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(54) **NEW STRAIN OF SALMONELLA ENTERICA S. TYPHIMURIUM, ITS USE AND A METHOD TO OBTAIN A THERAPEUTIC VACCINE VECTOR**

NEUER SALMONELLA ENTERICA S. TYPHIMURIUM STAMM, DESSEN VERWENDUNG SOWIE EIN VERFAHREN ZUR HERSTELLUNG EINES THERAPEUTISCHEN VEKTOR-IMPFSTOFFES

NOUVELLE SOUCHE DE SALMONELLA ENTERICA SEROVAR TYPHIMURIUM, SON UTILISATION, ET PROCÉDÉ DONNANT UN VECTEUR DE VACCINATION THÉRAPEUTIQUE

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Description

[0001] The present invention relates to the field of pharmacy, particularly the preparation of vaccines, especially of anti-cancer bacterial therapeutic vaccines.

[0002] Bacteria belonging to the genus Salmonella are Gram-negative bacteria from the *Enterobacteriaceae* family which are disease-causing in humans and animals, and represent a serious medical and veterinary problem.

[0003] Vaccination with live, attenuated strains of *Salmonella* is an effective way to prevent the infection. Attenuated strains of *Salmonella* were obtained by introducing specific irreversible mutations into certain genes in the chromosome of *Salmonella,* such as: *aroA* (1), *aroC* (2), *surA* (3), *htrA* (4), *rpoS* (5), and *galE* (6).

[0004] Current methods of *Salmonella* attenuation which are used to obtain vaccine material are based on the introduction of genetically stable deletions that reduce or eliminate the virulence of bacteria, while they do not change or intensify their immunogenicity.

[0005] Through attenuation of wild strain *Salmonella typhi,* a new strain Ty21a was created and used for vaccination against typhoid fever. Attenuation of this strain has been achieved through a number of mutations induced by chemical mutagens. This has resulted in a strain that is sensitive to galactose (a mutation in *galE* gene), auxotrophic for isoleucine and valine (mutations in *ilvD* genes), has reduced resistance to stress (a mutation in *rpoS*), and is also unable to produce the Vi polysaccharide. The plurality of mutations makes the strain genetically stable and safe. Revertant mutations within the virulence genes were not observed either *in vitro* or *in vivo* (7, 8).

[0006] The strain CVD908 has clearly defined mutations in the genes *aroC* and *aroD*, but causes fever and other adverse reactions in volunteers vaccinated with high doses. Further attenuation of this strain by the deletion of *htrA* gene, which encodes a serine protease essential for bacterial survival in macrophages, led to strain CVD908-htrA, which is well tolerated even at high doses and shows high immunogenicity (9).

[0007] Mutants of *Salmonella enterica* serovar Typhimurium, lacking the transcription regulator RfaH effectively prevent salmonellosis when used to vaccinate mice. Lack of RfaH affects the expression of genes involved in the synthesis of lipopolysaccharide core and O-antigen. Such mutants do not differ in their ability to proliferate, but show increased susceptibility to antibacterial peptides (10).

[0008] Due to the specificity of preventive vaccines, aiming to elicit immune memory after administration, their activity may be based on the ability of attenuated bacteria to stimulate a humoral immune response (production of neutralising antibodies). The generation of an effective humoral response does not require the ability to invade host cells by *Samonella* sp. vaccines. This means that even relatively large deletions in the chromosome of *Salmonella* sp. may be tolerated in vaccinting material prepared for use as a preventive vaccine. However, the relative ease of attenuation does not always coincide with the retention of the original (effective) immunogenicity.

[0009] A therapeutic vaccine vector should be highly attenuated and possibly poorly immunogenic to provide effectiveness of repeated administration into a patient. In the case of conjugated heterogeneous (allo- or xenogenic) antigens (epitopes), the vector should demonstrate adjuvant properties (while maintaining its low immunogenicity). A therapeutic vector should be capable of stimulating a specific type of immune response, that is to induce a humoral (Th2) or cellular (Th1) response as needed.

[0010] The VNP20009 strain was developed as a vaccine vector whose task was to deliver non-toxic cytostatic drug precursors (prodrugs) to tumour tissue (9, 10). Deletions within the genes *purI* and *msbB* result in auxotrophism of the strain with a reduced capacity to stimulate TNF in the infected organism. Preliminary studies have shown the ability of preferential accumulation of VNP20009 within tumours in mice (11). VNP20009 was also found to have an inhibitory effect on tumour development. Phase I clinical studies have not confirmed the effects observed in small mammals. So far, there are no studies on the immunogenicity of VNP20009 in mice nor humans (12).

[0011] Surface expression of the variable fragment of a CEA-specific antibody (T84.66) improved the ability of selective accumulation of bacteria in malignant tumours (13). However, the stress caused by overexpression of the fusion protein OmpA-scFv (CEA-specific) substantially (100-fold) reduced the invasiveness of transformed VNP20009 (designated VNP/scFv). Despite the reduced invasiveness, an increase in therapeutic effects was observed for VNP/scFv versus VNP20009 in murine models of transplantable tumours stably transfected with the human *CEA* gene (MC38CEA and CT26CEA adenocarcinomas). Another reason for the reduced effectiveness of VNP/scFv is probably genetic instability of the strain involving the loss of the plasmid (and hence the loss of ability to express OmpA-scFv) in the absence of antibiotic selection pressure. Reversion to wild type VNP20009 in the patient would not promote the targeting of the bacteria towards CEA-rich sites (tumour tissue).

[0012] In the case of a *Salmonella* sp., vector it is important to retain the invasive features of the bacteria, which are related to the quality of the immune mechanisms stimulated by those bacteria.

[0013] There is still a great need to develop expertise in the field of therapeutic vaccines. A particularly important issue is the impact of attenuation-related effects on the strength and type of immune response dependent on the type of microorganism.

[0014] The purpose of this invention is to provide an effective vaccine vector, particularly effective for the treatment

of cancer.

[0015] The aim of the present invention is therefore to obtain the appropriate strain of *Salmonella* sp., pursuant to demand in the field of therapeutic vaccines as a vector for delivery of therapeutic material to target cells, particularly cancer cells, cells infected with a virus, etc., with immunogenicity resulting from the physiological intracellular location of bacteria.

[0016] The subject of the present invention is a strain of *Samonella enterica* serovar Typhimurium VNP20009 deposited in the Polish Collection of Microorganisms under access no. B/00024. Another subject of the present invention is the use of the strain of *Samonella enterica* serovar Typhimurium VNP20009 deposited in the Polish Collection of Microorganisms under access no. B/00024 to produce a vaccine, especially anti-cancer vaccine.

[0017] Another subject of the present invention is a method of obtaining a therapeutic vaccine vector, characterized in that a genetic modification is introduced into the vector strain *Salmonella enterica* serovar Typhimurium VNP20009 specific to cancer cells, resulting in a delayed overexpression of a gene encoding a protein responsible for the invasive features of this strain, wherein an expression cassette is obtained, containing sipB gene under the control of PsifB promoter that controls its delayed overexpression, and then the cassette is integrated into the bacterial chromosome.

[0018] Preferably, the expression cassette is integrated into the bacterial chromosome is the cassette *PsifB-sipB* according to SEQ ID 1 or the cassette *ushA*-5'-*PsifB-sipB-ushA*-3' according to SEQ ID 2.

[0019] In a preferred embodiment of the present invention, the method includes an additional final step of removing a gene for antibiotic resistance from the bacterial genome.

[0020] The subject of the present invention in an embodiment is made clear in the figure, in which:

Figure 1A shows the electrophoretic separation of a PCR product in 1% agarose gel in the presence of ethidium bromide for the *sipB* gene sequence (1782 bp) obtained from the genomic DNA of a reference strain, *S. typhimurium* SL5319;
Figure 1B shows the results obtained using Western blotting confirming the functionality of the cloned *sipB* gene;
Figure 2 shows the results obtained as a result of the isolation of the *sifB* gene promoter and the results of functionality assays of the *PsifB*-GFP construct:
Figure 2A shows the image of the electrophoretic separation of the PCR product in 1.2% agarose gel in the presence of ethidium bromide;
Figure 2B shows a microscopic image of the RAW264.7 macrophage cell line infected with VNP20009 transformed with the pBR322-*PsifB*-GFP plasmid;
Figure 2C shows the results of the cytofluorimetric analysis of bacterial cultures. *VNP20009*/*PsifB-sipB-GFP:* induction of the expression of the SipB-GFP fusion protein (right panel), GFP-negative bacteria (left panel);
Figure 3 shows a diagram of the integration of the plasmid into the genome and DNA fragment exchange, stimulated by the cleavage of both DNA strands;
Figure 4 shows the relative position of the *sipB* gene sequence, *PsifB* promoter and the insertion site of the *PsifB-sipB* cassette (*ushA* gene) in the genome of *Salmonella typhimurium.* LT2;
Figure 5 shows a diagram of the pSG76C plasmid including cloned cassette *ushA-5'-PsifB-sipB-'ushA-3';*
Figure 6 shows a diagram of the genomic sequence generated through the recombination of the *ushA* gene with a homologous region 5'-*ushA* of the plasmid. Primer sequences used for the analysis of chloramphenicol-resistant clones were indicated on the figure;
Figure 7 shows the electrophoretic separation of RT-PCR products in 1% agarose gel in the presence of ethidium bromide: DNA of the mutated clone was amplified with the following primers: 1 - A and B (2350 bp) 2 - A and G (3755 bp) 3 - A and F (4560 bp) 4 - A and D, 5 - C and D (2411 bp); M - mass standard;
Figure 8 shows the images obtained by electrophoretic separation of RT-PCR products in 1% agarose gel in the presence of ethidium bromide:

- in Figure 8A the image of the product of cDNA amplification with primers specific for *PsifB-sipB* (1222 bp): 1 - VNP20009, 2 - modified VNP20009, M - mass standard;
- in Figure 8B the image of the the product of cDNA amplification with primers specific for *sipB* (389 bp; on the upper panel): 1 - VNP20009, 2 - modified VNP20009; product of cDNA amplification of 16S rRNA (350 bp; on the bottom panel); M - mass standard;

Figure 9 shows the image of electrophoretic separation of the PCR product with primer sequences complementary to sequences flanking the *ushA* gene performed on the genomic DNA of a clone obtained through the procedure of *PsifB-sipB* integration into the VNP20009 chromosome; 1 - amplification product with primers E and D (1526 bp), 2 - product amplification with primer A and D (4709 bp), M - mass standard;
Figure 10 shows a graph representing the results of the invasion of RAW264 cells by VNP20009 and VNP/sipB;
Figure 11 shows the results of an experiment performed on lung metastasis model CT26CEA in Balb/c mice. The

upper row shows the lungs of the control mice isolated on day 15 after the intravenous administration of 5 x 10^5 CT26CEA cells. White spots visible on the dark background are small tumour foci. The bottom row shows lungs explanted from mice, which were administered *Salmonella typhimurium* VNP/sipB bacteria intranasally in the amount of 2 x 10^7 CFU/mouse 48 hours after the injection of cancer cells;

Figure 12 presents the result of an experiment performed on lung metastasis model CT26CEA in Balb/c mice. The upper row shows lungs of the control mice isolated on day 15 after intravenous administration of 5 x 10^5 CT26CEA cells. White spots visible on the dark background are small tumour foci. The bottom row shows lungs taken from mice, which were administered various modifications of *Salmonella typhimurium* intranasally in the amount of 2 x 10^7 CFU/mouse 96 hours after the injection of cancer cells; the mean numbers of tumour foci in the lungs are shown on the graph in the lower part of the figure.

