



(51) International Patent Classification:
C12N 1/20 (2006.01) C12R 1/185 (2006.01)
C07K 14/245 (2006.01)

(21) International Application Number:
PCT/EP2009/006871

(22) International Filing Date:
23 September 2009 (23.09.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
08016660.6 23 September 2008 (23.09.2008) EP

(71) Applicant (for all designated States except US): **ETH ZÜRICH** [CH/CH]; Raemistrasse 101/ ETH transfer, CH-8092 Zurich (CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **HARDT, Wolf-Dietrich** [DE/CH]; Segantinistrasse 6, CH-8049 Zürich (CH). **STECHER, Elfriede** [DE/CH]; Mühlegasse 21, CH-8001 Zürich (CH).

(74) Agent: **ETH ZÜRICH**; ETH transfer HG E 43-49, Raemistrasse 101, CH-8092 Zürich (CH).

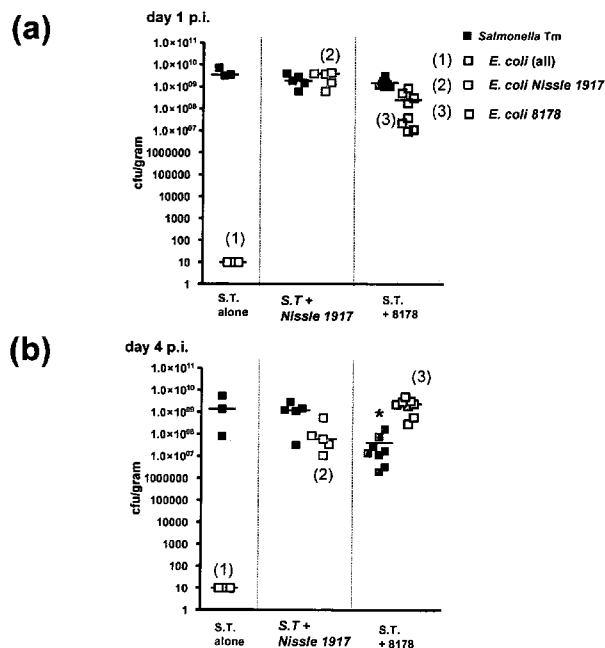
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: PROTECTIVE E. COLI STRAINS FOR REDUCING THE SALMONELLA TYPHIMURIUM PATHOGEN LOAD IN THE INTESTINES

Fig. 2



(57) Abstract: Genetically modified probiotic E. coli strains and methods for producing such E. coli strains comprising at least one first genetic modification introducing an exogenous protein and/or enhancing enzymatic activity of an endogenous protein, the first genetic modification resulting in an enhanced level of biosynthesis of at least one colicin immunity protein or precursors are provided. Also provided is an E. coli strain, which is designated 8178. Said strain corresponds to International Deposition No: DS M21844. A probiotic composition comprising an effective amount of said novel E. coli strains is used for treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals. Said gastrointestinal disorder may be Salmonella enterica induced acute diarrhea, chronic enteric Salmonellosis, and Typhoid fever (chronic carrier state). In an advantageous embodiment the composition according to the invention is a food product, a food additive or supplement, an animal feed additive or supplement, a pharmaceutical preparation or a biotherapeutic composition.

**Published:**

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

Protective *E. coli* Strains for Reducing the *Salmonella* Typhimurium Pathogen Load in the Intestines

Field of the Invention

- 5 The present invention relates to novel bacterial strains, compositions comprising the same, methods of treating or preventing gastrointestinal disorders in vertebrate, and using such strains in probiotic treatment of gastrointestinal disorders, such as diarrhea.

State of the art

- 10 Most mucosal surfaces of the mammalian body are colonized by microbial communities ("microbiota"). A Particularly high density of commensal microbiota is present the intestine. Commensal microbial colonization shields the intestines from infection with bacterial pathogens, said effect being called "colonization resistance".
- 15 The virulence strategies allowing enteropathogenic bacteria to successfully compete with the microbiota and to overcome colonization resistance are poorly understood. In a recent study looking at the manipulation of the intestinal microbiota by the enteropathogenic bacterium *Salmonella enterica* subspecies 1 Serovar Typhimurium (*S. typhimurium*) in a mouse colitis model, it was found that
- 20 an inflammatory host response induced by *S. typhimurium* was necessary and sufficient for the pathogen to overcome colonization resistance, changing both microbiota composition and suppressing its growth (Bärbel Stecher et al., "*Salmonella enterica* Serovar Typhimurium exploits inflammation to compete with

the intestinal microbiota", PLoS Biology, vol. 5, 2007, pp. 2177-2187). The disclosure of the above mentioned reference is incorporated herein by reference in its entirety.

The *Escherichia coli* strain Nissle 1917 is one of the best-studied probiotic strains. It is commercially available from ARDEYPHARM GmbH, Herdecke, Germany, under the trademark 'Mutaflor'. This particular *E. coli* strain was isolated in 1917 by Alfred Nissle based on its potential to protect from infectious gastroenteritis. The Nissle 1917 strain has been shown to combine efficient intestinal survival and colonization with the lack of virulence. This makes it a safe and effective candidate in the treatment of chronic inflammatory bowel diseases as well as diarrheal diseases in young children.

E. coli Nissle 1917 strain reduces the severity of the disease symptoms and duration. However, it cannot completely block the disease. Therefore, new probiotic strains with superior protective properties are needed.

15 **Objects of the Invention**

An object of the invention is to provide alternative and enhanced probiotic *E. coli* strains with increased colonization resistance properties. Another object of the invention is to provide an advantageous method of treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals.

