Quorum-sensing in *Pseudomonas aeruginosa* and *Salmonella*: Active natural compounds as antagonists.

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The cell to cell communication and regulation systems in bacteria are generally known as quorum sensing. The basic principles of such processes consist of a simplified two-step flow: first, some bacteria emit certain signalling molecules. These are then recognised by the bacteria present in the close neighbourhood, allowing them to develop the production of substances and other functions in the cells, which facilitates a quick transference of the signal to produce or regulate substances in all the cells in a synchronised manner. Specifically, there are some regulation mechanisms by quorum sensing that permit the colonisation and virulence in *Salmonella*, as well as the colonisation and formation of biofilms in *Pseudomonas* according to the model of Cystic Fibrosis. Some of these processes are inhibited by diverse substances which would provide new perspectives in the fight against certain illnesses.

Keywords: quorum-sensing, *Pseudomonas aeruginosa*, Cystic fibrosis, *Salmonella.*

1.- Introduction

1.1.- Quorum sensing (QS).

Bacteria are entities which associate and behave as assemblies of individuals growing independently. This "individuality" feature has traditionally been taken as the standard truth, and therefore, has excluded any possibility of cell-to-cell communication as described for eukaryotic cells.

Nonetheless, it has been observed that many bacteria are capable of communicating within themselves, and also controlling the density of their own population. In some cases, they have also been seen as capable of functioning on a cooperative basis. This phenomenon, described in 1966 by J. W. Hastings and his collaborators, studying the luminescent bacteria *Vibrio fisheri*, was originally called " autoinduction" [1]

Hastings verified that large quantities of luciferase were observed when the cultivation of *V. fisheri* was at an advanced point of the logarithmic phase. Subsequently, these findings suggested the existence of a very primitive system of communication among bacteria that permits them to optimise the production of certain substances, so they will not be wasted in mid- process.

The phenomenon was termed *quorum sensing* [2]. The discovery that bacterial cells can communicate among each other shows that bacteria are capable of exhibiting much more complex patterns of co-operative behaviour than would be expected for simple unicellular microorganisms. Nowadays, the generically termed *'quorum sensing'*, bacterial cell-to-cell communication, enables a bacterial population to trigger a unified response that is advantageous to its survival.

This response improves access to complex nutrients or environmental niches, and develops collective defence mechanisms against alternative competitive microorganisms (eukaryotic or not). Furthermore, it also optimises the population's survival by differentiation into morphological forms better adapted to combating environmental threats.

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The principle of QS encompasses the production and release of signal molecules by bacterial cells within a population. Such molecules are released into the environment. As cell numbers increase, so does the extracellular level of signal molecules, until the bacteria sense that a threshold has been reached and gene activation, or in some cases depression or repression, occurs via the activity of sensor-regulator systems [3]. The concentration increases until a critical level is reached (the quorum). At this point, the bacteria express the dependent genes of the quorum.

