

BACTERIAL INTER-SPECIES COMMUNICATION MEDIATED BY THE AUTOINDUCER-2 SIGNAL

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During the last few decades, scientists have come to appreciate the immense complexity in bacterial signaling interactions that sustain microbial communities. Quorum-sensing (QS) is a cell-cell communication process whereby single cell bacteria regulate gene expression synchronously in a population in response to self-produced extracellular signal molecules, called autoinducers. Autoinducer-2 (AI-2), the synthase of which, LuxS, is present in both Gram-negative and Gram-positive bacteria, was proposed to represent a non-species-specific signal that mediates inter-species communication. In enteric bacteria, extracellular AI-2 levels peak in late exponential phase and rapidly decline as bacteria continue to grow. This depletion occurs because AI-2 activates the expression of an operon, *lsr* (for LuxS Regulated), encoding the Lsr transporter and enzymes that degrade the signal. As the Lsr system imports self and non-self AI-2, *lsr*-containing bacteria can interfere with AI-2 signaling of other species and shut off group behaviors regulated by this molecule: this system represents the first example of interference with a bacterial inter-species QS signal.

The main goal of the research reported in this thesis was to characterize the Lsr system and its regulatory networks for AI-2 detection and interference, with the long term aim of increasing understanding of the role of AI-2 inter-species communication in multi-species environments.

To identify regulators of the *lsr* operon a genetic screen was performed in *Escherichia coli* (**Chapter III**). This led to the identification of the phosphotransferase system (PTS) as a central player in the regulatory network of the Lsr system. Mutants of components of PTS prevent AI-2 internalization and processing. This phenotype is overcome when the Lsr system is constitutively expressed. Overall, these results indicate that in order to activate its specific transport system, Lsr, PTS-dependent uptake of AI-2 must first occur. As Lsr transport is dependent on the PTS, this suggests a mechanism through which information about the physiological state of bacteria and regulation of AI-2 signal uptake is integrated.

There are two known AI-2 receptors: LuxP, found in the *Vibrio* genus and LsrB, first identified in *Salmonella enterica* subspecies *enterica* serovar Typhimurium. The research in **Chapter IV** was designed to determine the occurrence of the Lsr system within all the available bacterial genome sequences. Based on sequence analysis and structure prediction, a set of criteria was established to identify functional LsrB-like AI-2 receptors. Bioinformatic predictions were confirmed experimentally by assaying selected species for AI-2 internalization *in vivo*, and testing their LsrB orthologs for AI-2 binding *in vitro*. The presence of functional AI-2 receptors in the phylogenetically distant families *Enterobacteriaceae*, *Rhizobiaceae*, and *Bacillaceae* was thus demonstrated. Furthermore, it was shown that two residues that interact with AI-2 (D166 and A222) were conserved in all the LsrB orthologs analyzed and proved essential for AI-2 binding ability. Additionally, the evolutionary history of the *lsrB* gene was studied by phylogenetic analysis which suggests a single LsrB origin in a common ancestor of the *Enterobacteriales* and *Pasteurellales* families with two subsequent events of lateral gene transfer. These results provide a method for identifying and validating functional LsrB-type AI-2 receptors, an important step to the understanding of the molecular basis of AI-2-mediated behavioral regulation in a variety of new species.

Chapter V details the characterization of the Lsr system from a non-AI-2 producer, the plant symbiont *Sinorhizobium meliloti*. Sequence analysis revealed that *S. meliloti* has orthologs of all the proteins of the AI-2-interference Lsr system found in enteric bacteria: this operon could be similarly involved in AI-2 sequestration and degradation. Accordingly, transcription of this operon is induced by AI-2 and this microorganism can completely eliminate the AI-2 secreted by *Erwinia carotovora*, a plant pathogen shown to use AI-2 to regulate virulence, while *lsrK* mutant cannot. Moreover the transcriptional profile shows that, in the conditions tested, apart from inducing transcription of its own incorporation and processing system, the presence of the AI-2 signal in *S. meliloti* cultures does not regulate expression of any other genes. These findings suggest that *S. meliloti* is capable of

'eavesdropping' on the AI-2 signaling of other species and interfering with AI-2-regulated phenotypes, possibly gaining a benefit in colonization of niches containing multiple species of bacteria

The mechanisms by which microorganisms interfere with other species ability to communicate, for example, through the Lsr system studied in this research, can be exploited to design new clinical and biotechnological strategies towards the manipulation of bacterial behaviors such as virulence.

Nas últimas décadas, a noção de comportamento bacteriano alterou-se drasticamente. Até recentemente, as bactérias eram estudadas como populações de células individuais que atuavam de forma independente. Atualmente, sabe-se que formam comunidades complexas, existindo uma intensa comunicação e interação entre células bacterianas.

Através de um mecanismo de comunicação celular, denominado Detecção de Quórum, moléculas sinal designadas autoindutores, são produzidas e detetadas pelas células bacterianas em função da sua densidade populacional. O mecanismo de Detecção de Quórum permite deste modo uma regulação da expressão genética da população bacteriana de forma coletiva e, conseqüentemente, comportamento de grupo sincronizado. O Autoindutor-2 (AI-2) é o único autoindutor identificado que é produzido por bactérias filogeneticamente distantes, desde Gram-positivas a Gram-negativas, e por este motivo, pensa-se que possa ser utilizado em processos de comunicação entre espécies diferentes. Em bactérias entéricas, a concentração de AI-2 no meio extracelular atinge o seu máximo no final da fase exponencial e é rapidamente removido do ambiente exterior através de um mecanismo de transporte designado Lsr (do inglês LuxS Regulated) que é induzido pelo AI-2. Dado que o sistema de transporte Lsr incorpora a totalidade do AI-2 presente no meio extracelular, espécies que contêm este sistema de transporte podem inativar os comportamentos regulados por AI-2 de outras espécies em co-cultura. Destarte, o sistema Lsr representa o primeiro mecanismo identificado de interferência com sistemas de Detecção de Quórum inter-espécies.

A investigação levada a cabo nesta tese visa a caracterização do mecanismo de transporte e interferência Lsr, com o objetivo global de compreender a função do sinal AI-2 na comunicação inter-espécies em comunidades bacterianas habitadas por várias espécies.

Para identificar reguladores do operão *lsr* realizou-se um *screen* genético em *Escherichia coli* (**Capítulo III**). Desta forma o sistema de fosfotransferase foi identificado como componente essencial da ativação do sistema de transporte Lsr. Mutantes no sistema fosfotransferase não internalizam nem processam intracelularmente o AI-2, mas este fenótipo não se observa quando o sistema Lsr é expresso constitutivamente. No seu conjunto, estes resultados indicam que para o sistema Lsr ser ativado é necessário que o sinal AI-2 seja incorporado na célula através de um mecanismo que é dependente do sistema fosfotransferase. Esta dependência sugere uma estratégia celular que visa a integração da informação acerca do estado fisiológico da célula e, de acordo com esta, regular a incorporação do sinal AI-2 de Detecção de Quórum.

Até à data foram identificados dois tipos de recetores do AI-2: o LuxP no género *Vibrio* e o LsrB primeiramente identificado em *Salmonella enterica* subespécie *enterica* serovar Typhimurium. No **Capítulo IV** é analisada a presença do sistema Lsr na totalidade dos genomas sequenciados. Através da análise de sequências e previsões da estrutura proteica, estabeleceram-se uma série de critérios que visam a identificação de recetores funcionais do tipo LsrB. Estes critérios bioinformaticos foram testados experimentalmente numa seleção de espécies através de análises *in vivo* à incorporação de AI-2 e análises *in vitro* de ligação proteína-ligando/AI-2. Estas análises experimentais confirmaram a presença de recetores funcionais do tipo LsrB em espécies pertencentes a famílias filogeneticamente distantes tais como *Enterobacteriaceae*, *Rhizobiaceae*, e *Bacillaceae*. Demonstrou-se ainda que dois resíduos que interagem com o ligando AI-2 (D166 e A222) estão conservados na totalidade dos ortólogos de LsrB previstos como funcionais e são fundamentais para a capacidade de ligação da proteína LsrB ao sinal AI-2. Adicionalmente, a história evolutiva do gene *lsrB* foi inferida através de análise filogenética indicando que este gene teve origem no ancestral comum de *Enterobacteriales* e *Pasteurellales*, ocorrendo posteriormente dois eventos de transferência horizontal de genes. Em conjunto, estes resultados apoiam fortemente os critérios estabelecidos para a identificação de recetores

funcionais do tipo LsrB. Este é um passo fundamental para a compreensão dos mecanismos moleculares que estão na base da detecção celular do sinal AI-2 em variadas espécies de microrganismos.

No **Capítulo V** foi caracterizado o sistema Lsr de *Sinorhizobium meliloti*, uma espécie simbiote de plantas que não produz AI-2. Demonstrou-se que a espécie *S. meliloti* possui no seu genoma ortólogos de todos os genes do operão *lsr* e, em co-cultura, tem a capacidade de eliminar completamente o AI-2 produzido por *Erwinia carotovora*, uma espécie patogénica de plantas que utiliza este sinal para regular virulência. Adicionalmente, o perfil transcricional analisado por *microarrays* demonstra que, nas condições testadas, para além da sua própria incorporação e degradação, o AI-2 não altera a expressão genética da espécie *S. meliloti*. Estes resultados sugerem que, apesar de não produzir sinal, *S. meliloti* consegue 'ouvir' o sinal AI-2 produzido por outras espécies e interferir com os comportamentos regulados através de AI-2 nessas mesmas espécies produtoras.

A compreensão dos mecanismos utilizados pelos organismos para interferir com a capacidade de outras espécies comunicarem, como o sistema Lsr, pode ser usada no desenho de estratégias clínicas e biotecnológicas intencionadas para manipular comportamentos bacterianos.

Acknowledgements	v
Abstract	ix
Resumo	xiii
Table of Contents	xvii
Thesis Outline	xxi
Abbreviations and Acronyms	xxiii

Chapter I: Bacterial Chemical Communication

1 – Let there be light: a historical perspective of bacterial chemical language	3
2 – Bacterial speech: Quorum-sensing and its role in cell-cell communication	5
2.1 – Classified information: Species-specific bacterial signaling	6
2.1.1 – Acyl homoserine lactones	7
2.1.2 – Autoinducing peptides	15
2.1.3 – <i>Pseudomonas</i> quinolone signal	17
2.1.4 – Diffusible signal factor	18
2.1.5 – γ-butyrolactones	19
2.2 – Worldwide broadcast: Non-species-specific signaling	19
2.2.1 – Orphan AHL receptors	20
2.2.2 – Autoinducer-3	21
2.2.3 – Non-species specific DSF signaling	21
2.2.4 – Autoinducer-2	22

Chapter II: Autoinducer-2 Quorum-sensing

1 – The molecule that has been talked about: AI-2 Quorum-sensing	25
1.1 – “Universal” signal: a historical perspective on AI-2 signal	25
1.2 – The chemical nature of the signal: AI-2 synthesis, structure and activity	27
1.3 – AI-2 in metabolism vs. AI-2 signaling: Are they mutually exclusive?	29
1.4 – What has AI-2 been saying? Phenotypes regulated by AI-2 and/or <i>luxS</i>	30
1.4.1 – AI-2 signaling in bacterial species containing identified AI-2	32
1.4.1.1 – LuxPQ-type AI-2 sensing system	32
1.4.1.2 – LsrB-type AI-2 sensing systems	33
1.4.2 – AI-2 signaling in Gram-negative pathogens	37
1.4.3 – AI-2 signaling in Gram-positive pathogens	39
1.4.4 – AI-2 signaling in polymicrobial communities	41
1.4.4.1 – AI-2 signaling in the nasopharyngeal microflora	41
1.4.4.2 – AI-2 signaling in the oral cavity microflora	42
1.4.5 – AI-2 signaling in non-AI-2 producers	43
1.5 – LuxS role in AI-2 synthesis: experimental considerations	43
1.6 – Concluding remarks	45
2 – Objectives of the present research work	46
3 – Supplementary data	47

Chapter III: Activation of autoinducer-2 internalization and processing in enteric bacteria requires the phosphotransferase system	55
1 – Abstract	56
2 – Introduction	57
3 – Materials and Methods	58
3.1 – Bacterial strains, plasmids and media	58
3.2 – Genetic and molecular techniques	59
3.3 – Plasmid construction	59
3.4 – Construction of <i>crp*</i> gain of function mutation	60
3.5 – Screen for regulators of <i>lsr</i> transcription	60
3.6 – AI-2 activity assay	60
3.7 – β -galactosidase assays	61
3.8 – Time course of LsrK production	61
3.9 – <i>In vivo</i> ^{13}C NMR	61
4 – Results	62
4.1 – Analysis of the Lsr mutants phenotypes	62
4.2 – <i>ptsIcrr</i> mutation affects the regulation of Lsr transport system	63
4.3 – Both components of the PTS: EI and EIIA ^{glc} are required for AI-2 incorporation and <i>lsr</i> expression	65
4.4 – The PTS contributes to AI-2 incorporation and <i>lsr</i> expression in the presence of an <i>lsr</i> operon and catabolite repression	66
4.5 – Impairment in AI-2 incorporation and <i>lsr</i> activation in the PTS mutant is independent of AI-2 internal production by LuxS	67
4.6 – Lsr activation and AI-2 incorporation require phosphotransferase activity of	68
4.7 – Inhibition of <i>lsr</i> expression in a <i>ptsIcrr</i> mutant requires the repressor LsrR	69
4.8 – <i>ptsIcrr</i> mutation do not affect expression or activity of LsrK	71
4.9 – The <i>ptsIcrr</i> phenotype can be rescued by AI-2 internalization through a PTS-independent mechanism	73
5 – Discussion	74
6 – Acknowledgments	79
7 – Supplementary Material	79
Chapter IV: Identification of functional LsrB-like autoinducer-2 receptors	85
1 – Abstract	86
2 – Introduction	87
3 – Materials and Methods	89
3.1 – Bacterial strains and growth conditions	89
3.2 – Databases analysis	90
3.3 – Structure Prediction	91
3.4 – AI-2 activity in bacterial cultures	91
3.5 – Protein expression and purification	92
3.6 – AI-2 binding assay	92
3.7 – <i>B. anthracis</i> mutagenesis	92
3.8 – Phylogenetic analyses	93

4 – Results	94
4.1 – LsrB orthologs in completely sequenced bacterial genomes	94
4.2 – Profiles of AI-2 removal from extracellular medium	96
4.3 – <i>In vitro</i> AI-2 binding to LsrB orthologs	100
4.4 – The amino acids Aspartate 166 and Alanine 222 are required for AI-2	101
4.5 – Evolution of functional LsrB-like AI-2 receptors	103
5 – Discussion	107
6 – Acknowledgments	112
7 – Supplementary material	114
 Chapter V: <i>Sinorhizobium meliloti</i>, a bacterium lacking the autoinducer-2 synthase, responds to AI-2 supplied by other bacteria	 115
1 – Abstract	116
2 – Introduction	117
3 – Materials and methods	121
3.1 – Protein production	121
3.2 – AI-2 binding assay	122
3.3 – Crystallization and structure determination	122
3.4 – AI-2 synthesis	124
3.5 – Bacterial strains and growth conditions	124
3.6 – AI-2 activity in <i>S. meliloti</i> and <i>E. carotovora</i> cultures	125
3.7 – Quantitative real-time PCR analysis	126
3.8 – Microarray analysis	127
4 – Results	128
4.1 – <i>S. meliloti</i> contains an AI-2 binding protein	128
4.2 – Structure of the <i>S. meliloti</i> AI-2-receptor complex	129
4.3 – <i>S. meliloti</i> removes exogenously supplied AI-2 from extracellular medium	132
4.4 – Increased transcription of the <i>lsr</i>-like operon <i>ait</i> occurs in response to AI-2	134
4.5 – <i>S. meliloti aitK</i> and <i>aitA</i> mutants are impaired in the ability to remove AI-2 from the medium	134
4.6 – <i>S. meliloti</i> does not grow with AI-2 as a sole carbon source	136
4.7 – Identification of genes regulated by AI-2 in <i>S. meliloti</i>	137
4.8 – <i>S. meliloti</i> clears AI-2 produced by <i>Erwinia carotovora</i> in co-cultures of these two species	138
5 – Discussion	139
6 – Acknowledgements	143
7 – Supplementary material	144
 Chapter VI: Discussion	 147
 References	 159

To succeed in nature all organisms need to perceive their surrounding environment. Bacteria are not an exception; they recognize and constantly adjust to environmental changes by sensing external signals and altering gene expression accordingly. Quorum sensing is a general mechanism for gene regulation in which bacteria produce and detect small molecules called autoinducers to coordinate a wide variety of behaviors at a population level.

This work was designed to elucidate the occurrence, molecular mechanism of action and role of one specific signaling system in bacteria: the AI-2 Lsr transport system.

Chapter I contains a general introduction including a historical perspective of the important findings in the Quorum-sensing field.

Chapter II focuses on the more recent developments in the inter-specific AI-2 signal literature, with an emphasis on enteric bacteria; the motivation and major aims of this dissertation are also stated at the end of this chapter.

Chapter III presents data on regulation of the AI-2 system in enteric bacteria, revealing the phosphotransferase system (PTS) as a previously unidentified player involved in activation of the Lsr transport system. These findings link two of the most important sensing systems in bacteria: PTS and the Quorum-sensing system, providing support for existing evidence that bacterial populations are integrating common regulatory pathways to activate a coordinated and finely tuned recognition of the AI-2 signal.

Chapter IV analyses the occurrence and functionality of the AI-2 receptor, LsrB. This study was performed using all bacteria genomes currently sequenced; experimental and bioinformatic tools identified functional LsrB orthologs in the *Enterobacteriaceae*, *Rhizobiaceae*, and *Bacillaceae* families. In this chapter the evolution of functional LsrB-like AI-2 receptors is also explored.

In **Chapter V** a species-specific analysis of the *lsr* transport system was performed. The study focuses on the microorganism *Sinorhizobium meliloti* and its ability to 'eavesdrop' on the AI-2 signaling of other species and its consequent potential to interfere with AI-2-regulated behaviors, such as virulence, in these species.

Chapter VI summarizes the key findings of this study and integrates them with the latest results obtained by others on the subject of interference in inter-species signaling. It also proposes the major questions that should be addressed by further research.

3OC12HSL	<i>N</i> -3-oxo-dodecanoyl homoserine lactone
3OC6HSL	<i>N</i> -3-oxo-hexanoyl homoserine lactone
3OC8HSL	<i>N</i> -3-oxo-octanoyl homoserine lactone
3OHC4HSL	<i>N</i> -3-hydroxybutanoyl homoserine lactone
ABC	ATP-binding cassette
AHL	Acyl homoserine lactones
AI-2	Autoinducer-2
AI-2-P	AI-2-phosphate
AI-3	Autoinducer-3
AIP	Autoinducing peptides
AMC	Activated methyl cycle
Amp	Ampicillin
APEC	Avian pathogenic <i>Escherichia coli</i>
C4HSL	<i>N</i> -butanoyl homoserine lactone
Cm	Chloramphenicol
CSP	Competence-stimulating peptide
DNA	Deoxyribonucleic acid
DPD	4,5-dihydroxy-2,3-pentanedione
DSF	Diffusible signal factor
Ecc	<i>Erwinia carotovora</i> subs. <i>carotovora</i>
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EI	Enzyme I
EII	Enzyme II
EPEC	Enteropathogenic <i>Escherichia coli</i>
FDR	False Discovery Rate
FRET	Fluorescence resonance energy transfer
GC	Guanine-cytosine
GST	Glutathione-S-transferase
IPTG	Isopropyl beta-D-1-thiogalactopyranoside
Kan	Kanamycin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani
LGT	Lateral gene transfer

MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum likelihood
mRNA	Messenger ribonucleic acid
NMR	Nuclear magnetic resonance
OD	Optical density
PBP	Periplasmic binding protein
PCR	Polymerase chain reaction
PCWDEs	Plant cell wall -degrading enzymes
PDB	Protein Data Bank
PHYRE	Protein Homology/analogY Recognition Engine
PM	Perfect Match
PQS	<i>Pseudomonas</i> quinolone signal
P-TPO	3,4,4-trihydroxy-2-pentanone-5-phosphate
PTS	Phosphotransferase system
qRT-PCR	Quantitative real time polymerase chain reaction
QS	Quorum-sensing
RNA	Ribonucleic acid
RR	Response regulator
R-THMF	<i>R</i> -2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
SAH	<i>S</i> -adenosylhomocysteine
SahH	<i>S</i> -adenosylhomocysteine hydrolase
SAM	<i>S</i> -adenosylmethionine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SK	Sensor kinase
SRH	<i>S</i> -ribosylhomocysteine
SSDB	Sequence Similarity Data Base
<i>S</i> -THMF borate	<i>S</i> -2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate
Ti	Tumor inducing
UPEC	Uropathogenic <i>Escherichia coli</i>
WT	Wild-type

CHAPTER I

Bacterial Chemical Communication

"The limits of my language are the limits of my universe"

- Ludwig Wittgenstein

1 – Let there be light: a historical perspective of bacterial chemical language

By observing a colony of *Sinorhizobium meliloti*, it appears surprising that bacterial social interactions passed unnoticed by microbiologists for so long (**Figure 1A**). Such an ordered sculpted composition is likely to arise from some sort of cellular organization, and, as in any society, such organization is only achieved with exchange of information between members. One can consider that the description of microorganisms as part of chained communities extends back to the first man who ever observed bacteria. Antonie van Leeuwenhoek (1632-1723), the father of microbiology, didn't describe single solitary *animalcules*, but following observation through the original microscope lens, he sketched chains of what were most likely to be interacting bacteria (**Figure 1B**). However, it took three centuries for scientists to acknowledge that bacteria are not solitary and asocial beings, but are in fact quite the opposite; it is now broadly accepted that, bacteria promote intra- and inter-species interactions due to their ability to use molecules as source of information.



Figure 1 – Evidence for the existence of bacterial interactions. **A)** *Sinorhizobium meliloti* structured colony (C. S. Pereira, unpublished). **B)** Bacteria from the dental plaque (drawings by Antonie van Leeuwenhoek, September 17, 1683).

The view of bacteria as individuals probably lasted for so long because most of commonly used microbiology techniques favored the development of such phenotypes rather than those of a community nature, for example the isolation of single species in single colonies or the growth of these species in shaking liquid cultures. Furthermore, scientists were enthusiastically pursuing

other interesting new areas of research, such as genetics and biochemistry, using bacteria as tools rather than studying their behavior in its own right. As a result, knowledge on gene expression and regulation increased enormously during the 20th century, but until the 1960's signals activating or repressing changes in gene expression, were attributed to environmental cues and rarely to the bacterium itself.

The first article referring to cell density-dependent bacterial signals as modulators of behaviors was by Tomasz and Hotchkiss in 1964 (314). He and his coworkers observed that competence in pneumococcal cultures (now known as *Streptococcus pneumoniae*) was regulated by self-produced macromolecular cell products. What he called the "activator substance" was described as a signaling molecule that pneumococcus exports to the environment, which upon recognition coordinates the bacterial population to become competent at a particular stage of growth (314). It wasn't until 1970, however, that the term autoinduction was first used by Nealson *et al.* (215) to describe the phenomenon in which bioluminescent bacteria produce a compound, known as autoinducer, that accumulates in the medium until it reaches a certain concentration which induces the synthesis of the luciferase enzyme (215). Jointly, the results of Tomasz, Nealson and coworkers suggested that bacteria can adjust their behavior in response to variations in cell number by producing, releasing and detecting signaling molecules.

Until the late 1980's this idea that certain bacterial behaviors were regulated by self-produced autoinducers, which allowed individuals in a population to interact, was considered esoteric. By general scientific consensus it was confined to uncommon behaviors in few bacterial species, such as bioluminescence in marine bacteria (85) or sex pheromones in enterococci (83).

The crucial event that detonated the explosion of research into microbial cell-cell interactions was the findings by Fuqua, Winans and Greenberg which coined the appealing and "broadcastable" term Quorum-sensing (QS) (101). Furthermore, based on amino-acid sequences, they used the newly available

bioinformatic tools to demonstrate that homologues of the proteins involved in the production and recognition of autoinducers were present in diverse bacterial species, including those of important pathogenic bacteria, indicating an important role of this mechanism in bacterial adaptation to the environment (101). This brought to light that QS was not an anomalous occurrence, on the contrary it was a widespread mechanism within the bacterial kingdom (101). Bacteria could recognize their social environment through chemical signaling, and change gene expression accordingly. Suddenly scientists around the world wanted to know if their favorite bacteria were able to talk.

Years of intensive QS research followed: signals were chemically characterized; receptor structures were solved through crystallography and several phenotypes were proven to be controlled by QS in diverse species of bacteria. Furthermore, QS systems were shown to rely on a varied chemical lexicon which integrates environmental cues, such as temperature and pH, into a versatile molecular sensing mechanism facilitating bacteria adaptation to the many environments to which they are exposed.

Today, searching in Entrez Pubmed, there are over 3000 articles on QS, all of which contributed to the recognition that, through the use of autoinducers, bacteria engage in multicellular behaviors enabling them to thrive in the most distinct scenarios.

2 – Bacterial speech: Quorum-sensing and its role in cell-cell communication

Like any other organism, bacteria need to recognize their surroundings in order to better adapt, exploit and grow in a certain habitat. Quorum-sensing (QS) is a cell-cell communication mechanism in which bacteria produce, release and detect signaling molecules, called autoinducers, to access the bacteria in its vicinity and change gene expression accordingly. The canonical definition of QS is the production and accumulation in the extracellular media of an autoinducer which directly correlates with the

number of individuals in the population. When a threshold concentration of autoinducer is achieved, corresponding to a 'quorum' of bacteria, a signal transduction cascade is triggered which culminates in a synchronized modification in the gene expression of the population. Thus, QS allows bacteria to coordinate gene expression of the population and change their behavior as a group response to their social surroundings.

We now know that QS is "more than just a numbers game" (344): autoinducers can also provide information about the species composition of the local environment, whether or not bacteria are inside the host, their stage of development, and thus coordinate their communities on a multi-cellular and multi-species level. Based on this array of information, bacteria can further regulate gene expression as part of an ecological community, leading to a fine-tuning of group behaviors like biofilm formation, competence and production of toxins.

2.1 – Classified information: species-specific bacterial signaling

To regulate a variety of behaviors with enormous specificity and fine control, bacteria need to produce unambiguous autoinducers. Microorganisms have evolved QS systems which rely on species, or even strain, specific signals with high affinity for their cognate receptor (142), such that the autoinducer is primarily and most efficiently recognized by the producing species. Furthermore bacteria often integrate more than one QS mechanism, that possess regulatory devices at distinct levels in the signal-transduction cascade, which permits an intrinsic regulation causing a high fidelity in the QS regulated phenotypic responses.

This section describes selected examples of the species-specific bacterial signaling systems. A brief description of the most common QS signals, the acyl homoserine lactone and autoinducer peptides, of Gram-negative and Gram-positive bacteria respectively, will be presented and followed by

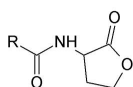
highlighting the particularities of these well studied QS systems. Furthermore, other classes of more recently recognized chemical signals are here documented. The increasing number and heterogeneity in QS signals is a relatively recent discovery, indicating that much is still unknown with many advances to be made in the coming years.

2.1.1 – Acyl homoserine lactones

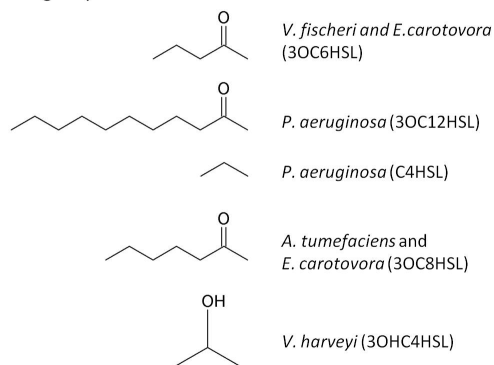
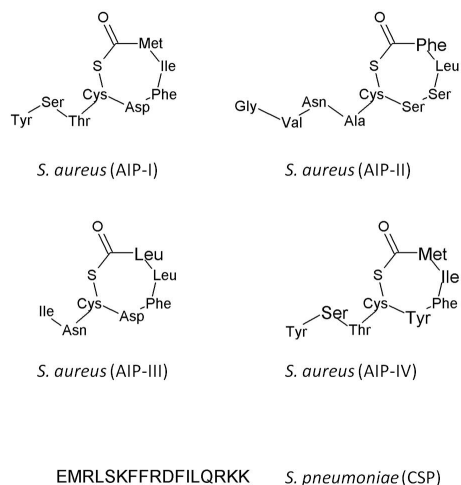
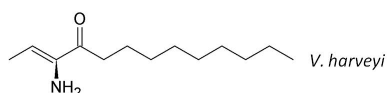
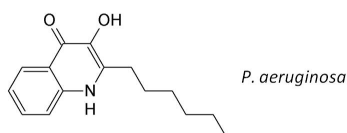
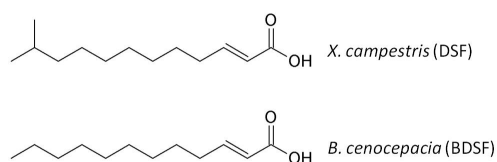
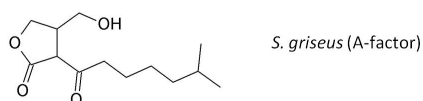
In Gram-negative proteobacteria the most widespread group of species specific autoinducer molecules are different forms of acyl homoserine lactones (AHL) (**Figure 2A**) (196). The AHL autoinducers share a common core, the homoserine lactone ring upon which specificity is conferred by the addition of structurally diverse acyl chain length (R group) which range from C4 to C18. Further specificity is added by the substituent at the third carbon (100) (**Figure 2A**). QS systems based on AHL signals are known as LuxI-LuxR circuits because these two proteins, LuxI and LuxR, or their homologues, are essential for the synthesis and response, respectively, of the AHL autoinducer. The LuxI-type enzyme catalyzes the synthesis of AHL through ligation of a specific acyl moiety, from a fatty acyl carrier protein, to the lactonized methionine moiety of *S*-adenosylmethionine (SAM) (213, 231). AHL can also be produced by a distinct family of AHL synthases the AinS/LuxM family, found exclusively in *Vibrio fischeri* and *Vibrio harveyi* respectively (24, 108). Most of the known AHLs, due to their size and polarity, freely diffuse across the cellular membrane and are released into the extracellular media. The few AHLs that are not able to diffuse through the membrane are actively transported (196, 232). As environmental concentrations of AHL increase, so will cellular concentrations (155) enabling recognition by the cytoplasmatic response regulator LuxR and forming the LuxR-AHL complex (**Figure 3**). Upon binding, this complex recognizes a consensus binding sequence and, by doing so, regulates the transcription of the QS target genes (reviewed in (23, 42, 142, 219, 330)).

AAcyl homoserine lactones (AHL)

Core molecule:



R groups:

**B**Autoinducer peptides (AIP)**C**Ea-C8-CAI-1**D**Autoinducer-2**E***Pseudomonas* quinolone signal (PQS)**F**Diffusible signal factor (DSF)**G** γ -butyrolactones**Figure 2 – Chemical structure of bacterial signal molecules.**

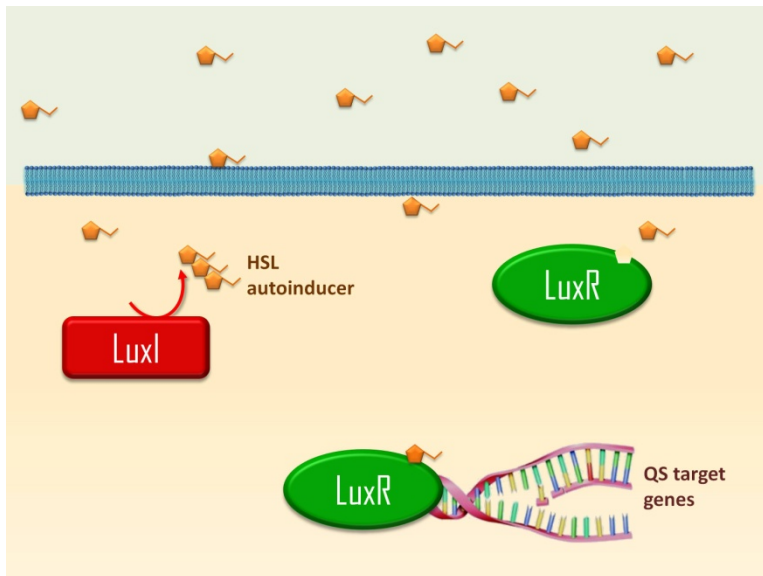


Figure 3 – Canonical AHL LuxI-LuxR QS circuit from Gram-negative bacteria.

Vibrio fischeri set the paradigm for Gram-negative QS because it was the microorganism where the molecular mechanisms of QS were first demonstrated (85, 89, 90, 155, 214). This bioluminescent bacteria lives in symbiosis with a eukaryotic organism, the squid *Euprymna scolopes*, commonly known as the bobtail squid which evolved an extremely specialized organ whose only function appears to be a vehicle for colonization by *V. fischeri* and, consequently, the production of light. In this organ *V. fischeri* encounters a nutrient-rich habitat that promotes the growth of the microbial population up to high numbers. Consequently, using a LuxI-LuxR-type QS system, the luciferase gene is activated and bioluminescence is achieved. Squids use the light produced by *V. fischeri* to mask their shadow and evade predation (264). *V. fischeri* LuxI synthesizes the *N*-3-oxo-hexanoyl homoserine lactone (3OC6HSL) (90) (**Figure 2A**) that freely diffuses in and out of the membrane accumulating in the media. At a certain concentration the 3OC6HSL is recognized by the cognate receptor LuxR and the LuxR-AHL complex binds to a consensus binding site activating the transcription of the operon *luxICDEABE* (90, 155, 292). As *luxI* is the first gene of the operon

this positive feed-back loop upregulates expression of the LuxI synthase and floods the system with signal giving a rapid response upon the change from a low cell density to a high cell density state (89).

QS systems are not always found in isolation. ***Pseudomonas aeruginosa*** is a highly versatile pathogen colonizing most man-made environments and is associated with chronic infections in the cystic fibrosis lung. One of the key regulatory systems of *P. aeruginosa* is a refined multi-signal QS system that coordinates the control of virulence, biofilm formation, swarming, antibiotic release and interaction with the host (336). As a result it is one of the QS systems to which most attention has been given. It comprises two LuxI/LuxR pairs: LasI/LasR and RhII/RhIR which, produce and recognize *N*-3-oxo-dodecanoyl homoserine lactone (3OC12HSL) and *N*-butanoyl homoserine lactone (C4HSL) respectively (**Figure 2A**) (102, 226). Among the genes activated by LasR is *rhII* which encodes the synthase RhII. Thus, the two systems have the particularity of functioning sequentially, so that LasI/LasR controlled genes are induced prior to RhII/RhIR-regulated genes. This hierarchical network circuit allows a temporal ordered sequence of gene expression that is critical for an efficient colonization (336). It is noteworthy that many players are involved in the broad regulation of the *las/rhI* systems. For instance, the LuxR homologue QscR has been shown to be part of the QS regulatory network but details of its action mode are still under investigation (53, 229).

There are QS systems that require signals from the environment. In the case of ***Agrobacterium tumefaciens***, which causes crown gall tumors in susceptible plants through transfer of part of the tumor-inducing (Ti) plasmid into the plant cell (91), this signal is provided by its host. Therefore, Ti-conjugation requires the presence of the autoinducer signal *N*-3-oxo-octanoyl homoserine lactone (3OC8HSL) (**Figure 2A**) (138) and a plant opine signal (26). This feature makes *A. tumefaciens* QS system remarkable since its activation is only achieved when bacteria encounter the plant cell opines

producer. It has been shown that the basic components of the *A. tumefaciens* QS system are the TraI/TraR pair of LuxI/LuxR homologues located in the Ti plasmid (138, 356). Conjugation of the Ti-plasmid occurs through the action of the TraR, which is thought to be transcriptionally induced by opines via AccR (26) and active when it binds to the AHL produced by the synthase TraI (335). Infection of the plant cell subsequently causes the overproduction of opines which, additionally to promote conjugation of the Ti plasmid, are also a source of nutrients for the colonizing bacteria, providing an advantageous environment where this plant pathogen can proliferate (335). Another important feature of this system is that TraR requires its specific ligand, the 3OC8HSL, for stability and accumulation *in vivo*. In the absence of its ligand TraR is a rapid target for proteolysis and it has been suggested that 3OC8HSL stabilizes TraR during translation being integral to the folding process (246, 335, 362). The *A. tumefaciens* system represents an example where the bacterial QS mechanism is only operational in the presence of a host signal and more examples like this are being discovered (271) exemplifying how bacteria can detect the presence of their host and regulate behavior accordingly.

Some QS systems differ from the paradigm since they rely on receptors with recognition capacity for broad range of AHLs. *Erwinia carotovora* subs. *carotovora* (Ecc), a Gram-negative enterobacterium that causes soft-rot in several plant hosts, including potatoes, possess one of these promiscuous AHLs receptors. Ecc infects the plant tissue by releasing a massive amount of extracellular plant cell wall -degrading enzymes (PCWDEs) (21). To establish successful infections the production and secretion of PCWDEs is controlled by a complex regulatory network, including a LuxI/LuxR-type QS circuit (21). Different Ecc strains produce different types of AHL and, accordingly, possess distinct cognate pairs of LuxI/LuxR homologues (49). As the strain SCC3193 was used in this study, discussion will focus on this organism; yet, the variety observed in the QS regulatory networks of closely related Ecc strains is

notable, exemplifying that QS circuits are custom-made systems for specific environmental requirements and colonization of specific niches. The Ecc strain SCC3193 synthesizes mainly 3OC8HSL, along with smaller amounts of 3OC6HSL (**Figure 2A**) via ExpI and has two LuxR homologues, the ExpR1 and ExpR2 (21, 283). It has been shown that ExpR1 has specificity for the cognate 3OC8HSL while ExpR2 is promiscuous as it can sense several acyl chain lengths. Perhaps the presence of two ExpR proteins with different binding capacities could permit Ecc to sense neighboring bacteria by detecting and responding to foreign AHLs (283). Both ExpR1 and ExpR2, without ligand, activate the transcription of the global regulator RsmA which inhibits PCWDEs production. However, in the high cell density mode, when AHL accumulates in the media, ExpR-AHL complexes are formed and there is no transcriptional activation of *rsmA*. As a consequence expression of downstream genes such as those for PCWDEs is activated and virulence is enhanced (21, 283).

Although phylogenetically *Vibrio harveyi* is closely related to *V. fischeri*, its QS circuit deviates significantly from the LuxI-LuxR canonical outline (**Figure 3** and **4**). This bioluminescent free-living bacteria species is an important pathogen of several marine animals. Bioluminescence, metalloprotease production and type III secretion, are controlled by production and detection of three different signals: the species-specific *N*-3-hydroxybutanoyl homoserine lactone (3OHC4HSL) (**Figure 2A**), the Ea-C8-CAI-1 that is also produced by many *Vibrio* species (**Figure 2C**) and autoinducer-2 (AI-2) that can be synthesized by a wide range of different bacterial species (**Figure 2D**) (41, 44, 125, 128, 219, 220, 274). The *V. harveyi* QS system (**Figure 4**) can be considered a hybrid between Gram-positive and Gram-negative canonical QS systems; detection of the three autoinducers occurs via membrane-bound histidine kinases similar to that in Gram-positive bacteria (described in the next section) as opposed to LuxR-type receptors present in Gram-negative bacteria (219). The autoinducers 3OHC4HSL, CAI-1 and AI-2

are produced by LuxM, CsqA and LuxS synthase, and detected by the membrane receptors LuxN, CqsS and LuxPQ, respectively. At low cell density, in the absence of ligand binding, the cognate membrane sensors, which under this condition act as histidine kinases, converge upon and phosphorylate a single phosphotransferase cytoplasmatic protein, LuxU (330). Phosphorylated LuxU then transfers the phosphate to the response regulator, LuxO, that in turn activates the transcription of five small regulatory RNAs, Qrr1-5. These together with the chaperone Hfq destabilize *luxR* mRNA (315). Therefore, at low cell density, the QS master transcription regulator LuxR is not expressed. As cell density increases and more autoinducer is present, binding of these ligands to their cognate receptors switches their activity from that of kinase to phosphatase, reversing the phosphate flow through the pathway and allowing accumulation of the LuxR protein (219) (**Figure 4**). (Note that the *V. harveyi* LuxR is not homologous to the LuxR described previously in canonical LuxI/LuxR-type QS circuit). One aspect that is remarkable in *V. harveyi* QS system is the Qrr1-5 *modus operandi*. These five small RNAs work additively, regulating LuxR with different strength: single deletion of one sRNA alters the phenotypic output. Furthermore, expression is intrinsically regulated by feedback loops ensuring a precise timing of the transition from low to high cell density (315-317).

The convergent detection mechanism that *V. harveyi* uses to respond to its multiple signals raises the question of how and whether the different autoinducers can be distinguished by and relate to distinct phenotypic outputs for this organism. Several studies pointed out that various amounts and combinations of the three autoinducers cause different phenotypic responses, which can be a reflection of the signal strength and its impact in the signal transduction cascade, for example at the level of LuxR activation and promoter affinity (126, 188, 212, 331). These evidences together with the different source of the three *V. harveyi* autoinducers indicate that this microorganism has the tools to monitor the species community not only in terms of numbers but also in terms of composition.

