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(54) Title: MARKER OF PATHOGENICITY IN SALMONELLA

(57) Abstract: The invention relates to marker(s) of pathogenicity in *Salmonella* and the uses thereof.

MARKER OF PATHOGENICITY IN SALMONELLA

FIELD OF THE INVENTION:

The present invention relates to marker(s) of pathogenicity in *Salmonella* and the uses thereof.

BACKGROUND OF THE INVENTION:

Salmonellae are Gram-negative bacterial pathogens that are capable of infecting a wide range of animals.

The bacterial genus *Salmonella* consists of two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella* are common causes of food poisoning in humans and can also cause more severe disease such as typhoid fever. Most of the *Salmonella* that cause disease in humans and animals are members of *S. enterica*. On the other hand, *S. bongori* is largely associated with reptiles but can cause disease in humans, albeit rarely.

The *Salmonella* genus comprises only two species: *bongori* and *enterica*. Whereas all *Salmonellae* are able to invade intestinal epithelium, *Salmonella bongori* is unable to survive and replicate intracellularly.

On contrary, *Salmonella enterica* is able to spread from the intestinal tissue via the lymphatics into the bloodstream and to multiply within the macrophages of the liver and spleen.

A critical difference between the two Salmonella species is the acquisition by *Salmonella enterica* of additional virulence genes.

Thus, the acquisition of *Salmonella* pathogenicity island 2 (SPI 2) has been shown as an important step during colonization of this intracellular niche.

The host specificities and the disease symptoms are caused by some of the thousands of *Salmonella* subspecies (Haraga et al., 2008).

So there is still a need for identifying new pathogenicity markers and in particular pathogenicity markers that allow differentiation between different *Enterica* subspecies.

Indeed, while *Salmonella enterica* subspecies (group I) that infect warm-blooded mammals are known to be responsible for 99% of mammalian infections (Baumler et al., 1997), *Salmonella enterica* subspecies arizonae (group IIIb) are isolated from reptiles and are rarely associated with human infection.

So, the identification of new pathogenicity markers that is to say markers that are associated with pathogenicity or pathways in *Salmonella* will lead to a new method for detecting pathogenic strains of Salmonella.

Methods based on such pathogenicity markers will allow discriminating pathogenic strains of *Salmonella* from apathogenic strains.

Further, these markers will also give lead to develop new drugs capable of impacting the viability of *Salmonella* but not of other bacteria present for instance in the gut flora.

Lastly, these markers will also allow designing a new model for bacterial pathogenesis.

SUMMARY OF THE INVENTION:

Now, the Applicant has found new markers that are associated with pathogenicity of *Salmonella*.

More precisely, the inventors have discovered that theses markers of pathogenicity of *Salmonella* are from two toxin-antitoxin systems.

Therefore, a subject of the present invention relates to a toxin:

-having the amino acid sequence set forth as SEQ ID NO: 5 or SEQ ID NO: 7 or

-a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 5 or SEQ ID NO: 7.

A subject of the present invention also relates to the antitoxin cognate to the toxin of the invention in the toxin-antitoxin system.

Therefore, the present invention also relates to an antitoxin:

-having the amino acid sequence set forth as SEQ ID NO: 6 or SEQ ID NO: 8 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 6 or SEQ ID NO: 8.

Toxin-antitoxin systems are known in many biotechnogical and medical applications (Gerdes et al.,2005).

First, toxin-antitoxin systems are used in stabilization or selection systems.

Therefore, the present invention also relates to a cassette of expression comprising a nucleic acid molecule encoding a toxin according to claim 1 operatively linked to a promoter.

Toxin-antitoxin system may also provide useful targets for the development of antibacterial drug.

Thus, the present invention relates to the toxin of the invention for use in the treatment of an *Enterobacteriaceae* infection.

The present invention also relates to an inhibitor of the antitoxin of the invention and it use in the treatment of *Salmonella* infection.

Due to their relation with the pathogenicity of *Salmonella*, these toxin-antitoxin systems may also be used to detect pathogenic strains of *Salmonella* in warm blood animals including humans.

Therefore, the present invention also relates to a method for detecting the presence of a pathogenic strain of *Salmonella* in a sample comprising the step of detecting the presence of a toxin of the invention or an antitoxin of the invention and it related kit.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Toxin-antitoxin (T/AT) systems, also called herein toxin-antitoxin module, are derived from genes located in the bacterial chromosome (as described in the invention) and in bacterial plasmids involved in plasmid stability. This phenomenon called addiction was described when a daughter cell lacks its plasmid. The fast degradation of the antitoxin leads to the release of the toxin, which causes bacterial growth inhibition (Hayes, 2003). T/AT systems form bicistronic operons, where generally, the first and second genes encode for an antitoxin and a toxin, respectively (Gerdes et al., 2005). Three types of T/AT systems have been described based on the protein or RNA nature

of the antitoxin (Blower et al., 2010; Bukowski et al., 2011; Yamaguchi and Inouye, 2011). These toxins might be interferases or ribonucleases that stop the translation by mRNA cleavage in the presence (Li et al., 2009; Zhang et al., 2009) or absence of ribosomes (Kamada and Hanaoka, 2005; Christensen-Dalsgaard and Gerdes 2008; Prysak et al., 2009), respectively.

In contrast to type I and type III systems, type II antitoxins are proteins that interact with their toxin counterpart to neutralize their toxic effects and that act as a transcriptional repressor of their own expression (Gerdes et al., 2005). A database is dedicated to Type 2 toxin–antitoxin systems (TADB: http://bioinfo-mml.sjtu.edu.cn/TADB/).

Studies about the link between some identified toxin-antitoxin systems and the pathogenicity of their host have been carried out. According to these studies, a combination of factors determines whether a given TA system is a good target. Then, nothing can be concluded on a given TA system without studying prevalence and functionality within clinical isolates (William J. et al., 2012).

Toxin and antitoxin

Protein of the invention:

The present invention relates to a toxin:

-having the amino acid sequence set forth as SEQ ID NO: 5 or SEQ ID NO: 7 or

-a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 5 or SEQ ID NO: 7.

The present invention also relates to an antitoxin:

-having the amino acid sequence set forth as SEQ ID NO: 6 or SEQ ID NO: 8 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 6 or SEQ ID NO: 8.

Indeed, the inventors have identified two new toxin/antitoxin systems, called hereinafter respectively SehT1-SehA1 and SehT2-SehA2 in *Salmonella* and have isolated the nucleic acid molecules encoding each toxin and antitoxin of these systems. Interestingly, the inventors have further found that SehT1-SehA1 and SehT2-SehA2, the two new toxin-antitoxin systems, are both homologous to the host inhibition of growth (higBA) toxin/antitoxin system of *E. coli* and *Vibrio cholerae* (Christensen-Dalsgaard and Gerdes, 2006; Christensen-Dalsgaard et al., 2010) and present a atypical organization in the chromosome barely found in other bacteria so far: the toxin was upstream the antitoxin gene in the operon.

Strikingly, this DNA signature was found in all sequenced serotypes of *Salmonella enterica* and absent in *Salmonella bongori* indicating that these T/AT modules are related with pathogenicity.

The toxin/antitoxin system SehT1-SehA1 comprises a toxin called herein sehT1 and an antitoxin called herein sehA1.

The toxin/antitoxin system SehT2-SehA2 comprises a toxin called herein sehT2 and an antitoxin called herein sehA2.

The correspondence between theses toxins and antitoxins and their "SEQ ID NO" is summarized in the table below.

Table 1: Summary of SEQ ID NO

	Toxin/Antitoxin System 1 Seh1		Toxin/antitoxin System 2 Seh2	
	Toxin sehT1	Antitoxin sehA1	Toxin sehT2	Antitoxin sehA2
Nucleic acid sequence	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO:3	SEQ ID NO: 4
Amino acid sequence	SEQ ID NO: 5	SEQ ID NO: 6	SEQ ID NO:7	SEQ ID NO:8

As used herein, the percentage of sequence identity refers to comparisons among amino acid sequences, and is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the amino acid sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical amino acid residue occurs in both sequences or an amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and

Waterman, 1981, Adv. Appl. Math. 2:482, by the homology alignment algorithm of Needleman and Wunsch, 1970, J. MoI. Biol. 48:443, by the search for similarity method of Pearson and Lipman, 1988, Proc. Natl. Acad. ScL USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection (see generally, Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel)). Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., 1990, J. MoI. Biol. 215: 403-410 and Altschul et al., 1977, Nucleic Acids Res. 3389-3402, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as, the neighbourhood word score threshold (Altschul et al, supra). These initial neighbourhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTP program (for amino acid sequences) uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, 1989, Proc Natl Acad Sci USA 89: 10915). Exemplary determination of sequence alignment and %

sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

Typically, the toxin of the invention has the amino acid sequence set forth as SEQ ID NO: 5 or SEQ ID NO: 7 or an amino acid sequence that has at least 90%, 95%, 96%, 97%, 98%, or at least 99% identity with the amino acid sequence set forth as SEQ ID NO: 5 or SEQ ID NO: 7.

In the same way, typically, the antitoxin of the invention has the amino acid sequence set forth as SEQ ID NO: 6 or SEQ ID NO: 8 or an amino acid sequence that has at least 90%, 95%, 96%, 97%, 98%, or at least 99% identity with the amino acid sequence set forth as SEQ ID NO: 6 or SEQ ID NO: 8.

In a preferred embodiment, the toxin has the amino acid sequence set forth as SEQ ID NO: 5.

In another embodiment, the toxin has the amino acid sequence set forth as SEQ ID NO: 7.

In a preferred embodiment, the antitoxin has the amino acid sequence set forth as SEQ ID NO: 6.

In another embodiment, the antitoxin has the amino acid sequence set forth as SEQ ID NO: 8.

Nucleic acid molecule of the invention:

The present invention also relates to a nucleic acid molecule encoding a toxin of the invention.

Preferably, the nucleic acid molecule has the sequence set forth as SEQ ID NO: 1 or SEQ ID NO: 3.

More preferably, the nucleic acid molecule has the sequence set forth as SEQ ID NO: 1.

The present invention also relates to a nucleic acid molecule encoding an antitoxin of the invention.

Preferably, the nucleic acid molecule has the sequence set forth as SEQ ID NO: 2 or SEQ ID NO: 4.

More preferably, the nucleic acid molecule has the sequence set forth as SEQ ID NO: 2.

Cassette of expression and vectors of the invention:

Toxin-antitoxin systems may be used in plasmid stabilization or positive selection vectors.

Examples of such biotechnological application are disclosed in Haigermoser et al., 1993, Gerdes et al, 1988, Pecota et al., 1997.

Therefore, a further object of the invention relates to a cassette of expression comprising a nucleic acid molecule encoding a toxin of the invention operatively linked to a promoter.

This cassette of expression may be used as a selection system.

Indeed, by introducing unique restriction sites for cloning into toxin gene in a way that the gene still encodes the active toxin, the toxin gene is disrupted when DNA restriction fragment is introduced into the vector and thus the vector can be maintained in host cells that are sensitive to the toxin whereas the vector harbouring a functional toxin gene can not be maintained.

