Microbial Pathogenesis 99 (2016) 178–190

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Novel insights from molecular docking of SdiA from *Salmonella* Enteritidis and *Escherichia coli* with quorum sensing and quorum quenching molecules

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ARTICLE INFO

Article history:
Received 24 May 2016
Received in revised form
4 July 2016
Accepted 22 August 2016
Available online 24 August 2016

Keywords:
1-Octanoyl-*rac*-glycerol
Acyl homoserine lactone
Autoinducer
Furanone
LuxR family proteins
Molecular modeling

ABSTRACT

Quorum sensing is a cell-to-cell communication mechanism leading to differential gene expression in response to high population density. The autoinducer-1 (AI-1) type quorum sensing system is incomplete in *Escherichia coli* and *Salmonella* due to the lack of the AI-1 synthase (LuxI homolog) responsible for acyl homoserine lactone (AHL) synthesis. However, these bacteria encode the AHL receptor SdiA (a LuxR homolog) leading to gene regulation in response to AI-1 produced by other bacteria. This study aimed to model the SdiA protein of *Salmonella enterica* serovar Enteritidis PT4 578 based on three crystallized SdiA structures from Enterohemorrhagic *E. coli* (EHEC) with different ligands. Molecular docking of these predicted structures with AHLs, furanones and 1-octanoyl-*rac*-glycerol were also performed. The available EHEC SdiA structures provided good prototypes for modeling SdiA from *Salmonella*. The molecular docking of these proteins showed that residues Y63, W67, Y71, D80 and S134 are common binding sites for different quorum modulating signals, besides being conserved among other LuxR type proteins. We also show that AHLs with twelve carbons presented better binding affinity to SdiA than AHLs with smaller side chains in our docking analysis, regardless of the protein structures used. Interestingly, the conformational changes provided by AHL binding resulted in structural models with increased affinities to brominated furanones. These results suggest that the use of brominated furanones to inhibit phenotypes controlled by quorum sensing in *Salmonella* and EHEC may present a good strategy since these inhibitors seem to specifically compete with AHLs for binding to SdiA in both pathogens.

1. Introduction

Quorum sensing is a mechanism of communication among cells leading to differential gene expression in response to changes in population density [1,2]. In phylum Proteobacteria, a pair of proteins LuxI (acyl homoserine lactone synthase) and LuxR (transcription activator) or their homologous proteins is responsible for this mechanism in which LuxI derivatives synthesizes the autoinducer-1 (AI-1) called *N*-acyl homoserine lactones (AHLs) [1,3–5]. Some proteobacteria belonging to the family Enterobacteriaceae, such as *Escherichia coli* and *Salmonella*, do not synthesize AHL due to the absence of LuxI homologues. However, these microorganisms encode a transcription factor of the LuxR family, named SdiA [6], which responds to AHLs produced by other bacterial species and synthetic AHLs [7–11]. In this regard, quorum sensing in *Salmonella* and *E. coli* resembles the paracrine signaling found in mammalian systems [12].

In AI-1 type quorum sensing system, when the population reaches a high density, the AHLs are internalized and bind to the ligand-binding domain (LBD) of the LuxR type proteins which dimerize and bind to DNA by using their DNA-binding domain (DBD) regulating expression of target genes [13–16]. In *Salmonella*, AHLs regulate phenotypes such as adhesion to HeLa cells, biofilm formation on polystyrene, invasion of HEp-2 epithelial cells and survival in rabbit and guinea pig serum [17–19]. Similarly in *E. coli*, these molecules regulate adhesion to HEp-2 epithelial cells, biofilm formation on polystyrene and resistance to acidic pH [20–22].

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http://dx.doi.org/10.1016/j.micpath.2016.08.024
0882-4010/

On the other hand, there have been reports of phenotypes regulated by SdiA protein in the absence of AHLs in *Salmonella* and *E. coli* [8,10,16,23,24]. For instance, SdiA of Enterohemorrhagic *E. coli* O157:H7 (EHEC) is constitutively activated by the binding of molecule 1-octanoyl-*rac*-glycerol (OCL) in the absence of AHLs [16]. The OCL molecule is a monoglycerol present in prokaryotes and eukaryotes and is used as an energy source, and substrate for the synthesis of membrane and a signaling molecule [25,26]. However, the activation of SdiA from EHEC by AHLs conferred greater stability and affinity to DNA, albeit not affecting *sdiA* gene transcription [16]. Additionally, these authors observed conformational changes of EHEC SdiA protein complexed with different ligands such as: OCL in the absence of AHLs; *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) and; *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL). More studies are needed to elucidate the exact functions of AHLs on the physiology of these microorganisms since a great level of complexity is seen as revealed by these previous works.

The structures of SdiA protein of EHEC crystallized with different ligands contain information about the atomic coordinates, structural factors, ligands and cofactors [16]. For this reason, these structures become interesting prototypes for the modeling of SdiA protein from *Salmonella* which has not been crystallized yet, and subsequently conduct studies of molecular docking with quorum sensing and quorum quenching molecules.

Molecular docking and protein modeling prediction are commonly used in quorum sensing studies in the search for auto-inducer and inhibitors as well as to predict their binding sites [27–41]. Gnanendra et al. [30] performed molecular modeling of *Salmonella enterica* serovar Typhimurium SdiA protein by using the structure of CviR from *Chromobacterium violaceum*, which was the available model at the time. These authors then used molecular docking to predict the AHL binding sites. Gnanendra et al. [32] also conducted a molecular docking study with halogenated AHLs, suggesting that those could be potential quorum sensing inhibitors in *Salmonella* Typhimurium.

Furanones are AHL antagonistic compounds in gram-negative bacteria, since they present structural similarity to these auto-inducers due to the homoserine lactone ring, but hinder transcriptional regulation by a mechanism still not fully understood [42–48]. The inhibitory effect of different brominated and non-brominated furanones on biofilm formation by *Salmonella* Typhimurium [47], *Salmonella enterica* serovar Agona [48] and *Salmonella enterica* serovar Enteritidis [19] has been reported as well as their effect on biofilm formation and motility in *E. coli* [49].

In the present study, SdiA protein of *Salmonella* Enteritidis PT4 578 has been modeled on the basis of three crystallized SdiA structures from EHEC with different ligands. We then performed molecular docking of these different structures with OCL, AHLs and furanones in order to predict their binding affinity and to identify the potential binding residues. Due to the importance of the phenotypes regulated by AHLs in *Salmonella* and EHEC, this study provides insights for the guided search for quorum sensing inhibitors as well as contributes to the understanding of the inhibitor and autoinducer binding mechanisms in *Salmonella* Enteritidis.

2. Materials and methods

The experimental strategy employed in this study in order to create the SdiA molecular model of *Salmonella* Enteritidis, from available EHEC SdiA crystal structures, and to evaluate the molecular docking of different quorum sensing and quorum quenching molecules is depicted on Fig. 1 and fully described herein.

2.1. Target amino acid sequence of SdiA protein

The amino acid sequence of SdiA of *Salmonella enterica* serovar Enteritidis PT4 578 (GenBank: AGZ95694.1 [50]) was obtained from the National Centre for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov/).

2.2. Search of homologous proteins to Salmonella Enteritidis SdiA

The tools "BLAST" and "Find and Model Structure" of the CLC Drug Discovery Workbench 2.5 software (http://www.clcbio.com/products/clc-drug-discovery-workbench/) were used to search homologous amino acid sequences with the SdiA protein of *Salmonella* Enteritidis PT4 578 (GenBank: AGZ95694.1) in the RCSB Protein Data Bank database (PDB; http://www.rcsb.org/pdb/home/home.do). The five parameters: resolution (Å), free R-value, E-value, percentage of identity and coverage were evaluated in this step. The amino acid sequences of proteins with more than 50% identity were extracted and aligned by "ClustalW" tool of the CLC Drug Discovery Workbench 2.5 software.