Figure 13 shows the results of an experiment performed on lung metastasis model B16F10 in C57B1/6 mice. The upper row shows lungs of the control mice isolated on day 28 after intravenous administration of 5 x 10^5 B16F10 cells. Tumour sites are visible as dark spots.

The middle and lower rows shows lungs isolated from mice, which were administered VNP (middle row) or VNP/sipB (bottom row) bacteria intranasally in the amount of 2 x 10^7 CFU/mouse 96 hours after the injection of cancer cells;

Figure 14 shows the effect of antibody isotype skewing caused by selective stimulation of a Th1-type immune response by VNP/sipB. The results were produced by vaccination of Swiss mice (outbred) with VNP20009 strains: mice vaccinated with "wild type" VNP20009 (upper panel), mice vaccinated with VNP/sipB (lower panel);

Figure 15 shows the result of DNA sequencing performed on a recombinant genomic DNA fragment of the modified strain of *Salmonella enterica* s. Typhimurium VNP20009:

Figure 15A shows a DNA sequence, which includes sequences flanking *ushA* gene and complete sequence of the integrated *PsifB-sipB* construct that in VNP/sipB bacteria is located within the *ushA* gene.

Figure 15B presents the results which confirm the identity of the sequence from VNP/sipB with template DNA from a model strain of *Salmonella typhimurium* LT2. Lower strand: template (lower strand of DNA), the location of the each element of the construct is indicated (*sifB* promoter, *sipB* ORF, *ushA* integration site) in accordance with the numbering of LT2 strain deposited in GenBank (NCBI). Upper strand: marked as "Query", shows the sequence obtained from the VNP/sipB.

Figure 16 shows the sequences of primers used in the sequencing of genomic DNA. A schematic location of the primers is indicated in Figure 15 within the flanking sequences and the integrated construct.

Figure 17 shows the Apoptosis of VNP20009- or VNP/sipB-infected MC38CEA cells, measured as a percentage of annexin V binding cells. Bacteria used for infection were expressing RFP. Annexin V-APC fluorescence was analyzed in RFP-positive population of MC38CEA cells.

[0021] In the present invention the VNP/sipB vector was obtained with the full retention of invasive ability (Fig. 10).

[0022] The prepared vaccine vector utilised the natural ability of *Salmonella* sp. to infect host cells. The invasiveness of *Salmonella* sp. is a multifactorial process, which involves components of both the bacterial cells and the host cells (14). Adhesion of bacteria to a host cell causes the activation of Type III Secretion Systems genes (TTSS) grouped into the "*Salmonella* Pathogenicity Island-1" (SPI-1). Products of those genes, including the SipB protein, allow bacteria to penetrate the cell (15). Expression of another set of genes is activated inside the host cell. Those genes are located within the SPI-2 and their products facilitate the proliferation of the bacteria within the cytoplasm. The expression of SPI-2 genes is regulated by interaction of endoplasmatic factors with the SPI-2 promoters. The promoter of the *sifB* gene is one of those promoters (16).

[0023] Proliferation of the bacteria in the cytoplasm leads to apoptosis of the host cell, which in turn allows the bacteria to escape and subsequently infect other cells. One of the factors that induce apoptosis is the already mentioned SipB protein (14).

[0024] It was assumed that delayed (by using the promoter of *sifB* gene) overexpression of SipB (*PsifB-sipB*) will result in intracellular elimination of infective bacteria with simultaneous apoptosis/necrosis of the infected cells. Interim infection will form a strong signal for leukocyte migration to the infection site, but will not result in the infection of the population of newly-migrating immune cells.

[0025] The result produced by such a vaccine vector will be the eradication of tumours through the concerted action of the bacteria and the immune system.

[0026] The present invention is illustrated by the following embodiments:

Example 1: Cloning of the *sipB* gene

[0027] The sequence of the *SipB* gene (1782 bp) (Fig. 1A) was obtained from the genomic DNA of a reference strain *S. typhimurium* SL5319 using a PCR technique with the following primers:

5'AACTGCAGAACCAATGCATTGGTTTCTCCCTTTATTTTGGCA
3'CGGGATCCCGAAGTAGCATTAGCCGTAGCG,

which contain restriction site *Pst*II *Bam*HI.

cDNA for *sipB* was cloned into the expression cassette of plasmid pQE30 to obtain a sequence encoding the fusion protein RGS-6His-SipB. The correctness of the *sipB* sequence was confirmed by sequencing of both strands of *sipB* cDNA.

[0028] The functionality of the cloned *sipB* gene was confirmed using a standard Western blot technique (Fig. 1B) performed on lysates of *E. coli* M15 incubated in the presence of IPTG (0.5 mM), using anti-His-tag antibodies.

[0029] The results of Western blot analysis showed that the expression of SipB with an inducible promoter was toxic to bacteria, as induced in the *E. coli* M 15 strain (containing the plasmid p*REP*-4 with the *lacI* gene encoding the repressor protein). Protein expression was induced with 0.5 mM IPTG in *E. coli* M15 grown in TB medium at 25°C up to an optical density of OD600≈1.0. Bacterial lysate was prepared from 120 ml of culture after a 2.5-hour induction and was purified on an IMAC chromatography resin, TALON BD. The collected fractions were separated with SDS-PAGE on 10% gel and the blots were probed with α-RGS-6His antibodies (Qiagen).

[0030] Molecular weight of the obtained product corresponded to the mass of SipB protein (62 kDa).

Example 2: Isolation of *sifB* gene promoter and construction of *sipB* gene controlled by *PsifB*

[0031] The coding sequence of the promoter region of *sifB* gene was obtained through PCR on genomic DNA of *Salmonella typhimurium* VNP20009, using the following primers: "forward" **CCCAAGCTTGGGCCTTAGCCATTCT-GACTG** with a *Hind*III restriction site and "reverse" **GAAGATCTTCACTTCATTACTGGAATAGGTGGT** with a *Bgl*II restriction site. Concurrently, a sequence encoding GFP was obtained from the pGFPuv plasmid (Clontech) using PCR and the following primers: "forward" **GAAGATCTTCTCACACAGGAAACAGCTATGAC** with a *Bgl*II restriction site and "reverse" **GAAGATCTTCGCGCTCAGTTGGAATTCA,** also with a *Bgl*II restriction site. PCR products were cloned into the plasmid pGEM-TEasy (Promega) and propagated in *Escherichia coli* DH5α. Upon confirmation of sequence identity with a sequence obtained from GenBank database, the *gfp* gene was cloned into the *BglII* restriction site of the pGEM-TEasy-*PsifB* plasmid, which had been previously obtained from plasmid pGEM-TEasy-*gfp*. Intracellular induction of the *PsifB* promoter was confiirmed *in vitro* by infection of the RAW264.7 macrophage cell line with VNP20009 bacteria containing the pGEM-TEasy-*PsijB-gfp* plasmid, with the *gfp* sequence cloned in forward or reverse orientation to the promoter. The *sipB* coding sequence with the RBS and 6His sequences was obtained from the pQE-*sipB* plasmid using PCR and the following primers:

"forward" **GGAAGATCTTCCAGAGGAGAAATTAACTATGAGA**
,
"reverse" **GAAGATCTTCGGAGTCCAAGCTCAGCTA**

(both primers with a *Bgl*II restriction site).

[0032] The PCR product was cloned into the pGEM-TEasy-*PsifB* plasmid into the *BglII* site. *PsifB-sipB* cassette was excised from pGEM-TEasy-*PsifB-sipB* plasmid with the restrictase *Not*I and after filling the sticky ends, it was cloned into a low-copy pBR322 plasmid (in *Eco*RV-*Nru*I cloning site) and into pMoPac2-*lpp-ompA-scFv* plasmid.

[0033] Figure 2 presents the results of *sifB* gene promoter isolation and functional tests of *PsifB-GFP* construct.

[0034] Figure 2A shows an electrophoretic image of PCR product separation in 1.2% agarose gel in the presence of ethidium bromide. The coding sequence of the promoter region of *sifB* gene (603 bp) was obtained using PCR from the genomic DNA of *Salmonella typhimurium.* The identity of the sequence was confirmed with the sequence obtained from GenBank (*Salmonella typhimurium* LT2, 1 691 572 - 1 692 152 bp). To confirm the functionality of the obtained promoter sequence, a transcriptional fusion protein *PsifB*-GFP was produced (*gfp* sequence amplified from the pGFPuv plasmid (Clontech) using PCR). Concurrently, the *RBS-RGS-6His-sipB* sequence was obtained from plasmid pQE30-*sipB* via PCR.

[0035] Figure 2B shows a microscopic image obtained of the RAW264.7 macrophage cell line infected with VNP20009 transformed with the pBR322-*PsifB-gfp* plasmid. Induction of the *PsifB* promoter in intracellular bacteria was evaluated microscopically. VNP20009 transformed with low-copy plasmid (pBR322) or high-copy plasmid (pGEM-TEasy), both including the *PsifB-gfp* cassette, did not demonstrate any microscopically detectable GFP expression when grown in TB medium.

[0036] Figure 2C presents the results of cytofluorimetric analysis performed on the bacterial culture. VNP20009/*PsifB-sipB-gfp* (but not *E. coli* DH5α/*PsifB-sipB-gfp*), cultured for 12 hours at 30°C with shaking (180 RPM) in eukaryotic cell culture medium OPTIMEM (Invitrogen), showed the induced expression of the fusion protein SipB-GFP (right panel).

The same bacteria cultured in TB were GFP-negative (left panel).

[0037] The use of plasmid constructs is limited *in vivo* due to the frequent loss of plasmids from bacteria in the absence of selection pressure (antibiotics). Yet genetic stability is one of the fundamental features of bacteria that allows their use as vaccine material. A method of obtaining genetically stable bacterial strains is the integration of functional cassettes (promoter-gene) into the bacterial chromosome.