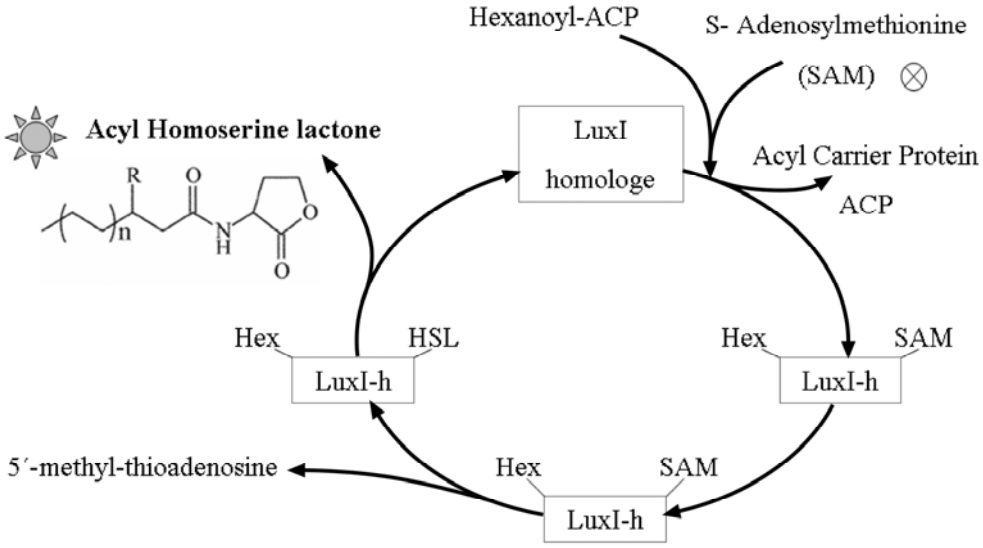

Fig 1. Scheme for homoserine lactone synthesis in Gram-negative bacteria. The hexanoyl group is transferred from ACP (Acyl-acyl Carrier Protein) to an active site cysteine on LuxI homologe, and SAM (S adenosin-methionine) binds to the active site. The hexanoyl group is released from the cysteine to form an amide bond with the amino group of SAM. 5'-Methylthioadenosine is released, and a lactonization reaction results in the synthesis of hexanoyl homoserine lactone.

In gram-negative organisms, the classical QS system is illustrated by the the LuxR/LuxI system of *Vibrio fischeri*. In this system, LuxI is the signal synthase that synthesises *N*-(3-oxo-C_6)-L-homoserine lactone, while LuxR is the signal receptor. There are LuxI homologues in other bacterial species that synthesise different *N*-acyl homoserine lactones (figure 1). The latter have various acyl chain lengths (C_4 to C_{18}), different degrees of saturation, or modifications at the third carbon of the acyl chain. Upon the interaction of these bacterial species with the AHL signal, the LuxR homologues are either activated or inhibited as a transcription factor capable of modulating the expression of the target genes. They then regulate various numbers of important biological functions, including biofilm formation, bioluminescence and virulence. In this context, different bacterial signalling molecules have been identified, and therefore, in Gram-negative bacteria, many diverse physiological functions are quorum-regulated (figure2). The most common molecules are *N*-acyl-homoserine lactones (AHL). In addition to AHLs, there are other quorum signalling molecules that have been identified in Gram-negative bacteria such as 2-heptyl-3-hydroxy-4-quinolone (PQS) in *Pseudomonas aeruginosa* and diketopiperazines in *P. aeruginosa* [4]; [5].

On the other hand, Gram-positive bacteria commonly utilise amino acids and short post-translationally processed peptides for cell density-dependent gene regulation [6]. Autoinducer-2 (AI-2), first discovered in *Vibrio harveyi* [7], has been described as a new quorum signal used by both Gram-negative and Gram-positive bacteria [8].

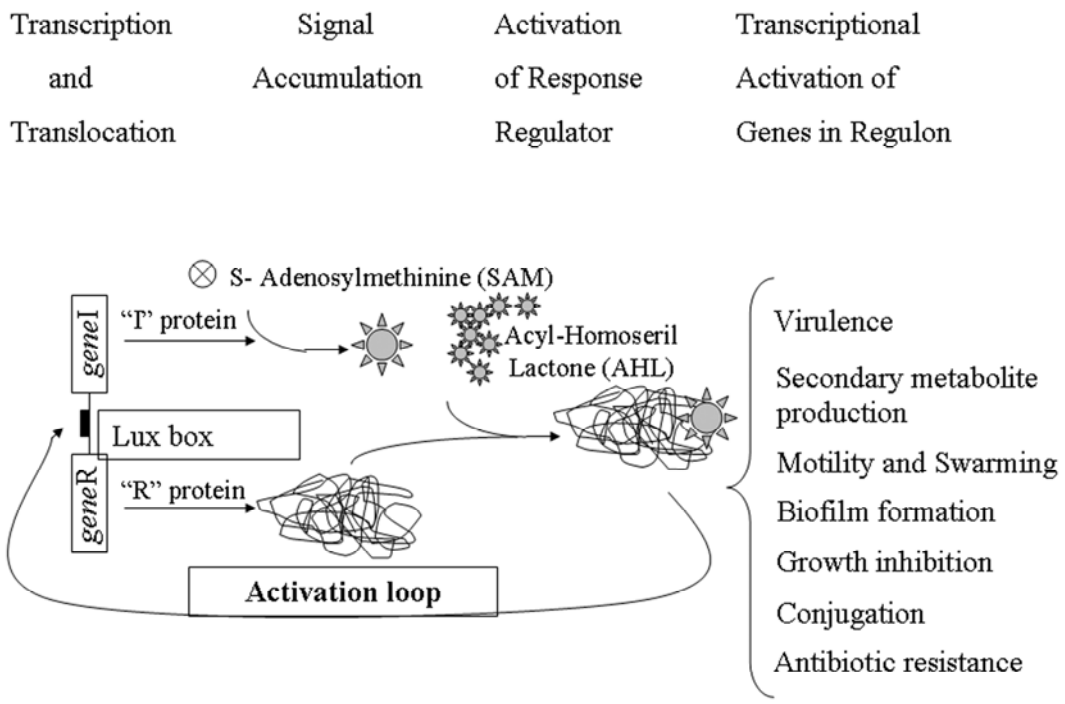

Fig 2. Model for the regulation of Gram-negative bacteria by quorum sensing system.