In other embodiment, the cassette of expression further comprises a nucleic acid molecule encoding an antitoxin of the invention operatively linked to a promoter.
In one embodiment, the cassette of expression comprises the operon set forth as SEQ ID NO: 9 or SEQ ID NO: 10.

The nucleic acid sequence of the operon sehT1A1 is set forth as SEQ ID NO: 9.

The nucleic acid sequence of the operon sehT2A2 is set forth as SEQ ID NO: 10.

In a preferred embodiment, the operon is set forth as SEQ ID NO: 9.

Further to be used as a selection system, the vector of the invention may also be used as stabilization vector.

Indeed, the toxin/antitoxin genes can efficiently stabilize vector. Toxin/antitoxin genes can be inserted into unstably inherited vectors, and thereby lead to an increased genetic stability of producer strains.

Therefore, another object of the invention relates to a vector comprising a cassette of expression of the invention.

The term "vector" means the vehicle by which a DNA or a RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence.

In a preferred embodiment, the vectors of the invention are plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989.

Such vectors may comprise regulatory elements, such as an enhancer, a terminator and the like.

In one embodiment, the cassette of expression encoding the toxin and the cassette of expression encoding the antitoxin may be comprised in different vectors, in a bacterial chromosome or one in a vector the other on a bacterial chromosome.

Table 2: Summary of sequences

	SEQ ID NO	Sequence
Nucleic acid sequence sehT1	1	GTGCATGTTATCAGCCGAAAACCGTTCAATG AAGCGATGCTCATGTATCCAAACCATGAGCT TGCTCTCACTGAGTTGCTGAATGTTCTGGAG AAAAAAACATTCACTCAGCCTGAAGAGATG AAGCGGTATATTCCTTCACTGGATAATTTTA AGTACAGAGATAAATGGTGGGTGATTGATGT TTCTGGTAACAGCCTGAGACTCATTTCCTAC ATAGATTTCAGGTTACATAAGATATTTGTGA AACATATCGTTTCCCATGCCGAATATGACAA ACTGACCGCATACTATCGGGGTAATAAAGAA TGA
Nucleic acid sequence sehA1	2	ATGGATGCAACCAGCGCAAAAAAGATCGTT GATACCTTCAGCGATGCGGTAAAAACCGTCC CACTGATGGGGGAAGACCGAAATGACAATG AGTATCGCAGGGCACTAGCGCTAGTGGAGTT TCTGGTCGACCACGACGATCTTGAAAACCCA

		CTATTTGAATTGCTCTGTGCCCGAATCAGTG AATACGAAAAACATGCGCCGGAATTCAAAG CACTCAACCAACACCTGGAGAAAACGCCCCC TGGCGTTTCAGTATTGCGAACGCTCATGGAT CAATACGGTCTCAAAGCAGCAGATCTTGCCA ACGAACTTGGTTCTAAATCGAACGTCAGCAA CATCTTAAATGGCCGCAGAGCACTAACGGTT AATCATATTAAAGCGCTTACACAACGCTTCA AACTACCAGCAGATGCCTTCATCGAGTAG
Nucleic acid sequence sehT2	3	ATGCAATTTATAGAAACGGAACTCTTTACTG AAGATGTTAAAAAACTGCTCGATGATGATGA ATACCATAAGCTTCAGGTTTTTATGGCTCAG CATCCAGATTGTGGTGATGTCATTCAGGAAA CGGGCGGCCTGAGAAAAATGCGCTGGGGAG CGCGAGGCAAAGGAAAGCGTAGTGGCGTGC GAATTATCTATTTTCACCGTAGTCAACGGTAT GAGATTCGCTTGCTTCTGATTTATCAAAAAG GCATTAAAGATGATCTCACGCCGCAGGAAAA AGCGGTGCTTCGTATGCTGAATGAGAGGTGG TAG
Nucleic acid sequence sehA2	4	ATGGATAAAGTGTTATTTGAGCGATTAACTC AAAGTATGTCTCAAATGAATGAAATCATTGA AGGGACCCGTGAACCTTCTCGTACCTTTCAT ATTGATGCAATGAAGATTAAAGAAATACGGC AGGCATCTGGGTTGTCGCAATCTAAGTTTGC AGAGCTGATTTCGGTCAACGTGGATACGCTG CGCAACTGGGAGCAAGGAAGACGTTCACCG ACAGGGCCAGCCAAAGCGTTACTTCGCGCCA TTGCCAACGACCCGAGAAACGTTATACAAGC ATTGCGTTATTGAG

Amino acid sequence sehT1	5	VHVISRKPFNEAMLMYPNHELALTELLNVLEK KTFTQPEEMKRYIPSLDNFKYRDKWWVIDVSG NSLRLISYIDFRLHKIFVKHIVSHAEYDKLTAYY RGNKE
Amino acid sequence sehA1	6	MDATSAKKIVDTFSDAVKTVPLMGEDRNDNE YRRALALVEFLVDHDDLENPLFELLCARISEYE KHAPEFKALNQHLEKTPPGVSVLRTLMDQYGL KAADLANELGSKSNVSNILNGRRALTVNHIKA LTQRFKLPADAFIE
Amino acid sequence sehT2	7	MQFIETELFTEDVKKLLDDDEYHKLQVFMAQH PDCGDVIQETGGLRKMRWGARGKGKRSGVRII YFHRSQRYEIRLLLIYQKGIKDDLTPQEKAVLR MLNERW
Amino acid sequence sehA2	8	MDKVLFERLTQSMSQMNEIIEGTREPSRTFHID AMKIKEIRQASGLSQSKFAELISVNVDTLRNWE QGRRSPTGPAKALLRAIANDPRNVIQALRY
sehT1A1 operon	9	AATTCAATTTTGTTTTCAACTTGAAAACTGGA TAACGTTGCGCTAAATTGTTTTCAGGTTGAA AACTTTGTAATGGAACTACATTGTGCATGTT ATCAGCCGAAAACCGTTCAATGAAGCGATGC TCATGTATCCAAACCATGAGCTTGCTCTCACT GAGTTGCTGAATGTTCTGGAGAAAAAAACAT TCACTCAGCCTGAAGAGATGAAGCGGTATAT TCCTTCACTGGATAATTTTAAGTACAGAGAT AAATGGTGGGTGATTGATGTTTCTGGTAACA GCCTGAGACTCATTTCCTACATAGATTTCAG GTTACATAAGATATTTGTGAAACATATCGTT TCCCATGCCGAATATGACAAACTGACCGCAT ACTATCGGGGTAATAAAGAATGAGAACTCAT CGTCAGATGGATGCAACCAGCGCAAAAAAG ATCGTTGATACCTTCAGCGATGCGGTAAAAA

		CCGTCCCACTGATGGGGGAAGACCGAAATG ACAATGAGTATCGCAGGGCACTAGCGCTAGT GGAGTTTCTGGTCGACCACGACGATCTTGAA AACCCACTATTTGAATTGCTCTGTGCCCGAA TCAGTGAATACGAAAAACATGCGCCGGAATT CAAAGCACTCAACCAACACCTGGAGAAAAC GCCCCCTGGCGTTTCAGTATTGCGAACGCTC ATGGATCAATACGGTCTCAAAGCAGCAGATC TTGCCAACGAACTTGGTTCTAAATCGAACGT CAGCAACATCTTAAATGGCCGCAGAGCACTA ACGGTTAATCATATTAAAGCGCTTACACAAC GCTTCAAACTACCAGCAGATGCCTTCATCGA GTAG
sehT2A2 operon	10	ATAACGCTCCTTTGACTGTATGTATGTACAGT TTATAACCTGGTTCGTTGGGGATGGAAAGTG GTGAAACGAGGGGATAATGATTCTGGATAG AGGTTCGCAAAAAATGGAATGCGGTTGGCGC TGGGTTTTTAATTTTTGTACTGTGAAACGATG ATGCTATACGCCATTGACGTATAGATTATTTT CACTACTATAAGCCAATGGCGTATGGAATAT GCAATTTATAGAAACGGAACTCTTTACTGAA GATGTTAAAAAACTGCTCGATGATGATGAAT ACCATAAGCTTCAGGTTTTTATGGCTCAGCA TCCAGATTGTGGTGATGTCATTCAGGAAACG GGCGGCCTGAGAAAAATGCGCTGGGGAGCG CGAGGCAAAGGAAAGCGTAGTGGCGTGCGA ATTATCTATTTTCACCGTAGTCAACGGTATGA GATTCGCTTGCTTCTGATTTATCAAAAAGGC ATTAAAGATGATCTCACGCCGCAGGAAAAA GCGGTGCTTCGTATGCTGAATGAGAGGTGGT

		AGATGGATAAAGTGTTATTTGAGCGATTAAC TCAAAGTATGTCTCAAATGAATGAAATCATT GAAGGGACCCGTGAACCTTCTCGTACCTTTC ATATTGATGCAATGAAGATTAAAGAAATACG GCAGGCATCTGGGTTGTCGCAATCTAAGTTT GCAGAGCTGATTTCGGTCAACGTGGATACGC TGCGCAACTGGGAGCAAGGAAGACGTTCAC CGACAGGGCCAGCCAAAGCGTTACTTCGCGC CATTGCCAACGACCCGAGAAACGTTATACAA GCATTGCGTTATTGAGCAA

Cells of the invention:

The present invention also relates to a host cell transformed by a nucleic acid molecule of the invention, a cassette of expression of the invention and/or the vector of the invention.

The term "transformation" means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence into a host cell, so that the host cell will express the introduced gene or sequence. A host cell that receives and expresses introduced DNA or RNA has been "transformed".

The host cell is preferably a recombinant bacterium, more preferably a recombinant Gram-negative bacterium and most preferably a recombinant *Salmonella*.

Inhibitor of antitoxin:

The present invention also relates to an inhibitor of the antitoxin of the invention.

The expression "**inhibitor of antitoxin**" should be understood broadly, it encompasses inhibitors of the antitoxin activity, and inhibitors of the expression of antitoxin.

An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit or significantly reduce the expression of a gene. Consequently an "inhibitor of antitoxin expression" refers to a natural or synthetic compound that has a biological effect to inhibit or significantly reduce the expression of the gene encoding for the antitoxin.

In "Artificial activation of toxin-antitoxin systems as an antibacterial strategy" (2012), Williams et al., disclose different ways to exploit the toxin-antitoxins systems to kill bacteria. It is shown that a way to develop an antibacterial drug is to relieve antitoxin inhibition of the toxin. This can notably be achieved by disruption of the toxin-antitoxin complex or the prevention of complex formation.

In one embodiment the antitoxin inhibitor is a compound that can partially or completely blocks the ability of antitoxin to inhibit it cognate toxin.

Examples of compounds that inhibit toxin-antitoxin interaction are given in Agarwal et al. (2010) and Chopra et al. (2011).

Agarwal et al. have shown how to design peptides able to perturb PemK-PemI interaction and that lead to bacterial cell death.

These peptides were notably 7-8 residues of PemI determined to be in the region necessary for the toxin binding.

