A new repertoire of informations about the quorum sensing system in *Salmonella enterica* serovar Enteritidis PT4

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ABSTRACT. *Salmonella* spp are among the main causative agents of foodborne diseases. Some phenotypes associated with increased drug resistance and virulence are regulated by quorum sensing (QS). In the present study, the autoinducer (AI)-1- and -2-mediated QS mechanisms were characterized in *Salmonella enterica* serovar Enteritidis PT4 for the first time. *Salmonella* Enteritidis did not produce AI-1. Phylogenetic analysis of nucleotides encoding the SdiA protein, the response regulator of AI-1-mediated QS, and comparative alignment of its amino acids showed that the gene and protein are conserved within the same bacterial genus. Thus, bacteria of the same genus respond to the same AIs. However, this finding did not preclude the possibility that *Salmonella* Enteritidis might respond to AIs released from bacteria of a different genus, which might confer a competitive advantage to this pathogen. We found that the regulation of AI-2-mediated QS in *Salmonella* Enteritidis is similar to that in serovar Typhimurium. The elucidation of

the AI-1- and AI-2-mediated QS mechanisms in *Salmonella* Enteritidis will contribute to the development of new control strategies for this pathogen by indicating new targets for antimicrobial drugs.

Key words: Quorum sensing; *Salmonella* Enteritidis PT4; SdiA

INTRODUCTION

Salmonella spp are the causative agents of a wide variety of diseases in humans and animals, including enteric fever and gastroenteritis (De Oliveira et al., 2014). The most frequent infections caused by eating food contaminated with *S.* result in gastroenteritis and are attributed to *Salmonella enterica* serovars Typhimurium and Enteritidis (Ahmer and Gunn, 2011; De Oliveira et al., 2014). The *Salmonella enterica* serotype Enteritidis is the most common cause of foodborne disease worldwide. This serovar is closely associated with consumption of eggs, poultry, and pork (Maciel et al., 2011).

The economic losses caused by *Salmonella* spp infections have attracted increasing attention in developed countries, particularly the United States, Canada, and European countries, although their impact may be much higher in developing countries, where little information is available. The financial costs are not only related to research, prevention, and treatment of human diseases but also to the maintenance of adequate sanitary conditions throughout the food production chain, and they affect both the public and private sectors (Sockett, 1991). Therefore, scientific research contributes to a solution to the problem by investigating pathogenic mechanisms and pathogen control.

A cell density-dependent signaling system was first described by Nealson and Hastings (1979), who reported the bioluminescent phenotype produced by *Vibrio fischeri*. Later, this quorum-dependent cellular communication mechanism, or quorum sensing (QS), was characterized in many other genera of bacteria. With this system, bacterial cells are able to monitor the presence of other bacteria surrounding them based on the production and response to low-molecular-mass signaling molecules termed autoinducers (AIs) (Fuqua et al., 1994; Kalia, 2013).

QS-mediated intracellular communication is used for regulating a wide variety of processes in bacterial communities. These include biofilm formation, resistance to antibiotics, induction of virulence factors, cell differentiation, nutrient flow, and collective defense against other competing microorganisms or against eukaryotic host defense mechanisms (Miller and Bassler, 2001; March and Bentley, 2004; Kalia, 2013). Furthermore, QS optimizes population survival through regulating differentiation into morphological forms that are best adapted to environmental adversities and other bacterial physiological events (Swift et al., 2001).

The QS system has been extensively studied in pathogenic and spoilage bacteria with the aim of elucidating the mechanisms involved in the regulation of infectious processes and in food spoilage (Jamuna Bai and Ravishankar Rai, 2011). Among the *Salmonella* genus, the QS systems have been examined in *Salmonella* Typhimurium, although different serovars of *Salmonella* spp presumably also exhibit the same three QS systems previously identified in this serovar (Halatsi et al., 2006).

The first QS system is incomplete in *Salmonella* spp because it fails to encode the AI-1 synthase homolog, termed LuxI, and expresses only the response regulator protein (SdiA), which is homologous to LuxR (Ahmer et al., 1998). *Salmonella* Typhimurium SdiA activity has been detected in the intestine of a turtle colonized by *Aeromonas hydrophila* (Smith

et al., 2008) and in mice colonized by *Yersinia enterocolitica* (Dzsel et al., 2010). Both of these bacteria are pathogenic and produce AIs. In these competitive situations, *Salmonella* Typhimurium found in these animals' intestines, producing the QS response regulator protein, intercept and decode the AI-mediated messages from their competitors and develop strategies to survive and win the competition (Ahmer and Gunn, 2011; Kalia, 2013).

The second QS system identified in *Salmonella* is mediated by AI-2, whose internalization is dependent on an ATP-binding cassette (ABC) transporter designated the Lsr transporter. The *lsr* operon contains seven genes, *lsrACDBFGE*, and its transcription is induced by AI-2 (Taga et al., 2001).

The third QS system found in *Salmonella* spp is mediated by AI-3, but little is known about this system. This communication mechanism exhibits characteristics that are shared with the AI-2 system, but unlike AI-2, the AI-3-mediated QS system also uses human hormones, epinephrine and norepinephrine, as signals (Walter and Sperandio, 2006).

The characterization of QS has revealed different levels of conservation in the three communication systems found among Gram-negative bacteria. According to Lerat and Moran (2004), the genes encoding the AI-1-, AI-2-, and AI-3-mediated QS systems are not fully conserved and can show variation within the same species. This fact suggests that losses or gains of genes have occurred, presumably through horizontal gene transfer (Lerat and Moran, 2004). However, recent studies have indicated that there is conservation of the gene sequences involved in QS within genera of bacteria and even between bacteria belonging to the same family (Gray and Garey, 2001).

Determining the molecular-level relationships between species is essential for making numerous biological inferences, and a proportionately large effort has been made to reconstruct this type of relationship among study organisms (Mcquiston et al., 2008). In this context, the aim of the current study was to characterize the AI-1- and AI-2-mediated QS systems of *Salmonella* Enteritidis PT4 578. A phylogenetic analysis of the nucleotide sequence encoding the response regulator protein of the AI-1-mediated QS mechanism (SdiA protein) was performed in an attempt to identify relationships between genera, species, and/or different serovars. Furthermore, a comparative analysis of the deduced amino acid sequence of the SdiA protein was performed with the aim of revealing how conserved these protein domains are.