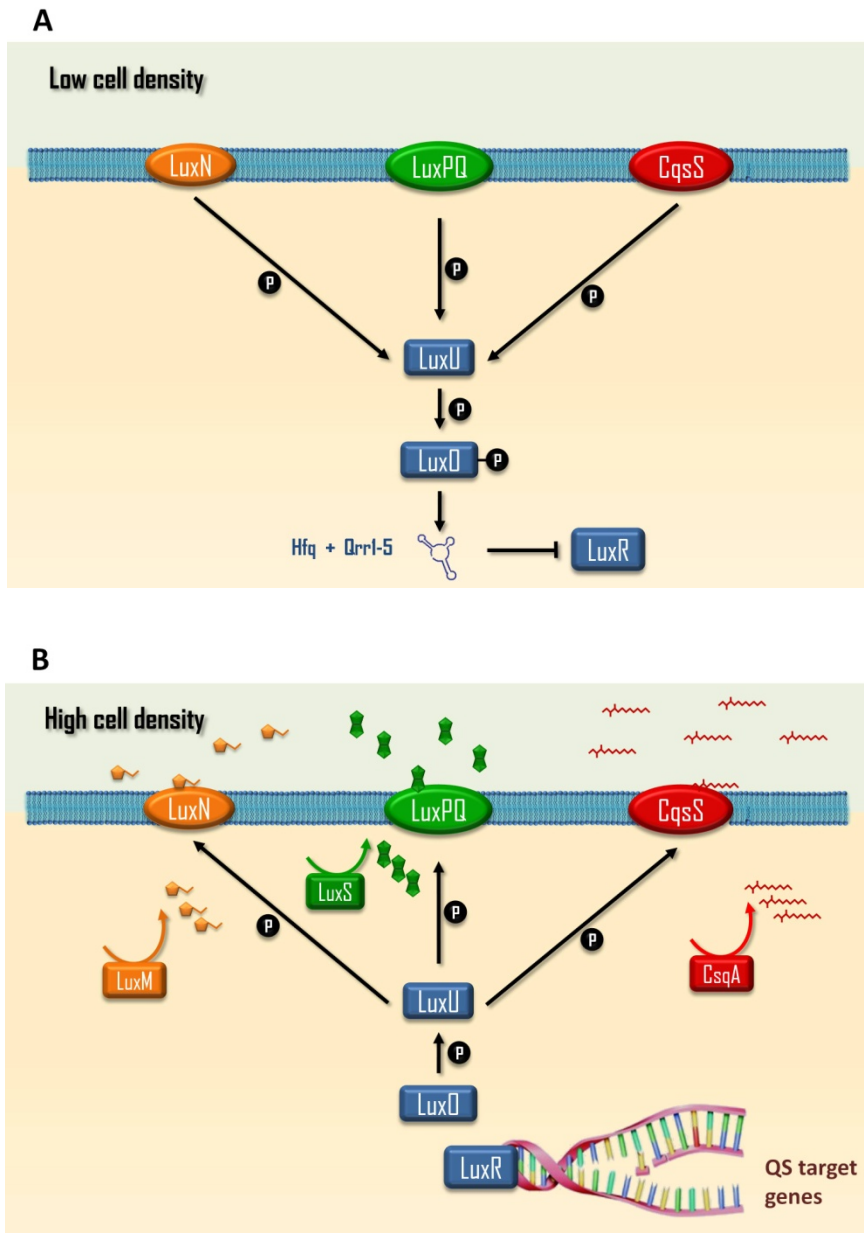


Figure 4 – *V. harveyi* QS system. **A)** At low cell density the membrane receptors LuxN, LuxPQ and CqsS work as kinases phosphorylating LuxU and then LuxO that activates the transcription of the five regulatory small RNAs, Qrr1-5 which in turn destabilize the LuxR mRNA. **B)** At high cell density the autoinducers accumulate in the media and bind the membrane receptors, which then function as phosphatases draining the phosphate from LuxO. Unphosphorylated LuxO does not induce the transcription of Qrr1-5 allowing the production of LuxR protein and regulation of the QS regulon.

2.1.2 – Autoinducing peptides

Gram-positive bacteria communicate primarily using modified oligopeptides as autoinducers that are extremely specific for each species or even strain (**Figure 2B**). These autoinducing peptides (AIP) typically consist of 5-17 amino acids often posttranslationally modified by the incorporation of lactone and thiolactone rings, lanthionines and isoprenyl groups. AIPs cannot freely diffuse through the membrane and are secreted via a specific exporter (**Figure 5**). During transport the signal precursor is processed and modified; AIPs can then be detected by a membrane-bound two-component system. Signal detection through binding to a transmembrane sensor kinase (SK) alters the enzymatic activity of this protein. Following autophosphorylation of a histidine residue, this phosphate is then transferred to an aspartate on a second protein, the response regulator. Phosphorylation of this response regulator (RR) alters its DNA binding affinity and regulates expression of QS target genes (**Figure 5**) (reviewed in (23, 42, 142, 219, 330)).

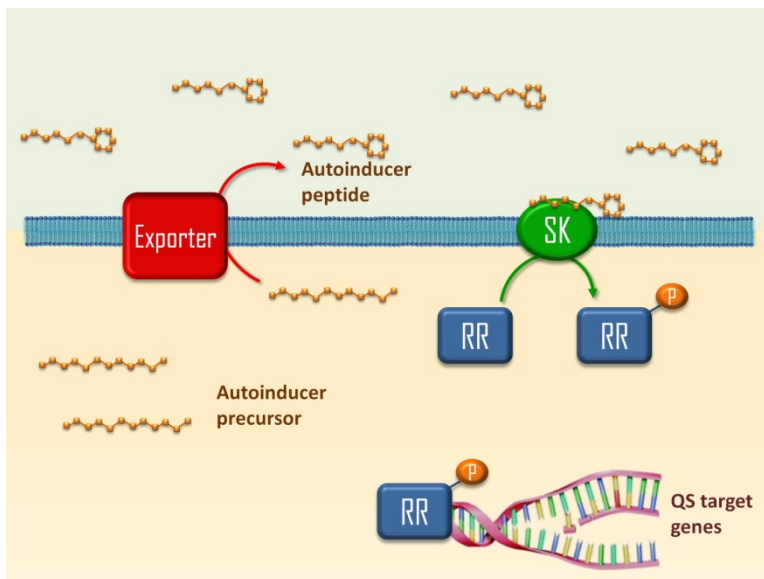


Figure 5 – Canonical AIP two-component QS circuit from Gram-positive bacteria.

QS in *Staphylococcus aureus* occurs at the strain level and is thought to be a speciation factor. This organism is a common inhabitant of the human microbiota present in 30% of the adult population. Nevertheless, under the right conditions, such as injured skin, it becomes a lethal pathogen frequently associated with antibiotic resistance (107). The expression of several virulence factors is regulated by QS through the *agr* (for accessory gene regulator) locus (82). The AIP (**Figure 2B**) is encoded by the *agrD* gene (147) and exported with concomitant processing by the AgrB protein (222). Detection of AIP by the AgrC histidine kinase activates its own phosphorylation and subsequent phosphate transfer to AgrA. Next, active AgrA induces the expression of the regulatory factor RNAIII. As in most QS systems, AgrA also activates the expression of its own promoter causing a positive feedback loop (222), however, unlike any other QS system, the final effector molecule is the regulatory RNAIII rather than the response regulator AgrA (223). RNAIII represses the expression of surface proteins such as cell adhesion factors and upregulates the expression of secreted factors (82). This allows a strategic transition from early colonization to late infection phase (reviewed in (106, 107, 221)). One important aspect of *S. aureus* QS system is the existence of four AIP specific groups categorized as I-IV (**Figure 2B**). These groups are defined by the allelic region *agrBDC* and consequent variation in AIP sequence. AIPs and their cognate receptors AgrC coevolved in such a way that a single amino-acid substitution can alter group specificity (105). Remarkably, besides activating its own set of genes, AIPs produced by one group inhibit the signal transduction cascade in all other groups, presumably by competitive antagonism of the AgrC receptor (146, 191, 192). The *S. aureus* group that first activates its own QS system presumably outcompetes secondary *S. aureus* groups through consequent effects on virulence gene expression and invasion, benefiting its own siblings to the detriment of non-kin. In addition, clinical isolates show that each *S. aureus* group causes predominantly one specific type of infection (221). This evidence indicates that the *agr* QS system has been a major determinant of strain divergence throughout *S. aureus* evolution.

Some QS circuits allow transient phenotypes that are followed by reversion to the original state. *Streptococcus pneumoniae* is a typical commensal of the nasopharyngeal flora that can occasionally become pathogenic and cause mortality in humans. One aspect that contributes to *S. pneumoniae* virulence is its ability to incorporate free DNA into its genome. This process, called competence, was the first phenotype described to be QS regulated. The macromolecular cell product which Tomasz and his colleagues in 1964 reported to regulate the absorption of external DNA (314) was proven to be an AIP that Havarstein and his coworkers designated competence-stimulating peptide (CSP) (**Figure 2B**) (120). CSP is produced from the ComC precursor and is modified through export by ComAB (137). CSP detection and response again occurs via a two component system, with autophosphorylation a sensor kinase, ComD and downstream phosphorylation of the response regulator, ComE (238). Phospho-ComE directly regulates gene expression of a series of downstream QS targets termed the early and late competence genes. Early genes include *comAB* and *comCDE* causing a positive feedback loop, as well as *comX*, the expression of which activates transcription of the late genes. What is remarkable about the *S. pneumoniae* QS system is that the competent state is transient and occurs only during a brief period in the exponential phase of bacterial growth. The positive feedback loop of *comAB* and *comCDE* enables a quick entry into the competent state (238) however the regulators responsible for the transition out of this state are not yet fully characterized.

2.1.3 – *Pseudomonas* quinolone signal

Together with the two previously described AHL signals, the *Pseudomonas* quinolone signal (PQS) (**Figure 2E**) also plays a key role in the QS structural design of *P. aeruginosa*. PQS, in conjunction with Las and Rhl QS systems, controls the expression of multiple virulence factor such as elastase (8). The expression of the *pqsABCDE* operon, required for the synthesis of PQS, is indirectly activated by the LasR/AHL complex, and PQS

sequentially induces transcription of *rhIR*. Consequently the PQS-dependent QS acts as a regulatory link between the two AHL-induced QS systems; impairment of any of these systems attenuates *P. aeruginosa* virulence (336). One important feature of the PQS is its high hydrophobicity and consequent mechanism of trafficking within a *P. aeruginosa* population. The PQS is packaged into membrane vesicles that arise from the interaction of the signal with the cell envelope. These membrane vesicles fuse with the recipient cell where they deliver the signal (199). These findings represent a resourceful bacterial strategy of signaling traffic that further widens potential vocabulary for cell-cell communication.

2.1.4 – Diffusible signal factor

Diffusible signal factor (DSF) (**Figure 2F**) is a family of *cis*-unsaturated fatty acids first described to regulate exopolysaccharide production and virulence in *Xantomonas campestris* (20, 327). Data suggests that the DSF is produced by the synthase, RpfF from this organism, and recognized in the periplasm by the two-component system, RpfCG (284). Recent evidence showed that other DSF related molecules are produced by the phylogenetically distant bacteria *Burkholderia cenocepacia* (**Figure 2F**), *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Xylella fastidiosa* (33, 65, 98), suggesting a broad distribution of DSF signaling in the bacterial kingdom. Interestingly, several lines of evidence have shown that each species can produce a family of DSF structurally related fatty acids (133). The chemical structure, number and concentration of the DSFs produced has been shown to be dependent on the culture medium (122). The physiological importance of the multiple DSFs production by a single species remains unknown, but could be a part of an exquisitely regulated system to combine intra-specific signaling, with a mechanism that reflects the environmental conditions in which bacteria are inhabiting (reviewed in (265)).

2.1.5 – γ -butyrolactones

Streptomyces is a genus of Gram-positive, soil-inhabiting bacteria that possess a productive secondary metabolism and a complex morphological development. In ***Streptomyces griseus*** this morphological differentiation is regulated, among other factors, by the autoinducer molecule 2-isocapryloyl-3R-hydroxymethyl- γ -butyrolactone, called A-factor (**Figure 2G**) (158). γ -butyrolactone biosynthesis is not well-characterized; however studies indicate that in *S. griseus* the enzyme AfsA is essential for the A-factor production. The recognition of the A-factor and, other γ -butyrolactones so far studied, involves the binding of the molecule to a cytoplasmic receptor called ArpA in *S. griseus*. These receptors are usually repressors, consequently when the receptor- γ -butyrolactone complex is formed, inhibition of the DNA target is released and induction of transcription occurs. Several other γ -butyrolactones have been found in at least seven different *Streptomyces* species; all regulate secondary metabolism, including the production of pharmaceutically valuable antibiotics. Although the chemical structure of γ -butyrolactones and AHLs differ only in the carbon-side-chain at present no evidence for cross-communication between these two signals exists (reviewed in (131, 307)).

2.2 – Worldwide broadcast: Non-species-specific signaling

Bacteria inhabit very diverse niches, from thermal vent environment to plant root surroundings and can even be found in clouds. These niches are frequently shared by an amazing variety of bacterial species that often rely on each other to maintain their normal physiological functions. With this in mind, it would be unlikely that members within a multispecies population would disregard their neighbor's actions. Despite this, the study of inter-species signaling is still in its initial stage. The increasing recognition of the astonishing bacterial variety within microbial communities highlights the importance and arising need for new creative approaches in microbiology. It seems plausible that in order to better understand inter-species signaling systems, microbiologists will shift from the mono-species experimental setups to poly-species cultures or natural consortia, such as those occurring within the bacterial hosts.

2.2.1 – Orphan AHL receptors

As complete bacterial genomes are being sequenced it is increasingly evident that numerous microbial species possess homologues of the AHL receptor LuxR but lack a cognate LuxI AHL synthase (47). This group of unpaired proteins has been called the orphan LuxR family and their role in bacterial inter-species signaling has been subject of recent research (296).

For example, though bacterial species of the genera *Escherichia*, *Salmonella* and *Klebsiella* lack the LuxI synthase and, therefore, do not produce any AHL, they possess a LuxR homologue called SdiA (286). Because these organisms are not able to produce AHLs it was suggested that SdiA could have a role in inter-species signaling since it could detect AHLs produced by other microbial species. Supporting this hypothesis, it has been shown that, in *Salmonella*, SdiA recognizes AHL generated by other bacterial species (205), regulating the *rck* (for resistance to complement killing) operon (6) and a gene of unknown function, *srgE* (286), which have no homologues in *E. coli* genome. Based on the observed inhibition of polymerization of a complement component by Rck, it has been suggested that this outer membrane protein could have a role in protecting the bacterium from mucosal complement in the gastrointestinal tract (55). Furthermore, Dyszel and coworker showed that *Salmonella* SdiA is active in mice infected with the AHL-producing pathogen *Yersinia enterocolitica* (287), however, deletion of the *sdiA* gene had no effect in *Salmonella* virulence towards mice, chicken or bovine models of disease (287) which makes it unclear whether responding to AHL from another organism is in fact important in the pathogenicity process. For *E. coli*, the identification of genes regulated by SdiA has been somewhat problematic: several independent studies identified genes that showed to be regulated by *E. coli* *sdiA* when it was overexpressed from a plasmid (154, 249, 328, 332). However, recently it was demonstrated that the genes that respond to plasmid-based expression of *sdiA* are different than those that respond to *sdiA* expressed from its chromosomal locus, indicating that physiological expression levels are important (84). The same study

demonstrated that *sdiA*, transcribed from its natural position in the chromosome, regulates several genes involved in glutamate dependent acid resistance regulation and flagella assembly (84), however the role of SdiA in inter-species signaling requires further investigation.

2.2.2 – Autoinducer-3

Autoinducer-3 (AI-3) has been extensively studied by Sperandio and colleagues in enterohemorrhagic *E. coli* and in *S. Typhimurium*. Though the chemical structure of this molecule has not yet been determined, it was demonstrated that AI-3 activates virulence genes through two two-component systems, QseBC and QseEF (57, 136, 290). The fact that AI-3 is produced by many bacteria frequently found amongst the intestinal flora (324) and the finding of several Qse homologues in the genome sequences of many bacterial species (251) led to the proposal that AI-3 has a function in inter-species communication. Production of AI-3 was initially associated with the *luxS* gene, however current results have shown that, although *luxS* mutants are affected in signal production, this is due to the alter metabolism in the *luxS* mutant strains that leads to decreased levels of AI-3 (324). Furthermore, this signal-response system also respond the eukaryotic hormones epinephrine and norepinephrine; it has been therefore described as an inter-kingdom system (57).

2.2.3 – Non-species specific DSF signaling

P. aeruginosa and *S. maltophilia* frequently share environmental niches such as the rhizosphere or the cystic fibrosis lung. In studies with *P. aeruginosa*, biofilm architecture was strongly affected by coculture with *S. maltophilia* or exogenous addition of synthetic *S. maltophilia* DSF. This effect was depended upon the synthesis of this diffusible signal, as deletion of *rpff* gene, encoding the DSF synthase, abrogated the structural defects of the pathogen's biofilm (266). Signal perception by *P. aeruginosa* was also shown to occur via the two-component system PA1396/PA1397 (266).

2.2.4 – Autoinducer-2

AI-2 is undoubtedly the best studied inter-species signal. To date, it is the only signaling molecule identified that is produced by many bacteria including Gram-negative and Gram-positive species (300). Many species of bacteria have been shown to control a variety of niche-specific behaviors in response to this signal, and so far, two classes of AI-2 receptors have been identified (51, 210). The next chapter will exclusively focus on AI-2 signaling, first by giving a brief historic overview and further by discussing the use of AI-2 signal in QS by various bacteria.

CHAPTER II

Autoinducer-2 Quorum-sensing

*"He who knows no foreign language, knows nothing of his own."
- Johann Wolfgang von Goethe*

1 – The molecule that has been talked about: AI-2 Quorum-sensing

The first example of a signal producing enzyme with homologues found in phylogenetically diverse bacteria (both Gram-negative and Gram-positive) is the LuxS enzyme (300), which catalyzes the synthesis of autoinducer-2 (AI-2). The initial evidence for inter-species communication emerged following the work of Greenberg in which he “contaminated” his *Vibrio harveyi* cultures with cell-free supernatant from other bacterial species. *V. harveyi* was not oblivious to his marine neighbors and responded by producing light (115). The Greenberg experiment was one of many breakthroughs in microbial chemical ecology which have lead to a better understanding of bacterial inter-species signaling.

1.1 – “Universal” signal: a historical perspective on AI-2 signal

The term AI-2 was first used by Bassler and colleagues to describe the second Quorum-sensing (QS) autoinducer found in *V. harveyi* (24). The existence of a second signal-response system became evident after the observation that *V. harveyi* mutants defective in the synthesis of AHL remained capable of activating the expression of QS dependent genes (24). In addition, the production of light by *V. harveyi* in response to culture supernatants from other unrelated bacteria led to the hypothesis that, this system, involving AI-2, was widespread amongst bacterial species (22, 115). In the late 1990’s, the gene responsible for AI-2 activity, designated *luxS*, was finally identified and homologues were found in several sequenced genomes (300). In every *luxS*-containing species studied, AI-2 activity was detected in the extracellular media, promoting the idea that bacteria use AI-2 to communicate between species (22, 300). Two years later the AI-2 biosynthetic pathway was determined using a genomic approach (274). Locations of the various *luxS* genes were analyzed and shown to be in the

proximity of *pfs* and *metK*, genes which encode enzymes involved in the utilization of *S*-adenosylmethionine (SAM) through the activated methyl cycle (AMC) (274). In this cycle a toxic product, *S*-adenosylhomocysteine (SAH), is formed and converted by Pfs to *S*-ribosylhomocysteine (SRH). Schauder and colleagues showed that LuxS was the previously unidentified enzyme that catalyzes the reaction of SRH to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), having the latter AI-2 activity (274). Subsequently, AI-2 could be produced *in vitro* using purified LuxS and Pfs proteins with SAH as a precursor (274, 338). Thereafter the AI-2 chemical structure was revealed. Purification and chemical characterization of the signal molecule did not yield success, but trapping the signal in the *V. harveyi* membrane receptor revealed AI-2 to be a furanosyl borate with no similarity to previously characterized autoinducers (51) (**Figure 2**). Later, a second AI-2 receptor crystal structure from *S. Typhimurium* identified a non-borated *R*-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) ligand (210). These differences demonstrated that the LuxS enzyme, irrespective of the producing bacterial species, generates a common molecule, DPD, which spontaneously interconverts into an array of AI-2 signaling molecules, providing a mechanism by which communication between different bacterial species can occur. Characterization of the sensing and signal transduction mechanisms in *V. harveyi*, *V. cholerae* and *E. coli* (25, 209, 306, 345) led to the first study showing that AI-2 produced by one species induces gene expression of another confirming that AI-2 can be use to communicate between different species (343).

Since the discovery of *luxS*, hundreds of studies have been published to further understand AI-2 signaling: mutants of *luxS* have been made in more than forty bacterial species; microarrays studies have been performed by several independent groups; and five LuxS protein structures have been determined. Despite this wealth of research, the question as to how widely in nature AI-2 signaling is used to promote inter-species communication remains unanswered.

1.2 – The chemical nature of the signal: AI-2 synthesis, structure and activity

AI-2 is produced by the enzyme LuxS that functions in the AMC (206, 274), an important metabolic pathway that recycles SAM, the major methyl donor in cell metabolism. The release of the activated methyl group from SAM to an acceptor molecule gives rise to SAH, a toxic intermediate that has to be converted in one of two distinct pathways. In eukaryotes, archaea and some bacteria SAH is directly hydrolyzed to adenosine and homocysteine in a reaction catalyzed by SAH hydrolase (SahH). In bacteria that possess LuxS, SAH is first converted by the Pfs enzyme into SRH that serves as intracellular source of substrate to the LuxS enzyme, which forms DPD and homocysteine (51, 274, 321) (**Figure 1**). DPD is a very reactive molecule that, in solution, spontaneously rearranges into a collection of chemically distinct molecular forms which contain AI-2 activity (**Figure 2**).

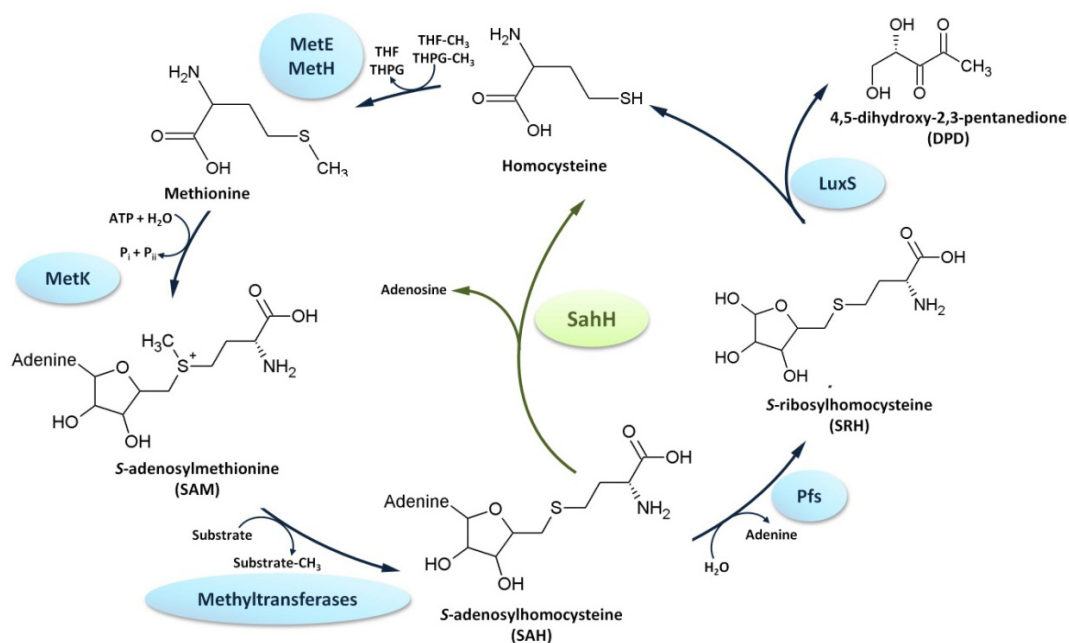


Figure 1 – The activated methyl cycle and DPD production. The methyl donation from SAM to an acceptor leads to the formation of the toxic intermediate SAH. Some bacteria, archaea and eukaryotes convert SAH directly into homocysteine using SahH (depicted in green). In bacteria that possess LuxS homologues SAH is converted in two steps: first into SRH and adenosine by the Pfs enzyme and secondly SRH is catalyzed by LuxS leading to the formation of homocysteine and DPD.

So far, two distinct AI-2 forms derived from DPD were identified by trapping the signal into the membrane receptors, LuxP and LsrB, from *V. harveyi* and *S. Typhimurium* respectively (51, 210, 306) (**Figure 2**). The crystal structure of the *V. harveyi* LuxP-AI-2 complex revealed that its ligand is a furanosyl borate diester, S-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (S-THMF borate) (51), a cyclic form of DPD bound to borate. Conversely, in *S. Typhimurium*, AI-2 is detected by the periplasmic binding protein LsrB in the form of R-THMF, a molecule that does not contain boron. These two molecules are an example of the array of different chemical configurations that DPD can easily interconvert to (**Figure 2**) but at the moment only two of these forms have been shown to be detected by AI-2 receptors (210).

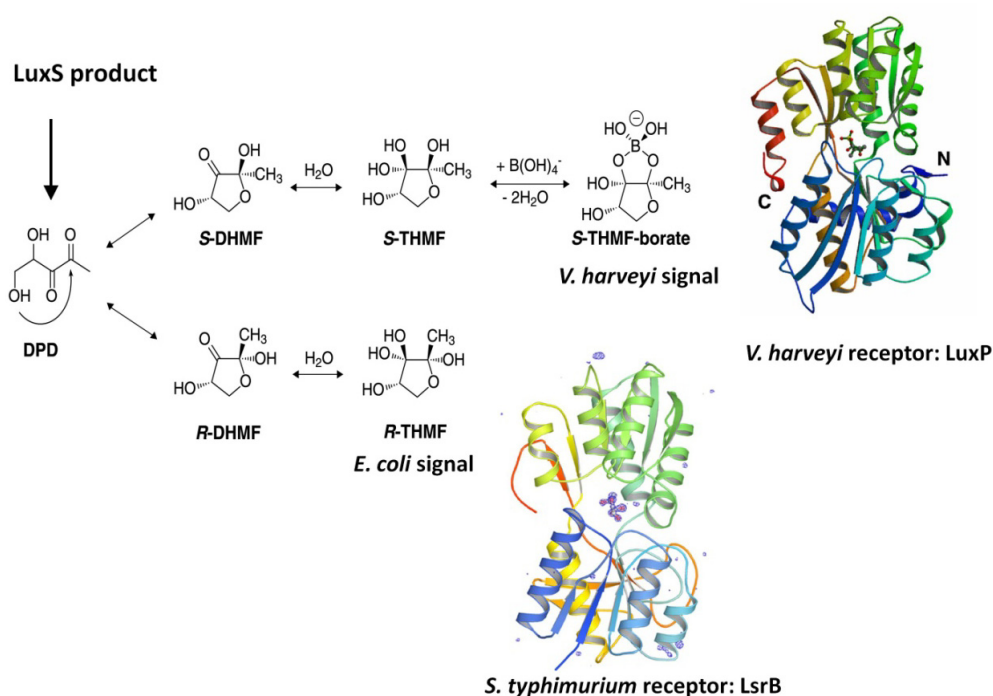


Figure 2 – Collection of AI-2 chemical structures and their *V. harveyi* and *S. typhimurium* receptors. The LuxS product DPD spontaneously undergoes cyclization and hydration to form R-THMF (the AI-2 form detected by *S. Typhimurium*) and S-THMF which can, by borate addition, lead to the formation of S-THMF-borate (the AI-2 form detected by *V. harveyi*). The structure of *V. harveyi* LuxP (51) and *S. Typhimurium* LsrB AI-2 receptors in conjunction with their respective AI-2 ligands (210) is shown. The ribbon diagrams are colored in rainbow order from N- to C terminus

The presence of boron is essential for the AI-2 response in *V. harveyi* and when boric acid is supplied in the media the equilibrium is shifted towards borated forms of the signal, which inhibits AI-2 signaling in *S. Typhimurium*: hence, borate availability alters the equilibrium of AI-2 interconversion and its signaling activity (210). These facts indicate that the state of equilibrium of AI-2 molecules reflects the environmental conditions and could be a method for bacteria to interpret both their biotic and abiotic context. For example, *V. cholerae*, a species that uses AI-2 to control biofilm formation and production of virulence factors, has two extremely distinct lifestyles in two distinct niches: the human intestine and the aquatic environment. The different concentration of boron in these two environment could be inferred by *V. cholerae* in the detected AI-2 molecule and used as source of information to decide between different life cycle stages (92).

To date, the findings on AI-2 chemistry indicate that a conserved biosynthetic pathway produces a molecule, DPD, which can interconvert into different chemical configurations such that different bacteria recognize distinctly rearranged DPD moieties ultimately defined by the chemistry of the surrounding environment.

1.3 – AI-2 in metabolism vs. AI-2 signaling: Are they mutually exclusive?

As LuxS is an enzyme in a crucial metabolic pathway, it is plausible that in some species, AI-2 does not act as a signal molecule *per se* but is actually an extracellular waste product. This does not, however preclude a role for AI-2 in signaling. In fact, it is clear that in *V. harveyi* and *V. cholerae*, AI-2 is used as a QS signal. Other species also appear to do so, but further and more accurate investigation is required to clarify the AI-2 function in these species. Inactivation of *luxS* could result in changes in gene expression as a consequence of defective methionine metabolism. Therefore, it is extremely important, in any given *luxS* mutant strain, to discriminate between metabolic and signaling defects. The experimental tools to clarify this dual effect of the *luxS* mutation have only recently become available. Several organic synthesis methods have been developed to synthesize DPD (11, 68, 99, 153, 203, 276), and this chemical is also commercially available (Omm Scientific). Chemical

complementation with pure DPD has been a successful method in determining the role of AI-2 as a QS molecule. Selected examples of the best studied cases are given in the next section. In the future it is expected that the increasing number of experiments with chemical complementation will clarify the AI-2 signaling or metabolic functions in most of the bacterial species. Furthermore, as the molecular mechanisms of AI-2 recognition and signal transduction are discovered, the manipulation of strains in AI-2 perception ability will allow the discrimination between the metabolic and signaling functions of LuxS.

It is predicted that the role of LuxS will not be the same in all bacteria. Based on the current knowledge it is likely that, in some bacteria, LuxS will act as a sole metabolic component of the AMC while in others it will have a dual function in signaling and metabolism. Additionally, in the latter case, since AI-2 results from a product of a metabolic process, it can provide information about the dimension, growth phase and physiological status of the bacterial population (337, 344). In fact studies in *Salmonella* suggest that AI-2 production is a reflection of metabolic state of the cell (27). And even in species where the role of *luxS* appears solely restricted to the AMC, AI-2 produced by these bacteria could be used as an information source by other bacterial species within a mixed community, illustrating the complexity of determining the contribution of LuxS and AI-2 in the bacterial behavior.

1.4 – What has AI-2 been saying? Phenotypes regulated by AI-2 and *luxS*

Elucidating the possible role of AI-2 in regulating inter-species communication has been approached with great enthusiasm by the scientific community and prompted scientists to study *luxS*/AI-2 phenotypes in the most diverse bacteria (**Table S1**). Here several examples of *luxS*/AI-2 regulated phenotypes are described. The focus will be on AI-2 signaling in species containing LuxPQ or LsrB-like receptors and on studies where, although the mechanisms of sensing and signal transduction remain to be identified, it was demonstrated that AI-2 acts as a signaling molecule (**Table 1**).

Table 1 – Functions regulated by AI-2 signal ^a

Species	Functions regulated by AI-2	References
<i>Actinobacillus pleuropneumoniae</i>	Cell adherence, growth in iron limited medium and biofilm formation ^b	(181)
<i>Actinomyces naeslundii</i> and <i>Streptococcus oralis</i>	Mutualistic biofilm formation	(260)
<i>Bacillus cereus</i>	Biofilm formation ^b	(12)
<i>Borrelia burgdorferi</i>	Expression of several protein including the outer surface lipoprotein VlsE ^b	(322)
<i>Escherichia coli EHEC</i>	Chemotaxis towards AI-2, motility and HeLa cell attachment	(19)
<i>Escherichia coli K12</i>	AI-2 incorporation , biofilm formation ^b , motility ^b and chemotaxis towards AI-2 ^b	(111, 123, 345)
<i>Helicobacter pylori</i>	Motility	(248, 280)
<i>Moraxella catarrhalis</i>	Biofilm formation and antibiotic resistance	(10)
<i>Moraxella catarrhalis</i> and <i>Haemophilus influenza</i>	Mutualistic biofilm formation and <i>Moraxella catarrhalis</i> persistence in rodent model	(10)
<i>Mycobacterium avium</i>	Biofilm formation ^b	(104)
<i>Pseudomonas aeruginosa</i>	Virulence factor production	(80)
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium	Pathogenicity island 1 gene expression and invasion into eukaryotic cells	(52, 305, 306)
	AI-2 incorporation	
<i>Staphylococcus aureus</i>	Capsular polysaccharide gene expression and survival rate in human blood and macrophages	(358)
<i>Staphylococcus epidermidis</i>	Expression of phenol-soluble modulins peptides, acetoin dehydrogenase, gluconokinase, LrgB, nitrite extrusion protein and fructose PTS system subunit	(183)
<i>Streptococcus anginosus</i>	Biofilm formation in the presence of antibiotics	(3)
<i>Streptococcus intermedius</i>	Hemolytic activity, antibiotic susceptibility and biofilm formation	(4, 5)
<i>Vibrio cholerae</i>	Virulence factors production, biofilm production and protease production	(116, 148, 209, 360)
<i>Vibrio harveyi</i>	Bioluminescence, colony morphology, siderophore production, biofilm formation, type III secretion and protease production	(24, 25, 184, 188, 212, 331)

^a Whole genome expression profiles such as microarrays analysis were not included in this table

^b DPD supplemented to WT strain cultures

1.4.1 – AI-2 signaling in bacterial species containing identified AI-2 receptors

1.4.1.1 – LuxPQ-type AI-2 sensing system

V. harveyi and *V. cholerae* possess the most characterized AI-2 signal transduction pathways. In the periplasmic space, borated-AI-2 is recognized by LuxP (**Figure 2**) and the resulting LuxP-AI-2 complex interacts with the sensor histidine kinase LuxQ, converting its enzymatic activity from kinase to phosphatase. This alteration in LuxQ protein function changes the flux of phosphate in the downstream phosphorylation cascade affecting the activity of the global regulator (as described in section 2.1.1 – Acyl homoserine lactones of Chapter I). This allows the system to change from a low cell density to a high cell density state with the corresponding changes in expression of genes involved in processes such as bioluminescence in *V. harveyi*, and production of virulence factors, in *V. cholerae*. As the multiple QS systems present in *V. harveyi* and *V. cholerae* respond to their distinct signals through convergent signaling pathways, the effect of the *luxS* mutation in these organisms is not always obvious. Nevertheless, it is well established that the presence of AI-2 modulates the activity of the global QS regulators LuxR and HapR in *V. harveyi* and *V. cholerae*, respectively, and thus the downstream expression of QS-controlled genes (126, 212, 331). Interestingly, LuxP antibodies were detected in people convalescing from cholera infection, indicating that AI-2 signaling might be active during infection (118). The components of the AI-2 signal transduction pathway have homologues in other *Vibrio* species, such as *Vibrio parahaemolyticus* and *Vibrio vulnificus*, and their QS systems have so far been shown to function in a similar fashion to those described above (125, 277, 351).

1.4.1.2 – LsrB-type AI-2 sensing systems

In enteric bacteria, like *S. Typhimurium* and *E. coli*, AI-2 released to the extracellular media is incorporated into the cell by the Lsr (for LuxS regulated) ATP-binding cassette (ABC) transport system at late exponential phase (305, 306, 345). Analogous to well-studied ABC transporters such as the ribose and maltose transport systems (34, 35), AI-2 bound to the ABC transporter through the interaction with the periplasmic binding protein LsrB, passes through the two transmembrane domains, LsrC and LsrD, and into the bacterial cell. Energy from ATP hydrolysis catalyzed by the ATPase, LsrA, drives this transport. Once inside the cell, the signal is phosphorylated by the Lsr kinase (LsrK) producing AI-2-phosphate (AI-2-P). Genes encoding the *lsr* ABC transport system are in an operon which is regulated by the Lsr repressor (LsrR): in the absence of AI-2-P, LsrR represses the transcription of the *lsr* operon (347), however, when AI-2-P accumulates in the cell, it binds to LsrR (347, 349), allowing de-repression of the operon and thus causing rapid depletion of AI-2 from the extracellular media (**Figure 3**). Intracellular AI-2-P is further processed by two enzymes, the isomerase, LsrG, and the putative aldolase, LsrF (73, 198, 347). LsrG catalyses the isomerization of AI-2-P to 3,4,4-trihydroxy-2-pentanone-5-phosphate (P-TPO); neither the metabolic fate of this compound nor the function of LsrF are known. Nevertheless it is clear that LsrG and LsrF are involved in the processing of the intracellular AI-2-P and thus can terminate Lsr induction (198). It was shown by Nuclear magnetic resonance (NMR) that AI-2-P accumulates in all cell extracts of *lsrG* mutant and that overexpression of *lsrG* results in degradation of AI-2-P. Because the levels of intracellular AI-2-P correlate directly with induction of the *lsr* operon transcription, these results constitute another evidence that AI-2-P is the main inducer of the *lsr* operon.

The physiological function of the Lsr system is not yet clear: why *E. coli* produces AI-2 to then incorporate and process it is still a matter of debate, nevertheless, it was shown that *E. coli* can use the Lsr system to interfere with AI-2 controlled behaviors of other species of bacteria which inhabit the same environment (343).

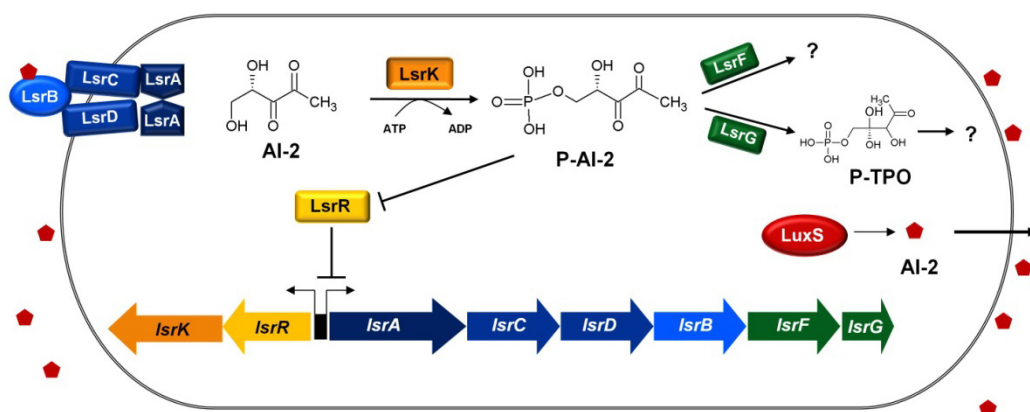


Figure 3 – *S. Typhimurium* and *E. coli* Lsr-mediated transport and processing of AI-2. In enteric bacteria AI-2 is produced by LuxS and accumulates in the extracellular medium. As its concentration increases, AI-2 binds to the periplasmic binding protein LsrB and is internalized by an ABC-type transport system: the Lsr system. Upon internalization, AI-2 is phosphorylated by LsrK. AI-2-P induces Lsr by binding and inactivating the transcriptional repressor LsrR. This causes a rapid increase in the production of the Lsr transporter and uptake of extracellular AI-2. AI-2-P is further processed by LsrG and LsrF.

Several studies have been conducted using *E. coli* and *S. Typhimurium*, including pathogenic strains as well as other bacterial species that have homologues of the Lsr system, which aimed to elucidate the AI-2-mediated signaling in these organisms. Addition of DPD to WT *E. coli* K12 stimulated biofilm formation, motility and chemotaxis towards AI-2. Addition of AI-2 to *lsrK* mutants failed to stimulate biofilm formation, however additional experiments, such as testing biofilm formation in *luxS* and *lsrB* mutants, are necessary to fully clarify this process (111, 123). Recently it was shown that deletion of *luxS* in *E. coli* K12 increases swimming motility and flagella synthesis (185). AI-2 was also shown to attract **enterohemorrhagic *E. coli*** (EHEC) *luxS* mutant in a concentration dependent manner, and to regulate motility and attachment to HeLa cells by this strain (19). Similarly, an **enteropathogenic *E. coli*** (EPEC) *luxS* mutant was also affected in motility but when associated with epithelial cells. Curiously, growth of this mutant in epithelial cell-free preconditioned medium restored motility and ability to produce flagella, suggesting the presence of a signal provided by mammalian cells is able to rescue the effect of the *luxS* mutation upon flagella function (109). Upon infection of rabbits, the *luxS* mutant showed reduced clinical

illness and adherence to the intestinal mucosa, demonstrating that AI-2 signaling may play a role in colonization and pathogenesis (359). Overall, these studies show that in *E. coli*, *luxS* gene expression and AI-2 signaling are important for proper regulation of several phenotypes, many of them crucial for virulence; however, further clarification of the role of the Lsr system, specifically the LsrB receptor, in the signal transduction pathways that regulate the described traits is necessary.

***Salmonella enterica* subspecies *enterica* serovar Typhimurium**

luxS mutants are impaired in several behaviors, namely biofilm formation, swarming, swimming and chick infection (68, 145, 156, 161, 244, 289). Exogenously supplied signal did not recover the biofilm impairment phenotype in ***S. Typhimurium*** (68), and unfortunately no pure DPD was used to verify whether the other phenotypes could be rescued by signal complementation, so it remains unclear whether such behaviors are due to AI-2 signaling or disruption of the SAM metabolism (145, 161, 289). Interestingly, constitutively expressed *luxS* could not rescue the biofilm formation in *S. Typhimurium* whereas the introduction of a functional gene driven by its own promoter in a plasmid was successful (68). This suggests that regulatory elements in the promoter region of *luxS* are important for biofilm formation and is an indication that the concentration and timing of AI-2 production could be crucial for a successful DPD complementation in this organism. The potential complex complementation of AI-2 signal in the species *S. Typhimurium* could explain the contradictory studies on AI-2 function in this species. While Choi *et al.* showed that *luxS* mutant phenotypes such as induction of virulence genes, invasion of epithelial cells and pathogenicity in the mouse model of infection, could be restored by supplying the signal to the cultures (52), Perrett and colleagues could not rescue the same *luxS* mutant phenotypes by chemical complementation (237). The fact that *S. Typhimurium* has an LsrB-like AI-2 receptor and it demonstrates several reported LuxS-dependent phenotypes that could be related either to metabolism or signaling, together with its genetic tractability and sequence

availability makes this organism an attractive system to further clarify the potential role of AI-2 signaling.

Photorhabdus luminescens, a species that contains the orthologues for the Lsr system, possesses a peculiar life cycle with two host-associated stages: a symbiotic stage, in which the microorganism lives in the gut of a nematode; and a pathogenic stage, in which the bacteria in association with the nematode infects and kills a wide variety of insect larvae. Studies in this organism showed that *luxS* plays a role in the regulation of carbapenem production, a broad-spectrum antibiotic thought to prevent contamination of the insect by other microorganisms (71). A global expression profile study, comparing WT with a *luxS* mutant grown in the presence of enzymatically synthesized AI-2, showed that the AI-2 signal regulates more than 108 targets in *P. luminescens luminescence*. Among the regulated genes were homologues of all the genes of the *E. coli lsr* operon, indicating that *P. luminescens* has the ability to import and process AI-2. Other genes that encode outer membrane-associated proteins, such as flagella and pili, that mediate virulence and that promote resistance to oxidative stress were also AI-2 sensitive; however, the corresponding phenotypic assays were performed without signal complementation (166). Therefore, the role of AI-2 signaling in *P. luminescens* remains to be explored, and it is important to determine whether AI-2 regulates motility, biofilm formation and resistance to oxidative stress through the LsrB receptor. Importantly, these phenotypes play a crucial role in the early steps of insect invasion; therefore such studies may also shed a light on the importance of AI-2 signaling in both the symbiosis and pathogenicity of *P. luminescens*.

