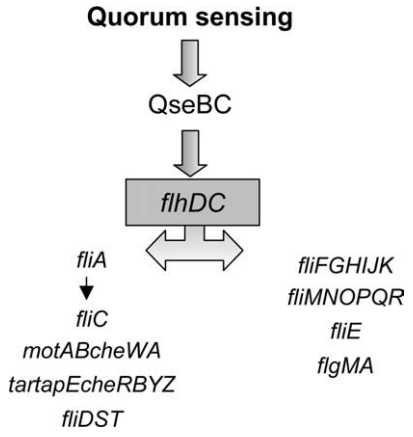

Fig. 10. Model of quorum sensing regulation in Enterohaemorrhagic *E. coli* (EHEC) cells. Quorum sensing activates transcription of *qseBC*, which in turn activates transcription of the flagella regulon (adapted from [162]).

regard to signal molecules as AI-2 but also in many other aspects [151]. Further study is required to determine whether AI-2 fulfils all the requirements for a cell-to-cell signal molecule in *E. coli* as it does in *V. harveyi*.

4. Interspecies signalling and interference with quorum sensing-mediated swarming

Different signal molecules either produced by bacteria (such as other AHLs and diketopiperazines) or excreted by plants (such as furanones) might influence the quorum sensing-regulated swarming behaviour in other bacteria different from the producer. On the other hand, several unrelated bacterial genera belonging to the α-Proteobacteria [163], the β-Proteobacteria [34,35,164], the γ-Proteobacteria [34], the low-G + C Gram-positive bacteria [165] and the high-G + C Gram-positive bacteria [34] have been demonstrated to reduce the local available concentration of signal molecules by enzymatic degradation of the AHLs produced by others. Recently, the efficacy of using a wild-type soil bacterium *Bacillus* species A24 with AHL-degrading capability for the biocontrol of plant diseases has been demonstrated [166]. *Bacillus* sp. strain A24 is able to degrade AHLs produced by plant pathogenic *E. carotovora* and *A. tumefaciens*, and exhibits broad-spectrum activity by significantly reducing diseases of potato and tomato caused by these phytopathogenic bacteria. In line with these results, wild-type *Rhodococcus erythropolis* degrading AHLs markedly reduces the pathogenicity of the plant pathogen *E. carotovora* in potato tubers [34].

4.1. Mixed swarming colony

Complementation of the AHL-deficient *S. liquefaciens swrI* mutant in a binary swarming colony, demonstrated that exogenous AHLs trigger biosurfactant synthesis in the population of AHL-deficient cells [167]. Such a swarming culture can be formed between *Serratia ficaria* and *S. liquefaciens* MG44, the *swrI* mutant, but also among more distantly related species such as *P. aeruginosa* and the *swrI* mutant of *S. liquefaciens* [75]. The appearance of bright green *swrI* cells harboring a plasmid-borne AHL monitoring system in which expression of *gfp* is controlled by LuxR, is indicative of interspecies communication [167].

4.2. Diketopiperazines

Diketopiperazines (DKPs) (Fig. 1), originally extracted from cell-free *P. aeruginosa*, *P. mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* supernatants, have high biological and pharmacological effects on cells of higher organisms [168], suggesting their role in communication with plant and animal cells rather than with other bacteria. DKPs activate some AHL-biosensors. To obtain this induction, often a much higher concentration from these non-AHLs is required when compared with natural AHLs [169]. Cyclo (L-Pro-L-Met) produced by *E. coli* stimulates the swarming motility of the *swrI* mutant as effective as C_4-HSL [75]. In contrast with this, DPKs such as cyclo (L-Pro-L-Tyr) antagonize the quorum sensing regulated swarming of *S. liquefaciens* at a significantly lower concentration than those required to induce an *E. coli* AHL-biosensor [169].