MATERIAL AND METHODS

Bacteria and growth conditions

S. enterica serovar Enteritidis PT4 578 was isolated from chicken breast tissue and provided by FIOCRUZ (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil). The other strains employed were *Hafnia alvei* and *A. hydrophila* 069, which were used as positive controls for acyl homoserine lactone production, and *Chromobacterium violaceum* CV026, as reporter bacterium for AI-1 (AHL)-mediated QS. Stocks of bacterial cultures were prepared in Luria Bertani (LB) broth medium (1% tryptone, 0.5% yeast extract, and 0.4% NaCl), pH 7.4, containing a 33% glycerol 50 (w/v) solution in sterile water, and frozen at -80°C. Prior to each experiment, the cells were reactivated in LB or LB supplemented with specific antibiotic media, when needed, via incubation for 18 h at 37°C for *Salmonella*, or 30°C for *A. hydrophila* and *C. violaceum*.

Screening for AHL production

The *Salmonella* Enteritidis PT4 578 strain was incubated in 200 mL LB medium at 37°C for 24 h, followed by centrifugation at 10,000 *g* for 10 min in a Sorvall microcentrifuge (model MC12V; Dupont, USA). Extraction of AHL from the supernatant was performed according to the method of Ravn et al. (2001), with some modifications. The supernatant was poured into a separation funnel, supplemented with 200 mL ethyl acetate acidified with 0.5% formic acid, homogenized, and then left to stand for phase separation. The last two procedures were repeated three times. The organic phase was removed and added back to the separation funnel, together with an additional 200 mL ethyl acetate, and the stirring and standing procedures were repeated. The phase containing ethyl acetate was removed and transferred to a sterilized Erlenmeyer flask for further concentration in a rotary evaporator (model Q344B; Quimis, Brazil) at 42°C, followed by resuspension in 600 µL ethyl acetate and freezing at -20°C. The *A. hydrophila* 069 strain was used as a positive control for AHL production, and the procedure for the extraction of AHL from the culture supernatant was performed as described above. After the signaling molecules were obtained, they were identified by thin-layer chromatography (TLC).

The following AHLs were used as standards in the TLC analysis: N-hexanoyl homoserine lactone and N-butanoyl-DL-homoserine lactone (Fluka, Switzerland). The standards and samples were applied to reverse-phase TLC plates (C_{18}, RP 254s, Merck, Germany), measuring 20 x 20 cm, in volumes ranging from 10 to 20 µL. The chromatograms were developed in a methanol/water (60:40, v/v) solvent system. At the end of the run time, the solvent was evaporated and the dry plates were coated with a bacterial culture prepared as follows: 20 mL *C. violaceum* 026 culture that had been activated for 24 h was used to inoculate 200 mL 0.65% LB agar. Following homogenization, the culture was evenly spread over the plate surface and enclosed with a safeguard to prevent agar spillage. Upon solidification of the medium, the plates were incubated at 37°C for 16 to 24 h in a properly closed humidified plastic container (Ravn et al., 2001; Christensen et al., 2003). In this assay, the production of violacein by *C. violaceum* resulted in violet spots on the plates, which is considered a positive result for the presence of AHLs (Shaw et al., 1997).

Effect of AHL on *Salmonella* Enteritidis PT4 growth

The effect of synthetic AHLs on *Salmonella* Enteritidis PT4 578 growth was assessed via a microplate assay using LB medium. *Salmonella* Enteritidis PT4 578 was grown for approximately 18 h at 37°C with stirring in 10 mL LB medium. The cells were collected by centrifugation in a Sorvall microcentrifuge (model MC12V, Dupont) at 4300 *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 1.5 mL 0.85% saline solution and then centrifuged again at the same speed for the same time. Following this step, the supernatant was discarded and the absorbance of cells was standardized to prepare a population equivalent to 10^7 colony-forming units/mL. A 20-µL volume of this suspension was added to 180 µL LB medium distributed on a 96-well polystyrene microplate and supplemented with 100 nM concentrations of the following AHLs: N-hexanoyl homoserine lactone (Fluka), N-octanoyl homoserine lactone (Fluka), N-decanoyl homoserine lactone (Sigma), and N-dodecanoyl homoserine lactone (Fluka). The microplate was subsequently incubated at 37°C without stirring and the absorbance was measured at 630 nm in a VERSA MAX enzyme-

linked immunosorbent assay (ELISA) reader (Molecular Devices; Sunnyvale, CA, USA) every 30 min until the stationary phase was reached. Eight replicates and three repetitions of this experiment were conducted.

Touchdown polymerase chain reaction (PCR) for amplification of AI-1- and AI-2-mediated QS genes

Characterization of the QS system was performed in *Salmonella* Enteritidis PT4 578 based on data from *Salmonella* Typhimurium. Genomic DNA extraction from *Salmonella* Enteritidis PT4 578 was performed using the Wizard Genomic DNA Purification kit (Promega, USA). Four Touchdown PCR programs were created according to Korbie and Mattick (2008) to amplify the 11 genes involved in the AI-1 and AI-2-mediated QS mechanisms in *Salmonella* Enteritidis PT4 578, which were initially identified in *Salmonella* Typhimurium (Taga et al., 2001), corresponding to the operon *lsr* genes (10 genes) and *sdiA*. Pairs of oligonucleotides (Table 1) were designed based on the *Salmonella* Enteritidis PT4 P125109 genome deposited in GenBank (accession No. AM 933172.1) by Thomson et al. (2008) and *Salmonella* Typhimurium LT2 sequences (accession No. AE 006468.1).

