(54) Title: PEPTIDES AND DERIVATIVES THEREOF FOR DETECTION AND CONTROL OF SALMONELLA

(57) Abstract: The present subject matter relates to isolated polypeptide having one of the sequences SEQ ID No 8, 9, 10, 11, 12, 13, 14 or fragments or derivatives thereof, having the ability to recognize and bind to a broad range of Salmonellae strains. Moreover, the present subject matter further relates to a nucleic acid molecules having one of the SEQ ID No 1, 2, 3, 4, 5, 6, 7 encoding said recombinant polypeptide or fragments or derivatives thereof, vectors comprising said nucleic acid molecules and host cells comprising either said nucleic acid molecules or said vectors.
DESCRIPTION

PEPTIDES AND DERIVATIVES THEREOF FOR DETECTION AND CONTROL OF SALMONELLA

FIELD OF THE INVENTION

The present subject matter relates to isolated polypeptides having the sequences SEQ. ID. No 8, 9, 10, 11, 12, 13, 14 or fragments or derivatives thereof, having the ability to recognize and bind to a broad range of Salmonella strains. Moreover, the present subject matter further relates to nucleic acid molecules SEQ. ID. No 1, 2, 3, 4, 5, 6, 7 encoding said recombinant polypeptide or fragments or derivatives thereof, vectors comprising said nucleic acid molecules and host cells comprising either said nucleic acid molecules or said vectors.

In addition, the present subject matter relates to said polypeptide, fragments or derivatives thereof for use as a detection tool or a kit for the identification of Salmonella strains in foodstuff, food processing equipment, food processing plants, food processing surfaces, medical devices, surfaces in hospitals and surgeries, human stools, environmental samples or any other samples where Salmonella might be present.

The present subject matter also relates to said polypeptide, fragments or derivatives thereof for use as a medicament, in particular for the treatment or prevention of Salmonella spp. infections.

The present subject matter also relates to the use of said polypeptide, fragments or derivatives thereof for the
treatment or prevention of bacterial contamination, particularly of Salmonella spp. contamination, of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries or other places where Salmonella might be present. Furthermore, the present subject matter relates to a pharmaceutical composition comprising said polypeptide, fragments or derivatives thereof.

BACKGROUND OF THE INVENTION
Foodborne diseases are of major concern due to their worldwide impact. The Center for Disease Control and Prevention (CDC) estimates that 76 million cases of foodborne diseases occur every year in the United States, causing roughly 5000 deaths. From the reported cases, it has been observed that Salmonella is the most common and widely distributed causing agent constituting thus a major public health burden with significant impact in the society costs worldwide. Besides the effort that has been done to control this pathogen, including the general improvement of food safety knowledge and good practices, they are still a major cause of the increasing occurrence of foodborne diseases.

Moreover, the production and distribution of foodstuff increased dramatically in volume and in number of consumers, increasing the risk of mass epidemics. Consequently, it is critical to identify and quantify the presence of such pathogen to monitor the safety of foodstuff and also to define strategies to reduce the number of outbreaks.
The golden standard for *Salmonella* detection is still the bacteriological culture (ISO 6579:2002) which is time-consuming, laborious, expensive and ineffective in detecting non-cultivable organisms. *Salmonella* detection methods typically take 3-5 days to obtain a result and during this long period portions of the food may have been distributed, marketed, sold, and eaten before a problem is even detected. A number of methods aiming at reducing the time required for the identification of foodborne pathogens have been developed, which include the use of antibodies to detect microbial antigens such as the enzyme-linked immunosorbent assay (ELISA) and nucleic acid-based techniques such as the polymerase chain reaction (PCR), DNA microarrays/DNA chips, sequencing-based identification and DNA hybridization. However, the sensitivity of immunological methods has been found to be low, with a variable specificity depending on the antibody used, commonly with the occurrence of cross-reactivity phenomena. Often, these methods do not allow the discrimination between dead and living microorganisms. Also, the feasibility of the method depends on the origin of samples. Despite the high sensitivity of the nucleic acid-based techniques and their increased value in the detection of fastidious microorganisms, they usually require extensive sample preparation, often including a DNA extraction step. These procedures are laborious, expensive and time consuming and may not be enough to remove all the contaminants, commonly found in the samples, that may inhibit (or influence) the reactions needed for the microorganisms detection. Moreover, the nucleic acid-based techniques such as the PCR and microarrays demand a high initial investment in equipment and expertise personnel.
Bacteriophages (phages) are naturally evolved entities that due to their intrinsic characteristics present high potential in the control and detection of bacteria, namely foodborne pathogens. Phages are viruses that only infect bacteria and are obligate intracellular parasites lacking their own metabolism. They are extremely specific, usually infecting a single species or even strain and consequently they have been found to present potential in the rapid detection of bacteria.