2.3. Molecular modeling and validation of Salmonella Enteritidis SdiA protein

The molecular modeling of SdiA protein of *Salmonella* Enteritidis was performed by the CLC Drug Discovery Workbench 2.5 software based on the protein with the higher percentage of convergence. The structures of *Salmonella* Enteritidis PT4 578 modeled from EHEC were identified with their template code of PDB plus the letter "S". Then, the generated macromolecular structure of SdiA protein of *Salmonella* Enteritidis (4Y13-S, 4Y15-S and 4Y17-S) and its template from EHEC (PDB: 4Y13, 4Y15 and 4Y17 [16]) were superposed and validated by using three different approaches. First we validated the structures by using the Ramachandran Plot Analysis (RAMPAGE [51]) at Crystallography and Biocomputing Group of the University of Cambridge, Department of Biochemistry server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php), then at the Verify3D [52], and finally by ERRAT [53] at Structure Analysis and Verification server version 4 (SAVES server; http://services.mbi.ucla.edu/SAVES/).

2.4. Comparison the generated macromolecular structure of SdiA protein with its template

The generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis and their templates of EHEC [16] were superposed to compare the ligand-binding domain (LBD) and the DNA-binding domain (DBD).

2.5. Amino acid conservation of SdiA proteins

The amino acid sequences of SdiA protein of *Salmonella* Enteritidis PT4 578 (GenBank: AGZ95694.1) and EHEC (UniProtKB: Q8XBD0) were aligned by "ClustalW" tool of the CLC Drug Discovery Workbench 2.5 software.

2.6. Molecular docking of SdiA proteins with different molecules

The molecular docking of SdiA proteins of *Salmonella* Enteritidis and EHEC were performed with quorum sensing and quorum quenching molecules (Table 1) by using the "Dock Ligands" tool of the CLC Drug Discovery Workbench 2.5 software, with 1000 interactions for each ligand being performed. The generated score mimics the potential energy change when the protein and the ligand come together based on hydrogen bonds, metal ions and

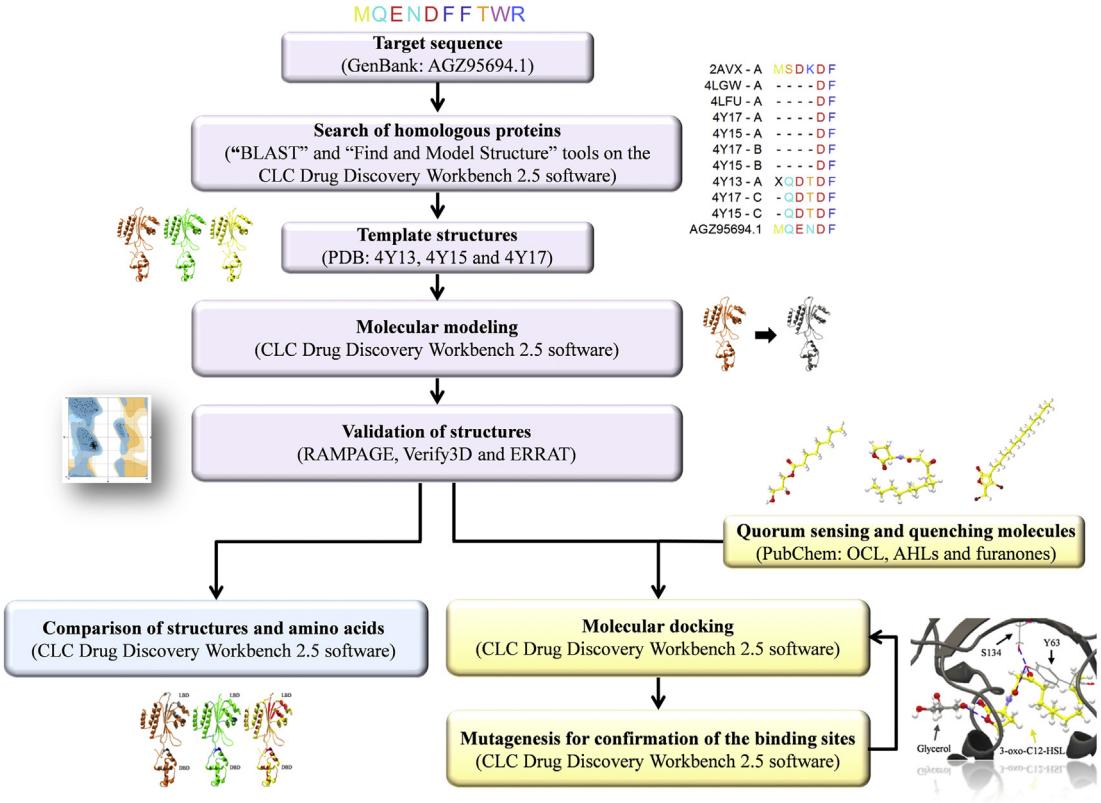

Fig. 1. Experimental strategy used to evaluate the molecular docking of SdiA from *Salmonella* Enteritidis and *Escherichia coli* with quorum modulating molecules. Different steps are color coded as: purple - molecular modeling, blue - comparison of the structures and the amino acid sequences and, yellow - molecular docking. The databases, tools and softwares used in the different steps are indicated in brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

steric interactions, where lower scores (more negative) correspond to higher binding affinities. The five best scores of the docking were used in each macromolecular structure, allowing the inspection of the binding sites of SdiA with ligands and cofactors.

2.7. Mutagenesis prediction of SdiA binding sites

An *in silico* leucine scanning mutagenesis was performed for each binding site of SdiA proteins with ligands and cofactors in the CLC Drug Discovery Workbench 2.5 software. Mutated proteins were docked with quorum sensing and quorum quenching molecules by using the above described software in order to confirm the binding sites as performed in previous studies [54–56].

3. Results and discussion

3.1. SdiA homologous proteins

The search for homologous proteins to the SdiA protein of *Salmonella* Enteritidis PT4 578 (GenBank: AGZ95694.1) by the "Find and Model Structure" and "BLAST" tools showed that 10 proteins from the PDB database have more than 63.7% identity, all of which are SdiA proteins from *E. coli* (Table 2). It is noteworthy that *Salmonella* SdiA structure is not available in the PDB database. The amino acid sequences of the C chains of 4Y15 and 4Y17 structures and the A chain of 4Y13 structure of EHEC had the highest percentage of coverage, 99.58%, and greater than 69.04% identity with SdiA protein of *Salmonella* Enteritidis PT4 578 (Table 2). This result was confirmed by alignment of the amino acid sequences of all proteins listed in Table 1 (please see Fig. S1, supplementary material). Gnanendra et al. [30] previously showed that the A chain of CviR, a LuxR type protein from *C. violaceum* (PDB: 3QP5), was the sequence available at that time with the most homology to SdiA protein of *Salmonella* Typhimurium, exhibiting 40% identity and E-value of 4e-15 by "BLASTP" tool.

Thus, the A chain of 4Y13 structure and the C chains of 4Y15 and 4Y17 structures of EHEC were selected for the molecular modeling of SdiA protein of *Salmonella* Enteritidis because they had the highest percentage of coverage, providing more refined information about the atomic coordinates, structural factors, ligands and cofactors. These structures are SdiA protein of EHEC (UniProtKB: Q8XBD0) complexed with different ligands. For instance, 4Y13 structure is bound to OCL; the 4Y15 structure is bound to 3-oxo-C6-HSL; and the 4Y17 structure is bound to 3-oxo-C8-HSL [16].

3.2. Molecular modeling and validation of SdiA predicted structures

The generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis from molecular modeling were superposed to the respective template of EHEC, which showed a good alignment of both the LBD in *N*-terminal end and DBD in C-terminal end (Fig. 2). Furthermore, these structures were validated by checking the stereo chemical parameters with three softwares: RAMPAGE, Verify3D and ERRAT (Table 3). The Ramachandran Plot Analysis showed that more than 95.9% of the residues of all structures analyzed were in favored region (Table 3 and Fig. 3). In addition, all the structures had more than 85.7% of the amino acid residues with scores greater than or equal to 0.2 in the Verify3D (Table 3). These results indicate that there is compatibility between an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location, environment and comparing the results to good structures [52].