Example 3: Integration of the *PsifB-sipB* expression cassette into the VNP20009 chromosome

[0038] In order to obtain a genetically stable, attenuated strain of *Salmonella typhimurium* with the overexpression of endogenous SipB protein induced inside an infected cell, the *PsifB-sipB* expression cassette was integrated into VNP20009 genome. Integration was performed by the homologous recombination method, based on the natural recombination and repair system of the bacteria (Rec-A protein activity) and using a conditionally replicating plasmid as a vector for delivering a mutant allele into genomic DNA (17). At a temperature not permitting multiplication of the plasmid, clones which integrated the plasmid (as a result of recombination with wild and mutant copies of the gene) and acquired chromosomal resistance are selected in the presence of an antibiotic. At this stage, homologous regions of the modified gene are duplicated in the genome. In the next stage, an exchange occurs between the duplicated sequences, stimulated by cleavage of DNA sequences within the plasmid sequence present in the genome. The result is a return to the wild allele form or replacement into the mutated form, with simultaneous excision of the antibiotic resistance gene. A diagram of gene exchange by recombination of homologous segments stimulated by DNA cleavage is shown in Figure 3.

[0039] The region of the *ushA* gene (STM0494) was chosen as the target site for integration, as it has a high homology with the *ushA* gene of *Escherichia coli,* but very low expression and low activity of the encoded enzyme in *Salmonella typhimurium* (UDP-glucose hydrolase with a point mutation, "silent gene"). The *ushA* gene has been inactivated in strains of *S. typhimurium* and its active functional homologue is *ushB.* The DNA sequences of these genes do not show a significant homology (18, 19). Considerable distance between *ushA* and sequences *PsifB* and *sipB* in *S. typhimurium* genome (Fig. 4) should reduce the genomic instability of the modified strain, associated with the introduction of additional copies of those sequences, which may be a substrate for homologous recombination.

[0040] A conditionally replicating plasmid, pSG76-C, was used for the integration. The *PsifB-sipB* sequence was cloned into the plasmid, flanked with regions of homology, i.e. segments of sequence identical to the target sequence of integration within the bacterial chromosome (5'-*ushA* and 3'-*ushA*); chloramphenicol resistance gene for selection of clones, in which crossing-over occurred; *ori* R6Kγ and an extremely rare restriction site for endonuclease I-*Sce*I (Fig. 4).

[0041] Replication of the pSG76-C plasmid requires protein Π, supplied with an auxiliary plasmid pPIR-A (with thermosensitive *ori* pSC101). Plasmid *pSG76C-ushA-PsifB-sipB* can be built into the genome by a single crossing-over involving a region of homology and the corresponding chromosomal region. At a temperature preventing the replication of the plasmid pPIR-A (37-42°C), and thus also plasmid pSG76C, in the presence of the antibiotic, there is a selection of clones with the plasmid sequence integrated into the genome. At this stage, flanking regions of homology are duplicated in the genome. Then, expression of I-SceI meganuclease is induced from the pSTKST auxiliary plasmid. The cleavage of both DNA strands within the integrated plasmid sequence stimulates the Rec-A-dependent intramolecular recombination (double strand break-stimulated gene replacement) - a repair of the DNA break occurs, with the use of adjacent flanking regions of homology. Single crossing-over could happen with the participation of the regions of 5'-*ushA* or 3'-*ushA.* In the first case the region of integration returns to the wild form (restoration of the *ushA* gene sequence integrity); in the second case, a productive re-arrangement occurs, with simultaneous removal of the antibiotic resistance gene. A modified bacterial strain is obtained as a result, without any selection marker in the form of antibiotic resistance gene or any other exogenous sequence.

Example 4: Cloning the *ushA-5'-PsifB-sipB-ushA-3'* cassette into the pSG76-C plasmid

[0042] Complementary DNA for *ushA* was obtained from the genomic DNA of VNP20009 using PCR and the following primers:

forward FushA GGGGTACCCC**GCGATGTTGGAGATAGTAGG,**
reverse RushA GGGGTACCCC**TACAGCCAGCTCACCTCA**,

both containing a restriction site for the enzyme *Kpn*I. The PCR product (1825 bp) was cloned into the pGEM-T*Easy* plasmid (Promega) and propagated in *Escherichia coli* DH5α. Upon confirmation of sequence identity with a sequence obtained from the GenBank database, the *PsifB-sipB* sequence (obtained from pGEM-TEasy-*PsifB-sipB*) was cloned into the restriction site *Hpa*I, located within *ushA.* The orientation of the *PsifB-sipB* sequence was the reverse of the orientation of the *ushA* gene.

[0043] In the thusly obtained construct, the *PsifB-sipB* sequence is flanked with a 1091 bp fragment of *ushA* at 5'-end

and a 732 bp fragment at 3'-end. Then, the *ushA*-5'-*PsifBsipB-ushA*-3' cassette was cut out with *Kpu*I enzyme and cloned into pSG76-C in *Kpn*I site, yielding pSG76C-USS (Fig. 5).

Example 5: Integration of the pSG76C-USS plasmid into VNP20009 chromosome

[0044] The plasmid was amplified in *Escherichia coli* DH5α.pir (with the genomic copy of *pir* gene, encoding n protein) and transformed into the VNP20009 strain (by electroporation), which had been previously transformed with the pPIR-A plasmid. Then, the VNP20009 bacteria were cultured on solid medium as follows: 40 hours at 30°C with ampicillin and chloramphenicol, 5 hours at 30°C with chloramphenicol, 17 hours at 42°C with chloramphenicol and 7 hours at 37°C with chloramphenicol, for the selection of clones that have acquired the chromosomal antibiotic resistance. Among the transformants resistant to chloramphenicol, 5 large colonies of bacteria were selected, transferred to solid medium with chloramphenicol and cultured for additional 20 hours at 37°C. Then the selected clones were tested for integration of the plasmid into chromosome, that is, whether the plasmid was inserted into the *ushA* gene. PCR was carried out with a pair of primers complimentary to sequences flanking the insertion site and to the plasmid sequence and *PsifB-sipB* (Fig 5, Table 1), with the following conditions: 94°C 90 sec, 94°C 45 s, 58°C 30 s, 72°C for 2 min 30 sec, 28 cycles.

[0045] Accordingly, a diagram of genomic sequence generated by recombination between *ush*A gene and homology region 5'-*ush*A of the plasmid in shown Figure 6. The position of the primers used for the analysis of chloramphenicol-resistant clones is also indicated.

[0046] The primers used in the experiment are listed in Table 1:

Table 1. Primer sequences used for analysis of recombinant clones and mass of PCR products confirming the integration of the plasmid into the genome within the *ushA* gene sequence.

PRIMER	SEQUENCE (5' - 3')	PRODUCT-WILD TYPE	PRODUCT AFTER INTEGRATION
FushA	GGGGTACCCCGCGATGTTGGAGATAGTAGG	1825 bp	8366 bp
RushA	GGGGTACCCCTACAGCCAGCTCACCTCA		4366 bp (after removal of resistance gene)
A	GCGACTGGATCATATCGT	-	2350 bp
B	CGCCTCACTATGCTCATG		
C	CTGAACGGTCTGGTTATAGG	-	2411 bp
D	CTGGATATTGAACTGGCG		- (after removal of resistance gene)
A	GCGACTGGATCATATCGT	2168 bp	8709 bp
D	CTGGATATTGAACTGGCG		4709 bp (after removal of resistance gene)
E	CCCAAGCTTGGGCCTTAGCCATTCTGACTG	-	5140 bp
D	CTGGATATTGAACTGGCG		1526 bp (after removal of resistance gene)
A	GCGACTGGATCATATCGT	-	4560 bp
F	GCAGGTCGACTCTAGAGGAT		- (after removal of resistance gene)
A	GCGACTGGATCATATCGT	-	3755 bp
G	CCCAAGCTTGGGCCTTAGCCATTCTGACTG		

[0047] In the case of plasmid integration into *ushA* gene in PCR with primers A and B, a product of 2350 bp was obtained and with primers C and D - a 2411 bp product, while the standard PCR with primers A and D did not yield any product (Fig. 7).

[0048] Figure 7 shows an electrophoretic image of the separation of PCR in a 1% agarose gel in the presence of ethidium bromide. DNA of the mutated clone was amplified using the following primers: 1 - A and B (2350 bp) 2 - A and G (3755 bp) 3 - A and F (4560 bp) 4 - A and D, 5 - C and D (2411 bp).

[0049] Total RNA was isolated from wild-type VNP20009 bacteria and a clone positive for the integration, cultured in

conditions that induced the *PsifB* promoter activity. In a RT-PCR with primer specific for the *sipB* copy newly introduced into the genome (complementary to the sequence including the synthetic RBS sequence) performed on a template derived from the mutant clone, a product was obtained with molecular mass corresponding to 1222 bp (Fig. 8).

[0050] Figure 8 shows the images obtained by electrophoretic separation of RT-PCR products in 1% agarose gel in the presence of ethidium bromide. RNA was isolated from bacteria cultured under *PsifB* promoter-inducing conditions. (A) Product of cDNA amplification with primers specific for *PsifB-sipB* (1222 bp): 1 - VNP20009, 2 - modified VNP20009; (B) product of cDNA amplification with primers specific for *sipB* (389 bp): 1 - VNP20009, 2 - modified VNP20009 and product of cDNA amplification for 16S rRNA (350 bp).

Example 6: Removal of antibiotic resistance gene from the VNP20009 genome

[0051] The above-mentioned genetic manipulations required the presence of an antibiotic resistance gene, which is undesirable in the final vaccine material. Therefore, this gene was deleted in the following stage. The cleavage of genomic DNA at the restriction site for I-*Sce*I enzyme, which had been introduced into the genome together with the plasmid sequence, stimulated recombination with the participation of neighbouring regions of homology and selection of the clones, in which the DNA break was repaired. Clones with confirmed integration of the pSG76C-USS plasmid were subsequently transformed with plasmid pSTKST (with thermosensitive *ori* sequence pSC101) containing a gene for I-*Sce*I meganuclease under the control of the tetracycline promoter. The clones were cultured on solid medium with kanamycin at 30°C. Then, individual colonies were transferred into liquid LB medium with kanamycin (20 μg/ml) and autoclaved chlortetracycline (cTc, 30 μg/ml, induces the expression of I-*Sce*I by inactivating the tetracycline repressor) and incubated for 24 hours at 30°C. The culture was diluted $1:10^6$, transferred to solid medium with kanamycin and cTc and cultured at 30°C for 20 hours. Then, the clones were tested for the deletion of the resistance gene and for planned recombination with a PCR using pair of primers E and D - in this case the obtained product had 1526 bp (Fig. 9, lane 1). PCR was performed using the primers A and D and a programme as follows: 94°C 2 min, 94°C 30 s, 58°C 30 s, 70°C 4 min, 10 cycles; 94°C 30 s, 58°C 30 s, 70°C 4 min + 10 sec/cycle, 20 cycles. The resulting product was longer than 4500 bp and corresponded to the length of the *ushA* sequence with the integrated *PsifB-sipB* cassette (Fig. 9, path 2).