The Touchdown PCR program is based on three phases. The first phase consisted of a denaturation step at 95°C (3 min), followed by 10 cycles of another denaturation step at 95°C (30 s), an annealing step with an increase of 10°C from the desired annealing temperature (Tm), and a final extension step at 72°C (70 s). The second phase comprised a denaturation step at 95°C (30 s), followed by a sequence of annealing cycles for 40 s each starting at the desired Tm plus 5°C and decreasing by 1°C each cycle for six consecutive cycles until the desired Tm was reached, with an extension cycle at 72°C (70 s); the entire sequence was repeated 19 times when the desired Tm was reached. The third phase consisted of one cycle at 72°C (5 min). The programs differed according to the desired Tm of 48, 50, 56, or 59°C. The PCR amplifications were performed in a 25-μL volume with 100 ng DNA, 1 μL each oligonucleotide, 10 pmol forward and reverse primer, 12.5 μL GoTaq Colorless Master Mix (Promega), and 9.5 μL H_2O. The obtained PCR products were purified using the Wizard SV Gel and PCR Clean-up System (Promega) and sent for sequencing at Macrogen Inc. (Seoul, South Korea).

In silico analysis

AI-2-mediated QS model

The targeted genes were amplified using the Touchdown PCR technique (described above) and sequenced to assess whether the organization of the genes belonging to the AI-2-mediated QS system of *Salmonella* Enteritidis PT4 578 corresponded to those of *Salmonella* Typhimurium reported by Taga et al. (2001). These sequences were identified in the *Salmonella* Enteritidis PT4 P125109 (AM933172.1) genome deposited by Thomson et al. (2008) using the BLASTn tool (Altschul et al., 1990). The positions and directions of the genes were identified using the National Center for Biotechnology Information (NCBI) Graphics tool for sequenced genomes.

Table 1. Amplified QS system genes of *Salmonella* Enteritidis PT4 578, based on the sequences of the correlated genes of *Salmonella* Typhimurium LT2 (AE 006468.1) and named in agreement with the genome of *Salmonella* Enteritidis PT4 P125109 (AM 933172.1). The gene functions and GenBank accession Nos. from the nucleotide sequences of *Salmonella* Enteritidis PT4 578 are also shown.

QS genes of *Salmonella* Typhimurium LT2	QS genes of *Salmonella* Enteritidis PT4 578[a]	Gene function	GenBank accession No. from nucleotide sequence of *S.* Enteritidis PT4 578*	Oligonucleotides
sdiA	sdiA	Cell division regulator protein and AI-1-mediated QS system response regulator protein	KF381283	F: 5' ATTGTCGACAAATGATTTCTTCACCTGGCGACGC 3' R: 5' ATTTCTAGATCATATCAGACCTGTCGCCGCA 3'
luxS	luxS	AI-2 synthase	KF381282	F: 5' ATTGTCGACAAAATGCAATTCCTGCAGTTT 3' R: 5' ATTTCTAGAGCCATTATTAGATAGCTTCGCAGT 3'
lsrA	ego	Putative ATP-binding protein, ABC transport system	KF381281	F: 5' TGCAAATCAGTCACAATACTGC 3' R: 5' CTCCGACGCACCATGTTC 3'
lsrB	yneA	Putative protein, ABC transport system	KF381289	F: 5' TGGCAAGACACAGCATTAAAA 3' R: 5' TCATATTTGTCGATATTGTCTTTGTTG 3'
lsrC	ydeY	Putative permeate protein, ABC transport system	KF381287	F: 5' TGTTGAAATTCATCCAAAATAACC 3' R: 5' TCATGCCACCTCTTTGTTTT 3'
lsrD	ydeZ	Putative membrane protein, ABC transport system	KF381288	F: 5' GATGAATCCATGGCGACGCTATAGC 3' R: 5' TCATGCCTGATGCCATTGCGA 3'
lsrE	sen3870	Putative ribulose-phosphate 3-epimerase	KF381284	F: 5' ACAGCCAGTTTGCCGGATTA 3' R: 5' TTATGCTGTGGAGGGTAAGAAA 3'
lsrF	yneB	Putative aldolase	KF381290	F: 5' TGGCTGATTTAGATGATATTAAAGATG 3' R: 5' CTTTCTCACTCAAAAACAGCTCAT 3'
lsrG	yneC	AI-2-modifying protein	KF381291	F: 5' TCCATGGCGAGCTATAGCTGG 3' R: 5' GCCTGATGCGCATTGCGACG 3'
lsrK	ydeV	Putative sugar kinase	KF381285	F: 5' TTACAGGCCTGGCGCTTTCCA 3' R: 5' TGGCTCGACTCTGTACCCATACTG 3'
lsrR	ydeW	Putative regulatory protein	KF381286	F: 5' TTGAATTATTTTCCCTGCGGTTT 3' R: 5' ATGAGCGATAATACGTTGGTATCTG 3'

[a]QS system genes of *Salmonella* Enteritidis PT4 578 named in agreement with the genome of *Salmonella* Enteritidis PT4 P125 109. *Comparative percentage identity between nucleotide sequence of genes of *Salmonella* Enteritidis PT4 P125 109 was 99% to luxS and 100% for the other genes.

Phylogenetic analysis of the sdiA gene and comparative analysis of the deduced amino acid sequence of the SdiA protein

A phylogenetic analysis was performed with the aim of comparing the nucleotide sequences encoding the response regulator protein of the QS systems (SdiA). The sequences used in the analysis were obtained from the GenBank database at the NCBI web page, and the included bacterial species, followed by their accession Nos. (Table 2). The nucleotide sequence encoding the SdiA protein of the *Salmonella* Enteritidis PT4 578 study isolate was also included.

Table 2. Bacterial species and GenBank accession Nos. of nucleotide sequences used in phylogenetic analysis, encoding the SdiA protein, a response regulator of the QS systems.