4.3. Furanones

It has been demonstrated that several exogenously added halogenated furanones (Fig. 1) with structural similarity to short-chain AHLs, isolated from the marine algae *Delisea pulchra*, negatively regulate swarming in *S. liquefaciens* [74]. The transcription of the quorum sensing-regulated gene *swrA* in *S. liquefaciens* is decreased in the presence of halogenated furanones. This in turn results in a reduced production of the surface-active compound serrawettin W2, which is crucial for surface translocation of the differentiated swarmer cells [74,170]. The presence of non-fluorescent wild-type *S. liquefaciens* cells, containing a plasmid-borne *luxR* based *luxI gfp* promoter fusion [167], after addition of algal metabolites, indicates that halogenated furanones shut down the intercellular communication [170]. The *D. pulchra* furanones do not influence *S. liquefaciens* flagellar synthesis, cell elongation or growth rate [74]. The concentrations used to inhibit swarming are well within the range of concentrations presented at the surface of the plant [171]. The inhibitory effect exerted by these metabolites is not limited to *S. liquefaciens*, that does not encounter *D. pulchra* naturally, but swarming of several marine bacterial isolates is also inhibited by furanones [74]. Preliminary work with marine algae also yielded novel compounds that appear to interfere with AHL based systems [172]. Recently, it has been suggested that oxidized halogens may interfere with 3-oxo-AHLs. Experiments with the marine alga *Laminaria digitata* demonstrated that natural haloperoxidase systems are capable of mediating the deactivation of AHLs [173].

When looked into more detail at the quorum sensing shut down-mechanism, halogenated furanones were found to have activity in an in vivo ligand-binding assay to monitor displacement of AHLs from the LuxR protein [174]. A recent study suggested that the reduction in *V. fischeri* LuxR stability is the mechanism by which furanones control expression of AHL-dependent phenotypes [175]. This observation rejects the previous model that furanones compete with AHLs for a common binding site on LuxR homologues [174]. Whilst a stable interaction between the algal metabolite and the

V. fischeri LuxR was not found, it was noted that the half-life of the protein is reduced up to 100-fold in the presence of furanones [175]. Once degradation of LuxR is initiated, it is not reversible by addition of AHLs. However, prior addition of the AHL offers some protection [175]. Studies revealed that a synthetic, modified furanone specifically targets *P. aeruginosa* quorum sensing systems [112,176]. Comparative analysis of this furanone's target genes and the quorum sensing regulon shows that 80% of the furanone-repressed genes are also quorum controlled. The furanone-repressed genes include the *lasB* gene, *lasA*, the *rhlAB* operon for rhamnolipid production and *phnAB* involved in PQS synthesis [112,176]. Among the activated genes is the MexEF multidrug efflux transporter that may result in decreased PQS, C_4-HSL and 3O,C_{12}-HSL levels. Transcription of the *lasIR* and *rhlIR* quorum sensing genes was not significantly affected by the furanone suggesting a regulation at the post-transcriptional level. However, there are indications that the furanone represses genes correlated with acyl-ACPs, the proposed acyl donors for synthesis of AHLs [112]. Although repression of *rhlAB* was clear, the effect of the synthetic furanone on swarming was not yet studied. Uncoordinated swarming without normal consolidation of the opportunistic human pathogen *P. mirabilis* was seen when crude extract of *D. pulchra* was added to the medium. Microscopic inspections revealed that *P. mirabilis* swarmer cell formation is not affected by the *D. pulchra* crude extract whilst close cell contact is abolished [177]. Only one of the four major halogenated furanones from *D. pulchra* inhibits swarming motility of *P. mirabilis* [177]. Other structurally similar furanones had no effect, suggesting considerable specificity in the effects on swarming motility by *P. mirabilis.* A synthetic furanone was also found to inhibit swarming in *E. coli* without affecting growth rate or swimming motility [178]. In addition, this furanone reduce the AI-2 activity in *E. coli* (screened with the *V. harveyi* reporter) [178]. The same synthetic furanone influences the growth rate and inhibits swarming of *B. subtilis*. Once swarming is initiated, the swarm colony becomes more resistant to the halogenated furanone [179]. According to Kjelleberg, furanone analogues interfering with AI-2-dependent quorum sensing have yet been identified in a number of gram-positive and gram-negative bacteria; efforts are underway to learn which components of that pathway are being affected [180].