Phage PVPSE1 belongs to the family Myoviridae and is characterized by an icosahedral head of 84 nm in apical diameter and a contractile tail of 120 x 18 nm with short tail fibers. This phage has the ability to bind to a very broad range of Salmonella strains which makes it an interesting phage to be used in the detection of Salmonella. In addition the lytic spectrum of this phage is broader than that described by Felix Ol. We observed that PVPSE1 is able to infect Salmonella several mutants defective in core polysaccharide assembly suggesting that the receptor for this phage is the conserved LPS inner core region and explains its broad lytic spectrum. The use of tail proteins (e.g. tail fibers and base plate) as recognition elements has been suggested by others, however, the present invention respects to a group of tail proteins that can cover a very broad range of Salmonella strains.

SUMMARY OF THE INVENTION

An embodiment of the present invention relates to new polypeptides isolated from phage PVPSE1 able to specifically bind to different Salmonella species and useful in the detection and control of Salmonella spp.,
namely an isolated polypeptide having the sequence of one of SEQ ID No 8, 9, 10, 11, 12, 13, or 14, or fragments or derivatives thereof, or combinations thereof, for recognizing or binding to Salmonella.

Namely:

SEQ ID No. 8 - Protein sequence of wt PYPSE1gp40 (604 amino acids)
MYPIPCFLTLSSGGTEPLPPGSGVKKAFTRGLVGGATKRSMAILLIDGRLYTQGDNAW SECANGNISPFPDKHWHLAANGVADVFGGGRAFVVKYNNGGWQYCGDTSQFTGSGSIYSS WTSFPSSITGTVSPLANQLVSCALGNLTQWQVMDGRSLYSGSNTGCLGSGNTTVVISIPR SISASSVRALSNCVTLINILPVRVCAGTHQIDGTSTTQTQNDIVSDASVTEVYV KEWLANETNSMAIASTGVDDTEHYLRTQGTAQYSKKEGIGPETFTRVIGGQSHFLI ADNKLZGLDLSAQLGLGTPSTMVELPVTLPVFPTGRDWDLKTYIVDMDKTDVLNQGNS ISHWMVYDNLYAGNLYGFFGSTDSTGGEFTNPEASFGGTADAITGSIYAIKGRS QSLTWTVEPADAEIYDISFTSSAPNIAVTDGNGMTFLEEGDFDITMTAKTSGAGADMT LTDTSGGYVYISFSTDSIPQKEVGDVVFVMDKNSPDYTPGPNVVGMEISPANVTDNFI DGETTTNPDVVMIDEGGFLSCIAVDARCGLYRILYREGQVEADFSDYSVSDFDTPPD PVDPEGVVPVPQPO

SEQ ID No. 9 - Protein sequence of wt PYPSE1gp41 (602 amino acids)
MADMTQFEQAVDQVVEDSERLHKVNVGTASETVTEDGSTIPTVRKALDLNFKTPPM PWIAGTQTTVFNQLYAFNGTNGVQQWYAPTATASAPVVPQPANNVSNRLYANDAMAS IYAPINSPLTGNPQAPTPAANSNSTTIATTAFVTAIASALSSISGVSFTAFANLSVT GATTNSLVWGGTIDNLNPVANADNSTGRFQNLILTEKELSSLFVFTDADNPTFIFKTRLID PYAIQTHSIQTDIINVGTVAEDDTTMSTGVTGNNFDVYVIRGNSKADPLTPARLKVSGT TEVENLNGITGNVITGIFSVPNLSVTADGTVGGDLQVSVGLNVGSTIGLIGGGLDI TSDLTVNNGNTLEDFAANIGNITGPFTVGGLTLSNGGFRTTGADTGKGGKSVTGTSEF IEDLSVLADVTVQDNLTNGDVLNLNATGGTTTVNLQITGTGTVLSVLDTQGINVQSL SSTGAVTANSLTVQDSAILTKFVEFLTLAEDIDSSTAASPSGSNNINYNTVDADLT IGAWEPEPTDAFSAVIYLTDQTGGHTVTDLPNLYVNLSETINETAGSVTILQLTYNGVE GGVIDTVIVRRP
SEQ ID No. 10 - Protein sequence of wt PVPE1gp46 (373 amino acids)
MAAPTVPFIEIWAYGDVLNPThelnkarFiddlwnkgwdlgekptveefnyvlnmlta
Akyitgeqipgldsrflrvqnladladkaartnldvksktedtryvnisgdmtga
Lsvpnlqpgesdysayiturnnafadttffdfvvgdgnignaptssidsmsrfrfvpsg
Siftmmelnaigtaalcrvtgniiasgsigasgvtatattanftnttvsgtlnapitiqst
Tirtgltltatgnvqgfnvvatsltpyasvngqcnnvlvnnnsatvggrgvrain
Gataodgnvntlnsgfsvqiqrlgraffgvesrfyaghvmwafgnkkelerayty
Taplqylinqqwvtvnsld