Therefore, across distant genera, there are many types of signalling molecules regulating diverse phenotypes, including the *N*-acylhomoserine lactones (AHLs), fatty acid derivatives, 4-quinolones, furanones (such as autoinducer-2 (AI-2) and oligopeptides. For instance, some bacteria produce multiple and chemically distinct signal molecules which control complex regulatory networks. Here, there is a hierarchical organisation of the genes involved in quorum-sensing signal molecule synthesis (LuxI family of AHL synthases) and signal transduction (e.g. LuxR family of response regulators activated by binding AHLs).

So far, the "universal" QS system remains elusive. Only the AI-2 synthase, LuxS is found in the Gram-negative/Gram-positive borderline [9]. For Gram-negative bacteria, the most common signalling molecules found are N-acyl derivatives of homoserine lactone (acyl HSLs). The processes controlled by acyl HSLs are modulated in a cell density- and growth phase-dependent manner. Therefore, the term 'quorum-sensing' has been related to describing the bacterial ability to monitor cell density prior to expressing a phenotype [10].

Overall, the perception of *quorum* has an especially practical meaning for bacterial economy. For example, in the production and liberation of enzymes, it would be highly impractical that few bacteria free a small quantity of enzyme, because it would diffuse and be diluted quickly, making this liberation ineffective. Therefore, if the production intends to reach the *quorum*, the enzyme would be released to the adequate concentration. When a critical concentration is reached, the signal can be sensed by the bacteria and prompts them to activate or repress certain target genes.

On the other hand, the attachment of bacteria to each other, as well as to wet surfaces, and the subsequent differentiation into highly structured biofilms, is considered a major virulence trait. In this matrix-enclosed sessile growth, bacteria are protected from adverse environmental conditions and from

biological and chemical antibacterial agents. This protection is given out by cells that are periodically shed from established biofilms, so that new habitats can be colonised with the formation of new biofilms. The knowledge of the molecular mechanisms involved in this phenomenon opens new doors to the therapeutic approach to many infections, which perhaps can be resolved some day without the use of antibiotics, not always efficient. The formation of biofilms by *Pseudomonas aeruginosa* in people affected by Cystic fibrosis and the *Salmonella* colonisation are some of the problems that could be resolved with the knowledge of quorum sensing.

2.- Bacterial biofilms: Gene regulation and formation of biofilms in *Pseudomonas*.

2.1.- Bacterial biofilms

Biofilms are complex communities of microorganisms embedded in a self-produced matrix and adhered to either inert or living surfaces [11]. Therefore, biofilms are absorbed in a headquarters of exopolysaccharides and adhere to a surface, either inert or alive and weaving (figure3).

This phenomenon is quite common in the bacterial world. When the environmental conditions are optimal and adequate, most of the microorganisms are capable of forming these structures. In fact, biofilms have been observed on a variety of surfaces and in a variety of niches, and are considered the prevailing microbial lifestyle in most environments.

The medical implications derived from this process are important. Biofilm-associated bacteria on implants or catheters can cause serious infections. In the case of recurrent and chronic infections, it is evident that bacteria are producers of biofilms. Therefore, the bacteria inside them would be protected from the action of antibodies, phagotypes and antibiotics.

Biofilm producing bacteria are ubiquitous in nature and can also colonise very specific environments, such as the respiratory mucous membrane in patients of Cystic fibrosis by *Pseudomonas aeruginosa*, the average otitis in determined stumps of *Haemophilus influenzae* or the endocarditic decay by *Streptococcus* of the viridans group. It becomes clear that the bacteria resposible for these types of recurrent and chronic infections are more difficult to treat.

We can cite several mechanisms responsible for the resistance. For instance, the presence of exopolysaccharides makes both the physical diffusion and the chemistry of compound penetration very difficult. Other mechanisms are the slow growth of the bacteria of the biofilm because of the limitation of nutrients, the existence of antagonists of the action of antibiotics, etc.