Table 1
Quorum sensing and quorum quenching molecules used in the molecular docking of SdiA proteins.

Group	Type	Molecule	IUPAC nomenclature	PubChem CID[a]	Reference
OCL	–	1-octanoyl-*rac*-glycerol	2,3-dihydroxypropyl octanoate	3033877	[16]
AHL	Unmodified in 3-oxo	*N*-butyryl-DL-homoserine lactone	*N*-(2-oxooxolan-3-yl)butanamide	443433	[44]
		N-hexanoyl-DL-homoserine lactone	*N*-(2-oxooxolan-3-yl)hexanamide	3462373	[19,44,50]
		N-octanoyl-DL-homoserine lactone	*N*-(2-oxooxolan-3-yl)octanamide	3474204	[19,44,50]
		N-decanoyl-DL-homoserine lactone	*N*-(2-oxooxolan-3-yl)decanamide	11644562	[19,44,50]
		N-dodecanoyl-DL-homoserine lactone	*N*-(2-oxooxolan-3-yl)dodecanamide	11565426	[19,50]
	Modified in 3-oxo	*N*-(3-oxohexanoyl)-L-homoserine lactone	3-oxo-*N*-[(3S)-2-oxooxolan-3-yl]hexanamide	688505	[16,44,45,64]
		N-(3-oxooctanoyl)-L-homoserine lactone	3-oxo-*N*-[(3S)-2-oxooxolan-3-yl]octanamide	127293	[16,44]
		N-(3-oxodecanoyl)-L-homoserine lactone	3-oxo-*N*-[(3S)-2-oxooxolan-3-yl]decanamide	10221060	[44]
		N-(3-oxododecanoyl)-L-homoserine lactone	3-oxo-*N*-[(3S)-2-oxooxolan-3-yl]dodecanamide	3246941	[64]
Furanone	Non-brominated	3-methyl-2(5H)-furanone	3-Methyl-2(5H)-furanone	30945	[19]
		3-butyl-2(5H)-furanone	4-butyl-2H-furan-5-one	11768654	[47]
		2-methyl tetrahydro-3-furanone	2-methyloxolan-3-one	18522	[19]
		2,2-dimethyl-3(2H)-furanone	2,2-dimethylfuran-3-one	147604	[19]
		2(5H)-furanone	2H-furan-5-one	10341	[19]
	Brominated	4-bromo-5-(bromomethylene)-2(5H)-furanone	4-bromo-5-(bromomethylidene)furan-2-one	67228456	[45,47,64]
		5-bromomethylene-2(5H)-furanone	5-(bromomethylidene)furan-2-one	67228360	[44,45,47,64]
		5-dibromomethylene-2(5H)-furanone	5-(dibromomethylidene)furan-2-one	315069	[47]
		3-ethyl-5-(dibromomethylene)furan-2(5H)-one	5-(dibromomethylidene)-3-ethylfuran-2-one	11140550	[47]
		4-bromo-3-butyl-5-(dibromomethylene)furan-2(5H)-one	4-bromo-3-butyl-5-(dibromomethylidene)furan-2-one	362385	[47]
		4-bromo-5-(bromomethylene)-3-ethyl-2(5H)-furanone	(5Z)-4-bromo-5-(bromomethylidene)-3-ethylfuran-2-one	12051732	[47]
		4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	(5Z)-4-bromo-5-(bromomethylidene)-3-butylfuran-2-one	9839657	[47]
		4-bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone	(5Z)-4-bromo-5-(bromomethylidene)-3-hexylfuran-2-one	16127328	[47]
		4-bromo-5-(bromomethylene)-3-octyl-2(5H)-furanone	(5Z)-4-bromo-5-(bromomethylidene)-3-octylfuran-2-one	52950214	[47]
		4-bromo-5-(bromomethylene)-3-decyl-2(5H)-furanone	(5Z)-4-bromo-5-(bromomethylidene)-3-decylfuran-2-one	52946441	[47]
		4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone	(5Z)-4-bromo-5-(bromomethylidene)-3-dodecylfuran-2-one	10180544	[47]

International Union of Pure and Applied Chemistry (IUPAC).
1-octanoyl-*rac*-glycerol (OCL).
Acyl homoserine lactone (AHL).
[a] Compound Identifier of PubChem database (PubChem CID; https://pubchem.ncbi.nlm.nih.gov/).

Moreover, all the structures showed a threshold higher than the cut off of 91% in ERRAT, except the generated 4Y15-S structure of SdiA protein from *Salmonella* that showed 87.0% of the protein above the acceptable limit (Table 3). Gnanendra et al. [30] considered values of 92.6% at Verify3D and 73.2% at ERRAT as reliable and with good quality in the validation of generated structure of SdiA protein of *Salmonella* Typhimurium based on the CviR protein from *C. violaceum*. Furthermore, the results of the structures of SdiA of EHEC used as template in molecular modeling of the present study are in agreement with those found by Nguyen et al. [16]. Thus, all the results of validation by Ramachandran Plot Analysis, Verify-3D and ERRAT confirm that the generated macromolecular structures of SdiA from *Salmonella* Typhimurium and their templates were acceptable to be used in further studies.

3.3. Comparison the generated macromolecular structures of SdiA proteins with their templates

All the generated macromolecular structures of the SdiA protein of *Salmonella* Enteritidis (Fig. 4A and B) and their EHEC templates (Fig. 4C and D) were superposed separately. These superpositions showed that the LBD and DBD had slight conformational changes depending upon the presence or absence of AHL, as well as in relation to the size of the AHL carbon chain (Fig. 4). These results corroborate with those found for the same protein of EHEC [16] and for TraR, another LuxR type protein [57,58]. In addition, Nguyen et al. [16] showed the conformational change of DBD of SdiA protein of EHEC in the presence of AHLs increased its stability and affinity for DNA. In fact, they showed that this stability and DNA affinity were even greater for AHLs with longer carbon chains. However, these authors reported that the conformation of the DBD in the presence of OCL and the absence of AHLs is also capable of regulating gene expression, confirming previous studies performed in the absence of AHLs [8,10,23,24,59,60].

3.4. Amino acid conservation of SdiA proteins

The amino acid sequences of SdiA protein of *Salmonella* Enteritidis PT4 578 and EHEC were aligned (Fig. 5). This comparison showed that 71.66% (172) of the amino acid residues were conserved between SdiA protein of *Salmonella* Enteritidis and EHEC. Besides, many of these conserved residues were also described as conserved among other LuxR type proteins participating in interactions between the domains of these proteins (Fig. 5) [6,16,30,50,61]. In addition, 15 amino acid residues were described by Gnanendra et al. [30], Yao et al. [62] and Nguyen et al. [16] as binding sites for different ligands between SdiA protein of *Salmonella* Enteritidis, *E. coli* and EHEC, respectively (Fig. 5). However, only the residues W67, Y71, and D80 were common binding sites among these studies while residue Y63 was common among *E. coli* studies. Moreover, it is noteworthy that the binding of OCL in the SdiA protein of EHEC may be mediated by glycerol as a cofactor [16].

Table 2
SdiA homologous proteins to *Salmonella* Enteritidis PT4 578 obtained from PDB database.