4.4. Non-AHL plant compounds

Recently, several varieties of pea and a number of other higher plants were reported to confuse bacterial invaders. The concentration of putative AHL-mimicking compounds at the plant surface may be high enough to affect AHL-regulated gene expression in bacteria in natural encounters [181]. For instance, by stimulating swarming, the plant may prevent bacteria from concentrating in sufficient numbers to attach the host successfully [182]. Although the chemical nature of the active mimicking compounds is currently unknown, it appears that the substances with AHL-mimicking signal activity are chemically different from bacterial AHLs [181]. The effect of these AHL-signal mimics on *S. liquefaciens* swarming was analyzed. Firstly, it was shown that *S. liquefaciens* swarming is strongly affected by substances secreted by pea seedlings. Furthermore, a methanol extract of these seedling exudates strongly stimulates swarming of the *swrI* mutant, unable to make its own AHLs. As a control, pea seedlings do not stimulate the *swrA* mutant, deficient in its own serrawettin biosurfactant production, indicating that pea does not secrete a biosurfactant capable of stimulating swarming but rather a signal molecule [181].

In addition to the early observations with pea exudates, various species of higher plants, including rice, soybean, tomato, crown vetch, and *M. truncatula*, secrete AHL-mimicking activities inducing swarming in *S. liquefaciens*. Neither lettuce, nor *Arabidopsis thaliana* stimulate activity in the tested reporter strains [181]. In addition, AHL inhibitory activities are particularly strong in extracts from a number of fruits, including grape and strawberry [183]. Preliminary results indicate that purified fractions of *M. truncatula* could stimulate LasR. Although the same fractions significantly affect biofilm initiation and the involvement of swarming in attachment is well known (see further), their effect on swarming is not yet known [184]. The synthesis of AHL-signal mimics is not constitutive: little activity was found in pea seedlings less than 4 days old. Bauer and Teplitski [182] speculated that secretion of AHL-mimicking compounds might be inducible by microorganisms. In a recent publication, the authors demonstrate that the secretion of particular AHLs by a bacterium may lead, in turn, to the secretion of different amounts or kinds of signal-mimicking compounds by the host [38]. They also provide evidence that the legume plant *M. truncatula* secretes compounds that affect AI-2-dependent quorum sensing in bacteria [184]. Moreover, the profile of stimulatory activity seen with an AHL biosensor was almost a mirror image of the inhibitory activities seen with the AI-2 reporter suggesting that both reporters may be responding in opposite ways to the same set of plant compounds [38]. Chemical identification of signal molecules present in the plant fractions with stimulatory and inhibitory activity is clearly needed to understand their activity towards both the AHLs and AI-2 reporter systems.

4.5. Enzymatic degradation of AHLs

Acyl homoserine lactonase activity (AiiA) that hydrolyzes the lactone ring of AHLs, has been demon-

strated for the first time in a *Bacillus* soil isolate [185–187]. Screening a large collection of rhizosphere bacteria for interference with the quorum sensing system of *P. aeruginosa* identified two *Bacillus* spp. with AHL-degrading activity. This activity is encoded in both isolates by a single *aiiA* gene. Apparently, effective intracellular degradation of the diffusible signal molecules C_4-HSL and C_6-HSL produced by *P. aeruginosa*, reduces the local signal concentration [187]. Strains of *B. thuringiensis* and the closely related species *B. cereus* and *B. mycoides* produce also AHL-inactivating enzymes [165,188]. A similar enzymatic activity controls signal turnover in *A. tumefaciens* [163]. Recently, the consequences of such a degradation on the infection process of plant pathogens were studied: transgenic plants expressing such an AHL-lactonase showed significantly enhanced resistance to *E. carotovora* infection [183,186]. Expression of the *Bacillus aiiA* gene in *P. aeruginosa* completely prevents the accumulation of C_4-HSL. Furthermore, expression of a translational *rhlA* gene fusion is severely reduced. There is a decreased production of rhamnolipids (and probably also HAA) and as a result, a strongly reduced swarming [187]. However, no effect was observed on flagellar swimming or on twitching motility [187].

Apart from the above described lactonase, an aminoacylase capable of inactivating AHLs has been described in *V. paradoxus* [35] and recently also in *Ralstonia* [164]. The enzyme, designated AiiD, hydrolyzes the AHL amide, releasing HSL and the corresponding fatty acid. Heterologous expression of *aiiD* in *P. aeruginosa* quenches quorum sensing, significantly reducing its ability to swarm [164]. Contradictory observations were obtained for commercial preparations of porcine kidney acylase [164,189]. Although this eukaryotic enzyme has been reported to transform AHLs into the corresponding homoserines with opened ring structure at pH above 9, no data are available for lower pH values [189]. However, under such alkaline conditions non-enzymatic degradation of the AHLs into homoserines has also been demonstrated [190–192].