Chopra et al. (2011), thank to knowledge guided protein-protein docking, molecular dynamics simulation, and energy minimization, have obtained the structure of the toxin-antitoxin MoxXT complex. These studies have been exploited for the de novo

design of a peptide capable of binding to MoxT. Chopra et al. found that the designed peptide caused a decrease in MoxX binding to MoxT

In one embodiment, the inhibitor of the antitoxin inhibits the formation of the complex toxin-antitoxin.

In another embodiment, the inhibitor of the antitoxin disrupts the toxin-antitoxin complex.

Typically, the inhibitor of the antitoxin may be a fragment of the antitoxin located in the toxin-antitoxin interface.

Preferably, the inhibitor is a fragment of 5 to 10 amino acids of the antitoxin.

As disclosed in William et al. 2012, the inhibitor of antitoxin may also play a role by modulating the expression of the toxin-antitoxin complex, for example at the promoter level.

Therefore, another aspect of the invention relates to an inhibitor of antitoxin expression.

The antitoxin is quickly degraded by cellular proteases. Therefore, a molecule that binds promoter DNA and inhibits transcription at the TA locus would prevent replenishment of the antitoxin. The non replenishment of the antitoxin would lead to the liberation of the latent toxin and to the killing of the cell.

The antitoxin of the invention negatively regulates it own expression.

Thus, the compound having homology with antitoxin that keeps the ability to bind to the promoter of the antitoxin promoter region with having an antitoxin activity may be efficient inhibitors of the antitoxin.

The inhibitors of antitoxin expression for use in the present invention may also be based on anti-sense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of antitoxin mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of antitoxin. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding antitoxin can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of antitoxin expression for use in the present invention. Antitoxin expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that antitoxin expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

In one embodiment, the inhibitor is a compound that prevents the antitoxin to repress the expression of the toxin.

In a preferred embodiment, the inhibitor of antitoxin is an inhibitor of the antitoxin set forth as SEQ ID NO: 6.

The inventors have found that only SehA1 plays a role in *S. Typhimurium* virulence.

Therefore for application related to typhoid fever, it will be more interesting to use inhibitor of SehA1 alone or in combination with an inhibitor of SehA2.

In a more preferred embodiment, the inhibitor of antitoxin is an inhibitor of the antitoxin set forth as SEQ ID NO: 6 and SEQ ID NO: 8.

Indeed, the inventors have found that SehA2 was able to act as molecular backup of SehA1. It is therefore more efficient to inhibit the two antitoxins.

Method for screening an inhibitor of antitoxin

The present invention also relates to a method for screening an inhibitor of an antitoxin of the invention.

The method for screening an inhibitor may comprise the step of contacting a test compound with a nucleic acid molecule encoding the antitoxin.

Then, the level of expression of the antitoxin is measured.

For example, a plasmid with an operon *toxin-antitoxin* where the *antitoxin* gene is replaced with a fusion of the gene of antitoxin with a reporter gene such as *gfp* may be used to transform strains.

The strains are contacted with a test compound and the level of expression of reporter is measured indicating the level of expression of antitoxin when the strains are in contact with the test compound.

If the level of expression of the antitoxin is significantly higher with the test compound than the level of expression in the control without the test compound then the test compound is an inhibitor of the antitoxin.

The method for screening an inhibitor may comprise the step of contacting a test compound with said antitoxin.

Then, the antitoxin activity of the antitoxin is measured.

Other examples of method for screening an inhibitor of antitoxin may be found in William 2012 and in the references cited therein.

The method of screening of the invention may, in particular, allow the identification of compounds that affect specifically the growth of pathogenic strains of *Salmonella*.

Indeed, the toxin-antitoxin systems of the invention are associated with pathogenicity in *Salmonella*.

More particularly, the inventors have shown that the deletion of SehA1 low the bacterial burden of mammals infected with *Salmonella* and therefore that an inhibitor of antitoxin SehA1 impacts the *Salmonella* virulence.

Method of treatment

The emergence and spread of pathogenic bacteria that have become resistant to multiple antibiotics through lateral gene transfer have created the need of novel antimicrobials. Toxin–antitoxin (TA) modules are foreseen as targets to fight against

antibiotic-resistant strains by using the toxin or disrupting the toxin-antitoxin system (for example see Engelberg-Kulka, et al. 2004 and Virginia S. Lioya, et al., plasmid 2010, William et al. 2012).

As used herein "**treatment or treating**" refers to both curative treatment and prophylactic or preventive measures, wherein the object is to prevent or slow down the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. Hence, the subject to be treated herein may have been diagnosed as having the disorder or may be predisposed or susceptible to the disorder.

The present invention also relates to a toxin of the invention for use in the treatment of *Enterobacteriaceae* infection.

Indeed, the inventors have shown that the toxin of the invention acts as a novel antibiotic by affecting the growth of bacteria and may be used in the treatment of bacterial infection.

Enterobacteriaceae may be selected from the group consisting of *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella* and *Shigella*. Preferably, *Enterobacteriaceae* infection is *Salmonella* infection.

More preferably, bacterial infection is an infection with *Salmonella enterica* and more preferably *Salmonella enterica* of group I.

More preferably, bacterial infection is an infection with Salmonella of *enterica* subspecies (group I) selected from the group consisting such as Typhi, Paratyphi, Enteritidis, Gallinarum, Heidelberg, Dublin, Agona and Newportin subspecies.

Salmonella infection may be entiritis, typhoid fever, blood stream infection, or focal infection such as meningitis, osteomyelitis, or abscess caused by *Salmonella*.

The present invention also relates to a method for the treatment of a bacterial infection in a subject in need thereof comprising administering to said subject an effective amount of the toxin of the invention.

The present invention also relates to a use of the toxin of the invention for the preparation of a medicament for the treatment of a bacterial infection.

The present invention also relates to an inhibitor of the antitoxin of the invention for use in the treatment of *Salmonella* infection.

Salmonella infection may be intestinal infection, typhoid fever, blood stream infection, or focal infection such as meningitis, osteomyelitis, or abscess.

The present invention also relates to a method for the treatment of a *Salmonella* infection in a subject in need thereof comprising administering to said subject an effective amount of an inhibitor of the antitoxin of the invention.

The inhibitor of the antitoxin by disrupting the toxin-antitoxin system acts as an antibiotic.

Further, the antitoxin of the invention is specific of the *Salmonella enterica* species. Therefore, the inhibitor of the antitoxin of the invention would not provide side effects on the gut flora of the subject.

The present invention also relates to a use of an inhibitor of the antitoxin of the invention for the preparation of a medicament for the treatment of a *Salmonella* infection.

Method for detecting a pathogenic strain of *Salmonella*

The present invention also relates to a method for detecting the presence of a pathogenic strain of *Salmonella* in a sample comprising the step of:

-detecting the presence of a toxin of the invention or an antitoxin of the invention.

Indeed, the inventors have shown that the *sehT1-sehA1* and *sehT2-sehA2* T/AT systems are present in the genus *Salmonella enterica* that affects mainly mammals and not *Salmonella bongori* that affect mainly cold-blood species.

Therefore, detecting the toxin-antitoxin systems of the invention allow the specific detection of *Salmonella enterica* strains.

Sample may be any sample taken for the realization of *in vitro* assays in animals or humans or collected from food whatever the nature or from any liquid medium or solid gas may contain pathogens sought.

Preferably, sample may be human or warm blood animal tissue homogenates, blood, feces and nasal and urethral mucous.

Preliminary enrichment of the bacteria present in the sample by cultivation may be possible before the identification test.

Preferably, sehT1 or sehA1 are detected.

Indeed, the inventors have shown that *sehT1-sehA1* T/AT system is present in the serotypes belonging to the *enterica* subspecies (group I) such as Typhi, Paratyphi, Enteritidis, Gallinarum, Heidelberg, Dublin, Agona and Newportin but not the *arizonae* subspecies.

Enterica subspecies (group I) infect warm-blooded mammals, while *Salmonella enterica* subspecies *arizonae* (group IIIb), also known to encode for SPI2, has been isolated from reptiles and is rarely associated with human infection.

The *enterica* subspecies group I are known to be responsible for 99% of mammalian infections (Baumler et al., 1997).

The present invention also relates to a kit for detecting the presence of a pathogenic strain of *Salmonella* in a sample comprising means for detecting the presence of the toxin of the invention or the antitoxin of the invention.

In one embodiment, the presence of the toxin of the invention or the antitoxin of the invention may be detected at the nucleic acid level.

Then, the means for detecting the presence of the toxin of the invention or the antitoxin of the invention are nucleic acid probes or primers that hybridize specifically to the nucleic acid molecule encoding the toxin or the antitoxin of the invention

As used herein, the terms "primer" and "probe" refer to the function of the oligonucleotide. A primer is typically extended by polymerase or ligation following hybridization to the target but a probe typically is not. A hybridized oligonucleotide may function as a probe if it is used to capture or detect a target sequence, and the same oligonucleotide may function as a primer when it is employed as a target binding sequence in an amplification primer. It will therefore be appreciated that any of the target binding sequences disclosed herein for amplification, detection or quantisation of the nucleic acid molecule encoding the toxin or the antitoxin of the invention may be used either as hybridization probes or as target binding sequences in primers for detection or amplification, optionally linked to a specialized sequence required by the selected amplification reaction or to facilitate detection.

Probes or primers may contain at least 10, 15, 20 or 30 nucleotides. Their length may be shorter than 400, 300, 200 or 100 nucleotides.

The method for detecting the presence of a pathogenic strain of *Salmonella* in a sample may comprise the step of:

-contacting the sample with the nucleic acid probe or primer that selectively hybridizes to the nucleic acid molecule encoding the toxin or the antitoxin of the invention,

-detecting the hybridization of the nucleic acid probe or primer with the nucleic acid molecule.

For example, the nucleic acid molecule may be extracted using any methods known in the art, such as described in Sambrook et al., 1989.

Then nucleic acid molecule may be amplified by polymerase chain reaction (PCR) using specific amplification primers.

Examples of method for detecting *Salmonella* strains at the nucleic acid level are disclosed in U.S. Patent No. 4,358,535, U.S. Patent Nos. 4,486,539 and 4,563,419.

In one embodiment, the presence of the toxin of the invention or the antitoxin of the invention may be detected at the protein level.

Then, the means for detecting the presence of the toxin or the antitoxin comprise a binding partner able of selectively interacting with respectively the toxin or the antitoxin.

The method for detecting the presence of a pathogenic strain of *Salmonella* in a sample may comprise the step of:

-contacting the sample with the binding partner able of selectively interacting with respectively the toxin or the antitoxin,

-detecting the binding of the binding partner with the toxin or the antitoxin.

The binding partner may be generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

Polyclonal antibodies directed against toxin of the invention, antitoxin of the invention or a fragment thereof can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred.

Monoclonal antibodies against toxin of the invention, antitoxin of the invention or a fragment thereof can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler et al. Nature. 1975;256(5517):495-7; the human B-cell hybridoma technique (Cote et al Proc Natl Acad Sci U S A. 1983;80(7):2026-30); and the EBV-hybridoma technique (Cole et al., 1985, In Monoclonal Antibodies and Cancer Therapy (Alan Liss, Inc.) pp. 77-96). Antibodies useful in practicing the present invention also include binding partners against toxin of the invention, antitoxin of the invention or a fragment thereof including but not limited to F(ab')2 fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments.

