Mini-Review

Communication and autoinduction in the species *Listeria monocytogenes*

A central role for the *agr* system

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In order to withstand changes in their environment, bacteria have evolved mechanisms to sense the surrounding environment, integrate these signals and adapt their physiology to thrive under fluctuating conditions. Among these mechanisms, the ability of bacteria to exchange information between cells has become a dynamic field of interest for microbiologists over the past four decades. First described by Nelson et al.,[1] this phenomenon often referred as either cell-cell communication, Quorum Sensing and/or AutoInduction involves the synthesis of small signal molecules called autoinducers. These signal molecules may be sensed by the bacterial population in the vicinity and induce regulation of gene expression. To date, three major communication systems have been described in bacteria. In this mini-review, we discuss the involvement of known communication systems in the transmission of information in the species *Listeria monocytogenes*. We will also discuss the latest findings on the role of communication in the regulation by *Listeria monocytogenes* of major adaptive strategies.

In Gram-negative bacteria, signal molecules belong to the homoserine lactone (HSL) family. The system, elucidated in 1983, involves the synthesis by the enzyme LuxI of the signal molecule that diffuses freely.[2] A cytoplasmic transcriptional regulator LuxR binds the HSL and affects transcription of target genes. In Gram positive bacteria, signal molecules are usually short peptides (linear or cyclic) processed by a transmembrane protein and other proteases.[3] Once secreted, these autoinducers accumulate and interact with a two-component system. The signal is translocated from the outside to a cytoplasmic regulator via a phosphorylation cascade after binding of the autoinducer to the sensor histidine kinase. The phosphorylated regulator affects transcription of target

genes. A third system has been described more recently.[4] The signal molecule, called AutoInducer 2 (AI-2), is a family of furanone derivatives synthesized by LuxS. The AI-2 system has been proposed as an interspecies communication system as AI-2 production has been reported in both Gram negative and Gram-positive bacteria. *Listeria monocytogenes* is the causative agent of listeriosis.[5] Its capacity to withstand stressful conditions, to colonize surfaces and grow as biofilm contribute to its ability to adapt and survive in many habitats.[6]

AI-2 System: The Metabolic Hypothesis

Fifteen years ago, the study of *Vibrio harveyi* a marine bacterium led to the elucidation of a new Quorum Sensing system based on the release of a furanone derivative called Auto Inducer 2 (AI-2).[7] In this system, two enzymes, Pfs and LuxS catalyze the conversion of S-adenosyl homocystein (SAH), a toxic by-product of the Activated Methyl Cycle (AMC), into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). The latter spontaneously rearranges into various cyclic compounds commonly called AI-2 (Fig. 1).