Bacterial species	GenBank (accession No.) SdiA
Citrobacter freundii 4747CFAA	ZP_09336017.1
Citrobacter rodentium ICC168	YP_003365526.1
Citrobacter youngae ATCC 29220	ZP_06352425.1
Cronobacter turicensis Z3032	YP_003210982.1
Escherichia coli BW2952	YP_002926921.1
Escherichia coli DH1	YP_006129257.1
Escherichia coli E101	EH_O00167.1
Escherichia coli O103 H2	YP_003222098.1
Escherichia coli O55 H7 CB9615	YP_003499911.1
Escherichia coli TW14359	YP_003078403.1
Enterobacter cloacae ATCC 13047	YP_003611882.1
Enterobacter hormaechei ATCC 49162	ZP_08498664.1
Erwinia billingiae Eb661	YP_003740953.1
Klebsiella pneumoniae MGH 78578	YP_001336067.1
Pantoea ananatis LMG 20103	YP_003519711.1
Salmonella bongori NCTC 12419	YP_004730634.1
Salmonella Choleraesuis SCSA50	ZP_09760024
Salmonella Dublin SD3246	ZP_09763785.1
Salmonella Dublin CT02021853	YP_002215136
Salmonella Agona	YP_002146073
Salmonella Enteritidis P125109	YP_002243187
Salmonella Enteritidis PT4 578	KF381283.1[▲]
Salmonella Gallinarum	YP_002226156
Salmonella Heidelberg B182	AF_H45905.1
Salmonella Heidelberg SL476	YP_002046001
Salmonella Newport SL254	AC_F63987.1
Salmonella Paratyphi A	YP_150210
Salmonella Paratyphi C	YP_002637347
Salmonella Schwarzengrund	ZP_02664244
Salmonella Schwarzengrundstr CVM19633	YP_002114983
Salmonella Typhi	CA_D05699
Salmonella Typhi Ty2	NP_804754
Salmonella Typhimurium LT2	LT2 NP_460903
Salmonella Typhimurium SL1344	SL1344 YP_005181773
Shigella boydii	YP_001879705
Shigella sonnei	YP_310157
Yokenella regensburgei ATCC 43003	ZP_09390474

[▲]Nucleotide sequence encoding the SdiA protein of the *Salmonella* Enteritidis PT4 578 study isolate.

Manual alignment of these nucleotide sequences was performed using the Mega 5.0 software (Kumar et al., 2008; Tamura et al., 2011) according to the deduced amino acid sequence corresponding to the SdiA protein. These sequences were pooled to perform phylogenetic inference through Bayesian inference (Yang and Rannala, 1997).

Bayesian inference was performed using the MrBayes 3.1 software (Huelsenbeck and Ronquist, 2001), and the evolutionary model SYM+I+G was selected using the MrMod-

eltest v2 software (Nylander, 2004). Two independent runs with four Monte Carlo Markov chains were performed for 10 million generations, and the trees were sampled and retained at every 1000th generation. The first 1 million tree samples were discarded in the burn-in phase, and the remaining trees were summarized to generate a consensus of the majority tree.

The alignment and a comparative analysis of the deduced amino acid sequence of the SdiA protein from 12 isolates and the study isolate *Salmonella* Enteritidis PT4 578 were performed using the ClustalW2 software (Larkin et al., 2007). The 12 isolates, obtained from GenBank were as follows: *Citrobacter youngae* ATCC 29220 (ZP_06352425.1); *Citrobacter turicensis* Z3032 (YP_003210982.1); *Escherichia coli* O55H7 CB9615 (YP_003499911.1); *Enterobacter cloacae* ATCC 13047 (YP_003611882.1); *Klebsiella pneumoniae* MGH 78578 (YP_001336067.1); *Pantoea ananatis* LMG 20103 (YP_003519711.1); *Salmonella bongori* NCTC 12419 (YP_004730634.1); *Salmonella* Enteritidis P125109 (YP_002243187); *Salmonella* Typhi (CA_D05699); *Salmonella* Typhimurium LT2 (NP_460903); *Shigella boydii* (YP_001879705); and *Yokenella regensburgei* ATCC 43003 (ZP_09390474)]. Characterization of the protein functional domains was performed using the Conserved Domain Database tool (Marchler-Bauer et al., 2011).

RESULTS AND DISCUSSION

### Quorum sensing in *Salmonella* Enteritidis PT4 578

No gene homologous to *luxI* was found in the *Salmonella* Enteritidis PT4 578 genome using the BLASTn tool. PCR was performed with pairs of oligonucleotides targeting the *luxI* homologs of the *H. alvei*, *E. cloacae*, and *Salmonella* Enteritidis PT4 578 enterobacteria, and no resultant DNA fragment was detected on 1.5% agarose gels. The same procedure was performed with *A. hydrophila* 069, in which a *luxI* gene fragment was amplified. These results indicate that *Salmonella* Enteritidis PT4 578 lacks AI-1 synthase. Confirmation that this serovar failures to produce the signal molecule (AI-1) was performed through TLC, as no spot relative to induction of violacein production by *C. violaceum* was detected (Figure 1).
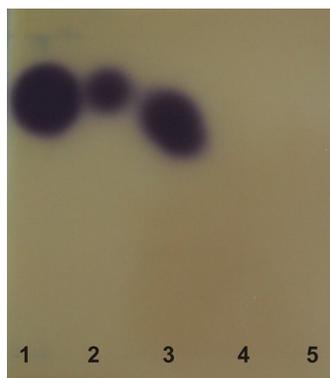
Figure 1. Thin-layer chromatography (TLC) of AHL extracts prepared from *Salmonella* Enteritidis PT4 578 and *Aeromonas hydrophila* 069 grown in Luria Bertani medium for 18 h, revealed using the reporter strain *Chromobacterium violaceum* CV026. *Lane 1* = 1 mg/mL N-butanoyl homoserine lactone standard (6 μL); *lane 2* = 0.1 mg/mL N-hexanoyl homoserine lactone standard (6 μL); *lane 3* = *A. hydrophila* 069 cell-free supernatant extract (60 μL); *lane 4* = Culture medium extract (60 μL); and *lane 5* = *Salmonella* Enteritidis PT4 578 cell-free supernatant extract (60 μL).

An incomplete AI-1-mediated QS system was first described in *E. coli,* which lacks LuxI, an AI-1 synthase but has the LuxR homolog, termed SdiA (Sitnikov et al., 1996). The presence of *sdiA* in the *Salmonella* Typhimurium genome was identified through alignment analyses performed by Ahmer et al. (1998). The lack of a *luxI* gene homolog in the *Salmonella* Enteritidis PT4 578 genome and confirmation that this serovar fails to produce AHL do not preclude it from responding to AHLs produced by other microorganisms.