The binding partners of the invention may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

As used herein, the term "labeled", with regards to the binding partner is intended to encompass direct labeling of the binding partner by coupling (i.e., physically linking) a detectable substance, such as a radioactive agent or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)) to the binding partner, as well as indirect labeling of the probe or antibody by reactivity with a detectable substance. A binding partner of the invention may be labeled with a radioactive molecule by any method known in the art. For example radioactive molecules include but are not limited radioactive atom for scintigraphic studies such as I123, I124, In111, Re186, Re188.

In a preferred embodiment, the toxin or the antitoxin of the invention are detected by Western blot.

Western blot technique is well known technique. It is in particular described in Burnette WN., Analytical Biochemistry, vol. 112, no 2, Avril 1981, p. 195-203.

Typically, cells from sample are lysed, for example with RIPA, and then sonicated. Then total proteins from sample are separated using gel electrophoresis, for example a SDS-PAGE. The proteins are transferred out of the gel and onto a membrane, typically polyvinyldiflroride or nitrocellulose, where they are probed using a labeled binding partner capable of selectively interacting with the toxin of the invention, the antitoxin of the invention or a fragment thereof.

In another embodiment, the toxin of the invention, the antitoxin of the invention is detected by ELISA.

Typically, cells from sample are lysed, sonicated and microcentrifuged. After suitable dilution, the supernatant is added to wells of a microtiter plate that are coated with a set of primary antibodies.

Then, after a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES

Figure 1 shows that the atypical HigBA-type T/AT modules are present in pathogenic *Salmonella*. A) Genetic organization of the HigBA-type T/AT modules in *S.* Typhimurium chromosome. SehT1 and SehT2 encode for toxins and SehA1 and SehA2 for antitoxins. Both T/AT modules presented A+T rich sequences. These T/AT modules diverge being separated by the STM4032 unrelated gene. B) Phylogenetic tree showing the presence or the absence of both Seh T/AT modules in *Salmonella* species. All sequenced genomes were taken of NCBI and Colibase databases. The tree shown here was modified of Boyd et al., 1996.

Figure 2 shows that the SehA1 antitoxin represses its own expression and controls the *Salmonella* growth. A) Growth kinetic using different *S.* Typhimurium strains: wild type (12023), antitoxin mutant (Δ*sehA1*), toxin mutant (Δ*sehT1*) and toxin antitoxin mutant (Δ*sehT1* Δ*sehA1*). Strains were grown in LB medium for optical density measurements. B) Expression of the *sehT1-gfp* fusion in S. Typhimurium wild type (12023), Δ*sehA1*, Δ*sehT1* and Δ*sehT1* Δ*sehA1*. The strains were grown in LB medium and the samples were taken at 4 h post-inoculation. The fluorescence levels shown on the graphics were calculated as the GFP values reported to the OD600. C) Electrophoretic mobility shift assays were carried out with the *sehT1-sehA1* promoter region (100 ng), using His6-tagged SehA1 at the indicated concentrations. STM3559-

STM3558 promoter region was used as a negative control. The complexes were separated on 6% polyacrylamide gels.

Figure 3 shows that the SehA2 antitoxin can act as a molecular backup of SehA1. A) Expression of the *sehT2-gfp* fusion in S. Typhimurium wild type (12023), Δ*sehA2*, Δ*sehT2* and Δ*sehT2* Δ*sehA2*. The strains were grown in LB medium and the samples were taken at 4 h post-inoculation. The fluorescence levels shown on the graphics were calculated as the GFP values reported to the OD600. B) Electrophoretic mobility shift assays were carried out with the *sehT2-sehA2* promoter region (100 ng), using His6-tagged SehA2 at the indicated concentrations. STM3559-STM3558 promoter region was used as a negative control. The complexes were separated on 6% polyacrylamide gels. C) Growth kinetic using *S.* Typhimurium wild type (12023) and Δ*sehA1* antitoxin mutant, overexpressing both SehA1 (pBAD-SehA1) and SehA2 (pBAD-SehA2). 0.1 % arabinose was added at 2 h post-inoculation. Strains were grown in LB medium for optical density measurements.

Figure 4 shows that seh1 and seh2 are dispensable for replication in cultured cells. Mouse RAW 264.7 macrophages (A) or human HeLa cells (B) were infected with wild-type or various seh1 or seh2 mutant strains. ΔsifA and ΔssaV strains were used as controls. Cells were lysed at 2 and 16 hr post-infection for enumeration of intracellular bacteria. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 16 and 2 hr and normalized to that of the wild-type strain. Values are means ± SD of 3 independent experiments. One way ANOVA tests were used to determine whether the values were significantly different. P-values: ns, not significant; *, $P<0.05$; ***, $P<0.0005$.

Figure 5 shows that seh1 is transiently involved in Salmonella virulence upon peroral inoculation. Mice were inoculated intraperitoneally (A) or perorally (B & C) with a 1:1 mixture of two strains as indicated. Spleens were harvested two days (A), five days (C) or at various time points (B) post-inoculation and bacteria were enumerated. Each

symbol represents a mouse, and horizontal bars correspond to the means ±SD. A one-sample t-test was used to determine whether a CI was significantly different of one, and unpaired t-tests to determine whether values were significantly different. P-values: ns, not significant; *, P<0.05; **, P<0.01; ***, P <0.0005.

Figure 6 shows that seh1 and seh2 antitoxins bind to their promoter region and repress their own expression. Expressions of seh1-gfp and seh2-gfp are derepressed in minimum medium and inside RAW 264.7 mouse macrophages. Wildtype *S. Typhimurium* strains carrying a transcriptional fusion seh1-gfp, seh2-gfp or sifA-gfp (control) were grown in LB, minimum medium (MM) or inside RAW 264.7 mouse macrophages. Bacteria were collected at 4 (LB) or 16 (MM) hrs postinoculation or extracted from cells 16 hr post-infection and the relative fluorescence intensity of bacteria was determined by flow cytometry.

EXAMPLES

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Strains were cultured in LB broth (Difco) or minimal medium (M9, glycerol 0.2%, $MgSO_4$ 1mM, $CaCl_2$ 200 mM, thiamine 1 mg/ml, casamino acids 1 mg/ml. Ampicillin (50μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml) and chloramphenicol (50 μg/ml) were added when required. Bacterial suspensions were prepared from overnight LB cultures that were centrifuged and resuspended in fresh LB or minimal medium to an OD_{600} of 1. Then, 250 ml-flasks containing 50 ml of LB or minimal medium were inoculated with 1 ml of the bacterial suspensions and incubated at 37°C in a shaking incubator at 200 rpm.

Toxin-antitoxin databases and identification

The inventors used the RASTA webtool and BLASTP of known bacterial toxins to detect putative TA modules in the S. Typhimurium genome. Finally, we selected genes organized into a putative operon of two genes.

Construction of mutant strains

Non-polar gene-deletion mutants were generated by the lambda Red recombinase system, using gene-specific primer pairs to amplify pKD4 kanamycin or pKD3 chloramphenicol resistance genes. All mutagenesis was performed in the 12023 wild-type strain. When required, Salmonella mutants were transformed with the pCP20 plasmid to excise the antibiotic cassette. Gene deletions were checked by PCR.

Construction of plasmids

The seh1-gfp and seh2-gfp transcriptional fusions were generated by cloning PCR products corresponding to seh1 and seh2 promoter regions, respectively, into pFPV25. These PCR products were digested with EcoRI and BamHI and ligated to pFPV25 digested with the same enzymes. For E. coli expression, PCR products corresponding to Salmonella toxins were cloned in pDEST-17 using the Gateway system (Invitrogen, Ltd., Paisley, U.K.). seh1 and seh2 antitoxin genes were cloned in both pBAD-DEST49 and pMPM-K6. For pMPM-K6, primers contained the NcoI (5')/HindIII (3') restriction sites. The resulting PCR products were digested with NcoI and HindIII and ligated into pMPM-K6 digested with the same restriction enzymes, generating the plasmids pK6-sehA1 and pK6-sehA2. These plasmids contained the seh1 and seh2 antitoxin genes under an arabinose inducible promoter. All constructs were confirmed by DNA sequencing.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed as described previously. PCR products corresponding to seh1 and seh2 promoter regions were the same as used to generate seh1-gfp and seh2-gfp. These fragments (100 ng), were mixed with increasing

concentrations of [His]6-tagged sehA1 or sehA2 in PBS/50% glycerol. They were incubated 30 min at room temperature and then separated by electrophoresis in 6% polyacrylamide gels in 1X Tris-borate-EDTA buffer. The DNA bands were visualized by staining with ethidium bromide.

Eukaryotic cells and culture conditions

RAW 264.7 and HeLa cell lines were grown in DMEM (GibcoBRL) supplemented with 10% foetal calf serum (FCS; GibcoBRL), 2 mM nonessential amino acids, and glutamine (GibcoBRL) at 37°C in 5% CO2.

Bacterial infection and replication assays

HeLa and RAW 264.7 macrophage cell lines were grown, infected and treated as previously described (Schroeder N et al. (2010)).

Competitive index

Eight- to ten-week-old C57BL/6 or 129S2 mice were inoculated intraperitoneally or perorally with equal amounts of two bacterial strains for a total of 105 bacteria per mouse. The spleens were harvested 2 or 5 days after inoculation, as indicated, and homogenized. Bacteria were recovered and enumerated after plating a dilution series onto LB agar with the appropriate antibiotics. Competitive indexes (CI) were determined for each mouse. The CI is defined as the ratio between the mutant and wild-type strains within the output (bacteria recovered from the mouse after infection) divided by their ratios within the input (initial inoculum). Statistical analyses were performed using Prism (GraphPad, San Diego, CA, USA).

Flow cytometry analysis of bacteria extracted from synthetic medium, macrophages or mouse organs

Bacteria grown in synthetic medium (LB or MM) or extracted from infected cells or mouse organs were treated and analyzed by flow cytometry as previously described.

RESULTS:

The inventors have done a bioinformatics analysis several sequenced genomes of *bongori* and *enterica* species, subspecies and serotypes using the RASTA program (Sevin and Barloy-Hubler, 2007) and identified 2 toxin-antitoxin (T/AT) modules in the *Salmonella* chromosome.

Further analyzing these two T/AT modules, the inventors have found they are homologous to *higBA* of *E. coli* and *Vibrio cholerae* (Christensen-Dalsgaard and Gerdes, 2006; Christensen-Dalsgaard et al., 2010).

Theses T/AT modules presented opposite orientations and were separated by a gene, which encodes an esterase. The modules showed an atypical organization rarely found in other bacteria so far: the toxin was upstream the antitoxin gene in the operon (Fig. 1A). The inventors named these genes as *sehT* (*S. enterica* HigB toxin) for the toxins and *sehA* (*S. enterica* HigA antitoxin) for the antitoxins. Hence, the modules were named the *sehT1-sehA1* and *sehT2-sehA2* T/AT modules, respectively.