In fact, biofilm grown cells can become 10 to 1,000-fold more resistant to the effects of antimicrobial agents than their planktonic counterparts [12]; [13]. Biofilms show resistance to a wide range of antibiotics (including ampicillin, streptomycin, tetracyclines, gentamicin, and many others) and biocide oxidants such as ozone, chlorine and iodine.

But not only bio-medical domains are concerned about this. For instance, in the food industry, the formation of biofilms on food and food-processing surfaces, and in potable water distribution systems, constitutes an increased risk of contamination with spoilage or pathogenic microbiota [14]; [15].

Due to the aforementioned characteristics, biofilms are extremely difficult to control in both medical and industrial scenarios. Although traditional antibiotic therapy can eliminate sensitive planktonic bacteria, these same organisms can survive previously proven efficient treatment when growing in a biofilm. For example, the dosage levels of antibiotic needed to eliminate biofilm easily reach toxic levels [16] on surfaces of medical implants which require antibiotic treatment, Therefore, biofilm-based infections may become chronic and untreatable.

Cell-to-cell signalling plays a role in the differentiation of *Ps. aeruginosa* in biofilms [17] and in cell attachment. Furthermore, it is involved in the development of survival of *Ps. aeruginosa* biofilms[18]. However, it remains unclear how common quorum signalling occurs in biofilm-forming bacteria, and whether quorum sensing is generally required for biofilm-forming capacity.

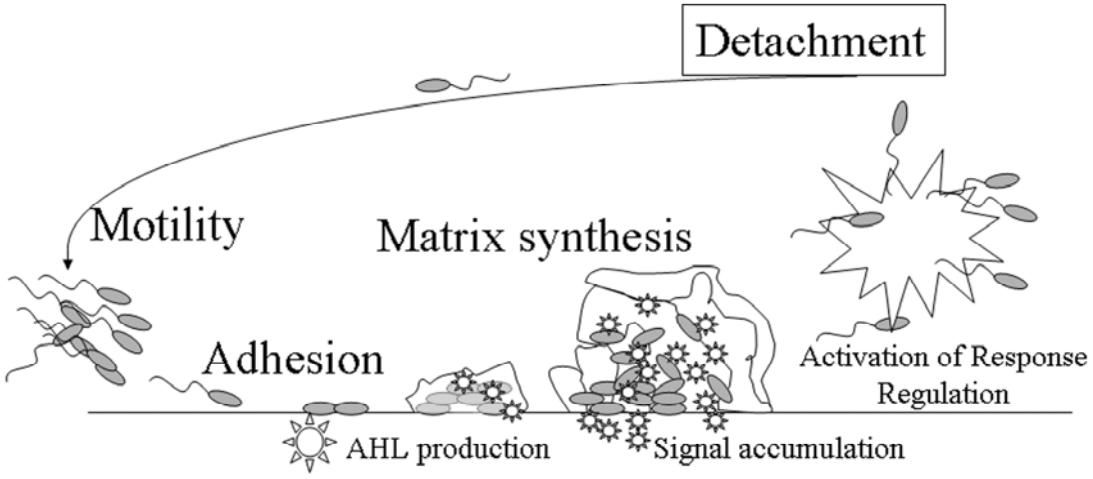

Fig 3. Steps to biofilm formation. It directly depends on the AHL´s production and their accumulation. They regulate adhesion, matrix synthesis and finally the multiple gene activation or repression.

2.2.- Gene Regulation and formation of biofilm by *Pseudomonas* in Cystic Fibrosis (CF).

CF manifests as a clinical syndrome characterised by chronic sinopulmonary infection as well as by gastrointestinal or nutritional infections and other abnormalities. In fact, CF is the most common homozygotic recessive illness that affects Caucasians. The genetic basis for CF is a well-characterised severe monogenic disorder. The mutation causes the dysfunction of a membrane channel for chlorides, the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), which, at the same time, is the cause of a deficiency in the secretion of water and ions in diverse secretory systems. The most serious consequence is the dysfunction of the defence mechanisms of the pulmonary mucous membrane, due to the excessive viscosity of bronchial secretions, so that the patient suffers repeated pulmonary infections. But the formation of biofilm is also important for the colonisation of inert materials, like prostheses. In any case, biofilm provides high resistance to antimicrobial factors: phagocytes, antibodies and antimicrobials. Therefore, inhibitory therapeutics of the formation of biofilm could be efficient in the eradication of *P. aeruginosa* in infected implants or in the lung of the patient of CF.