5. Swarming in the real world

Swarmer cells are exclusively located in the perimeter of the growing colony where their activity creates a thin, motile biofilm in advance of the growing cell mass. The biomass of the colony increases and ultimately the population colonizes the available surface. Firstly, the role for and regulation of a surface-associated movement (swarming and twitching) in biofilm formation is discussed (Section 5.1). This section also describes how stimulation of swarming disperses existing biofilms. Swarming was believed to have important consequences for the interaction between bacteria and higher organisms [170]. Moreover, the requirement for swarming in invasion of the host was suggested. The particular role for this surface movement in the bacterial-plant interaction is mentioned in Section 5.2.

5.1. Dual role for surface-associated movement in biofilms

The motility requirement and the involvement of quorum sensing in biofilm formation were analyzed. Studies in *V. cholerae*, *E. coli* and *P. aeruginosa*, show that the formation of a mature biofilm proceeds through an ordered series of steps. The present model for *P. aeruginosa* biofilm formation (5-steps) is in accordance with the earlier described 3-steps model (reviewed in [48,193,194]), and contains the following stages: reversible attachment, irreversible attachment, maturation 1 and 2, and dispersion [195]. Biofilm cells were shown to change the regulation of motility and the quorum sensing status during the process of development. Sauer et al. [195,196] showed the importance of motility in *P. aeruginosa* biofilm formation and in the dispersion stage. When planktonic cells were compared with cells in the last step of biofilm maturation, more than 800 *P. aeruginosa* proteins (over 50% of the proteins on the SDS page) were shown to have a 6-fold or greater change in expression level [195]. Furthermore, 3O,C_{12}-HSL accumulates in a *P. aeruginosa* biofilm to a 45-fold higher concentration as compared to the planktonic phase [90].

Possibly, the flagellum plays a direct role as an adhesin. Moreover, for *P. aeruginosa*, *V. cholerae*, and *E. coli*, flagella-mediated motility is believed to overcome repulsive forces at the surface of the substratum and as a consequence, a monolayer of cells forms on the abiotic surface. Once the initial contact is established, cells are thought to move over the surface, aggregate and then form microcolonies that are dispersed throughout the monolayer of cells [193,197]. Subtractive hybridization indicated differential expression of pili and flagella genes following adhesion of *Pseudomonas putida* to a surface, suggesting a surface-regulated switch from flagellum-based motility (swimming) to swarming or twitching (type IV pili) motility. In contrast to the *pil* genes (required for twitching in *P. putida*), genes involved in flagellum production were found to be downregulated following initial adhesion [196]. Apart from this, no *rhl gfp* dependent fluorescence was observed in *P. aeruginosa* during the initiation of biofilm development [198].

After this first stage, a period of irreversible attachment was observed. Now *P. aeruginosa* cell clusters commence their development. Motility ceases in the attached cells and at this stage, the *las* quorum sensing system becomes active [195].

Once the cell clusters become progressively layered, the *P. aeruginosa rhl* quorum sensing system becomes active (maturation 1). Finally, the microcolonies

differentiate to become a mature biofilm (maturation 2) [195]. Detailed analysis revealed that biofilms are open, highly hydrated structures consisting of cells embedded in an extracellular matrix filled with large void spaces. These void spaces or channels allow fluid to flow throughout the biofilm (nutrients, oxygen, metabolic end products) [199].

Quorum sensing-regulated biofilm maturation was shown for the *P. aeruginosa las* system [195,200], and recently for *P. putida* [201], *B. cepacia* (*cepI*; [58]), *Aeromonas hydrophila* (*ahyI*; [202]) and the gram-positive *Streptococcus mutans* [203] and *Staphylococcus* species [204,205]. Remarkably, *P. aeruginosa* residing as biofilms in the lung is undergoing anaerobic metabolism. Here, the *rhl* system is required for optimal anaerobic biofilm viability by regulation of NO reductase preventing metabolic NO suicide [206,207]. Transcriptome analysis of the quorum regulons supported the observation that quorum sensing plays a pivotal role in the anaerobic growth of *P. aeruginosa* [21]. In addition to AHLs, also LuxS-dependent intercellular communication controls structured biofilm development [208]. Neither form of quorum sensing-regulated biofilm maturation will be discussed in this review. Noteworthy, biofilm formation is multifactorial and complex. Hence, differentiated biofilms may be the net result of many independent interactions, rather than being determined by a particular global quorum sensing system [199,209].