SEQ ID No. 11 - Protein sequence of wt PVPE1gp48 (497 amino acids)
Maaqyglnzychafipsldldiadtktqslirtfgenqatvvdktlltlinereyqli
Lllaaavysaqtlagaegyldeggrrg5yrgrkrgsrgtqrmvvnntvpynmisst
Ysidsngflvlqdtpvagniliqnilqdwlgnyticqminqdngdstskmnltltnv
Nspqlnafmsisiqfivdnstqnedrifdsasaggamydydnnkkmlgnrsdfnrss
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Teeiselayntialsnatygnvydfvdteqdquzryhskaqrelavrvrvgkykllsv
Teqntikdlkavvdplniadltylniqvlsavgsisspgfrtqllvdvkntdqpsaysyt
Nsdvvgmtevfaalddttidftqqii

SEQ ID No. 12 - Protein sequence of wt PVPE1gp50 (929 amino acids)
Maqlrhnidnvfqgldghiqflrknkgdtdptvqhyilnqlonvannhrfinwtaqea
Qpngdattegsqslilgcaayaladndpkylealaedfwaqayidwwffagqipdpavaur
Pnwiingkeprlahypltddgypthfhkggfsmswtnktpilphgahweyldkawfa
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AKLGDCTIIDYKTNSLHYTPGIPFISNISDPNTALYDGWRGLFYPGAQFYPTLYCFKGQN
IDWTRLNNFTDFLYDSMQWYNSFNFPVMGPMQAFVWDRWDACKYGEPNTTFTMKWHNE
KAWDGYEARAFFCVRCVCYYLSQRGSEAIPSKLFTVAKNWVDYLKWQFDNNEGVRPTIFN
PDGTIVLAPIDDFTSHEMSALPMMGGCSLNMAGMDDRIPNIDLVAERCDFDLIQENYIIILTP
NHPMNGCSVAPRATDNMGFFGHAGELLGRRFGLYAMYRNLTR

SEQ ID No. 13 - Protein sequence of wt PVPSE1gp51 (224
amino acids)
MADVSFPFTVKSQSDLPSAVTSGGVDYVMDQADTRRASKLDTIMTRGMKMVVFSEGGF
LESKDKLAFDNTGKYWTNGVYPKTIPMSSSPSTTGISENAQFEGASGGGSGTGV
VNLGNVQGTATCNLRGDGFSVANLTGGQCQVITTNPSTAQDSQSFITSLQTGTGANLV
SWPSNIKWNYGRVPSLQYKTGVRDFQFVTDGGNSWFGSLIMAGVE

SEQ ID No. 14 - Protein sequence of wt PVPSE1gp69 (918
amino acids)
MADYNKDVKVNAEPIILPEGTGGMGTFVDDGQFHTEDTTTTLGNNSTTPLAAVVDVPA
SGNALVAGPDGLSVGVSPSLDNVMLAGKYVAPPQIVVIPSTKTGNVLVEVTDGEE
GLFYPKPPKGGDGDGKDGQPVGYYPIQIQIGIQIGIDPSQLPAAATMQEGDTYVIGTHYWTVV
KGTWADLDGDFAGPQGQDGIQLVRGAFSDTLLPTQGNTEGDTYIIOQDMWVWWTGADV
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QPGEGQMGAVELGLKDNTTELSTPGEQYQGILIEGDFWGTGTYYENLQIPQPGD
VPGQPGQPGQPIQGIQIQIKGDQGTLWNLSNFRNPAGDPGRIGDYFINKSTLEYFQKTSAT
AWASLGYMGGGMVYDASTIPQARNTAGWVDVPHLEAPDATGGYVVRDNWAAKLDYDL
LVTSTTGAMDGGVSVQFVKDGTTANKTMSFTNLPAANRTIVIVFSGSAGSLTWFPGNLAW
SNGTAVTLGTTRTVTVTILWDGTNTLTTSLTVD
Another preferred embodiment of the present invention provides new polypeptides that bind to *Salmonella* lipopolysaccharide(s) (LPS) comprising an isolated polynucleotide encoding the said polypeptide.

Another preferred embodiment of the present invention provides new polypeptides that bind to *Salmonella* lipopolysaccharide(s) (LPS) comprising the amino acid sequence encoded preferably by an isolated nucleotide sequences of SEQ ID No 1, 2, 3, 4, 5, 6, 7. Namely:

SEQ ID No. 1 - Nucleotide sequence of wt PVPSElgp40 (1815bp)