20 These and other objects are achieved by an *E. coli* strain, a composition comprising said *E. coli* strain, and a method of treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals, as given in the independent

claims. Advantageous embodiments of the invention are given in the dependent claims.

Summary of the invention

According to a first aspect of the invention, there are provided genetically modified probiotic *E. coli* cells comprising at least one first genetic modification introducing an exogenous protein and/or enhancing enzymatic activity of an endogenous protein, the first genetic modification resulting in an enhanced level of biosynthesis of at least one colicin immunity protein or precursors thereof compared to the respective wild type cell.

According to a second aspect of the invention, there are provided modified probiotic *E. coli* cells, wherein the colicin immunity protein is selected from a group comprising: colicin A, colicin B, colicin D, colicin Ia, colicin Ib, colicin K, colicin M, colicin N, colicin E1, colicin E2, colicin E4, colicin E5, colicin E6, colicin E7, colicin E8, colicin E9 and colicin S4 immunity protein preferably from a group consisting of: colicin Ia, colicin E1, colicin E2, colicin B, colicin M, colicin K and colicin S4 immunity protein.

According to a third aspect of the invention, the probiotic cells comprise plasmids configured to express a gene encoding said colicin immunity protein. Alternatively genes encoding colicin resistance can be introduced into the genome of the cells as transgene.

According to a fourth aspect of the invention the plasmid is configured to express a gene encoding said colicin immunity protein and the corresponding colicin protein.

According to a further aspect of the invention, there is provided an isolated *E. coli* strain, which is designated 8178. It corresponds to International Deposition No: DSM21844.

According to a further aspect of the invention, there are provided probiotic compositions comprising an effective amount of *E. coli* strain 8178 and/or of enhanced genetically modified probiotic *E. coli* cells comprising at least one first genetic modification introducing an exogenous protein and/or enhancing enzymatic activity of an endogenous protein, the first genetic modification resulting in an enhanced level of biosynthesis of at least one colicin immunity protein compared to the respective wild type cell.

In an advantageous embodiment the composition according to the invention is a food product, a food additive or supplement, an animal feed additive or supplement, a pharmaceutical preparation or a biotherapeutic composition. Preferably the composition according to the invention is formulated for oral delivery. In another advantageous embodiment of the composition according to the invention the bacterial strain is provided in a lyophilized form.

According to a further aspect of the invention the above mentioned composition is used for treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals. Said gastrointestinal disorder may be for example *Salmonel-*

la enterica induced acute diarrhea, chronic enteric Salmonellosis, and Typhoid fever (chronic carrier state).

According to a fourth aspect of the invention the above mentioned composition is used for the manufacture of a medicament for treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals. Said gastrointestinal disorder may be *Salmonella enterica* induced acute diarrhea, chronic enteric Salmonellosis, and Typhoid fever (chronic carrier state).

According to a further aspect of the invention, there is provided a method of treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals, the method comprising the administration to a subject in need thereof an therapeutically effective amount of the above mentioned *E. coli* cells or *E. coli* strain 8178 or a composition as described above, comprising an effective amount of said *E. coli* cells or the *E. coli* strain 8178. Said gastrointestinal disorder may be *Salmonella enterica* induced acute diarrhea, chronic enteric Salmonellosis, and Typhoid fever (chronic carrier state).

In an advantageous embodiment of the method according to the invention the bacteria, the bacterial strain or the composition is administered orally. In another advantageous embodiment of the method according to the invention the bacterial strain is provided in a lyophilized form.

Preferably the administration is effected at a concentration of said bacterial strain between 10^5 and 10^9 viable cells per dose.

According to a further aspect of the invention, there is provided a method for enhancing colonization resistance properties in *E. coli* strains comprising the steps of:

- 5 i) selection of a colicin-producing *Salmonella* spp. strain or serovar (short for serovarietas), preferably human pathogenic *Salmonella typhimurium* or *Salmonella enteritidis* strains,
- ii) co-infection of said colicin-producing *Salmonella* with at least one *E. coli* strain, preferably a *E. coli* strain Nissle 1917,
- 10 iii) in vivo transfer of a plasmid encoding for at least a colicin immunity protein from the *Salmonella* strain to the *E. coli* strain, and
- iv) isolation of co-infected *E. coli* strains carrying the plasmid encoding for at least a colicin immunity protein.

15 According to a further aspect of the invention, there is provided a method for enhancing colonization resistance properties in *E. coli* strains comprising the steps of:

- 20 i) producing a plasmid configured to express a gene encoding at least one colicin immunity protein, preferably selected from: colicin A, colicin B, colicin D, colicin Ia, colicin Ib, colicin K, colicin M, colicin N, colicin E1, colicin E2, colicin E4, colicin E5, colicin E6, colicin E7, colicin E8, colicin E9 and colicin S4, and
- ii) in vivo or in vitro transfer of said plasmid into an *E. coli* strain.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. All publications mentioned hereunder are incorporated herein by reference.

- 5 Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

I. *E. coli* strain 8178

Described herein is the isolation of strains of *E. coli* according to the present
10 invention for use as probiotic treatments for the prevention of Salmonella enterica *S. typhimurium* diarrhea. One of these *E. coli* strains was isolated from Mouse and designated as 8178 (corresponding to International Deposition No. DSM21844) It has to be noted that the above identified strain may be used alone or in combination, and may also be combined with other treatments
15 known in the art.