The research of Demuth and colleagues has advanced on understanding AI-2 signaling in (94, 95, 141, 278, 279) ***Aggregatibacter actinomycetemcomitans***. This microorganism is an inhabitant of the oral commensal flora, and is associated with severe infections in the oral cavity. It is the only species so far where successful chemical complementation and lack of complementation by expression of SahH have clearly shown that LsrB is acting as a signal receptor controlling AI-2-dependent behaviors, such as

biofilm formation. The *A. actinomycetemcomitans luxS* gene is required for leukotoxin production and growth under iron limiting conditions, regulating the expression of several genes involved in iron transport and storage (94, 95). Biofilm formation, under iron-replete conditions, is defective in a *luxS*-deficient strain and this impairment can be rescued by addition of partially purified AI-2. Interestingly, the genetic introduction of the enzyme SahH did not complement the *luxS* mutation, indicating that in this organism the LuxS function is not strictly metabolic. This bacterium appears to have two AI-2 receptors, LsrB and RbsB. RbsB was proposed as a second receptor because purified periplasmic protein from this species inhibits bioluminescence of *V. harveyi* reporter strain and competes with the AI-2 receptor LsrB. By using phenotypic and *in vitro* protein signal binding competition assays, the Demuth group has shown that RbsB interacts with AI-2, playing a role in the response of *A. actinomycetemcomitans* to the signal (141, 278, 279). Accordingly, inactivation of both AI-2 receptors, LsrB and RbsB revealed a biofilm formation defect similar to a *luxS* mutant strain. These results strongly indicate that in *A. actinomycetemcomitans* the AI-2 signal is indeed necessary for biofilm assembly and that its detection in the periplasmic space occurs by LsrB and RbsB.

1.4.2 – AI-2 signaling in other Gram-negative pathogens

Given the expectation that QS manipulation might provide a therapeutical approach to control bacterial infections, understanding the role of AI-2 in the regulation of virulence related traits is of extreme importance. ***Helicobacter pylori***, an agent of gastric diseases, such as stomach ulcers and gastric cancer, is one of the few organisms where the dual role of LuxS in metabolism and signaling has been addressed. *H. pylori* depends on the *luxS* gene for biofilm formation and motility (58, 248). Investigations of the signaling effect of *luxS* using qRT-PCR, phenotypic assays and chemical complementation showed that *H. pylori* uses AI-2 signaling to control motility via flagellar gene transcription (248, 280). *H. pylori luxS* mutants also exhibit a reduced ability to infect the gastric mucosa in a Mongolian gerbil model of

infection (230). It would be interesting to explore whether this reduction in infectivity is due to the motility impairment in a *luxS* mutant strain and, if exogenous AI-2 could restore infection in the animal model. Ultimately, the development of AI-2 chelators could be a novel therapeutic approach to control *H. pylori* associated diseases. The role of LuxS in metabolism was also explored in *H. pylori* where it was demonstrated that *luxS* has a previously undescribed metabolic function in cysteine production rather than the previously described classical metabolic role in the AMC (75). This study also ruled out the possibility that the motility impairment observed in a *luxS* mutant originated from a disruption of cysteine metabolism because exogenously added cysteine could not restore motility to the *luxS* mutant whereas DPD did (75). Overall this data shows that LuxS, besides being a central metabolic enzyme, can in some organisms, act as a synthase of signaling molecules.

Borrelia burgdorferi is one of the causative agents of Lyme disease, a chronic relapsing infection that affects multiple body systems and produces a range of symptoms in humans. Although some studies reveal that *luxS* expression is not required for *B. burgdorferi* infection (32, 135), others show that addition of AI-2 to WT cultures results in differential protein expression, including VlsE (293, 294), an outer surface lipoprotein thought to play a major role in the immune response to Lyme disease (86). Since *B. burgdorferi* is not able to convert homocysteine (the other product of LuxS enzyme) into methionine, it would be important to clarify if LuxS has an alternative role in metabolism such as in *H. pylori*, where LuxS is required for cysteine production. If that is not the case this could be an example of a species where LuxS function is restricted to AI-2 signal synthesis (15, 261, 322).

The bacterium ***Actinobacillus pleuropneumoniae*** is the causative agent of porcine pleuropneumonia and, therefore, a pathogen of major economic importance to the swine industry. In this organism, *luxS* inactivation causes impaired biofilm formation, cell adherence, growth under iron limited medium and virulence towards mice. Biofilm experiments showed

that signal complementation could not rescue the *luxS* phenotype, however supplying DPD to WT cultures increased biofilm formation (181). These results could be explained by the fact that the metabolic effect of the *luxS* mutation is prevailing over the signaling effect of AI-2. In contrast, impaired cell adherence and growth defects observed for the *luxS* mutant in iron restricted conditions was rescued by DPD complementation. However this result was not consistent with the fact that the impairment in cell adherence was not rescued by co-infection with a mixture of WT and *luxS* mutant bacteria. It is possible that complementation by WT was unsuccessful because the AI-2 levels were not high enough (181). The *A. pleuropneumoniae* experiments performed by Li *et al.* show that AI-2 has a broad signaling effect in this microorganism and also highlight the need of meticulous experiments to elucidate the AI-2/*luxS* role in bacteria.

1.4.3 – AI-2 signaling in Gram-positive pathogens

So far no AI-2 sensing mechanism has been identified in Gram-positive bacteria; however, there is evidence that bacteria can respond to AI-2, as described in the examples below.

Streptococcus is a genus containing several clinically significant species responsible for numerous infections such as pharyngitis and scarlet fever. In the species ***Streptococcus anginosus*** and ***Streptococcus intermedius***, *luxS*-deficient strains have shown lower cell densities when exposed to antibiotics such as ampicillin. Chemical complementation restored the levels of antibiotic susceptibility to those of the WT strain (3, 4). Additionally, biofilm production and hemolytic activity in *S. intermedius* was shown to be AI-2 dependent (5, 234). These results indicate that AI-2 works as a QS molecule in *Streptococcus* and call for a comprehensive investigation on the molecular bases of AI-2 detection and signal transduction in this Gram-positive genus.

In a study conducted in ***Staphylococcus aureus***, the most common agent of staphylococcal infections, *luxS* mutants grown in a sulfur-limited

defined medium exhibited a growth defect but as signal complementation could not restore growth to the WT level, it was suggested that in this organism, the LuxS effect was due to metabolism and not signaling (74). Additionally, *luxS* gene inactivation did not alter any of the virulence-associated traits analyzed (74). In contrast, Zhao and colleagues showed using qRT-PCR that genes related to the synthesis of the virulence determinant, capsular polysaccharide (*cap* gene cluster), and the two-component system genes *kdpDE*, were downregulated by LuxS and AI-2 in a dose-dependent manner. As KdpDE downregulates *cap* gene expression, and the regulatory protein KdpE can bind to the promoter region of the *cap* operon, this suggests that the KdpDE two-component system may mediate the AI-2 QS regulation of *cap* genes transcription (311). Research directly addressing if AI-2 regulation of *cap* gene expression is via the KdpDE is yet to be reported. Given that expression of *cap* genes enhances bacterial virulence (311) and the KdpDE system is upregulated during infection (323) the authors also examined the effects AI-2 signaling on the survival of *S. aureus* in human whole blood and human monocytic cells. They demonstrated that survival and growth of the *luxS* mutant was higher in both types of culture and that signal complementation restored the WT phenotype. These results indicated that AI-2 signaling, possibly through the KdpDE system, is involved in bacterial virulence in invasive *S. aureus* infection (358). A comprehensive investigation of KdpDE is crucial to understand if this two-component system represents a third class of AI-2 detection mechanism.

Staphylococcus epidermidis is a skin commensal that can cause severe infections in immune-suppressed patients. In this organism, AI-2 function was assessed using microarrays for a primary global analysis, with confirmation of a selection of the differentially transcribed genes by qRT-PCR. Transcription of genes encoding acetoin dehydrogenase, gluconokinase, LrgB, nitrite extrusion protein and a fructose PTS subunit was affected in the *luxS* mutant and subsequently restored to WT levels by exogenous addition of AI-

2, thus this signal regulates the transcription of these genes. Additionally, it was shown by liquid chromatography/mass-spectrometry of culture filtrates that the phenol-soluble modulins peptides, key virulence factors of *S. epidermidis*, are also under AI-2 signal control (183).

1.4.4 – AI-2 signaling in polymicrobial communities

In nature, bacteria are more likely to grow in polymicrobial communities containing a range of species than in mono-species cultures. It is thus important to elucidate the inter-species signal interactions between the community members that are required for such communities to develop and be maintained.

1.4.4.1 – AI-2 signaling in the nasopharyngeal microflora

Otitis media is a frequent pediatric disease in which the composition of the polymicrobial infection impacts treatment efficacy (129, 194). In a study addressing polymicrobial communities in disease, Armbruster *et al.* have shown that ***Haemophilus influenza*** promotes ***Moraxella catarrhalis*** persistence in biofilms and suggests that this effect is due to AI-2 (10). *M. catarrhalis* (a non-AI-2-producing species) showed increased biofilm biomass in the presence of AI-2 and, consequently, higher resistance to antibiotic treatment. Furthermore, using a rodent model to follow nasopharyngeal infection, it was shown that animals inoculated with *M. catarrhalis* alone had bacterial loads much lower than those coinfecting with both species. Additionally and most importantly, no increase in *M. catarrhalis* bacterial load was observed during coinfection with *H. influenza luxS* mutant, evidence in support of AI-2 secreted by *H. influenza* as the factor inducing biofilm formation, which increased persistence and antibiotic resistance during coinfection (10). It would be interesting to administrate DPD to the nasopharynx of the rodents to evaluate if this signal can mimic the effect of

H. influenza upon *M. catarrhalis* persistence, although this may be technically difficult. This is a good example where AI-2 analogues which would compete with the bacterially produced signal could be used in the future as an alternative therapeutic approach to antibiotics against polymicrobial infections.

1.4.4.2 – AI-2 signaling in the oral cavity microflora

The role of AI-2 signaling in the **oral cavity microflora** has been intensively explored. The surface of the teeth was the source of the first bacterial community observed by van Leeuwenhoek. He wrote: “The number of these *animalcules* in the scurf of a man’s teeth is so many that I believe they exceed the number of man in the kingdom”. These bacteria, described more than three centuries ago, comprise multispecies communities that exchange intra- and inter-species signals. AI-2 is one of these compounds and has been shown to be an essential player in the mutualistic biofilm formation of two human oral commensal bacteria: ***Actinomyces naeslundii***, a *luxS* deficient species, and ***Streptococcus oralis*** (260). Contrasting to biofilms containing WT bacteria from both species, mixtures of WT *A. naeslundii* and *S. oralis luxS* mutant produced biofilms with lower biomass which were rescued by chemical complementation in a concentration-dependent manner (260). Furthermore, a critical AI-2 concentration for biofilm production was determined, above or below which biofilm production was impaired. This fact is of extreme importance to the AI-2 research field as it emphasizes that exquisite signal sensitivity can be crucial in AI-2-regulated QS. Bacterial AI-2 communication might occur at a particular signal molecule concentration that is neglected in a standard experimental setup, in which a single AI-2 concentration is frequently tested.

A related study using mixed species was performed by McNab *et al.* where the *luxS* gene in ***Streptococcus gordonii*** was shown to be essential

for mutualistic biofilm formation, in this case with the periodontal pathogen *Porphyromonas gingivalis* (201). Together, these studies demonstrate that AI-2 acts as an inter-species signal and is critical in the development of mutualism between species of oral microflora.

1.4.5 – AI-2 signaling in non-AI-2 producers

Pseudomonas aeruginosa inhabits the oropharyngeal flora with a variety of other species and can cause mortal pulmonary failure in patients with cystic fibrosis. In a study performed by Surette and coworkers, the sputum of cystic fibrosis patients was analyzed to investigate the interactions between pathogenic *P. aeruginosa* and the common oropharyngeal microflora (80). Interestingly, the presence of the avirulent microflora exacerbated the lung injury caused by *P. aeruginosa* in a rat model. A significant amount of AI-2 was identified in the sputum samples and AI-2-mediated QS accounted for some upregulation of virulence factor production (80). This is one of the few published studies that explores the contribution of inter-species communication in bacterial pathogenicity in the context of the host environment. Furthermore, given that *P. aeruginosa* is a species that lacks *luxS* in its genome and does not produce AI-2, this study highlights that even in non-signal producers, AI-2 mediated QS could exist, broadening the range of potential partakes in the AI-2 conversation.

1.5 – LuxS role in AI-2 synthesis: experimental considerations

The fact that the AI-2 synthase LuxS is an enzyme involved in an important metabolic pathway raises concerns in the conclusions drawn from analysis of phenotypes of *luxS* mutants: these could either be a consequence of interruption of the AMC metabolic pathway or absence of the AI-2 QS signal. Genetic complementation by inserting the *luxS* gene *in trans* is insufficient to clarify its role in AI-2 signaling, since this approach also rescues the interruption of the methyl cycle and any potential metabolic defects. The use of cell-free culture media has been another strategy frequently used to

rescue the AI-2 signaling defects of *luxS* mutants. Nevertheless, data resulting from this procedure should be interpreted with great caution: as Winzer *et al.* suggested the conditioned media of WT EHEC and the corresponding *luxS* mutant is likely to differ significantly since the two strains have approximately 10% differences in gene expression (291, 318, 338, 339). Altered chemistry or/and concentration of extracellular compounds other than AI-2 could be responsible for the rescue of the *luxS* mutant phenotype. In fact this was the rationale behind the discovery of the AI-3 signaling molecule, which has been suggested to regulate several phenotypes in EHEC, independently of AI-2 (324, 325). To definitely show that AI-2 is a QS signal, chemical complementation with pure DPD must restore any defect attributed to the signaling function of the *luxS* gene mutation (as in the multiple cases reported above).

Chemical complementation is not always that straightforward: DPD addition to the extracellular media might need to reproduce *in vivo* conditions such as the timing of signal production, the signal concentration, any potential chemical modifications of the signal, and the percentage of the population responding to the signal (92). Furthermore, in certain bacterial species and growth conditions, the metabolic effects of deleting *luxS* could be so dramatic that any AI-2 signaling complementation which did occur would pass unnoticed. In these situations, one experimental possibility could be to supplement WT cultures with the signal. Cells are exposed to higher concentrations of AI-2 than they themselves produce; this might have a physiological relevance as in certain niches containing a mix of species, several AI-2-producing species naturally occur. An additional experimental complication that can occur in AI-2 signaling studies derives from potential signal redundancy in some QS systems. In these situations the effect of a *luxS* mutation in the phenotypic output can pass unnoticed, not only because of redundancy due to other signals converging upon shared transduction machinery, but also due to the use of phenotypic assays that are frequently qualitative and disregard slight changes. Examples of this situation occur with light production in *V. harveyi*, and virulence in *V. cholerae* (24, 92).

Together, these data point out that unsuccessful complementation of a *luxS* mutant with DPD does not necessarily provide evidence for an exclusive role of the LuxS enzyme in metabolism, and calls for a complete understanding of LuxS, AI-2 and AMC in bacterial physiology.

1.6 – Concluding remarks

Ten years have passed since the AI-2 synthase LuxS was shown to be present in a widespread range of bacterial species; however in early AI-2 investigations, there was no method for production of AI-2, and genetic or biochemical complementation with gene expression or conditioned media, respectively, were misleading methods in the investigation of LuxS function in AI-2 signaling. Nowadays, with the availability of commercially synthetic DPD, it is of great importance to confirm and clarify some of the previously reported phenotypes associated with *luxS* mutant strains of bacteria.

Most studies on AI-2, although referring to a signal that is produced by a wide range of bacterial species, analyzed the effects of the signal in an intra-species setup. This is not surprising given that AI-2 is produced in a culture composed of single species; however it does not address the role of AI-2 as a source of information between species. The information collected from such mono-species experiments could be distinct from the output when several species are cocultured. For instance, quorum could be reached earlier, or the amount of the detected species-specific signal (AHL, AIP or others) relative to AI-2 could reveal the numbers of a given species in a multi-species community. Novel experimental setups, such as the study of microbial communities in their natural host, will contribute to elucidation of AI-2 QS in polymicrobial environments. Upcoming studies using DPD in natural microbial consortia are likely to further this research into AI-2 as an inter-species signal.

2 – Objectives of the present research work

Despite the wealth of literature on AI-2 questions remain; it is still far from evident how this compound is generally used in bacterial communication. The fact that the molecular mechanisms of AI-2 recognition and signal transduction are still unidentified in most of the species responsive to AI-2 is one of the obstacles yet to be removed. In enteric bacteria a receptor for AI-2, LsrB, has been described and shown to be part of an ABC transport system that delivers AI-2 from the extracellular environment into the cell. The overall aim of this work was to gain a deeper understanding of AI-2 signaling by focusing on the Lsr recognition and processing system, not only in enteric bacteria but also in several other microorganisms with particular attention to *Sinorhizobium meliloti*. In summary research presented in this thesis has focused on the following topics:

- Identification of the PTS as a regulatory component contributing to the activation of the *lsr* operon;
- Identification of functional LsrB-like autoinducer-2 receptors and its phylogenetic analysis;
- Study of the Lsr transport system in *Sinorhizobium meliloti* and its role in inter-species signaling.

3 – Supplementary data

Table S1 – Functions regulated by LuxS and/or AI-2^a.

Species	Functions regulated by <i>luxS</i>	Phenotype rescued by			Functions regulated by AI-2	References
		Gene complementation	Supernatants	AI-2		
<i>Actinobacillus pleuropneumoniae</i>	Cell adherence	Yes	No	Yes	Cell adherence	(181, 182)
	Biofilm formation	Yes	Not shown	No	Inconclusive	
	Growth in iron limited medium	Yes	Yes	Yes	Growth in iron limited medium	
	Virulence in mouse infection model	Not shown	Not shown	Not shown	Inconclusive	
	-	-	-	Yes	Supplementation of WT culture media with DPD modulated biofilm formation	
<i>Actinomyces naeslundii</i> and <i>Streptococcus oralis</i>	Mutualistic biofilm formation	Yes	Not shown	Yes	Mutualistic biofilm formation	(260)
<i>Aeromonas hydrophila</i>	Biofilm formation, motility and virulence in mice model	Yes	Not shown	Not shown	Inconclusive	(165)
<i>Aggregatibacter actinomycetemcomitans</i>	Leukotoxic activity and iron transport	Not shown	Yes	Not shown	Inconclusive	(94, 95, 141, 279)
	Growth under iron limiting conditions	Yes	Not shown	Not shown	Inconclusive	
	Biofilm formation	Yes	Yes	Not shown	Inconclusive	
<i>Bacillus anthracis</i>	Growth	Yes	Not shown	Not shown	Inconclusive	(149, 150)

CHAPTER II

<i>Bacillus cereus</i>	Not shown	Not shown	Not shown	Not shown	Supplementation of WT culture media with DPD modulated biofilm formation	(12)
<i>Bacillus subtilis natto strain</i>	Biofilm formation, swarming and aerial architecture	Not shown	Not shown	Not shown	Inconclusive	(187)
<i>Borrelia burgdorferi</i>	Expression of several protein including the outer surface lipoprotein VlsE	Not shown	Not shown	Yes	Expression of several protein including the outer surface lipoprotein VlsE	(15, 261, 293, 294, 322)
<i>Campylobacter jejuni</i>	Motility, agglutination proprieties, toxin production, colonization of the chick and chemotaxis	Not shown	Not shown	Not shown	Inconclusive	(59, 87, 121, 130, 143, 144, 247, 253)
	Biofilm formation	Not shown	Yes	Not shown	Inconclusive	
	Adherence to chicken hepatoma cells.	Yes	Not shown	Not shown	Inconclusive	
<i>Clostridium perfringens</i>	Toxin production	Not shown	Yes	Not shown	Inconclusive	(45, 228)
<i>Edwardsiella tarda</i>	Growth	Not shown	No	Not shown	Inconclusive	(357)
	Type III secretion and biofilm formation	Not shown	Yes	Not shown	Inconclusive	
	Virulence	Not shown	Not shown	Not shown	Inconclusive	
<i>Eikenella corrodens</i>	Biofilm formation	Not shown	Yes (unpublished results)	Not shown	Inconclusive	(13, 200)
<i>Erwinia amylovora</i>	Motility, extracellular polysaccharide production, tolerance for hydrogen peroxide and virulence on pear leaves.	Not shown	Not shown	Not shown	Inconclusive	(103, 211, 257)
<i>Erwinia carotovora ssp. atroseptica SCRI1043</i>	Motility	Not shown	Not shown	Not shown	Inconclusive	(61)

<i>Erwinia carotovora</i> ssp. <i>carotovora</i> strain ATtn10	Virulence in plants	Not shown	Not shown	Not shown	Inconclusive	(61)
<i>Erwinia carotovora</i> ssp. <i>Carotovora</i> strain SCC3193	Plant tissue maceration and pectinolytic enzymes production	Yes	Not shown	Not shown	Inconclusive	(167)
<i>Escherichia coli</i> EHEC	Chemotaxis towards AI-2, motility and HeLa cell attachment	Not shown	Not shown	Yes	Chemotaxis towards AI-2, motility and HeLa cell attachment	(19, 288)
<i>Escherichia coli</i> K12	AI-2 incorporation	Yes	Yes	Yes	AI-2 incorporation	(70, 111, 185, 326, 345)
	Not shown	Not shown	Not shown	Not shown	Supplementation of WT culture media with DPD modulated biofilm formation and motility	
	Motility	Not shown	Not shown	Not shown	Inconclusive	
<i>Escherichia. coli</i> EPEC	Adherence to cultured HeLa cells	No	Not shown	Not shown	Inconclusive	(109, 359)
	Virulence in the rabbit model	Yes	Not shown	Not shown	Inconclusive	
	Motility	Not shown	Not shown	Not shown	Inconclusive	
<i>Fusobacterium nucleatum</i> and <i>Porphyromonas gingivalis</i>	Mutualistic biofilm formation	Yes	Not shown	Not shown	Inconclusive	(267)
<i>Haemophilus ducreyi</i>	Virulence	Not shown	Not shown	Not shown	Inconclusive	(168)
<i>Haemophilus influenza</i> and <i>Moraxella catarrhalis</i>	Mutualistic biofilm formation and <i>Moraxella catarrhalis</i> persistence in rodent model	Not shown	Not shown	Not shown	Inconclusive	(10)
<i>Haemophilus influenzae</i>	Invasion of human cells, biofilm formation, virulence	Not shown	Not shown	Not shown	Inconclusive	(9, 63)

CHAPTER II

<i>Helicobacter pylori</i>	Biofilm formation and infection in mice model	Not shown	Not shown	Not shown	Inconclusive	(58, 97, 151, 186, 230, 248, 280)
	Motility	Yes	Yes	Yes	Motility	
<i>Klebsiella pneumoniae</i>	Biofilm formation	Not shown	Not shown	Not shown	Inconclusive	(17, 67)
<i>Lactobacillus acidophilus.</i>	Adherence to Caco-2 cells	Not shown	Not shown	Not shown	Inconclusive	(39)
<i>Lactobacillus reuteri</i>	Biofilm formation	Not shown	No	Not shown	Inconclusive	(309)
<i>Lactobacillus rhamnosus</i>	Growth	Yes	Not shown	No	Inconclusive	(172, 173)
	Biofilm formation	Yes	No	No	Inconclusive	
	Persistence in mice and EPS production	Not shown	Not shown	Not shown	Inconclusive	
<i>Listeria monocytogenes</i>	Biofilm formation	Not shown	No	Not shown	Inconclusive	
<i>Mannheimia haemolytica</i>	Virulence genes expression, level of encapsulation and adherence to bovine tracheal cells	Not shown	Not shown	Not shown	Inconclusive	(195, 320)
<i>Moraxella catarrhalis</i>	Not relevant	Not relevant	Not shown	Yes	Biofilm formation, antibiotic resistance and persistence in a rodent model	(10)
<i>Mycobacterium avium</i>	Not shown	Not shown	Not shown	Yes	Supplementation of WT culture media with DPD modulated biofilm formation	(104)
<i>Neisseria meningitidis</i>	Growth	Yes	Not shown	No	Inconclusive	(79, 127, 273, 341)
	Bacteremia	Yes	Yes	Not shown	Inconclusive	

<i>Photorhabdus luminescens</i>	Carbapen production, motility, biofilm formation and virulence	Not shown	Not shown	Not shown	Inconclusive	(71, 166)
<i>Porphyromonas gingivalis</i>	Hemin acquisition and arginine-specific protease production	Not shown	Not shown	Not shown	Inconclusive	(40, 54, 140, 353)
	Expression of hemin acquisition genes	Yes	Yes	Not shown	Inconclusive	
<i>Pseudomonas aeruginosa</i>	Not relevant	Not relevant	Not shown	Yes	Virulence factor production	(80)
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium	Flagellar Phase Variation	Yes	No	Not shown	Inconclusive	(37, 52, 68, 145, 156, 161, 237, 289, 305, 306)
	Virulence in the mice model	Not shown	Not shown	Not shown	Inconclusive	
	Pathogenicity island 1 gene expression and invasion into eukaryotic cells	Yes	Not shown	No	Inconclusive	
	Pathogenicity island 1 gene expression and invasion into eukaryotic cells	Yes	Not shown	Yes	Pathogenicity island 1 gene expression and invasion into eukaryotic cells	
	Persistence in the intestine, spleen and feces of chicks	Not shown	Not shown	Not shown	Inconclusive	
	AI-2 incorporation	Yes	Yes	Yes	AI-2 incorporation	
	Swarming and swimming	Not shown	Not shown	Not shown	Inconclusive	
	Biofilm formation	Yes	Not shown	Not shown	Inconclusive	

CHAPTER II

<i>Serratia marcescens</i>	Virulence in the <i>Caenorhabditis elegans</i> model	Not shown	Not shown	Not shown	Inconclusive	(60)
	Prodigiosin and haemolysin production	Yes	No	No	Inconclusive	
	Carbapenem antibiotic production	Yes	Yes	Not shown	Inconclusive	
<i>Shewanella oneidensis</i>	Biofilm formation	Yes	Not shown	No	Inconclusive	(171)
<i>Shigella flexneri</i>	Virulence factor VirB	Not shown	Yes	Not shown	Inconclusive	(66)
<i>Staphylococcus aureus</i>	Growth defect in a sulfur-limited defined medium	Yes	No	Not shown	Inconclusive	(74, 358)
	Capsular polysaccharide gene expression and survival rate in human blood and macrophages	Yes	Not shown	Yes	Capsular polysaccharide gene expression and survival rate in human blood and macrophages	
<i>Staphylococcus epidermidis</i>	Biofilm formation	Yes	Yes	Not shown	Inconclusive	(183, 348)
	Virulence in a rat model	Not shown	Not shown	Not shown	Inconclusive	
	Intercellular adhesion operon expression	Yes	Not shown	Not shown	Inconclusive	
	Expression of phenol-soluble modulins, peptides, acetoin dehydrogenase, gluconokinase, LrgB, nitrite extrusion protein and fructose PTS subunit	Not shown	Not shown	Yes	Expression of phenol-soluble modulins, peptides, acetoin dehydrogenase, gluconokinase, LrgB, nitrite extrusion protein and fructose PTS subunit	
<i>Streptococcus anginosus</i>	Biofilm formation	Not shown	Not shown	Not shown	Inconclusive	(3, 239)
	Biofilm formation in the presence of antibiotics	Not shown	Yes	Yes	Biofilm formation in the presence of antibiotics	

<i>Streptococcus gordinii</i> and <i>Candida albicans</i>	Mutualistic biofilm formation	Yes	Not shown	No	Inconclusive	(18)
<i>Streptococcus gordonii</i>	Biofilm formation	Yes	Not shown	Not shown	Inconclusive	(31)
<i>Streptococcus gordonii</i> and <i>Porphyromonas gingivalis</i>	Mutualistic biofilm formation	Yes	Not shown	Not shown	Inconclusive	(50, 201)
<i>Streptococcus intermedius</i>	Hemolytic activity, antibiotic susceptibility and biofilm formation	Not shown	Not shown	Yes	Hemolytic activity, antibiotic susceptibility and biofilm formation	(3, 5, 234)
<i>Streptococcus mutans</i>	Bacteriocin production	Not shown	Not shown	Not shown	Inconclusive	(134, 204, 333, 334, 352)
	Biofilm formation	Yes	Yes	Not shown	Inconclusive	
<i>Streptococcus pneumoniae</i>	Virulence in the mice model	Not shown	Not shown	Not shown	Inconclusive	(152, 262, 295)
	Competence	Not shown	Yes	Not shown	Inconclusive	
	LytA-dependent autolysis	Not shown	No	Not shown	Inconclusive	
<i>Streptococcus pyogenes</i>	Virulence factors production and virulence proprieties: hemolytic and proteolytic activity	Yes	Not shown	Not shown	Inconclusive	(197) (193, 282)
	Acidic stress tolerance and survival in epithelial cells and macrophages	Yes	Not shown	Not shown	Inconclusive	
<i>Vibrio alginolyticus</i>	Virulence in a fish model, growth, protease, EPS and biofilm formation	Not shown	Not shown	Not shown	Inconclusive	(313, 350)
	Motility and flagella biosynthesis	Yes	Yes	Not shown	Inconclusive	

CHAPTER II

<i>Vibrio cholerae</i>	Virulence factors production, biofilm formation, protease production	Yes	Yes	Yes	Virulence factors production, biofilm production, protease production	(116, 148, 209, 360)
<i>Vibrio fischeri</i>	Luminescence and colonization	Not shown	Not shown	Not shown	Inconclusive	(190)
<i>Vibrio harveyi</i>	Bioluminescence, colony morphology, siderophore production, biofilm formation, type III secretion, protease production	Yes	Yes	Yes	Bioluminescence, colony morphology, siderophore production, biofilm formation, type III secretion, protease production	(24, 25, 184, 188, 212, 331)
<i>Vibrio vulnificos</i>	Haemolysin and protease production	Yes	Yes	Not shown	Inconclusive	(159, 160)

^a Whole genome expression profiles such as microarrays analysis were not included in this table.

CHAPTER III

Activation of autoinducer-2 internalization and processing in enteric bacteria requires the phosphotransferase system

Autoinducer-2 (AI-2) is a signal molecule produced by a wide range of phylogenetically distant microorganisms which enables inter-species cell-cell communication and regulates several bacterial phenotypes. Certain bacteria can interfere with AI-2-regulated behaviors of neighboring species by incorporating extracellular AI-2 using the Lsr transport system (encoded by the *lsr* operon). Once inside the cell AI-2 is phosphorylated by the LsrK kinase. AI-2-phosphate (AI-2-P) is the inducer of the *lsr* operon: it acts by binding the LsrR transcriptional repressor which leads to derepression of the operon. Here we show that the phosphotransferase system (PTS) is required for Lsr activation and is essential for AI-2 internalization. Phosphorylation of Enzyme I (EI) from PTS is necessary for AI-2 incorporation but is not required for AI-2 phosphorylation, as even in the presence of PTS LsrK is essential for this phosphorylation to occur. We also show that the requirement for PTS in the activation of *lsr* transcription is via LsrR and is AI-2-dependent. Overall our results suggest that to initiate AI-2 internalization and intracellular processing, AI-2 has to be first incorporated by a PTS-dependent mechanism, whether directly or indirectly, which relieves *lsr* repression by intracellular AI-2-P. The Lsr transporter is expressed, which starts a positive feedback loop with the consequent fast removal of AI-2 from the extracellular medium. The fact that AI-2 internalization is dependent on both the AI-2-induced Lsr transporter and the PTS could represent a cell strategy to integrate information about its physiological state and, according to that, regulate AI-2 signal incorporation.

2 – Introduction

Bacteria rely on self-produced signals to coordinate behaviors at a population level. This mechanism of cell-cell communication, known as Quorum-sensing (QS), comprises the production, release and detection of extracellular signals, called autoinducers and culminates in the regulation of gene expression in a synchronized manner. Numerous chemically distinct molecules have been described as autoinducers but only Autoinducer-2 (AI-2) is produced by an enzyme, LuxS, that is widespread across the bacterial kingdom (92, 235, 274). In some species, it has clearly been shown that AI-2 is a QS signal molecule responsible for the regulation of several phenotypes, (3-5, 219, 260) in others, however, a restricted role as an extracellular waste product of central metabolism has been proposed (257). A more comprehensive investigation is required in most species to discriminate between metabolic and signaling effects of disrupting the AI-2 synthase.

Though LuxS-related phenotypes have been reported in more than 40 bacterial species, only two AI-2 receptors have been identified so far. The fact that the molecular mechanisms of AI-2 signal transduction and its regulatory networks remain largely uncharacterized is a major obstacle to understanding the role of this molecule as a signal in bacteria. Orthologues of the *Salmonella enterica* subspecies *enterica* serovar Typhimurium AI-2 receptor, LsrB, were found in organisms belonging to phylogenetically distinct families such as *Enterobacteriaceae*, *Rhizobiaceae*, and *Bacillaceae* (235). In these species AI-2 accumulates in the extracellular milieu as bacteria grow: during the late exponential phase it is recognized by the periplasmic ligand binding protein LsrB and internalized by the ATP-binding cassette (ABC) transporter, Lsr (for LuxS regulated). Upon internalization, intracellular AI-2 is phosphorylated by the kinase, LsrK. As a consequence, AI-2-phosphate (AI-2-P) is sequestered in the cytoplasm where it binds the transcriptional repressor, LsrR, and inhibits its binding to the *lsr* promoter as well as that of the *lsrRK* operon (326, 347, 349) (**Figure S3**). Thus expression of the genes encoding Lsr is induced, creating a positive feedback loop which causes a

rapid depletion of AI-2 from the extracellular media. Therefore, AI-2 induces its own internalization and phosphorylation (235, 305, 306, 345).

Several phenotypes, such as biofilm formation, motility and attachment to HeLa cells, have been hypothesized to be regulated through *luxS*/AI-2 QS via the Lsr system in *E. coli* (19, 111, 185), however the physiological consequences of AI-2 incorporation are still a matter of debate and will be subject of discussion in the last chapter of this thesis. Characterization of all the components involved in the network architecture controlling the AI-2 incorporation system is an essential step towards improved general understanding of the Lsr system's role in the bacterial behavior.

To gain a better understanding on the regulation of AI-2 internalization we performed a screen for genes encoding proteins involved in AI-2 internalization or its regulation in *E. coli*. The findings reported here show that these processes are dependent upon a functional phosphoenolpyruvate phosphotransferase system (PTS). In many bacterial species the PTS provides a mechanism for the translocation of a wide range of carbohydrates through the bacterial cell membrane, coincident with their phosphorylation which is required for intracellular sequestration. Our data suggests that, in order to initiate AI-2 internalization and subsequent induction of the Lsr transport system, AI-2 has to be first incorporated by a PTS-dependent mechanism. Phosphorylation of AI-2, however, requires LsrK. These results shed a light on the processes governing AI-2 internalization in enteric bacteria, bringing together two of the most important sensing systems in bacteria: the PTS and detection of the Quorum-sensing signal AI-2.

3 – Materials and Methods

3.1 – Bacterial strains and growth conditions

The *E. coli* strains and plasmids used in this study are listed in Supplementary material **Table S1** and **Table S2**, respectively. Wild-type (WT) *E. coli* K-12 strain MG1655 (30) was used as the parental strain for all subsequent genetic manipulations. *E. coli* strains were grown at 37°C with

aeration in Luria–Bertani (LB) broth supplemented with 100 mM MOPS buffer pH 7 (LBMOPS), except where otherwise mentioned. When necessary, 0.1 mM of Isopropyl beta-D-1-thiogalactopyranoside (IPTG) or 40 μ M of synthetically produced AI-2 (276) were supplied to the media at the time of inoculation, and antibiotics were used at the following concentrations (mg l⁻¹): ampicillin (Amp), 100; kanamycin (Kan), 50, and chloramphenicol (Cm), 25. MacConkey–lactose plates were prepared as described previously (207).

3.2 – Genetic and molecular techniques

Chromosomal deletions carrying the specified antibiotic resistance markers, from the keio collection single deletion mutants (14) and from previous work, were introduced by phage P1 transduction as described previously (281). All deletions were confirmed either phenotypically or by polymerase chain reactions (PCR) using Taq DNA polymerase (New England Biolabs). The *ptsIcrr::kan* double mutation was constructed using the red swap protocol described by Datsenko and Wanner (64) using the primers: fw *ptsI* wanner and rv *crr* wanner (**Table S3**). To construct in frame deletions, the Kan_R cassette was eliminated by introducing the FLP recombinase expressing plasmid pCP20 into the strains (64).

3.3 – Plasmid construction

To express the Lsr transport system under an IPTG inducible promoter, *lsrACDB* genes were cloned into pCA24N-no GFP from ASKA library (162). A 4.7 kb DNA fragment comprising the *lsrACDB* genes was amplified by PCR with using Bio-X-Act DNA polymerase (Bioline) to ensure greater accuracy using the primers: *lsr* operon fw 3 and *lsr* operon rv 3 (**Table S3**) and digested with NotI and SalI (New England Biolabs). The expression vector was also digested with NotI and SalI followed by ligation using T4 DNA ligase (New England Biolabs) to the *lsrCDBA* PCR product to construct the plasmid pCSP184 (**Table S2**). The nucleotide sequence was confirmed by sequencing one strand of the *lsrCDBA* genes carried out by the IGC sequencing facility. We introduced the plasmid pCSP184 into the strain AS108 by electroporation (269).

3.4 – Construction of *crp** gain of function mutation

To construct strains insensitive to catabolite repression, we added a *crp** gain of function mutation to a *cya* deletion background. Because a *cya* mutant cannot grow on glycerol, we used the P1 lysate from KX1468 to transduce the *crp** mutation into AS7 (*lsr-lacZ*, Δ *cya*) by selecting for growth on M63 medium containing glycerol. This step produced strain AS8 (*lsr-lacZ*, Δ *cya*, *crp**).

3.5 – Screen for regulators of *lsr* transcription

To identify genes involved in the regulation of the Lsr transport system, a transposon with a kanamycin resistance gene EZ-Tn5 was introduced into strain AS4. The transposon insertion was performed according to the manufacturers' instructions (Epicenter). The cells were infected with a multiplicity of infection of 1 and plated on LB plates containing kanamycin (50 mg.ml⁻¹). We screened approximately 5 000 mutant colonies for low levels of β -galactosidase activity when AI-2 was exogenously supplied in MacConkey-lactose agar plates. One mutant was selected to study. The transposon chromosome fusion junctions of the selected mutant were amplified by two step PCR using arbitrary primers and Tn5 specific primers. The insertion site was identified by DNA sequencing coupled with BLAST analysis.

3.6 – AI-2 activity assay

To measure extracellular AI-2 activity in *E. coli* cultures, overnight cultures were diluted (1:100) into LBMOPS medium in Erlenmeyer flasks and its growth was monitored. Aliquots were collected at the times indicated and used to analyze the optical density at 600 nm (OD₆₀₀) and to prepare cell-free culture fluids. The AI-2 detection and quantification in the cell-free culture fluids was measured using a LuxP-fluorescence resonance energy transfer (FRET) assay as previously described (250). Cell-free culture fluids were prepared by filtration of liquid cultures through 96-well filtration plates. For the determination of AI-2 concentration, 2.5 μ l of the cell-free fluid was added

to 280 μ l of purified CLPY FRET protein to a final concentration of 0.0125 mg/ml diluted in phosphate buffer. Results were compared to a calibration curve obtained from CLPY response to known concentrations of synthetic AI-2 prepared dilutions. Each sample was assessed in duplicates.

3.7 – β -galactosidase assays

Overnight cultures of *E. coli* were diluted 1:100 into LBMOPS and grown to the OD₆₀₀ indicated. Cells were harvested and resuspended in 1 ml of Z-buffer, and β -galactosidase assays were performed as described previously (285). β -galactosidase units are defined as $(OD_{420} \text{ min}^{-1} * 10^{-4}) / [OD_{600} * \text{volume (ml)}]$. All assays are reported as the mean β -galactosidase activity from triplicate data and error bars represent the standard error.

3.8 – Time course of LsrK production

To measure LsrK expression in *E. coli* strains during growth, overnight cultures were diluted (1:100) into LB medium and grown at 37°C for 4.5 hours. All cultures were then normalized to OD₆₀₀=1.0. Each culture was centrifuged and re-suspended in H₂O. Equal volumes were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were loaded on a 10% SDS-PAGE gel (269). Proteins were separated on the gel and blotted onto a nitrocellulose filter (Biorad). After blocking with 10% skimmed milk for 1 h at room temperature, the blot was incubated with anti-LsrK antiserum diluted 1:2000 in 10% skimmed milk for 16 h at 4°C with gentle mixing. After three washes of 10 minutes in TBS containing 0.1% Tween 20, the filter was incubated for 40 minutes at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgGs (Promega), which were diluted 1:10000 in TBS containing 0.1% Tween 20. The blot was then washed three times in TBS containing 0.1% Tween 20. Binding of the antibodies to the blot was probed by using the enhanced chemiluminescence light-based detection system ECL plus (GE healthcare) and visualized by phospho-imager (Storm 860).

3.9 – *In vivo* ^{13}C NMR

Each strain was cultured in LB to $\text{OD}_{600}=3$ and harvested by centrifugation at $7000 \times g$ for 10 min at 4°C . Cells were washed twice with 100 mM PIPES buffer, pH 6.6, containing 1 mM MgCl_2 ; cell pellets were resuspended in the same buffer to a final volume of 3 ml and a calculated OD_{578} of 70 to 80, which was transferred to a 10-mm NMR tube. D_2O was added to a final concentration of approximately 5% (vol/vol) in order to provide a lock signal. This mixture was incubated at 30°C with aeration. Efficient mixing and supply of oxygen to the cell suspension was achieved by bubbling oxygen and using an airlift system (96). At the initial time point [$1\text{-}^{13}\text{C}$] DPD (with a final concentration of 2 mM) was added to the cell suspension and the tube was introduced in the spectrometer. ^{13}C NMR spectra were acquired sequentially after the addition of [$1\text{-}^{13}\text{C}$]DPD and run on a Bruker AVANCE II 500 spectrometer (Bruker) equipped with a quadruple nucleus-probe head. The acquisition of ^{13}C NMR *in vivo* spectra was performed as previously described (218). Spectra were analyzed using Topspin (Bruker).