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atgtaccttatctatgtgctcttcttgacattatcttggaggaggaacagaacacactacc
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SEQ ID No. 2 - Nucleotide sequence of wt PVPSElgp41 (1809bp)
gtgccagacatgactcaatattgaaacggctgtcgatcaggtgtggtgaagactctgaaacgc
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SEQ ID No. 4 - Nucleotide sequence of wt PVFSE1gp48 (1494bp)
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SEQ ID No. 5 - Nucleotide sequence of wt PVPSE1gp50 (2790bp)

atggcacagttacgcacacacatagtcatctggatgctacataca
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The nucleotide sequences coding for the polypeptides can be genetically fused to genes coding for fluorescent, bioluminescent or other biomarker proteins, or to a gene coding for a biotinylatable peptide or alternatively the polypeptides can be chemically conjugated to the above mentioned peptides. These fused recombinant proteins are able to specifically bind to Salmonella strains, to exhibit a fluorescent color (for instance green when excited at 475nm (emission maximum of 505nm)) and/or covalently bind to streptavidin-coated surfaces (which can be surfaces of detection devices or of magnetic beads). The polypeptides or derivatives thereof may additionally comprise of an affinity tag, e.g. histidine-tags, Strep-tags, Avi-tags, Myc-tags, Gst-tags, JS-tags, cysteine-tags, 6 FLAG-tags or other tags known in the art, thioredoxin or maltose binding
proteins (MBP) or other tags able to facilitate or increase protein expression and/or purification. Another preferred embodiment of the present invention provides an isolated nucleic acid molecule encoding the polypeptides. Another embodiment of the present invention relates to a vector (e.g. plasmids or phages, or alike) comprising a nucleic acid molecule according to the present invention. An even more preferred embodiment of the present invention relates to a host cell comprising a nucleic acid molecule according to the present invention or a vector according to the present invention. Another preferred embodiment of the present invention relates to the use of the polypeptide(s), or derivative(s) or a fusion protein or multiple combinations thereof according to the present invention for use as human medical, agro-food, environmental and veterinary diagnostic tool and/or antimicrobial agent. In a more preferred embodiment, said polypeptide(s) or derivative(s) according to the present invention is to be used in the detection/identification of Salmonella spp. in foodstuff, food processing equipment, food processing plants, food processing surfaces, medical devices, surfaces in hospitals and surgeries, human stools, environmental samples or any other samples where Salmonella might be present and also with specific antimicrobial properties, when fused to antimicrobial compounds, in food or on cosmetics, as disinfecting agent or in the environmental field. Another preferred embodiment, said polypeptide(s), a fragment(s) or derivative(s) or a fusion protein according to the present invention is(are) used in the production of a medicament for treatment or prevention of Salmonella spp. infections. Another preferred embodiment relates to the use of said polypeptide(s), a fragment(s) or derivative(s) or a fusion
protein according to the present invention for the treatment or prevention of Salmonella spp. contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries or other places where Salmonella might be present. An isolated nucleic acid molecule encoding the polypeptide according to any one of claims 1 to 7.

Another preferred embodiment relates to a detection tool for recognizing Salmonella spp. comprising one of the polypeptides, or derivatives thereof, or combinations disclosed in the present subject matter; and described but it is not limited in figures 1 and 2. Another preferred embodiment of the present subject matter relates to a kit comprising the polypeptides or derivatives thereof, or combinations disclosed in the present subject matter.

Another preferred embodiment of the present subject matter relates to composition comprising the polypeptides or derivatives thereof described in the present invention. The pharmaceutical composition includes, but is not limited to, the fusion of antimicrobial compounds or cell-wall degrading enzymes and/or penetrating enzymes with polypeptides or derivatives described in the present invention in order to direct the antimicrobial compounds or cell-wall degrading enzyme to specific bacteria, that is, enabling specificity to the said antimicrobial compound or cell-wall degrading enzymes.

DETAILED DESCRIPTION
The following figures provide preferred embodiments for illustrating the description and should not be seen as limiting the scope of invention.

Figure 1 - shows the schematic representation of two examples of detection tools based on the peptides and derivatives covered by the present invention where in:

(Bi) represents a biotin;
(S) represents a streptavidin,
(pei) represents peptide,
(sup) represents solid support,
(B) represents a target bacteria,
(MN) represents a magnetic nanoparticle,
(R) represents a reporter strain,
(C) represents a control,
(Sa) represents a sample,
(BP) represents a biochip platform,
(SE) represents a sensor,
(H) represents an external magnetic field,
(M) represents a label magnetization,
(a) represents a peptide immobilized on biochip platform,
(b) represents a biotinylated peptide immobilized on streptavidin modified magnetic nanoparticles
(1) represents a derivative immobilized on the surface of a microtiter plate;
(2) represents a target bacterium bound to the functionalized solid support;
(3) represents a reporter strain bound to free binding sites and the signal is measured and compared to that of the control
(4) represents a capture of the target bacteria by the biotinylated peptide immobilized on the magnetic nanoparticles;
(5) represents the peptide and target bacteria which are complementary leading to a biomolecular reaction;
(6) represents an external magnetic field that is applied and the sensor detects the fringe field due to label magnetization.

Figure 2 - shows the schematic representation of a non-specific antimicrobial fused to the peptides and derivatives covered by the present invention. where in:

(prep) represents peptide,
(B) represents a target bacterium,
(NB) represents a non-target bacterium,
(AMC) represents an antimicrobial compound,
(7) represents the use of non-specific antimicrobials in the control of Salmonella spp,
(8) represents the use of non-specific antimicrobials fused to a tail protein in the control of Salmonella spp.