[0052] Figure 9 shows the image of electrophoretic separation of the PCR product with primer sequences complementary to sequences flanking, the *ushA* gene performed on the genomic DNA of a clone obtained with the procedure of *PsifB-sipB* integration into the VNP20009 chromosome. Amplification products were obtained with primers E and D (1526 bp) and with primers A and D (4709 bp).

[0053] Integration of the *PsifBsipB* functional cassette into the VNP20009 chromosome is such a significant genetic modification of the bacteria, that it results in obtaining of a new bacterial strain.

Example 7: Testing of the VNP/sipB (INT) functionality

Example 7A: Comparison of the invasive ability of VNP20009 and VNP/sipB bacteria

[0054] The invasiveness VNP20009 and VNP/sipB strains was tested on RAW264.7 cells. No statistically significant differences in the invasiveness were found between "wild" and recombinant strains of VNP. RAW264.7 macrophage cell line was cultured on 48-well plates at the density of 2.5×10^4 in DMEM medium with 10% serum for 24 hours. The cells were infected with VNP20009 and VNP/sipB in 100 μl of OPTIMEM medium (Invitrogen) without serum at MOI 5, determined by measuring optical density at 600 nm. After 1-hour of co-incubation of the cells with bacteria (37°C, 5% CO_2), another 100 μl of OPTIMEM supplemented serum and gentamicin (2% serum, 100 μg/ml gentamicin) was added and incubated for 3 hours (37°C, 5% CO_2) to eliminate extracellular bacteria. Then, the cells were harvested and transferred to LB/agar plates. The number of live intracellular bacteria was assessed based on the number of bacterial colonies present after 24-hour incubation and referred to the number of cells in the specific well.

[0055] Concurrently, the number of CFU per well of the start of infection was determined, based on the amount of bacterial colonies after 24-hour culturing on LB/agar plates, obtained from an appropriately diluted bacterial suspension used for the infection. Invasiveness of the bacteria was defined as the fraction of intracellular bacteria from the number of bacteria used for the infection (Fig. 10).

Example 7B: Therapeutic effects of VNP/sipB in a mouse tumour model

[0056] The experiment was performed on the CT26CEA lung metastasis model in Balb/c mice. Lungs were stained with Indian ink. Figure 11 (upper row) shows lungs of the control mice isolated on day 15 after the intravenous administration of 5×10^5 CT26CEA cells. White spots visible on the dark background are small tumour foci and their number (> 300 per lung) and randomly assessed volume illustrate the effectiveness of metastasis. The bottom row shows lungs taken from mice, which were administered *Salmonella typhimurium* VNP/sipB bacteria intranasally in the amount of 2 x

10^7 CFU/mouse 48 hours after the injection of cancer cells. In those lungs, an average of <20 tumours/lung were found. In the group of mice that were vaccinated with VNP/sipB, no detectable tumours were found in 50% of the examined lungs.

Example 7C: Comparison of therapeutic effects of various VNP20009 modifications in a mouse tumour model

[0057] The experiment was performed on the CT26CEA lung metastasis model in Balb/c mice. Lungs were stained with Indian ink. Figure 12 (upper row) shows lungs of the control mice isolated on day 15 after intravenous administration of 5 x 10^5 CT26CEA cells. White spots visible on the dark background are small tumour foci and their number (> 300 per lung) and randomly assessed volume illustrate the effectiveness of metastasis. The bottom row shows lungs taken from mice, which were administered various modifications of *Salmonella typhimurium* bacteria intranasally in the amount of 2 x 10^7 CFU/mouse 96 hours after the injection of cancer cells. In the lungs VNP/sipB vaccinated animals an average of 54 tumours/lung was found and 50% of the examined lungs did not have any detectable tumours; the mean numbers of tumour foci in the lungs are shown on the graph in the lower part of the figure.

Example 7D: Comparison of therapeutic effects of VNP20009 and VNP/sipB in mouse tumour model of B16F10 melanoma

[0058] The experiment was performed on the B16F10 lung metastasis model in C57B1/6 mice (Fig.13). Lungs were stained with picric acid. Figure 13 (upper row) shows lungs of the control mice isolated on day 28 after intravenous administration of 5 x 10^5 B16F10 cells. Tumours are shown as dark dots and their number and randomly assessed size illustrate the effectiveness of metastasis. The middle and lower rows shows lungs isolated from mice, which were administered VNP (middle row) or VNP/sipB (bottom row) bacteria intranasally in the amount of 2 x 10^7 CFU/mouse 96 hours after the injection of cancer cells. In these lungs, less than 2 tumours/lung were found on average. In the group of mice that were vaccinated with VNP/sipB, no detectable tumours were found in 75% of the examined lungs.

Example 7E: Selective stimulation of Th1-type response by VNP/sipB

[0059] As an effect of the vaccination of Swiss mice (outbred) with the modified VNP20009 strains, immune response skewing towards a Th1-type response is found in half of the vaccinated animals (Fig. 14). While administration of the "wild type" VNP20009 (top panel) elicited all analysed antibody isotypes (IgG1, IgG2a and IgM), only high levels of IgG2a and the almost complete absence of IgG1 were found in the serum of mice vaccinated with VNP/sipB (bottom panel). It is very likely that the observed isotype skewing forms an important link in the chain of immune responses stimulated by VNP/sipB, which result, inter alia, in growth inhibition (or elimination) of tumours in the vaccinated mice.

Example 7F: Apoptosis of VNP/sipB infected MC38CEA cells

[0060] Apoptosis induction in MC38CEA adenocarcinoma cells infected with VNP20009 or VNP/sipB was analyzed by flow cytometry of annexin V-stained cells. Bacteria transformed with the pDsRed2 plasmid encoding RFP (Red Fluorescent Protein) were used for the infection.

[0061] Cells were seeded on 24-well plate at 7.5-104 per well in DMEM containing 10% FBS and cultured at 37°C in 5% CO2. Infection was carried out with bacteria suspended in 200 μl Opti-MEM at the multiplicity of infection equal to 20 bacteria per cell. Bacteria were allowed to invade cells for 45 min at 37°C in 5% CO2. Then, 200 μl of gentamicin-containing Opti-MEM was added to kill extracellular bacteria (final concentration of gentamicin was 100 μg/ml). Incubation was continued for 1.5 h (37°C, 5% CO2). The media were then replaced with 0.5 ml Opti-MEM with 20 μg/ml gentamicin. After 20 h of incubation, cells were collected, stained with APC-labeled annexin V for 15 min at room temperature and analyzed by flow cytometry. Fig. 17 shows the percentage of annexin V binding cells gated on an RFP-positive, bacteria-infected population. There was around 10% more annexin V positive MC38CEA cells following infection with VNP/sipB than VNP20009.

Example 8: Sequencing of the recombinant fragment of genomic DNA of the modified *Salmonella enterica* s. Typhimurium VNP20009 strain

[0062] The following DNA sequences were analysed: the *ushA* gene with its flanking sequences and the entire sequence of the integrated *PsifB-sipB* construct that in VNP/sipB bacteria is located within the *ushA* gene (Fig. 15). The identity of the sequence from VNP/sipB with template DNA from a model strain of *Salmonella typhimurium,* LT2, was confirmed using BLAST software (NCBI). On the template (lower strand of DNA) in Figure 15B, the location of the each element of the construct is indicated (*sifB* promoter, *sipB* ORF, *ushA* integration site) in accordance with the numbering of the LT2 strain deposited in GenBank (NCBI). Upper strand, marked as "Query", shows the sequence obtained from

the VNP/sipB. The *PsifB* sequence in the genome of *Salmonella typhimurium* LT2 is located in the position 1 691 578 - 1 692 147 bp. The *sipB* sequence in the genome of *Salmonella typhimurium* LT2 is located in the position of 3 029 114-3 030 895 bp. The *ushA* sequence in the genome of *Salmonella typhimurium. LT2* is located in the position 553 634 - 555 286 bp.

[0063] Oligonucleotides used for sequencing of genomic DNA are listed in Figure 16. The schematic location of the primers is indicated in Figure 15 within the flanking, sequences and the integrated construct.

[0064] As a result of the production of a properly modified strain of *Salmonella enterica* s. *Typhimurium,* a favourable therapeutic vaccine vector was produced, which is particularly suitable for use as an anti-cancer bacterial vaccine vector.

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[0065]

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SEQUENCE LISTING

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Claims

1. A strain of *Samonella enterica* serovar Typhimurium VNP/sipB deposited in the Polish Collection of Microorganisms under access no. B/00024.

2. Application of the strain *Samonella enterica* serovar Typhimurium VNP/sipB deposited in the Polish Collection of Microorganisms under access no. B/00024 to obtain a vaccine, especially an anti-cancer vaccine.

3. Method of obtaining a therapeutic vaccine vector, **characterized in that** a genetic modification is introduced into the vector strain of *Salmonella enterica* serovar Typhimurium VNP20009 specific to cancer cells, resulting in the delayed overexpression of a gene encoding a protein responsible for the invasive ability of this strain, wherein an expression cassette is obtained, containing gene *sipB* under the control of *PsifB* promoter that controls its delayed overexpression, and then the cassette is integrated into the bacterial chromosome.

4. A method according to claim 3, **characterised in that** the expression cassette integrated into the bacterial chromosome is the *PsifB-sipB* cassette according to SEQ ID 1 or the *ushA-5'-PsifB-sipB-ushA-3'* cassette according to SEQ ID 2.

5. A method according to claim 4, **characterised in that** the method includes an additional final step of removing a gene for antibiotic resistance from the bacterial genome.

Patentansprüche

1. *Salmonella enterica* serovar Typhimurium VNP/sipB-Stamm, der in der Polnischen Sammlung für Mikroorganismen unter der Hinterlegungsnummer B/00024 hinterlegt ist.

2. Verwendung des *Salmonella enterica* serovar Typhimurium VNP/sipB-Stamms, der in der Polnischen Sammlung für Mikroorganismen unter der Hinterlegungsnummer B/00024 hinterlegt ist, um einen Impfstoff, insbesondere einen Anti-Krebs-Impfstoff, zu erhalten.

3. Verfahren zum Erhalt eines therapeutischen Impfstoff-Vektors, **dadurch gekennzeichnet, dass** eine genetische Modifikation in den *Salmonella enterica* serovar Typhimurium VNP20009-Vektor-Stamm, der spezifisch für Krebszellen ist, eingeführt wird, die zu einer verzögerten Überexpression eines Gens führt, welches ein Protein kodiert, das für die invasive Fähigkeit des Stamms verantwortlich ist, wobei eine Expressionskassette erhalten wird, die das *sipB-Gen* unter Kontrolle des *PsifB*-Promoters, der dessen verzögerte Überexpression kontrolliert, umfasst, und dann die Kassette in das bakterielle Chromosom integriert wird.