The inventors detected the presence of the *sehT1-sehA1* and *sehT2-sehA2* T/AT modules in all sequenced serotypes belonging to the *enterica* species and specifically in the *enterica* subspecies (group I) such as Typhi, Paratyphi, Enteritidis, Gallinarum, Heidelberg, Dublin, Agona and Newport (Fig. 1B). The inventors did not find these T/AT modules in *bongori* species. *Enterica* subspecies (group I) infect warm-blooded mammals, while *Salmonella enterica* subspecies *arizonae* (group IIIb), also known to encode SPI2, has been isolated from reptiles and is rarely associated with human infection. Therefore, *Salmonella enterica* subspecies *arizonae* has been suggested to be an intermediate in evolution between the *bongori* and *enterica* species (Baumler et al., 1997). Strikingly, the inventors detected, in the *arizonae* subspecies the presence of the *sehT2-sehA2* module but not the *sehT1-sehA1* module (Fig. 1B). These observations suggest that the *sehT2-sehA2* module was initially acquired, followed by the *sehT1-*

sehA1. The SehT1-SehA1 module is present in *enterica* subspecies (group I) known to be responsible for 99% of mammalian infections (Baumler et al., 1997). Altogether these data suggest that the presence of the two Seh T/AT modules is linked to the acquisition of *Salmonella* virulence.

The inventors generated mutants of *sehT1-sehA1* and *sehT2-sehA2* T/AT modules and the inventors first evaluated the impact of these deletions on bacterial growth. While deletion of the *higA* antitoxin gene of *Mycobacterium tuberculosis* and *Vibrio cholerae* has not been possible because of lethality problem (Budde et al., 2007; Fivian-Hughes and Davis, 2010), the inventors were able to generate an antitoxin mutant in *S.* Typhimurium (Δ*sehA1*). The Δ*sehA1* antitoxin mutant grew slower than the wt, mainly at the entrance of the stationary phase (Fig. 2A). This mutant growth could be complemented by the pBAD-SehA1 plasmid. The attenuated phenotype of the Δ*sehA1* mutant could not be observed when the inventors deleted both the *SehA1* antitoxin gene and *sehT1* toxin gene (Fig. 2A). This result demonstrates the importance of the SehA1 antitoxin in the control of the toxicity of SehT1. One explanation would be that in the absence of the antitoxin, the toxin inhibits the bacterial growth.

In addition to interact with its cognate toxin, the HigBA antitoxin is a transcriptional repressor of its own expression. Thus, the inventors evaluated the expression of the *sehT1-sehA1* operon by using a transcriptional fusion (*sehT1-gfp*) that contained the promoter region of the *sehT1-sehA1* operon. Different strains were transformed with the *sehT1-gfp* plasmid and grown in LB medium. Then the transcriptional expression was measured by flow cytometry. The expression of the *sehT1-gfp* fusion was 50-fold de-repressed in the absence of SehA1 antitoxin (Fig. 2B), indicating that SehA1 negatively regulated its own expression. To investigate if this repression was direct, the inventors purified a $[His]_6$ tagged version of SehA1 protein and performed an electrophoretic mobility shift assay experiments. The SehA1 antitoxin specifically bound to the *sehT1* promoter region (Fig. 2C), but not to the *STM3559-STM3558 (*another toxin-antitoxin found in *Salmonella)* promoter region used as negative control. SehA1 bound with high affinity to its promoter region, while *Mycobacterium*

tuberculosis HigA binding affinity is above 50 µM (Fivian-Hughes and Davis, 2010). The inventors found two palindromic sequences in the *sehT1* promoter region, which overlapped with the -35 box and the putative transcription start site that are similar to those described for *Mycobacterium tuberculosis* HigA (Fivian-Hughes and Davis, 2010). These sequences are likely recognized by the antitoxins to repress their own expression. Future studies will determine the nucleotide sequence relevant in the SehA1-DNA interaction. These data indicated that in *S.* Typhimurium, the *sehT1-sehA1* system encoded a T/AT functional module, where the presence of the antitoxin was determinant to control the function of the toxin in *S.* Typhimurium.

Similarly to *sehT1-sehA1*, the inventors characterized *S.* Typhimurium *sehT2-sehA2* module. Deletion of either gene did not impact significantly bacterial growth (data not shown). To corroborate the role of the SehA2 antitoxin as a transcriptional repressor, the inventors analyzed a transcriptional fusion (*sehT2-gfp*) containing the promoter region of the *sehT2-sehA2* operon. In the absence of the SehA2 antitoxin, the expression of *sehT2-gfp* was 10-fold derepressed with respect to the wild type strain (Fig. 3A). EMSA experiments showed that SehA2 antitoxin specifically bound to its promoter region indicating that *sehA2* encodes an antitoxin, which directly repressed the expression of the *sehT2-sehA2* operon. Similarly to the *sehT1-sehA1* module, the inventors found two palindromic sequences in the promoter region of the *sehT2-sehA2* operon. However, the palindromic sequences and the nucleotide sequences between the palindromes were both different from those found in the *sehT1-sehA1* promoter region. The inventors analyzed a possible cross-regulation between both T/AT modules. SehA1 and SehA2 only directly repressed their own expressions. However, overexpressed SehA2 was able to complement the absence of SehA1 (Fig. 3C). These data showed that SehA2 was able act as a molecular backup of SehA1 *in vitro*.

The inventors sought to determine if the transcription of seh loci is altered in response to conditions encountered during interactions with animal cells. Stressful conditions increase the proteolytic cleavage of antitoxins that have a higher susceptibility to degradation than toxins. This results in both the freeing of toxins and the increased

transcription-rate of TA operons. Wild-type *S. Typhimurium* carrying transcriptional reporters were grown in minimal medium known to induce the expression of genes necessary for intracellular survival/replication or were used to infect RAW 264.7 mouse macrophages. As a control, the inventors used a transcriptional reporter for sifA, which is required for intracellular replication and up regulated in these growth conditions (Fig. 6). As compared to bacteria grown in LB, the fluorescence resulting from the expression of seh1-gfp and seh2-gfp increased significantly in minimal medium and in macrophages (Fig. 6). The inventors concluded that both she loci respond to conditions encountered inside host cells, therefore suggesting these modules might influence the intracellular growth of *S. Typhimurium.*

To test this hypothesis human HeLa cells and RAW 264.7 mouse macrophage were infected with different strains and the fold increase of intracellular bacteria between 2 and 16 h after infection was determined. As expected, the inventors observed in 12 RAW 264.7 cells a dramatic replication defect for the control ΔssaV and ΔsifA mutant strains (Fig. 4A). By contrast, bacteria deleted of seh-A1 presented only a minor intracellular replication defect (Fig. 4A), with respect to the wild-type strain.

Mutants deleted for the toxin (ΔsehT1) and for the full module (Δseh1) replicated as well as the wild-type strain, indicating that the phenotype observed with the ΔsehA1 mutant is toxin-dependent and also that Seh1 is dispensable for the replication inside cultured cells. Deletion of either or both genes of seh2 had no effect on intracellular replication (Fig. 4A). Strains over-expressing either antitoxin replicated more than twice as much as the control, in the absence or not of their cognate toxin or module. The inventors concluded that i) Seh1 and Seh2 are dispensable for replication inside HeLa and RAW 264.7 cells; ii) SehT1 but not SehT2 restricts weakly the growth of *S. Typhimurium* in cultured cells; iii) that over-expression of either antitoxin confers to Salmonella an intracellular hyper-replicative phenotype.

Next, the inventors compared the virulence of wild-type and mutants strains in C57BL/6 mice by performing mixed infections. As controls, the inventors used again ΔsifAand ΔssaV strains that are moderately and highly attenuated in this model,

respectively. C57BL/6 mice were inoculated intraperitoneally with a 1:1 mix of two strains. Bacteria were recovered from mouse spleens after 2 days and the competitive index (CI) was determined. The inventors found that the ΔsehA1 mutant is highly attenuated with respect to the wild-type strain (CI=0.02±0.01). Remarkably, this attenuation is comparable to that of the *Δ*ssaV strain (CI=0.01±0.003) (Figure 5A). Complementation with psehA1 allowed the *ΔsehA1* mutant to recover virulence (CI=0.75±0.2). ΔsehT1 and Δseh1 mutant strains displayed a wild-type level of virulence. These results demonstrate that SehT1 exerts in vivo a very strong toxic action that is neutralized by its cognate antitoxin (Fig. 5A). It indicates that *Salmonella* encounters in the mouse conditions under which the toxin is highly active, thus severely limiting the growth of the ΔsehA1 strain and also that the seh1 module is dispensable for systemic infections.

Therefore, the inventors tested if this module could play a role in mice inoculated by the natural route. The inventors found that after mixed peroral inoculation a Δseh1 strain was significantly attenuated (CI=0.52±0.18) (Fig. 5B). The inventors also examined the role of seh1 in mice in which bacteria undergo a harsher challenge from the immune system. For this, the inventors used 129S2 mice carrying the wild-type Nramp1 allele as a model of persistent *S. Typhimurium* infection. In addition, the inventors took advantage of the prolonged survival of 129S2 mice to *Salmonella* challenges to analyze the evolution of the CI with time. At five days post-inoculation, a Δseh1 strain presented an attenuation (CI=0.64±0.24) similar to that observed in C57BL/6 mice. This value did not decrease to a great extent with time but rather tended to reach a plateau (CI=0.41±0.05 at day eight-teen post-inoculation, Fig. 5B). These results suggest that after an initial deficiency in establishing an infection by the natural route, a Δseh1 mutant is able to persist as well as the wild-type strain. It confirms that seh1 does not play a significant role in the systemic infection. Rather, this module is involved in the early phase of the peroral infection. Strains deleted of Δseh1 or of both Δseh1 and Δseh2 and given perorally to C57BL/6 mice were not significantly different in their virulence attenuation (Fig. 5C). It shows that the seh2

module, whose toxin does not seem to be active in mice (seh2-A$^-$ in Fig. 5A), does not contribute significantly to *Salmonella* virulence in the mouse model (Fig. 5C).

The attenuation of a Δseh1 mutant upon peroral inoculation of mice suggested that *Salmonella* encounters conditions favouring the degradation of the antitoxin and consequently an enhanced transcription of the locus. In order to confirm this point, the inventors inoculated mice with wild-type *S. Typhimurium* carrying a transcriptional reporter for seh1. Bacteria extracted from organs were examined for the expression seh1-gfp by flow cytometry and compared to reference strains grown in LB. Most *Salmonella* extracted from inoculated mice showed GFP fluorescence levels that were comparable to that of wild-type *S. Typhimurium* grown in LB. This indicates that the majority of bacteria presented a basal or slightly increased level of seh1 expression. However, we also observed a second population with a mean GFP fluorescence similar to that observed for the ΔsehA1 strain. This population, thereafter named GFP^{high} bacteria, corresponded to *Salmonella* in which expression of seh1 is de-repressed. Using this method the inventors analyzed bacteria extracted from mesenteric lymph nodes (MLN) and from the spleen. GFP^{high} bacteria were hardly detected the spleen of mice inoculated intraperitoneally, while this population was significantly more prevalent in the spleen and MLNs of mice inoculated perorally. Yet, in these conditions, the percentage of GFP^{high} *Salmonella* did not exceed 5%. Also, the mean fluorescence of total bacteria was significantly increased in populations extracted from perorally but not from intraperitoneally inoculated mice. Finally, in perorally inoculated mice, the mean fluorescence of GFP^{high} bacteria was significantly higher in bacteria extracted form MLNs in comparison to spleens. Considering that after having crossed the intestinal barrier the bacteria reach MLNs before the spleen, these results confirm that activation of the seh1 module is an early event in the process of infection through oral ingestion.