In addition to swarming, *P. aeruginosa* is also able to move across the solid surfaces by twitching, a process necessary to form multicellular aggregates in static *P. aeruginosa* biofilms [197,210]. This movement is the consequence of the extension and retraction of type IV pili. Initially, both *las* and *rhl* quorum sensing systems were thought to control twitching motility in *P. aeruginosa* [211]. Today, several arguments can be cited to reject this hypothesis. Recently, twitching-defective variants were found to accumulate during culturing of *lasI* and *rhlI* mutants as a consequence of spontaneous secondary mutations in *vfr* and *algR*, respectively, both of which encode key regulators affecting a variety of phenotypes, including twitching motility [187]. These results indicated that mutations in one regulatory system create distortions that select during subsequent culturing for compensatory mutations in other regulatory genes within the cellular network. This problem may have affected some past studies of regulatory hierarchies controlled by quorum sensing and of bacterial regulatory systems in general [212]. Furthermore, Reimmann et al. [187] showed that destruction of C_4-HSL by a lactonase in another *P. aeruginosa* background does not influence twitching. In line with this, recent analysis showed that neither the *las* nor the *rhl* quorum sensing system is activated in initial stages of biofilm development [195]. Taken together, these data indicated that a functional quorum sensing system is not required for twitching in *P. aeruginosa*. The *cep* quorum sensing system, which regulates swarming in *B. cepacia*, is not involved in initial attachment, but rather controls the maturation of the biofilm. Complementation with biosurfactants restores swarming, while biofilm formation is not significantly increased. This suggested that swarming motility per se is not essential for biofilm formation [58].

In contrast to the above-described role for surface motility in the initial stage of biofilm formation, swarming can also disperse a biofilm. Various surface-active compounds or biosurfactants have the capacity of regulating the attachment and detachment of bacteria to and from surfaces [213]. What feature of a biofilm allows adherence in one case and expansion in another? At least one difference between adherent and moving biofilms may lie in the surfactant composition of the slime, since the absence of biosurfactants such as serrawettin or LPS, inhibits swarming of *S. marcescens* and *S. enterica*, but promotes biofilm formation and vice versa [214]. Furthermore, surfactin from *B. subtilis* disperses preformed biofilms without affecting cell growth and prevents biofilm formation by organisms such as *S. enterica*, *E. coli*, and *P. mirabilis*. Biofilms formed by *P. aeruginosa* were not affected by the biosurfactants tested [214]. Recently, a new role for rhamnolipids has been reported. High levels of rhamnolipids can impede the formation of biofilms. This means that rhamnolipids produced in major biofilms may be able to maintain open (non-colonized) channels surrounding macrocolonies by affecting both cell–cell interactions of "self" and also other planktonic microbes and attachment to surfaces [198,215].

5.2. Swarming during bacterium-plant interaction

5.2.1. AHL-mediated swarming in Rhizobium etli

The gram-negative nitrogen-fixing soil bacterium *Rhizobium etli* is the bacterial symbiotic partner of the common bean plant. The symbiosis is characterized by a signal exchange between the rhizobia and the legume [216,217]. Rhizobia in the rhizosphere are chemotactically attracted towards the legume roots, by certain compounds in the root exudates, such as flavonoids, phenolics, sugars, dicarboxylic acids and amino acids. Following chemotaxis, the rhizobia adhere to and colonize the root surface. Certain flavonoid and nonflavonoid compounds in the root exudates induce a specific response in the rhizobia. Together with these compounds, the rhizobial NodD protein activates the nodulation genes [218]. The nodulation genes encode gene products that synthesize and transport a class of molecules called Nod factors (NFs) or lipo-chitin oligosaccharides. NFs induce several responses on the legume root [219]. Thirdly, signal exchange between different rhizobia in the rhizosphere is based on the production of

quorum sensing signal molecules such as AHLs allowing the whole population to initiate a concerted action once a critical concentration has been reached e.g. [43,220–225]. Recently, a new class of quorum sensing molecules involved in symbiotic gene regulation was identified in *Bradyrhizobium japonicum* (Fig. 1M). Population and iron control of bradyoxetin in the nodule can result in increased signal production, and hence elevated NolA and NodD2 expression, and the subsequent repression of the *nod* genes [226].