4. Verfahren nach Patentanspruch 3, **dadurch gekennzeichnet, dass** die Expressionskassette, die in das bakterielle Chromosom integriert ist, die *PsifB-sipB*-Kassette gemäß SEQ ID NO: 1 oder die *ushA-5'-PsifB-sipB-ushA-3'*-Kassette gemäß SEQ ID NO: 2 ist.

5. Verfahren nach Patentanspruch 4, **dadurch gekennzeichnet, dass** das Verfahren einen zusätzlichen finalen Schritt umfasst, der darin besteht, ein Gen für Antibiotikaresistenz aus dem bakteriellen Genom zu entfernen.

Revendications

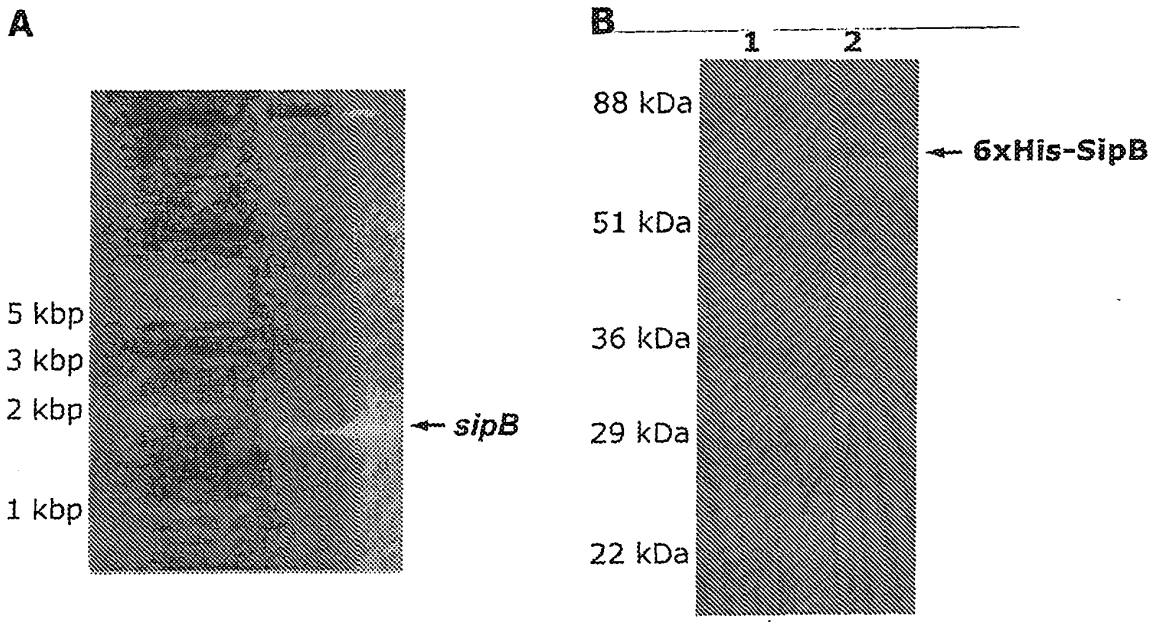
1. Souche de *Salmonella enterica* sérovar Typhimurium VNP/sipB déposée à la Collection Polonaise de Microorganismes sous le numéro d'enregistrement B/00024.

2. Application de la souche de *Salmonella enterica* sérovar Typhimurium VNP/sipB déposée à la Collection Polonaise de Microorganismes sous le numéro d'enregistrement B/00024 pour obtenir un vaccin, en particulier un vaccin anti-cancer.

3. Méthode d'obtention d'un vecteur vaccinal thérapeutique, **caractérisée en ce qu'**une modification génétique est introduite dans la souche vecteur de *Salmonella enterica* sérovar Typhimurium VNP20009 spécifique des cellules cancéreuses, entraînant la surexpression retardée d'un gène codant pour une protéine responsable de la capacité invasive de cette souche, dans laquelle une cassette d'expression est obtenue, contenant le gène *sipB* sous le contrôle du promoteur *PsifB* qui contrôle sa surexpression retardée, et puis la cassette est intégrée dans le chromosome bactérien.

4. Méthode selon la revendication 3, **caractérisée en ce que** la cassette d'expression intégrée dans le chromosome bactérien est la cassette *PsifB-sipB* selon SEQ ID 1 ou la cassette *ushA-5'-PsifB-sipB-ushA-3'selon* SEQ ID 2.

5. Méthode selon la revendication 4, **caractérisée en ce que** la méthode comprend une étape finale supplémentaire d'élimination d'un gène de résistance à un antibiotique à partir du génome bactérien.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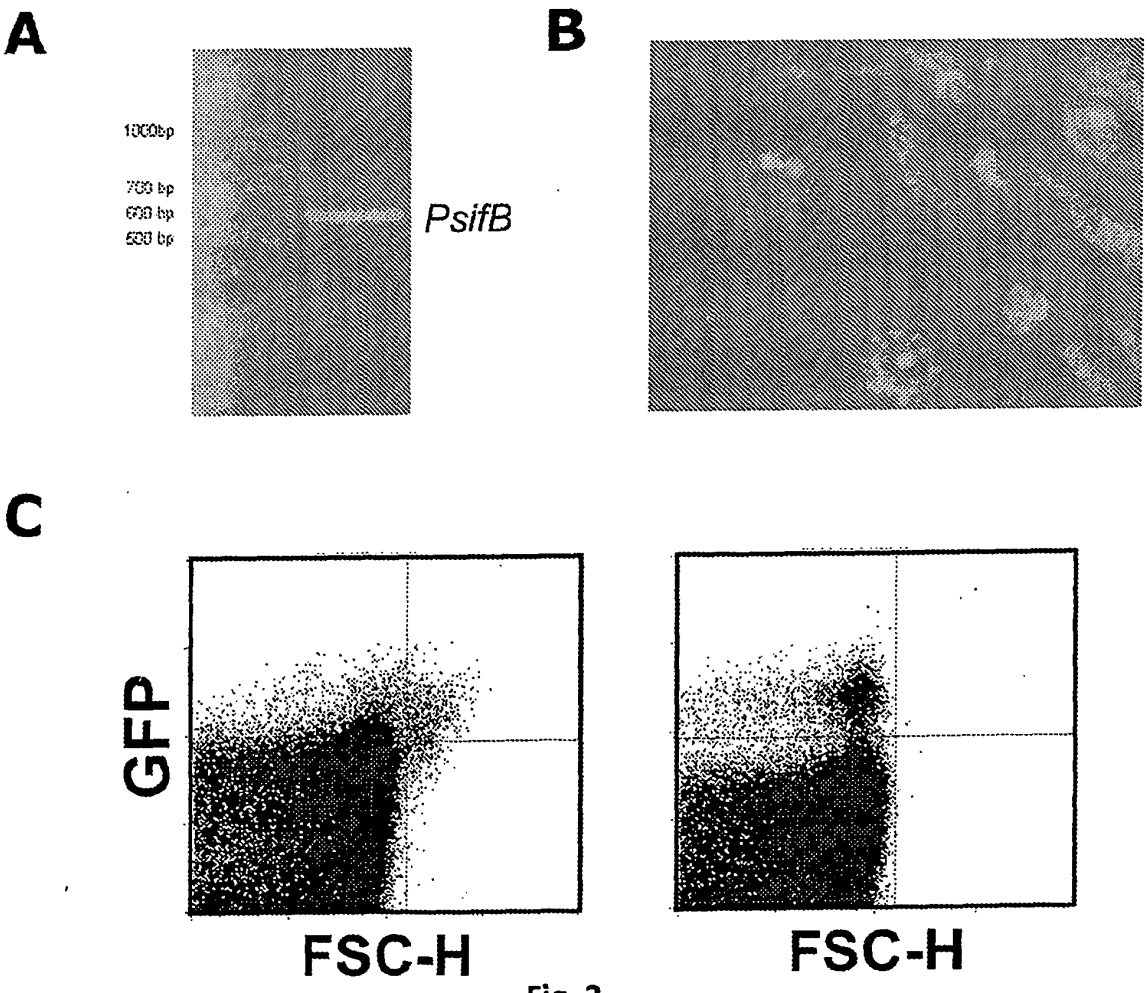
3 kbp
2 kbp
1 kbp
sipB
B
1
2
88 kDa
51 kDa
36 kDa
29 kDa
22 kDa
6xHis-SipB

Fig. 1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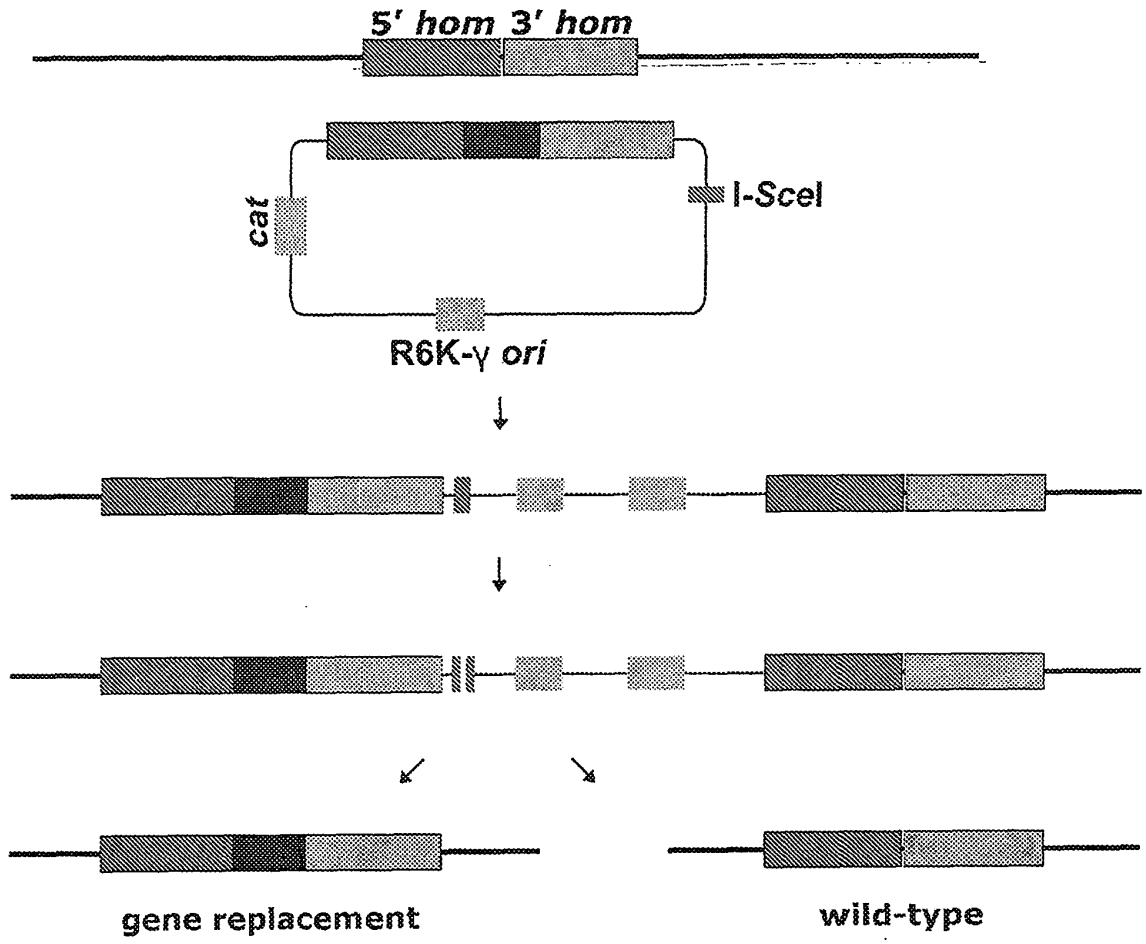
500 bp
PsifB
B
C
GFP
FSC-H
FSC-H