Since it has been shown that SPI2 is the main virulence determinant of *S.* Typhimurium, the inventors analyzed whether attenuation mediated by SehA1 was SPI-2-dependent. The inventors first studied the fate of PipB2 and SseJ and showed

that the translocation of these two SPI2 effector proteins into macrophages was not altered in the absence of SehA1. Moreover, an Δ*sehA1* Δ*ssaV* double mutant was even more attenuated than the Δ*sehA1* mutant. The inventors concluded that the attenuation of the Δ*sehA1* mutant was not dependent on SPI-2.

DISCUSSION

Type II TA modules are inhibitors of translation that induce bacterial dormancy. Being in a dormant state help bacteria to survive harmful environments. This study presents evidence that virulent strains of Salmonella possess specific TA modules and shows that one of these is beneficial in the early phase of the infection by the natural route.

The inventors identified eleven toxin/antitoxin systems in *S. Typhimurium* and examined the presence of homologous genes across Salmonellae. The heatmap generated from clustering by both strains and TA modules genes highlighted a set of four toxin/antitoxin systems that are found across serovars of *S. enterica* infecting warm-blooded animals, while the nonpathogenic species and strains of *S. enterica*, which are associated with coldblooded animals, have no or a low number of TA modules, respectively.

The inventors found that a mutant deleted of Seh1 is attenuated for virulence in mice inoculated perorally but fully virulent in mice inoculated intraperitoneally. This is reminiscent to what has been observed for the SP1-encoded type three secretion system, which helps Salmonella to cross the intestinal barrier. Thus, Seh1 plays a role in an early step of the infection that is temporally localized between the arrival in the stomach and the triggering of a systemic infection. Interestingly, the CI in perorally inoculated mice was the same at 5 and 18 days post-infection, thus confirming that Seh1 plays a transient role and is not involved in the systemic phase of the infection. The stomach and the gut are aggressive environments in which the activation of Seh1 might favour persistence. Peyer's patches are preferential sites for Salmonella to cross the intestinal barrier. They are also central sites for the induction of the mucosal immune response and consequently have an important antibacterial arsenal. Thus,

another possibility is that Seh1 helps to survive the biochemical stresses (NO, antibacterial peptide...) and/or the innate immune cellular defences present in the gut and the subepithelial environment. Indeed, this possibility is supported by the presence of Salmonella with a high activation profile of the seh1 promoter in MLNs, into which Peyer's patches are drained.

At five days post-inoculation the inventors detected in MLNs a maximum of five percent of GFPhigh bacteria. The inventors cannot exclude that the activation of Seh1 occurs earlier and in a larger part of the bacterial population. However, our attempts to detect fluorescent bacteria in mice earlier than five days post-inoculation failed because the number of bacteria present in spleen, MLNs and even the small intestine was too low.

The HigB toxin of E. coli K12 cleaves mRNAs positioned at the ribosomal A-site and stops the translation. The mechanism of action of SehT1 is unknown but, considering that SehT1 presents 40% identity with HigB, these toxins are likely to have identical targets. However, the above results indicate that SehT1 activity is dependent on an additional mechanism. Indeed, a ΔsehA1 strain, which expresses a high level of free toxin, presents only a partial growth defect in LB or in cultured cells. In contrast, the virulence of a ΔsehA1 mutant is severely impaired in the mouse model as its defect is comparable to the strongly attenuated ΔssaV mutant.

Interestingly, a previous screen in the mouse model selected STM4030 (sehA1) among the most important genes for virulence. Altogether, it indicates that the toxin itself is necessary but not sufficient to limit the bacterial growth and that an additional bacterial factor synergizes the SehT1 activity in conditions of mouse infection. This might be an activator/inhibitor factor whose association/dissociation with the toxin is controlled by the environmental conditions encountered in mice. This factor is operational in intraperitoneally inoculated mice though, in these conditions of infection, expression of SehT1 is not increased. It shows that synergization of the SehT1 toxin activity by the putative factor and activation of the *seh1* locus transcription are not triggered by the same environmental conditions. In general toxins and antitoxins form hetero-

oligomers that are toxin-inhibiting complexes and that bind their promoter region. The studies of the inventors have shown that SehA1 forms dimers whereas SehT1 forms tetramers and aggregates. The aggregation of SehT1 might result from a non-native folding and explain its lack of interaction with the antitoxin. However, is it also possible that SehA1 and SehT1 do not interact. The dimer of SehA1 binds a palindromic stretch of its promoter and could well be the functional unit that represses seh1 transcription. In support of this hypothesis is the observation that SehA1 inhibits effectively the operon expression even in absence of its cognate toxin. Moreover, as discussed above, the activity of the toxin in mice seems to be regulated by the aforementioned additional factor rather than by SehA1.

Stress conditions leading to afully active SehT1 are poorly experienced in cultured cells. This is shown by the limited activation of the seh1 promoter and the weak growth defect of a Δseh1 mutant. This suggests that Seh1 does not support the survival/replication of intracellular bacteria. Another possibility is that HeLa and RAW264.7 cells are not representative of cell types Salmonella encounters during the early phase after oral inoculation. Seh1 might help to survive the bactericidal activity of immune cells that are recruited to the infection sites. Supporting this observation is the fact that Salmonella are not killed and survive in dendritic cells and that this survival is independent of virulence factors important in macrophages.

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CLAIMS

1. A method for detecting the presence of a pathogenic strain of *Salmonella* in a sample comprising the step of detecting the presence of:

-a toxin having the amino acid sequence set forth as SEQ ID NO: 5 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 5

and/or

- an antitoxin having the amino acid sequence set forth as SEQ ID NO: 6 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 6.

2. The method for detecting the presence of a pathogenic strain according to claim 1 wherein the presence of the toxin or the antitoxin is detected at the nucleic acid level.

3. The method for detecting the presence of a pathogenic strain according to claim 1 wherein the presence of the toxin or the antitoxin is detected at the protein level.

4. A kit for detecting the presence of a pathogenic strain of *Salmonella* in a sample comprising means for detecting the presence of:

-a toxin having the amino acid sequence set forth as SEQ ID NO: 5 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 5

and/or

- an antitoxin having the amino acid sequence set forth as SEQ ID NO: 6 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 6.

5. An inhibitor of an antitoxin having the amino acid sequence set forth as SEQ ID NO: 6 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 6.

6. The inhibitor according to claim 5 for use in the treatment of *Salmonella* infection.

7. A method for screening the inhibitor according to claim 5.

5

8. A toxin having the amino acid sequence set forth as SEQ ID NO: 5 or a variant thereof having at least 90% identity with amino acid sequence set forth as SEQ ID NO: 5 for use in the treatment of an *Enterobacteriaceae* infection.

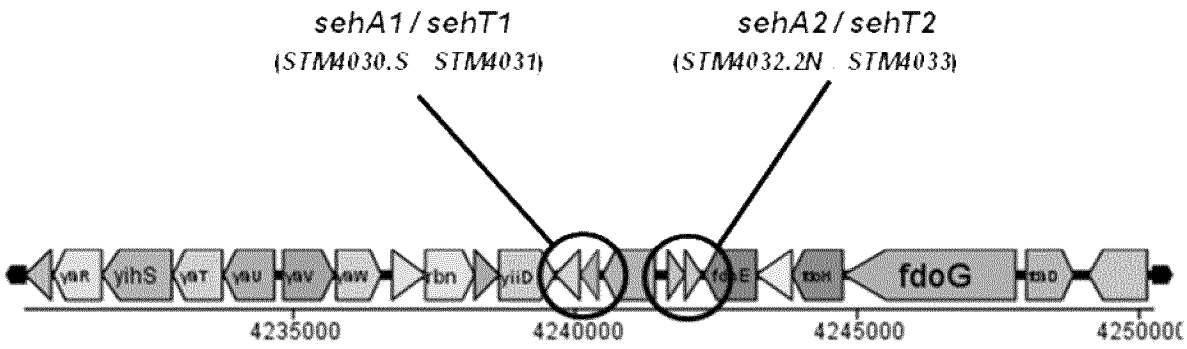

sehA1 / sehT1
(STM4030.S STM4031)
sehA2 / sehT2
(STM4032.2N STM4033)
ydR
yihS
ydT
ydU
ydV
ydW
fbn
yiiD
fdoG
fdoI
4235000
4240000
4245000
425000(

FIG. 1A

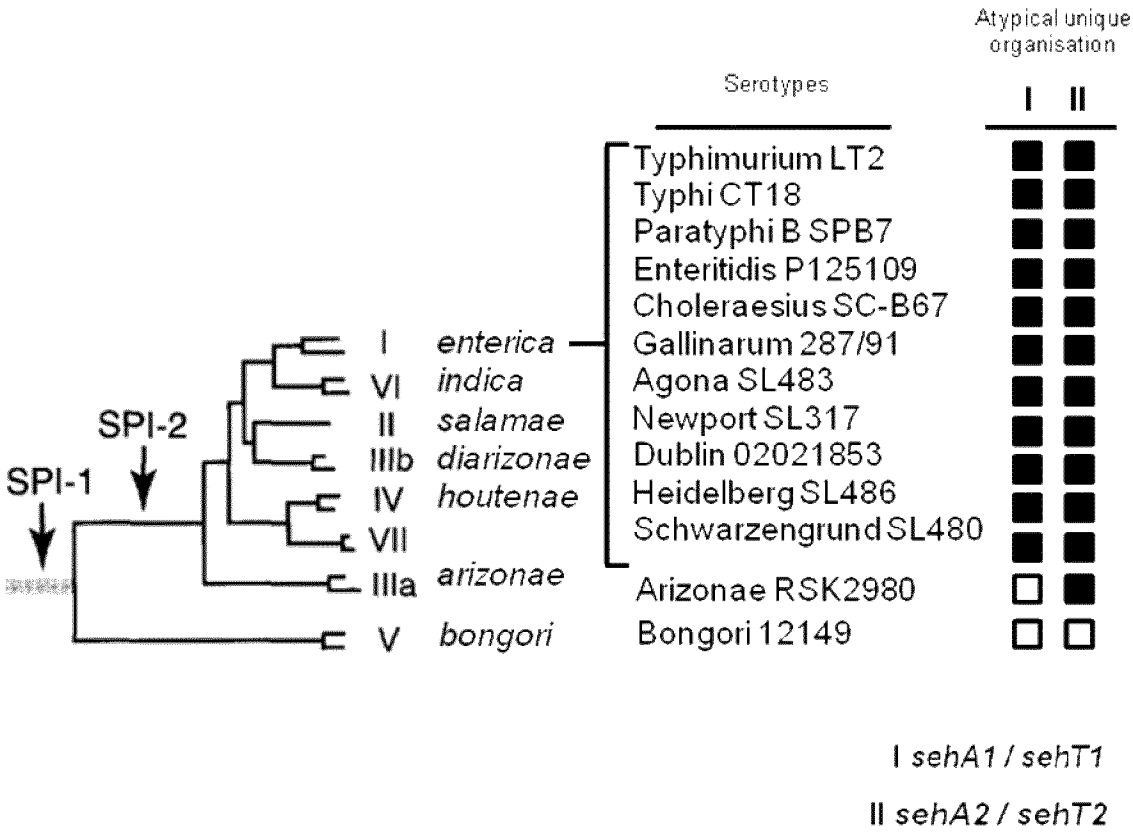

Atypical unique organisation
Serotypes
I II
Typhimurium LT2
Typhi CT18
Paratyphi B SPB7
Enteritidis P125109
Choleraesius SC-B67
Gallinarum 287/91
Agona SL483
Newport SL317
Dublin 02021853
Heidelberg SL486
Schwarzengrund SL480
Arizonae RSK2980
Bongori 12149
SPI-1
SPI-2
I enterica
VI indica
II salamae
IIIb diarizonae
IV houtenae
VII
IIIa arizonae
V bongori
I sehA1 / sehT1
II sehA2 / sehT2