Infancy and early childhood are the critical stages for infection or colonisation of susceptible lungs in CF patients, who often get infections from agents such as *Staphylococcus aureus* and *Haemophilus influenzae*. These infections may damage epithelial sufaces, leading to an increase of the attachment of further *Pseudomomas aeruginosa.* As a sign of urgent need of therapy, the recovery of these organisms from a bronchoalveolar lavage (BAL) fluid sample from the lung indicates that the infection is advanced. Nonetheless, the precise role that *S. aureus* and non-typeable *H. influenzae*, isolated from oropharyngeal cultures, play in the progression of CF patients to respiratory failure has not been clarified. In fact, the development of lung disease in CF patients due to *S. aureus* and non-typeable *H. influenzae* lacks any solid evidence from studies in literature [19].

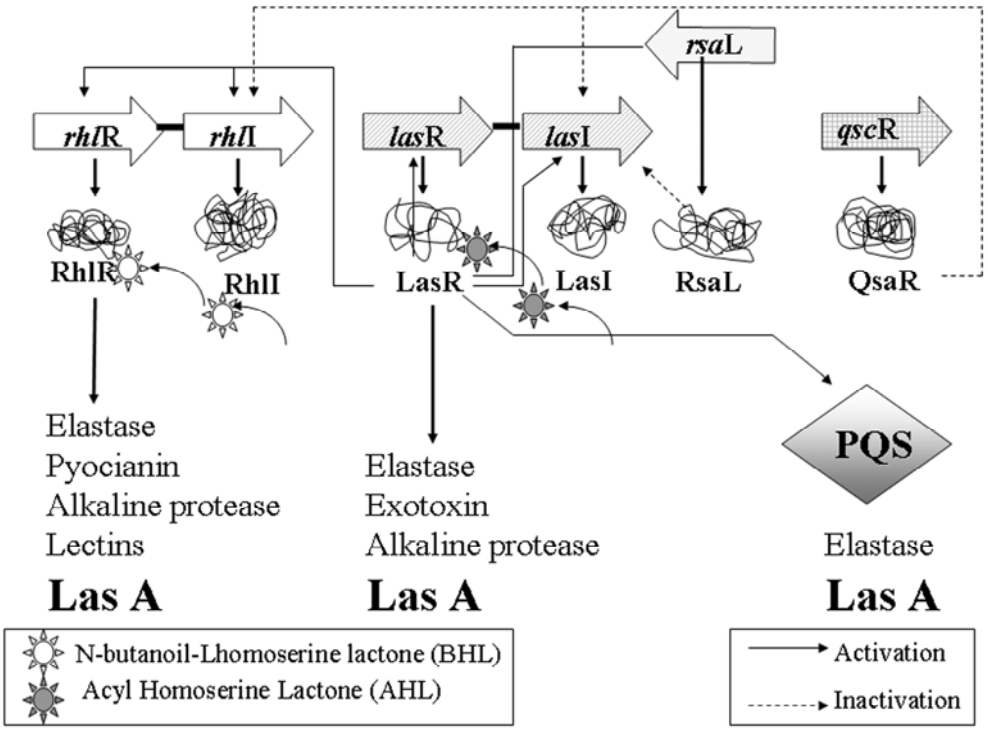

Fig 4. The use of hierarchical quorum-sensing to control virulence in P. aeruginosa. The Las and Rhl quorum-sensing systems of P. aeruginosa integrate with other global regulators to control the production of multiple virulence factors. RpoS positively and negatively regulates a further group of physiological processes, some of which are shown.

The basis of the pathogenicity of *P. aeruginosa* is its ability to produce and secrete multiple extracellular virulence factors such as proteases, haemolysins, exotoxin A, exoenzyme S and pyocyanin (figure 4). These exofactors are collectively capable of causing extensive tissue damage in humans and other mammals. QS controls not only virulence factor production, but also biofilm formation in *P. aeruginosa* and thus contributes significantly to the pathogenesis and persistence of infection. The QS system in *P. aeruginosa* comprises two hierarchically organised systems, each consisting of an autoinducer synthetase (LasI/RhlI) and a corresponding regulator protein (LasR/RhlR). Each system produces its own AHL synthetase (LasI and Rhli) and its regulating place (LasR and RhlR). Thus, Acyl Homoseril Lactones (AHL) have an important influence in the development of *Pseudomonas*.