Fig. 2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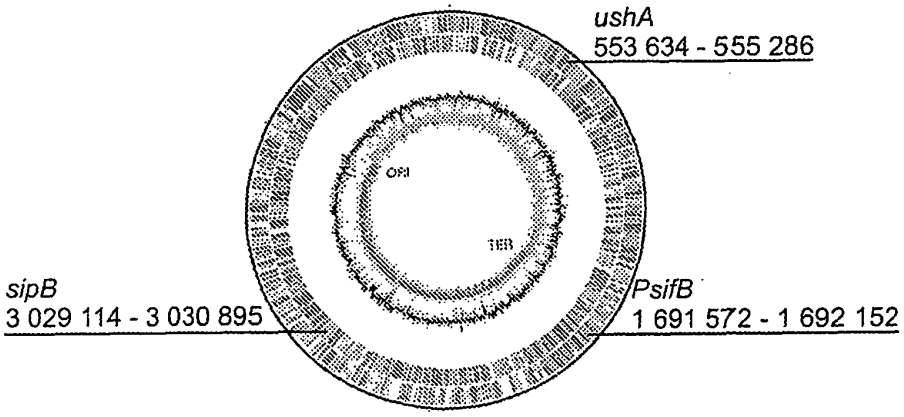
cat
I-SceI
R6K-γ ori
gene replacement
wild-type

Fig. 3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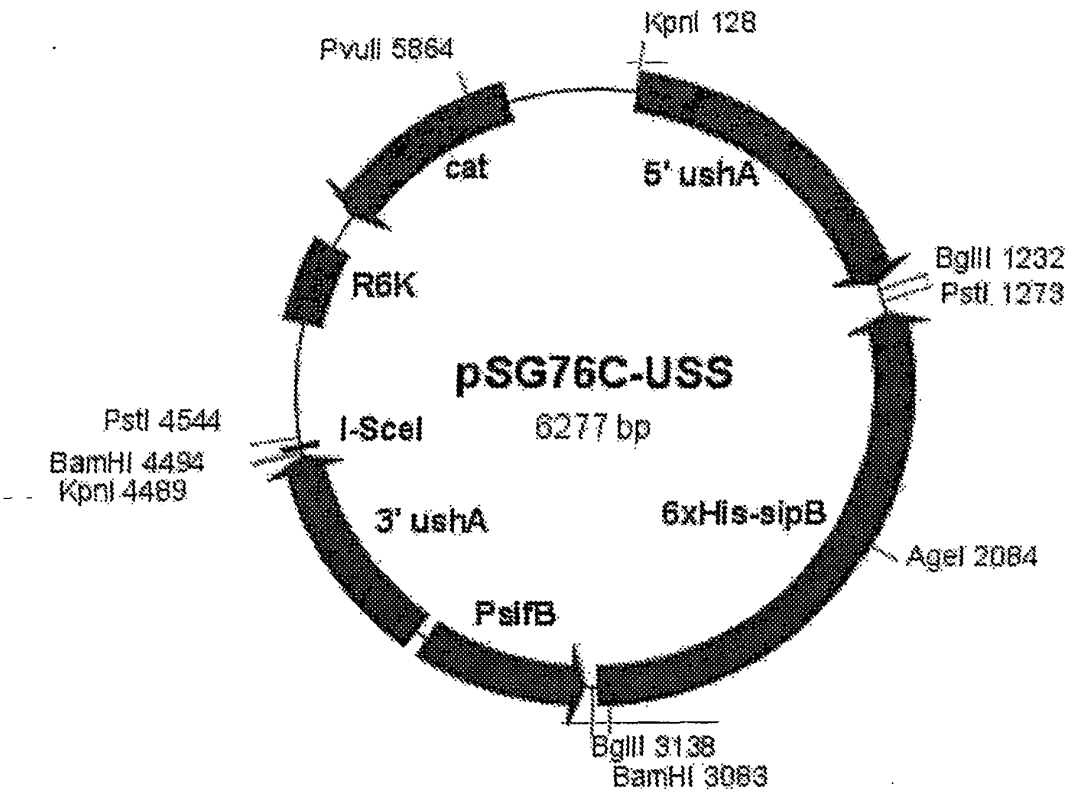
553 634 - 555 286
sipB
3 029 114 - 3 030 895
PsifB
1 691 572 - 1 692 152

Fig. 4



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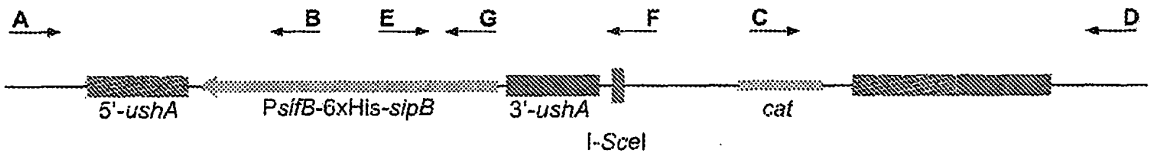

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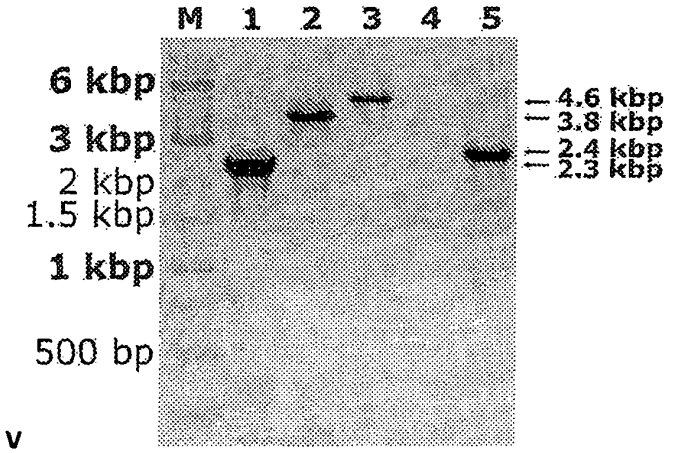

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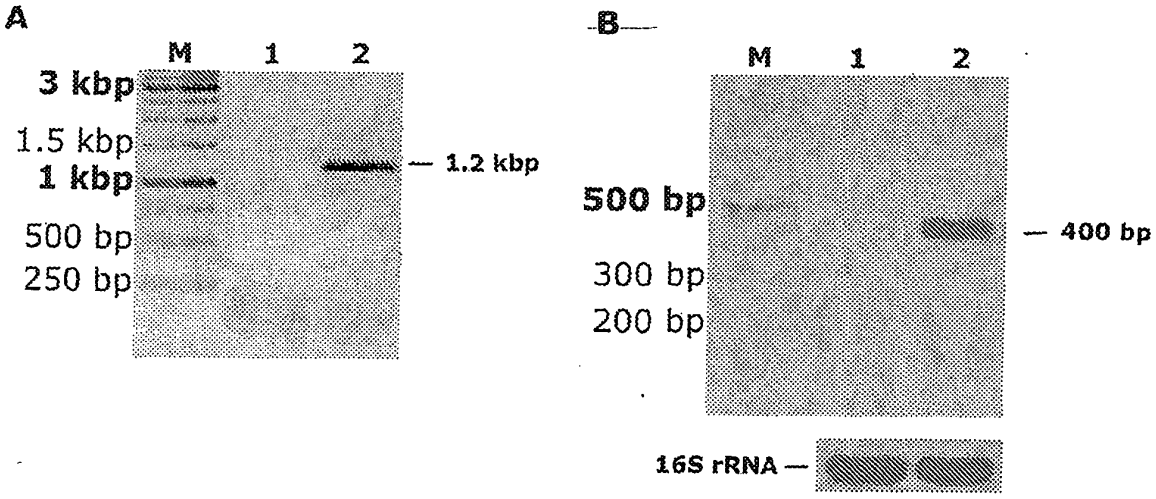

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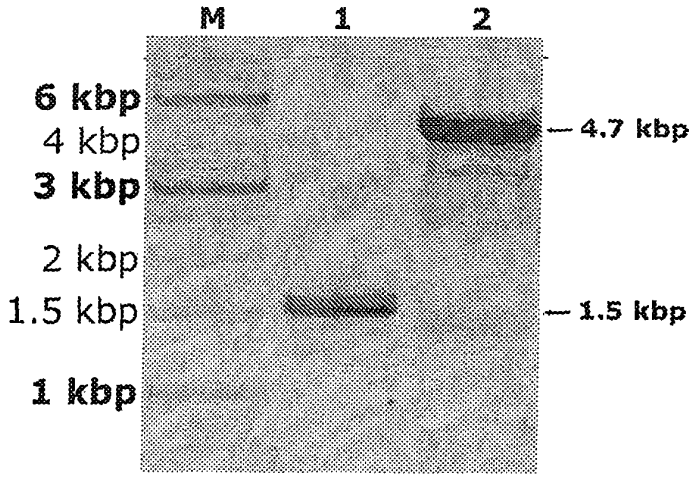
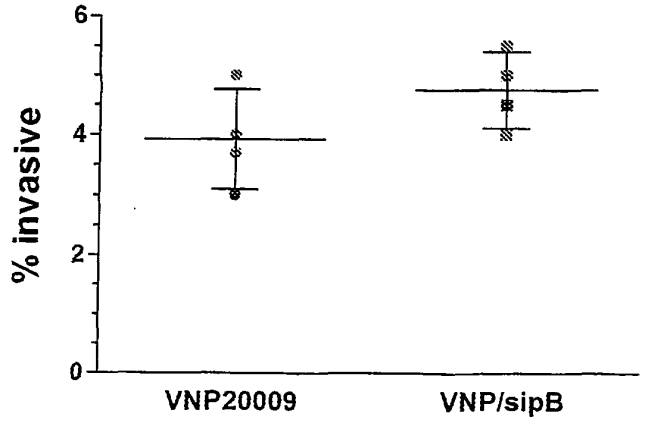