Growth curves for this pathogen in the presence of different AHLs were designed with the aim of assessing whether the AHLs affect *Salmonella* Enteritidis PT4 578 growth. In these assays, a 100 nM concentration of the signaling molecules did not noticeably affect bacterial growth (Figure 2).
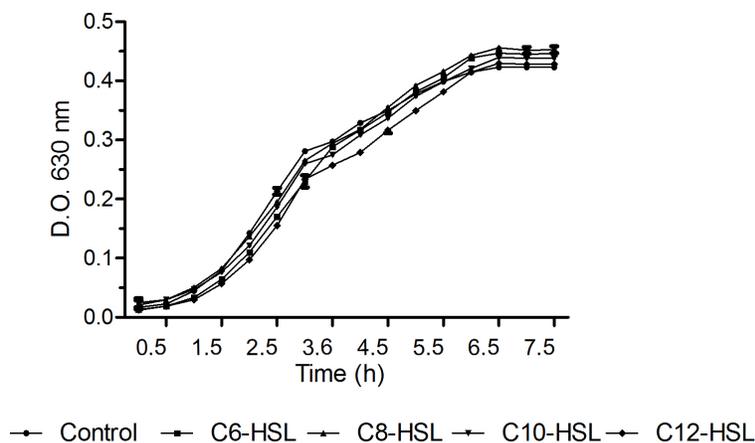
Figure 2. *Salmonella* Enteritidis PT4 578 growth over 7.5 h in Luria Bertani medium (control) and Luria Bertani media with different AHLs at a concentration of 100 nM. AHLs: C6-N-hexanoyl homoserine lactone, C8-N-octanoyl homoserine lactone, C10-N-decanoyl homoserine lactone, and C12-N-dodecanoyl homoserine lactone.

Michael et al. (2001) showed that, depending on the AHL assessed, a concentration of 1 nM suffices to induce *sdiA* gene expression in *Salmonella* Typhimurium. Confirmation that a 100 nM concentration of different AHLs does not negatively affect the microorganism's growth is essential for studying phenotypes regulated by these AIs because it enables the detection of phenotypes stimulated by this signal.

Genes encoding products belonging to AI-1- and AI-2-mediated QS systems were identified in the *Salmonella* Typhimurium LT2 (AE 006468.1) genome, and the corresponding genes were recognized in *Salmonella* Enteritidis PT4 P125109 (AM 933172.1) using the BLASTN tool (Table 1). The same genes were found in *Salmonella* Enteritidis PT4 578 after sequencing (Table 1). All sequenced gene products from *Salmonella* Enteritidis PT4 578 were deposited and showed a greater than 99% identity to gene sequences of *Salmonella* Enteritidis PT4 P125109 (AM 933172.1).

The *sdiA* gene, encoding the response regulator protein SdiA, is present in the *Salmonella* Enteritidis PT4 P125109 (AM 933172.1) genome sequenced by Thomson et al. (2008) and this gene was also identified in *Salmonella* Enteritidis PT4 578, containing 723 bp (Table 1).

The SdiA protein is a sensor of AHLs produced by other bacterial species, possibly in mammalian gastrointestinal tracts, as described by Ahmer and Gunn (2011). SdiA positively

regulates two loci in *Salmonella* Typhimurium, the *rck* operon and the *srgABC* operon, both of which were found in a virulence plasmid. The first is responsible for increasing resistance to host defense responses, whereas the latter operon lacks a given function (Ahmer et al., 1998; Michael et al., 2001).

Another gene potentially regulated by SdiA is *srgE*, which was acquired by horizontal transfer and has an unknown function (Sabag-Daigle et al., 2012). However, according to *in silico* nucleotide sequence analyses performed by the same authors, the SrgE protein may be involved in type III secretion systems (Sabag-Daigle et al., 2012).

The other genes identified in the genome of *Salmonella* Enteritidis PT4 578 (Table 1) belong to the AI-2-mediated system, in which *luxS* encodes AI-2 synthase, and the other genes belong to an operon responsible for transporting AI-2. An *in silico* analysis based on the identification and position of the AI-2-mediated QS system genes was performed to predict the model of genome organization in *Salmonella* Enteritidis PT4 578 from the genome of *Salmonella* Enteritidis P125109 (AM 933172.1). These genes are found in the region extending from 4.1485 to 4.1585 Mbp, and these data were subjected to comparison with the model of the AI-2-mediated QS system in *Salmonella* Typhimurium proposed by Taga et al. (2001).

Following a detailed analysis, the organization of the AI-2-mediated QS system was concluded to match in the two serovars. In addition, the model suggested for *Salmonella* Enteritidis PT4 578 matched the model proposed for the organization of the *lsr* operon in *Salmonella* Typhimurium by Taga et al. (2001) and these results show that the AI-2-mediated mechanism is conserved between these serovars (Figure 3). The suggested model (Figure 3) will enable the investigation of whether the AI-2-regulated phenotypes observed in *Salmonella* Typhimurium exhibit the same mode of regulation in *Salmonella* Enteritidis PT4 578.
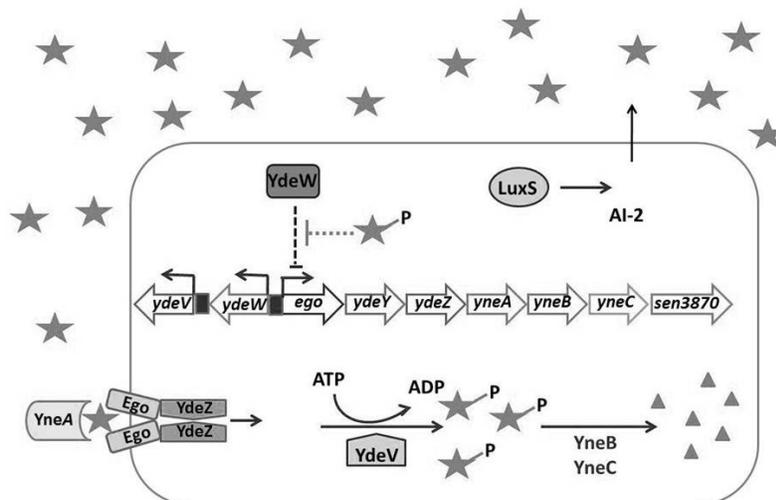
Figure 3. Suggested model for the regulation of the AI-2-mediated QS system in *Salmonella* Enteritidis PT4 578. AI-2 (star) is synthesized by LuxS and accumulates in the extracellular medium. The basal expression of the ABC transport system (YneA, Ego, and YdeZ), encoded by the operon found in this system, enables some AI-2 to enter the cytoplasm, wherein it is phosphorylated by YdeV. The phosphorylated AI-2 induces operon transcription through YdeW inactivation. At high concentrations, phosphorylated AI-2 is processed by YneB and YneC (modified model of Taga et al., 2001). The copyrights of the model proposed by Taga et al. (2001) were kindly provided for publication in this study.