Number PDB	Chain	Protein complex	Organism	Resolution (Å)	Free R-value	Tools				
						"BLAST"		"Find and model structure"		
						E-value	% Identity	E-value	% Identity	% Coverage
4Y15	C	SdiA in complex with 3-oxo-C6-homoserine lactone	EHEC	2.84	0.25	2.20e-109	71.55	1.34e-129	71.55	99.58
4Y17	C	SdiA in complex with 3-oxo-C8-homoserine lactone	EHEC	2.84	0.27	2.38e-109	71.55	1.83e-129	71.55	99.58
4Y13	A	SdiA in complex with 1-octanoyl-*rac*-glycerol	EHEC	3.10	0.24	4.58e-103	69.04	1.10e-122	69.04	99.58
4Y15	B	SdiA in complex with 3-oxo-C6-homoserine lactone	EHEC	2.84	0.25	1.25e-108	72.03	9.22e-129	72.03	98.33
4Y17	B	SdiA in complex with 3-oxo-C8-homoserine lactone	EHEC	2.84	0.27	1.25e-108	72.03	9.22e-129	72.03	98.33
4Y15	A	SdiA in complex with 3-oxo-C6-homoserine lactone	EHEC	2.84	0.25	1.56e-108	72.03	1.04e-128	72.03	98.33
4Y17	A	SdiA in complex with 3-oxo-C8-homoserine lactone	EHEC	2.84	0.27	1.56e-108	72.03	1.04e-128	72.03	98.33
4LFU	A	Crystal structure of *Escherichia coli* SdiA in the space group C2	*E. coli*	2.26	0.28	1.62e-108	72.03	1.30e-128	72.03	98.33
4LGW	A	Crystal structure of *Escherichia coli* SdiA in the space group P6522	*E. coli*	2.70	0.27	1.35e-108	72.03	1.49e-128	72.03	98.33
2AVX	A	Solution structure of *E. coli* SdiA1-171	*E. coli*	Pending	Pending	3.48e-67	63.74	3.41e-78	63.74	71.25

3.5. Molecular docking of SdiA proteins of Salmonella Enteritidis and EHEC

The generated 4Y13-S structure of SdiA protein of *Salmonella* Enteritidis modeled from the SdiA protein of EHEC complexed with OCL, in the absence of AHL, showed the highest binding affinity to *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) with a score of −70.32 (Table 4). This molecule bound to Y63 and S134 residues of the protein and with glycerol (Fig. 6A). The molecules with greater affinity to this structure were AHLs with twelve carbons with or without 3-oxo modification, followed by AHLs with ten and eight carbons (Table 4). These data corroborate those found by Campos-Galvão et al. [19] that showed enhanced biofilm formation by *Salmonella* Enteritidis PT4 578 growing in the presence of 50 nM of AHLs with six, eight, ten and twelve carbons. However, the effect of *N*-dodecanoyl-DL-homoserine lactone (C12-HSL) on this phenotype was statistically higher than the other AHLs evaluated. Gnanendra et al. [30] showed that 3-oxo AHLs with six carbons had higher binding affinities to SdiA of *Salmonella* Typhimurium when compared AHLs with eight carbons without 3-oxo modification. This result might reflect the use of CviR structure by those authors when modeling SdiA, since CviR is more specific to AHLs with shorter carbon side chains, which would likely interfere in the modeled SdiA structure. We, however, have modeled *Salmonella* SdiA from a more conserved protein from EHEC which supposedly generates a more robust model. Moreover, Gnanendra et al. [30] showed that the R60, W67, Y71, D80 and W95 residues were conserved binding sites of the different AHLs unlike what we have observed in this study, where the Y63 residue was the most conserved (Table 4).

The brominated furanones with twelve, ten, eight, six and four carbons had higher scores in relation to all other furanones (Table 4). Furthermore, four out of five non-brominated furanones bound to W67 residue of 4Y13-S structure *Salmonella* Enteritidis PT4 578 (Table 4). Campos-Galvão et al. [19] showed that when a mixture of four non-brominated furanones was added concurrently with C12-HSL, biofilms by *Salmonella* Enteritidis PT4 578 were not observed. Thus, the results of the molecular docking showed that three of these four furanones used by Campos-Galvão et al. [19] bound in the modeled 4Y13-S structure of SdiA protein of *Salmonella* Enteritidis. This result indicates a competition between the AHL and furanones for the W67 residue which is a common binding site for these molecules, despite furanones havin lower score than C12-HSL. Similar results were found by Yang et al. [27] and Husain et al. [41] in which the molecular docking of LasR of *Pseudomonas aeruginosa* presented free binding energy higher with 3-oxo-C12-HSL than with quorum sensing inhibiting compounds such as chlorzoxazone, ceftazidime, nifuroxazide, and salicylic acid.

Corroborating our previous results, the 4Y15-S and 4Y17-S structures of SdiA protein of *Salmonella* Enteritidis PT4 578 modeled from the SdiA protein of EHEC complexed with 3-oxo-C6-HSL and 3-oxo-C8-HSL showed a similar pattern of binding affinities to the molecules evaluated in comparison with 4Y13-S structure (Table 4). The modeled 4Y15-S and 4Y17-S *Salmonella* structures also showed higher binding affinities to AHL with twelve carbons, such as −85.00 between 4Y15-S structure and C12-HSL and −86.30 between 4Y17-S structure and 3-oxo-C12-HSL (Table 4). The molecules with the second highest score affinity to these structures were brominated furanones with twelve carbons, followed by those AHL with ten, eight and six carbons and finally OCL (Table 4). In addition, the W67 and Y71 residues were the most common binding sites for brominated furanones in these structures (Table 4). Thus, the conformational changes of 4Y15-S and 4Y17-S structures of *Salmonella* Enteritidis resulting from the binding with AHLs resulted in structures with higher predicted affinities to brominated furanones. This is likely due to optimization of the binding site in the presence of AHL which forms a tight pocket in these structures as compared with that model with OCL which would show an open chamber formation in the absence of AHL [16] and possibly not perfectly accommodating the bulky inhibitor. The Y63, W67, Y71, D80 and S134 residues were common binding sites when the five best scores of each molecule resulting from molecular docking with the three structures of SdiA protein of *Salmonella* Enteritidis were compared (Table 4). The only difference was the presence of glycerol in the 4Y13-S structure as a binding site, which may bind at Q72 residue. Gnanendra et al. [30] also showed by molecular docking that the R60, W67, Y71, D80, V82, L83, W95 and V119 residues of modeled structure of SdiA protein of *Salmonella* Typhimurium were binding sites of different AHLs. Thus, the W67, Y71 and D80 residues are common binding sites between that and