Figure 3 - shows the SDS-PAGE analysis of the recombinant expression and purification of PVPSE1gp51. SDS-PAGE analysis shows the elution protein fractions (E1, E2, E3) relative to the protometrics reference marker (M), as well as the flow through (FT) and waste fractions (W). Exp and ExpC are the expression culture before and after concentration by centrifugation respectively. The thick bands around 24 kDa indicate the high purity of the recombinant protein.

Figure 4 - shows the fluorescence intensity measured at 520 nm in the wells coated with different concentrations of PVPSE1gp40 and PVPSE1gp51 in PB (phosphate buffer 0.1 M, pH
7.5; 3.1 g NaH₂PO₄·H₂O, 10.9 g Na₂HPO₄) or SM buffers (sodium magnesium buffer 100 mM NaCl, 8mM MgSO₄, 50 mM Tris-HCl pH 7.5).

where in:
(51SM) represents PVPSE1gp40 in SM buffer,
(51PB) represents PVPSE1 gp40 in PB buffer at 0.1M,
(40PB) represents PVPSE1gp40 in PB buffer at 0.1M.

Figure 5 - shows the fluorescence intensity measured at 520 nm in the wells coated with 200μg/ml PVPSE1gp40 and PVPSE1gp51. Negative control is BSA (5%) and positive controls are phage PVPSE1 (1x10⁹ pfu’s/ml) and Salmonella Polyclonal Antibody.

where in:
(I) represents Samples,
(II) represents BSA 5%,
(III) represents polyclonal antibody anti-Salmonella at 200 μg/ml,
(IV) represents phage PVP-SE1 at 1.0x10⁹,
(V) represents PVP-SE1 gp40 at 200 μg/ml in PB buffer at 0.1M,
(VI) represents PVP-SE1 gp51 at 200 μg/ml in SM buffer.

Figure 6 - shows spots of immobilized PVPSE1gp51 on gold substrates specifically recognizing Salmonella Enteritidis cells (A: PVPSE1gp51 at 200 μg/ml, B: PVPSE1gp51 at approximately 10 mg/ml, C: PVPSE1gp51 at 10 mg/ml) and a Negative control of Salmonella Enteritidis cells detection using a spot of unspecific protein (Bovine Serum Albumin-BSA) (Image D). The Positive Control (E) shows spots of immobilized PVPSE1 phage on gold substrate specifically recognizing Salmonella Enteritidis cells.
Description

If appearing herein, the following terms shall have the definitions set out below.

The term "protein" as used herein refers synonymously to the term "polypeptide".
The term "protein/ polypeptide" as used herein refers to a linear polymer of amino acid residues linked by peptide bonds in a specific sequence. The amino acid residues of a protein may be modified by e.g. covalent attachments of various groups such as carbohydrates and phosphate. Other substances may be more loosely associated with the polypeptide chains, such as heme or lipid, giving rise to the conjugated proteins which are also comprised by the term “protein” as 20 used herein.

The term "fusion protein" as used herein refers to an expression product resulting from the fusion of two nucleic acid sequences. Such a protein may be produced, e.g., in recombinant DNA expression systems. Moreover, the term “fusion protein” as used herein refers to a fusion of a first amino acid sequence as e.g. tail protein, with a second or further amino acid sequence. Preferably, the second or further amino acid sequence is foreign to and not substantially homologous with any domain of the first amino acid sequence.

The term “tag” as used herein refers to a peptide which can be useful to facilitate expression and/or affinity purification of a polypeptide, to immobilize a polypeptide to a surface or to serve as a marker or a label moiety for
detection of a polypeptide e.g. by antibody binding in different ELISA assay formats.

The term “peptide” as used herein refers to short polypeptides consisting of from about 2 to about 100 amino acid residues, more preferably from about 4 to about 50 amino acid residues, more preferably from about 5 to about 30 amino acid residues, wherein the amino group of one amino acid residue is linked to the carboxyl group of another amino acid residue by a peptide bond. A peptide may have a specific function. A peptide can be a naturally occurring peptide or a synthetically designed and produced peptide. The peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)).

The term “tail proteins” as used herein refers to a protein which is able to bind or adsorb to bacterial cell surfaces. Generally, tail proteins are able to bind different components on the surface of bacteria.

The term "wild type" or "wt" as used herein refers to the amino acid sequences of the PVPSE1 predicted tail proteins as depicted in SEQ ID No.: 8 to 14. The nucleic acid sequence encoding the wild PVPSE1 tail proteins are depicted in SEQ ID No. 1 to 7.

The term “derivative” as used herein refers to "fusion protein", an expression product resulting from the fusion
of two nucleic acid sequences or shorter versions of sequences that encode the tail proteins. The derivatives according to the present invention are fusion proteins.

The present invention relates to seven tail proteins of phage PVPSE1. This phage presents a very broad lytic spectrum and thus its tail proteins are useful for the construction of a diagnostic tool for the fast detection of Salmonella spp. and its specific control. The genes encoding for the polypeptides (SEQ ID No. 8 to 14) comprise preferably one of the sequences SEQ ID No.1, 2, 3, 4, 5, 6 7.