The alignment of *Salmonella* Enteritidis PT4 578 genes performed with the genome of the P125109 serotype enabled us to identify AI-2-mediated QS genes using the BLASTn tool and to confirm the presence of only one copy of the genes in the genome of *Salmonella* Enteritidis PT4 578. In this serovar, the *lsr* operon is represented by the *ydeV*, *ydeW*, *ego*, *ydeY*, *ydeZ*, *yneA*, *yneB*, *yneCv*, and *sen3870* genes (Figure 3), which are responsible for encoding the AI-2 transport and modulation proteins. The *luxS* gene encoding the AI-2 synthase is also present in addition to these genes, although situated in another portion of the genome.

In the AI-2-mediated QS system, this signaling molecule is synthesized by LuxS and released into the extracellular medium. High concentrations of this molecule in the external environment and basal expression of the genes encoding the ABC transport system enable AI-2 entry into the cytoplasm. The transport system is formed by YneA, a homolog of the LsrB protein; Ego, homologous to LsrA; and YdeZ, homologous to LsrD, from the *lsr* operon of *Salmonella* Typhimurium (Taga et al., 2001). The YdeV protein, corresponding to the LsrK protein of *Salmonella* Typhimurium, is responsible for phosphorylating AI-2, and this molecule can therefore act as a transcriptional regulator, inducing the transcription of this operon by inactivating YdeW, corresponding to LsrR. The AI-2 concentration is decreased in the presence of YneB and YneC, which are homologous to the LsrF and LsrG proteins, respectively, through unknown mechanisms. No product from the other genes belonging to the *lsr* operon of *Salmonella* Typhimurium (Taga et al., 2001) was directly correlated with the AI-2-mediated system of *Salmonella* Enteritidis PT4 578. However, possible functions of these genes are highlighted in Table 1.

AI-2 is regulated by the *lsrACDBFGE* operon in the model proposed for *Salmonella* Typhimurium (Taga et al., 2001). The first four genes, *lsrACDB*, encode the AI-2 import components. The function of the other three *lsrFGE* genes is unknown, although nucleotide sequence analyses suggest that *lsrF* encodes an aldolase, while *lsrG* encodes a conserved protein of unknown function, and *lsrE* is homologous to *rpe*, encoding a ribulose-phosphate isomerase (Taga et al., 2001). The *lsrR* gene, located upstream of *lsrA*, encodes a transcriptional repressor and is transcribed differently than the *lsr* operon. LsrR represses *lsr* operon transcription in the absence of AI-2, and its presence results in derepression (Taga et al., 2001).

Phylogenetic analysis of the *sdiA* gene and comparative analysis of the deduced amino acid sequence of the SdiA protein

A phylogenetic analysis was performed to obtain evidence of how conserved the nucleotide sequences of the gene encoding the response regulator protein SdiA (homologous to LuxR) are and to predict relationships between the bacterial genera, species, and serovars available in the database.