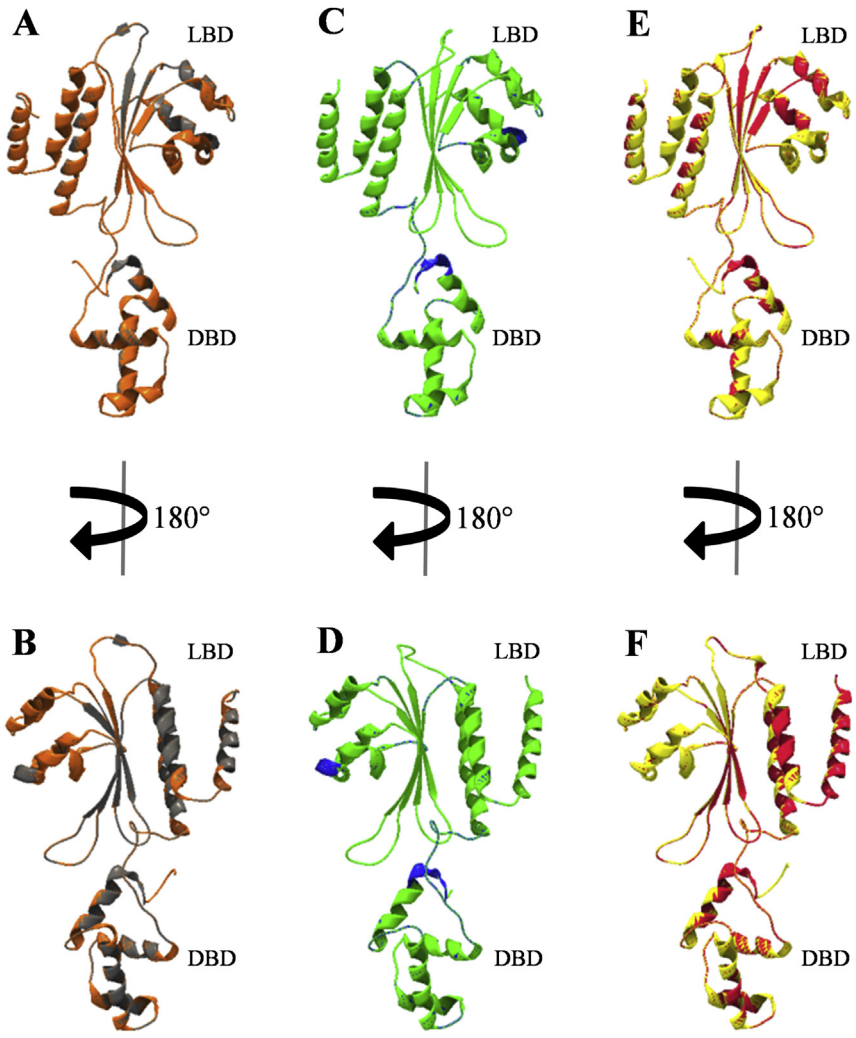

Fig. 2. Superposition of the generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis PT4 578 with their respective templates of EHEC. The structures 4Y13-S of *Salmonella* Enteritidis (gray) and 4Y13 of EHEC (orange) (**A** and **B**), the structures 4Y15-S of *Salmonella* Enteritidis (blue) and 4Y15 of EHEC (green) (**C** and **D**), and the structures 4Y17-S of *Salmonella* Enteritidis (pink) and 4Y17 of EHEC (yellow) (**E** and **F**). Ligand-binding domain (LBD); DNA-binding domain (DBD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Validation of the generated macromolecular structures of SdiA proteins and their templates of EHEC.

Organism	Structure [a]	Chain	Ramachandran plot analysis			Verify3D (%) [d]	ERRAT (%) [e]
			Residues in the region (%)				
			Favored [b]	Allowed [c]	Outlier		
Salmonella Enteritidis PT4 578	4Y13-S	A	96.2	3.8	0.0	89.17	92.672
Salmonella Enteritidis PT4 578	4Y15-S	C	97.7	2.1	0.0	93.31	87.013
Salmonella Enteritidis PT4 578	4Y17-S	C	97.0	3.0	0.0	92.89	91.775
EHEC	4Y13	A	95.9	4.1	0.0	85.78	95.413
EHEC	4Y15	C	98.3	1.7	0.0	89.58	91.379
EHEC	4Y17	C	95.9	3.7	0.4	91.36	91.845

[a] The structures of *Salmonella* Enteritidis PT4 578 modeled from EHEC were identified with their template code of PDB plus the letter "S".
[b] Approximately 98.0% expected.
[c] Approximately 2.0% expected.
[d] At least 80% of the amino acid residues with score $\geq$ 0.2 indicating compatibility between an atomic model (3D) with its own amino acid sequence (1D) [52].
[e] Expressed as the percentage of the protein for which the calculated error value falls below the 95% rejection limit. Good high resolution structures generally produce values around 95% or higher. For lower resolutions (2.5 to 3 Å) the average overall quality factor is around 91% [53].

the present study. These residues would present great targets for site directed mutagenesis studies.

The 4Y13, 4Y15 and 4Y17 structures of SdiA proteins of EHEC used as templates in this study showed the highest binding affinity to 3-oxo-C12-HSL with scores of −75.66, −82.30 and −86.16, respectively (Table 5). This molecule bound to S43 and S134

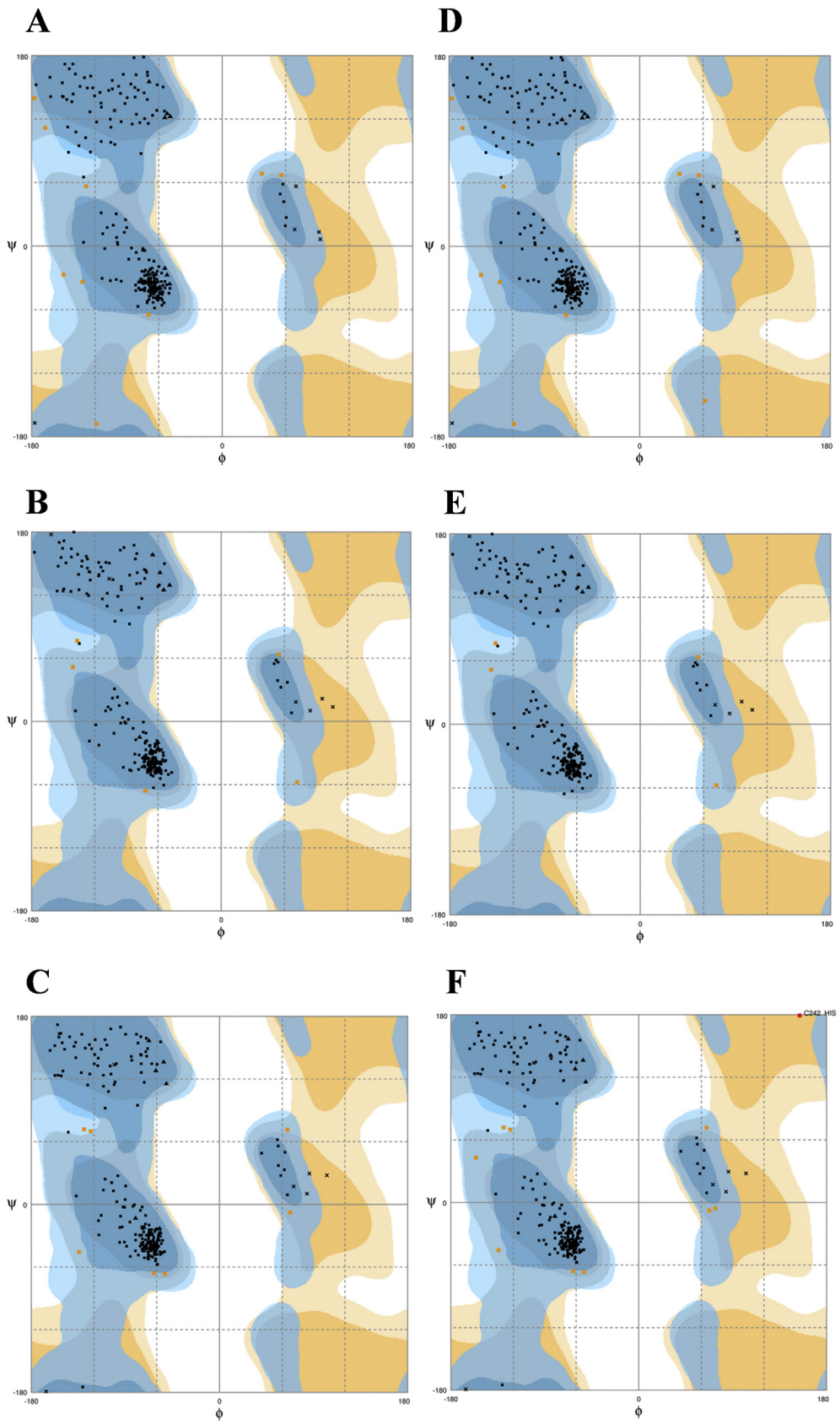

Fig. 3. Ramachandran Plot Analysis of the generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis PT4 578: A chain of 4Y13-S structure **(A)**, C chain of 4Y15-S structure **(B)**, C chain of 4Y17-S structure **(C)** and their templates of EHEC: A chain of 4Y13 structure **(D)**, C chain of 4Y15 structure **(E)**, C chain of 4Y17 structure **(F)**. Black square with dark blue background, general amino acid in favored region; black triangle with dark blue background, pre and pro-proline in favored region; black cross with dark pink background, glycine in favored region; orange square with light blue background, general amino acid in allowed region; orange triangle with light blue background, pre and pro-proline in allowed region; orange cross with light pink background, glycine in allowed region; Red square or triangle or cross, general amino acid or pre and pro-proline or glycine in outlier region, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