4 – Results

4.1 – Analysis of the *Lsr* mutants phenotypes

In *E. coli* extracellular AI-2 activity peaks and promptly declines in late exponential phase. While in the parent strain culture extracellular AI-2 is not detected by 5 h, the *lsrCDB* mutant strain takes more than 8 h to reach similar levels. Therefore, the loss of AI-2 from the culture media is only partially a consequence of its import by the *Lsr* transporter: mutants in the genes for the transport components (*lsrCDB*) remove AI-2 significantly slower than wild type (WT) but are still capable of removing the molecule from to culture fluid. This observation suggests that another AI-2 transport system exists (**Figure 1A**). In contrast, extracellular AI-2 reaches a higher concentration and AI-2 internalization is drastically reduced in the *lsrK* mutant (**Figure 1A**) thus, *LsrK* activity, that is, AI-2 phosphorylation, is required to sequester the signal in the cytoplasm independently of the mechanism of AI-2

internalization. Consistent with these results, transcription of the *lsr* promoter (measured using an *lsr-lacZ* promoter fusion inserted at the lambda attachment site), is only slightly impaired in the *lsrCDB* mutant in comparison to that which occurs in the WT strain, whereas no induction of the promoter occurs in the *lsrK* mutant strain (**Figure 1B**). The lack of activation of the Lsr system in the absence of LsrK (**Figure 1B**) confirms that all phosphorylation of AI-2 is through LsrK, independent of which transport system is functioning. Overall, these results indicate that, besides the Lsr transporter, another AI-2 transport system exists and even the alternative AI-2 internalization system requires LsrK for intracellular sequestering.

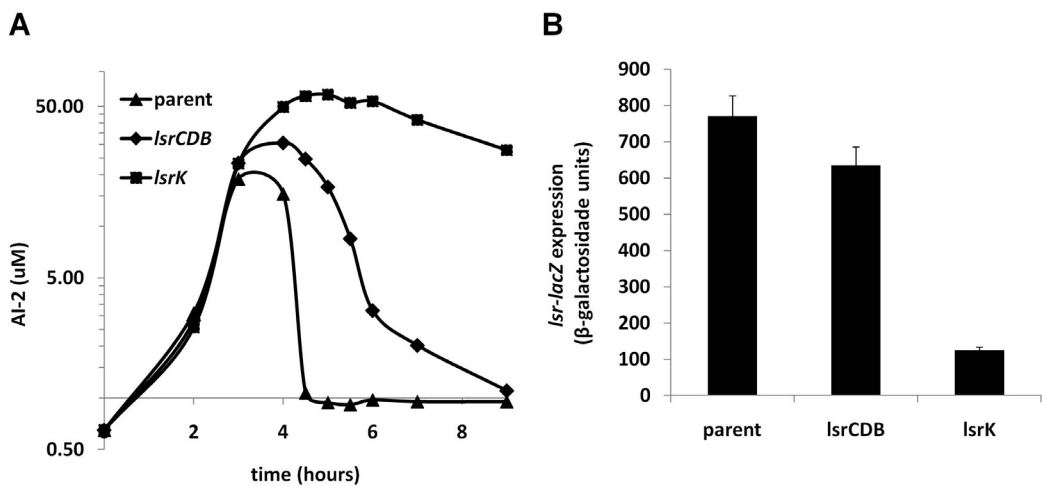


Figure 1 –Phenotypes of Lsr mutants in *E. coli*. Extracellular AI-2 activity **A)** and transcription of the *lsr* operon at OD₆₀₀=4 **B)** was analyzed in the following strains: parent (KX1123 – *lsr-lacZ*), *lsrCDB* (CJG23 – *lsr-lacZ*, *lsrCDB*), *lsrK* (KX1186 – *lsr-lacZ*, *lsrK*).

4.2 – *ptsIcrr* mutants are impaired in AI-2 internalization

To identify new genes involved in internalizing and sequestering AI-2 in the cytoplasm, we constructed a *Tn5::Kan* transposon library using the strain AS5 (*lsr-lacZ*, *cya*, *crp**, *luxS*, *lsrCDB::Cm*) as the parental strain. It is known that the *lsr* regulon of *E. coli* is catabolite-sensitive (345), so we used a *cya*, *crp** double mutant to avoid obtaining mutants strains affected in *lsr-lacZ* regulation due to altered cAMP levels: the *crp** gain of function mutation does not require cAMP for activation. An *lsrCDB* deletion background was used so

that AI-2 incorporation could occur only via the secondary transporter. Using MacConkey lactose indicator plates, we screened for transposon mutants with low *lacZ* expression in the presence of exogenously supplied AI-2, that is, in genes that are required to activate the *lsr* promoter by direct regulation, or indirectly by AI-2 internalization through an alternative transport mechanism. Selected candidates were transduced into an AI-2-producing strain, AS9 (*lsr-lacZ*, *cya*, *crp**, *lsrCDB::cm*), and tested for their ability to internalize AI-2.

One transposon insertion that resulted in low *lsr-lacZ* expression measured in MacConkey lactose indicator plates was in the *ptsI* gene (**Figure 2**) which encodes Enzyme I (EI) of the PTS. As shown in **Figure 2** transduction of the *ptsI::Tn5* mutation to an AI-2 producing strain results in a AI-2 incorporation profile similar to the *lsrK* mutant. Although this strain has a growth defect (**Figure S1**), it is insufficient to explain the severe defect in AI-2 internalization, however it is likely to explain the slight delay in AI-2 accumulation when compared to the *lsrK* mutant strain. Thus in the *ptsI* mutant strain there is either no internalization or phosphorylation of AI-2, and the molecule is not sequestered intracellularly.

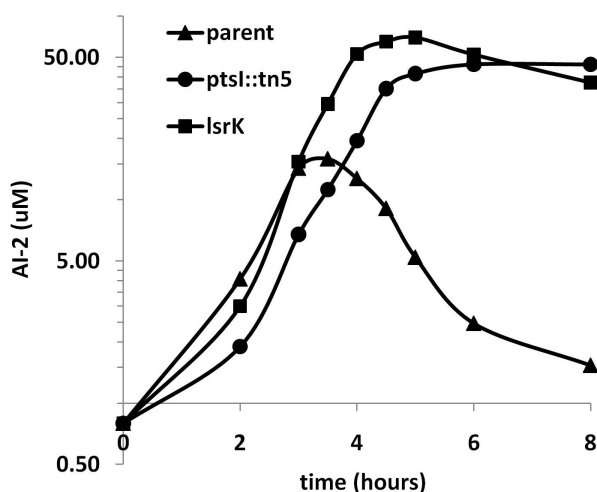


Figure 2 – *ptsI* transposon mutant is impaired in AI-2 incorporation. Extracellular AI-2 activity in cell free culture fluids of the following strains: parent (AS9 – *cya*, *crp**, *lsrCDB*), *ptsI::tn5* (AS70 – *cya*, *crp**, *lsrCDB*, *ptsI::tn5*) and *lsrK* (AS90 – *cya*, *crp**, *lsrCDB*, *lsrK*).

4.3 – Both components of the PTS: EI and EIIA^{glc} are required for WT AI-2 incorporation and *lsr* expression

EI is a general PTS protein participating in the phosphorylation of all PTS sugars, whereas the role of the EIIA^{glc} role is restricted to the translocation of certain sugars such as glucose and trehalose. The *ptsI* and *crr*, genes (coding for EI and EIIA^{glc} respectively) are cotranscribed as an operon with a promoter region located upstream of *ptsI* (243). Therefore, the phenotype of the *ptsI*::Tn5 mutation could be the result of the disruption in *ptsI*, polar effects on *crr* expression, or both. To test these possibilities the accumulation of extracellular AI-2 was measured in *ptsI* and *crr* single mutants and in a *ptsIcrr* double deletion mutant (Figure 3). Both single *ptsI* and double *ptsIcrr* mutants showed a significant impairment in AI-2 removal from the culture media (Figure 3A) as well as a decrease in *lsr* transcription (Figure 3B). Complementation of the *ptsI* single mutant by expression from a plasmid demonstrated that this mutation did not have polar effects on *crr* (data not shown). As the *crr* mutant showed an intermediate phenotype both in terms of AI-2 uptake and *lsr* expression (Figure 3), this demonstrates that *E. coli* requires both a functional EI and EIIA^{glc} for WT levels of AI-2 internalization and activation of *lsr* expression.

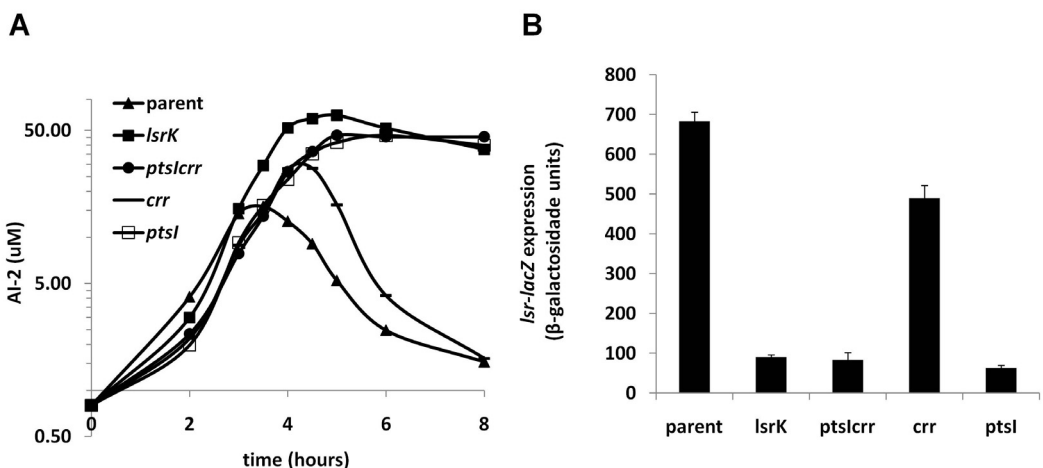


Figure 3 – EI and EIIA^{glc} are impaired in AI-2 incorporation and repress the *lsr* operon transcription. Extracellular AI-2 activity **A**) and transcription of the *lsr* operon at OD₆₀₀=4 **B**) was analyzed in the following strains: parent (AS9 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*), *lsrK* (AS40 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*, *lsrK*), *ptsIcrr* (AS90 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*, *ptsIcrr*), *crr* (AS39 – (*lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*, *crr*), *ptsI* (CSP114 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*, *ptsI*).

4.4 – The PTS contributes to AI-2 incorporation and *lsr* expression in the presence of an *lsr* operon and catabolite repression

To access the relevance of the *ptsIcrr* mutation on AI-2 internalization impairment in a WT situation we tested AI-2 incorporation and *lsr-lacZ* expression first in a strain carrying a functional Lsr transporter and second in a background with the presence of catabolite repression (*i. e.* with the WT *crp* gene).

Comparing the strains carrying a functional Lsr transporter with those harboring the *lsrCDB* gene disruption (**Figure 3A** and **4A**, respectively), we conclude that, where internalization occurs, AI-2 uptake is faster in the presence of its specific ABC transport system. For example, the parent strain internalizes most of the AI-2 by 4h whereas the *lsrCDB* mutant requires up to 8h for the equivalent internalization to occur (parent strains in **Figure 4A** and **3A**, respectively). Despite this observation, even in the presence of a functional Lsr system, *ptsI*, *crr* and *ptsIcrr* remained unable to remove AI-2 from the extracellular medium to the same extent as WT (**Figure 4A**). The reduced expression of *lsr-lacZ* in these mutant strains was also unalleviated by the restoration of the Lsr system (**Figure 4B**). This shows that the PTS is crucial for AI-2 internalization and *lsr-lacZ* induction and is not only required when the specific AI-2 transport mechanism, the Lsr transporter, is absent.

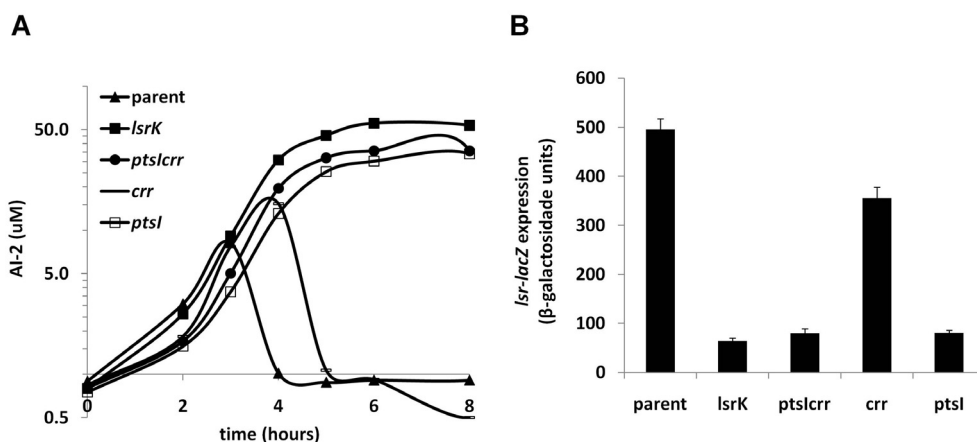


Figure 4 – The PTS mutant’s impairment in AI-2 incorporation and repression of the *lsr* operon transcription is observed in strains with a functional Lsr transport system. Extracellular AI-2 activity **A)** and transcription of the *lsr* operon at OD₆₀₀=4 **B)** was analyzed in the following strains: parent (AS8 – *lsr-lacZ*, *cya*, *crp**), *lsrK* (AS82 – *lsr-lacZ*, *cya*, *crp**, *lsrK*), *ptsIcrr* (AS108 – *lsr-lacZ*, *cya*, *crp**, *ptsIcrr*), *crr* (AS106 – *lsr-lacZ*, *cya*, *crp**, *crr*), *ptsI* (CSP108 – *lsr-lacZ*, *cya*, *crp**, *ptsI*).

Next we examined if the previously observed phenotype was still present in strains where catabolite repression was restored. Again, impairment of AI-2 incorporation and low induction of the *lsr* promoter was observed in all the mutants (data not shown), demonstrating that the PTS system is required for AI-2 incorporation in a WT background.

4.5 – Impairment in AI-2 incorporation and *lsr* activation in the PTS mutant is also observed with exogenously supplied AI-2

In *E. coli* AI-2 is produced intracellularly by the LuxS synthase and subsequently released to the extracellular environment. Therefore, the persistence of AI-2 in the media observed in the *ptsIcrr* mutant might be due to changes in AI-2 production and release. To examine this possibility we analyzed the incorporation of exogenously supplied AI-2 in a *luxS* mutant background. We determined the incorporation of AI-2 by cells growing in the presence of exogenously supplied AI-2 (**Figure 5A**) and observed that by 4 h AI-2 was not detected in WT cultures whereas it remained at high concentrations in the *ptsIcrr* mutant strain cultures (**Figure 5A**). We also analyzed this process in non-growing cell suspensions in response to a pulse of extracellular AI-2 (**Figure 5B**) where cells were grown to late exponential phase, when incorporation is usually observed, harvested and washed to remove extracellular AI-2. ¹³C-labelled AI-2 was provided to the cells and the decrease of ¹³C-labelled AI-2 was followed by *in vivo* NMR. Incorporation of ¹³C-labelled AI-2 was lower in the *ptsI* and *lsrK* mutant strain when compared to the parent strain (**Figure 5B**). The *lsr* expression was also measured using the β -galactosidase assay (**Figure 5C**) and the phenotypes were in agreement with the AI-2 internalization data: low levels of β -galactosidase activity were observed in the *lsrK* and *ptsIcrr* mutant strains (**Figure 5C**). This demonstrates that PTS is required for internalization of extracellular AI-2 and that this phenotype is not due to any effects on AI-2 synthesis and export.

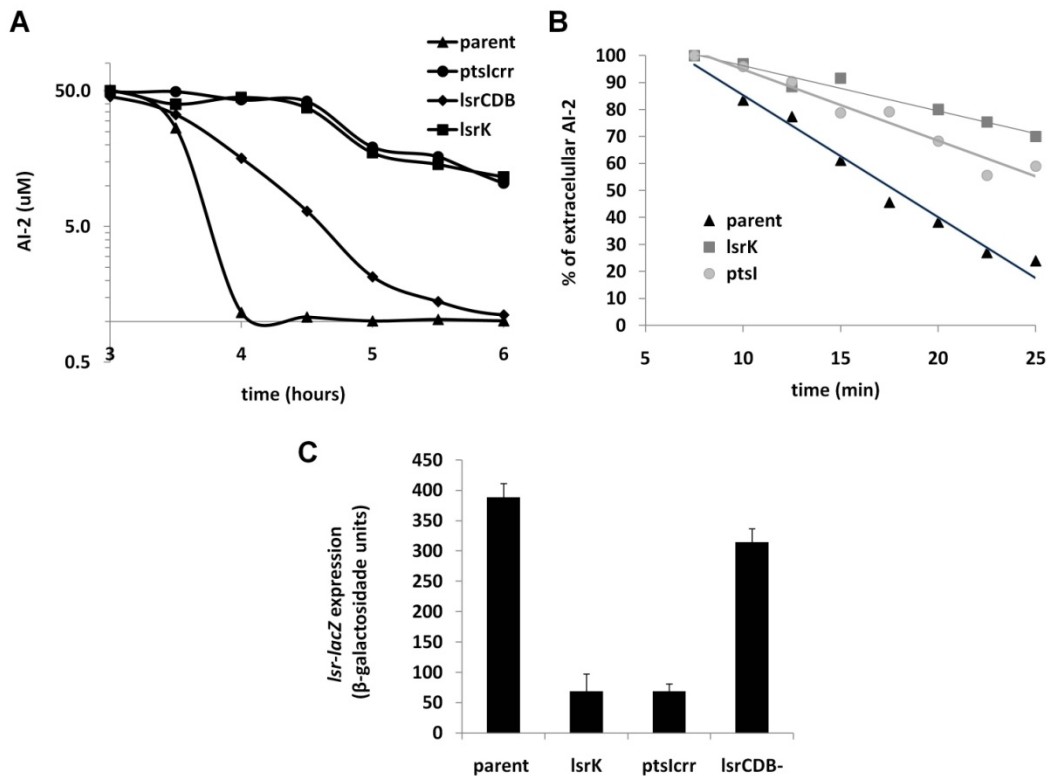


Figure 5 - The *ptsIcrr* mutant impairment in AI-2 incorporation and repression of the *lsr* operon transcription is independent of AI-2 production by *LuxS*. Extracellular AI-2 activity measured using FRET **A**) and *in vivo* NMR **B**) and transcription of the *lsr* operon at OD₆₀₀=4 **C**) was analyzed in the following strains: **A**) and **C**) parent (AS4 - *lsr-lacZ*, *cya*, *crp**, *luxS*), *lsrK* (CSP160 - *lsr-lacZ*, *cya*, *crp**, *luxS*, *lsrK*), *ptsIcrr* (CSP150 - *lsr-lacZ*, *cya*, *crp**, *luxS*, *ptsIcrr*) and *lsrCDB* (CSP162 - *lsr-lacZ*, *cya*, *crp**, *luxS*, *lsrCDB*). **B**) parent (AS8 - *lsr-lacZ*, *cya*, *crp**, *lsrK*) and *ptsI* (AS148 - *lsr-lacZ*, *cya*, *crp**, *ptsI*).

4.6 - Lsr activation and AI-2 incorporation requires the phosphorylated form of EI

Although most functions attributed to EI are absolutely dependent upon the phosphotransferase activity of this enzyme, in at least one case it has been reported that the EI phosphorelay is not necessary: activation of BglG, the regulator of the β -glucoside utilization system in *E. coli* (252). To confirm if the EI phosphorylation plays a role in the AI-2 uptake phenotype of the *ptsI* mutant we tested for complementation with an IPTG-inducible multicopy plasmid expressing the *ptsI*-WT gene or a mutant allele which cannot be phosphorylated (EI^{H189A}). As shown above, AI-2 internalization and *lsr*

transcription were low in the *ptsI* mutant strain in comparison with the parent strain. This impairment was recovered by the presence of the WT allele of the *ptsI* gene; however the phosphorylation deficient protein (EI^{H189A}) was not able to rescue AI-2 internalization or induction of *lsr* transcription (**Figure 6**). Therefore the phosphorylated form of EI is required for AI-2 internalization by the PTS.

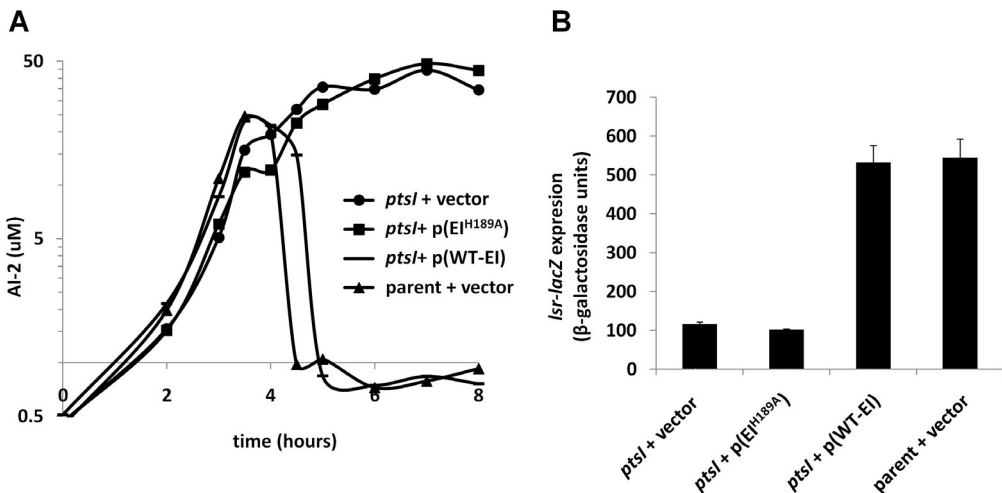


Figure 6 – EI phosphotransferase ability is required for AI-2 incorporation and for transcription of the *lsr* operon. Extracellular AI-2 activity **A)** and transcription of the *lsr* operon at OD₆₀₀=4 **B)** was analyzed in the following strains: *ptsI* + vector (PBC07 – *lsr-lacZ*, *cya*, *crp*^{*}, *ptsI* + pQE32-*lacIq*), *ptsI* + p(EI^{H189A}) (PBC35 *lsr-lacZ*, *cya*, *crp*^{*}, *ptsI* + pQELL-EI^{H189A}), *ptsI* + p(WT-EI) (PBC09– *lsr-lacZ*, *cya*, *crp*^{*}, *ptsI* + pQELL-EI) and parent + p() (PBC19 – *lsr-lacZ*, *cya*, *crp*^{*} + pQELL-EI).

4.7 – Inhibition of *lsr* expression in a *ptsIcrr* mutant requires the repressor LsrR

The fact that the *ptsIcrr* mutant has a strong impairment in AI-2 incorporation even in a strain with an intact Lsr transport system indicates that the regulation/activity of the *lsr* system is also being affected by the absence of a functional PTS; otherwise AI-2 should be internalized, at least to some extent, by the Lsr transporter with consequent induction of the *lsr-lacZ* in a *ptsIcrr* mutant strain.

To determine whether the PTS phenotype was linked to the function of LsrR, the major regulator of the *lsr* operon, or if it is a consequence of a different level of regulation, we determined *lsr* transcription of the *ptsIcrr* mutant in a *lsrR* null background. As has been published (345), in the *lsrR* mutant as well as in the *lsrR*, *luxS* double mutant repression of *lsr-lacZ* no longer occurs, its expression is very high and no longer responsive to AI-2 (**Figure 7**). This was also the case for all the PTS mutants tested: *ptsI*, *crr* and *ptsIcrr*, which showed similar levels of *lsr-lacZ* expression to the *lsrR* mutant (**Figure 7**), thus the *ptsIcrr* mutation is affecting Lsr transport expression via LsrR.

As shown previously, LsrR-independent regulation of the *lsr* also occurs (345). Specifically, *lsr* transcription is regulated by catabolite repression thus in *cya* mutant, which is required for making cAMP, *lsr* transcription is low, but this effect is not rescued in a *cya*, *lsrR* double mutant (**Figure S2**) because it is not LsrR-dependent. The results in **Figure 7** show that this is not the case for PTS-dependent regulation because it is abolish in a *lsrR* mutant. Thus PTS is required for LsrR-dependent de-repression of the *lsr* operon.

Although AI-2-P is the main regulator of LsrR, it is possible that components of the PTS could act directly on this protein and relieve LsrR-mediated repression, or alternatively that a second co-regulator is required which is absent in a *ptsIcrr* mutant. PTS could also influence AI-2-P levels inside the cell, either by regulating AI-2 phosphorylation through LsrK or by AI-2 uptake via the alternative transport mechanism.

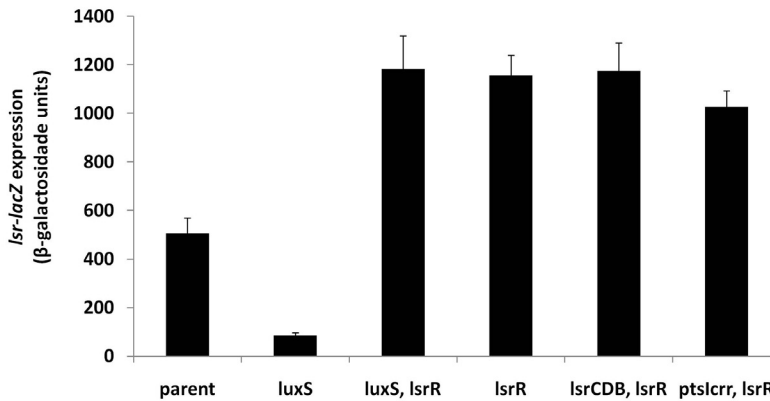


Figure 7 – The PTS regulates the *lsr* operon transcription via the LsrR repressor.

Transcription of the *lsr* operon was analyzed at OD₆₀₀=4 in the following strains: parent (AS8 – *lsr-lacZ*, *cya*, *crp**), *luxS* (AS4 – *lsr-lacZ*, *cya*, *crp**, *luxS*), *lsrR* (AS110 – *lsr-lacZ*, *cya*, *crp**, *luxS*, *lsrR*), *lsrR* (AS112 – *lsr-lacZ*, *cya*, *crp**, *lsrR*), *lsrR, lsrCDB* (MBS120 – *lsr-lacZ*, *cya*, *crp**, *lsrR*, *lsrCDB*) and *lsrR, ptsIcrr* (AS122 – *lsr-lacZ*, *cya*, *crp**, *lsrR*, *ptsIcrr*)

4.8 – *ptsIcrr* mutation do not affect expression or activity of LsrK

To test if PTS could be involved in regulating AI-2 phosphorylation we determined if this system could be regulating LsrK. As shown in **Figure 8**, by western blots with antibodies against LsrK, protein expression is lower in a *ptsIcrr* mutant in comparison to WT (upper blot). However, this phenotype is again rescued in a *lsrR* mutant (lower blot) as the *lsrR, ptsIcrr* triple mutant shows the same levels of expression as the *lsrR* single mutants. The same result is observed with the *lsrCDB* single mutant and *lsrR, lsrCDB* mutants. Similar to what was observed in the regulation of *lsr* operon, the PTS-dependent regulation on LsrK protein expression is indirect, and LsrR dependent, and one can conclude that PTS is not directly regulating LsrK expression. As transcription of *lsrK* is again regulated by LsrR, low expression of LsrK in the *ptsIcrr* mutant could be explained by internalization of AI-2. This would prevent inactivation of LsrR repression and give rise to the observed low levels of LsrK and thus consequent low levels of AI-2-P. This is likely to be the case for the *lsrCDB* mutant, however the above analysis does not preclude a role for PTS in promoting LsrK activity.



Figure 8 – LsrK protein levels. LsrK protein levels were determined by western blotting of the whole cell lysates of the following strains at OD₆₀₀=4: parent (AS8 – *lsr-lacZ*, *cya*, *crp*^{*}), *lsrK* (AS82 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrK*), *luxS* (AS4 – *lsr-lacZ*, *cya*, *crp*^{*}, *luxS*), *ptsIcrr* (AS108 – *lsr-lacZ*, *cya*, *crp*^{*}, *ptsIcrr*), *lsrCDB* (AS9 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*), *lsrR*, *lsrK* (MBS182 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrR*, *lsrK*), *lsrR* (AS112 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrR*), *luxS*, *lsrR* (AS110 – *lsr-lacZ*, *cya*, *crp*^{*}, *luxS*, *lsrR*), *lsrR*, *ptsIcrr* (AS122 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrR*, *ptsIcrr*) and *lsrR*, *lsrCDB* (MBS120 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrR*, *lsrCDB*).

We next used a *ptsIcrr*, *lsrR* triple mutant to determine if the LsrK protein is functional and able to sequester AI-2-P intracellularly: *E. coli luxS* mutants were grown in the presence of synthetically synthesized AI-2 and the AI-2 activity in the corresponding cell-free culture fluids was followed over time. As expected, due to derepression of the *lsr* operon in this strain, the *lsrR* mutant removed AI-2 from the extracellular medium faster than the parent strain (**Figure 9**). Notably, introduction of the *ptsIcrr* deletion to the *lsrR* background did not prevent AI-2 internalization (in contrast to what is observed in the *ptsIcrr* mutant). The *lsrR*, *ptsIcrr* mutant is fully capable of removing AI-2 from the extracellular media (**Figure 9**); incorporation is delayed one hour in comparison with the *lsrR* mutant but this difference is consistent with the previously described growth defect associated with *ptsIcrr* mutant strain. The fact that the *lsrR*, *ptsIcrr* mutant showed a clear ability to internalize AI-2 demonstrates that this molecule can be sequestered inside the cell even in the absence of the PTS (**Figure 9**). We can further conclude that intracellular sequestration of AI-2 observed in the *lsrR*, *ptsIcrr* mutant is due to active LsrK because in a *ptsIcrr*, *lsrR*, *lsrK* background AI-2 is no longer removed from the external media. Thus PTS is not essential for LsrK expression or activity.

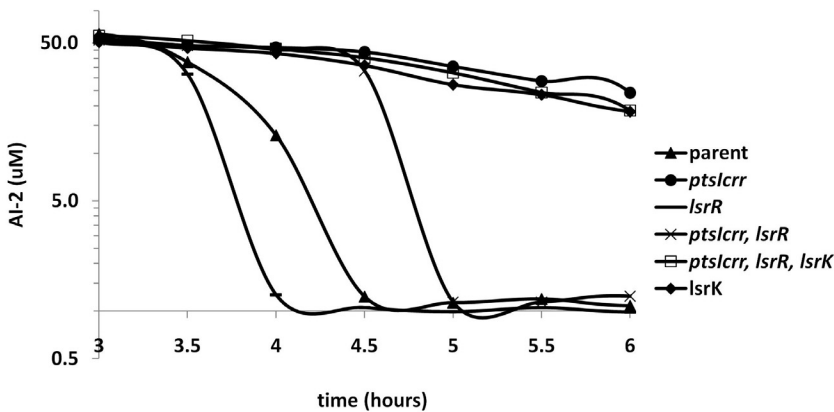


Figure 9 – LsrK is functional in a *ptsIcrr*, *lsrR* double mutant. Extracellular AI-2 activity was analyzed in the following strains: parent (AS4 – *lsr-lacZ*, *cya*, *crp**, *luxS*), *ptsIcrr* (CSP150 – *lsr-lacZ*, *cya*, *crp**, *luxS*, *ptsIcrr*), *lsrR* (AS110 – *lsr-lacZ*, *cya*, *crp**, *luxS*, *lsrR*), *ptsIcrr*, *lsrR* (CSP258 – *lsr-lacZ*, *cya*, *crp**, *luxS*, *ptsIcrr*, *lsrR*), *ptsIcrr*, *lsrR*, *lsrK* (CSP260 – *lsr-lacZ*, *cya*, *crp**, *luxS*, *ptsIcrr*, *lsrR*, *lsrK*) and *lsrK* (CSP160 – *lsr-lacZ*, *cya*, *crp**, *luxS*, *lsrK*).

4.9 – The *ptsIcrr* phenotype can be rescued by AI-2 internalization through a PTS-independent mechanism

Our data shows that in a *ptsIcrr* mutant LsrR is strongly repressing the *lsrACDB* transport genes and *lsrK*. Thus, these results indicate that in this mutant AI-2 cannot be internalized by the Lsr transporter and even if AI-2 enters the cell via the secondary transport (**Figure 1**), because this other transporter also requires LsrK for intracellular sequestration, AI-2 is not removed from the extracellular medium. So it is possible that the *ptsIcrr* mutation can be justified only by a direct dependence of PTS for LsrR de-repression or that in this mutant the LsrR-independent internalization is impaired and thus LsrR is acting as a repressor only because intracellular AI-2 is absent. If the second hypothesis is correct, uptake of AI-2 by a transport mechanism that is not regulated by LsrR in a *ptsIcrr* mutant should restore the intracellular pool of AI-2-P and *lsr* induction. If the phenotype is linked to a direct interaction with LsrR, then no rescue of the *ptsIcrr* phenotype should be observed. We tested *lsr-lacZ* expression, as a reporter for AI-2 internalization and phosphorylation, in a *ptsIcrr* mutant complemented with a plasmid expressing the components of the Lsr transporter under the control of an IPTG inducible promoter (p(*lsrACDB*)). In this situation, AI-2 can enter via

the Lsr transporter without requiring the activation of its own transcription by AI-2-P. As shown in **Figure 10**, *lsr-lacZ* expression was induced even in a *ptsIcrr* mutant when *lsrACDB* is ectopically expressed and controlled by an IPTG inducible promoter. This result excludes the hypothesis that another co-regulator, other than AI-2-P is required to inactivate LsrR, but also that the possibility that the EI enzyme is required for inactivating LsrR activity. We propose that PTS is essential for the initial AI-2 internalization, which is crucial for the inactivation of LsrR, increased expression of LsrK, subsequent production of the main inducer of the system: AI-2-P, and induction of the positive feedback loop that drives the rapid internalization of AI-2 from the extracellular medium.

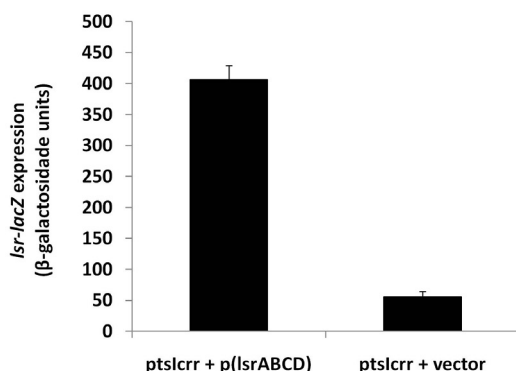


Figure 10- The *ptsIcrr* mutant phenotype is overcome when *lsrACDB* is expressed by an IPTG inducible promoter. The following strains were analyzed at OD₆₀₀=4: *ptsIcrr* + p(*lsrACDB*) (CSP195 – *lsr-lacZ*, *cya*, *crp**, *ptsIcrr* + pCSP184) and *ptsIcrr* + vector (CSP197 – *lsr-lacZ*, *cya*, *crp**, *ptsIcrr* + pCA24N-no GFP).

5 – Discussion

In this work we set out to investigate the molecular mechanisms for AI-2 internalization in *E. coli*. Specifically, we performed a genetic screen to identify components involved in AI-2 internalization or the regulation of this process. The Lsr transport system is one of the few AI-2 recognition systems identified so far, and the only system which has been linked to interference with AI-2 related behaviors. Understanding the regulation of the *lsr* operon and AI-2 synthesis, export and uptake is crucial in uncovering what the

physiological role of this system is and how it might be exploited in the manipulation of AI-2-regulated phenotypes.

Previous work indicated that other components besides the Lsr system were required for AI-2 internalization by *E. coli* and *S. Typhimurium* (305, 306, 345). In Lsr transport mutants AI-2 internalization is slower but not completely absent; according to the data from Zhu *et al.*, passive diffusion of AI-2 through the membrane does not occur (361), therefore an alternative transport mechanism must exist. Importantly, this alternative transporter also requires the LsrK kinase for intracellular AI-2 sequestration, thus this secondary system is also dependent on a component of the *lsr* regulon, LsrK, which is in turn regulated by the LsrR repressor in a AI-2 dependent manner.

We identified the PTS, specifically the EI component as a critical activator of AI-2 internalization, additionally deletion of EI^{IAGlc} also has a partial effect on the system. A wide variety of bacteria take up carbohydrates through the PTS, which consists of two general proteins, EI and HPr, and a number of carbohydrate-specific enzymes, collectively known as the EII. Carbohydrates translocation across the membrane is coupled to a phosphorylation from the phospho group donor, phosphoenolpyruvate, to the intermediates, EI, HPr and EII, in this order, until the final phosphate transfer to the imported carbohydrate molecule (243).

Here we have shown that AI-2 internalization requires the PTS. We determined that the observed impairment of AI-2 incorporation and transcription of the *lsr* operon in a *ptsIcrr* mutant is LsrR dependent and that, in this mutant, the *lsr* operon and the LsrK kinase that phosphorylates AI-2 are repressed. This regulation could occur by multiple mechanisms. PTS may be required for the expression of LsrK or its kinase activity upon AI-2. However, we showed that PTS is not directly required for LsrK expression and that LsrK can sequester AI-2 in a *ptsIcrr* mutant if repression by LsrR is absent (**Figure 8** and **9**). It could be that inactivation of LsrR repression was not strictly dependent on AI-2-P levels and required a component of the PTS system or a co-regulator that was not present in PTS mutants, however

activation of the system has not been observed in the absence of AI-2. Also arguing against this hypothesis is the fact that when AI-2 enters the cell by an Lsr transporter expressed by a IPTG inducible promoter the PTS mechanism is no longer necessary to de-repress *lsr* transcription (**Figure 10**). Thus, we concluded that the impairment observed in the *ptsIcrr* mutant is caused by inability of AI-2 to enter the cell. Therefore, by understanding the process that causes Lsr to require PTS for activation we were able propose a model that explains the overall dependence and role of PTS in AI-2 internalization (**Figure 11**).

We suggest that for the AI-2 signal transduction pathway to be activated, extracellular AI-2 needs to be first transported by a PTS-dependent mechanism and then phosphorylated by LsrK. Based on the data reported here we propose that the early AI-2 import is achieved exclusively by a PTS-dependent transport system. In this model the *lsr* operon would be tightly repressed (by the LsrR repressor) to such an extent that no AI-2 uptake occurs through the Lsr system. We further suggest that LsrK is expressed at a basal level enabling the phosphorylation of the intracellular AI-2 resulting from PTS-dependent uptake (**Figure 11**). Additionally, our results indicate that is this pool of intracellular AI-2-P that binds to LsrR, releasing the *lsr* repression, and allowing the start of the positive feedback loop (**Figure 11**). Once the Lsr transport system is expressed it causes the rapid uptake of extracellular AI-2 observed during the late exponential phase. Interestingly, *lsrK* transcription is regulated by the *lsrR* promoter and is not part of the *lsr* operon, supporting the possibility for differences in timing and level of expression and that *lsr* transcription is more tightly regulated than *lsrK*.

The mechanism by which PTS promotes AI-2 transport remains an unsolved question. Given the role of PTS identified here, and the chemical nature of AI-2, one possibility is that it enters the cell by one of the 20 carbohydrate specific permeases characterized in *E. coli* (243). However, we tested single mutants in all the PTS permeases and did not observe a strong impairment in AI-2 uptake for any of the single permease mutants. Thus, the impairment observed with the *ptsI* mutant cannot be exclusively explained by

the loss of any of the single PTS permeases (**Figure S4** and **S5**); this analysis does not rule out that transporters might contribute additively to AI-2 incorporation in a redundant manner. Supporting this hypothesis is the fact that *ptsI* phenotype is more pronounced than the *crr* phenotype (**Figure 3** and **4**). As there are several PTS independent of EIIA^{glc}, this result could indicate that several PTS permeases contribute to the non-specific transport of AI-2, including those that do not use EIIA^{glc}. The *treB* mutant, which encodes for the PTS permease for trehalose, has a significant impairment in AI-2 incorporation (**Figure S4**). Trehalose is one of the most abundant carbohydrate in LB media (346) and thus *treB* should be one of the most induced PST permeases in the growth conditions used in this study. Thus, this permease could be one of the PTS permeases contributing for AI-2 uptake, or simply trehalose uptake could influence the levels of phosphorylated EI and indirectly interfere with the PTS-regulated transporter.

An alternative hypothesis, is that the observed PTS-dependent transport is accomplished by a transporter that it is not a PTS type of transporter but is regulated by components of the PTS. Examples of that are regulation by EIIA^{glc} by a mechanism called inducer exclusion but this is not the case for AI-2 because AI-2 incorporation phenotypes are observed in *crr* and *ptsIcrr* mutants, that is, in the absence of EIIA^{glc}. As the regulatory networks controlled by PTSs are complex comprising multiple different molecular mechanisms, the existence of alternative transport systems regulated by PTS are possible.

The phosphorylation state of the PTS proteins reflects the availability of carbohydrates and consequently is an indirect readout of the cellular metabolic status. Consistently, the protein components of the PTS have been implicated in the regulation of processes related to metabolism such as chemotaxis, inducer exclusion and catabolite repression. Recently, the function of PTS as a crucial regulator of cellular behaviors has been reported to extend past its direct metabolic association. Processes such as virulence in *S. Typhimurium* and biofilm formation in *Vibrio cholerae* have been shown to be regulated by the PTS. Presumably PTS monitors the metabolic capability of

the cells and, only when the right metabolic conditions are encountered, are other physiologically appropriated actions turned on. Therefore, the PTS could be a global sensory system which integrates information about nutrient availability and metabolic status of bacteria and modulates behaviors accordingly (174). Here we demonstrate that the activation of AI-2 internalization requires a functional PTS. We speculate that in enteric bacteria, and potentially in other *Isr*-containing bacteria, the cell has to perceive a specific metabolic condition (given by the PTS) to activate the AI-2 incorporation and processing by its specific mechanism Lsr. In this sense, this is an example of a cell integrating information by two distinct sensory pathways, reinforcing the importance of investigating the role of the AI-2 QS signal in different growth conditions to understand the amplitude to which the phosphorylation state of the PTS components affects QS interference by the Lsr transporter.

Elucidation of the natural strategies, such as the Lsr system, which organisms use to interfere with other species' ability to communicate, could provide important tools in the design of clinical and biotechnological strategies for the exploitation of bacterial behaviors, whether in the development of novel therapies or economically important compounds, further illustrating the relevance and future applicability of this research.

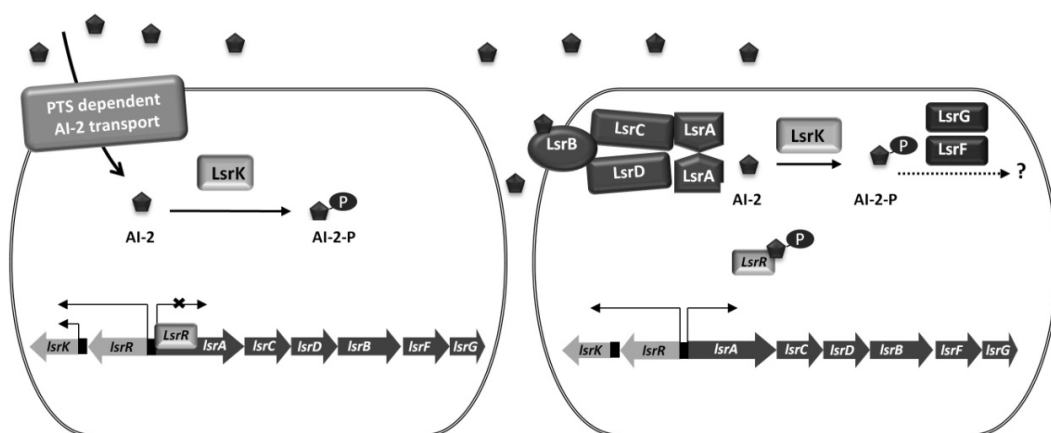


Figure 11 – Proposed model for AI-2 incorporation and Lsr transport system activation.