Using a mouse model for acute *S. typhimurium* induced diarrhea (Hapfelmeier and Hardt, TiM 2005; Stecher and Hardt TiM 2008) it was observed that some *E. coli* strains, being low frequency members of the normal gut flora, grow up to high densities specifically in cases of severe gut inflammation. This phenome-
20 non has been evident in 10-30% of all mouse experiments.

One such competing *E. coli* strain was isolated from mouse and termed strain 8178. Said strain has been deposited at Deutsche Sammlung von Mikroorga-

nismen und Zellkulturen (DSMZ), Braunschweig, Germany, and was assigned Deposition No. DSM21844. *E. coli* strain 8178 was assigned to ECOR group B2.

The competition of *E. coli* strain 8178 as well as *E. coli* strain Nissle 1917 with wild type strain *S. typhimurium* SL1344 in the intestinal lumen was specifically
5 tested in the above mentioned *S. typhimurium* diarrhea mouse model. It was found that *E. coli* strain 8178 can significantly reduce wild type *S. typhimurium* colonization in the infected inflamed gut lumen at day 4 post infection. In contrast, *S. typhimurium* levels in the intestine were not significantly decreased by Nissle 1917 within the same time. *S. typhimurium* densities in the caecal con-
10 tent were significantly lower in strain 8178-coinfected mice than in control mice. Similarly, the *E. coli* Nissle 1917 strain was able to grow, but could not to diminish *S. typhimurium* levels in streptomycin-treated mice.

The experimental data therefore show that the newly isolated *E. coli* strain 8178 is a more effective agent than *E. coli* Nissle 1917 for treating diarrheal diseases
15 in vertebrates, particularly in mammalian organisms including humans.

Experimental results

It was observed that after *S. typhimurium* infection of streptomycin-treated mice (mouse model for acute *S. typhimurium* induced diarrhea, Barthel, 2003 #1300) certain *E. coli* strains, being low frequency members of the normal gut flora,
20 grow up to high densities.

Intestinal contents of infected mice were plated on MacConkey-agar plates (selective culture media for enterobacteriaceae) without antibiotics, on which *E. coli*

colonies ("red colonies") and *Salmonella* colonies ("white colonies") can be distinguished. **Figure 1** shows the percentage of "red colonies" in relation to *Salmonella* colonies for a selection of streptomycin-treated mice infected with wt *S. typhimurium*, at day 1 and 4 after infection. As is evident in a considerable number of experiments (10-30% of all mouse experiments) the percentage of *E. coli* colonization in relation to *S. typhimurium* colonization in the intestines on day 4 after infection reaches up to 80 %.

One of the *E. coli* strains with high colonization percentage was isolated from mouse (strain 8178), and was tested for its competition with wild type strain *S. typhimurium* SL1344 in the intestinal lumen, and compared to coli strain Nissle 1917.

Animals: All aspects of animal procedures were approved by the responsible local authorities and performed according to the Swiss legal requirements. Sex and age matched specified pathogen free (SPF) C57Bl/6 mice were held under barrier conditions at the rodent center (RCHCI, ETH Hönggerberg, Wolfgang-Pauli-Str. 10, CH-8093 Zürich) of the Swiss Federal Institute of Technology Zurich.

Infection: Mice were treated with streptomycin (20 mg i.g.) and infected 24 h later, with *S. typhimurium* strain SL1344 (5×10^7 cfu i.g., streptomycin-resistant), as a control group, (2) with a 1:1 mixture of *S. typhimurium* wild type strain SL1344 and streptomycin-resistant *E. coli* Nissle 1917 (a total of 5×10^7 cfu i.g.), (3) with a 1:1 mixture of *S. typhimurium* wild type strain SL1344 and streptomycin-sensitive *E. coli* strain 8178 (a total of 5×10^7 cfu i.g.).

Fecal bacterial loads at day 1 post infection (p.i.) were determined by plating on respective MacConkey-agar plates. Mice were sacrificed at day 4 p.i. and caecal bacterial loads were determined by plating on respective MacConkey-agar plates with or without streptomycin. The results are shown in **Figure 2**, (a) for
5 day 1 post infection, and (b) for day 4 post infection, on a logarithmical scale.

The experimental results clearly show that *E. coli* strain 8178 significantly reduces wild type *S. typhimurium* colonization in the infected inflamed gut lumen at day 4 post infection. In contrast, *S. typhimurium* levels in the intestine were not significantly decreased by *E. coli* Nissle 1917 within the same time. The de-
10 termined *S. typhimurium* densities in the caecal content were significantly lower in *E. coli* strain 8178-coinfected mice than in the control group (*S. typhimurium* infection only). Similarly, the *E. coli* Nissle 1917 strain was able to grow, but could not to diminish *S. typhimurium* levels in streptomycin-treated mice.

E. coli strain 8178 is thus a more effective agent than Nissle 1917 for treating
15 diarrheal diseases in mammalian organisms as it has enhanced colonization resistance properties and is therefore a better competitor against *S. typhimurium*. Furthermore it can be concluded that *E. coli* strain is also a more effective probiotic agent for preventing diarrheal diseases.

20 II. Screen for genes of 8178 that enhance its colonization resistance properties

A screen was conducted for genes that are present in *E. coli* 8178 and make - after transfer into *E. coli* Nissle 1917 - this probiotic strain a better competitor against *S. typhimurium*. A cosmid library containing partially digested genomic

DNA of *E.coli* 8178 was constructed. As host vector for the library pM1423 was used which is based on the cosmid vector Supercos1 (Stratagene) but contains an origin of transfer (oriT) that enabled mobilization of the cosmid between different *E.coli* strains by conjugation with a helper strain. The library having a
5 complexity of 1000 individual cosmids was transferred into *E. coli* Nissle 1917.