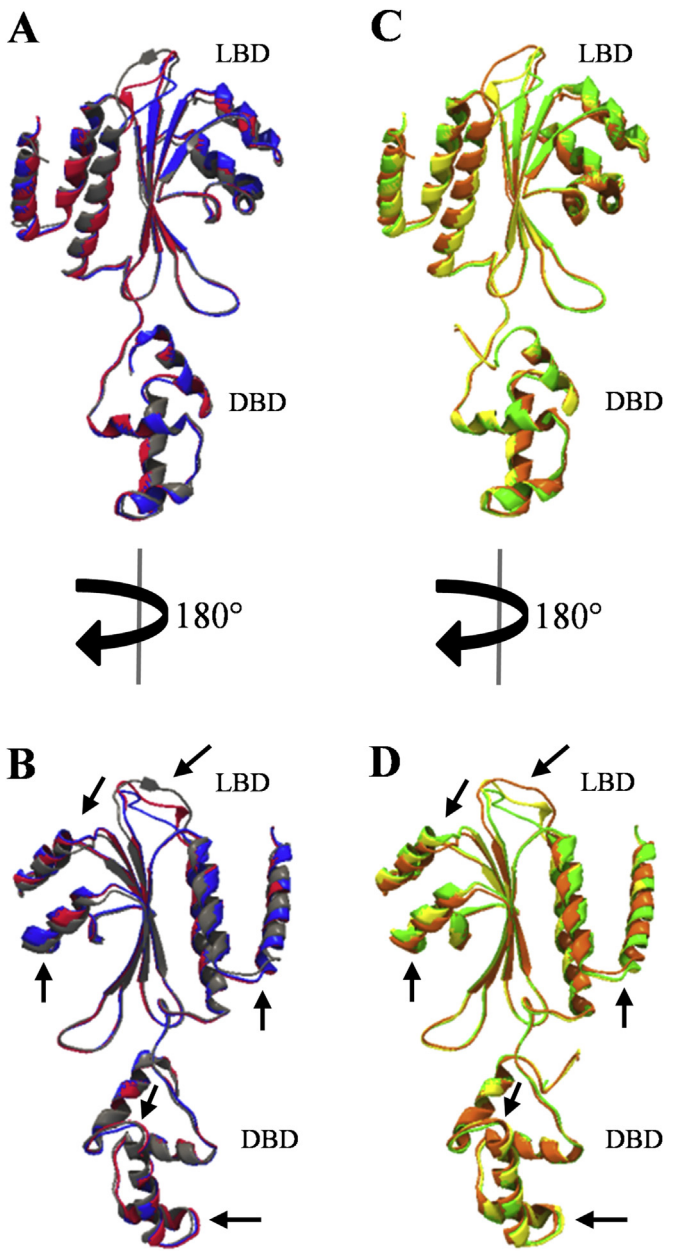

Fig. 4. Superposition of all the generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis PT4 578 and their templates of EHEC separately. The 4Y13-S (gray), 4Y15-S (blue) and 4Y17-S (pink) structures of *Salmonella* (**A** and **B**), and 4Y13 (orange), 4Y15 (green) and 4Y17 (yellow) structure of EHEC (**C** and **D**). Ligand-binding domain (LBD); DNA-binding domain (DBD); Black arrows show conformational changes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

residues of the protein and with glycerol bounded at Q72 residue (Fig. 6B). Moreover, the S43, Y63, W67, D80 and S134 residues of 4Y15 and 4Y17 structures were common binding sites for 3-oxo-C12-HSL (Table 5). In addition, the binding affinity of the AHLs by 4Y13 structure of SdiA protein of EHEC was similar to 4Y13-S structure of SdiA protein of *Salmonella* Enteritidis (Table 5). Differently, Kim et al. [61] showed that the binding affinity of C8-HSL to SdiA of *E. coli* is stronger than that of C4-HSL and C10-HSL. Furthermore, only one brominated furanone and another non-brominated furanone were not predicted to bind to EHEC SdiA (Table 5).

The molecules with greater binding affinities to 4Y15 and 4Y17 structures of EHEC SdiA were AHLs with twelve and ten carbons with or without 3-oxo modification as well as brominated furanones with twelve and ten carbons, following by same molecules with eight carbons and OCL (Table 5). Moreover, the conformational changes of 4Y15 and 4Y17 structures of EHEC resulting from the binding with AHLs also increased the affinity to brominated furanones as observed in *Salmonella* Enteritidis predicted structures. The S43, Y63, W67, Y71, D80 and S134 residues were common binding sites when comparing the five best scores of each molecule resulting from the molecular docking with the three structures of SdiA protein of EHEC (Table 5). Yao et al. [62] showed that the Y63, W67, Y71, D80 and W95 residues of SdiA protein of the *E. coli* were binding sites for C8-HSL. Moreover, Nguyen et al. [16] also showed that the S43, F59, T61, Y63, W67, Y71, L77, D80, W107 and S134 residues of SdiA protein of the EHEC were binding sites for different AHLs and OCL, with Y63 being the most specific and presenting the highest affinity. Thus, all binding residues found in this study corroborate with those described Yao et al. [62] and Nguyen et al. [16]. In addition, we show that AHLs with longer acyl chains may also be good activators of SdiA in EHEC, and to our knowledge, this has not been tested. Interestingly, AHLs with longer chain are more stable in slightly alkaline environment such as that found in the gut where these microorganisms cause infection [63]. It would be interesting to test whether EHEC SdiA responds to C12-HSL or 3-oxo-C12-HSL as *Salmonella* does.

In general, the comparison of molecular docking results of different structures of SdiA protein of *Salmonella* Enteritidis and EHEC with the results of Gnanendra et al. [30], Yao et al. [62] and Nguyen et al. [16] showed that the W67, Y71 and D80 residues are common binding sites for different quorum sensing and quorum quenching molecules. In addition, the Y63 and S134 residues are common binding sites for SdiA proteins as described by Nguyen et al. [16], as well as the Y63 residue for Yao et al. [62]. Furthermore, these residues are conserved among many LuxR type proteins [6,16,30,50,61].

3.6. Site directed mutagenesis of predicted binding sites of the SdiA

All SdiA residues of *Salmonella* Enteritidis and EHEC found as binding sites for OCL, AHLs and furanones were point mutated to leucine residues. Then, new molecular docking for each mutant protein was performed and we finally showed that the signaling molecules did not bind to the proteins with mutated residues. These results confirm that the Y63, W67, Y71, Q72, D80 and S134 residues of the SdiA protein of *Salmonella* Enteritidis are binding sites for quorum sensing and quenching molecules (Fig. 7A and B). In EHEC, the S43, Y63, W67, Y71, Q72, D80, R111, F132 and S134 residues were also confirmed as binding sites in this mutagenic approach (Fig. 7C and D).

4. Conclusion

We have shown that the available structures of SdiA protein of EHEC provided good prototypes for modeling SdiA from *Salmonella* Enteritidis PT4 578. The molecular docking of these proteins showed that the Y63, W67, Y71, D80 and S134 residues are common binding sites for different quorum sensing and quorum quenching molecules and these residues are conserved among LuxR type proteins. In addition, the S43 residue of EHEC SdiA is a common binding site, but this residue is not conserved. The AHLs with twelve carbons have better binding affinities to SdiA, regardless of the evaluated structure. The models generated from SdiA bound to AHLs presented increased affinity to brominated furanones, indicating strong possibility for competition for binding to SdiA among