AI-2 is first incorporated by *E. coli* cell via an PTS-dependent transport. Upon entering the cell, cytoplasmic AI-2 is phosphorylated by basal levels of LsrK. The AI-2-P binds to the LsrR repressor allowing the transcription of the *Isr* operon. The Lsr transport system is subsequently responsible for the rapid AI-2 internalization.

6 – Acknowledgments

Catarina S. Pereira, António Santos and Karina B. Xavier planned the experiments, analyzed the data and wrote the manuscript. Michal Bejerano-Sagie performed the Western blots, João Marques performed the NMR experiments and Paulo B. Correia performed the EI complementation and constructed some of the strains used here. Catarina S. Pereira and António Santos performed all the other experiments.

7 – Supplementary Material

Table S1 – Strains used in this study

strain	genotype	source
<i>E. coli</i> strains		
AS4	<i>lsr-lacZ, ΔluxS, Δcya, crp*</i>	This study
AS5	<i>lsr-lacZ, ΔluxS, Δcya, crp*, ΔlscDB::Cm</i>	This study
AS6	<i>lsr-lacZ, ΔluxS, Δcya::Cm</i>	This study
AS7	<i>lsr-lacZ, Δcya</i>	This study
AS8	<i>lsr-lacZ, Δcya, crp*</i>	This study
AS9	<i>lsr-lacZ, Δcya, crp*, ΔlscDB::Cm</i>	This study
AS39	<i>lsr-lacZ, Δcya, crp*, ΔlscDB::Cm, Δcrr::Kan</i>	This study
AS40	<i>lsr-lacZ, Δcya, crp*, ΔlscDB::Cm, ΔlscK::Kan</i>	This study
AS70	<i>lsr-lacZ, Δcya, crp*, ΔlscDB::Cm, ΔptsI::Tn5Kan</i>	This study
AS82	<i>lsr-lacZ, Δcya, crp*, ΔlscK::Kan</i>	This study
AS90	<i>lsr-lacZ, Δcya, crp*, ΔlscDB::Cm, ΔptsIcrr::Kan</i>	This study
AS98	<i>lsr-lacZ, Δcrr::Kan</i>	This study
AS100	<i>lsr-lacZ, ΔptsIcrr::Kan</i>	This study
AS106	<i>lsr-lacZ, Δcya, crp*, Δcrr::Kan</i>	This study
AS108	<i>lsr-lacZ, Δcya, crp*, ΔptsIcrr::Kan</i>	This study
AS110	<i>lsr-lacZ, ΔluxS, Δcya, crp*, ΔlscR::Cm</i>	This study
AS112	<i>lsr-lacZ, Δcya, crp*, ΔlscR::Cm</i>	This study
AS122	<i>lsr-lacZ, Δcya, crp*, ΔptsIcrr::Kan, ΔlscR::Cm</i>	This study
AS148	<i>lsr-lacZ, Δcya, crp*, ΔptsI::Kan</i>	This study
AS160	<i>lsr-lacZ, Δcya, crp*, ΔlscDB::Cm, ΔtreB::Kan</i>	This study

AS161	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta glvC::Kan$	This study
AS162	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta mngA::Kan$	This study
AS196	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta ptsN::Kan$	This study
AS202	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta ptsP::Kan$	This study
AS218	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta sgcB::Kan$	This study
AS220	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta manY::Kan$	This study
AS221	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta agaW::Kan$	This study
AS222	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta agaC::Kan$	This study
AS223	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta gatC::Kan$	This study
AS224	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta ulaA::Kan$	This study
AS226	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta bglF::Kan$	This study
AS227	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta nagE::Kan$	This study
AS228	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta fruB::Kan$	This study
AS229	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta srlA::Kan$	This study
AS230	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta fruA::Kan$	This study
AS231	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta frvB::Kan$	This study
AS232	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta celB::Kan$	This study
AS233	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta frwB::Kan$	This study
AS234	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta fryB::Kan$	This study
AS235	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta ulaB::Kan$	This study
AS236	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta mtIA::Kan$	This study
CJG23	<i>Isr-lacZ</i> , $\Delta IsrCDB$	Lab collection
CSP90	<i>Isr-lacZ</i> , $\Delta IsrK::Kan$	This study
CSP108	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta ptsI$	This study
CSP110	<i>Isr-lacZ</i> , $\Delta ptsI$	This study
CSP114	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta ptsI$, $\Delta IsrCDB::Cm$	This study
CSP120	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta malX::Kan$	This study
CSP122	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta ptsG::Kan$	This study
CSP124	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta murP::Kan$	This study
CSP126	<i>Isr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta IsrCDB::Cm$, $\Delta ascF::Kan$	This study
CSP150	<i>Isr-lacZ</i> , $\Delta luxS$, Δcya , <i>crp</i> [*] , $\Delta ptsIcrr::Kan$	This study
CSP160	<i>Isr-lacZ</i> , $\Delta luxS$, Δcya , <i>crp</i> [*] , $\Delta IsrK::Kan$	This study
CSP162	<i>Isr-lacZ</i> , $\Delta luxS$, Δcya , <i>crp</i> [*] , $\Delta IsrCDB$	This study
CSP164	<i>Isr-lacZ</i> , $\Delta luxS$, $\Delta cya::Cm$, $\Delta IsrR::Kan$	This study

CSP195	Strain AS108 carrying the plasmid pCSP184	This study
CSP197	Strain AS108 carrying the plasmid pCA24N-no GFP	This study
CSP258	<i>lsr-lacZ</i> , $\Delta luxS$, Δcya , <i>crp</i> [*] , $\Delta lsrR::Cm$, $\Delta ptsIcrr::Kan$	This study
CSP260	<i>lsr-lacZ</i> , $\Delta luxS$, Δcya , <i>crp</i> [*] , $\Delta ptsIcrr::Kan$, $\Delta lsrR::Cm$, $\Delta lsrK::Kan$	This study
KX1086	MG1655 carrying the plasmid pKD46	Lab collection
KX1123	<i>lsr-lacZ</i>	(345)
KX1186	<i>lsr-lacZ</i> , $\Delta lsrK::Cm$	(345)
KX1322	<i>lsr-lacZ</i> , $\Delta lsrR::Cm$	Lab collection
KX1468	<i>lsr-lacZ</i> , $\Delta cya::Cm$, <i>crp</i> [*]	Lab collection
MBS120	<i>lsr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta lsrCDB::Cm$, $\Delta lsrR::Kan$	This study
MBS128	<i>lsr-lacZ</i> , Δcya , <i>crp</i> [*] , $\Delta lsrR::Cm$, $\Delta lsrK::Kan$	This study
PBC07	Strain CSP108 carrying the plasmid pQE32- <i>lacI</i> ^q	This study
PBC09	Strain CSP108 carrying the plasmid pQELL-EI	This study
PBC19	Strain AS8 carrying the plasmid pQE32- <i>lacI</i> ^q	This study
PBC35	Strain CSP108 carrying the plasmid pQELL-EI ^{H189A}	This study
PBC105	<i>lsrR-lacZ</i> , <i>cyaA::Cm</i> , <i>crp</i> [*]	This study
PBC107	<i>lsrR-lacZ</i> , $\Delta luxS$, <i>cyaA::Cm</i> , <i>crp</i> [*]	This study
PBC109	<i>lsrR-lacZ</i> , $\Delta lsrK$, <i>cyaA::Cm</i> , <i>crp</i> [*]	This study
PBC125	<i>lsrR-lacZ</i> , $\Delta lsrR$ <i>cyaA::Cm</i> , <i>crp</i> [*]	This study

Table S2 – Plasmids used in this study

Plasmids		
pCSP184	<i>lsrACBD</i> expression vector with iptg inducible	This study
pCA24N-no GFP		(162)
pKD4	vector containing kanamycin resistance cassette	(64)
pKD46	ts vector containing arabinose-inducible phage I Red recombinase (Amp _R)	(64)
pCp20	ts vector expressing heat-inducible FLP recombinase (Amp _R , Cm _R)	(64)
pQE32-<i>lacI</i>^q		(252)
pQELL-EI	EI expression vector with IPTG inducible promoter	(252)
pQELL-EI^{H189A}	EI ^{H189A} expression vector with IPTG inducible promoter	(252)

Table S3 – Primers used in this study

Primer name	Oligonucleotide
fw ptsI wanner	CCCGGGTTCCTTTTAAAAATCAGTCACAAGTAAGGTAGGGTTATGATTTTCAGTGTAG GCTGGAGCTGCTTC
rv crr wanner	GGCGCCGATGGGCGCCATTTTTCAGTGCAGCAAGAATTACTTCTTGATGCCATATG AATATCCTCCTTAGT
Lsr operon fw3	ACCTAGCGGCCGCGTTATGAACAAATTAAGCAGAAATACAT
Lsr operon rv3	ACCTAGTCGACTAATATCGTCTAAATCTTGCCATAACTTACT

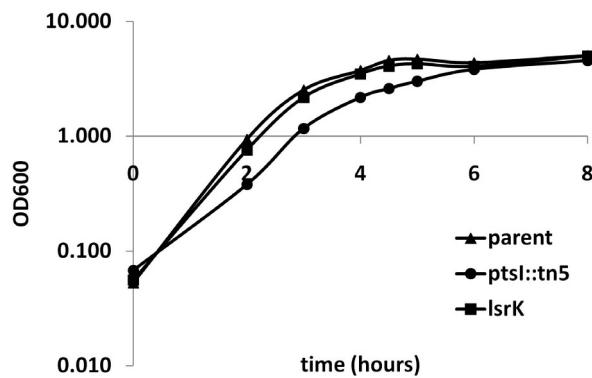


Figure S1 – Growth curves of the Lsr transport mutants. OD₆₀₀ was measured in the following strains: parent (AS9 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*), *ptsI*::tn5 (AS70 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*, *ptsI*::tn5) and *lsrK* (AS90 – *lsr-lacZ*, *cya*, *crp*^{*}, *lsrCDB*, *lsrK*).

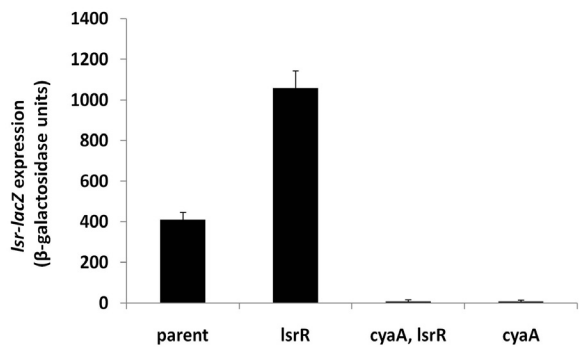


Figure S2 – LsrR-independent *lsr* regulation. Transcription of the *lsr* operon was analyzed at OD₆₀₀=4 in the following strains: parent (KX1123 – *lsr-lacZ*), *lsrR* (KX1322 – *lsr-lacZ*, *lsrR*), *lsrR* (CSP164 – *lsr-lacZ*, *cya*, *lsrR*) and *cya* (AS6 – *lsr-lacZ*, *cya*).

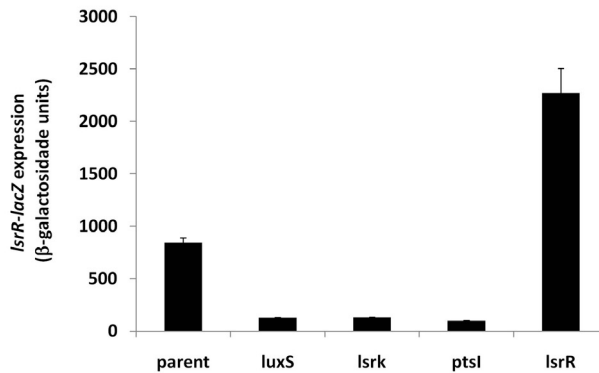


Figure S3 – *lsrRK* operon is regulated by the *LsrR* transcriptional repressor. Transcription of the *lsrRK* operon was analyzed at $OD_{600}=4$ in the following strains: parent (PBC105 – *lsrR-lacZ*, *cyaA*, *crp**), *luxS* (PBC107 – *lsrR-lacZ*, *cyaA::cm*, *crp**, *luxS*), *lsrK* (PBC109 – *lsrR-lacZ*, *cyaA*, *crp**, *lsrK*), *ptsI* (PBC111 – *lsrR-lacZ*, *cyaA*, *crp**, *ptsI*) and *lsrR* (PBC125 – *lsrR-lacZ*, *cyaA*, *crp**, *lsrR*).

AI-2 incorporation in permeases from PTS transporters. All these experiments were performed in LB (Figure S4 and S5). In this media a pH increase is associated with the entry into stationary phase; AI-2 is unstable above LB 7.5 explaining the decrease in extracellular AI-2 activity at late stages of *ptsIcrr* and *lsrK* mutant strains.

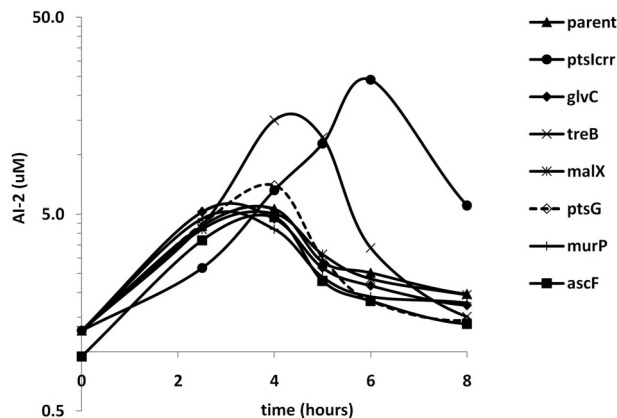


Figure S4 – AI-2 incorporation of EIIA^{glc} dependent permease mutants. Extracellular AI-2 activity was analyzed in the following strains: parent (AS9 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*), *lsrK* (AS90 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *lsrK*), *glvC* (AS161 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *glvC*), *treB* (AS160 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *treB*), *malX* (CSP120 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *malX*), *ptsG* (CSP122 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *ptsG*), *murP* (CSP124 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *murP*) and *ascF* (CSP126 – *lsr-lacZ*, *cya*, *crp**, *lsrCDB*, *ascF*)

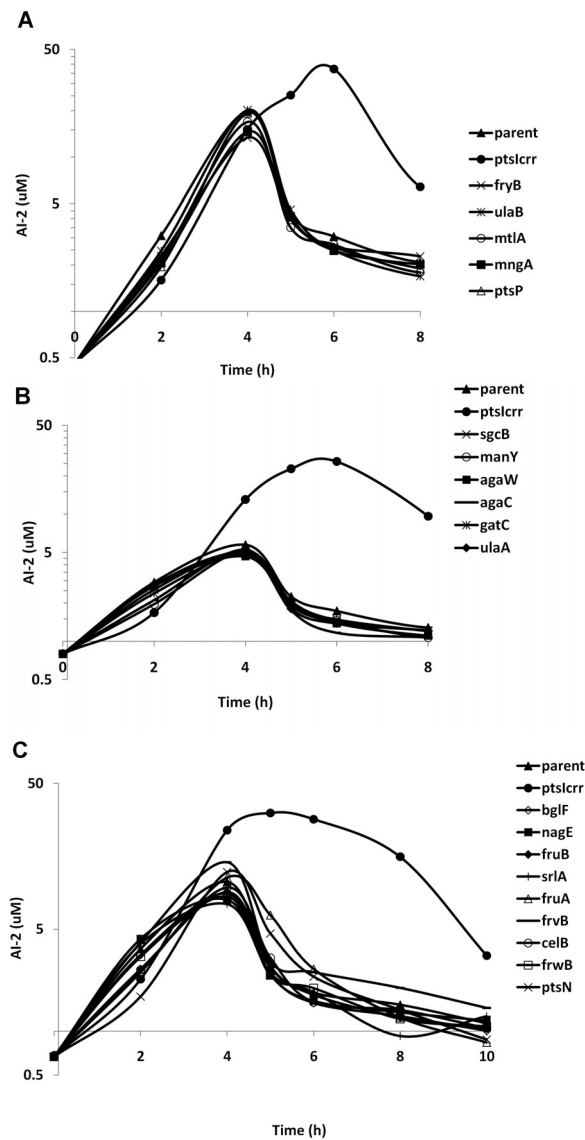


Figure S5- AI-2 incorporation of PTS permease mutants. Extracellular AI-2 activity was analyzed in the following strains **A)** parent (AS9 - *lsr-lacZ*, *cya*, *crp**, *lslCDB*), *lslK* (AS90 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *lslK*), *fryB* (AS234 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *fryB*), *ulaB* (AS235 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *ulaB*), *mtlA* (AS236 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *mtlA*), *mngA* (AS162 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *mngA*) and *ptsP* (AS202 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *ptsP*); **B)** *sgcB* (AS218 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *sgcB*), *manY* (AS220 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *manY*), *agaW* (AS221 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *agaW*), *agaC* (AS222 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *agaC*), *gatC* (AS223 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *gatC*) and *ulaA* (AS224 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *ulaA*); **C)** *bglF* (AS226 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *bglF*), *nagE* (AS227 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *nagE*), *fruB* (AS228 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *fruB*), *srlA* (AS229 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *srlA*), *fruA* (AS230 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *fruA*), *frvB* (AS231 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *frvB*), *celB* (AS232 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *celB*), *frwB* (AS233 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *frwB*) and *ptsN* (AS196 - *lsl-lacZ*, *cya*, *crp**, *lslCDB*, *ptsN*).

CHAPTER IV

Identification of functional LsrB-like autoinducer-2 receptors

Data published in:

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Although a variety of bacterial species have been reported to use the inter-species communication signal autoinducer-2 (AI-2) to regulate multiple behaviors, the molecular mechanisms of AI-2 recognition and signal transduction remain poorly understood. To date, two types of AI-2 receptors have been identified: LuxP, present in *Vibrio* spp, and LsrB, first identified in *Salmonella enterica* subspecies *enterica* serovar Typhimurium. In *S. Typhimurium*, LsrB is the ligand binding protein of a transport system that enables internalization of AI-2. Here, using both sequence analysis and structure prediction, we establish a set of criteria for identifying functional AI-2 receptors. We test our predictions experimentally, assaying key species for their ability to import AI-2 *in vivo* and test their LsrB orthologs for AI-2 binding *in vitro*. Using these experimental approaches, we were able to identify AI-2 receptors in organisms belonging to phylogenetically distinct families such as *Enterobacteriaceae*, *Rhizobiaceae*, and *Bacillaceae*. Phylogenetic analysis of LsrB orthologs indicates that this pattern could result from one single origin of the functional LsrB gene in a γ -*proteobacterium*, suggesting possible posterior independent events of lateral gene transfer to the α -*proteobacteria* and *Firmicutes*. Finally, we used mutagenesis to show that two AI-2 interacting residues are essential for AI-2 binding ability. These two residues are conserved in the binding site of all the functional AI-2-binding proteins but not in the non-AI-2-binding orthologs. Together, these results strongly support our ability to identify functional LsrB-type AI-2 receptors, an important step in investigations of this inter-species signal.

2 – Introduction

Autoinducer-2 (AI-2) is a small molecule produced and secreted by a large number of bacterial species belonging to very widespread branches within the Bacteria kingdom (93, 274, 344). AI-2 or its synthase, LuxS, has been implicated in the regulation of many bacterial behaviors including biofilm formation, virulence, competence, and production of secondary metabolites like antibiotics (119, 321, 344). While in some cases AI-2 is clearly acting through a canonical Quorum-sensing mechanism (330), in others a role in central metabolism has been proposed (340). One of the obstacles to understanding the function of AI-2 in any given species is a lack of knowledge of the molecular mechanisms of AI-2 recognition, signal transduction, and/or processing.

Undoubtedly, one of the major difficulties in identifying AI-2 receptors is the complexity of the chemistry of this signal molecule. The product of the reaction catalyzed by LuxS is 4,5-dihydroxy-2,3-pentadione (DPD) which, in solution, spontaneously re-arranges into a variety of chemically distinct forms collectively called AI-2 (210, 274). We have shown that these forms are in equilibrium and can thus interconvert and that the availability of the different forms of AI-2 is highly dependent on the chemistry of the environment (210). Additionally, different organisms recognize distinct forms of this molecule (51).

So far, two types of AI-2 receptors have been identified and are classified by their ability to bind chemically distinct DPD derivatives, the LuxP- and LsrB-type of receptors characterized first in *Vibrio harveyi* and *Salmonella enterica* subspecies *enterica* serovar Typhimurium, respectively (51, 210). The crystal structure of the *V. harveyi* LuxP-AI-2 complex revealed that the ligand recognized by this receptor is a furanosyl borate diester (51), a cyclic form of DPD bound to borate, while crystal structures of the LsrB-AI-2 complexes from *S. Typhimurium* and *Sinorhizobium meliloti* show that these species recognize a DPD adduct that does not contain boron and has different stereochemistry ((2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-

THMF)) (210, 236). The structures of the LsrB-type receptors bound to AI-2 further showed that six residues were responsible for hydrogen-bonding with AI-2 and that these residues were completely conserved between the two species (210, 236). These residues are distinct from those in the LuxP AI-2 binding site, contributing to the specificity of each receptor for the form of AI-2 recognized by a given species.

LuxP is a periplasmic binding protein (PBP) that, upon binding to AI-2, modulates the activity of a membrane sensor histidine kinase, LuxQ. Together, LuxPQ regulate a signal transduction cascade which controls the AI-2 Quorum-sensing regulon in organisms belonging to the Vibrionales like *V. harveyi*, *Vibrio cholerae* and *Vibrio anguillarum* (25, 62, 216, 217); to date, however, LuxP-type receptors have not been found outside of the Vibrionales.

The LsrB-type receptors also belong to the large family of PBPs but have a low homology to LuxP (the sequence identity between the *V. harveyi* LuxP and the *S. Typhimurium* LsrB AI-2 receptors is only approximately 11 %). The function of the LsrB protein has been characterized in the two closely related enteric bacteria *S. Typhimurium* (305, 306) and *Escherichia coli* (345), the plant symbiont *S. meliloti* (236), and the oral pathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (278). In all these organisms it is thought that LsrB acts as the substrate binding protein of an ABC (ATP-binding cassette) transport system responsible for AI-2 internalization. Due to the homology with other ABC transport systems, it is predicted that the Lsr transporter is composed of LsrB, two transmembrane proteins (LsrC and LsrD) which form a channel, and a cytoplasmic protein (LsrA) that contains an ABC-binding motif and is thought to be responsible for ATP hydrolysis during transport. Once inside the cell, AI-2 is phosphorylated by the kinase LsrK and further processed by the enzymes LsrG and LsrF (305, 347). The genes encoding these proteins (with the exception of LsrK) are all in the same operon, which is regulated by the repressor LsrR. In the absence of AI-2-phosphate (AI-2-P), LsrR represses the transcription of the *lsr* operon; however, when AI-2 is internalized and phosphorylated by LsrK, AI-2-P binds LsrR causing the de-repression of the operon. Thus, increased expression of

the Lsr system leads to increased AI-2 import, resulting in a rapid depletion of AI-2 from the extracellular medium.

It does not appear that AI-2 taken up by this system is used as a carbon source, since cultures of *S. Typhimurium* and *S. meliloti* were unable to grow when AI-2 was used as the sole carbon source (236, 306). Rather, AI-2 removal via the Lsr system enables these organisms to terminate their own AI-2 signaling system and to regulate the AI-2-dependent gene expression of other organisms in the vicinity. Thus, in cultures composed of different species, bacteria with a functional Lsr system are capable of interfering with AI-2-mediated group behaviors of the other species (343).

Recently, two studies have undertaken database sequence analysis to identify LsrB orthologs (256, 297). These studies showed that orthologs to the Lsr system are not broadly conserved across the Bacteria Kingdom, while identifying hypothetical LsrB receptors in some organisms belonging to very distinct families such as *Enterobacteriaceae*, *Pasteurellaceae*, *Rhizobiaceae*, *Rhodobacteraceae* and *Bacillaceae*.

Here, we expand upon the previous bioinformatic studies (256, 297) with additional analysis, based not only on sequence but also on structure prediction, that allow us to establish a set of criteria for predicting which orthologs of LsrB are functional AI-2 receptors. We then present experimental evidence that confirms a set of these predictions and demonstrates the presence of functional AI-2 receptors in the *Enterobacteriaceae*, *Rhizobiaceae* and *Bacillaceae* families.

3 – Materials and Methods

3.1 – Bacterial strains and growth conditions

The strains used are listed in **Table 1**. Bacteria from the *Enterobacteriaceae* family (*E. coli* MG1655 and UTI89 UPEC) and the *Bacillaceae* family (*Bacillus cereus* ATCC 10987 and *Bacillus anthracis* Sterne 34F2 vaccine strain) were grown in Luria-Bertani (LB) medium with shaking at

37°C. The bacteria from the *Rhizobiaceae* family (*S. meliloti* Rm1021, *Agrobacterium tumefaciens* C58, *Rhizobium etli* CFN42 and *Rhizobium leguminosarum* bv viciae 3841) were cultured with shaking at 30°C in their optimal cultured medium respectively: LBMC (LB supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂), LB, YEM (10 g L⁻¹ mannitol, 0.5 g L⁻¹ yeast extract, 0.2 g L⁻¹ MgSO₄·7H₂O and 1 g L⁻¹ NaCl) and TYC (5 g L⁻¹ tryptone, 3 g L⁻¹ yeast extract, 0.5 g L⁻¹ CaCl₂).

Table 1 – Bacterial strains used in this study.

Strain	Source and Reference
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar <i>Typhimurium</i>	ATCC 14028
<i>Escherichia coli</i> K-12 MG1655	(30)
<i>Escherichia coli</i> UTI89 (UPEC)	Jeffrey I. Gordon (254)
<i>Bacillus anthracis</i> Sterne 34F2 (vaccine strain)	Martin J. Blaser (149)
<i>Bacillus cereus</i> (ATCC 10987)	Adriano O. Henriques
<i>Sinorhizobium meliloti</i> Rm1021	(202)
<i>Agrobacterium tumefaciens</i> C58	James P. Shapleigh (16)
<i>Rhizobium leguminosarum</i> bv. viciae 3841	Gladys Alexandre (208)
<i>Rhizobium etli</i> CFN42	ATCC 51251

3.2 – Databases analysis

The KEGG SSDB (Kyoto Encyclopedia of Genes and Genomes - Sequence Similarity Data Base, <http://www.genome.jp/kegg/ssdb/>) was used to search for protein orthologs of LuxS and the Lsr operon from *S. Typhimurium* LT2 in January 2009. This database provides amino acid sequence similarities between all protein-coding genes in the complete genomes in the GENES database and all possible pairwise genome comparisons are performed by the SSEARCH proGram (233) available at <http://www.genome.jp/kegg/ssdb/>). In this study, we have selected gene pairs that were best bidirectional hits and had a Smith-Waterman similarity score of at least 100. To be considered a best bidirectional hit, the relationship of gene x in genome A with gene y in genome B must be such

that, when x is compared against all genes in genome B, y is found as the top scoring and the reverse is also true. Pairs that met these criteria were scored as orthologs proteins.

3.3 – Structure Prediction

All LsrB protein orthologs were submitted to the fold-recognition server PHYRE (157) for structure prediction. In the majority of cases, *S. Typhimurium* LsrB was identified as one of the top ten fold templates and, thus, the server returned a structure-based sequence alignment between LsrB and the query sequence. Alignments were examined to determine if residues previously shown to form hydrogen bonds with R-THMF in *S. Typhimurium* LsrB (K35, D116, D166, Q167, P220, and A222, (210)) were conserved in the predicted structure. For the one-third of group II orthologs where PHYRE did not return an alignment with LsrB, simple sequence alignments were calculated using NCBI-blastp (7, 124) and checked for conservation of the residues listed above. Such cases are noted in **Table S1** (<http://jb.asm.org/cgi/content/full/191/22/6975/DC1>).

3.4 – AI-2 activity in bacterial cultures

To monitor AI-2 activity in *E. coli* and *Bacillus* cell cultures during growth, overnight cultures were grown to saturation and diluted (1:100) into 25 ml of LB medium in 250 ml Erlenmeyer flasks. In *Rhizobiaceae* species, cultures in exponential phase were diluted to optical density at 600 nm (OD_{600}) = 1 into the appropriate medium with 80 μ M chemically synthesized AI-2 (276, 347). In both cases, aliquots were collected at the indicated times and cell-free culture fluids were prepared by filtration of liquid cultures (298, 299) which were analyzed in duplicate for AI-2 activity using the *V. harvey* BB170 bioluminescence reporter assay, as described previously (24, 25). AI-2 activity is reported as fold induction of light production compared with the background light obtained with the appropriate growth medium (as previously explained in (236)).

3.5 – Protein expression and purification

The genes encoding LsrB orthologs in *R. etli*, *R. leguminosarum*, *A. tumefaciens*, *E. coli* MG1655 and *E. coli* UTI89 were cloned from genomic DNA into the plasmid pProEX HTb for expression as polyhistidine-tagged proteins. The *B. anthracis* LsrB ortholog was cloned into the plasmid pET151/D-TOPO using The Champion pET Directional TOPO Expression Kit (Invitrogen) for expression as a polyhistidine-tagged fusion protein. N-terminal signal peptides for secretion, as determined by the proGram SignalP 3.0 (29), were excluded from the constructs. Plasmids were transformed into *E. coli* strains BL21 and FED101 (BL21 *luxS* null mutant) and expression was induced with 0.1 mM Isopropyl beta-D-1-thiogalactopyranoside (IPTG) when the cultures reached an OD₅₉₅ of 0.9. The bacteria were harvested after expressing for 5 hours at 22°. Pellets were resuspended in 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM DTT, 0.36 mg/ml leupeptin, 0.36 mg/ml aprotinin, 0.36 mg/ml DNase and lysed using an M-110Y Microfluidizer (Microfluidics). The lysate was centrifuged and the tagged protein purified using Ni-NTA affinity chromatography (Qiagen). Protein was eluted from the column using 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole and then buffer swapped using Sephadex-G25 agarose into 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 1 mM DTT. Purified protein was concentrated to 10 mg mL⁻¹. The genes encoding the *S. Typhimurium* and *B. cereus* LsrB orthologs, were cloned in pGEX-4T1, transformed, expressed and purified as described previously (210, 236). The primers used for cloning the respective genes are listed in **Table 2**.

3.6 – AI-2 binding assay

Proteins tested for AI-2 binding were denatured (70°, 10 min) to release any bound ligand and pelleted (51). The *V. harveyi* strain BB170 was used to test for the presence or absence of AI-2 in the resulting supernatants as previously described (24, 25).

3.7 – *B. anthracis* mutagenesis

The mutations D171N and A227T were introduced into two separate *B. anthracis*/pET151 constructs using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Primers used for creating the mutations are given in **Table 2**. The same kit was then used to create the double mutant D171N/A227T. The mutant proteins were expressed and purified as described above for the *B. anthracis* wild-type LsrB ortholog.

Table 2 – Primers used in this study.

Construct - purpose	5'/sense	3'/antisense
<i>R. leguminosarum</i> / pPro – PCR	CGCGGATCCGCCGACATCAAGATCG GT	CCGCTCGAGCGTCAGAAGACCTTGGA GAATCG
<i>A. tumefaciens</i> / pPro – PCR	CGCGGATCCGCAGACGTCAAGATCG C	CCGCTCGAGCAATCTTCGAGAACTGAT CGAT
<i>R. etli</i> / pPro – PCR	CGCGGATCCAAGGACATCAAGATCGG C	CCGCTCGAGTCAGAAGACCTTGAGAG ACTG
<i>E. coli</i> UPEC / pPro - PCR	CGGGATCCGCGGAAAAAGTCG	CCGCTCGAGTTAATAAAGTGAGTCGAT ATTGTC
<i>E. coli</i> MG1655 / pPro - PCR	CGCGGATCCGCAGAGCGTATTGCATT T	CCGCTCGAGTCAGAAATCGTATTTGCC GAT
<i>B. anthracis</i> / pET151 - D171N	CTCTAGTCCAACAGTAACGAATCAAA ACCAATGGGTAAC	GTTACCCATTGGTTTTGATTCTGTACT GTTGGACTAGAG
<i>B. anthracis</i> / pET151 - A227T	TATTAATGCAGTCATTTGTCCGGATAC GACGGCACTTCCAG	CTGGAAGTGCCGTCGTATCCGGACAA ATGACTGCATTAATA
<i>S. Typhimurium</i> / pGEX - PCR	(210)	
<i>B. cereus</i> / pGEX - PCR	CGGGATCCAAGAAAAAGCTGATGAT GT	GGAATTCCTAATCAATATTATCCTTCGT AAATACGAC
<i>B. anthracis</i> / pET151 – PCR	CACCGATAAGAAAAAGCGGA	CTAAAAATTATATTTATCAATAT

3.8 – Phylogenetic analyses

The evolutionary history of the *lsrB* gene was studied by analyzing the phylogenetic relationship of the functional orthologs identified in this study and contrasting it with the phylogeny of *rpoB* (RNA polymerase β -subunit). *rpoB* is generally accepted to provide a good representation of the phylogenetic relationships within Bacteria (46), as it provides a comparable phylogenetic resolution to that of 16S rRNA with the advantage of being a single-copy gene. To construct the organismal tree, the *rpoB* gene sequences

from all the organisms in **Table S1** (<http://jb.asm.org/cgi/content/full/191/22/6975/DC1>) and representative species of all major phyla of Bacteria were downloaded from the KEGG database and aligned with ClustalW (312) using the translated protein sequences. Alignments were carried out with default parameters and visually inspected in Molecular Evolutionary Genetics Analysis (MEGA), version 4 (308). Hypervariable regions with ambiguous alignment were excluded from analysis. The *LsrB* gene tree was made with all the sequences identified as functional *LsrB* orthologs (group I, **Table 3**) and the tree was inferred using maximum likelihood (ML) in PAUP* 4.0b10 (301) using heuristic searches, 10 random taxon-additions, and TBR branch-swapping. Mrbayes 3.1.2 (263) was used to infer branch support by running two simultaneous sets of four Markov chains for 1 million generations sampled every 100 generations. The distribution of the log likelihoods was used to evaluate the stationarity of this parameter and to determine burn-in values. Modeltest 3.7 (242) and MrModeltest 2.2 (224) were used to select the best-fitting evolutionary models for phylogenetic analyses. The *rpoB* phylogeny was estimated with a total dataset of 83 species. This dataset was translated to amino acids and analyzed using Neighbor-Joining (268) with the Poisson correction distances (363) and a gamma distribution rate variation among sites. Nodal support was estimated with non-parametric bootstrap (1000 replicates). The *rpoB* trees were rooted with *Thermotoga maritima* (Thermotogales). These analyses were carried out in MEGA.

4 – Results

4.1 – LsrB orthologs in completely sequenced bacterial genomes

To search for orthologs of LsrB we carried out a reciprocal best hit analysis against all 809 completely sequenced bacteria genomes present in the KEGG database as of January 2009 using the protein sequence of LsrB from *S. Typhimurium* (STM4077). The reciprocal best hit strategy of sequence similarity comparisons has been employed previously for this type of studies because it allows the distinction between orthologs and paralogs (43). The

organisms with proteins identified as orthologs are shown in **Table 3** (KEGG proteins identities and E-values are provided in **Table S1** <http://jb.asm.org/cgi/content/full/191/22/6975/DC1>). Sorting these organisms in order of percentage of identity of the LsrB orthologs with the *S. Typhimurium* AI-2 receptor clearly revealed two distinct groups of LsrB orthologs: a first group with high percentage identity (>60%, E-value bellow 1E-103), and a second group with percentage identity below 36% (E-value higher than 1E-44) which we termed group I and group II, respectively.

We then performed the reciprocal best hit analysis against all genomes using each LsrB protein sequence from group I as a reference (i.e. instead of the LsrB from *S. Typhimurium*). In all cases, the only hits with greater than 57 % identity were the other protein sequences included in group I from the first analysis. Thus, the group I orthologs are consistent regardless of the LsrB sequence used as reference.

The genomes of the organisms with LsrB orthologs were further analyzed to identify orthologs of the other proteins of the Lsr operon. As shown in **Table 3**, all the species of group I have orthologs of all the proteins in the Lsr operon (with the exception of LsrF, a putative AI-2 processing protein, in *Rhodobacter sphaeroides*), whereas none of the group II organisms have orthologs of the complete operon, lacking at least two proteins encoded by genes from this operon in all cases. LsrE was not included in this analysis because the protein seems to be exclusive to the *Salmonella* genus and a LsrE knockout mutant in *S. Typhimurium* showed no phenotype related to the regulation of the *lsr* operon or AI-2 production (305, 306).

Reasoning that conservation of the residues that formed hydrogen bonds with AI-2 (210) would be crucial to LsrB function, we next used a fold recognition-based server to predict structures for the LsrB orthologs. The sequences of the LsrB orthologs were submitted to the PHYRE web server (157), which returned structure predictions and structure-based alignments based on each of the ten best scoring template PDB structures available in the PHYRE library. For all of the orthologs in group I and two-thirds of the

orthologs in group II, the structure of *S. Typhimurium* LsrB was returned as one of these top ten templates. The alignments with *S. Typhimurium* LsrB were then examined to determine if residues previously shown to form hydrogen bonds with *R*-THMF in *S. Typhimurium* LsrB (K35, D116, D166, Q167, P220, and A222, (210)) were predicted to be structurally conserved. Strikingly, as shown in the last column of **Table 3**, these six residues were completely conserved in all of the orthologs in group I and differed in at least two positions in all cases for group II. Residue D166 (numbering based on *S. Typhimurium* LsrB) was not conserved in any of the group II orthologs, most typically being replaced with an asparagine. The other most common substitution was A222T (a full listing of the non-conserved amino acids is given in **Table S1** <http://jb.asm.org/cgi/content/full/191/22/6975/DC1>).

Based on these results, we hypothesize that the species in group I, which have >60% identity, orthologs to the proteins of the Lsr operon, and all six AI-2 binding site residues conserved, have functional LsrB-like AI-2 receptors, whereas group II proteins are likely to have a different function.

4.2 – Profiles of AI-2 removal from extracellular medium

Previous studies in *S. Typhimurium* (306), *E. coli* (345), *S. meliloti* (236), and *A. actinomycetemcomitans* (279) revealed that the *lsr* operon in these organisms encodes proteins involved in an ABC transport system that imports extracellular AI-2. Thus, in the presence of these organisms, AI-2 does not persist in the extracellular medium but is internalized by the cells and further modified. Our analysis, described above, indicated that all the organisms predicted to have functional LsrB receptors (group I, **Table 3**) also had orthologs to all the proteins in the Lsr operon. Thus, we predicted that the organisms in group I have a functional Lsr system for AI-2 internalization and that these organisms would rapidly remove AI-2 from culture fluids. In contrast, for organisms from group II, which lack orthologs to some of the proteins in the Lsr operon and presumably do not have a functional AI-2 transport system, we predicted that AI-2 would persist in the extracellular media. To test these predictions, we compared the profile of AI-2 removal of a set of organisms from groups I and II.

Table 3 - Orthologs of the LuxS and Lsr proteins from *S. Typhimurium* present in the complete genomes of the KEGG database (January, 2009).

Species	Orthologs ^b									LsrB identity ^c	Binding site residues ^d
	LuxS	LsrB	LsrA	LsrC	LsrD	LsrK	LsrR	LsrG	LsrF		
group I											
<i>Salmonella Typhimurium</i> LT2	+	+	+	+	+	+	+	+	+	100%	6
<i>Salmonella enterica</i> (13 strains)	+	+	+	+	+	+	+	+	+	100%	6
<i>Escherichia coli</i> (11 strains)	+	+	+ ^e	+	+	+	+	+	+	85%	6
<i>Escherichia fergusonii</i>	+	+	+	+	+	+	+	+	+	85%	6
<i>Yersinia pestis</i> (7 strains)	+	+	+	+	+	+	+	+	+	84%	6
<i>Yersinia pseudotuberculosis</i> (4 strains)	+	+	+	+	+	+	+	+	+	84%	6
<i>Yersinia enterocolitica</i>	+	+	+	+	+	+	+	+	+	83%	6
<i>Klebsiella pneumoniae</i> (2 strains)	+	+	+	+	+		+	+	+	82%	6
<i>Photorhabdus luminescens</i>	+	+	+	+	+	+	+	+	+	82%	6
<i>Enterobacter</i> sp. 638	+	+	+	+	+	+	+	+	+	82%	6
<i>Pasteurella multocida</i>	+	+	+	+	+	+	+	+	+	80%	6
<i>Haemophilus influenzae</i> PittEE	+	+	+	+	+	+	+	+	+	80%	6
<i>Haemophilus somnus</i> (2 strains)	+		+	+	+	+	+	+	+	76%	6
<i>Sinorhizobium meliloti</i>			+	+	+	+	+	+	+	72%	6
<i>Rhodobacter sphaeroides</i> (2 strains)			+	+	+	+	+	+		72%	6
<i>Bacillus anthracis</i> (4 strains)	+	+	+	+	+	+	+	+	+	63%	6
<i>Bacillus cereus</i> (7 strains)	+	+	+	+	+	+	+	+	+	63%	6
<i>Bacillus thuringiensis</i> (2 strains)	+	+	+	+	+	+	+	+	+	63%	6
group II											
<i>Rubrobacter xylanophilus</i>		+			+			+	+	36%	3
<i>Ochrobactrum anthropi</i>		+		+	+		+		+	35%	4
<i>Sinorhizobium medicae</i>		+		+	+		+	+	+	35%	4
<i>Roseobacter denitrificans</i>		+			+		+	+		34%	4
<i>Mesorhizobium loti</i>		+			+		+		+	34%	4
<i>Agrobacterium tumefaciens</i> C58 (2 strains)		+			+		+	+	+	33%	4
<i>Leptothrix cholodnii</i>		+			+		+		+	33%	4
<i>Dinoroseobacter shibae</i>		+		+	+	+	+	+		33%	4
<i>Verminephrobacter eiseniae</i>		+			+		+			33%	4
<i>Burkholderia phytofirmans</i>			+		+		+			33%	4
<i>Gluconacetobacter diazotrophicus</i> PAI 5 (JGI)		+			+					33%	2
<i>Rhizobium leguminosarum</i>		+		+	+		+	+	+	33%	4
<i>Rhizobium leguminosarum</i> bv. trifolii WSM2304		+		+	+		+		+	33%	4
<i>Gluconacetobacter diazotrophicus</i> PAI 5 (Brazil)		+			+					33%	1
<i>Rhodococcus</i> sp. RHA1		+		+	+		+	+		33%	4
<i>Streptomyces coelicolor</i>	+	+		+	+		+	+		33%	4
<i>Burkholderia xenovorans</i>		+		+	+		+		+	32%	3
<i>Rhizobium etli</i>		+			+		+	+	+	32%	4
<i>Dictyoglomus thermophilum</i>		+			+					32%	4
<i>Rhizobium etli</i> CIAT 652		+			+		+	+		32%	4
<i>Jannaschia</i> sp. CCS1		+		+	+					32%	4
<i>Dictyoglomus turgidum</i>		+			+					32%	4
<i>Acidiphilium cryptum</i> JF-5		+					+			31%	2
<i>Streptomyces avermitilis</i>		+		+	+		+			31%	4
<i>Burkholderia phymatum</i>		+		+	+		+		+	31%	4
<i>Deinococcus geothermalis</i>	+	+		+	+		+			31%	4
<i>Burkholderia ambifaria</i> MC40-6		+		+	+		+	+	+	31%	4
<i>Syntrophomonas wolfei</i>		+		+	+		+			30%	1
<i>Chloroflexus aggregans</i>		+		+	+					27%	4
<i>Escherichia coli</i> APEC O1	+	+			+	+	+			27%	0
<i>Escherichia coli</i> UT189 (UPEC)	+	+			+	+	+			27%	1

^a Organisms classified as group I are highlighted in black and group II in grey.