The first derivatives are according to the present invention, the polypeptides fused at the N or C terminal of polypeptides to a fluorescent, bioluminescent or other biomarker protein. These derivatives are able to exhibit a fluorescence color (for instance green, when excited at 475nm (emission maximum of 505nm)).

The second derivatives comprise the polypeptides (SEQ ID No. 8, 9, 10, 11, 12, 13, 14) fused to a biotinylated peptide at the N- or C-terminal end. These derivatives are able to simultaneously bind to the bacterium to be detected and to streptavidin-coated surfaces. The biotin-streptavidin bond, once formed, is very resistant to extremes of temperature, pH organic solvents, denaturants, detergents and proteolytic enzymes requiring thus very harsh conditions to break the biotin-streptavidin interaction, conditions that often denature the protein of interest being purified. These features led to the widely use of biotin-streptavidin system in molecular biology and bionanotechnology, namely in the purification and detection
of various biomolecules and to attach various biomolecules to one another or onto a solid support.
The third derivatives are fused with a non-specific peptide or polypeptide with antimicrobial activity at the N or C terminal.

The first and second derivatives can be used directly in the construction of detection tools according, but not limited, to the following examples:
The immobilization of the derivatives on solid supports with streptavidin (see figure 1) through the biotinylated end.

The method developed through this approach consists in adding the sample to the wells of a microtiter plate coated with the derivatives in order to enable the target bacteria to be trapped by the peptides. This step can be repeated to increase the number of bound bacteria and thus sensitivity. Afterwards, wells are washed with buffer to remove unbound bacteria and a suspension with reporter bacterial strains with affinity to the immobilized peptides will then be added. The reporter strain can be a Salmonella strain with affinity to all the peptides of the present invention which must have a reporter gene (e.g. GFP and beta-galactosidase (beta- Gal) but not limited to these). The reporter strains bind to the free available binding surface producing a signal. By comparing this signal with the signal generated from wells saturated with the reporter strains (control) it will be possible to determine the number of target bacteria present in the sample.
The immobilization of the derivatives on magnetic beads coated with streptavidin and to a platform also coated with streptavidin (see figure 1).

The method developed through this approach consists in mixing the functionalized beads with the sample. The target bacteria will bind to the beads. Subsequently, the targeted bacteria from the sample now bound to the beads will bind to the sensor platform via immobilized second derivatives. The detection tool consists of a magnetoresistive sensor that will be used to detect the fringe fields emanating from magnetic nanoparticles that will attach to the platform as described elsewhere (7).

The first derivatives will also be used by direct addition to the sample. The said first derivatives will be used to detect the targeted bacteria present in samples, possibly contaminated with Salmonella spp. When samples are contaminated with Salmonella spp., the said first derivatives will bind specifically to the targeted cells. The number of Salmonella cells specifically bound to the said first derivatives will be determined by direct observation of the sample under epifluorescence microscopy or by the determination of the intensity of the signal with a maximum emission at 475 nm when excited at maximum at 505 nm on a spectrofluorimeter, in case GFP was fused.

The third derivative can be used in medicament and pharmaceutical formulations in the treatment or prevention of Gram-negative bacterial infections, in particular for Salmonella spp. in cosmetic substance or sanitizing agents by increasing the specificity of antimicrobial agents to Salmonella. By fusing the third derivative to antimicrobial compound or protein (either recombinantly or chemically)
the resulting product will be directly guided towards *Salmonella* spp., and as such increase the efficiency and specificity of the antimicrobial compound or protein (Figure 2).

The following example explains the present invention but is not considered to be limiting to the disclosure or the appended claims.


**EXAMPLE 1. Cloning, expression and purification of the tail proteins of the *Salmonella* Enteritis phage PVPSE1**

Purified genomic DNA of phage PVPSE1 was used as a template for the amplification of the seven tail proteins in a standard PCR reaction with Phusion polymerase using the following PCR parameters: Start: 98°C, 2min; followed by 35 cycles of [98°C (20 sec) 50°C (30 sec) 72 °C (1 min)], followed by 72°C (7 min) & cooling to 4°C and storage. Primers used during standard PCR amplification were as follows:

- **ORF 40 Fw**

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GGGCCGGATCCGACGACGACGACAAGATGTATCTATTCCATGTCTTCTTCTTG
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3' - (SEQ ID No. 15)
• ORF 40 Rev 5' GGGCCGAGCTCTTTATGGCGTTGAGAAGGTACA 3' (SEQ ID No. 16)

• ORF 41 Fw 5' GGGCCGGATCCGACGACGACAAGTGTCGCGACATGACTCAATTTG 3' (SEQ ID No. 17)

• ORF 41 Rev 5' GGGCCGAGCTCTTTACGGACGAAGCACAATTACAG 3' (SEQ ID No. 18)