Fig. 9



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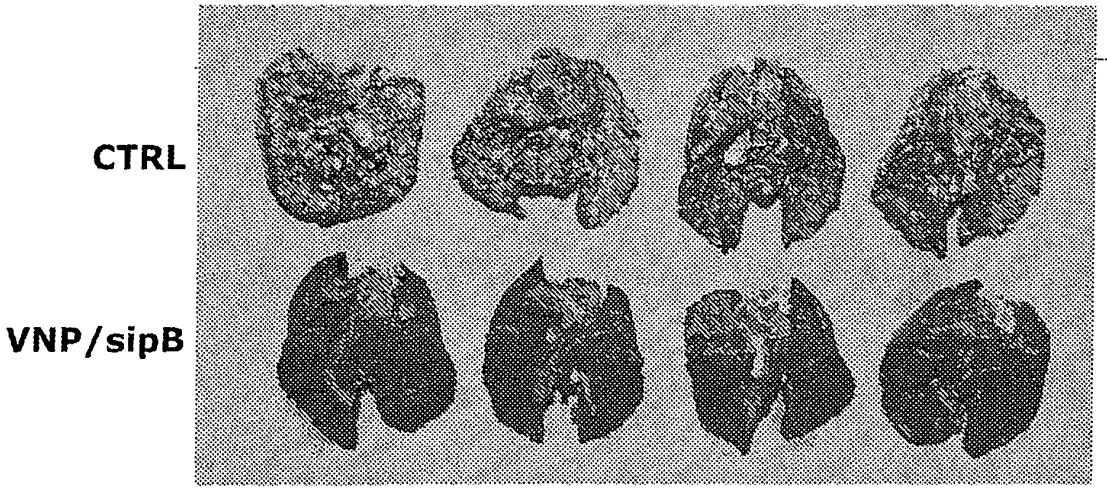
CTRL
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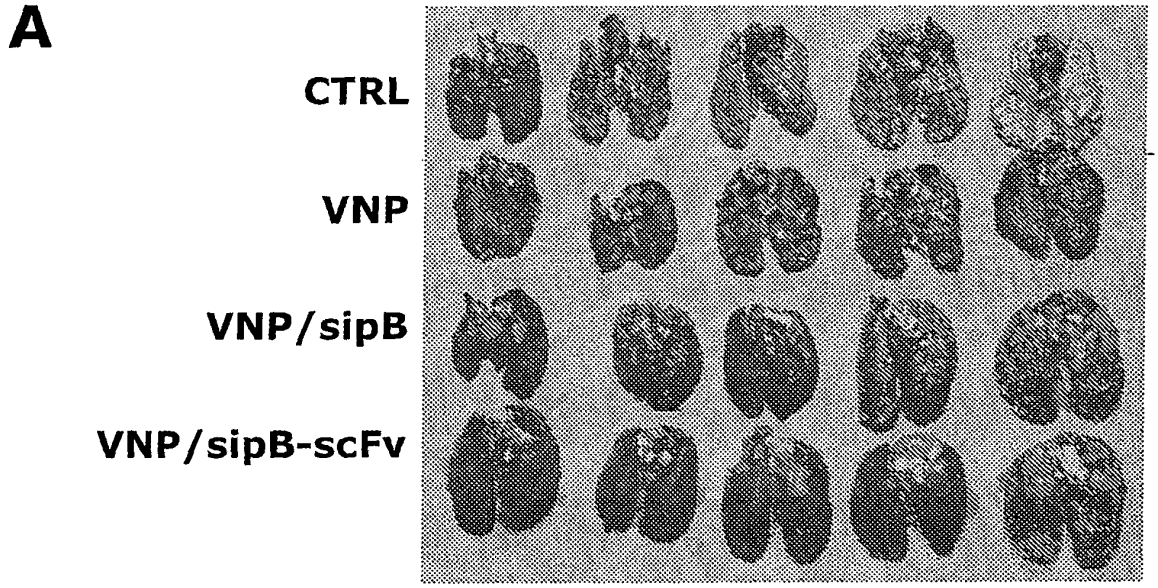
Fig. 11


A
CTRL
VNP
VNP/sipB
VNP/sipB-scFv

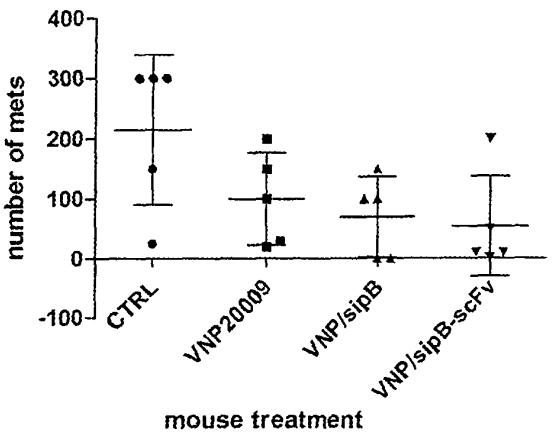

B
400
300
200
100
0
-100
number of mets
CTRL
VNP20009
VNP/sipB
VNP/sipB-scFv
mouse treatment

Fig. 12

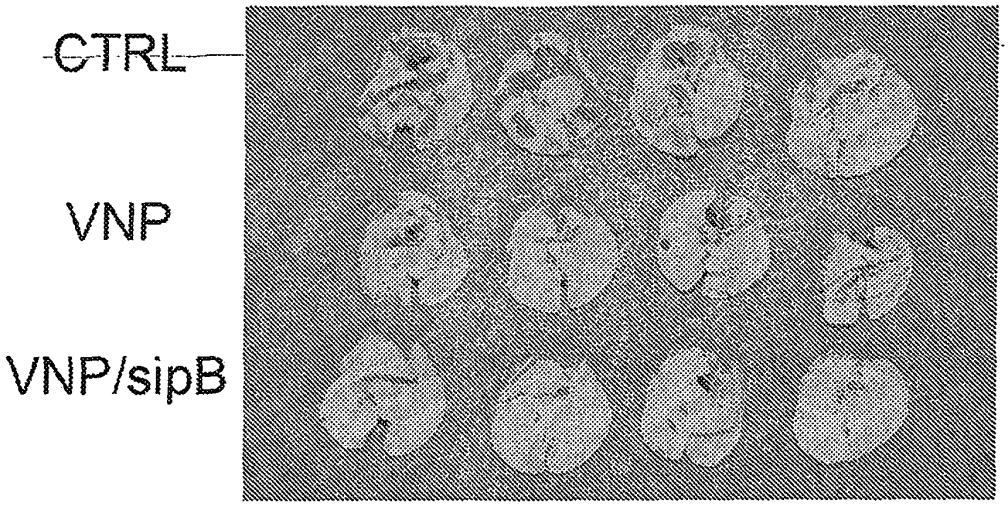


Fig. 13

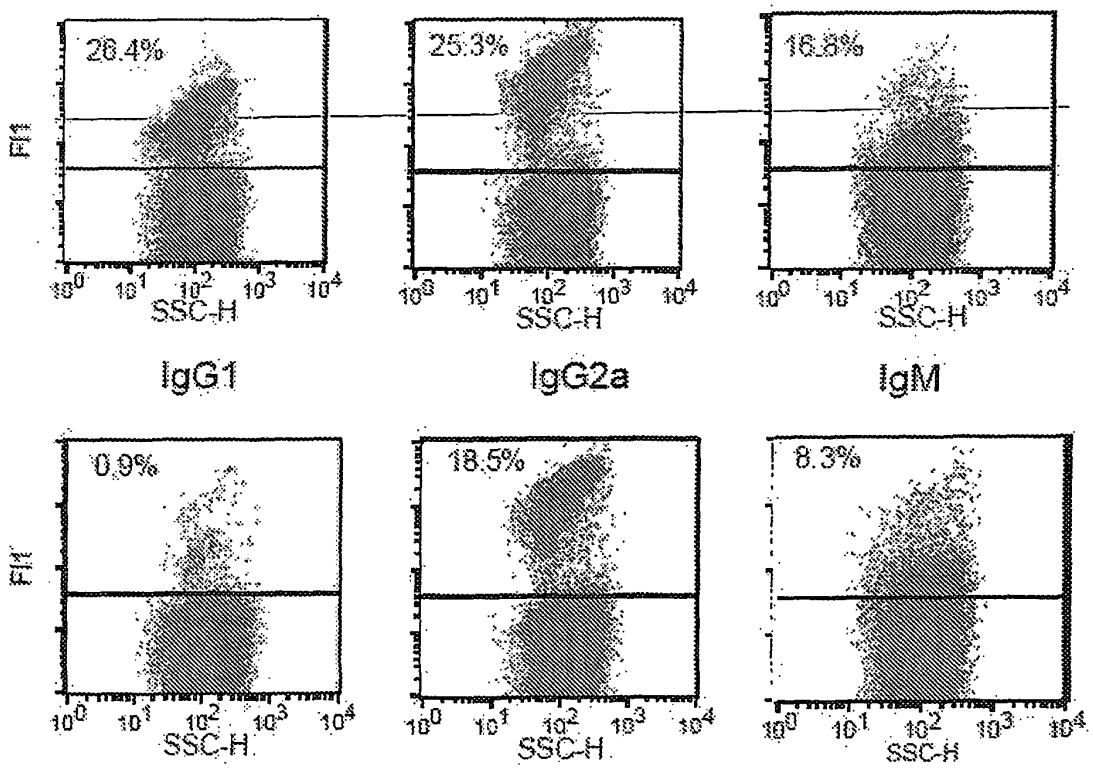


Fig. 14

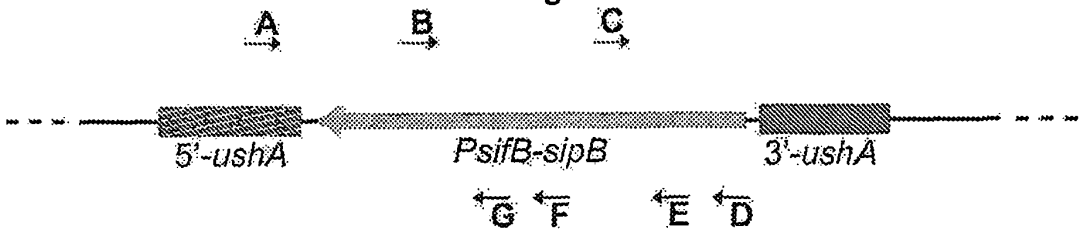


Fig. 15 A

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 gb|AE006468.1|  Salmonella typhimurium LT2, the whole genome
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Sbjct  3030667  CCCGGGCGGCGTCCGTAGGCGGCTTTAGACCCACCGTATTAATAGCGCTCTCGCCGGACT  3030726
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Query  240      GATTTTGGGTATATCCGCTACGGCTAATGCTACTT  274
                ||||||||||||||||||||||||||||||||||
Sbjct  3030847  GATTTTGGGTATATCCGCTACGGCTAATGCTACTT  3030881
Query  340      ACTTCATTACTGGAAATAGGTGGTATTCGAA-TATTATCCCATGTTGCCCATCGGTTTGC  398
                |||||||||||||||| ||||||||||||||| ||||||||||||||||||||||||||
Sbjct  1692153  ACTTCATTACTGGAA-TAGGTGGTATTCGAAATATTATCCCATGTTGCCCATCGGTTTGC  1692095
Query  399      CTATCGGTGAAACACCTGATTTTTGCTT-GTCCTGAACCGTCAACATTATTGTTCAATTG  457
                |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1692094  CTATCGGTGAAACACCTGATTTTTGCTTTGTCCTGAACCGTCAACATTATTGTTCAATTG  1692035
Query  458      TTCAATCCGACCCGTAGCTTTAGTCATGGccccccccTCCTGGGCCATGAAATATGTCCGA  517
                |||||  |||||||||||||||||||||| || | ||||||  ||||||||||||||| |
```