^b Orthologs of both group I and group II are defined as a complete match in the bidirectional best hits and are denoted with +.

^c Percentage of identity using *S. Typhimurium* LsrB as reference.

^d Number of conserved residues in the binding site based on structure prediction using *S. Typhimurium* LsrB as reference.

^e LsrA from *Escherichia coli* E24377A is truncated.

Our analysis revealed that almost all *E. coli* strains (11 out of 13) analyzed belong to group I. However, two *E. coli* strains (*E. coli* APEC and *E. coli* UT189 / UPEC) have LsrB orthologs with very low sequence identity and lack orthologs to several of the proteins from the *lsr* operon and therefore are classified as members of group II (**Table 3**). We tested an *E. coli* strain (MG1655) from group I for AI-2 uptake and found, as had been previously shown (345), that this strain removed AI-2 from culture fluids (**Figure 1A**). We then compared the AI-2 removal profile in *E. coli* UT189 / UPEC strain (from group II) with the profile from *E. coli* MG1655 strain and observed that, while *E. coli* MG1655 efficiently cleared AI-2 from culture fluids by 6 hours, the *E. coli* strain UT189 / UPEC strain cleared little, if any, AI-2 by 10 h (**Figure 1A**). This supports our prediction that the uropathogenic strain UT189 / UPEC, though belonging to the same species as MG1655, is a member of group II and accordingly does not have a functional Lsr transport system for AI-2 uptake.

Like *E. coli* MG1655, two *Bacillus* strains, *cereus* (ATCC 1087) and *anthracis* (vaccine Sterne 34F2), have orthologs classified as group I. Putative AI-2 receptors have been identified in these species previously (256, 297), but not confirmed experimentally. We tested these strains for AI-2 removal and, as expected, they were able to completely remove AI-2 from culture fluids (**Figure 1B**), supporting the premise that organisms in group I have functional AI-2 transporters.

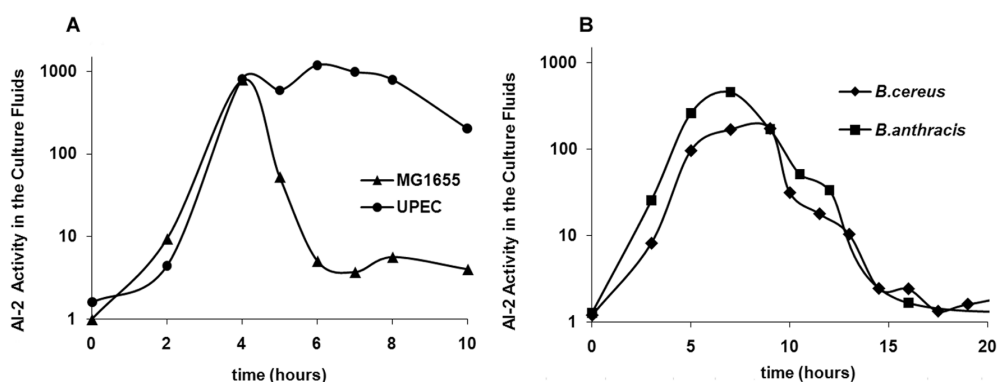


Figure 1 - AI-2 removal profile in bacteria producing AI-2. Extracellular AI-2 activity in cell-free culture fluids from LuxS⁺ strains **A)** *E. coli* MG1655 (triangles) and *E. coli* UPEC (circles) and **B)** *B. cereus* (diamonds) and *B. anthracis* (squares) cultures. Aliquots were taken at the specified times. AI-2 activity is reported as fold induction of light produced by *V. harveyi* BB170.

To further test the premise that group II organisms are unable to incorporate AI-2, we compared AI-2 removal in organisms from the *Rhizobiaceae* family from group II (*R. etli*, *R. leguminosarum*, and *A. tumefaciens*) with AI-2 in the only *Rhizobiaceae* from group I (*S. meliloti*). None of the *Rhizobiaceae* in **Table 3** has LuxS orthologs, and thus we expected that none of these species would produce AI-2. This was confirmed by the fact that cell-free culture fluids collected from these bacteria produced only low levels of bioluminescence induction in a *V. harveyi* BB170 bioassay (data not shown). However, as we have previously shown in the case of *S. meliloti*, non-AI-2 producing species can still be capable of taking up AI-2 produced synthetically or by other species (236). Thus, in order to compare AI-2 removal profiles in these species, we cultured these bacteria to the same cell density ($OD_{600}=1$), supplied chemically synthesized AI-2, and measured AI-2 activity in the culture fluids over time (**Figure 2**). Over the time of the measurements, *S. meliloti* effectively removed the exogenously provided AI-2 while the other three species did not, supporting the prediction that the bacteria from group II (*R. etli*, *R. leguminosarum* and *A. tumefaciens*), and likely all group II species, do not have Lsr systems capable of taking up AI-2.

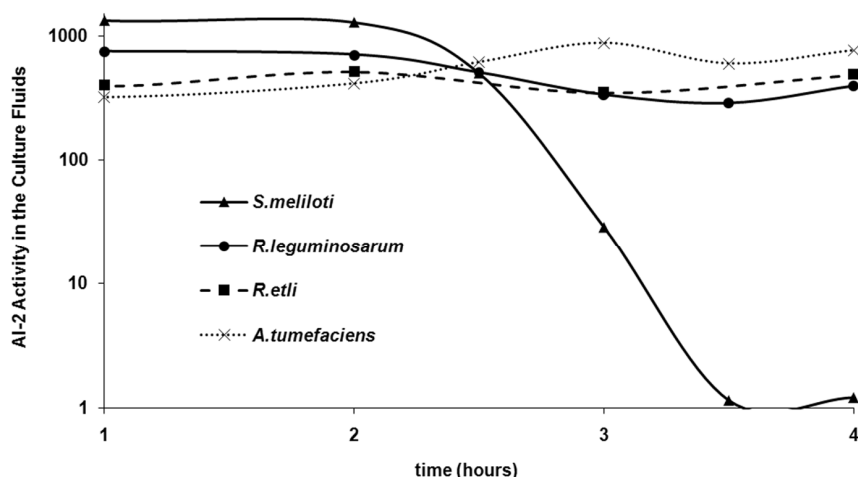


Figure 2 - Removal of exogenously supplied AI-2. *S. meliloti* (triangles), *R. leguminosarum* (circles), *R. etli* (squares), and *A. tumefaciens* (crosses) were cultured to $OD_{600}=1$ in their optimal culture media (LBMC, TYC, YEM and LB respectively). Chemically synthesized AI-2 was then added to all the cultures and aliquots were taken at the specified times. AI-2 activity in cell-free culture fluids is reported as fold induction of light produced by *V. harveyi* BB170.

4.3 – *In vitro* AI-2 binding to LsrB orthologs

While the above results support our ability to identify species with functional AI-2 transporters, they do not directly show that the identified LsrB ortholog is responsible for AI-2 binding. In order to directly test for AI-2 binding ability, we cloned the LsrB orthologs from the same organisms tested in the previous section (three belonging to group I and four belonging to group II) and compared their ability to bind AI-2 with that of LsrB from *S. Typhimurium*. The candidate proteins were overexpressed in both an *E. coli* strain that produces AI-2 and, as a negative control, in a *luxS* mutant *E. coli* strain that does not make AI-2. These proteins were then purified and tested for the ability to bind AI-2 using a previously developed assay (51) in which the protein is heat denatured to release any bound ligand. The denatured protein is then pelleted and the resulting supernatants are added to a reporter strain of *V. harveyi* that bioluminesces in response to AI-2. As shown in **Figure 3**, all three orthologs from group I (i.e. that have >60% identity, a complete set of orthologs to the *lsr* genes, and the six amino acids from the binding pocket conserved), *E. coli* MG1655, *B. cereus* and *B. anthracis*, showed a LuxS-dependent AI-2 binding ability similar to that observed for the previously characterized *S. Typhimurium* LsrB protein (first four pairs of bars in **Figure 3**). Conversely, no AI-2 binding activity was detected in the candidates from the group II (*R. etli*, *R. leguminosarum*, *A. tumefaciens*, *E. coli* UT189 / UPEC, last four pairs of bars in **Figure 3**). Thus, as predicted from sequence analysis and structure prediction (above), LsrB orthologs from group I demonstrate AI-2 binding ability while group II orthologs lack this ability.

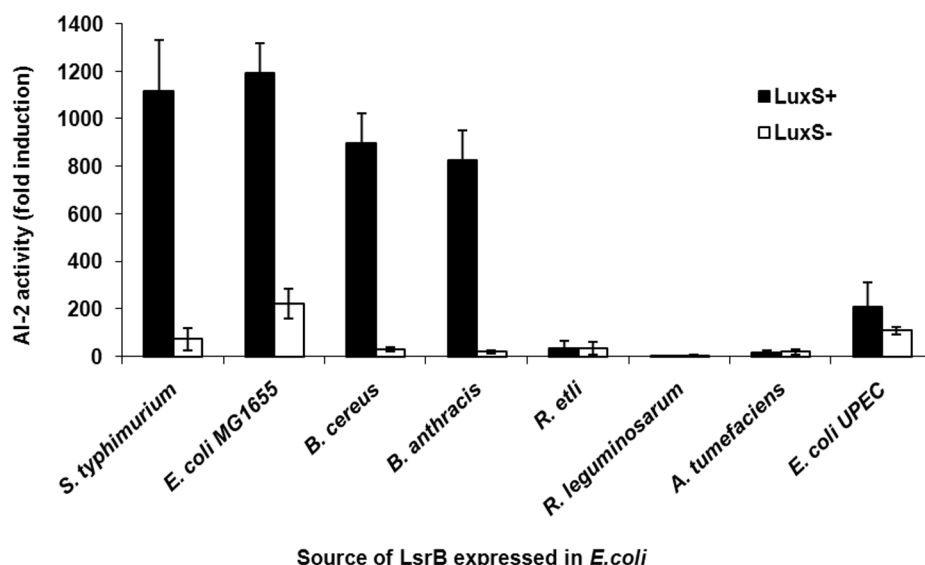


Figure 3 - Binding of AI-2 to potential LsrB-like orthologs. Proteins were expressed in either LuxS⁺ (black bars) or LuxS⁻ (white bars) *E. coli* strains (BL21 and FED101, respectively), purified, and denatured to release the ligand. The released ligand was added to a *V. harveyi* AI-2 reporter strain (BB170) to determine AI-2 activity. AI-2 activity is reported as fold induction of light production by *V. harveyi* BB170 supplemented with protein supernatant to that of the appropriate buffer. Error bars represent the standard deviations for three independent cultures.

4.4 – The amino acids Aspartate 166 and Alanine 222 are required for AI-2 binding

Based on predicted structure-based sequence alignments (above), the amino acids that form hydrogen bonds with AI-2 are completely conserved in all of the LsrB orthologs that demonstrated the ability to bind AI-2. In contrast, all the proteins that were unable to bind AI-2 in our *in vitro* assays lacked at least two of these residues. Specifically, in *R. etli*, *R. leguminosarum* and *A. tumefaciens*, there are predicted to be two substitutions: D166N and A222T (numbering follows LsrB from *S. Typhimurium*). Indeed, the majority of the proteins in group II have these substitutions, though other substitutions are observed (see **Table S1** <http://jb.asm.org/cgi/content/full/191/22/6975/DC1> for detailed information). The complete conservation of AI-2 hydrogen binding residues in orthologs of group I but not group II is apparent in a multiple sequence alignment of all of the LsrB orthologs for which we have experimental data (purple in Supplementary **Figure S1**). It is worth noting that 29 non-binding site residues

are completely conserved across all groups in this alignment (yellow in **Figure S1**). However, structural analysis shows that these residues are not clustered. Moreover, these residues are disproportionately Gly and Pro (10 and 4 conserved occurrences respectively) suggesting that, unlike the six residues in the binding site, these residues are conserved for structural rather than functional reasons.

We interpreted this to indicate that residues D166 and A222 are essential for AI-2 binding ability, and to test this idea we introduced the above mutations (D166N and A222T) into the *B. anthracis* LsrB ortholog, both individually and together, and assayed for AI-2 binding ability. As shown in **Figure 4**, while the wild type protein is capable of binding AI-2, no AI-2 activity was present in the binding pockets of any of the mutants as measured by the *V. harveyi* bioassay. As a complementary experiment, we tested the ability to create AI-2 binding capacity in the distantly related LsrB ortholog of *R. etli* by mutating the putative binding site residues to mimic the binding site of the proteins from group I. These mutants failed to show AI-2 binding in the *V. harveyi* bioassay (data not shown), indicating that these proteins have already diverged to such a degree that other aspects of the protein structure important for AI-2 binding are missing.

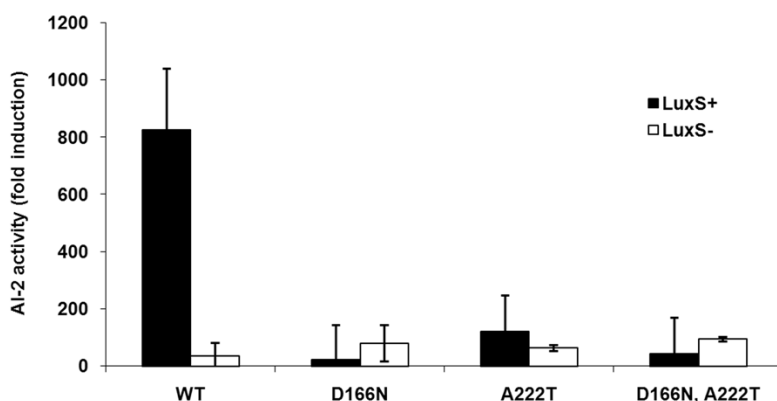


Figure 4 - Binding of AI-2 by *B. anthracis* wild type (WT) and mutants D166N and A222T LsrB-like proteins. *B. anthracis* wild type (WT), and mutant D166N and A222T proteins were expressed in either LuxS⁺ (black bars) or LuxS⁻ (white bars) as explained in Figure 3. AI-2 activity is reported as fold induction of light production by *V. harveyi* BB170 supplemented with protein supernatant to that supplemented with appropriate buffer. Error bars represent the standard deviations for three independent cultures.

These results show that D166 and A222, conserved in all the LsrB-orthologs we have shown to bind AI-2, are necessary (though not sufficient) for the ability of these proteins to bind AI-2, and thus provide a useful criterion for the identification of other LsrB-like AI-2 receptors. It is possible that more conservative mutations would still allow AI-2 binding, but such mutations are not observed in our list of orthologs. Further, these results support the hypothesis that the proteins in group II are incapable of AI-2 binding and are therefore very unlikely to function as AI-2 receptors *in vivo*.

4.5 – Evolution of functional LsrB-like AI-2 receptors

Our sequence/structural and functional studies lead us to predict that all the organisms from group I have LsrB orthologs that function as LsrB-AI-2 receptors. This group contains members from the evolutionary distant orders of the *Enterobacteriales*, *Pasteurellales*, *Rhizobiales*, *Rhodobacterales*, and *Bacillales*. To infer the evolutionary history of the *lsrB* gene we determined the phylogenetic tree of all the *lsrB* gene orthologs from group I (**Figure 5**) and compared it to the *rpoB* housekeeping gene organismal tree constructed with representatives of all major phyla of Bacteria (**Figure 6**). Importantly, the organismal tree recovers all major phyla and classes with high bootstrap support. The relationship among phyla has a lower bootstrap support but this does not influence our analysis because the phylogenetic relationship between all species with functional *lsrB* genes (highlighted in grey boxes **Figure 6**) is also well supported in this tree.

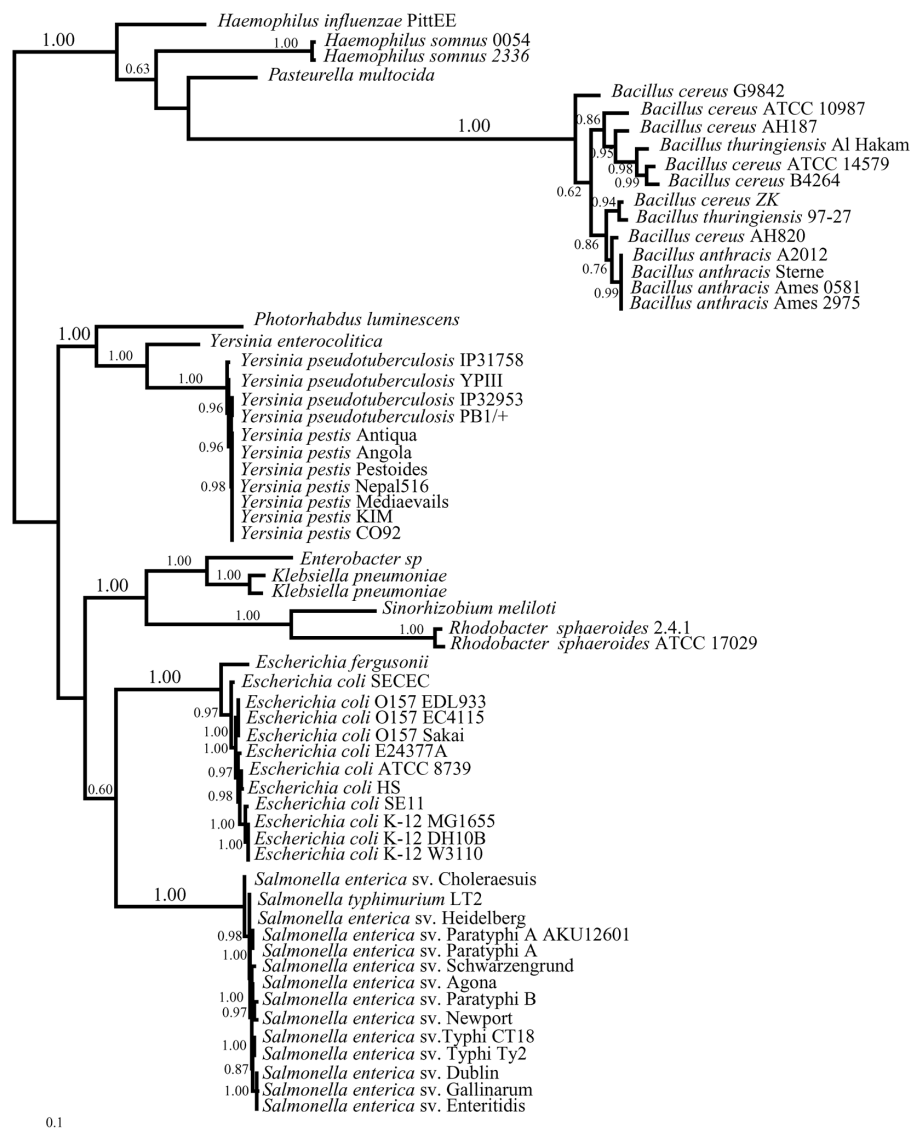


Figure 5 - Evolutionary history of genes encoding functional LsrB orthologs inferred with maximum likelihood. *IsrB* gene tree constructed with the sequences from all organisms in group I. This is an unrooted phyloGram oriented to show maximum congruence with the organismal tree. Numbers on the nodes indicate posterior probability as estimated with MrBayes.

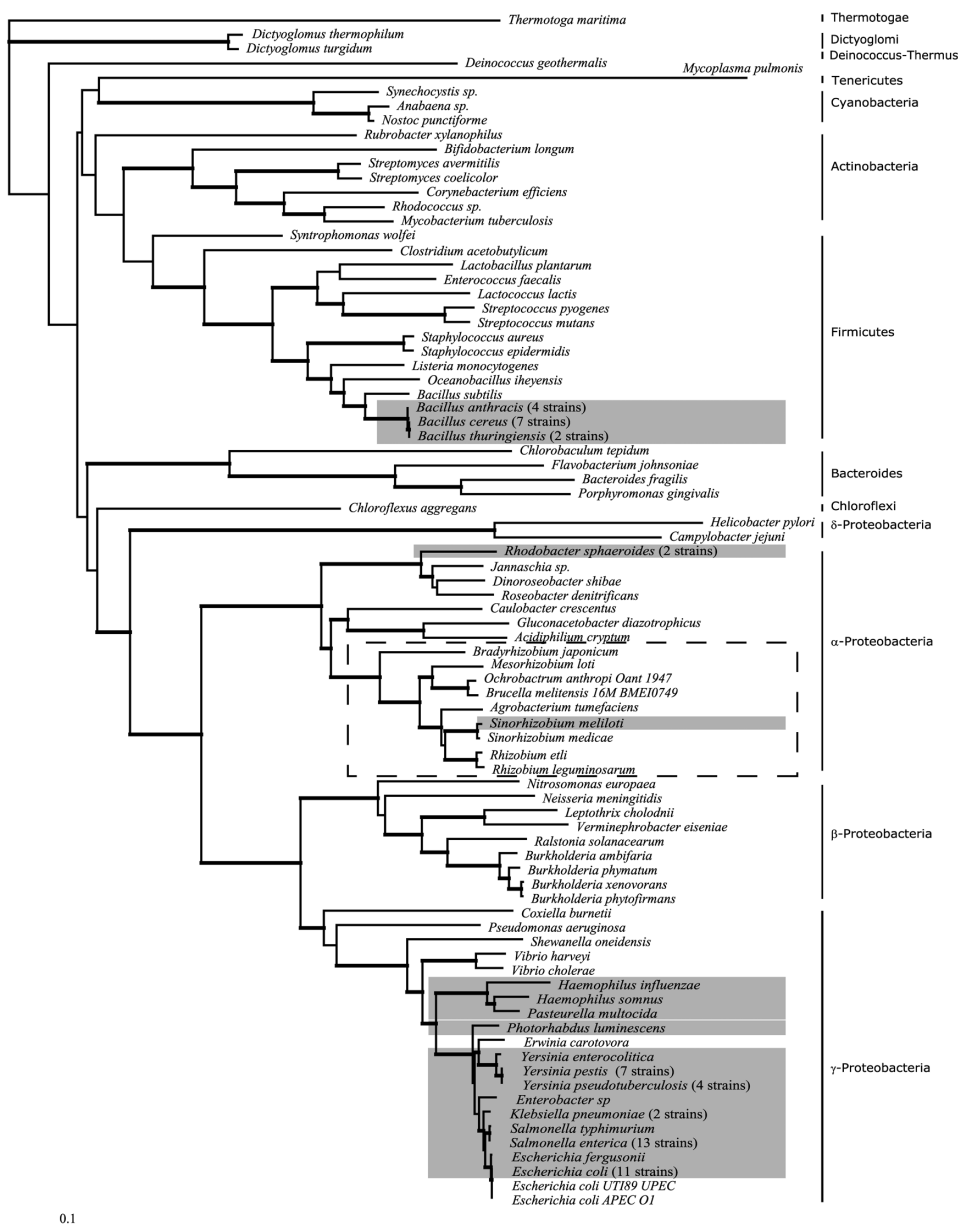


Figure 6 - Molecular phylogeny of Bacteria estimated with *rpoB* gene. *rpoB* gene tree constructed with all the organisms in Table 3 (group I and II) and representative species of all major phyla of Bacteria. This represents our best inference of the organismal tree. Grey boxes indicate species with functional *lsrB* genes (group I, Table 3), and dashed box locates the species with protein sequences in Table 3 likely to function as a rhamnose binding protein. The numbers after species names indicate the number of strains analyzed for the respective species. Taxonomic classifications (Phyla) are shown on the right. This tree was inferred with Neighbor-Joining and the branch lengths are scaled to the number of amino acid substitutions per site. Thickened branches indicate high bootstrap support (higher than 75 %). This is a measurement of phylogenetic strength between nodes and this value reflects a high confidence in the inferred relationships between species.

This analysis indicates that the phylogenies of *lsrB* and *rpoB* largely overlap in their diversification patterns, although with some important exceptions. The majority of the species included in group I of **Table 3** clustered within the *Enterobacteriales* and *Pasteurellales* (both γ -*proteobacteria*) and the diversification pattern of the *lsrB* gene mimics the phylogenetic relationships obtained in the *rpoB* organismal tree within this group (compare distributions in **Figures 5** and **6**); that is, the *lsrB* gene tree recovers all species groups and the relationship within *Enterobacteriales* and *Pasteurellales* is largely congruent between gene trees. Additionally, the widespread occurrence of LsrB within the *Enterobacteriales* and *Pasteurellales* strongly suggest a single origin for this AI-2 receptor that occurred in an ancestor of these organisms after the diversification of the *Enterobacteriales* and *Pasteurellales* from the *Vibrionales*. Nonetheless, the presence of *lsrB* genes in the *Enterobacteriales* and *Pasteurellales* is not ubiquitous, as shown by *Erwinia carotovora* and two *E. coli* (UTI89 and APECO1) suggesting independent events of gene loss (**Figure 6**).

The major discordance between the *lsrB* and *rpoB* phylogenies relates to the occurrence of functional LsrB in *S. meliloti* (*Rhizobiales*, α -*proteobacteria*), *R. sphaeroides* (*Rhodobacterales*, α -*proteobacteria*), and three species of *Bacillus* (*Bacillales*, *Firmicutes*). Specifically, *lsrB* genes from these species cluster with strong nodal support (Bayesian posterior probability of 1.0; **Figure 5**) with specific clades of the *Enterobacteriales* and *Pasteurellales*. Thus, these species appear “misplaced” in the *lsrB* gene phylogeny (**Figure 5**) in contrast with the organismal phylogeny (*rpoB* tree, **Figure 6**). This type of incongruence is consistent with LGT events (38, 302).

In the case of the *Bacillus* species, the phylogenetic pattern of the *lsrB* gene tree reveals that these species cluster with the *Pasteurellales*. Thus, the occurrence of the *lsrB* gene in the *Bacillus* lineage could be explained by a putative LGT event from bacteria of the family *Pasteurellaceae*. The occurrence of this gene within so many *Bacillus* species indicates that, if such a transfer occurred, enough time has passed for the lineage to diversify into at least three different species (**Figures 5** and **6**).

The two species from the *α-proteobacteria*, (*S. meliloti* and *R. sphaeroides*) are nested within the *Enterobacteriales* clustering with the *Klebsiella* and *Enterobacter*. Given the phylogenetic distance that separates *S. meliloti* and *R. sphaeroides* (**Figure 6**) it is surprising that the *lsrB* gene topology clusters these two species together. The most likely explanation for this occurrence requires at least more than one LGT event. Such pattern could be obtained if two sequential LGT events had occurred; for example first from one *Enterobacteria* (most likely an ancestor of *Klebsiella* and *Enterobacter*) to a *Sinorhizobium* and a second to a *Rhodobacter*, or from one *Enterobacter* first to *Rhodobacter* and then to *Sinorhizobium*. However, with the data at hand it is difficult to predict the specific order of these events. Furthermore, we predict that the proposed LGT to *S. meliloti* and *R. sphaeroides* must have been quite recent events, given that no further *α-proteobacteria* species were identified with group I LsrB orthologs. Alternatively, we could postulate one LGT event to the ancestor of these *α-proteobacteria* with a massive number of gene losses, but we find this possibility very unlikely.

5 – Discussion

A variety of bacterial species have been shown to be capable of responding to AI-2 by regulation of a range of niche-specific functions, but the mechanisms for AI-2 detection have been characterized in only a few cases (119, 344). This constitutes a major obstacle in work towards understanding of the function of AI-2. While sequence analysis of bacterial genomes reveals the presence of orthologs of LsrB-like AI-2 receptors in Gram-negative as well as Gram-positive bacteria (this study and (256, 297)), establishing which orthologs are, in fact, functional as AI-2 receptors is important for determining if and how these species use AI-2 as a chemical signal. Thus, after analyzing sequences and predicted structures of LsrB orthologs, we identified criteria for predicting which LsrB orthologs are functional AI-2 receptors and assayed the AI-2 binding ability of selected candidates to test our criteria. Our results not only support our predictions, but also provide the

first biochemical confirmation of the presence of functional AI-2 receptors in Gram-positive bacteria specifically in *B. anthracis* and *B. cereus*.

Our sequence and structural analyses allowed us to categorize the organisms with LsrB orthologs into two different groups. Members of group I have: 1) LsrB orthologs with greater than 60% sequence identity with *S. Typhimurium* LsrB, 2) orthologs to the other key transport proteins of the Lsr operon, and 3) complete conservation of all 6 residues which hydrogen bond with AI-2 in *S. Typhimurium* LsrB (based on structure prediction). On the other hand, in organisms belonging to group II the LsrB orthologs have a sequence identity below 36%, are missing orthologs to key proteins of the Lsr operon, and lack at least 2 of the 6 residues in the AI-2 binding pocket. These characteristics led us to hypothesize that the organisms from group I had functional AI-2 binding proteins, whereas the LsrB orthologs in group II were likely to have a different function. In all organisms where the function of either the LsrB protein or its gene has been studied, LsrB has been shown, along with other proteins that form the Lsr transport system, to participate in the uptake of AI-2 (236, 279, 306, 345); thus, we further predicted that organisms with a functional LsrB and orthologs to all the proteins from the Lsr system would take up AI-2. Accordingly, all the organisms from group I tested for binding of AI-2 by LsrB or for *in vivo* AI-2 removal (*S. Typhimurium*, *S. meliloti* (236), *E. coli* K-12 (MG1655), *B. cereus* and *B. anthracis*) were capable of both of these functions. None of the proteins from the organisms we tested from group II (*E. coli* UT189 / UPEC, *R. etli*, *R. leguminosarum*, *A. tumefaciens*) were capable of binding AI-2, nor were these organisms able to take up AI-2. In addition, our analysis of predicted structures of the LsrB orthologs identified key binding site residues that are not conserved in group II organisms. Mutagenesis of the *B. anthracis* LsrB ortholog (classified as group I and demonstrated to bind AI-2) with the two most common group II substitutions (D166N, and A222T) confirmed that these residues are critical for AI-2 binding. This result strongly supports our use of binding site conservation as a key criterion in identifying class I orthologs.

These results offer experimental evidence that functional LsrB-AI-2 receptors are present in particular members of the *Enterobacteriaceae* (*S. Typhimurium*, and *E. coli*), *Rhizobiaceae* (*S. meliloti*), and *Bacillaceae* (*B. cereus* and *B. anthracis*) and, given the correlation of our experimental results with our classification scheme, we predict that all the other LsrB orthologs from group I are functional AI-2 receptors and that these organisms are competent for AI-2 uptake. Accordingly, we expect that the members of the *Pasteurellaceae* and *Rhodobacteraceae* families in group I (**Table 3**) also have functional AI-2 transporters. On the other hand, we believe it is likely that all group II members have orthologs that are not involved in AI-2 transport, and thus that these organisms do not uptake AI-2 via an LsrB-type mechanism. The criteria described here can be used to predict the presence (or absence) of functional LsrB-like AI-2 receptors in newly sequenced species, and as new species are sequenced we expect the number of organisms in group I to increase.

The large majority of the organisms from group I belong to the *Enterobacteriales* and the *Pasteurellales*. This, coupled with the fact that the diversification pattern of the *lsrB* gene largely mimics the bacterial phylogenetic relationships within this group, is consistent with a single origin for the LsrB-AI-2 receptor that likely occurred in an ancestor of these organisms after the diversification of the *Enterobacteriales* and the *Pasteurellales* from the *Vibrionales*. Thus, the occurrence of LsrB receptors in one species of *Rhizobiales* (*S. meliloti*), *Rhodobacterales* (*R. sphaeroides*), and three species of *Bacillales* was very surprising and immediately raised the possibility of LGT. The hypothesis of LGT between organisms from the *Enterobacteriales* or the *Pasteurellales* and these three orders was supported by the comparison of the *lsrB* gene tree and the *rpoB* organismal tree. Specifically, in the *lsrB* gene tree the *Bacillus* are clustered with the *Pasteurellales*, and the *S. meliloti* and *R. sphaeroides* are nested within the *Enterobacteriales*. These are nested patterns where species appeared to be “misplaced” in the gene phylogeny and can be interpreted as an indication of events of LGT. Often, genes that have been acquired by LGT have atypical

nucleotide distribution (reflected in GC content or codon usage) when compared with the rest of the genome (169). However, in this case analysis of GC usage and codon bias provided no information to argue for or against the hypothesis of LGT (data not shown). Certainly, other occurrences such as convergent evolution by natural selection or ancient origin of *LsrB* at the base of the Bacteria tree with a large number of events of gene loss could also explain the observed patterns, but since we do not have specific data to support a particular explanation over the others, we favor LGT as the most parsimonious explanation as it requires the minimum number of assumptions. LGT events are now well accepted as a major force in the evolution of bacterial genomes (36, 163) leading to an increment in the number of genes (225) and pathways (139) and often enabling bacteria to acquire new functions, such as traits associated with pathogenicity, that allow adaptation to novel environments. In the specific cases of *S. meliloti* and *R. sphaeroides*, it is intriguing that that these organisms have acquired the AI-2 receptor but not its synthase (LuxS); thus, these organisms have potentially gained the ability to eavesdrop on their neighbors signal as previously suggested (236, 256). It will also be interesting to determine the adaptive value of this new function and explore its impact in the physiology of these organisms. LGT has been proposed for other autoinducer receptors and regulators from the LuxI/LuxR family of species-specific Quorum-sensing proteins, where it was proposed that the acquisition of this family of proteins has benefited certain bacterial species by allowing them to gain an efficient mechanism for regulating virulence genes (36, 114, 176).

Interestingly, the LsrB ortholog in *R. leguminosarum* bv trifolii, which we identified as belonging to group II, has been shown to be essential for rhamnose (a methyl-pentose sugar) uptake and growth in this sugar, and is thus likely to be a rhamnose binding protein (258, 259). Motivated by this finding, we used the protein sequence of *R. leguminosarum* bv trifolii (KEGG ID pRL110413) to carry out a reciprocal best hit analysis against all the genomes sequences used in the previous analysis. We found that there are 12 orthologs to the *R. leguminosarum* binding protein (along with the proteins

from the rhamnose transport operon) present in group II of **Table 3**. Thus, these 12 binding proteins are orthologs to both LsrB of *S. Typhimurium* and the rhamnose binding protein of *R. leguminosarum*. These proteins have more than 65% sequence identity with the *R. leguminosarum* protein but less than 36% identity with *S. Typhimurium* LsrB. We interpret this as strong evidence that these 12 proteins in group II are functioning as rhamnose binding proteins, in agreement with our prediction that they are not AI-2 receptors (these proteins are highlighted in the supplementary **Table S1**). These 12 organisms correspond to species belonging to *α-proteobacteria* that cluster together in the organismal *rpoB* tree (highlighted by the dashed box in **Figure 6**). Interestingly, *S. meliloti* is the only organism that has an LsrB ortholog belonging to group I and also a different set of proteins which are orthologs to the *R. leguminosarum* proteins from the rhamnose transport operon, further corroborating our hypothesis that the acquisition of LsrB occurred by LGT in *S. meliloti*.

While the presence of a functional LsrB ortholog does not prove that AI-2 import is involved in control of AI-2 mediated behavior, it is suggestive. Accordingly, the function of the Lsr system in AI-2 signaling has already been shown for a member of the *Pasteurellaceae*, the *A. actinomycetemcomitans*, (an organism not present in **Table 3** because, to date, its genome is not present in the KEGG database). Demuth and co-workers have shown that this oral pathogen is capable of internalizing AI-2 via the Lsr system and, importantly, that LsrB is required to mediate the complete AI-2-dependent activation of biofilm formation in this organism (279). In other cases like *Photobacterium luminescens*, an insect pathogen belonging to the *Enterobacteriaceae*, transcription of the *lsr* operon was shown to be induced by AI-2, and AI-2 has also been implicated in the regulation of biofilm formation and motility (166). However, it remains to be demonstrated whether or not the Lsr system is involved in mediating these AI-2 regulated behaviors. Likewise, it will be interesting to determine whether the Lsr system is involved in mediating AI-2 signal transduction in *B. cereus* and *B. anthracis*, where AI-2 has been implicated in regulating biofilm formation (12)

and growth rate (149). Certainly, the results presented here give support to that possibility.

This study, along with the two previous studies based on sequence analysis (256, 297), also reveals that certain bacteria like *Helicobacter pylori* (248), *Streptococcus mutans* (303), *Staphylococcus epidermidis* (183), *Porphyromonas gingivalis* (140, 353), *Pseudomonas aeruginosa* (80), *Bacillus subtilis* (187) which have been shown to respond to AI-2 do not have either of the known types of AI-2 receptors (neither LuxP nor LsrB), and thus we expect that other receptors for AI-2 remain to be discovered. These receptors may be of entirely new classes or may be promiscuous receptors for other small molecules. Novel receptor classes are likely to be identified by approaches that rely on genetic screens to isolate mutants involved in modulating AI-2-regulated phenotypes, and as shown here integration with approaches that use sequence analysis coupled with biochemical assays may prove very useful. Clearly, elucidation of the proteins involved in AI-2 recognition and signal relay is essential for studying the potential functions of this class of signal molecule in intra- and inter-species cell-to-cell communication and/or intra- and inter-cellular signal transduction. The identification and experimental confirmation of functional LsrB receptors in this study opens the door to the understanding of the molecular basis of AI-2 mediated behavioral regulation in a variety of new species.

6 – Acknowledgments

Catarina S. Pereira, Stephen T. Miller and Karina B. Xavier planned the experiments, analyzed the data and wrote the manuscript. Anna K. de Regt and Stephen T. Miller did the structure predictions and the AI-2 binding assays. Patrícia H. Brito performed the phylogenetic analysis. Catarina S. Pereira performed all the other experiments.

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7 – Supplementary material

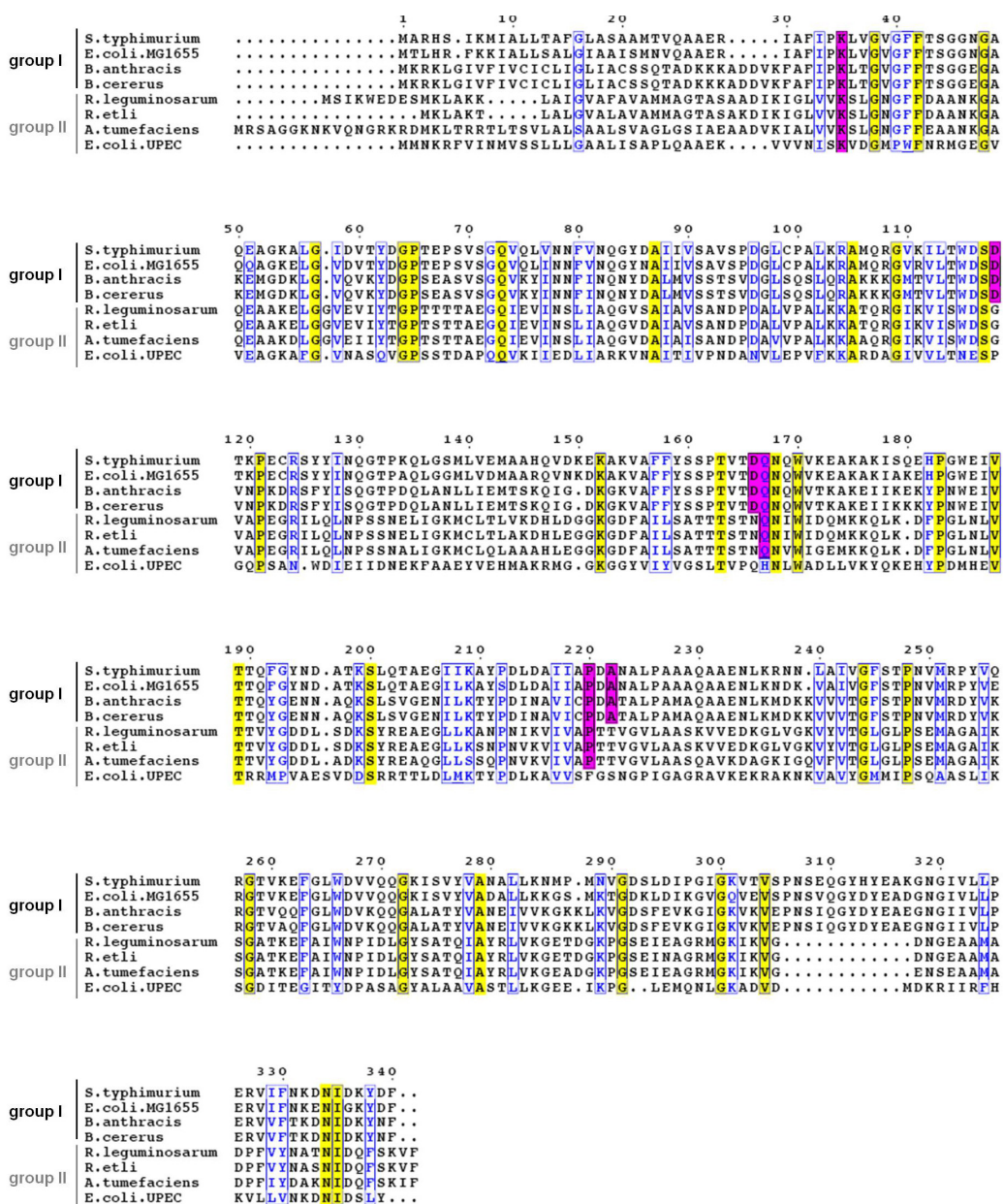


Figure S1 – Multiple sequence alignment of LsrB orthologs studied experimentally in this study. Residues identical to those that form hydrogen bonds with AI-2 in *S. Typhimurium* LsrB (Miller et al, 2004) are colored purple. Note that these six residues are completely conserved in all proteins from group I, but the group II proteins lack at least two of these residues. Non-binding site residues with complete identity across species are yellow, and chemically conserved substitutions are boxed in blue. Numbering follows *S. Typhimurium* LsrB.