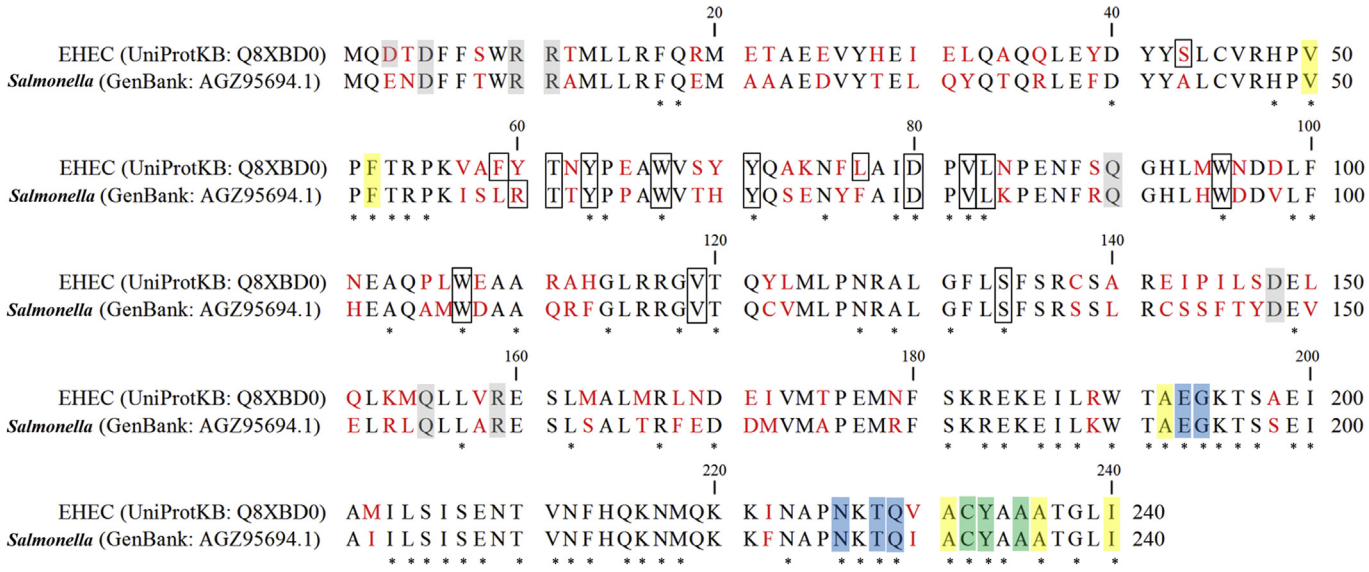

Fig. 5. Alignment of the amino acid sequences of SdiA protein of *Salmonella* Enteritidis PT4 578 (GenBank: AGZ95694.1) and EHEC (UniProtKB: Q8XBD0). Black letter, conserved amino acid residue; Red letter, non-conserved amino acid residue; asterisk, conserved amino acid residue between LuxR family protein described by Ahmer et al. [6], Gnanendra et al. [30], Kim et al. [61], Nguyen et al. [16] and Campos-Galvão et al. [50]; Gray background, amino acid residues involved in the interaction between two LBD described by Nguyen et al. [16]; Blue background, amino acid residue involved in the interaction between two DBD described by Nguyen et al. [16]; Yellow background, amino acid residue involved in the interaction between LBD-DBD described by Nguyen et al. [16]; Green background, amino acid residue involved in the interaction between DBD-DBD and LBD-DBD described by Nguyen et al. [16]; Boxed residue, amino acid residue involved in the interaction between LBD-ligand described by Gnanendra et al. [30], Yao et al. [62] and Nguyen et al. [16]. Ligand-binding domain (LBD); DNA-binding domain (DBD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Results from molecular docking of generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis PT4 578 with quorum sensing and quorum quenching molecules.

Molecule	Generated macromolecular structures of SdiA protein of *Salmonella* Enteritidis								
	4Y13-S			4Y15-S			4Y17-S		
	Binding residue	Score	Rank	Binding residue	Score	Rank	Binding residue	Score	Rank
***N*-(3-oxododecanoyl)-L-homoserine lactone**	**Y63, S134, Glycerol**	**−70.32**	**1**	Y71, S134	−83.28	3	**Y63, W67, D80, S134**	**−86.30**	**1**
***N*-dodecanoyl-DL-homoserine lactone**	Y63, W67	−69.67	2	**Y63, W67, S134**	**−85.00**	**1**	Y63, W67, D80	−83.89	3
N-decanoyl-DL-homoserine lactone	Y71	−69.29	3	Y63, W67, S134	−80.68	4	W67, S134	−80.05	4
N-(3-oxodecanoyl)-L-homoserine lactone	Y63, W67, D80, S134	−66.76	4	Y63, W67, S134	−79.21	5	W67, S134	−78.85	5
N-(3-oxooctanoyl)-L-homoserine lactone	Y63	−65.65	5	Y63, W67, Y71, D80, S134	−72.13	7	Y63, W67, Y71, D80	−72.03	7
N-octanoyl-DL-homoserine lactone	Y63	−64.26	6	Y63, W67, D80, S134	−71.34	8	Y63, W67, D80	−70.89	8
1-octanoyl-*rac*-glycerol	W67, D80, Glycerol	−60.82	7	Y63, D80, S134	−62.16	11	Y63, D80	−62.94	11
4-bromo-5-(bromomethylene)-3-decyl-2(5H)-furanone	Y71	−60.55	8	Y71	−76.82	6	Y71	−74.48	6
4-bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone	W67	−60.50	9	W67	−64.85	10	W67	−63.59	10
4-bromo-5-(bromomethylene)-3-octyl-2(5H)-furanone	S134	−59.40	10	Y71	−66.33	9	Y71	−68.29	9
3-butyl-2(5H)-furanone	W67	−57.55	11	–	–	–	–	–	–
N-(3-oxohexanoyl)-L-homoserine lactone	Y63	−57.39	12	Y63, W67	−60.66	12	Y63, W67	−60.71	12
N-hexanoyl-DL-homoserine lactone	Y63, W67	−56.38	13	Y63, W67	−60.42	13	Y63, W67, D80	−60.50	13
4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone	Glycerol (Q72)	−55.52	14	Y71	−83.51	2	Y71	−84.02	2
4-bromo-3-butyl-5-(dibromomethylene)furan-2(5H)-one	W67	−53.62	15	W67	−58.39	14	W67	−57.69	14
4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	W67	−52.17	16	Y71	−53.49	15	W67	−54.61	15
N-butyryl-DL-homoserine lactone	Y63	−45.43	17	–	–	–	–	–	–
5-bromomethylene-2(5H)-furanone	W67	−35.45	18	–	–	–	–	–	–
3-methyl-2(5H)-furanone	W67	−35.14	19	–	–	–	–	–	–
2(5H)-furanone	W67	−31.41	20	–	–	–	–	–	–
2-methyl tetrahydro-3-furanone	W67	−30.14	21	–	–	–	–	–	–
4-bromo-5-(bromomethylene)-2(5H)-furanone	–	–	–	Y71	−40.87	16	Y71	−42.32	16
4-bromo-5-(bromomethylene)-3-ethyl-2(5H)-furanone	–	–	–	–	–	–	–	–	–
5-dibromomethylene-2(5H)-furanone	–	–	–	–	–	–	–	–	–
3-ethyl-5-(dibromomethylene)furan-2(5H)-one	–	–	–	–	–	–	–	–	–
2,2-dimethyl-3(2H)-furanone	–	–	–	–	–	–	–	–	–
All binding residues of the five best score	Y63, W67, Y71, D80, S134, Glycerol (Q72), Glycerol			Y63, W67, Y71, D80, S134			Y63, W67, Y71, D80, S134		

No binding (–). The best result of all in bold.

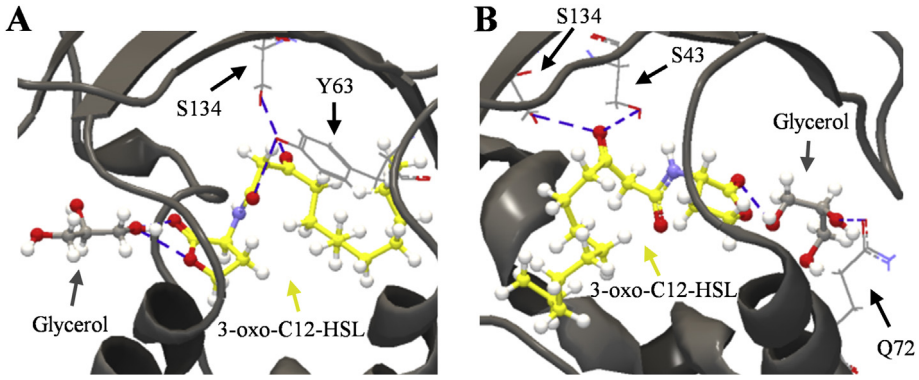

Fig. 6. Representative inspection of the binding sites the 4Y13-S structure of SdiA protein of *Salmonella* Enteritidis PT4 578 **(A)** and the 4Y13 structure of EHEC **(B)** with 3-oxo-C12-HSL and glycerol. Black arrow, the binding sistes; Gray arrow, glycerol; Yellow arrow, 3-oxo-C12-HSL; Blue dashed line, hydrogen bond. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Results from molecular docking of macromolecular structures of SdiA protein of EHEC with quorum sensing and quenching molecules.