FIG. 1B

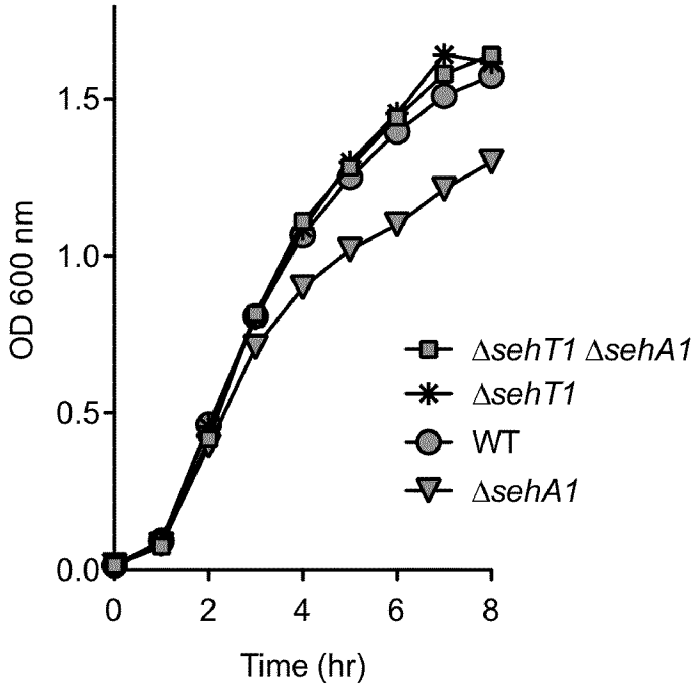

OD 600 nm
1.5
1.0
0.5
0.0
0
2
4
6
8
Time (hr)
ΔsehT1 ΔsehA1
ΔsehT1
WT
ΔsehA1

FIG. 2A

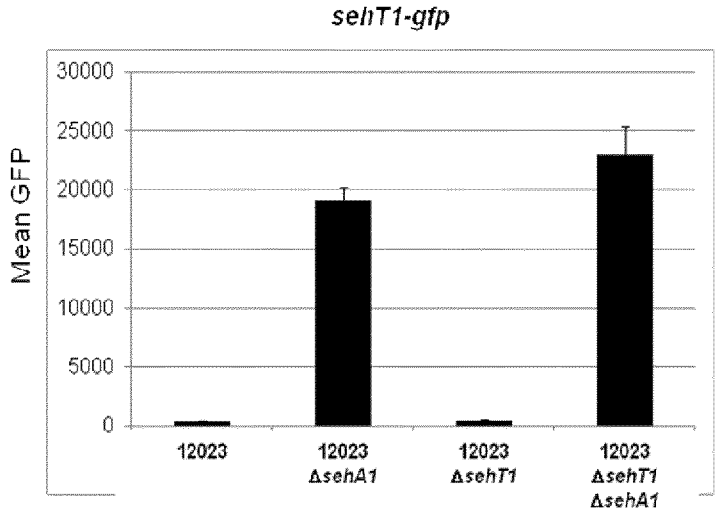

sehT1-gfp
Mean GFP
30000
25000
20000
15000
10000
5000
0
12023
12023 ΔsehA1
12023 ΔsehT1
12023 ΔsehT1 ΔsehA1

FIG. 2B

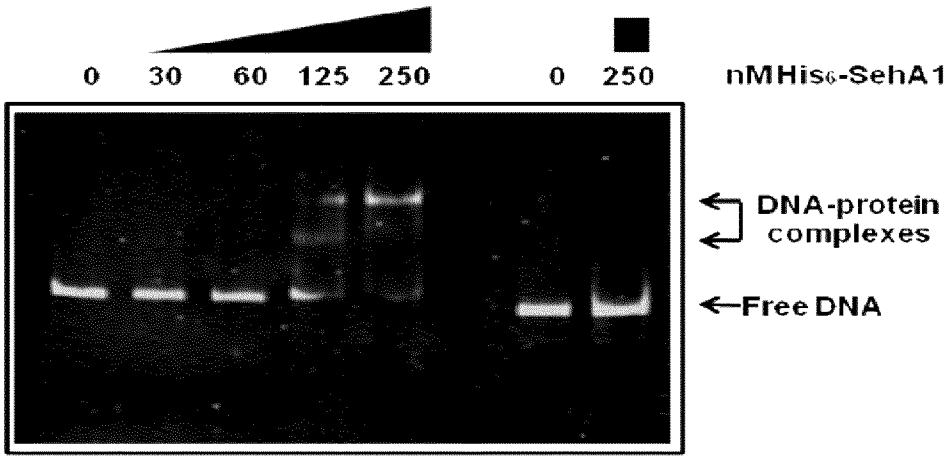

0
30
60
125
250
0
250
nMHis6-SehA1
DNA-protein complexes
Free DNA
sehT1-sehA1 promoter region
STM3559 promoter region

FIG. 2C

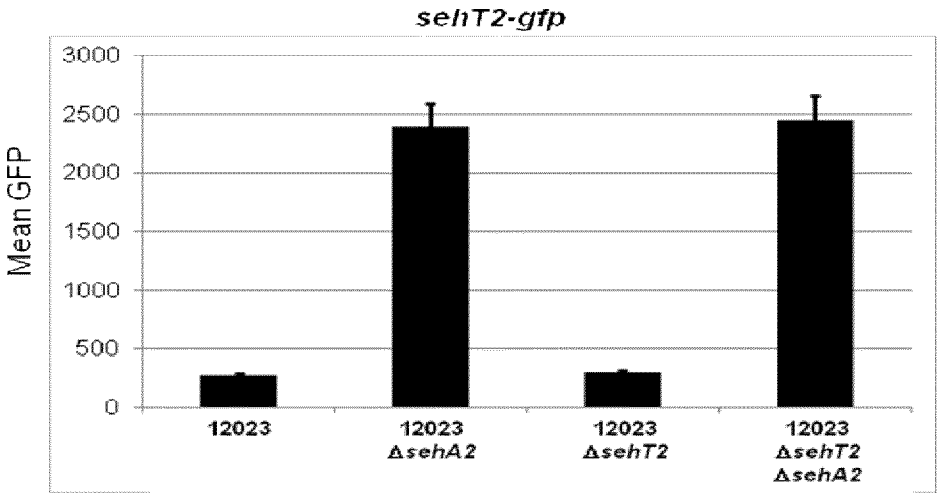

sehT2-gfp
Mean GFP
3000
2500
2000
1500
1000
500
0
12023
12023 ΔsehA2
12023 ΔsehT2
12023 ΔsehT2 ΔsehA2

FIG. 3A

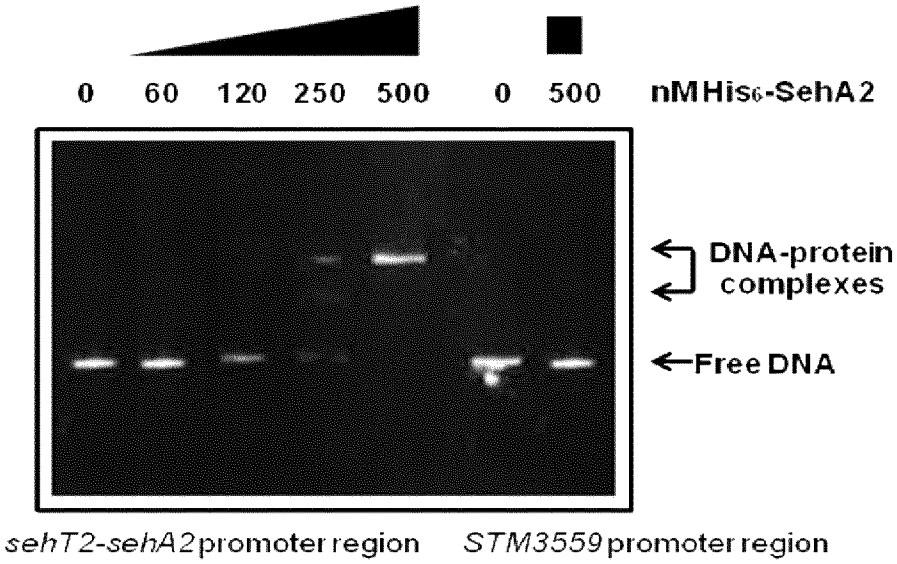

0 60 120 250 500 0 500 nM His6-SehA2
DNA-protein complexes
Free DNA
sehT2-sehA2 promoter region
STM3559 promoter region