As we have stated before, the processes of communication among bacteria are generalised in the case of Gram-negative bacteria. Nevertheless, a single model of gene activation does not exist. Each model presents its own proteins of these molecules for the perception of the quorum. Thus, the routes of activation and the genes involved in such routes differ substantially among themselves.

As explained before, quorum sensing is an important regulation mechanism in *Pseudomonas*. The regulation of genes encoding the exoproducts depends on a signalling system that encompasses at least two sets of LuxRI homologues. The first of these quorum-sensing systems includes LasI, which is responsible for the synthesis of an acil homoserine lactone, and the transcriptional activator, LasR. The LasRI system was initially believed to regulate the expression of LasA elastase, LasB elastase, exotoxin A and alkaline protease. The second quorum-sensing system of *P. aeruginosa* is controlled by the LuxRI homologues, RhlRI. RhlI directs the synthesis of N-butanoyl-Lhomoserine lactone (BHL). This acyl

HSL was initially shown to interact with RhlR to activate expression of rhlAB, an operon encoding a rhamnosyltransferase required for the production of rhamnolipid biosurfactants.

These compounds can reduce surface tension and thereby allow *P. aeruginosa* cells to swarm over semi-solid surfaces. Subsequent studies have revealed that a functional RhlRI system is also required to fully induce expression of other factors, including alkaline protease, pyocyanin, hydrogen cyanide, lectins and elastase. On the other hand, the presence of RhlR BHL enhances the transcription of RhlI, creating a further autoregulatory loop within the LasRI/RhlRI regulons. Thus, activated RhlR may be capable of activating transcription of rpoS [20].

At present, a third regulator is known, QscR. Although the QscR system does not participate in the synthesis of AHL by itself, it regulates it negatively. Recently, some room for regulation has been described for VqsR, but it is not clear whether or not it interacts directly because of the effect of the AHL. *Pseudomonas* also possesses another signalling cellular system integrated in the circuit of the AHL, the so-called PQS (Pseudomones Quinolone Signal). The combined effect of these systems is very complex, and in *Pseudomonas,* it is believed to affect the expression of 400 or more genes, many of which are implicated in virulence capabilities. There is ample evidence that the formation of biofilm is regulated by quorum sensing and it is confirmed that such capability is essential for the colonisation by *P. aeruginosa* of the pulmonary epithelia affected by cystic fibrosis.

In recent years, this view has frequently been resorted to to stump characterisation and descriptive population studies using molecular biology techniques and studies of gene regulation. But to the best of our knowledge, there are no data either addressing or proposing the inhibition of the quorum sensing process as an extensive application to eliminate the biofilm production from *Pseudomonas*.

Taking this into account, and considering the broad repertoire of possibilities that this approach might offer, we feel that there is still a need to conduct deeper experimental studies to delineate the usefulness and importance of the compounds affecting QS in microbial monitoring.

2.3.- Invasion and virulence of *Salmonella*.

Salmonella is a group of bacteria that can cause diarrhoeal illness in humans. They are gram-negative, motile bacilli belonging to the *Enterobacteriaceae* family. Even today, the taxonomy and nomenclature of *Salmonella* is still a subject of debate, as the genus *Salmonella* covers a large taxonomic group with over 2,463 recognised serotypes on the basis of O (somatic) and H (flagellar) antigens. Historically, serotypes of *Salmonella* were considered as different species, but molecular analyses have proved that typical *Salmonella*e might be considered as a single species, denominated *Salmonella enterica* by Le Minor and Popoff (1987) and divided into six subspecies [21]. Later, subspecies V was recognised as a species, *Salmonella bongori,* and, recently, a third specie, *Salmonella subterranea,* has been described. *Salmonella enterica* subsp. *enterica* (subspecie I) encompasses 1,454 serotypes that colonise warm-blooded animals (including humans). The remaining subspecies (II, IIIa, IIIb, and IV), as well as *S. bongori*, inhabit cold-blooded animals. However, *Salmonella* is a ubiquitous bacterium and any serotype can be isolated from very diverse environmental habitats [22].