Molecule	Macromolecular structures of SdiA protein of EHEC								
	4Y13			4Y15			4Y17		
	Binding residue	Score	Rank	Binding residue	Score	Rank	Binding residue	Score	Rank
***N*-(3-oxododecanoyl)-L-homoserine lactone**	**S43, S134, Glycerol (Q72)**	**−75.66**	**1**	**S43, Y63, W67, D80, S134**	**−82.30**	**1**	**S43, Y63, W67, D80, S134**	**−86.16**	**1**
N-(3-oxodecanoyl)-L-homoserine lactone	S43, S134, Glycerol (Q72)	−72.78	2	S43, Y63, W67, D80, S134	−77.65	4	S43, Y63, W67, D80	−80.46	3
N-dodecanoyl-DL-homoserine lactone	Y63, D80, Glycerol (R111)	−72.17	3	Y63, W67, D80	−81.88	2	Y63, W67, D80	−85.53	2
N-decanoyl-DL-homoserine lactone	Y71	−72.13	4	Y63, W67, D80, S134	−78.04	3	Y63, W67, D80	−79.25	4
N-(3-oxooctanoyl)-L-homoserine lactone	S43, Y63	−67.96	5	S43, Y63, W67, D80	−70.13	7	S43, Y63, W67	−71.22	7
N-octanoyl-DL-homoserine lactone	Y63	−67.12	6	Y63, W67, S134	−68.68	8	Y63	−69.04	9
1-octanoyl-*rac*-glycerol	W67, D80, Glycerol (R111)	−63.20	7	W67, Y71, D80	−60.52	11	Y71, D80	−61.86	11
4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone	S43	−62.19	8	W67	−72.98	5	W67	−78.97	5
4-bromo-5-(bromomethylene)-3-octyl-2(5H)-furanone	W67	−60.84	9	W67	−65.90	9	S43	−71.19	8
4-bromo-5-(bromomethylene)-3-decyl-2(5H)-furanone	W67	−60.68	10	S43	−72.58	6	S43	−78.75	6
4-bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone	W67	−60.52	11	W67	−60.54	10	S43	−59.95	12
N-(3-oxohexanoyl)-L-homoserine lactone	Y63, W67, Glycerol (Q72)	−60.24	12	S43, Y63, W67	−60.28	12	S43, Y63, W67	−61.96	10
N-hexanoyl-DL-homoserine lactone	Glycerol (Q72)	−58.12	13	Y63, W67	−58.46	13	Y63, W67	−59.87	13
4-bromo-3-butyl-5-(dibromomethylene)furan-2(5H)-one	W67	−57.01	14	–	–	–	–	–	–
4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	W67	−56.17	15	–	–	–	–	–	–
3-butyl-2(5H)-furanone	W67	−49.75	16	–	–	–	Q72	−52.42	14
N-butyryl-DL-homoserine lactone	S43	−48.33	17	S43, Y71	−45.81	14	–	–	–
3-methyl-2(5H)-furanone	Glycerol (Q72)	−38.01	18	–	–	–	–	–	–
5-dibromomethylene-2(5H)-furanone	W67	−37.99	19	S43	−40.41	17	S43	−40.02	18
2,2-dimethyl-3(2H)-furanone	Glycerol (Q72)	−37.32	20	–	–	–	S43	−33.27	20
4-bromo-5-(bromomethylene)-2(5H)-furanone	Glycerol (Q72)	−36.64	21	S43	−40.52	16	S43	−41.03	17
5-bromomethylene-2(5H)-furanone	W67	−34.62	22	S43	−34.30	18	Q72	−36.60	19
2(5H)-furanone	Glycerol (Q72)	−33.43	23	–	–	–	Q72	−32.02	21
2-methyl tetrahydro-3-furanone	Glycerol (Q72)	−32.61	24	S43	−29.58	19	–	–	–
4-bromo-5-(bromomethylene)-3-ethyl-2(5H)-furanone	–	–	–	S43	−45.04	15	S43	−48.77	15
3-ethyl-5-(dibromomethylene)furan-2(5H)-one	–	–	–	–	–	–	S43	−47.99	16
All binding residues of the five best score	S43, Y63, W67, Y71, Q72, D80, S134, Glycerol (Q72 and R111)			S43, Y63, W67, Y71, Q72, D80, F132, S134			S43, Y63, W67, Y71, D80, S134		

No binding (–). The best result of all in bold.

these molecules. Therefore, brominated furanones may be used to inhibit phenotypes controlled by quorum sensing in *Salmonella* and EHEC whenever external AHLs are present, since these inhibitors may compete with AHLs for binding to SdiA. The modeled protein from *Salmonella* as well as the approach here described may be used to test different types of quorum sensing inhibitors and guide future laboratory works.

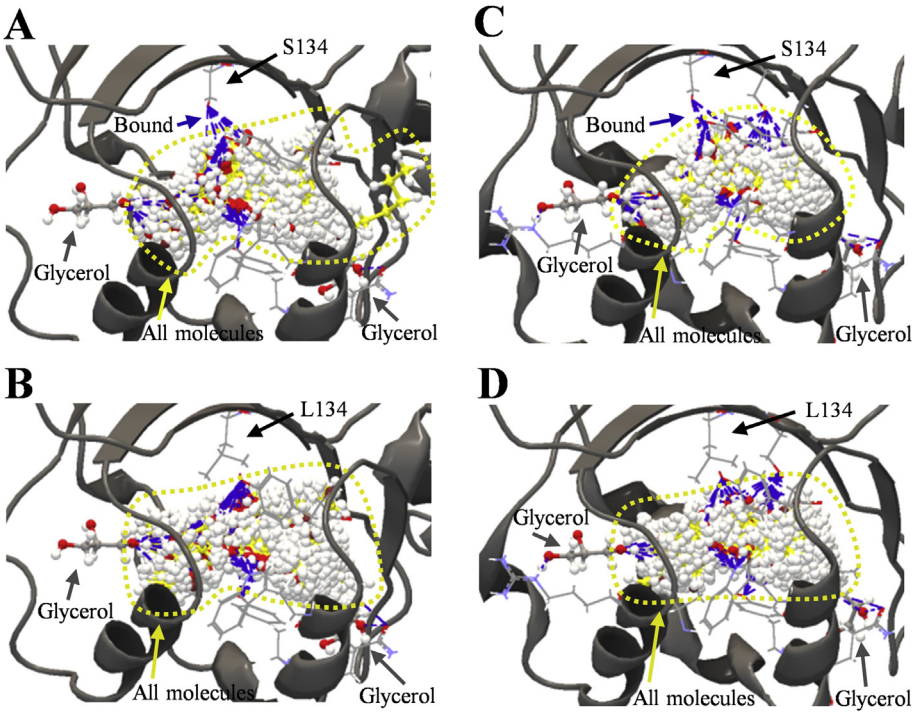

Fig. 7. Representative confirmation of the S134 residue of the 4Y13-S structure of SdiA proteins of *Salmonella* Enteritidis PT4 578 **(A** and **B)** and the 4Y13 structure of EHEC **(C** and **D)** as binding site of different molecules by *in silico* leucine scanning mutagenesis followed by new molecular docking. Black arrow, S134 or L134 residue; Gray arrow, glycerol; Yellow arrow and yellow dashed line, quorum sensing and quorum quenching molecules; Blue arrow, hydrogen bond between S134 residue and evaluated molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgements

Felipe Alves de Almeida was supported by a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and this research has been supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors acknowledge the CLC bio of the QIAGEN Company by license of the CLC Drug Discovery Workbench 2.5 software. UMP acknowledges the support from CNPq process number (457794/2014-3) for his research.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2016.08.024.

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