For construction of plasmid pM1423 the oriT sequence of pGP704 was amplified by PCR using primer sequences: OriTfwd: CCCAAGCTTTTTTGTCCGGTGTGTTGGGTTG (Sequ. ID. No. 5) and OriTrev: CCCAAGCTTAGCCGACCAGGCTTTCACGC(Sequ. ID. No. 6) and cloned
10 into the HindIII site of SuperCos 1 (Stratagene).

Genomic DNA was isolated from *E. coli* 8178 by standard methods. The preparation of the pM1423 vector for ligation reaction with genomic DNA fragment of *E. coli* 8178 was executed as indicated in the Cold Spring Harbor protocol (Sambrook et al., 1989). 20 µg of pM1423 DNA was digested with 50U XbaI in
15 a volume of 200 µl and incubated over night at 37 ° C. The digested cosmid was extracted once with phenol/chloroform and once with chloroform, precipitated with 100% ethanol/0.3M sodium acetate, washed in a solution of 70% ethanol, and dissolved in 180 µl ddH₂O. The digest was analyzed by 1% agarose gel electrophoresis. To the 180 µl pM1423 [XbaI], 20 µl 10x dephosphorylation buffer and 0.1 Unit of Alkaline phosphatase (AP) was added and incubated for 30min at 37 ° C. An additional aliquot (0.1 U) of AP was added, incubated again for 30min at 37 ° C and then the AP was inactivated at 65° C for 30min. The cosmid was extracted, precipitated, washed and dissolved in 180 µl ddH₂O as indicated above. To the pM1423 [XbaI, CIAP] DNA, 20 µl 10x BamHI
20 restriction buffer and 40U BamHI were added, incubated over night at 37 ° C. The BamHI treated DNA was extracted precipitated, washed and the DNA pellet was dissolved in 15 µl ddH₂O. The XbaI and BamHI digested cosmid was ana-

lyzed by 1% agarose gel electrophoresis. The ligation reaction was performed as indicated in the SuperCos1 cosmid vector kit protocol (Stratagene). The amount of digested chromosomal #8178 and SuperCos 1 DNA was measured; SuperCos 1: 127ng/ μ l and #8178 chromosomal DNA: 746ng/ μ l. The ligation
5 was done using the following components: 2.5 μ g partially digested #8178 genomic DNA [CIAP], 1.0 μ g SuperCos 1 DNA [XbaI, CIAP, BamHI], 2.0 μ l of 10x ligase buffer, 2.0 μ l 10mM rATP, 1.0 μ l T4 DNA ligase and water was added to a final volume of 20 μ l. Afterwards the ligation reactions were incubated over night at 16°C. The ligation was then packaged in lambda phage using the Stra-
10 tagene Gigapack® III XL Packaging Extract according to the manufacturer's instructions.

Cosmids were transferred from the host strain *E. coli* XL1blue MR to *E. coli* Nissle 1917 by conjugation.

By way of infection experiments it was screened for cosmids that contain genes
15 conferring competitive properties to *E. coli* Nissle 1917 against *S. typhimurium* in the gut. Streptomycin-treated mice were co-infected with a 1:1 mixture of *S. typhimurium* SL1344 and *E. coli* Nissle 1917 (+cosmid-library) (in total 10^7 cfu). 4 days post infection cosmid-bearing *E. coli* Nissle 1917 clones were isolated from intestinal content. When analyzing the cosmids by restriction enzyme
20 analysis it was found that in 2/3 of the mice one cosmid clone was enriched.

This clone was termed pB944-4. pB944-4 was re-transformed into *E. coli* Nissle 1917 and it was verified that it confers competitive properties against *S. typhimurium* in a co-infection experiment (**Figure 3**).

The sequence of the insert of pB944-4 derived from 8178 was determined and
25 found to be 35,657 kbp. To narrow further down the genes responsible for the

competitive property a subscreen of pB944-4 was performed. DNA of pB944-4 was partially digested with Bsp143I and fragments between 1 and 3 kbp were ligated into *Bam*HI digested pBluescript (Stratagene). The resulting plasmid library was transformed into *E. coli* Nissle 1917. Streptomycin-treated mice with
5 a 1:1 mixture of *S. typhimurium* SL1344 and *E. coli* Nissle 1917 (+plasmid library) (in total 10^7 cfu) were co-infected. 4 days post infection plasmid-bearing *E. coli* Nissle 1917 clones were isolated from intestinal content and the cosmids were analyzed by restriction enzyme analysis and it was found that in 3 mice one plasmid clone was enriched.

10 The enriched clone was termed pC831-2. It contains a 1,177 kbp long insert. The sequence is homologous to the gene for colicin IB immunity protein (Konisky and Richards, 1970; Varley and Boulnois, 1984). In **Figure 4** the Plasmid map of pC831-2 is shown. pC831-2 has pSK Bluescript (Stratagene) as backbone and contains a 1,177 kbp long insert (arrow between MCS). The sequence is homologous to the gene for promoter and coding sequence of colicin
15 Ib immunity protein (cii) and the c-terminal part of colicin Ib (marked as Colicin IB in **Figure 4**), which is non-functional.