R. etli CNPAF512 produces at least seven different quorum sensing signal molecules, as detected using the *A. tumefaciens tra* reporter [221] of which some are produced by the *cinIR* quorum sensing system. In our current model, *cinI* codes for the AHL synthase and *cinR* for the transcriptional regulator that binds this AHL. Expression of both genes is regulated as a function of the population density and reaches a maximal expression level in the stationary phase. Expression of *cinI* requires the CinR/CinI-dependent AHL complex. Furthermore, both genes are expressed under symbiotic conditions. Plants nodulated by *cin* mutant strains were shown to be limited in nitrogen fixation capacity, most likely because of arrested bacteroid differentiation [220]. It was recently observed that *R. etli* CNPAF512 swarms and promotes surface colonization of YEM soft agar (0.75%) [227]. The swarming colony neither showed terraces nor a typical pattern such as a dendritic pattern, as described for other bacteria in literature. A glistening film preceding the colony front suggests the production of a surface conditioning film. The *R. etli cinIR* mutants are no longer able to move over this solid surface [227]. In contrast, they form a regular colony at the inoculation point (Fig. 11). Detailed observation revealed that the *cinR* colony edges were smooth, without bacteria escape from the colony (Fig. 11). Microscopy of these mutants never revealed scalloping or finger-like extrusions. Because swarming of the *cin* mutants is restored on plates containing exogenous biosurfactant, the inability of the *cinIR* mutants to swarm is probably caused by a *cin*-dependent regulation of the biosurfactant synthesis. In the future, restoration of the *cinI* mutant for such a biosurfactant production by exogenously added AHLs is required to confirm this hypothesis.

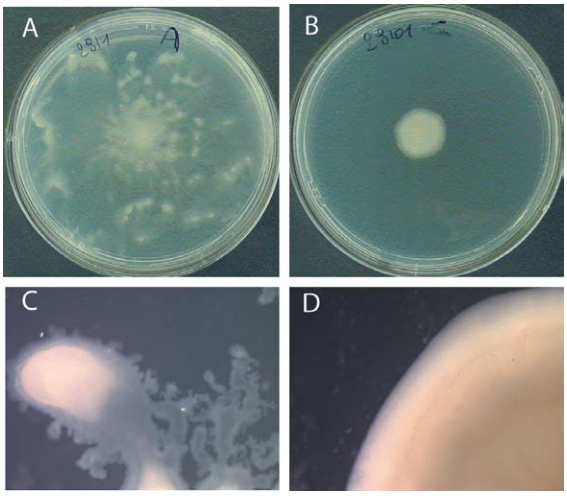

Fig. 11. *Rhizobium etli* swarming and colony morphology. (A, C, respectively) wild-type *R. etli*; (B, D, respectively) *cinR* mutant (FAJ4009).

R. etli is the first member of the *Rhizobiaceae* with a quorum sensing-regulated bacterial swarming behaviour. Recently, multicellular swarming was demonstrated for the *S. meliloti fadD* mutant. Although fatty acid derivatives were suggested to act as intracellular signals controlling motility, no evidence was found that AHLs play a role in the regulation of this bacterium's swarming behaviour [56].

5.2.2. Significance of surface motility during root colonization

It is striking to observe that in bacteria-plant interactions, attachment to plant roots proceeds through a similar mechanism as observed for initiation of biofilm formation. Firstly, mediated by a bacterial adhesin (rhicadhesin for *Rhizobium*), the bacteria adhere loosely as single cells to the plant root surface. In the second attachment step, bacteria become more firmly attached to the plant root, resulting in the formation of large bacterial clusters. Bacterial polysaccharides were found to be responsible for this strong adherence and agglutination through binding with the host lectins [228]. Whether swarming plays a role in the *R. etli* root colonization is not yet known.