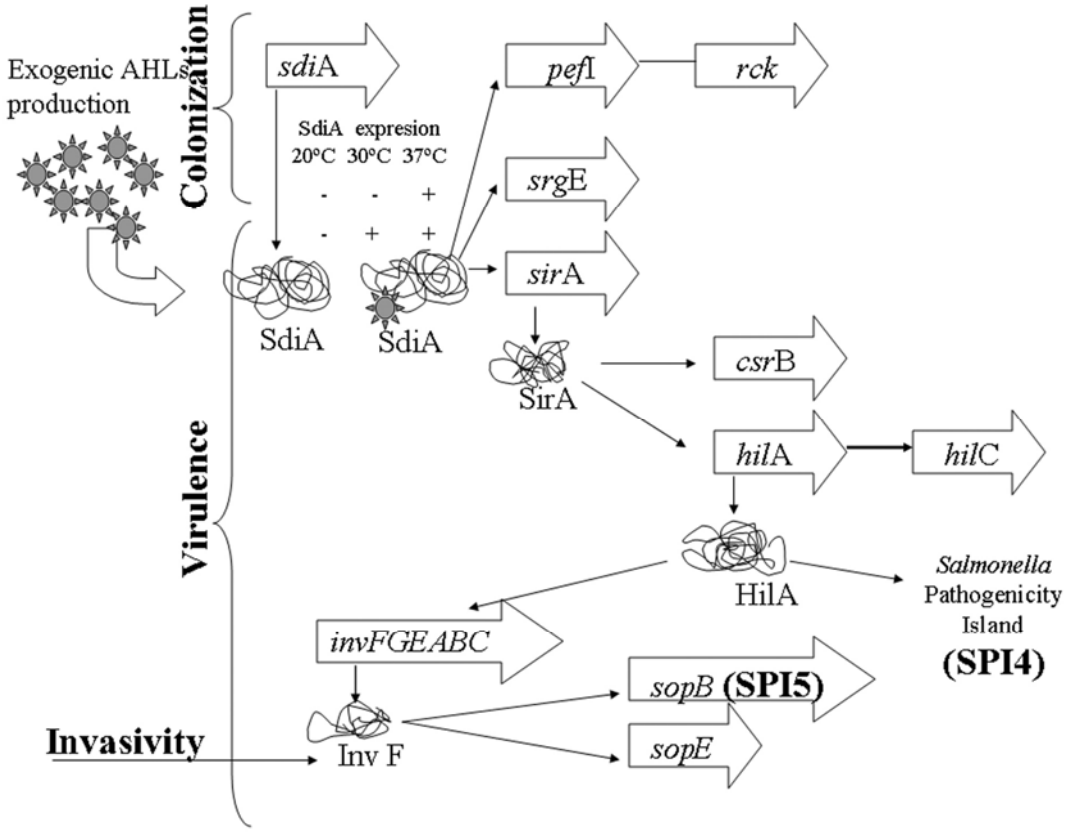

Fig 5. SdiA is a transcription factor that activates transcription in response to bacterial AHLs. Indeed, *sdiA* gene is shown activating the expression of *srgE* and the *rck* operon on the virulence plasmid pSLT. The *pefI* and *srgA* genes are encoded within the *rck* operon and affect the transcription and folding, respectively, of plasmid-encoded fimbriae that are encoded by the *pef* genes upstream of the *rck* operon. SirA (*Salmonella* invasion regulator) is a response regulator and an apparent "housekeeping" and also at the top of a regulatory cascade controlling virulence gene expression. In fact, *sirA* gene is a regulator of *Salmonella* pathogenicity island 1 (SPI1). The *sirA* gene is shown activating *hilA* and *hilC* expression. These activation events are known to be direct. The *hilA* and *hilC* genes are both regulatory genes encoded within the horizontal acquisition SPI1, which control other genes within SPI1 that encode a type III secretion system, genes within SPI4 that encode a type I secretion system and other smaller horizontal acquisitions that encode type III effector proteins. SirA also directly activates the evolutionarily conserved *csrB* gene, which encodes an RNA that antagonizes the activity of the CsrA such an RNA-binding protein.

Salmonella gastroenteritis, a food poisoning, caused by various serotypes of *Salmonella enterica*, is a health problem difficult to eradicate, even in the most industrialised countries. In fact, measures taken to eliminate the poultry disease caused by *Salmonella* Gallinarum-Pullorum seem to have increased the asymptomatic colonisation by *S.* Enteritidis, more difficult to detect. This is reflected in the increment of human cases produced by *S*. Enteritidis, surpassing *S*. Typhimurium, previously the main cause of human food poisoning. *Salmonella* invades enterocytes and phagocyte cells, directing its endocytosis through a route that avoids the final destruction of the bacteria in the phagosome. To this end, it injects several proteins in the host cell through a system codified by a cluster of genes located in the *Salmonella* Pathogenicity Island 1 (SPI-1). These proteins affect the polymerisation of actin, the membrane's phosphoinositide metabolism and the endocytic traffic. The invasion of the intestinal epithelia and the

survival of phagocytes cause an intense inflammatory answer with infiltration of leukocytes resulting in purulent diarrhoea [22].