Colicins are proteinous toxins produced by and toxic for bacteria of the Enterobacteriaceae family (Kerr et al., 2002). They can be encoded on colicinogenic
20 plasmids that bear the genetic determinants for colicin synthesis, immunity and sometimes also release. The gene for colicin Ib production and the respective immunity protein are also present in *S. typhimurium* strain SL1344. The colicin Ib immunity protein inserts into the bacterial membrane and protects the producer against being killed by its own colicin.

Experimental results

It was shown that colicin Ib production by *S. typhimurium* SL1344 inhibits *E. coli* Nissle 1917 in co-infection experiments in the gut. However, *E. coli* Nissle 1917 competed better against *S. typhimurium* SL1344 deficient for colicin production
5 (Δ colIB IR).

It is shown in **Figure 3** that *E. coli* Nissle pB944-4 out-competes *S. typhimurium* wild-type. Ampicillin-treated mice (n=5; 25mg i.g.) were co-infected with *S. typhimurium* wild type (*S. typhimurium* pWKS30) and *E. coli* Nissle 1917 (ECN pM1423 or pB944-4 (total of 5x10⁷; 1:1). *E. coli* (triangles) and Salmonella
10 (dots) colonization density in cecal content at day 4 pi. was determined (Log10 cfu/gram). Complementation of ECN with cosmid pB944-4, containing a 35kbp fragment of 8178 DNA confers out-competing phenotype. The dotted line indicates the detection limit.

Furthermore, it was shown that pC831-2 improves the fitness of *E. coli* Nissle
15 1917 as it renders it resistant against colicin Ib. In **Figure 5** it is shown that Colicin Ib plays a major role in competition between *S. typhimurium* SL1344 and *E. coli* Nissle 1917. In the experiments according to **Figure 5A** Ampicillin-treated mice (n=5; 25mg i.g.) were co-infected with *S. typhimurium* wild type (*S. typhimurium* pWKS30) and *E. coli* Nissle 1917 ECN pSK or pC831-2 (total of 5x10⁷;
20 1:1). *E. coli* (blue) and Salmonella (red) colonization density in cecal content at day 4 pi. was determined (Log10 cfu/gram). Complementation of ECN with plasmid pC831-2, encoding the colicin Ib (colIB) immunity protein improves the fitness of *E. coli* Nissle 1917 in competition against *S. typhimurium*. In the experiments shown in **Figure 5B** Streptomycin-treated mice (n=5; 25mg i.g.) were

co-infected with *S. typhimurium* wild type or colicin Ib (colIB) deficient mutant M990 and *E. coli* Nissle 1917 ECN (total of 5×10^7 ; 1:1). *E. coli* (blue) and *Salmonella* (red) colonization density in cecal content at day 4 pi. was determined (Log₁₀ cfu/gram). *E. coli* Nissle 1917 ECN competes significantly better against
5 the colicin-deficient *S. typhimurium* mutant. The dotted line indicates again the detection limit.

III. Improvement of probiotic *E. coli* Nissle by natural *in vivo* selection against *S. typhimurium* SL1344

The genes for colicin Ib production and immunity are encoded on a conjugative
10 86kb plasmid in *S. typhimurium* SL1344 ('plasmid II'). Plasmid II was tagged in *S. typhimurium* SL1344 with an antibiotic resistance marker (*S. typhimurium* M992; plasmid II::aphT) and streptomycin-treated mice were co-infected with *E. coli* Nissle 1917 and M992 (1:1 by gavage). After 4 days post infection *E. coli* Nissle 1917 harboring plasmid II that was *in vivo* transferred by conjugation
15 from *S. typhimurium* M992 was detected. The resulting *E. coli* Nissle 1917 strain (plasmid II::aphT) was now resistant to colicin IB as it contained now the genes for colicin Ib synthesis and immunity and showed improved competitiveness against *S. typhimurium* SL1344.

This *in vivo* selection can be done analogically with any other colicin-producing
20 *Salmonella enterica* serovars to improve the fitness of a probiotic *E. coli* strain. With this strategy, *E. coli* Nissle 1917 with higher efficacy against predominant epidemic *Salmonella enterica* strains can be produced 'naturally' without *in vitro* genetic manipulation.

IV. Improvement of probiotic *E.coli* Nissle by rational design

Colicin production by *Salmonella enterica* strains or serovars is an important factor for competition against commensal *E. coli* strains. It has been shown above, that the efficacy of *E. coli* Nissle 1917 against *S. typhimurium* SL1344
5 can be significantly improved by the introduction of a plasmid expressing the gene for colicin Ib immunity protein by enhancing colonization resistance properties in said *E. coli* strain.

Based on the disclosure above, plasmids can be designed that express immunity proteins for other colicins. Such colicins are for example: colicin A, colicin B,
10 colicin D, colicin Ia, colicin Ib, colicin K, colicin M, colicin N, colicin E1, colicin E2, colicin E4, colicin E5, colicin E6, colicin E7, colicin E8, colicin E9 and colicin S4. Immunity proteins for colicin Ia, colicin E1, colicin E2, colicin B, colicin M, colicin K and colicin S4 are encoded by the enclosed Sequences Sequ ID Nos. 1 to 4.

Said sequences were cloned into pSK Bluescript and afterwards introduced into
15 *E. coli*, preferably into *E. coli* Nissle.