As orthologs of *luxS* and *pfs* of the AI-2 production pathway have been identified in silico in the genome of most γ-, β- and ε-proteobacteria and firmicutes,[7,8] AI-2 has been postulated to be a universal signal involved in bacterial interspecies communication.[9] This is of a special interest in biofilm studies where cell-cell communication is postulated to play a key role. Both genes, *pfs* (*lmo1494*) and *luxS* (*lmo1288*) are present on the genome of *Listeria monocytogenes* (http://genolist.pasteur.fr/ListiList/) and two different studies reported that a *luxS* mutant shows a reduced AI-2 activity and an increased biofilm formation on stainless steel[10] and on glass.[11] Interestingly, complementation of the mutant strain with in vitro synthesized AI-2 has no effect on biofilm formation but the addition of exogenous S-ribosyl homocysteine (SRH) increased the number of attached cells. Moreover, it appears that *L. monocytogenes luxS* mutant, accumulates more SAH and SRH in its culture supernatant than the parental strains does.[10] These results are to be correlated with those obtained on *Neisseria meningitidis pfs* and *luxS* mutants[12] where growth defects are due
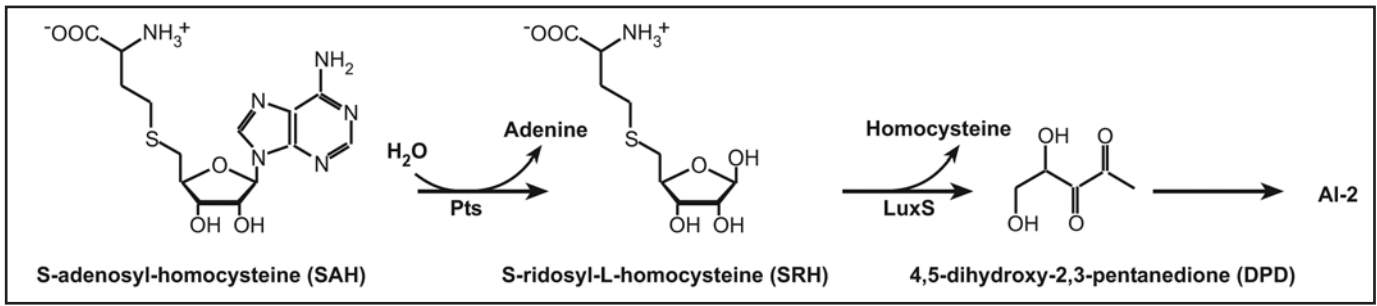
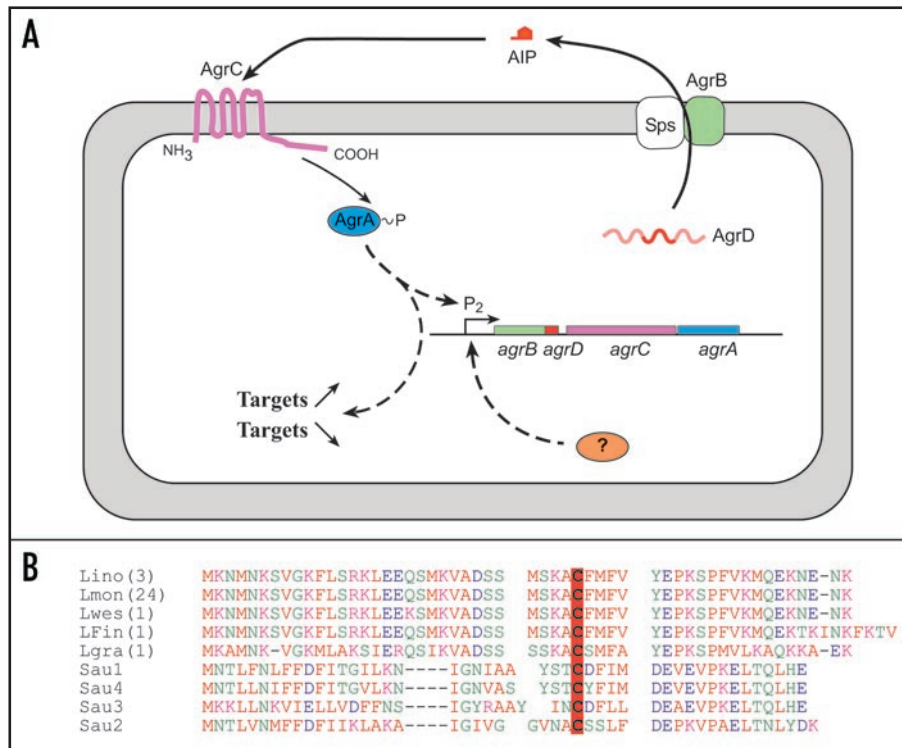Figure 1. Synthesis of AI-2 through the Pfs and LuxS pathway.

Figure 2. The *agr* system of *L. monocytogenes*. (A) Schematic of *agr* autoinduction. AgrB and Sps process AgrD into a secreted cyclic autoinducing peptide (AIP). The detection of AIP by the sensor kinase AgrC induces a phosphorylation cascade resulting in the activation of the regulator AgrA. (B) AgrD sequence alignments of Listeria and the four groups of staphylococcal AgrD. Lino: *Listeria innocua*; Lmon: *Listeria monocytogenes*; Lwes: *Listeria welshimeri*; LFin: *Listeria monocytogenes* Finland; Lgra: *Listeria grayi*. In brackets, number of genomes available online; identical sequences were observed in these genomes. In bold face and red-boxed, the conserved cystein residue critical for the thiolactone ring.

to metabolic imbalance instead of the absence of AI-2 signal and favors the "metabolic hypothesis" to explain such a phenotype. So far, these experimental evidence and the lack of any known receptor for AI-2 in *L. monocytogenes*[13] suggest that AI-2 is not a communication signal in the genus Listeria.