• ORF 46 Fw 5' GGGCCGGATCCGACGACGACAAGATGGCAGCCGACAACAGTAC 3' (SEQ ID No. 19)

• ORF 46 Rev 5' GGGCCGAGCTCTTATGCGATGATTGAAACAGTTAC 3' (SEQ ID No. 20)

• ORF 48 Fw 5' GGGCCGGATCCGACGACGACAAGGTTGGCTGCTCAATATGGATTTAA 3' (SEQ ID No. 21)

• ORF 48 Rev 5' GGGCCGAGCTCTTTAGATGATTGGCTGGAATGTATGT 3' (SEQ ID No. 22)

• ORF 50 Fw 5' GGGCCGGATCCGACGACGACAAGATGGCAGACCTACGCCACA 3' (SEQ ID No. 23)

• ORF 50 Rev 5' GGGCCGAGCTCTTTATCTTTGTAAGGGTTCTGTACATAG 3' (SEQ ID No. 24)

• ORF 51 Fw 5' GGGCCGATCCGACGACGACAAGATGGCAGATGTTCTTTTCCAACG 3' (SEQ ID No. 25)

• ORF 51 Rev 5' GGGCCGAGCTCTACTCAACTCTCTGATCCATAATAAGG 3' (SEQ ID No. 26)

• ORF 69 Fw 5' GGGCCGATCCGACGACGACAAGATGGCAGATGATAATTACAAGGTGC 3' (SEQ ID No. 27)
• ORF 69 Rev 5' GGGCGAGCTCTTAATCGACAGTCAAAGAGGTGTAC 3' (SEQ ID No. 28)

The obtained PCR fragments were then ligated in the commercial available pET28a(+) expression vector (Novagen), which was previously provided with the gene, encoding a fluorescent, bioluminescent or other biomarker protein and/or a biotinylated peptide, following digestion with BamHI and SacI restriction enzymes of both vector and PCR fragment. Ligation was carried out by adding the vector, PCR fragment, T4 ligase (New England Biolabs) and respective buffer followed by incubation at 16°C for 2 hours.

The obtained DNA and amino acid sequences for the recombinant tail proteins are listed in SEQ ID No. 1 to 7 and SEQ ID No. 8 to 14, respectively. Recombinant expression of tail proteins was performed in exponentially growing E. coli BL21 (DE3) pLysS cells after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 to 18 hours. The tail proteins were then purified by Ni2+ affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pET28a(+) expression vector. The Ni2+ affinity chromatography is performed in 4 subsequent steps, all at room temperature:

1. Equilibration of the Histrap HP 1 ml column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH2PO4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. Loading of the total lysate (with the wanted tail protein) on the Histrap HP 1 ml column at a flow rate of 0.5 ml/min.
3. Washing of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. Elution of bound tail protein from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH2PO4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.

The yield for the purification of recombinant tail protein per liter E. coli expression culture is determined by spectrophotometric measurement at 280 nm. Purified stock solution of recombinant tail protein in Elution Buffer (20 mM NaH2PO4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) is determined visually on an SDS-PAGE gel (see Figure 3). The protein concentration is determined spectrophotometrically at a wavelength of 280 nm.

Example 2 Binding ability of the tail proteins of the Salmonella Enteritidis phage PVPSE1 by immunofluorescence

The tail proteins prepared as described in Example 1 (at concentrations of 100µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500 µg/ml in PB (phosphate buffer 0.1 M, pH 7.5; 3.1 g NaH2PO4. H2O, 10.9 g Na2HPO4) or SM (sodium magnesium buffer 100 mM NaCl, 8mM MgSO4, 50 mM Tris-HCl pH 7.5) and the positive/negative controls were immobilized onto a polystyrene microtiter plate by passive adsorption and incubated during 2 hours at room temperature, followed by a three times washing procedure with PB or SM. Positive controls consist of Salmonella spp. Polyclonal Antibody (200 µg/ml) and PVPSE1 (phage stock at a concentration 1.0x10^10 pfu's/ml). Negative controls consisted of bovine serum albumin (BSA) 5% in PB and PB or SM.

After the immobilization of tail proteins, 1% of BSA in PB was added as a blocking agent to each well followed by a 40
min incubation at room temperature and a three times washing with PB.

One hundred milliliter of an exponential culture of *Salmonella enteritidis* S140013 grown in Luria Broth (37°C, 120 rpm) until an optical density at 600 nm of 0.4, which corresponds to 1.0x10^9 cells/ml, was added to each well followed by a 1-hour incubation period at room temperature. After a washing step with PB to remove unbound bacteria, the wells were filled with 100 µl of *Salmonella* spp. Polyclonal Antibody, Biotin conjugated at 200 µg/ml (Thermo Scientific, ref PA1-73022) and incubated during 1 hour. After, the wells were washed with PB and filled with 100 µl Streptavidin Rhodamine conjugated (TRITC, Pierce, ref 21724) at 20 µg/ml and incubated 40 minutes at room temperature. After several washes with PB, a volume of 100 µl of PB was added to each well and the fluorescence was measured by a Synergy H1 microplate reader at 520 nm of excitation and at 570 nm of emission.