Experimental data shows that the immunity proteins for said colicins can lead to improved efficacy of such enhanced strain against infections with *Salmonella* strains or serovars that produce the respective type of colicin.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM



ETH Zürich

Institute of Microbiology

Wolfgang-Pauli-Str. 10

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SWITZERLAND

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Isolate 8178	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 21844
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: ☐ a scientific description ☒ a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2008-09-17 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Inhoffenstr. 7 B D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2008-09-19

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE
INTERNATIONAL FORM



ETH Zürich
Institute of Microbiology
Wolfgang-Pauli-Str. 10
8093 Zürich
SWITZERLAND

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: ETH Zürich Institute of Microbiology Address: Wolfgang-Pauli-Str. 10 8093 Zürich SWITZERLAND	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 21844 Date of the deposit or the transfer ¹ : 2008-09-17
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2008-09-17 ² . On that date, the said microorganism was ☒ ³ viable ☐ ³ no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Inhoffenstr. 7 B D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2008-09-19

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
³ Mark with a cross the applicable box.
⁴ Fill in if the information has been requested and if the results of the test were negative.

Claims

1. Genetically modified probiotic *E. coli* cells comprising at least one first genetic modification introducing an exogenous protein and/or enhancing enzymatic activity of an endogenous protein, the first genetic modification resulting in an enhanced level of biosynthesis of at least one colicin immunity protein compared to the respective wild type cell.
2. Genetically modified probiotic *E. coli* cells according to claim 1, wherein the colicin immunity protein is selected from a group comprising: colicin A, colicin B, colicin D, colicin Ia, colicin Ib, colicin K, colicin M, colicin N, colicin E1, colicin E2, colicin E4, colicin E5, colicin E6, colicin E7, colicin E8, colicin E9 and colicin S4 immunity protein preferably from a group consisting of: colicin Ia, colicin E1, colicin E2, colicin B, colicin M, colicin K and colicin S4 immunity protein.
3. Genetically modified probiotic *E. coli* cells according to claim 1 or 2, wherein the cells express a genomically encoded transgene encoding said colicin immunity protein or comprise plasmids configured to express a gene encoding said colicin immunity protein.
4. Genetically modified probiotic *E. coli* cells according to claim 3, wherein the plasmid is configured to express a gene encoding said colicin immunity protein and the corresponding colicin protein.
5. A strain of bacteria having all the identifying characteristics of *E. coli* strain 8178 (International Deposition No: DSM21844).

6. A composition comprising an effective amount of probiotic *E. coli* according to one of claims 1 to 5.
7. A composition according to claim 6, the composition being a food product, a food additive or supplement, an animal feed additive or supplement, a pharmaceutical preparation or a biotherapeutic composition.
8. A composition according to claim 6 or 7 for treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals.
9. The composition according to claim 8, wherein the gastrointestinal disorder is selected from the group consisting of *Salmonella enterica* induced acute diarrhea, chronic enteric Salmonellosis, and Typhoid fever (chronic carrier state).
10. A use of a composition according to any of claims 6 to 9 for the manufacture of a medicament for treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals.
11. The use according to claim 10, wherein the gastrointestinal disorder is selected from the group consisting of *Salmonella enterica* induced acute diarrhea, chronic enteric Salmonellosis, and Typhoid fever (chronic carrier state).
12. A method of treating or preventing a gastrointestinal disorder in vertebrates, particularly mammals, the method comprising the administration to a subject in need thereof an therapeutically effective amount of bacteria

according to any of claims 1 to 5 or a composition according to any of claims 6 to 9.

- 5 **13.** The method according to claim 12, wherein said administration is effected at a concentration of said bacterial strain between 10^5 and 10^8 viable cells viable cells in one dose.
- 10 **14.** A method for enhancing colonization resistance properties in *E. coli* strains comprising the steps of: i) selection of a colicin-producing *Salmonella* strain or serovar, preferably *Salmonella typhimurium* or *Salmonella enteritidis* strains, ii) co-infection of said colicin-producing *Salmonella* with at least one *E. coli* strain, preferably a *E. coli* strain Nissle 1917, iii) in vivo transfer of a plasmid encoding for at least a colicin immunity protein from the *Salmonella* strain or serovar to the *E. coli* strain, iv) isolation of co-infected *E. coli* strains carrying the plasmid encoding for at least a colicin immunity protein.
- 15 **15.** A method for enhancing colonization resistance properties in *E. coli* strains comprising the steps of: i) producing a plasmid configured to express a gene encoding at least one colicin immunity protein, preferably selected from: colicin A, colicin B, colicin D, colicin Ia, colicin Ib, colicin K, colicin M, colicin N, colicin E1, colicin E2, colicin E4, colicin E5, colicin E6, colicin E7, colicin E8, colicin E9 and colicin S4 ii) in vivo or in vitro transfer of said plasmid into an *E. coli* strain.
- 20