```
Sbjct  1692034  TTCAAATCGACCCGTAGCTTTAGTCATGCCCACGCCTCCT-GGCCATGAAATATGTCAG-  1691977
Query  518      ATAAAACGAATGGAAGTAAAACGGGTTTTCTTAATTCCCCAATCCATCCGGGACCATTGA  577
                ||||||||||||| |||||||||| |||||||||||||| | || |||| | || | |||
Sbjct  1691976  ATAAAACGAATGAAAGTAAAAC-GGTTTTCTTAATTCTCACAT-CATCATGTACTA-TGA  1691920
Query  578      GTAAGGATTAA  588
                |||| ||||||
Sbjct  1691919  GTAATGATTAA  1691909
```

Primer E (FsipBgl):

```
>ref|NC_003197.1|  Salmonella typhimurium LT2, the whole genome
 gb|AE006468.1|  Salmonella typhimurium LT2, the whole genome
length = 4857432
Query  31       AAGTAGCATTAGCCGTCCCGGATATACCCAAAATCCGCGCCTCGCTGAGGCGGCTTTTGA  90
                |||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030881  AAGTAGCATTAGCCGTAGCGGATATACCCAAAATCCGCGCCTCGCTGAGGCGGCTTTTGA  3030822
Query  91       AGGCGTTCGTAAGAACACGGACTTTTTAAAAGCGGCGGATAAAGCTTTTAAAGATGTGGT  150
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030821  AGGCGTTCGTAAGAACACGGACTTTTTAAAAGCGGCGGATAAAGCTTTTAAAGATGTGGT  3030762
Query  151      GGCAACGAAAGCGGGCGACCTTAAAGCCGGAACAAAGTCCGGCGAGAGCGCTATTAATAC  210
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030761  GGCAACGAAAGCGGGCGACCTTAAAGCCGGAACAAAGTCCGGCGAGAGCGCTATTAATAC  3030702
Query  211      GGTGGGTCTAAAGCCGCCTACGGACGCCGCCCGGGAAAAACTCTCCAGCGAAGGGCAATT  270
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030701  GGTGGGTCTAAAGCCGCCTACGGACGCCGCCCGGGAAAAACTCTCCAGCGAAGGGCAATT  3030642
Query  271      GACATTACTGCTTGGCAAGTTAATGACCCTACTGGGCGATGTTTCGCTGTCTCAACTGGA  330
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030641  GACATTACTGCTTGGCAAGTTAATGACCCTACTGGGCGATGTTTCGCTGTCTCAACTGGA  3030582
Query  331      GTCTCGTCTGGCGGTATGGCAGGCGATGATTGAGTCACAAAAAGAGATGGGGATTCAGGT  390
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030581  GTCTCGTCTGGCGGTATGGCAGGCGATGATTGAGTCACAAAAAGAGATGGGGATTCAGGT  3030522
Query  391      ATCGAAAGAATTCCAGACGGGTCTGGGAGAGGCTCAGGAGGCGACGGATCTCTATGAAGC  450
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3030521  ATCGAAAGAATTCCAGACGGCTCTGGGAGAGGCTCAGGAGGCGACGGATCTCTATGAAGC  3030462
Query  451      CAGTATCAAAAAGACGGATACCGCCAAGAGTGTTTATGACGCTGCGACCaaaaaaaCTGA  510
                |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  3030461  CAGTATCAAAAAGACGGATACCGCCAAGAGTGTTTATGACGCTGCGACC-AAAAAACTGA  3030403
Query  511      CGCAGGCGCAAAATAAATTGCAATCGCTGGGACCCGGGCTGACCCCGGCTATGCCCAAGC  570
                |||||||||||||||||||||||||||| |||||| |||||||||||||||||| |||||
Sbjct  3030402  CGCAGGCGCAAAATAAATTGCAATCGCT-GGACCC-GGCTGACCCCGGCTATGCACAAGC  3030345
Query  571      TGAAACCCACGGTAAAACCAGGGCCGGAAAAGAAACGACCAGAGGNCGAAAGAGGCCTTT  630
                || || || ||||| ||| |||||||||||||| ||| |||||| ||||||||||||||
Sbjct  3030344  TG-AAGCCGCGGTAGAAC--AGGCCGGAAAAGAAGCGA-CAGAGG-CGAAAGAGGCCTT-  3030291
Query  631      anaanaaGGNCCCNGGAAGGCNACCGGGTTAAAGCCAGGCNcnnaaccccaaangcnaa  690
                   |||| | | |  | || ||  |||||||| |||| |  |  | |||| || ||
Sbjct  3030290  -AGATAAGGCCACGG--ATGCGAC--GGTTAAAG-CAGGCACAGA--CGCCAAA-GCGAA  3030240
Query  691      angccnnanaaaGGCGGAATAACATT  716
                | |||  | |||| |||| ||||||||
Sbjct  3030239  A-GCC-GAGAAA-GCGG-ATAACATT  3030218
```

Primer A (Fusha1):

```
>ref|NC_003197.1|  Salmonella typhimurium LT2, the whole genome
 gb|AE006468.1|  Salmonella typhimurium LT2, the whole genome
length = 4857432
Query  12       TGTGGGCCGTGCGGATTTCGAATTCCGTAACGGCGAGATG-AAATGGTT  59

Sbjct  554515  TGTGGGCCGTGCGGATTTCGAATTCCGTAACGGCGAGATGAAAATGGTT  554563
Query  138      TTTCTCCCTTTATTTTGGCAGTTTTTATGCGCGACTCTGGCGCAGAATAAAACGCGAAGC  197

Sbjct  3029090  TTTCTCCCTTTATTTTGGCAGTTTTTATGCGCGACTCTGGCGCAGAATAAAACGCGAAGC  3029149
Query  198      ATCCGCATTTTGCTGTACCGCAGAAGACATGGCTTTTTGCAGTTCCGCCGTTACCTTCTG  257

Sbjct  3029150  ATCCGCATTTTGCTGTACCGCAGAAGACATGGCTTTTTGCAGTTCCGCCGTTACCTTCTG  3029209
Query  258      GTTTTCACCAAATATTTCTACGGATTGTTTAAGCCACTGCTGAATCTGATCCATGGCAAA  317

Sbjct  3029210  GTTTTCACCAAATATTTCTACGGATTGTTTAAGCCACTGCTGAATCTGATCCATGGCAAA  3029269
Query  318      ACGGGCGAGCATAAAATCAGCAAGCGCCTCGCTGGCATTTTTAATAAATACGCCCCTCGG  377

Sbjct  3029270  ACGGGCGAGCATAAAATCAGCAAGCGCCTCGCTGGCATTTTTAATAAATACG-CCCTCGG  3029328
Query  378      CAACACCACCGGCTGACTGGGGCTGCGGTATTCGTGACTTCCATGCCCAACGCCACTTTA  437

Sbjct  3029329  CAACACCACCGGCTGACT-GGGCTGCGGTATTCGTGACTTCCATGCCCAACGCCACTTTA  3029387
Query  438      TTTAGGGTATTACCTACCAGCTCTTTACTTAAGGCATTCGTTTGCAGGCCCATCTTGCTA  497

Sbjct  3029388  TTTAGGGTATTACCTACCAGCTCTTTACTTAAGGCATTCGTTTGCAGGCCCATCTTGCTA  3029447
Query  498      CCCACATTACCCAGACCGCTAGTAATACGTTGCATCCCCCTGGGGTAAAAAAGTTGGCTG  557

Sbjct  3029448  CCCACATTACCCAGACCGCTAGTAATACGTTGCAT-CCCCTGGG--TAAAGAGTTTGCTG  3029504
Query  558      CCGTTTTGCGCCAACTGTTTCAGCCACGTTAGGCACCAAACTCCTTAATCCGTTTCGCCC  617

Sbjct  3029505  CCGTTTTGCGCCAACTGTTTCAG-CACGTTAGGCACC-AACTTCTTAAT-CGTTTCGCCC  3029561
Query  618      ATCAATTTTGGCTCAAGCNGGGTTACCCCAGTTT  651

Sbjct  3029562  ATCA--TTTTGCTC-AGC-GCGTTA-CCCAGTTT  3029590
```

Fig. 15 B

A Fusha1 AATGGCATCTGGATCGTG
B sipseq3 GTAATCGCCTTGCCAATC
C sipseq4 AGACGAGACTCCAGTTGAGA
D siff CCCAAGCTTGGGCCTTAGCCATTCTGACTG
E FsipBgl GGAAGATCTTCCAGAGGAGAAATTAACTATGAGA
F sipseq1 GAGGCGACGGATCTCTAT
G sipseq2 CGCCTCACTATGCTCATG

Fig. 16

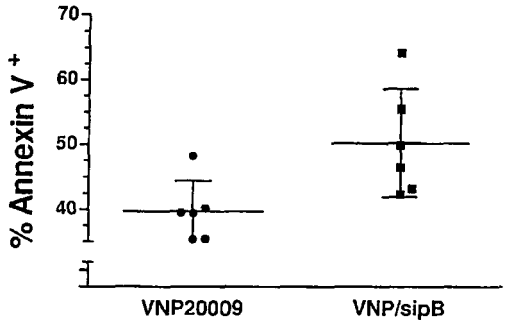

Fig. 17

REFERENCES CITED IN THE DESCRIPTION

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