In silico Investigation of Peptide-Based Communication Systems in the Genome of Listeria sp

Two communication systems have been extensively studied in Gram-positive bacteria. These are the development of competence of *Bacillus subtilis* associated with the autoinducer ComX,[14] and the *agr* system first described in *Staphylococcus aureus*.[15]

In silico analysis indicates that orthologs of most of the competence genes of *B. subtilis* are present in the genome of *Listeria monocytogenes* EGD-e (http://genolist.pasteur.fr/ListiList/), but the gene coding for the inducer (*comX*), the transmembrane protein (*comQ*) and the two component system (*comP/comA*) are absent in the genome. Indeed so far, natural genetic transformation of *L. monocytogenes* has not been observed experimentally.[16]

On the opposite, the four genes of the *agr* system are represented in the genome of Listeria sp. These four genes *agrBDCA* (Fig. 2) have a similar organization as the *agrBDCA* operon of Staphylococcus where *agrB* and *agrD* code for a transmembrane protein and the propeptide AgrD. AgrB is involved in the

processing of AgrD into a cyclic autoinducer peptide (AIP) and its exportation. The genes *agrC* and *agrA* code for the sensor kinase and response regulator of a two-component system AgrC/AgrA.

Comparison of the genomes available indicates a high level of conservation of the *agr* genes within the genus Listeria (Fig. 2). Unlike what is observed among isolates of *Staphylococcus aureus* where 4 classes of AIP are evidenced,[17] sequence analysis of Listeria AgrD showed 99% identity in the species *L. monocytogenes*, *L. innocua* and *L. welshimeri*. AgrD is different in the species *L. grayi*. Putative AIPs are identical in *L. monocytogenes*, *L. innocua* and *L. welshimeri* but is different in *L. grayi*; these differences in autoinduction and AIP sensing are in accordance with evolution in the genus Listeria.[18,19] Furthermore, in silico analysis failed to identify putative orthologs of the staphylococcal non-coding RNA regulator RNAIII in the genus Listeria and in ortholog systems of other Gram-positive bacteria. This suggests that, on an evolutionary point of view, RNAIII is an innovation specific to Staphylococcaceae.[20]

agr-Mediated Regulation of Virulence

The first studies on the role of the *agr* system in the regulation of virulence were performed by characterizing the virulence potential of a Tn*1545* insertion[21] and an in frame deletion *agrA* mutant.[22] The authors reported that their respective mutants were not affected in their intracellular infectious cycle assayed in vitro on Caco-2, HepG-2 and Cos-1 cell lines and in macrophages.[21,22] An increase in LD50 was observed with the Tn*1545* insertion *agrA* mutant during infection of Swiss mice,[21] however in-frame deletion of *agrA* did not affect virulence in a BALB/C mice infection assay.[22] These results suggested that AgrA was not directly involved in the regulation of virulence-associated gene expression.

In the latest report on the role of the *agr* system in the virulence of *L. monocytogenes*, the virulence potential of an in-frame *agrD* deletion mutant was investigated.[23] These authors evidenced internalin A-dependent differences invasion when in vitro assays were performed with exponential phase-grown inocula while differences were not significant when inocula were grown to stationary phase, the experimental condition used in the previous studies. Bioluminescent in vivo imaging confirmed a virulence defect of Δ*agrD* mutant when exponentially grown cells were injected intravenously. It is striking to notice how critical is the procedure of inoculum preparation (exponential versus stationary) on the outcome of these in vitro and in vivo virulence assays.

agr-Mediated Biofilm Formation

The growth of Δ*agrA* mutants was similar to the growth of the parental strain at temperatures ranging from 4°C to 43°C,[21,22] in the presence of 9% NaCl and 0.025% H_2O_2 thus demonstrating the absence of pleiotropic effects.[22]

However, in our laboratory we have demonstrated that in-frame deletion of *agrA* affects the ability of *L. monocytogenes* to adhere to glass, polystyrene and stainless steel. For example, adhesion to glass slides was decreased by 62% in comparison to the parental strain EGD-e. Strikingly, a similar phenotype was observed with an *agrD* in-frame deletion mutant.[24] This is indirect evidence that AgrD

is the precursor of an auto-inducing peptide (AIP). However, this AIP has yet to be isolated and sequenced. Nevertheless, the presence of a conserved cystein residue within the sequence of AgrD (Fig. 2) confirms that cyclic auto-inducing peptides can derive from this pro-peptide. This critical conserved cystein residue is observed in AIPs of the ortholog agr systems of *S. aureus*,[25] *Enterococcus faecalis*[26] and *Lactobacillus plantarum*.[27]