Fig. 1

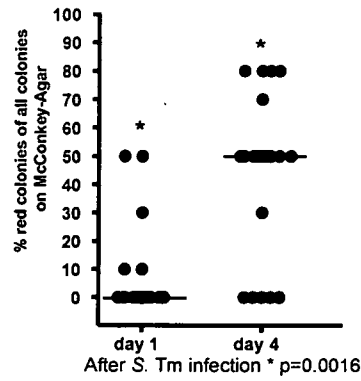
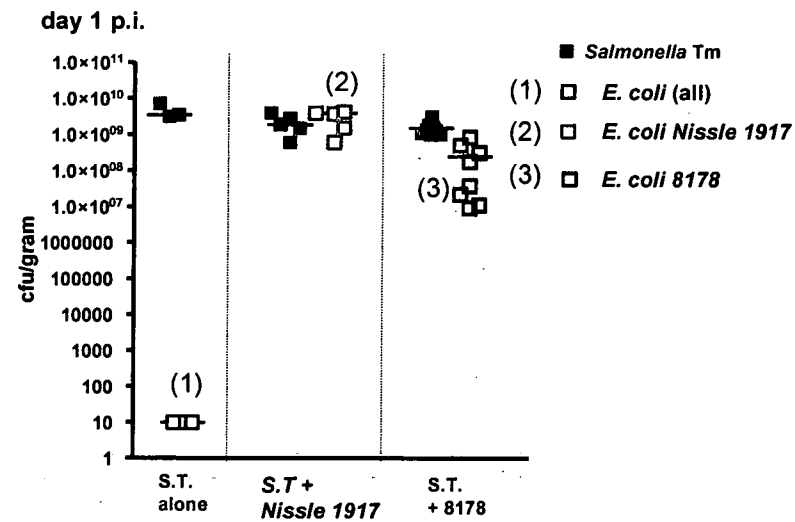
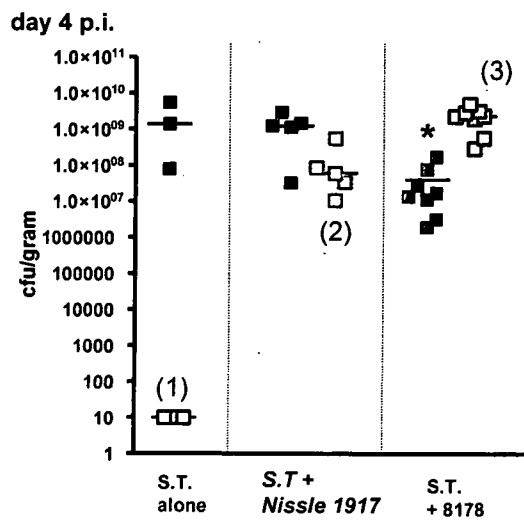


Fig. 2

(a)



(b)