CHAPTER V

Sinorhizobium meliloti, a bacterium lacking the autoinducer-2 (AI-2) synthase, responds to AI-2 supplied by other bacteria

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Many bacterial species respond to the Quorum-sensing signal autoinducer-2 (AI-2) by regulating different niche specific genes. Here, we show that *Sinorhizobium meliloti*, a plant symbiont lacking the gene for the AI-2 synthase, while not capable of producing AI-2 can nonetheless respond to AI-2 produced by other species. We demonstrate that *S. meliloti* has a periplasmic binding protein that binds AI-2. The crystal structure of this protein (here named SmLsrB) with its ligand reveals that it binds (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), the identical AI-2 isomer recognized by LsrB of *Salmonella typhimurium*. The gene encoding SmLsrB is in an operon with orthologs of the *lsr* genes required for AI-2 internalization in enteric bacteria and we have shown, using qRT-PCR, that increased transcription of the *lsr*-like operon, named *ait*, occurs in response to AI-2. Accordingly, *S. meliloti* internalizes exogenous AI-2, and mutants in the *ait* operon are defective in AI-2 internalization. Furthermore, we show that *S. meliloti* does not grow in AI-2 as a sole carbon source and that this organism can completely eliminate the AI-2 secreted by *Erwinia carotovora*, a plant pathogen shown to use AI-2 to regulate virulence. Transcriptional profile shows that, under the conditions tested, in the presence of AI-2 *S. meliloti* induces only the genes for AI-2 incorporation and processing and changes in gene expression of other genes was not observed. Altogether our findings suggest that *S. meliloti* is capable of 'eavesdropping' on the AI-2 signaling of other species and interfering with AI-2-regulated behaviors such as virulence.

2 – Introduction

Quorum-sensing is a cell-cell signaling process that enables bacteria to regulate gene expression as a function of population density. It is becoming increasingly apparent that Quorum-sensing signals, called autoinducers, can provide bacteria more information than simply the number of cells in the vicinity. By sensing combinations of various autoinducer signals in the environment, bacteria can determine, for instance, the species composition of the population or if they are inside or outside their host. Bacteria translate the information provided by the different autoinducers into specific gene expression responses leading to the promotion or inhibition of group behaviors such as bioluminescence, biofilm formation, and production of virulence factors. Additionally, some bacteria have mechanisms that enable them to interfere with other species' ability to correctly sense and respond to autoinducer signals. It is likely that this interference with Quorum-sensing provides a benefit during competition for colonization of a common niche.

Most autoinducers are species specific; however, one autoinducer, autoinducer-2 (AI-2), and its synthase, LuxS, have been identified in many bacteria including both Gram-negative and Gram-positive species. Likewise, bacterial species have been shown to respond to AI-2 with behaviors such as bioluminescence in *Vibrio harveyi* (212, 274, 331), motility in *Helicobacter pylori* (248), interference with AI-2 regulated Quorum-sensing in *Escherichia coli* (345), cell division and stress response in *Streptococcus mutans* (303), virulence and formation of biofilms in *Vibrio cholerae* (116, 117, 209, 345) and *Staphylococcus epidermis* (183), and mutualistic biofilm growth in co-cultures of *Actinomyces naslundii* and *Streptococcus oralis* (260). AI-2 is hypothesized to play an important role in enabling cross-species communication by allowing bacteria to regulate gene expression in response to the density and species composition of the bacterial populations they encounter. In some species (such as the examples given above), AI-2 has been shown to be the chemical signal responsible for inducing regulation of those specific phenotypes; in other species, the role played by AI-2 might be more complex and further studies are needed to distinguish between the

metabolic effect of disrupting the AI-2 synthase and the responses caused by the signal itself (318, 321).

Despite the large number of studies identifying AI-2 regulated phenotypes (reviewed in (119, 344)), the mechanisms of AI-2 detection and signal transduction have only been determined in two *Vibrio* species (*V. harveyi* and *V. cholerae*) (51, 175, 209, 212, 331) and the enteric bacteria *Salmonella enterica* subspecies *enterica* serovar Typhimurium and *E. coli* (305, 306, 345, 347). LuxS catalyzes the production of 4,5-dihydroxy-2,3-pentanedione (DPD) from *S*-ribosylhomocystine, however, DPD is not directly recognized by these species as AI-2 (51, 210, 274). Rather, crystal structures of the AI-2 receptor/ligand complexes revealed that these bacterial species recognize different adducts of DPD as AI-2 signals. In *V. harveyi*, the AI-2 signal is formed by cyclization of DPD, followed by hydration and addition of borate. In enteric bacteria, the LsrB protein recognizes an AI-2 moiety that lacks boron and is a different stereoisomer than the signal recognized by *V. harveyi* (**Figure 1A**). Importantly, although different bacterial species recognize chemically distinct molecules as AI-2, these molecules interconvert spontaneously in solution, allowing different bacterial species to respond to one another (210, 343).

In *S. Typhimurium* and *E. coli*, AI-2 induces the production of a transport apparatus responsible for internalizing, phosphorylating, and processing of the AI-2 signal (**Figure 1B**). The genes encoding this transport system are in the operon *lsr* (for LuxS Regulated), along with other genes involved in AI-2 processing and response (305, 306, 345). The Lsr transport system internalizes endogenously produced AI-2 as well as AI-2 produced by other bacterial species, eliminating the signal from the environment. Thus, in cultures composed of different species, these enteric bacteria are capable of interfering with the AI-2-mediated signaling of other species by disrupting their ability to regulate group behaviors (343). Recently, Demuth and co-workers have studied the function of the Lsr homologue in the oral pathogen *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and showed that this organism is also capable of internalizing AI-2 from the environment via the Lsr system. Further, they demonstrated that the LsrB homologue is required to mediate the complete, AI-2-dependent activation of biofilm formation in this organism (278, 279).

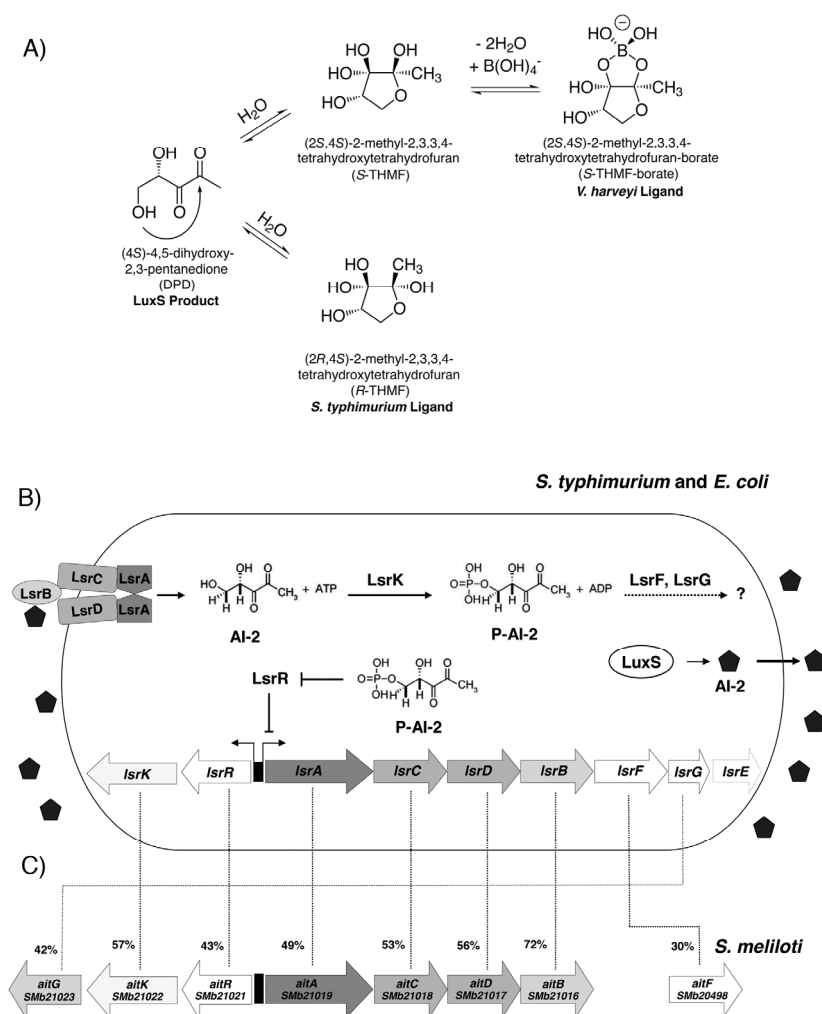


Figure 1 - The interconversion of DPD into the known AI-2 ligands and the AI-2-dependent internalization system.

A) Proposed equilibrium between the currently known forms of AI-2 and their common precursor, DPD. The *V. harveyi* and *S. Typhimurium* ligands have previously been shown to interconvert in solution.

B) *S. Typhimurium* and *E. coli* Lsr-mediated transport and processing of AI-2. In *S. Typhimurium* and *E. coli*, AI-2 is produced within the cell by LuxS and is secreted to the medium. As the concentration of extracellular AI-2 increases, AI-2 binds to the periplasmic binding protein LsrB and is internalized by the Lsr system, an ABC-type transport system. Once in the cytoplasm, AI-2 is phosphorylated (AI-2-P) by LsrK. AI-2-P binds to the repressor of the *lsr* operon, LsrR, inactivating LsrR, relieving repression, and inducing transcription of *lsr*. This causes a rapid increase in the production of the Lsr transporter and, consequently removal of AI-2 from the environment. AI-2-P is further processed by a mechanism not fully understood involving LsrG and LsrF. The *lsrE* gene is also present in the operon of *S. Typhimurium* but not in *E. coli* and its function is not known.

C) The *lsr* orthologs in *S. meliloti* operon. *S. meliloti* has orthologs to all the genes of the *lsr* operon except *lsrE*. We named the *S. meliloti* *lsr*-like operon *ait* (for autoinducer transporter). The percent identity to the Lsr proteins from *S. Typhimurium* is shown.

Of the bacteria that have a complete genome sequence in the KEGG database as of this writing, there are 16 different species with protein sequences that have greater than 60% sequence identity to the LsrB protein from *S. Typhimurium*. Interestingly, two of these bacterial species, *Sinorhizobium meliloti* and *Rhodobacter sphaeroides*, do not have orthologs to the LuxS protein from *S. Typhimurium*. This leads us to hypothesize that although these bacteria do not make their own AI-2 they could use their LsrB homologues to recognize AI-2 produced by other species. Additionally, sequence analysis revealed that *S. meliloti* also has orthologs to all the proteins of the Lsr systems from the enteric bacteria (**Figure 1B** and **1C**), except LsrE (which is present in *S. Typhimurium* but not in *E. coli* and has no known function), suggesting that this operon could be involved in AI-2 internalization in *S. meliloti*. It should be noted that the LsrF orthologue (*SMB20498*) has the lowest sequence homology to its *S. Typhimurium* counterpart and is not located in the same operon, raising the doubt as to whether it plays the same role as *E. coli* and *S. Typhimurium* LsrF.

S. meliloti is a soil bacterium well known for its capacity to establish a symbiotic relationship with legume plants from the genera *Medicago*, *Melilotus* and *Trigonella*. *S. meliloti* symbiosis is initiated under nitrogen-limiting conditions by the exchange and recognition of specific signals between the plant and the bacteria. *S. meliloti* has at least two, and in some strains three, Quorum-sensing systems dependent on homoserine lactone-type autoinducers (113). While it has been shown that these species-specific Quorum-sensing systems regulate functions crucial for symbiosis between this bacterium and its host, they are not expected to facilitate bacterial inter-species Quorum-sensing. Here, we show that *S. meliloti* does not produce the inter-species signal AI-2 but does contain a functional AI-2 receptor protein, and furthermore that *S. meliloti* recognizes the same form of AI-2 previously described for *S. Typhimurium*: (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF). *S. meliloti* is able to internalize exogenously supplied AI-2 from its environment, and it responds to the AI-2 signal by up-regulating transcription of its Lsr-like operon. By importing and

responding to a signal it does not produce, *S. meliloti* is apparently employing a different strategy for AI-2-based signaling than that employed by previously characterized species possessing the Lsr system, in effect eavesdropping on other bacteria rather than participating in the conversation.

3 – Materials and methods

3.1 – Protein production

AI-2 receptor proteins from *S. Typhimurium* and *S. meliloti*, and RbsB from *E. coli* were cloned from each species' genomic DNA (*S. Typhimurium* 14028, *S. meliloti* Rm1021, *E. coli* MG1655, respectively) into plasmid pGEX-4T1 for expression as glutathione-S-transferase (GST) fusion proteins. In all cases, the amino-terminal signal peptides, as determined by the proGram SignalP (29), were omitted from the construct. The primers used to PCR amplify the genes (Stfgex1 and Stfgex2 for the *S. meliloti* AI-2 receptor, Styl1 and Styl2 for *S. Typhimurium* LsrB, and Strbs1 and Strbs2 for *E. coli* RbsB) are shown in **Table S1**.

Plasmids were transformed into *E. coli* strains BL21 and FED101 (BL21, *luxS* mutant), and protein expression was induced by the addition of 0.1 mM isopropyl β -D-thiogalactopyranoside. After induction, the bacteria were grown for 6 hours at 22°C before harvesting.

Cells were resuspended in 25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT and lysed using a M-110Y Microfluidizer (Microfluidics). The lysates were then clarified by centrifugation and fusion proteins purified by affinity chromatography using glutathione agarose (Sigma-Aldrich). Proteins for luminescence assays were eluted from the affinity resin using 25 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT, 10 mM reduced glutathione. Eluted protein was concentrated to approximately 10 mg/ml for the luminescence assay.

To prepare the *S. meliloti* receptor protein for crystallization, the GST-fusion protein was digested with thrombin for 18 hours at 4°C while still bound to the glutathione agarose. The receptor protein was then eluted from the agarose in resuspension buffer and subsequently swapped into 25 mM

HEPES pH 7.0, 1mM DTT using G25 resin (GE Healthcare). The receptor protein was further purified by ion exchange chromatography using a SourceQ column (GE Healthcare) with a gradient from 0 to 350 mM NaCl. As a final purification step, the protein was subjected to size exclusion chromatography on an Superdex 75 column (GE Healthcare), eluting in 25 mM HEPES pH 7.0, 150 mM NaCl, 1mM DTT. The protein was then concentrated to approximately 10 mg/ml.

3.2 – AI-2 binding assay

Ligands were released from purified receptor proteins by heating the protein samples (10 min, 70°C). The denatured protein was then pelleted and the supernatants used in the luminescence assay. For this assay, the *V. harveyi* strain MM32 (*luxN::Cm*, *luxS::Tn5Kan*) was used as a reporter. Because this strain has an insertion in the AI-1 receptor (LuxN) it does not respond to autoinducer-1, and since it is a *luxS* mutant, it does not produce AI-2; thus the strain will only produce light in response to exogenous AI-2 and is effective for discriminating between the presence and absence of AI-2. *V. harveyi* MM32 was grown for 16 hr in AB medium at 30°C and subsequently diluted 1:5000 into fresh AB medium containing 10% released autoinducer sample or buffer. The bacteria were then grown at 30°C and luminescence measured using a Wallac Victor2 1420 multilabel counter. Bioluminescence produced by MM32 is reported as counts per second (c. p. s.) as measured by the instrument.

3.3 – Crystallization and structure determination

Crystals of the *S. meliloti* AI-2 receptor protein expressed in *E. coli* BL21 (LuxS⁺) were grown via the sitting drop method with a well solution of 0.1 M Tris-HCl pH 8.5, 0.2 M MgCl₂, 30% w/v PEG 4000 and developed in approximately two weeks at room temperature. Crystals were frozen in mother liquor, and data were collected at 100K using an R-Axis-IV image plate detector mounted on a Rigaku 200HB generator. The crystals (P2₁, a = 57.85, b = 71.49, c = 68.04, β = 98.69) diffracted to 1.8Å resolution. Data

were processed using MOSFLM (177) and CCP4 (1).

The structure of *S. meliloti* LsrB (SmLsrB) was solved via molecular replacement with PHENIX (2), using LsrB from *S. Typhimurium* (PDB ID: 1TJY) as a search model. The automatically generated partial structure was used as a starting point for building in Coot (88). The structure was refined using data to 1.8Å with PHENIX. To avoid bias, the ligand was omitted from the search model and not built until the protein structure had been completed and refined. The binding site electron density was clearly interpretable and modeled as *R*-THMF. A prime-and-switch map was also calculated via PHENIX to confirm the identity of the bound ligand. Refinement parameters for the ligand were generated with the eLBOW module of PHENIX. The final structure contains two copies of SmLsrB, including all residues in the expressed construct (29 - 343), two copies of the bound ligand, and 1113 water molecules. The structure has good geometry (**Table 1**), with only two residues per chain (Asp118 and Leu268) outside the allowed regions of the Ramachandran plot. Clear density exists for both of these residues. The final R_{cryst} and R_{free} were 0.168 and 0.214 respectively. All molecular images were generated using PyMOL (69).

Table 1 - Crystallographic data and refinement statistics

Data	
Resolution (Å)	1.8 (1.9-1.8)
Unique Reflections	50186 (7394)
Rmerge (outer shell)	0.059 (0.348)
Mean I/ σ I (outer shell)	15.3 (2.6)
Completeness (%)	98.8 (100)
Multiplicity	2.6 (2.5)
Refinement	
$R_{\text{cryst}}/R_{\text{free}}$	0.168/0.214
RMS deviation	
Bond Length (Å)	0.007
Bond Angle (°)	1.007
Dihedrals (°)	19.074
Average B factor	
Non-solvent	17.20
Waters	28.74
All atoms	19.26

3.4 – AI-2 synthesis

DPD protected with cyclohexylidene was synthesized as reported previously (276). The protective group was removed with H₂SO₄ followed by neutralization with potassium phosphate buffer, pH 7 as described in Xavier, 2007 (347).

3.5 – Bacterial strains and growth conditions

The *S. meliloti* strains used in this study are derived from the wild-type strain Rm1021 (202). To construct the *aitA*::pJH104 mutant (MET2000), a 300 bp internal fragment of *aitA* was amplified by PCR from an *S. meliloti* Rm1021 colony with the primers P113 and P114 (**Table S1**), ligated into the suicide plasmid pJH104 at the SpeI and XhoI restriction sites, and transformed into *E. coli* DH5 α . The resulting plasmid was introduced into Rm1021 by triparental mating, and integration of the plasmid was selected by growth on neomycin (0.2 mg/ml). To construct the in-frame deletion of *aitK* (MET2002), a 750 bp region upstream of the *aitK* open reading frame was amplified by PCR with primers P117 and P118 (**Table S1**), and a 500 bp region downstream of *aitK* was amplified with primers P120 and P121 (**Table S1**). The two PCR products were ligated in tandem into the plasmid pK18 mob sacB (272) at the BamHI, PstI, and HindIII restriction sites, transformed into *E. coli*, and introduced into Rm1021 by triparental mating. Neomycin resistant exconjugants were plated on TY with 10% sucrose to select for a second recombination event. Neomycin sensitive colonies were screened by PCR for deletion of *aitK*. The *E. carotovora* ssp. *carotovora* strains used are the wild type SCC3193 (241) and its isogenic *luxS*::Cm mutant SCC6063 (167). *S. meliloti* and *E. carotovora* strains were grown at 30°C with aeration in Luria-Bertani broth (LB) supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBMC).

For the studies of *S. meliloti* growth on different carbon sources, *S. meliloti* was grown overnight in LBMC at 30°C. The culture was washed three

times in M9 minimal medium (110) with no carbon source and used to inoculate M9 media with biotin and sucrose, glucose, ribose or AI-2 to a final concentration of 2 mM. As a control, a culture was grown with M9 media and biotin but no carbon source. Cultures were grown at 30°C with agitation for 72 hours and growth was monitored by optical density at 600 nm (OD₆₀₀). Each carbon source was tested in duplicate.

3.6 – AI-2 activity in *S. meliloti* and *E. carotovora* cultures

To monitor extracellular AI-2 activity in *S. meliloti* cultures during growth, overnight cultures were diluted to OD₆₀₀=0.08 into LBMC medium with and without 80 µM chemically synthesized AI-2. Aliquots were collected at the times indicated and used for measurement of OD₆₀₀ and preparation of cell-free culture fluids. The AI-2 activity in cell-free culture fluids was measured using the *V. harveyi* BB170 (*luxN::Tn5Kan*) bioluminescence reporter assay, as described previously (24, 25). Cell-free culture fluids were prepared by filtration of liquid cultures (298, 299). The filtered samples were analyzed in duplicate. A similar procedure was used to measure AI-2 activity in *E. carotovora* cultures, either in single cultures or in co-culture with *S. meliloti*. When *S. meliloti* was grown in co-culture with *E. carotovora*, or when single cultures of *E. carotovora* were tested, no synthetic AI-2 was supplied.

AI-2 activity in cell-free culture fluids is reported as fold induction of light produced by BB170 and is calculated as the ratio of light produced by BB170 supplemented with sample to light produced by BB170 supplemented with *S. meliloti* growth medium (LBMC). When required, serial dilutions of the cell-free fluids in LBMC were tested and the values were calculated from the dilution of each sample that resulted in half-maximal induction (approximately 500 fold). (In these experiments, BB170 was used because it allows determination of the fold induction of each sample in relation to the background luminescence when the response is not saturated (24) and is thus appropriate for quantifying the AI-2 in the cell-free fluids. The MM32 assay, used in the AI-2 receptor binding assay, above, is more effective in discriminating between the presence and absence of AI-2.)

3.7 – Quantitative real-time PCR analysis

To measure the induction of *aitB* transcription by AI-2 in *S. meliloti* culture, aliquots were collected throughout growth in the presence or absence of 80 μ M AI-2. Cells were collected by centrifugation at 16,000g for 10 min. 50 μ L of 5 mg/ml lysozyme was added to each sample, which were then incubated on ice for 5 minutes. Samples were frozen in liquid nitrogen and stored at -80°C until the RNA was extracted.

S. meliloti RNA was extracted with Trizol reagent (Invitrogen) and chloroform according to the manufacturer's protocol. RNA was precipitated using isopropanol, washed with 75% ethanol, and diluted in DEPC water. RNA samples were diluted to a concentration of 200 ng/ μ l and treated with DNase I (Roche). The RNeasy Mini kit from QIAGEN was used to clean the RNA. cDNA was generated in 100- μ L reactions, each containing 20 μ g of RNA, 5x First Strand Buffer (Invitrogen), 100 mM DTT (Invitrogen), 10 mM dNTPs (ABI), random hexamers (Roche), and SuperScript II reverse transcriptase (Invitrogen). The reverse transcriptase reactions were undertaken in a thermocycler with steps of 10 min at 25°C, 50 min at 42°C, and 15 min at 72°C. Identical reactions were performed without reverse transcriptase enzyme to ensure the absence of genomic DNA contamination. Quantitative RT-PCR reaction mixtures contained 5 μ l of cDNA template, 2 μ l gene-specific primers, 12.5 μ l of SYBR Green Mix (Applied Biosystems), and 5.5 μ l H₂O. For each reaction, 10 μ l of reaction mixture were loaded into 384-well optical reaction plates (Applied Biosystems) using a platemate 2x2 automated liquid pipettor (Matrix), with six replicates of each sample. Real-time PCR reactions were carried out on an ABI Prism 7900HT Sequence Detector (Applied Biosystems). Real-time PCR primers were designed using Primer Express 2.0 (ABI Software) and are listed in **Table S1**. *hfq* or *rpsL* transcripts were used as endogenous controls for the reactions, and RNA levels were quantified using absolute quantification (standard curve analysis).

3.8 – RNA extraction and Microarray analysis

To prepare cell cultures for RNA extraction, a single *S. meliloti* culture, grown until stationary phase in LBMC at 30°C, was used to inoculate three different Erlenmeyer flasks containing 30 ml of fresh LBMC media to a final OD₆₀₀=0.08. These three independent *S. meliloti* cultures were then grown until an OD₆₀₀=1. Then, each of the three cultures was split equally into two and DPD (80 µM final concentration) was supplied to one of the cultures and cyclohexanone (80 µM final concentration) to the other. Cyclohexanone is the protective group of DPD and was added as a control. Following DPD and cyclohexanone addition, the cultures were incubated for 1 hour and then placed on ice. Cells were harvested by centrifugation and RNA extraction was performed according to the Qiagen Midi kit manufacture procedures. DNase digestion was performed using the Ambion DNase rigorous treatment. RNA preparation for Affymetrix (Santa Clara, CA, USA) GeneChip *Medicago/Sinorhizobium* Genome Arrays was performed as described in (270). Scanned arrays were first analyzed using Affymetrix Expression Console software to obtain Absent/Present calls and guarantee that all quality parameters were in the suggested range. Further examination was carried out with DNA-Chip Analyzer 2008. Initially, a digital mask was applied, to leave out *Medicago truncatula* and *Medicago sativa* genes and let for analysis the 8305 probe sets on the array representing *Sinorhizobium meliloti* transcripts. The 6 arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Methodv (178). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a Perfect Match (PM)-only model (179). Replicate data (triplicates) for each of the samples with and without AI-2 were weighted gene-wise by using inverse squared standard error as weights. Genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was

above 1.2, resulting in 2 differentially expressed transcripts with a median False Discovery Rate (FDR) of 0%. The lower confidence bound criterion means that we can be 90% confident that the fold change is a value between the lower confidence bound and a variable upper confidence bound. Li and Wong (179) have shown that the lower confidence bound is a conservative estimate of the fold change and therefore more reliable as a ranking statistic for changes in gene expression. A second analysis where the fold change between experiment and baseline was reduced to be above 1.1 was performed using also DNA-Chip Analyzer 2008. In this analysis we obtained 6 genes differentially expressed but the FDR was of 67%.

4 – Results

4.1 – *S. meliloti* contains an AI-2 binding protein

The SMb21016 hypothetical protein from *S. meliloti* is 72% identical to the LsrB protein from *S. Typhimurium* and, significantly, the gene *SMb21016* is located in an operon that includes orthologs to all the genes necessary for AI-2 internalization in *S. Typhimurium* (**Figures 1B** and **1C**). To demonstrate that the protein encoded by *SMb21016*, the putative *S. meliloti* AI-2 receptor gene, is capable of binding AI-2, we cloned and overexpressed the protein in an *E. coli* strain producing AI-2. The candidate protein was purified and tested for AI-2 binding. Ligand was released from the receptor by thermal denaturation and the denatured protein removed from solution by pelleting. The resulting supernatant containing released ligand was subsequently tested for its ability to induce bioluminescence in a reporter strain of *V. harveyi*, MM32, which produces light only in the presence of exogenous AI-2.

Ligand released from the *S. meliloti* AI-2 receptor induced a light response similar to that of ligand released from *S. Typhimurium* LsrB (**Figure 2**, black bars). To confirm that the light response was specifically induced by

AI-2, receptor proteins from *S. meliloti* and *S. Typhimurium* were overexpressed in FED101, an *E. coli* strain lacking LuxS and thus unable to produce AI-2. As expected, denaturation of these proteins released no ligand capable of inducing light production in MM32 (**Figure 2**, white bars). As a further control, ribose binding protein from *E. coli* was overexpressed as a GST-fusion and tested for AI-2 binding ability; the protein showed no ability to bind AI-2 in this bioassay (**Figure 2**). Thus, the periplasmic binding protein (PBP) encoded by the *S. meliloti* **SMb21016** has the capacity to bind AI-2, and we named it SmLsrB (for *S. meliloti* LsrB).

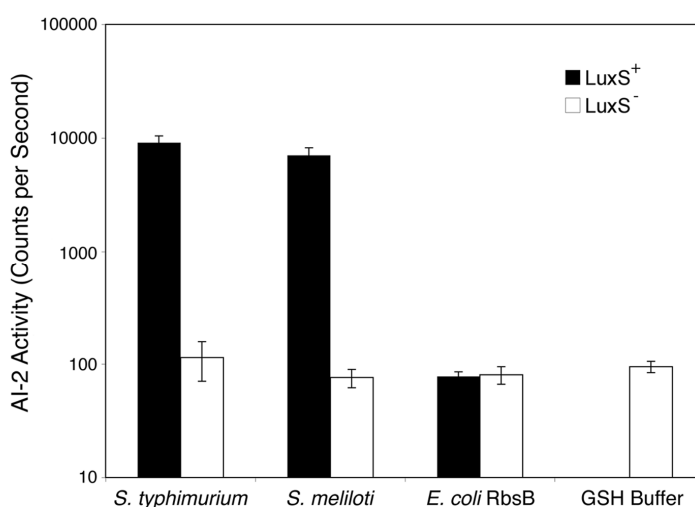


Figure 2 – Binding of AI-2 to potential receptor proteins. Light produced by *V. harveyi* strain MM32 (LuxN⁺, LuxS⁻) was assayed following the addition of ligand released from purified protein expressed in either LuxS⁺ (black bars) or LuxS⁻ (white bars) *E. coli* (strains BL21 and FED101, respectively). The *E. coli* ribose binding protein RbsB and protein-free GSH-buffer were included as negative controls. AI-2 activity is reported as c.p.s. of MM32 bioluminescence. Error bars represent the standard deviations for three independent cultures.

4.2 – Structure of the *S. meliloti* AI-2-receptor complex

To identify the form of AI-2 recognized by *S. meliloti*, we determined the crystal structure of the SmLsrB/AI-2 complex. SmLsrB crystallized with ligand bound, and the structure was solved at 1.8Å resolution by molecular replacement (bound ligand was omitted from the molecular replacement model). The *S. meliloti* AI-2 receptor has a classic PBP fold, with two α/β

domains connected via a three-stranded hinge. The structure is very similar to that of LsrB from *S. Typhimurium*, with an RMSD of 0.6Å. As with other PBPs, the receptor binds the ligand in a cleft between the two domains (**Figure 3A**). The structure has been deposited in the PDB with ID code 3EJW.

After the structure of the protein was modeled and refined, electron density corresponding to the ligand was easily interpretable. This density was modeled as *R*-THMF (**Figure 3B**), the same form of AI-2 recognized by *S. Typhimurium* (**Figure 1A**). The electron density does not make it possible to unequivocally rule out the enantiomer *S*-THMF as the ligand, but the assignment of *R*-THMF is consistent with the chemical environment of the binding site. If the *R*- form of AI-2 is bound, the methyl group on the cyclized ligand is positioned in a hydrophobic pocket, surrounded by residues Phe43, Ala224, Leu268, and Trp269 (**Figure 3C**). In contrast, modeling the *S*- form of the ligand into the binding site leads to the less energetically favorable positioning of a hydrophilic hydroxyl group in this hydrophobic pocket. Further, in the *S*- form, the nearest potential hydrogen bonding partner for this hydroxyl group is the backbone nitrogen from Ala224, but the interatomic distance of 4.12Å and poor geometry rule out a significant hydrogen bond interaction. When the *R*- form is modeled into the binding site electron density, this hydroxyl group is 3.35Å from the backbone oxygen of Pro222 (within hydrogen bonding distance) and 3.85Å from the side chain of Gln169 and thus reasonably positioned for a beneficial electrostatic interaction. Thus, we expect that these interactions selectively stabilize the receptor-ligand complex with bound *R*-THMF. Furthermore, these interactions are consistent with those observed previously in the *S. Typhimurium* LsrB/AI-2 complex; in that case, higher resolution electron density further supported the assignment of *R*-THMF as the bound form of AI-2. Thus, all the evidence supports the conclusion that *R*-THMF is the predominant species recognized by *S. meliloti* as AI-2.

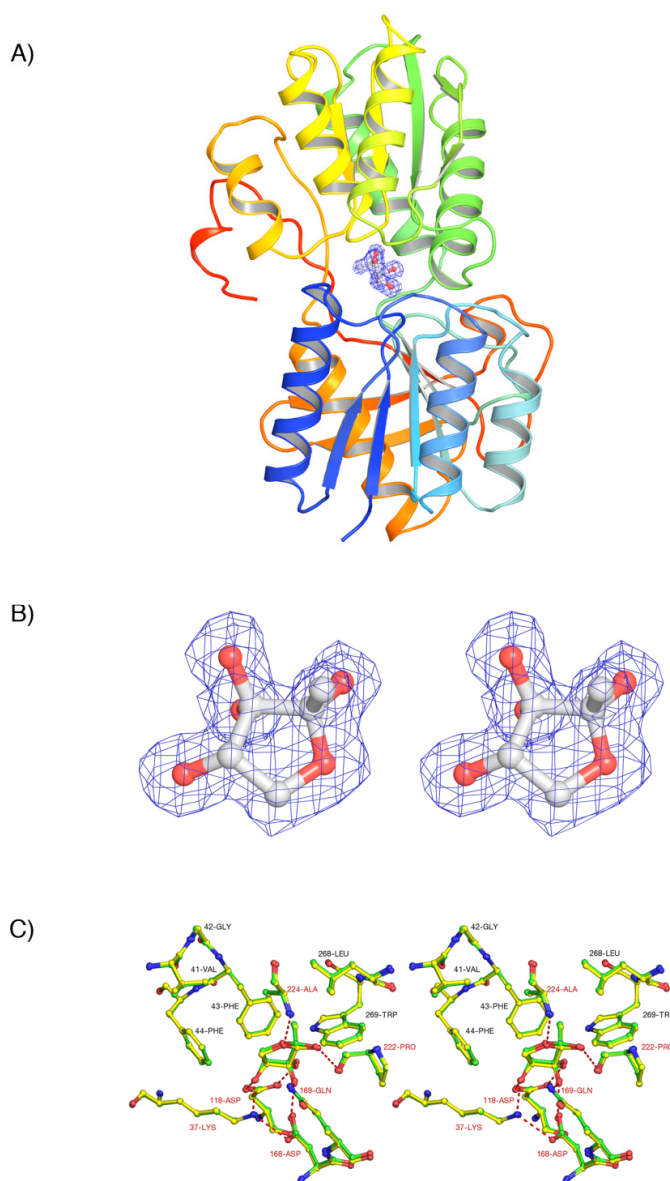


Figure 3 - Structure of *S. meliloti* LsrB and its ligand.

A) Ribbon diagram of SmLsrB. The ribbon diagram is colored in rainbow order from N- to C-terminus. The bound ligand and the corresponding electron density are shown.

B) Stereoview of 2Fo-Fc ligand electron density. The DPD isomer *R*-THMF is shown modeled into non-protein electron density in the receptor binding site.

C) Comparison of the *S. meliloti* and *S. Typhimurium* LsrB AI-2 binding sites. Overlay of the SmLsrB (green) and *S. Typhimurium* LsrB (yellow) binding sites based on overall alignment of the protein structures as calculated by PyMOL. Residue numbers are from the *S. meliloti* sequence. Dashed red lines indicate potential hydrogen bonds and the interacting residues are labeled in red.

4.3 – *S. meliloti* removes exogenously supplied AI-2 from extracellular medium

S. meliloti does not have an ortholog to the *luxS* gene from *S. Typhimurium*, and thus we expected that this bacterium does not produce AI-2. Accordingly, cell-free culture fluids collected from *S. meliloti* cultures grown on LBMC do not possess AI-2 activity, as measured by induction of bioluminescence in a *V. harveyi* BB170 AI-2-reporter assay (**Figure 4A**, squares).

We cultured *S. meliloti* in medium supplemented with *in vitro* synthesized AI-2 and followed the AI-2 activity in the cell-free culture fluids over time. As shown in **Figure 4A**, the initial AI-2 activity due to the addition of synthetic AI-2 results in 100,000-fold induction of bioluminescence in the *V. harveyi* assay (**Figure 4A**, black triangles). However, after 12 hours of growth, the AI-2 activity measured in the cell-free culture fluids decreases drastically to only 20-fold induction. This decrease in extracellular AI-2 activity is consistent with *S. meliloti* having a functional Lsr operon and, thus, the ability to remove exogenously supplied AI-2 from its environment.

The decrease in apparent AI-2 levels in *S. meliloti* cultures supplemented with AI-2 could also be due to accumulation of an inhibitor of the *V. harveyi* AI-2 response or to the degradation of AI-2 by some extracellular factor. To eliminate the first possibility, we added AI-2 to *S. meliloti* cell-free culture fluids collected after 24 hours of growth ($OD_{600}=6.0$). These samples of cell-free culture fluids were subsequently tested in the *V. harveyi* bioluminescence assay and found to give high-level light induction (**Figure S1**). Thus, no inhibitor was present. To control for the possibility of AI-2 degradation, we collected cell-free culture fluids from *S. meliloti* grown in the presence of AI-2 for 18 hours ($OD_{600}=3.6$). As shown in **Figure S2A**, no AI-2 activity can be detected in these cell-free culture fluids. We then supplemented this cell-free culture fluid with synthetic AI-2 and incubated this sample for 24 hours at 30°C to determine the stability of AI-2 in this cell-free

sample. Results from a BB170 bioluminescence assay show that AI-2 activity did not decrease during this incubation period (**Figure S2B**). Therefore, we conclude that the decrease of extracellular AI-2 activity observed during growth of *S. meliloti* results from AI-2 internalization by the cells and not from inhibition of AI-2 detection or degradation of AI-2 by an extracellular factor.

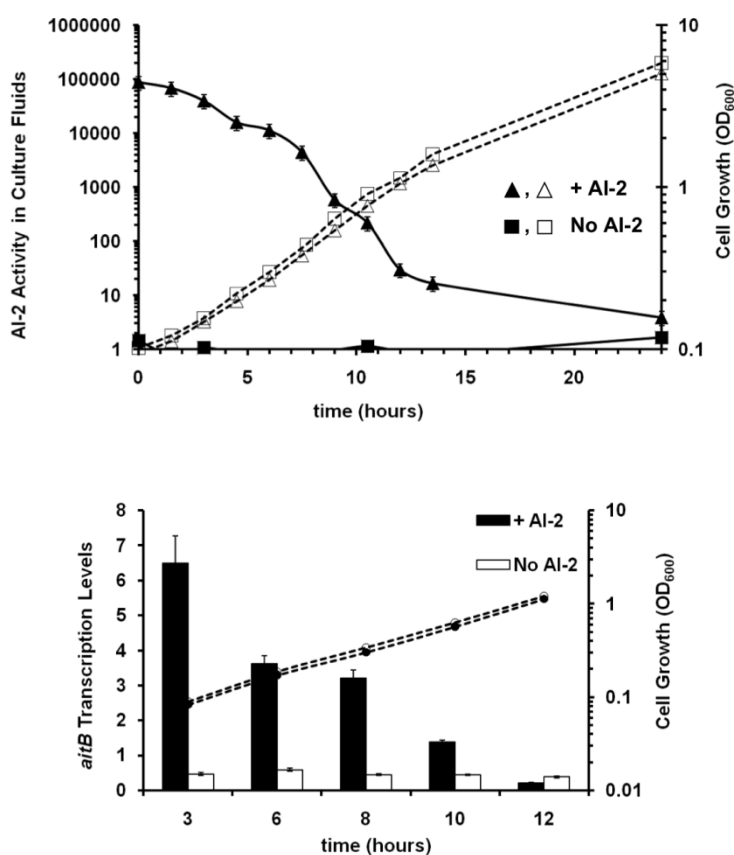


Figure 4 - *S. meliloti* internalization of exogenously supplied AI-2.

A) Extracellular AI-2 activity in *S. meliloti* cultures. Wild-type *S. meliloti* Rm1021 was cultured in LBMC in the presence (triangles) and absence (squares) of *in vitro* synthesized AI-2 and aliquots were taken at the specified times. AI-2 activity in cell-free culture fluids is reported as fold induction of light production by *V. harveyi* BB170 (solid lines). Samples with fold inductions above 1000 were diluted in LBMC and values shown were calculated taking account the dilution factor. Cell growth was monitored by OD (dashed lines).

B) Expression of the *S. meliloti* *aitB* transcript in the presence and absence of AI-2. RNA levels in cultures of wild-type *S. Meliloti* RM1021 grown in the presence (black bars) and absence (white bars) of *in vitro* synthesized AI-2 were measured using quantitative real-time PCR. *aitB* transcript levels are reported as fold increase of *aitB* transcript in relation to the *rpsL* transcript. Cell growth in the presence (black circles) and in the absence (white circles) of AI-2 was measured by OD.

4.4 – Increased transcription of the *lsr*-like operon *ait* occurs in response to AI-2

In *S. Typhimurium* and *E. coli*, the *lsr*-operon is induced by the presence of AI-2. Induction of *lsr* in these bacteria leads to increased production of the AI-2 transport proteins and, thus, a positive feedback loop resulting in increased removal of AI-2 from the extracellular medium. As shown in **Figure 1B** and **1C**, the *S. meliloti* orthologs to the proteins of the *S. Typhimurium* Lsr transport system (LsrA, LsrC, LsrD, and LsrB) have a sequence identity above 49% to their orthologs. Thus, we predicted that the *S. meliloti* *lsr*-like operon was involved in the removal of AI-2 from culture fluids reported above and we named the *S. meliloti* *lsr*-like operon *ait* (for autoinducer transporter). This name was chosen because the name *lsr* has already been given to another gene in *S. meliloti* with an unrelated function (189).

We anticipated that *S. meliloti* would respond to exogenously supplied AI-2 with a similar up-regulation of its *ait* operon. To test this premise, we used qRT-PCR to determine whether the addition of AI-2 to *S. meliloti* cultures would induce transcription of this operon. As predicted, levels of *aitB* (*SMB21016*) mRNA were higher in the culture that was supplemented with synthetic AI-2 than in AI-2-free cultures (**Figure 4B**, black and white bars respectively). Moreover, induction of *aitB* transcription decreases overtime, an observation in accordance with the decrease of extracellular AI-2 during growth shown in **Figure 4A** (triangles).

4.5 – *S. meliloti* *aitK* and *aitA* mutants are impaired in the ability to remove AI-2 from the medium

To verify if the *S. meliloti* Lsr-like system (Ait) was capable of internalizing AI-2, we constructed a mutant in *aitA* (previously *SMB21019*), the *S. meliloti* gene homologous to *lsrA* from *S. Typhimurium* (**Figure 1B**). In *S. Typhimurium* and *E. coli*, this gene is predicted to encode the ATP-binding

subunit of the Lsr transporter. Comparison of AI-2 activity in cell-free culture fluids of *S. meliloti* WT cultures with cultures of the *aitA* mutant shows that the mutant is defective in AI-2 internalization (**Figure 5**, diamonds and squares respectively), supporting our prediction that this operon encodes a functional AI-2 transporter that accounts, at least partially, for the observed AI-2 internalization.