The results showed that the buffer and protein concentration influence the signal intensity and thus these two variables need to be optimized for each tail protein. For example, the gp40 presents a higher signal in PB at 200 µg/ml, while gp51 gives better results in SM at 200 µg/ml (Figure 4).

Comparison of the fluorescence intensity obtained with the peptides, the entire phage PVpSE1, *Salmonella* Polyclonal antibody and the negative control (Figure 5) showed that the tail proteins present the same signal intensity of a *Salmonella* Polyclonal antibody and of the entire phage particle showing the high binding efficiency of said proteins.
Example 3 Binding affinity of tail proteins immobilized on gold substrates

Au substrates (7x7 mm) were incubated for 2 hours in Microstrip® 3001 (Fujifilm Electronic Materials, Belgium) at 65 °C for removal of the protective coating of photoresist polymer. The substrates were then washed three times with isopropanol and sterile distilled water and dried under N₂. After the cleaning step, the tail proteins at a concentration of 200 µg/ml, prepared in SM buffer, were immobilized by physical adsorption on the Au substrates in spots of 1 µl and incubated four hours at room temperature in a humidified atmosphere, in order to prevent evaporation. The unbound tail proteins were removed by washing three times with SM. The spots were then incubated with the blocking agent BSA 1% prepared in TE during 45 minutes and the excess of blocking solution was removed by washing the substrate with PB (0.1M, pH=7.5). All substrates were exposed to 2.8x10⁹ cells/ml during 1 hour and washed with PB (0.1M, pH=7.4) and distilled water. The bacterial coverage extent in spot and out spot was visualized by a Nikon SMZ 1500 microscope. Results are showed in figure 6 and were consistent with the results reported in Example 2, the tail protein concentration influences its binding ability, moreover the binding ability of the tail protein seems equal to that of the entire phage PVPSE1.

Reference List


its production in a nonpathogenic Escherichia coli strain. Applied and Environmental Microbiology, 76(21), 7338-7342.

The invention is of course not in any way restricted to the embodiments described and a person with ordinary skill in the art will foresee many possibilities to modifications thereof without departing from the basic idea of the invention as defined in the appended claims.

The following claims set out particular embodiments of the invention.
CLAIMS

1. An isolated polypeptide having the sequence of one of SEQ ID No 8, 9, 10, 11, 12, 13, or 14, or derivatives thereof, or combinations thereof, for recognizing or binding to Salmonella.

2. The polypeptide according to claim 1, wherein the polypeptide is fused with a biomarker protein at the N- or C-terminal end.

3. The polypeptides according to the previous claim wherein the said biomarker protein is a colored fluorescent protein or a bioluminescent protein, preferably GFP.

4. The polypeptides according to the previous claims, wherein the polypeptides are fused with a biotinylated peptide at the N- or C-terminal end.

5. The polypeptides according to the previous claims, wherein the polypeptides are fused to a compound having an antimicrobial activity at the N- or C-terminal end.

6. The polypeptide according to any of the preceding claims further comprising a tag.

7. The polypeptide according to the previous claim wherein the said tag is selected from histidine-tags, Strep-tags, Avi-tags, Myc-tags, Gst-tags, JS-tags, cysteine-tags, 6 FLAG-tags, and/or thioredoxin or maltose binding proteins.
8. The polypeptide according to any one of the preceding claims for use in medicine or as medicament.

9. The polypeptide according to the previous claim for use in treatment or prevention of Salmonella spp. infections.

10. The polypeptide according to any one of the claims 1 to 7 for use in the treatment or prevention of bacterial contamination, particularly of Gram-negative contamination, of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, surfaces in hospitals and surgeries, of environmental samples.

11. An isolated polynucleotide encoding the polypeptide according to any one of the claims 1-7.

12. An isolated nucleic acid molecule having the sequence of one of SEQ ID No 1, 2, 3, 4, 5, 6, 7.

13. An isolated nucleic acid molecule encoding the polypeptide according to any one of claims 1 to 7.

14. A vector comprising the nucleic acid molecule according to any one of the claims 11-13.

15. A host cell comprising the nucleic acid molecule according to claim 11-13 or the vector according to claim 14.

16. A detection tool for recognizing Salmonella spp. comprising one of the polypeptides, or derivatives
thereof, or combinations thereof according to claims 1 to 7.

17. A kit comprising the polypeptides or derivatives thereof according to any one of the claims 1 to 7.

18. A pharmaceutical composition comprising the polypeptides or derivatives thereof described in any one of claims 1 to 7.
Figure 1
INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2012/054113

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/64 C12Q1/70

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.

See patent family annex.

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search
26 November 2012

Date of mailing of the international search report
03/12/2012

Name and mailing address of the ISA/Authorized officer
European Patent Office, P.B. 5618 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Fax: (+31-70) 340-3016
Scheffzyk, Irmgard
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