2/3

Fig. 3

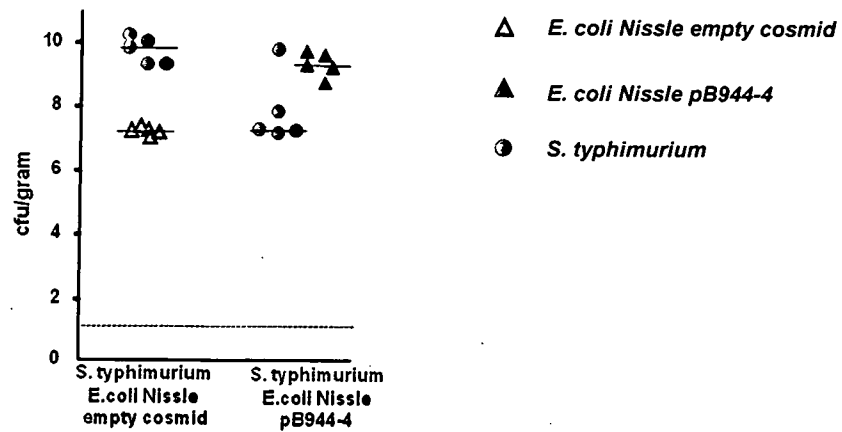


Fig. 4

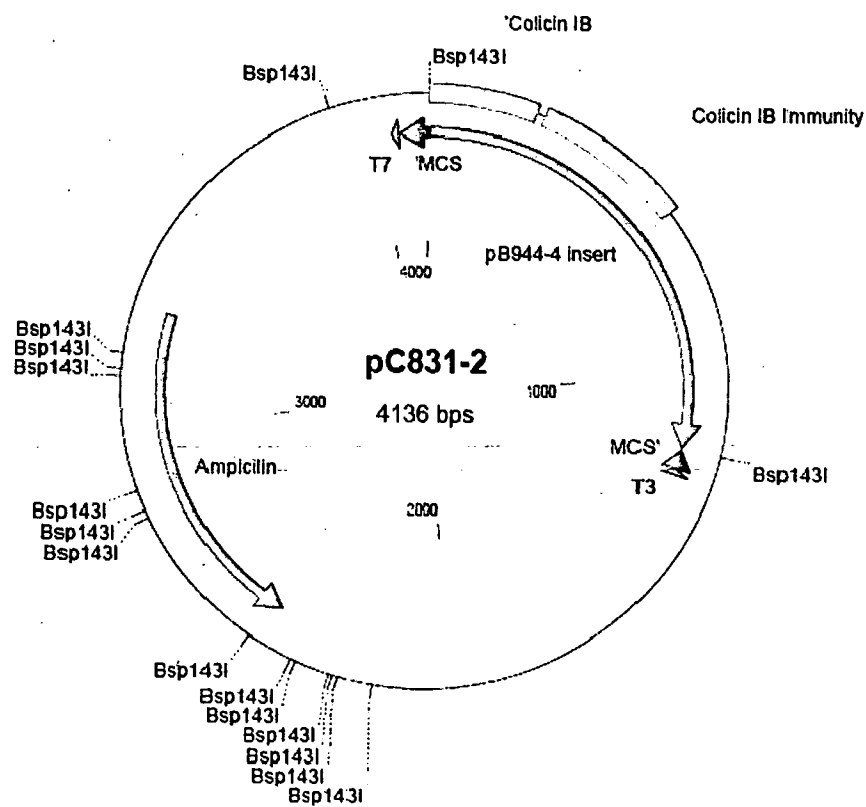
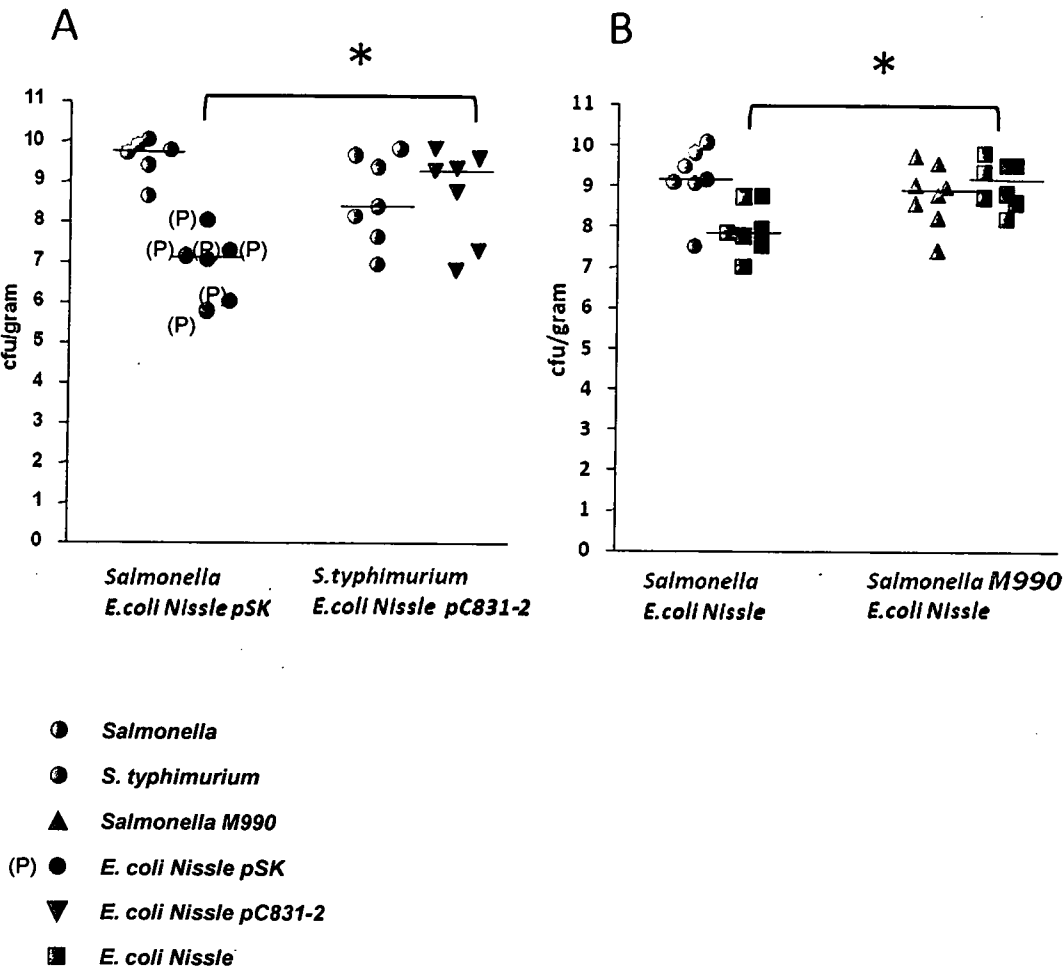


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/006871

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N1/20 C07K14/245
ADD. C12R1/185

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)

C12N C07K

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 practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHANAFELT A B ET AL: "Cloning and overexpression of the colicin E1 immunity gene" PLASMID, NEW YORK, NY, US, vol. 17, no. 3, 1 May 1987 (1987-05-01), pages 261-264, XP024799717 ISSN: 0147-619X [retrieved on 1987-05-01] the whole document ----- -/--	1-7

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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.

"&" document member of the same patent family

Date of the actual completion of the international search

3 February 2010

Date of mailing of the international search report

05/03/2010

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Schneider, Patrick

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/006871

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRICKMAN T J ET AL: "Colicins B and Ia as novel counterselective agents in interspecies conjugal DNA transfers from colicin-sensitive Escherichia coli donors to other Gram-negative recipient species" GENE, ELSEVIER, AMSTERDAM, NL, vol. 178, no. 1, 31 October 1996 (1996-10-31), pages 39-42, XP004043336 ISSN: 0378-1119 the whole document	1-7
X	O'BRIEN G J ET AL: "Colicin 24, a New Plasmid-Borne Colicin from a Uropathogenic Strain of Escherichia coli" PLASMID, NEW YORK, NY, US, vol. 31, no. 3, 1 May 1994 (1994-05-01), pages 288-296, XP024799067 ISSN: 0147-619X [retrieved on 1994-05-01] the whole document	1-7
A	WO 2007/124588 A (UNIV MANITOBA [CA]; KRAUSE DENIS O [CA]; NYACHOTI MARTIN [CA]) 8 November 2007 (2007-11-08) the whole document	
A	STECHER BAERBEL ET AL: "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota" PLOS BIOLOGY, vol. 5, no. 10, October 2007 (2007-10), pages 2177-2189, XP009128684 ISSN: 1544-9173(print) 1545-7885(ele cited in the application the whole document	
A	STECHER ET AL: "The role of microbiota in infectious disease" TRENDS IN MICROBIOLOGY, ELSEVIER SCIENCE LTD., KIDLINGTON, GB, vol. 16, no. 3, 14 February 2008 (2008-02-14), pages 107-114, XP022509717 ISSN: 0966-842X cited in the application the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/006871

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007124588 A	08-11-2007	CA 2649992 A1 US 2009324565 A1	08-11-2007 31-12-2009