The involvement of surface movement in a colonization process has been reported in other bacterial-plant associations. During colonization of the alfalfa rhizosphere, *P. fluorescens* F113 undergoes phenotypic variation, resulting in the appearance of colonies with different morphology. Three phase variants, C, F, and S, were observed and isolated, with the C variant presenting the wild-type phenotype [229]. Two phenotypic variants (F and S) were shown to swim faster than the wild-type C variant and to swarm under conditions that do not allow swarming of the wild type. Flagellin overproduction results in longer flagella, rather than more flagella [229]. Furthermore, they preferentially colonize distal parts of the roots that are not easily reached by the wild-type strain, reflecting specialization in colonizing different parts of the root [229]. Production of the cyclic lipopeptide amphisin, in combination with expression of flagella enables the fluorescent *Pseudomonas* sp. DSS73 to move rapidly over a surface. At present, this bacterium seems not to produce AHLs under the conditions tested [230]. Amphisin is a new member of a group of dual-functioning compounds such as tensin, viscosin, and viscosinamid that display both

biosurfactant and antifungal properties [231]. It is demonstrated that the containment of pathogenic microfungi in the rhizosphere of sugar beet requires amphisin-dependent surface translocation combined with a cocktail of antifungal agents [230].

6. Concluding remarks

Quorum sensing-regulated biosurfactant production has been demonstrated in *S. liquefaciens* [75], *B. subtilis* [232] and possibly in *B. cepacia* [58]. Moreover, in these bacteria the link between the biosurfactant production and swarming has also been shown. The inability of the *P. aeruginosa rhl* mutant to swarm could be the result of a reduced biosurfactant production containing both HAAs and rhamnolipids [45,97,98]. In addition, *R. etli* also displays a quorum sensing-regulated bacterial swarming behaviour. Besides AHL-regulated swarming, a link between AI-2-mediated quorum sensing and swarming was found for *P. mirabilis* and *V. parahaemolyticus* [62,154]. A bacterial signal molecule designated AI-3 regulates swarmer cell differentiation in *E. coli* [160,162]. Indirect evidence for quorum sensing-mediated swarming was obtained by the observation that a number of signal molecule-mimicking compounds such as diketopiperazines [169], halogenated furanones [170], and plant-secreted substances [181,182] can influence different swarming bacteria. Recently, it was demonstrated that plant compounds affect AHL and AI-2 signalling in opposite ways [38]. Apart from the interspecies signalling, degradation of AHLs may influence quorum sensing. Although at present, a rich lexicon of molecules is involved in communication, we expect even more, new molecular structures to be identified in the future controlling the social behaviour described in this review.

While the biochemical mechanisms underlying AHL-mediated quorum sensing have been well studied in culture, the functioning of this signalling mechanism under natural biological conditions is more difficult to assess. Recent studies have demonstrated that communication through the use of AHLs is not limited to recognition among cells of the same species. For example *P. aeruginosa* and *B. cepacia* are capable of forming mixed biofilms in the lungs of cystic fibrosis patients. During the co-infection period a dramatic reduction in the amounts of AHLs produced by the co-residing *P. aeruginosa* isolates was observed [233]. Another example demonstrating interpopulation signalling is the restoration of the AHL-deficient *S. liquefaciens* mutant in a binary swarming colony. The AHLs produced by the co-inoculated bacterium trigger biosurfactant synthesis in the population of AHL-deficient *S. liquefaciens* cells [167]. One must always be careful to extrapolate findings on bacteria grown in laboratory conditions to the in vivo situation [30], where the physiological situation may be different. For example, a study under natural biological conditions revealed anaerobic respiration in *P. aeruginosa* biofilms [206]. The latter condition requires the presence of the *rhl* quorum sensing system for bacterial survival.

From an applied point of view, influencing the swarming behaviour of bacteria may help to control root colonization and containment, and this may have important applications in agriculture. Quorum sensing-mediated swarming control may also have implications on biofilms in industrial and ecological settings (e.g. potable water distribution systems) and in environments more relevant for public health (such as indwelling medical devices, cystic fibrosis, periodontitis) [199] and may provide an alternative therapeutic strategy to combat microbial contamination.

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