The phylogenetic relationships of the nucleotide sequence of the SdiA protein are shown in the majority consensus tree generated via Bayesian inference (Figure 4). The values next to each ancestor node, ordered from left to right, correspond to the *a posteriori* probability value. The branches of trees whose *a posteriori* probability values were below 0.95 were omitted because this indicated a low reliability of the branch, as supported by inference.
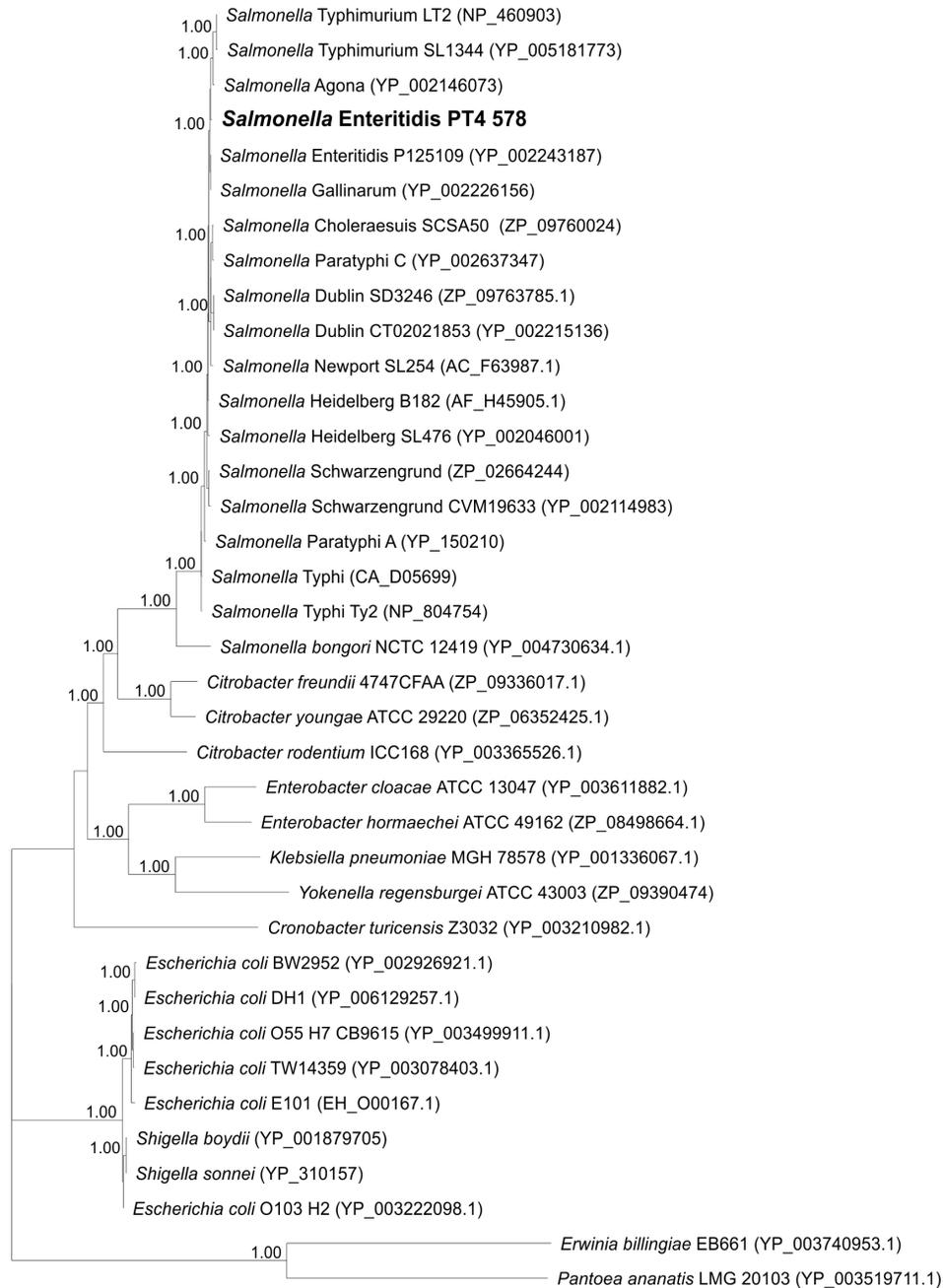
Figure 4. Phylogenetic tree constructed via Bayesian inference using the nucleotide sequences of the genes encoding the SdiA protein in 36 bacterial isolates found in GenBank (accession No. in parentheses) and the study isolate *Salmonella* Enteritidis PT4 578 (in bold). The *a posteriori* probability value obtained using Bayesian inference is shown, from left to right, beside each ancestor node. Values below 0.95 were omitted from the tree. The bar indicates a substitution estimated every 10 bp.

The constructed tree (Figure 4) shows that the nucleotide sequence of genes encoding the response regulator protein SdiA are conserved among organisms of the same genus (*Enterobacter*, *Escherichia*, *Salmonella*, and *Shigella*) based on the *a posteriori* probability value. An exception should be made for *E. coli* O103: H2, which is grouped with representatives of the genus *Shigella*. The *Pantoea* and *Erwinia* genera are phylogenetically so distant that they were inevitably used as an outgroup to highlight the differences among the other genera.

Similar to other QS proteins, the SdiA protein contains approximately 228 amino acids and consists of two domains: an N-terminal AI-binding domain (Figure 5) and a C-terminal domain (Figure 5), containing a helix-turn-helix motif involved in DNA binding (Nasser and Reverchon, 2007). AI binding in these proteins modulates the binding of the SdiA C-terminal protein domain to DNA. For example, in the case of the LuxR protein, an AHL binds to its N-terminal domain, leading to dimerization (Choi and Greenberg, 1991).

The SdiA protein N-terminal AI-binding domain is highly conserved within each bacterial genus (the *Salmonella* genus in this case, highlighted in white text in Figure 5). This indicates that each bacterial genus possibly responds to the same AIs. In the same way, the N-terminal binding domains for the AIs vary greatly among different bacterial genera, possibly indicating that each genus has an inherent ability to respond to different types of AIs. However, this does not mean that different bacterial genera can respond to the same AI.

The SdiA protein DNA-binding domain is quite conserved in all of the bacteria evaluated (Figure 5). This is shown by the high frequency of the asterisks and colons, which indicate that the amino acid residues in the column were identical in all of the aligned sequences and that there were substitutions, albeit conserved, of residues, respectively.

A plausible explanation for the conservation of this gene and its product in environments characterized by high selective pressure imposed by competition between bacterial species and/or genera is the ability of the SdiA protein to detect and respond to AHL signal molecules produced by different bacteria (Case et al., 2008). This ability confers advantages on the bacteria because it enables them to regulate their increase in virulence, as in the well-studied case of *Salmonella* Typhimurium and in biofilm formation by *E. coli*, both of which are crucial phenotypes to overcome competition and infect the host (Case et al., 2008).

QS response regulators similar to LuxR act simultaneously as sensors and transcriptional factors (Choi and Greenberg, 1991). The AI- and DNA-binding domains are fused in this signal transduction system. For this reason, it is referred to as a one-component signal transduction system, which has been shown to be the most common type of signal transduction system (Madan Babu and Teichmann, 2003).

A phylogenetic analysis performed by Case et al. (2008) showed that 45 bacteria of all of the bacterial genomes deposited in the database until 2008 included a LuxR homolog, albeit without the corresponding AHL synthase.