FIG. 3B

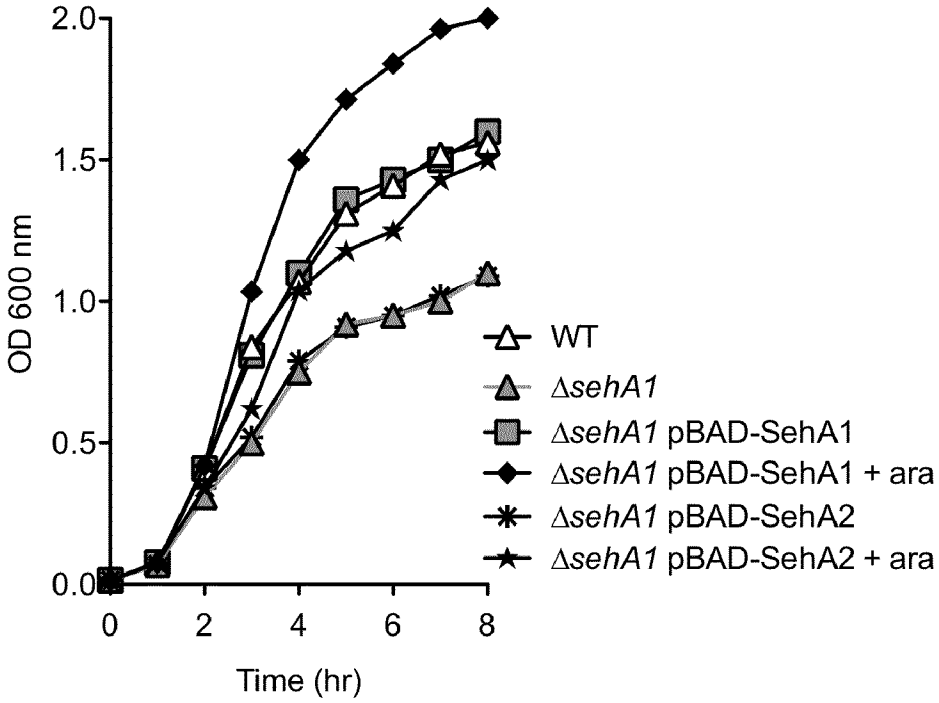
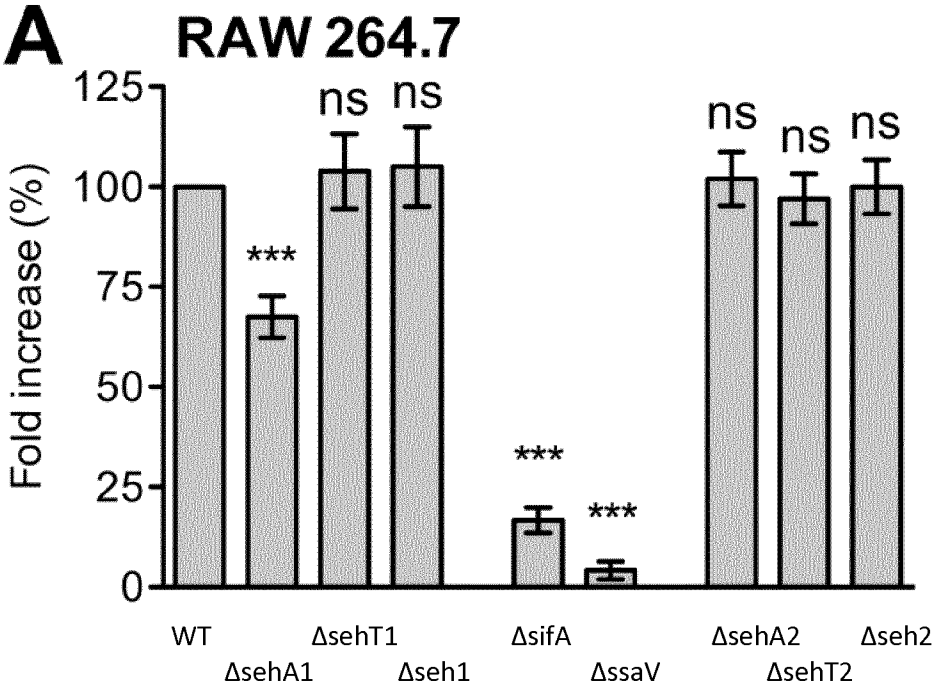

2.0
1.5
1.0
0.5
0.0
OD 600 nm
0
2
4
6
8
Time (hr)
WT
ΔsehA1
ΔsehA1 pBAD-SehA1
ΔsehA1 pBAD-SehA1 + ara
ΔsehA1 pBAD-SehA2
ΔsehA1 pBAD-SehA2 + ara

FIG. 3C


A
RAW 264.7
Fold increase (%)
125
100
75
50
25
0
***
ns
ns
***
***
ns
ns
ns
WT
ΔsehA1
ΔsehT1
Δseh1
ΔsifA
ΔssaV
ΔsehA2
ΔsehT2
Δseh2

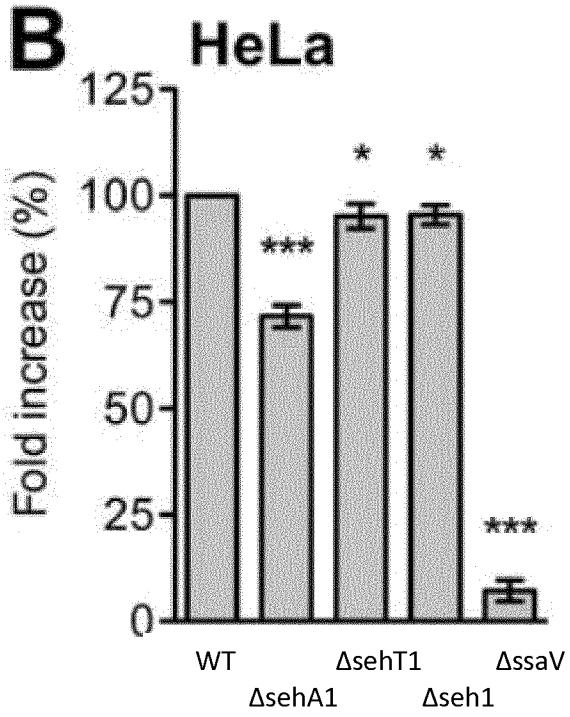

B
HeLa
Fold increase (%)
125
100
75
50
25
0
***
*
*
***
WT
ΔsehA1
ΔsehT1
Δseh1
ΔssaV

FIG. 4

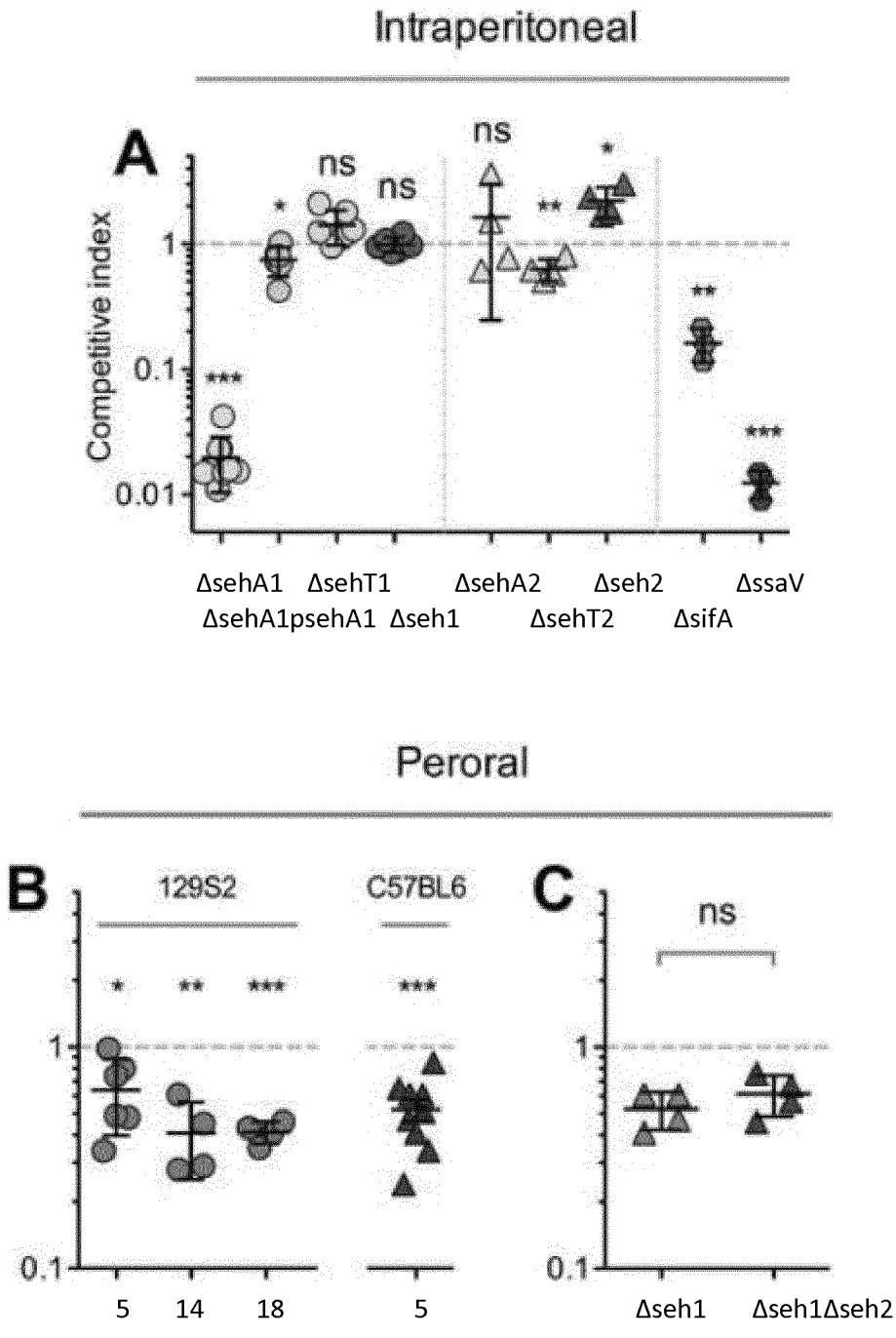

Intraperitoneal
A
Competitive index
1
0.1
0.01
ns
ns
ns
*
*
**
**
***
***
ΔsehA1
ΔsehA1psehA1
ΔsehT1
Δseh1
ΔsehA2
ΔsehT2
Δseh2
ΔsifA
ΔssaV
Peroral
B
129S2
C57BL6
*
**
***
***
1
0.1
5
14
18
5
C
ns
1
0.1
Δseh1
Δseh1Δseh2

FIG. 5

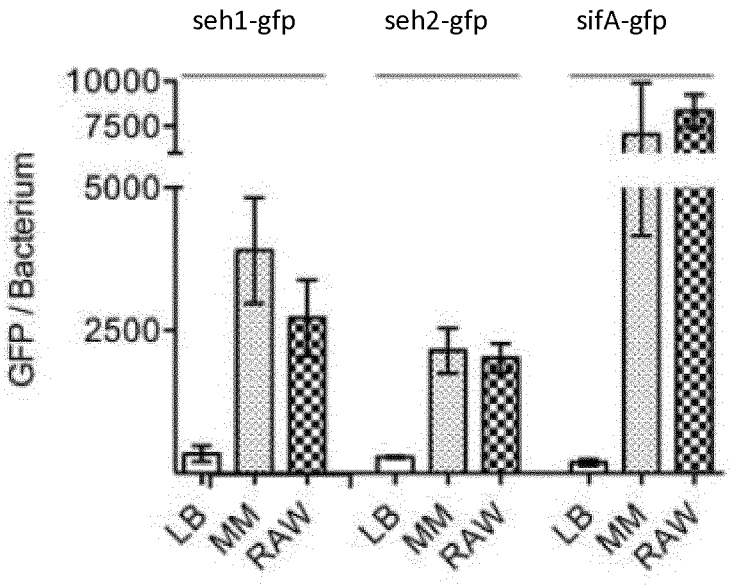

seh1-gfp
seh2-gfp
sifA-gfp
10000
7500
5000
2500
GFP / Bacterium
LB
MM
RAW
LB
MM
RAW
LB
MM
RAW

FIG. 6