Δ*agrA* and Δ*agrD* mutants were also affected during growth as biofilm within the first 24 h of incubation under static conditions in a microtiter plate assay[24] and under dynamic conditions in a flow-cell model of growth.[28] Similar conclusions were reached by other authors.[23]

Spatiotemporal expression of *agr* expression of a GFP reporter strain was followed in situ during biofilm development under batch and dynamic conditions.[28] Surprisingly, GFP expression, recorded with a confocal scanning laser microscope, depended on the growth conditions. It was low under static conditions. Unorganized multicellular layers of cells were observed, only a few expressing *agr* (less than 1% of the total number of cells in a 48 h biofilm). Under dynamic conditions, *agr* expression increased from 15% during adhesion to 80% in flow-cell mature biofilms. The *agr* expression was mainly recorded at the apex of the biofilm, in a complex network of cells organized in chains that surrounded ball-shaped microcolonies.

The *agr* System and the Regulation of Transcription

In order to refine our understanding of the significance of *agr* signaling in the physiology of *L. monocytogenes*, it is of importance to determine the targets of AgrA. A recent study gives some clues on the genes that might be regulated by this response regulator.[23] These authors compared global gene expression profiles of an *agrD* deletion mutant and its parental strain *L. monocytogenes* EGD-e. During exponential phase, 55 genes and 66 genes respectively were up and downregulated in the mutant. In our laboratory, similar results were observed with an *agrA* deletion mutant of *L. monocytogenes* EGD-e (Piveteau P, personal communication). The number of genes differentially regulated was higher during stationary phase; the number of genes up and downregulated respectively was 345 and 325 respectively.[23] All categories of genes were affected by the mutation. The transcription of a rather large number of genes (58) coding for regulatory proteins was affected.[23] These preliminary results suggest first of all that the *agr* system plays a major role in the adaptation of *L. monocytogenes* to environmental conditions, and secondly, that AgrA is probably involved in a complex regulatory network. However, it is necessary to know whether these expression differences are a direct consequence of a low abundance of AgrA in the Δ*agrD* background or rather an indirect consequence due to a disturbance within a complex regulatory network.

Autoinduction, Communication and Quorum Sensing: Where Does *agr* Stand?

Although there is no doubt that the *agr* system is indeed a communication system based on autoinduction, whether it is dedicated to Quorum Sensing (QS) remains on open question.

QS, first proposed by Fuqua et al. (1994)[29] can be defined as "the phenomenon whereby the accumulation of signaling molecules in the surrounding environment enables a single cell to assess the number of bacteria (cell density) so that the population as a whole can make a coordinated response."[30] Experimental evidence indicate that the transcription of the *agr* genes is lower in the *agrA* and *agrD* in-frame deletion mutants compared to the parental strain EGD-e thus confirming that the *agr* system is autoregulated.[24] However, during growth of *L. monocytogenes* in liquid cultures, the *agr* operon is transcribed to a similar level during mid exponential and early stationary phase,[21,24] suggesting that the *agr* system is not a mechanism to assess cell density in order to proceed in coordinated behavior of the whole population.

In situ expression also gave results that are not in agreement with the QS paradigm; heterogeneity of *agr* expression during biofilm growth[28] does not fit either with the definition of QS. On the contrary, our results corroborate with two theories presented more recently. The theory of Diffusion Sensing (DS) postulates that autoinduction enables the cell to evaluate the diffusion and mixing of autoinducers and other molecules in the cell's environment.[31] The second theory, Efficiency sensing (ES), is an elegant attempt to explain autoinduction and signaling in complex environments thus unifying QS and DS in a global theory of cell-cell communication.[32] The latter theory of ES is the most convincing in regard to our experimental results.

Conclusions

Information exchange and signaling are central to the adaptation of bacteria to environmental conditions. In the genus Listeria, AI-2 does not fit with the definition of a signaling molecule. So far, the *agr* system is the only communication system described in the genus Listeria. It is based on autoinduction but evidence suggests that Quorum Sensing does not apply to this communication system. Major adaptive responses are regulated through *agr* autoinduction; these include virulence and biofilm formation. It is tempting to propose that AgrA may be involved in a complex regulatory network in charge of coordinating gene expression for an optimal adaptation of *Listeria monocytogenes* and Listeria sp. to environmental conditions. A synthetic approach will have to be developed to decipher such a network.

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