Salmonella possesses at least two quorum sensing systems: one is the autoinductor AI-2 and the other, induced by acyl-homoserine-lactones (AHS´s). However, may author opine that AI-2 may represent a metabolite that has been adopted as a cell-to-cell signal molecule by some, but not all bacteria. (Winzer et al., 2002). According that, we considered that AHL´s is the most important cell to cell communication system in *Salmonella*. As figure 5 shows, AHL´s systems act through the regulating protein SdiA, a counterpart of LuxR, which activates the genes of the SPI-1 and other genes that also seem to be implied in the virulence [23]. Another important question is that *sdiA* gene is activated at 37ºC. It could explain the reason why *Salmonella* colonization and virulence its produced when the bacteria penetrate inside the human intestine and produce the gastroenteritis.

2.4.- Natural products as inhibiting of quorum-sensing.

Currently, 25% of common drugs proceed from medicinal plants, which were used for the same purpose in antiquity [24], and approximately half of the best-selling drugs are by-products of plants or developed from them (digoxin, taxol, artemisin, vincristine, vinblastine, Ginkgo, enalapril, ...). Many of those natural products are characterised by an enormous variety of new structures, often complex and difficult to obtain for chemical synthesis. Others have interesting pharmacological and biological activities and have been used either as chemotherapy agents or as a starting point in the development of new drugs. Favonoids, phenols, stilbenes, non-proteinic animo acids, and rough extracts are some of the groups of secondary metabolites that have shown anti quorum-sensing activity [25]; [26]; [27]; [28]; [29]. At present, there is an active, growing interest in the search for new principles, as well as in the pharmaceutical application of new molecules, reinforced by recent technological developments.

The emergence of antibiotic resistance strengthens the need for novel therapeutic drugs. It has been suggested that targeting the QS system, instead of killing bacteria, may provide a solution to antibiotic resistance [30]. There are a number of ways to block the QS flow, making anti-quorum sensing (anti-QS) compounds of great interest in the treatment of bacterial infections [31]. A number of quorum-quenching enzymes that hydrolyse AHLs have been identified in bacteria. To date, however, the only known anti-QS compounds of non-bacterial origin are halogenated furanones from the red alga *Delisea pulchra* [32]. A number of southern Florida seaweeds (Cumberbatch, 2002) and a few terrestrial plants [33] have also shown anti-QS activity.

With the promise of anti-QS compounds, one is compelled to search for these agents using the most efficient method possible. There have been many ethnobotanically directed searches for agents to treat infection, demonstrating not only the need for these drugs, but also the large number of plants used for bacterial conditions [34]; [35]. Although this antibacterial effect is important, it is not the only source of a plant's medicinal properties. Shifting the focus from the strictly antibacterial to anti-QS properties of plants may reveal new quorum quenching compounds and provide use-validation for traditional drugs [36].

The main chemical groups in these compounds are furanones, lactones and other poly-hydroxilated rings. Numerous authors have suggested that these chemical groups would be responsible for the inhibitory activities of bacterial growth either through quorum-sensing or through other still unknown mechanisms. Numerous products of chemical synthesis have an anti-QS activity. They are present in a great quantity of vegetable extracts of use in traditional medicine [36]. At present, despite a well- known existence, the science behind quorum-sensing is just emerging and in its infancy. As potential recipients profiting from the development of this line of research, controlling and preventing the formation of biofilms will help in many ways: the efficiency of cooling towers in buildings will be increased, therefore reducing the sources of Legionnaire's Disease; the food industry, which uses natural preservatives, and the health industry, which produces catheters, are good examples of other fields which may also benefit from this particular research [37].

In conclusion, thought the manipulation of quorum sensing by the use of inhibitors, or perhaps also activators, can affect the capacity of colonisation by *Salmonella* or *Pseudomonas,* we consider this approach an imaginative alternative to the use of antibiotics for the control of the colonisation and virulence.

Acknowledgements. We wish to thank Linda Hamalainen for linguistic assistance and to Rafael Rotger Anglada and Ana Rojas Mendoza for their precious collaboration.

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