In *S. Typhimurium* and *E. coli*, the phenotype of the *lsrK* mutant with respect to AI-2 internalization is even more pronounced than a transport mutant because phosphorylation of AI-2 is required for trapping the signal inside the cell (305, 306, 345). As shown in **Figure 5**, this is also true for the *S. meliloti* mutant in *aitK* (previously *SMB21022*) the *lsrK* orthologue (**Figure 5**, triangles). In cultures of the *aitK* mutant, AI-2 activity persists in the extracellular medium for much longer than in wild type *S. meliloti*. The fact that *aitA* transport mutant is less defective than the *aitK* mutant in AI-2 removal suggests that at least one more transport mechanism for AI-2 exists, a finding consistent with previous results in enteric bacteria (305, 306, 345). Nonetheless, our data indicate that in *S. Typhimurium*, *E. coli*, and *S. meliloti* the alternate system(s) for AI-2 uptake is less efficient in AI-2 transport than the Lsr and Ait systems.

We have also tested the ability of the *S. meliloti* *aitA* mutant to colonize its host, the plant *Medicago sativa*, and found that plants inoculated with the *aitA* mutant or the *aitK* mutant were indistinguishable from WT-inoculated plants. Additionally, the *aitA* mutant did not have a competitive defect when co-inoculated with the WT (data not shown). We interpret this result to mean that the *S. meliloti* Ait system is not essential in this single-species symbiosis process. This result is not surprising since, as we demonstrate in **Figure 4B**, the Ait system in *S. meliloti* is only induced in the presence of exogenously supplied AI-2.

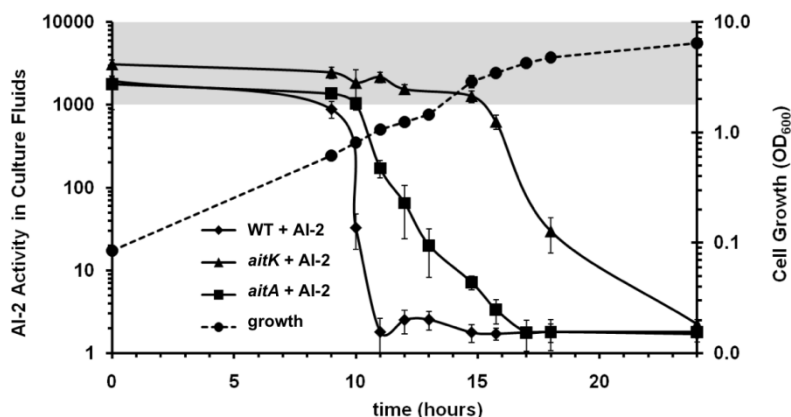


Figure 5 - Extracellular AI-2 activity in *S. meliloti* *ait* mutants. Cultures of the following *S. meliloti* strains, RM1021 (WT, diamonds), MET2000 (*aitA*, squares) and MET2002 (*aitK*, triangles), were grown in LBMC with *in vitro* synthesized AI-2. AI-2 activity in the cell-free culture fluids is reported as fold induction of light production by *V. harveyi* BB170 (solid lines) and cell growth was monitored by OD (dashed lines). The average OD of the three cultures is shown (circles) with the corresponding standard deviation. Fold inductions above 1000 correspond to AI-2 concentrations that saturate the *V. harveyi* bioassay and are highlighted by the grey shadow

4.6 – *S. meliloti* does not grow with AI-2 as a sole carbon source

Some bacteria, such as *Variovorax paradoxus*, are capable of degrading specific acyl-homoserine lactone autoinducers and using these molecules as a source of nitrogen and energy when grown in minimal medium (170). This led us to test whether, in conditions of low nutrient availability, *S. meliloti* could similarly use AI-2 as a sole carbon source. We grew cultures of *S. meliloti* in minimal medium (M9) using AI-2 as a carbon source and compared growth to cultures grown in M9 supplemented with a variety of sugars (glucose, sucrose, and ribose). *S. meliloti* was able to grow on glucose, sucrose, and ribose as a sole carbon source (at a concentration of 2 mM) but not when the same concentration of AI-2 was provided (**Figure S3**). After 72 hours of incubation the OD₆₀₀ of a culture of *S. meliloti* in M9 supplemented with 2 mM AI-2 remained as low as the control where no carbon source was added. Additionally, our results (**Figure 4A and 5**) show that in rich medium (LBMC) addition of AI-2 to cultures of *S. meliloti* WT or *ait* mutants does not affect the growth rate of these strains. Thus, we have no evidence indicating that *S. meliloti* can gain any metabolic benefit from internalizing AI-2.

4.7 – Identification of genes regulated by AI-2 in *S. meliloti*

Whole genome expression profiles conducted to elucidate the AI-2 signaling role in QS are frequently performed comparing pattern of transcription between WT and *luxS* deficient strains. In some relevant studies, pure DPD was supplied to the *luxS*-deficient strain conferring more accuracy to the conclusions related to AI-2 as a QS signal. Nevertheless, given the global cellular effect of a *luxS* mutation, the interpretation of microarrays results is to some extent complex.

Because *S. meliloti* does not possess the *luxS* gene, and therefore does not produce AI-2, the construction of mutants is not necessary, making this species a good candidate to explore the global transcriptional effect of adding the AI-2 signaling to the growing media. Using DNA microarrays we searched for AI-2 regulated genes by comparing three WT *S. meliloti* cultures grown with and without the AI-2 signal for one hour. Surprisingly even when the cut off value set to as low as 1.1 the only genes that showed regulation by AI-2 were the ones belonging to the *lsr* and *lsrRK* operons (**Table 2**). We were expecting these transcripts to be upregulated since, from this research work and previous investigations, we demonstrated that both the *lsr* and *lsrRK* operon expression are AI-2 dependent. Strikingly, however, these and the gene *Smb21015* were the only genes that showed to be differentially expressed in the presence of exogenously supplied AI-2.

Smb21015 is the gene downstream of *aitB* but initially we did not classify this gene as being part of the *ait* operon because it was not the best bidirectional hit of *lsrF* gene of *S. Typhimurium*. Thus, the fact that it is induced by AI-2 in the microarrays study was surprising, but indicates that it is indeed part of the *ait* operon. The orthologue *Smb20498* we identified based on our best bidirectional hit criteria might have a function unrelated to AI-2. Interestingly, *Smb21015* gene has an aldolase type II motif, contrasting with the *LsrF* from *E. coli* that is a class I aldolase (73). To clarify this issue, functional biochemical studies, which are being conducted in the laboratory, are required.

Table 2 – Genes responding to AI-2 in *S. meliloti*.

Gene name	Gene product and/or function	Fold change	Lower bound
<i>aitK</i> (<i>Smb21022</i>)	autoinducer-2 (AI-2) kinase	+1.35	1.11
<i>aitR</i> (<i>Smb21021</i>)	putative transcriptional regulator protein	+1.35	1.18
<i>aitD</i> (<i>Smb21017</i>)	putative sugar ABC transporter permease protein	+1.45	1.18
<i>aitB</i> (<i>Smb21016</i>)	putative sugar ABC transporter periplasmic solute-binding protein precursor	+2.45	2.12
(<i>Smb21015</i>)	short chain dehydrogenase	+ 1.55	1.25

4.8 – *S. meliloti* clears AI-2 produced by *Erwinia carotovora* in co-cultures of these two species

We have shown that *S. meliloti* is capable of removing exogenously supplied synthetic AI-2 from culture fluids and that the *S. meliloti* Ait system is involved in this process. Importantly, we also showed that the Ait system is induced only when exogenous AI-2 is supplied to the culture. To test if *S. meliloti* grown in the presence of an AI-2 producing bacterial species could use the Ait system to remove AI-2 produced by that species, we cultured *S. meliloti* in the presence of *Erwinia carotovora* (wild type strain SCC3193). *E. carotovora* is a Gram-negative plant pathogen that can co-exist with *S. meliloti* in the rhizosphere of several plants. *E. carotovora* has a *luxS* homolog and has been shown to produce AI-2 (167). Accordingly, AI-2 activity was detected in cell-free culture fluids of cultures of *E. carotovora* and in co-cultures with wild type strains of both *S. meliloti* and *E. carotovora* (**Figure 6**, triangles and circles respectively) but not when *S. meliloti* was co-cultured with an *E. carotovora luxS* mutant (strain SCC6023) incapable of producing AI-2 (**Figure 6**, crosses). In assays of mixed cultures of the wild type strains of *S. meliloti* and *E. carotovora*, we observed that AI-2 activity in culture cell-free fluids increased for 4 hours and then began to decrease (**Figure 6**, circles). After 6 hours, AI-2 activity in this culture was almost undetectable. In contrast, AI-2 activity in cell-free culture fluids remained high when *E. carotovora* is grown as a pure culture (**Figure 6**, triangles) or in

mixed cultures of *E. carotovora* and the *S. meliloti aitK* mutant (**Figure 6**, squares), showing that the disappearance of AI-2 from the extracellular medium in these mixed cultures requires *S. meliloti* with a functional Ait system.

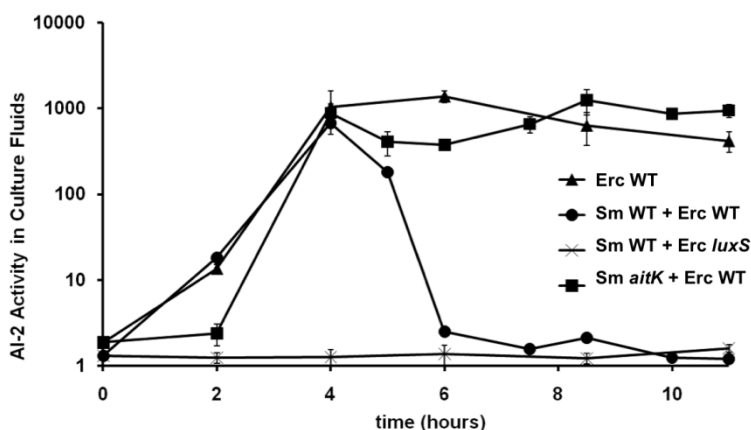


Figure 6 - Extracellular AI-2 activity in co-cultures of *S. meliloti* with *E. carotovora*.

Extracellular AI-2 activity was measured in a pure culture of *E. carotovora* WT strain SCC3193 (triangles) or co-cultures of the following combinations: *S. meliloti* (WT) with *E. carotovora* WT strain SCC3193 (circles), *S. meliloti* (WT) with *E. carotovora luxS* strain SCC6023 (crosses), and *S. meliloti aitK* strain MET2002 with *E. carotovora* WT strain SCC3193 (squares). All strains were grown in LBMC medium. AI-2 activity in the cell-free culture fluids is reported as fold induction of light production by *V. harveyi* BB170.

5 – Discussion

As of this writing, approximately 40% of the nearly 800 sequenced bacterial genomes contain the *luxS* gene (based on genomes with homologues to the *luxS* gene of *S. Typhimurium* with an e-value smaller than 10^{-12} (<http://www.genome.jp/kegg/>)), suggesting that there are a large number of bacterial species capable of producing DPD, the AI-2 precursor. Additionally, *luxS*/AI-2 has been implicated in the regulation of a variety of niche-specific functions. For these reasons, AI-2 has been proposed to function as a universal bacterial signal. Here we provide the molecular mechanism for AI-2 detection and response in *S. meliloti*, an organism that lacks the AI-2 synthase and thus is incapable of producing its own AI-2. We have determined the crystal structure of the AI-2/receptor complex in *S. meliloti* and have shown that transcription of this receptor is dependent on

exogenously supplied AI-2, produced either synthetically or by organisms capable of synthesizing AI-2.

This work demonstrates that AI-2 signaling can influence levels of gene expression in non-AI-2 producers, a fact that increases the range of species with the potential to be involved in exchange of, or response to, the AI-2 molecule beyond those that carry the *luxS* gene. The *S. meliloti* response to AI-2 emphasizes the role of AI-2 as an inter-species signal; because *S. meliloti* is incapable of producing AI-2, other organisms are the only source of AI-2 in the environment. Significantly, the *S. meliloti* case is not an isolated example; in fact, Surette and colleagues have shown (80) that expression of several genes in *Pseudomonas aeruginosa*, another organism lacking *luxS*, is influenced by the presence of AI-2, although the molecular mechanism involved in this process has yet to be defined. We predict that this phenomenon is not limited to these two species, and other species lacking LuxS will be shown to respond to AI-2.

Understanding the role played by AI-2 across species requires understanding of the molecular mechanisms by which different species recognize and respond to AI-2. To date, the AI-2 receptors of the marine bacterium *V. harveyi* and the human pathogen *S. Typhimurium* have been characterized. Here, by studying AI-2 response in a plant symbiont, we expand our understanding of the molecular mechanism of AI-2 detection into a new environmental niche, the soil. Previous work has shown that *S. Typhimurium* and *V. harveyi* recognize chemically distinct forms of the AI-2 molecule and that levels of the various forms of AI-2 present in a particular environment are dictated by the chemistry of that environment (51, 210). In this work, we demonstrate that *S. meliloti* recognizes the same form of AI-2 as the enteric bacterium *S. Typhimurium* despite the fact that these bacteria are usually isolated from chemically different niches (the soil and the human gut).

Previous work has shown that *S. Typhimurium* and *E. coli* have the ability to internalize AI-2 via their Lsr system, thus removing the molecule from the environment. These species can use this ability to interfere with AI-2 based signaling of other species (343). Here we show that *S. meliloti* also

has a functional AI-2-inducible Lsr-like system (Ait) capable of removing AI-2 from the environment. *S. meliloti* colonizes the rhizosphere of several legume plants and therefore it shares its habitat with many AI-2-producing bacterial species. Our results show that *S. meliloti* can use the Ait system to clear the AI-2 signal produced by *E. carotovora*, a plant pathogen that can co-exist with *S. meliloti* in the rhizosphere and that has been reported to regulate virulence by AI-2 Quorum-sensing (167). Thus, it is reasonable to presume that, like the enteric bacterium, *S. meliloti* can use the AI-2 internalization system for interference. However, this strategy of interference likely functions somewhat differently for *S. meliloti* than for the enteric species, since in *S. meliloti* the *ait* operon can only be induced in the presence of AI-2 produced by other bacterial species. Thus, unlike other previously characterized bacterial species, a population of *S. meliloti* cannot up-regulate AI-2 internalization in response to fluctuations in its own population density. Instead, a population of *S. meliloti* could sense the AI-2 produced by its neighbors, leading to induction of its *ait* operon and thus interference with the AI-2 mediated behaviors of other species in the vicinity. Moreover, *S. meliloti* presumably does so without allowing the other species to detect its presence via AI-2 mediated Quorum-sensing, effectively eavesdropping on its neighbors. It is tempting to speculate that the ability of *S. meliloti* to interfere with the Quorum-sensing of plant pathogens that use AI-2 to regulate virulence could be beneficial to the plant, decreasing the virulence of pathogens like *E. carotovora*. The identification and characterization of the *S. meliloti* AI-2 dependent Ait system has provided us an excellent tool to begin studying the influence of inter-species bacterial signaling on bacteria-plant interactions, both symbiotic and pathogenic.

It has been argued that some species gain mainly a metabolic benefit from internalization of AI-2 (339); if this were the case, a non-AI-2 producing species could be acting as a "free-rider" in a mixed-species environment where other species are producing AI-2. Although this remains a possibility, our results indicate that *S. meliloti* gains no metabolic benefit from metabolizing AI-2, at least under our growth conditions. We did not observe

an increase in the growth rate of *S. meliloti* cultured in the presence of AI-2 in either complex medium or in minimal medium with AI-2 as sole carbon source, nor did the *S. meliloti ait* mutants show a growth defect in the presence or absence of AI-2.

An hypothesis for the function of the AI-2-response in *S. meliloti* is that AI-2 is used to distinguish between being in the soil, a mixed-species environment where, presumably, it encounters AI-2 produced by bacteria such as *Erwinia* or any of several bacillus, and being in its plant host where it exists inside nodules colonized exclusively by a single-species culture of *S. meliloti* and, thus, in a niche where it will encounter no AI-2.

Some bacteria are capable of degrading acyl-homoserine lactone signals produced by other species (76, 170, 329, 354). While the producing species use these molecules for species-specific Quorum-sensing, at least one bacterium, *Variovorax paradoxus*, is able to use these signal molecules as an energy source (170). Although the benefit derived by *V. paradoxus* from removing autoinducer signals from the environment might be only metabolic, *S. meliloti* does not gain a metabolic benefit from internalizing AI-2 and therefore would be expected to gain another advantage. This supports the possibility that *S. meliloti* is using AI-2 internalization as a means to interfere with the Quorum-sensing of competitive species. Furthermore, our *S. meliloti* transcriptional profile shows that, in the presence of AI-2 signal, only genes belonging to the *lsr* and *lsrRK* operons were differentially expressed. Due to our knowledge of *lsr* genes regulation their higher expression in the presence of AI-2 is not surprising; however the lack of any other AI-2 targets is considerably remarkable. One hypothesis to explain the obtained absence of AI-2 regulated genes in *S. meliloti* is the possible exclusive role of the Lsr system as an interference mechanism in this microorganism. In this scenario the only benefit of AI-2 incorporation by *S. meliloti* would be to avoid the AI-2 regulated behaviors by neighbor bacteria and, by doing so, taking advantage of colonization on a polymicrobial species community. Nevertheless, it cannot be disregarded the possibility that potential complementary signals, that would work in coincidence with AI-2, would be absent under the condition

tested. For example in *Agrobacterium tumefaciens* the Ti plasmid transfer, a phenotype regulated by an intra-specific autoinducer, is only accomplished if opines (a plant produced molecule) is sensed by the bacteria. It is also possible that since we are measuring differential gene expression one hour after the addition of AI-2 to the extracellular, we cannot rule out the possibility that AI-2 regulated genes were earlier expressed and return to their control levels of expression. Arguing against this possibility is the fact that the receptor LsrB and other components of the AI-2 recognition system are still being transcribed at this time point (**Table 2**), and therefore we would expect that genes regulated downstream of the AI-2 sensor would also be expressed at this stage.

In any case, given that *S. meliloti* lacks the ability to produce its own AI-2, it is clear that any benefits derived from AI-2 recognition and transport must arise from inter-species interactions.

6 – Acknowledgements

Catarina S. Pereira, Stephen T. Miller and Karina B. Xavier planned the experiments, analyzed the data and wrote the manuscript. J. Randall McAuley and Stephen T. Miller did crystallization and structure determination of SmlsrB and Michiko E. Taga constructed the *S. meliloti* mutants. Catarina S. Pereira performed all the other experiments.

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7 – Supplementary material

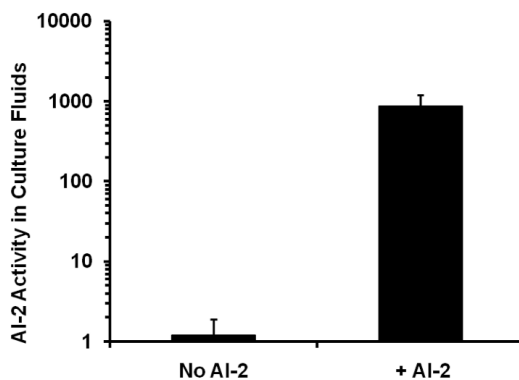


Figure S1 - AI-2 activity in *S. meliloti* late stationary cell-free culture fluids. Wild-type *S. meliloti* Rm1021 was cultured in LBMC in the presence of *in vitro* synthesized AI-2 and cell-free supernatants were tested for AI-2 activity with the *V. harveyi* BB170 bioassay. Cell-free culture fluids collected after 24 hours of growth ($OD_{600}=6$) had no AI-2 activity but when synthetic AI-2 was added to that cell-free culture sample AI-2 activity could be detected.

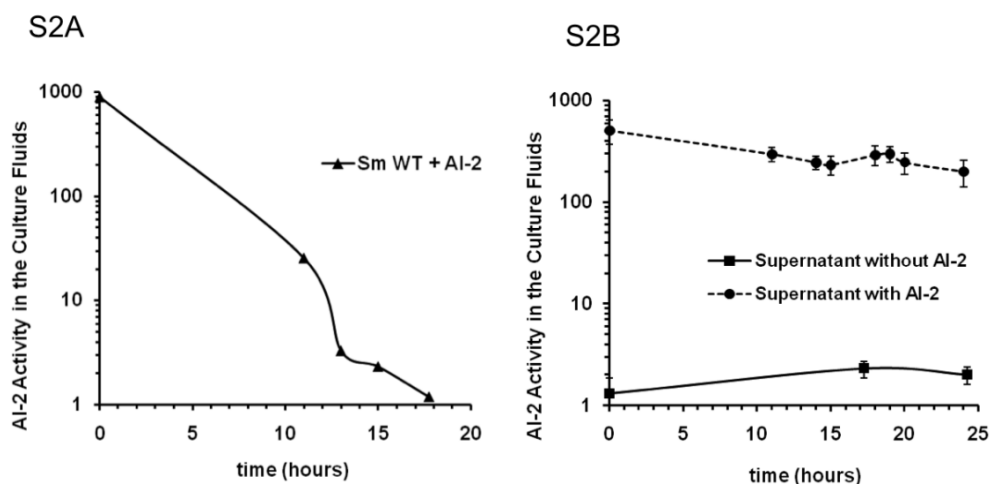


Figure S2 - AI-2 stability in *S. meliloti* cell-free culture fluids.

A) Wild-type *S. meliloti* Rm1021 was cultured in LBMC in the presence of *in vitro* synthesized AI-2 and aliquots were taken at the specified times. AI-2 activity in cell-free culture fluids is reported as fold induction of light production by *V. harveyi* BB170 (triangles).

B) To test the stability of AI-2 activity in late stationary phase cell-free culture fluids AI-2 was added to cell-free culture fluid collected from the experiment shown in A at 18 hours. This sample was then incubated for 24 hours at 30°C. Aliquots were taken and assayed for AI-2 activity via the *V. harveyi* BB170 bioassay.

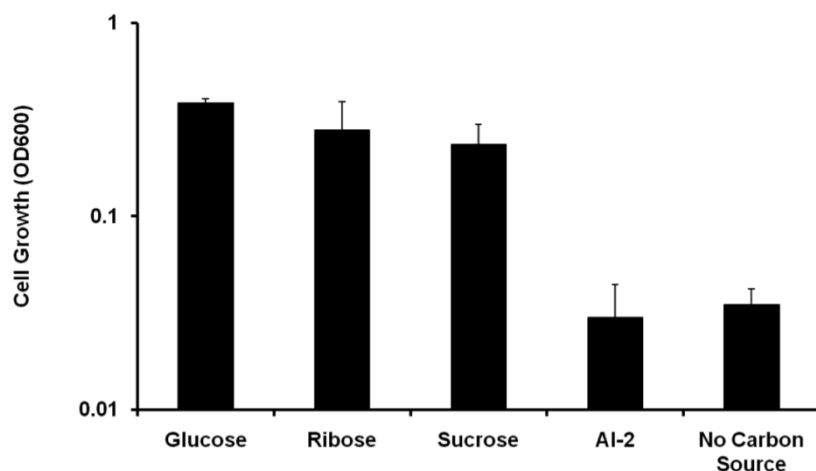


Figure S3 - Growth of *S. meliloti* in minimal medium supplemented with various carbon sources. *S. meliloti* was incubated in minimal medium M9 supplemented with either a sugar (glucose, ribose or sucrose) or AI-2 to a final concentration of 2 mM. A negative control with no carbon source added was also tested. OD₆₀₀ after 72 hours incubation are shown.

Table S1 - Primers used in this study.

Primer name	Sequence (5'-3')
P113	TGACTAGTTCTTCTTCATCTCGCACAAAGCT
P114	GTCTCGAGCGTTTCGGCGAACTCCGTGCGT
P117	GTGGATCCCTGTGCGCGCGGAAAAAGTCGT
P118	GTGCTGCAGTCGCGCTGCAAGTCGCGCACCT
P120	GTGCTGCAGGACCACAAGTTATCTACGACG
P121	GTGAAGCTTTGAACGAAGTAGTGTCAGCCGT
Smait1	ACGGAGCCGAGCGTTTC
Smait2	GAGACGATGAGCGCGTTGTAG
Smhfq1	CGTAACATCGTTTGACAATTTCTGT
Smhfq2	TGCTTGTAGACGAGCTGAGAATG
Smrpsl1	AACCTTACGCAGAGCCGAGTT
Smrpsl2	TTTGACCCGCGTCTACAC
Stfgex1	CATGGATCCAAGGACATCAAGATCGGCC
Stfgex2	CATCTCGAGTCAGAAGACCTTGAGAACTG
Strbs1	GTGGATCCAAAGACACCATCGCGCT
Strbs2	GTGAATTCCTACTGCTTAACAACCAAGTTTCAGATCAAC
Styl1	GCTGGATCCGCAGAGCGGATTGCTTTTA
Styl2	GCGAATTCTCAGAAATCATATTTGTCGATAT

CHAPTER VI

Discussion

To comprehend the chemical interactions upon which microbial communities are founded is one of the main challenges for microbiologists in the coming decades. It is now clear that bacteria rely on self-produced molecules to continuously adapt and thrive in natural populations. Giving the increasing amplitude of the chemical lexicon, and the intricate molecular mechanisms for the sensory, transduction and regulation of this 'language'; much research is still required in this area. Quorum-sensing is a cell-cell communication process in which single-celled bacteria produce, release and detect small molecules called autoinducers. The outcome of the detection of such molecules is a synchronized modification of gene expression, culminating in behavioral alterations at a population level. Autoinducer-2 is a low-weight signal molecule, produced by various phylogenetically distinct bacterial species, that has been shown to regulate multiple phenotypes, such as bioluminescence in *Vibrio harveyi* (25, 51, 212, 219, 331) and biofilm formation in *Streptococcus intermedius* (3-5). Unlike typical autoinducers that accumulate in the stationary phase of bacterial growth, in certain bacterial species, extracellular AI-2 activity declines at high cell densities. It was found that this depletion is due to signal uptake by the cell through the AI-2-specific ABC transporter, Lsr. Thus, in *Escherichia coli* and other Lsr-containing species, AI-2 induces its own incorporation and processing by activating the transcription of the *lsr*, encoding for the Lsr transporter, and the enzymes required for signal sequestration inside the cell and its subsequent processing. As the Lsr transport system incorporates all extracellular AI-2 signal, whether of self or non-self origin, Lsr-containing species can inhibit AI-2-regulated behaviors in other organisms in a multi-species environment. It thus represents the only natural system known so far for the interference of inter-species AI-2-mediated signaling.

Systems that interfere with AI-2-dependent QS could be exploited to manipulate bacterial behavior and thus provide an alternative to traditional antibiotics. Therapies that interfere with QS could inhibit virulence without killing the bacteria, a factor which is likely to create a lower selective pressure for the acquisition of resistance. A variety of approaches are being developed

that interfere with the QS at different steps of the pathways involved. One of these strategies, Quorum-quenching, involves altering, destroying, or sequestering the autoinducer signal. Most reports on Quorum-quenching approaches have employed naturally occurring enzymes that degrade species-specific signals to interfere with species-specific behaviors regulated by these signals (77, 112). However, many bacteria live in poly-species communities and, when treating infections caused by biofilms composed of multiple species, it is often not enough to target a single pathogen. In these cases, it is predicted that targeting inter-species communication, such as AI-2 signaling, is likely to be more effective in the ultimate resolution of such infections.

The overall aim of this research was to further elucidate the AI-2 signaling process, with a particular focus on the high affinity AI-2 receptor LsrB and the associated AI-2 transporter, Lsr. Understanding the mechanisms involved in regulating this process will contribute to the identification of useful targets for AI-2-based Quorum-quenching therapies.

Initially, we aimed to find components of the *lsr* system regulatory network using a molecular approach. In chapter III we identify the PTS as being essential for AI-2 internalization and induction of *lsr* operon. Although mutants in the PTS are very pleiotropic, deletion of *ptsIcrr* in an *lsrR* mutant background demonstrated that, any inhibition of *lsr* operon induction and AI-2 uptake by the PTS, is dependent on the LsrR repressor. Even though it could be hypothesized that *ptsIcrr* affected LsrR activity directly, or perhaps indirectly, through a second co-activator in an AI-2 independent manner, the fact that a constitutively expressed transporter (which allows AI-2 uptake by an PTS-independent mechanism) rescues the *ptsIcrr* mutant phenotype inducing the *lsr* operon excludes that possibility. Furthermore, we show that, although LsrK expression is affected in a *ptsIcrr* mutant strain, the rescue in *lsrR* mutant background demonstrate that this is not due to a direct effect upon LsrK by PTS itself. We argue that these data supports a model whereby the PTS is required for the initial internalization of AI-2, and this pool of intracellular AI-2, upon phosphorylation by LsrK, triggers derepression of LsrR

and induction of the Lsr transport system. PTS was identified in the 1960s as a machinery for sugar translocation across the bacterial membrane. Its function as a transporter of a wide variety of carbohydrates involves phosphotransfer from phosphoenolpyruvate across the individual PTS subunits to the internalized sugar (72, 243). The phosphorylation state of the PTS proteins reflects the availability of carbohydrates and consequently is an indirect readout of cellular metabolic status. Accordingly, the protein components of the PTS have been implicated in the regulation of carbohydrate metabolism related processes such as chemotaxis, inducer exclusion and catabolite repression (72, 164, 174, 243). Recently, the function of PTS as a crucial regulator of cellular processes has been reported to go further than these directly associated with carbohydrate metabolism. Processes such as virulence and biofilm formation in *Salmonella* Typhimurium and *Vibrio cholerae* respectively, have been shown to be regulated by the PTS (52, 132). Presumably, PTS is monitoring the metabolic capability of the cells and, only when the right metabolic conditions are achieved, are other physiological activities induced or repressed. This suggests that, at least for *lsr*-containing bacteria, the cell has to perceive a specific metabolic condition (given by the PTS) to regulate AI-2 incorporation and processing by its specific transport apparatus, Lsr. If this is the case, this is an example of how bacteria can integrate information through two distinct sensory pathways; suggesting that the Lsr system should be studied in diverse growth environments to elucidate the full extent to which it is affected by the phosphorylation state of the PTS components and the interplay between AI-2 signaling, metabolism and the environment.

In chapter IV an evolutionary strategy was employed to clarify the occurrence of Lsr transporter orthologs with the expectation that would also yield functional insights based on the ecology of the *lsr*-containing microorganisms identified. We analyzed the presence of the Lsr system amongst all the bacterial genomes sequenced and identified AI-2 receptors in organisms belonging to phylogenetically distinct families such as the *Enterobacteriaceae*, *Rhizobiaceae*, and *Bacillaceae*. Additionally, using both

sequence analysis and structural prediction tools, we established a set of criteria for successfully identifying functional LsrB AI-2 receptors. However, as the number of species identified containing a functional Lsr system was relatively low, and the realization that the genome sequences currently available are of a highly biased phylogenetic distribution compared to the extent of microbial diversity known today, it was too speculative to infer Lsr function from the ecology of the found *lsr*-containing bacteria. Nevertheless, it is worth noting that except for *Rhodobacter sphaeroides*, all the bacteria predicted to have a functional Lsr transporter also had a pathogenic or symbiotic host interaction as part of their life cycle. It was also apparent from our study that the presence of the *lsr* operon is not ubiquitous amongst *E. coli* strains: avian pathogenic *E. coli* O1 and uropathogenic *E. coli* UTI89 (UPEC) lack four of the genes encoded within the operon present on the other 11 *E. coli* strains analyzed. This surprising result raises questions as to whether the presence/absence of genes in the *lsr* operon of different *E. coli* strains genomes correlates with their phylogenetic history, adaptation to specific niches, pathogenicity or virotype. The criteria described in chapter IV to predict whether functional LsrB-like AI-2 receptors are present will provide a tool for the analysis of newly sequenced species. We expect that the numbers of organisms containing the Lsr transporter will increase with the number of sequences available, providing new opportunities for the elucidation of Lsr transporter function, with the likely identification of species or even strain-specific characteristics.

Based on the data obtained in chapter IV we selected a species, *Sinorhizobium meliloti* predicted to contain a functional Lsr transporter which we characterized in chapter V. This species was investigated because of two interesting features: it does not have *luxS* and it establishes a symbiotic relationship with leguminous plants, forming a root nodule where nitrogen fixation by the bacteria benefits the plant. We have shown that *S. meliloti*, while not capable of producing its own AI-2, responds to AI-2 produced by other species. Sequence analysis revealed that *S. meliloti* has orthologs (named *ait*) to all the proteins of the AI-2-interference Lsr system found in

enteric bacteria; it was thus likely that in *S. meliloti* this operon was also involved in AI-2 internalization and processing. In depth analysis showed that transcription of this operon by *S. meliloti* was dependent on exogenously supplied AI-2, whether synthetically produced or from other AI-2 producing organisms and *S. meliloti* completely removed the AI-2 secreted into culture by *Erwinia carotovora*. Importantly, mutants strains affected in the *ait* operon were impaired in AI-2 internalization. With these results we have demonstrated that AI-2 signaling can influence levels of gene expression in non-AI-2 producers, a fact that increases the range of potential species involved in exchange of, or response to, the AI-2 molecule beyond those that carry the *luxS* gene. We have also shown that, in the conditions tested, *S. meliloti* does not gain a metabolic benefit from internalizing AI-2, suggesting that AI-2 does not provide a suitable carbon source. Furthermore, *S. meliloti* transcriptional profile showed that, in the presence of AI-2 signal, only genes belonging to the *aitACDBF* and *aitRKG* operons were differently expressed. In light of the data reported in chapter III and chapter V, AI-2 regulation of *aitACDBF* and *aitRKG* was expected, however the lack of any other AI-2 targets is remarkable. One hypothesis to explain why the Lsr system is present and functional despite the absence of AI-2 regulated genes in *S. meliloti* is its potential role as an interference mechanism by which this microorganism could be using AI-2 internalization as a means to interfere with the QS of other species. In this scenario the main benefit of AI-2 destruction by *S. meliloti* would be to inhibit the AI-2-regulated behaviors of neighboring bacteria and, by doing so, gain competitive advantage in colonization within poly-species communities. Altogether, the findings reported in chapter V suggest that, through the acquisition of the Lsr transport system, *S. meliloti* is capable of 'eavesdropping' on the AI-2 signaling of other species and interfering with AI-2 regulated behaviors such as virulence.

The physiological consequences of AI-2 incorporation by the Lsr system are still a matter of debate. Given the resemblance of the *lsr* system to that of the ribose transporter, a restricted role of the *lsr* system to AI-2 catabolism was suggested (326). This hypothesis attributes a scavenging role to the Lsr

system that would allow the use of AI-2 as carbon and energy source when other preferred nutrients become limiting. This possibility is supported by the fact that one of the products of AI-2 incorporation and processing is 2-phosphoglycolate (347), a molecule that was suggested to be converted by the enzyme 2-phosphoglycolate phosphatase into glycolate (310) which is utilized as the sole source of carbon and energy by enteric bacteria (56). Furthermore, in *E. coli* cultures grown in LB, AI-2 is incorporated at late exponential phase, the stage of growth defined by the onset of nutrient depletion. Another analogous system reported in *E. coli* is the well-known "acetate switch" in which acetate secreted during exponential growth is later internalized, and can be metabolized as a sole carbon source (342). Very importantly, however, in the conditions tested it does not appear that AI-2 imported by the Lsr systems is used as a carbon source, as WT cultures of *S. Typhimurium* and *S. meliloti* were unable to grow when AI-2 was used as the sole carbon source (236, 306). Furthermore, the switch from acetate dissimilation to acetate assimilation requires a global change in *E. coli* gene expression, not only the activation of the *acs* operon that encodes Acetyl-CoA synthetase (which catalyzes the conversion of acetate to acetyl-CoA) is observed, but the expression levels of central metabolic pathway genes are considerably altered (227). In the presence of AI-2, at least for *S. meliloti*, the absence of regulation of any genes that encode for proteins with metabolic function does not indicate a major integration of AI-2 in the general metabolism of this microorganism. Characterization of the other AI-2 products resulting from processing by Lsr and elucidation of PTS regulation of this system may provide further evidence regarding the relationship between the Lsr degradation system and metabolism (304). However, the data reported in this thesis does not support the hypothesis of AI-2 being used as a carbon source.

A classical QS signal function has been proposed for the Lsr system (70, 180, 255). As a signaling molecule, AI-2 (or the intracellular AI-2-P) could trigger gene expression and therefore, function as a messenger in a transduction pathway. This hypothesis is supported by the results of Wood

and coworkers who demonstrated increased biofilm formation upon AI-2 addition to the growth media of WT *E. coli*, but not in a *lsrK*-deficient strain (111). To further investigate this phenotype and draw conclusions regarding the role of Lsr and AI-2 sensing in shaping *E. coli* biofilm formation, it would be necessary to test key players of this system such as *luxS*, *lsrB* and *lsrR*. This would be, to my knowledge, the first ABC transporter described to act as a signal transduction mechanism. Typically, bacteria employ two component systems, which consist of a membrane-bound histidine kinase, to sense a specific environmental or self-produced stimulus, and a response regulator, which upon activation by the sensor kinase, triggers a cellular response. Further investigations on genes regulated by AI-2 and the Lsr system as a signal transduction mechanism are required to provide more insights about the role of AI-2/*lsr* as a conventional QS system in *E. coli*. However, for *S. meliloti*, the data gathered in this thesis does not support the hypothesis of a classical AI-2 QS system.

Another theory is that the *lsr* system could work as a signal terminator analogous to the AHL lactonase of *Agrobacterium tumefaciens*, which has been shown to function as a mechanism for QS signal turnover (355). In this scenario, the Lsr system could enable bacteria to turn off their own QS signaling, providing internal regulation of the timing of the signaling process. However, for this negative feedback to be important, additional AI-2 regulated genes outside of the Lsr system would be expected to be found and those require further investigation. Nevertheless, based on previous data and on the results presented here, it is possible that in *E. coli* and other *luxS*- and *lsr*-containing species, production of AI-2 and subsequent internalization of the signal could be a mechanism for shutting off the signaling pathway.

The existence of species that do not produce AI-2 but nonetheless are able to internalize this signal, *S. meliloti* and *R. sphaeroides* for example, suggests another potential role for the Lsr system: inter-species signaling interference. Supporting this hypothesis, the microarray data reported in chapter V shows that, in the conditions tested, apart from its own incorporation and processing, *S. meliloti* does not modify gene expression in

the presence of the AI-2 signal. This would not be expected in a classical QS system, which is defined as a self-produced extracellular signal that is detected and ultimately triggers downstream transcription of genes. Other enzymes have been described to manipulate bacterial behavior by interfering with QS signals. One example is a lactonase produced by *Bacillus thuringiensis*, which suppresses the QS-dependent virulence of the plant pathogen *E. carotovora* without affecting growth (78). Another case of interference with QS signals is the *Arthrobacter nitroguajacolicus* Hod enzyme-mediated inactivation of PQS that was shown to reduce expression of virulence determinants in *P. aeruginosa* (245).

Although an interference role might appear more likely for Lsr system in *S. meliloti*, the fact that potential complementary signals, that would work in coincidence with AI-2, could be absent in the conditions tested must not be disregarded. For example in *A. tumefaciens*, Ti plasmid transfer, a phenotype regulated by AHL-QS, is only accomplished if plant-produced opines are sensed by the bacterium. In any case, given that *S. meliloti* lacks the ability to produce its own AI-2, it is clear that any benefits derived from AI-2 recognition and transport must arise from inter-species interactions. Additionally, taking into consideration the role of PTS in *lsr* activation in *E. coli* described in chapter III, the fact that the *S. meliloti* transcriptional response to AI-2 was only analyzed in single growth media should not be ignored. It is plausible that, since orthologs of PTS components have been found in the genome of *S. meliloti* (240), this mechanism of sugar translocation (and perception of cell energy status) could also be an important regulator of AI-2 incorporation in this organism; further growth conditions should be tested to fully characterize the role of Lsr in *S. meliloti*. Even so, it is tempting to speculate that the ability of *S. meliloti* to interfere with the QS systems of plant pathogens that use AI-2 to regulate virulence could be beneficial to the plant, decreasing the virulence of pathogens like *E. carotovora*. This would also benefit *S. meliloti* as protection of the plant preserves its preferred niche for growth; in the absence of the plant *S. meliloti* reproduces very slowly and has to compete with other soil bacteria for limited carbohydrate resources

(28). If this were the case, in an experimental setup that mimicked the rhizosphere, *S. meliloti* would have an *lsr*-dependent colonization advantage. In favor of this interpretation, bacterial symbionts have already been reported to provide protection for plants against infection: *Achromobacter xylosoxidans* protected the congo pea, *Cajanus cajan*, from wilt caused by fungal infection by the genus *Fusarium* (319). More research is required to confirm and consolidate the data presented in this thesis on Lsr system in *S. meliloti*, however, in light of the existing evidence, it is not expected that this transport system functions as a conventional QS mechanism, but is rather a system for interference in AI-2 dependent behaviors of neighboring species.

A crucial drawback which relates not only to the studies performed in this thesis but also in the experimental setup of most microbiological studies, is the fact that bacteria are taken out of the physiological context in which the system under analysis is relevant. The main reason to use laboratory setups such as the ones applied in this research is because they allow the maximum control of the multitude of possible variables. This enables reproducible and reliable experimental results to be obtained which is an essential feature of the scientific method. However, in contrast to molecular genetics for example, it is predicted that the research of the phenomenon of signaling interaction in bacteria is particularly affected by the body of techniques currently applied in microbiology. Bacteria use self-produced chemicals in conjugation with other environmental cues to change gene expression and adapt their behavior to fluctuations in their surroundings. If we are taking the bacteria out of their context, it is likely that a certain condition (of chemical or physical nature) necessary to activate the phenomenon under study is absent, therefore making its analysis impossible. One alternative experimental method, which is being more and more applied in microbiology, is to put the microorganisms back into their natural habitat to study them in association with their eukaryotic hosts, *e. g.* analysis of *E. coli* in the mouse intestine, or *S. meliloti* in the rhizosphere and the nodules of the plant *Medicago sativa*.

The work reported in this thesis suggests that in *S. meliloti*, the main function of the Lsr system is of signal interference, but that in *E. coli*, its role

is most likely to be environmentally dependent. It is expected that the molecular characterization of the Lsr regulation reported here will permit to address the role of this system in colonization of the natural *E. coli* habitat, the mammalian gut, and its subsequent effects upon interaction between *E. coli* and other bacterial species comprising the endogenous intestinal microflora. Moreover, the study of *S. meliloti* Lsr system has provided an excellent tool to study the influence of inter-species bacterial signaling on bacteria-bacteria interactions in the context of the plant rhizosphere that will give more insight on the function of AI-2 signaling. Future investigation into the Lsr system, including the analysis of the ecology of novel Lsr-containing species identified from newly sequenced genomes and the study of bacteria in their natural habitat, will fully clarify the role of Lsr system role in the bacterial behavior. Exploring the Quorum-quenching role of the Lsr system not only provides a potential therapeutic alternative for the control of several phenotypes in a broad spectrum of bacterial species, but also presents new challenges in the investigation of chemical communication among bacteria.

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