Although it is unknown whether all LuxR homologs are actually functional, this profile may be prevalent among proteobacteria, and 26% of all of the sequenced bacterial genomes from this group exhibit the complete QS system, including genes encoding the AI synthase and the response regulator protein, while 17% present an incomplete QS, failing to synthesize the signaling molecule but showing the potential to spy on the AHL-mediated QS system of other proteobacteria (Case et al., 2008). Some of the species chosen for the phylogenetic analysis performed here exhibited the complete AI-1-mediated QS system; that is, they presented both the synthase-encoding gene (*luxI*) and the response regulator protein-encoding gene (*luxR*). The nucleotide sequence of the LuxR protein, which belongs to the complete sys-

tem, despite being considered homologous to SdiA (the regulatory protein of the incomplete system), shows a low identity to SdiA (data not shown), which can be explained by the greater flexibility of the SdiA protein in detecting and responding to signal molecules produced by other bacterial species and/or genera (Gray and Garey, 2001).
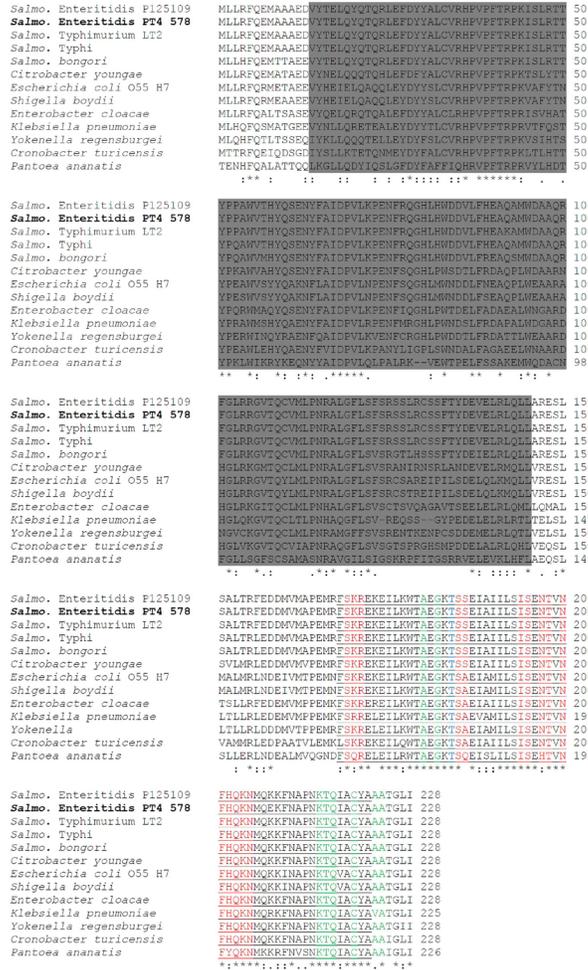
Figure 5. Deduced amino acid sequence alignment of the SdiA protein of 12 isolates from GenBank (for accession No., refer to the Material and Methods) and the study isolate in *Salmonella* Enteritidis PT4 578 (in bold) using ClustalW2 software. The two main domains of the SdiA protein were elucidated using the Conserved Domain Database tool. The autoinducer-binding N-terminal domain is shaded in dark gray, and the DNA-binding C-terminal domain, showing a helix-turn-helix motif, is shaded in light gray. The amino acid residues within the autoinducer-binding domain that were identical between species and serovar representatives of the genus *Salmonella* are highlighted in white. The DNA-binding amino acid residues within the DNA-binding domain are shown in red, while the residues involved in the dimerization interface are presented in green, and the residues involved both in DNA-binding and dimerization are indicated in blue. Symbols: asterisk means that the amino acid residues in the column were identical in all sequences aligned; colon means there were substitutions of residues, but the substitutions were conserved; and dot indicates semi-conserved substitutions.

The genetic exchange capacity of bacteria can be demonstrated by the QS mechanism they exhibit. The presence of two components (*luxI,* encoding an AI-1 synthase protein, and *luxR,* encoding a response regulator protein that acts as a transcriptional factor, activating or repressing gene expression) is observed when the AI-1-mediated QS mechanism is analyzed, as originally described in *V. fischeri* by Nealson and Hastings (1979). However, this system is incomplete in some microorganisms, and it may have arisen through horizontal gene transfer (Gray and Garey, 2001).

Bacteria of the *Citrobacter* genus are enteric pathogens that show a similar mode of action to enteropathogenic *E. coli* and exhibit the complete QS system, consisting of *croI* (*luxI*) and *croR* (*luxR*) (Coulthurst et al., 2007). *Erwinia* spp also belong to the family Entero-bacteriaceae, including species with a verified complete system, such as *Erwinia carotovora*, showing a QS mechanism that involves both *carI* (*luxI*) and *carR* (*luxR*). Only the gene encoding the SdiA-homolog regulator protein was detected in *Erwinia amylovora* (Smith et al., 2008), whereas the complete system consisting of *esaI*, *esaR*, *eagI*, and *eagR* was found in the genus *Pantoea* (Gray and Garey, 2001). However, there is little information available in the literature about the other genera analyzed, including *Cronobacter* spp (formerly *Enterobacter sakazakii*)*, Klebsiella,* and *Yokenella*, in which the complete and incomplete QS systems are still poorly understood (Araújo et al., 2012).

In conclusion, *Salmonella* Enteritidis PT4 578 exhibits AI-1- and AI-2-mediated QS systems. To our knowledge, these systems were characterized for the first time in *Salmonella* Enteritidis in the present study, and they are similar to the QS systems described for *Salmonella* Typhimurium.

The nucleotide sequences encoding the SdiA protein of *Salmonella* Enteritidis PT4 578 are grouped with the other serovars of *S. enterica*, including Typhimurium. Thus, previously elucidated phenotypes known to be regulated by AI-1-mediated QS in *Salmonella* Typhimurium may be extended to studies in *Salmonella* Enteritidis.

Data obtained from the phylogenetic analysis of the nucleotide sequence of the SdiA protein and from a comparative alignment of its deduced amino acid sequence clearly show that both the gene and the protein, including its domains, are conserved within a bacterial genus. Thus, bacteria from the same genus can respond to the same AIs. However, this does not preclude the possibility that different bacterial genera may respond to the same AI, which may confer a competitive advantage on these bacteria, given their ability to respond to the signal released by the quorum comprised of other organisms.

The characterization of the QS mechanisms of the human pathogen *Salmonella* Enteritidis performed in the current study will enable future clarification of new control strategies in potential AI-1- or AI-2-regulated phenotypes. Moreover, the combination of conventional antibiotics and the development of quorum inhibitors may result in greater control of these pathogenic agents by preventing biofilm formation, overcoming resistance to antibiotics, or even reducing the microorganism virulence.

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