(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 January 2021 (14.01.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/005059 A1

(51) International Patent Classification:
A61K 35/741 (2015.01) *C12N 1/20* (2006.01)

(21) International Application Number:
PCT/EP2020/069124

(22) International Filing Date:
07 July 2020 (07.07.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
19184867.0 08 July 2019 (08.07.2019) EP

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(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, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, 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, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title:** MODIFIED ESCHERICHIA COLI STRAIN NISSLE AND TREATMENT OF GASTROINTESTINAL DISORDER

(57) **Abstract:** The invention relates to the field of modified *Escherichia coli* strain Nissle 1917 (EcN) and its use for treating gastro-intestinal disorders. The invention is based on the study of the mechanisms implicated in the probiotic properties of the *Escherichia coli* strain Nissle 1917 (EcN). This study has allowed the inventors to decouple the probiotic activity of EcN from its genotoxic activity by demonstrating that EcN ClbP protein, the enzyme that activates the genotoxin colibactin, is also required for the siderophore-microcins activity of probiotic EcN, but interestingly, not its enzymatic domain that cleaves precolibactin to form active colibactin. Furthermore, inventors demonstrate in an *in vivo* animal model infected by a bacterial pathogen that administration of an EcN modified strain with clbP gene encoding ClbP protein inactive for the peptidase domain, is non-genotoxic (do not produce colibactin) but keeps the bacterial antagonist activity, and reduces colonization and virulence of the pathogen by maintaining the siderophore-microcin production. Thus this study opens the way to safe use of EcN and accordingly the present invention provides an *Escherichia coli* strain Nissle 1917 (EcN) bacterium carrying a gene encoding ClbP protein which is inactive for the peptidase domain, and its use as a drug and more particularly for use in the treatment of gastro-intestinal disease.

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MODIFIED ESCHERICHIA COLI STRAIN NISSLE AND TREATMENT OF GASTROINTESTINAL DISORDER

FIELD OF THE INVENTION:

The invention generally relates to the field of modified *Escherichia coli* strain Nissle 1917 (EcN) and its use for treating gastro-intestinal disorders.

BACKGROUND OF THE INVENTION:

The probiotic *Escherichia coli* strain Nissle 1917 (EcN) was isolated during World War I by Alfred Nissle in a soldier who resisted a severe diarrhea outbreak (1,2). EcN was initially studied for its ability to fight bacterial gastrointestinal infections. It was demonstrated to impede intestinal colonization by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (3,4) and to exhibit an antibacterial activity against enterohemorrhagic *E. coli* strains (5). EcN is an excellent colonizer of the human gut, and exhibits beneficial effects in various intestinal dysfunctions such as acute diarrhea in infants and toddlers (6), chronic constipation (7), and abdominal pain in patients with irritable bowel syndrome (8). It has been widely used in the treatment of inflammatory bowel diseases (1) and has proven to be as effective as the gold standard mesalazine for the maintenance of remission in ulcerative colitis in children and adults (9).

EcN probiotic activity is believed to be based on multiple peculiar properties and fitness determinants, including antibacterial activities against other bacteria (10). Thanks to an extensive list of siderophores (enterobactin, salmochelin, yersiniabactin, and aerobactin) and multiple siderophore receptors and iron transport systems, EcN reduces *S.* Typhimurium intestinal colonization by competing for iron (3). Enterobactin, salmochelin and yersiniabactin are nonribosomal peptides (NRP) or polyketide (PK)-NRP hybrids, which are synthesized by NRP synthetases and PK synthases (NRPS and PKS) activated by a cognate phosphopantetheinyl transferase (PPTase). In addition to this competition for a limiting nutrient, EcN exhibits a direct antibacterial activity linked to the production of two microcins (Mcc), H47 (MccH47) and M (MccM) (4,11–13). Mcc are secreted low-molecular weight peptides that are synthesized by ribosomes and posttranslationally modified, and which display a potent bactericidal activity against phylogenetically-related bacteria (14,15). MccH47 and MccM are called “siderophore-Mcc” because they are modified posttranslationally by the linkage of a catechol siderophore (13,16). The C-terminus of the Mcc peptide is covalently

bound with a linearized and glycosylated derivative of enterobactin (13,16,17). This siderophore moiety is recognized by the catecholate-siderophore receptors of the target bacterium (12,16). The siderophore-Mcc can therefore enter and kill the sensitive bacterium by a "Trojan Horse" stratagem, by mimicking the iron-siderophore complexes.

Comparative genomic analyses have shown that EcN is closely related to pathogenic *E. coli* strains such as the uropathogenic strain CFT073 (18–20). EcN and CFT073 share eight genomic islands, including the pks/clb island encoding a NRPS-PKS assembly line that synthesizes the genotoxin colibactin (21, 22). Colibactin is produced as a prodrug moiety that is exported in the periplasm by the efflux pump ClbM (23) and then hydrolyzed by the periplasmic membrane-bound ClbP protein with a peptidase activity, which releases the active colibactin (24,25). Colibactin is not only a bona fide virulence factor (26,27) but also a putative procarcinogenic compound. Colibactin alkylates the host cell DNA, resulting in DNA crosslinks, double-strand breaks, chromosome aberrations and gene mutations both in vitro and in vivo (21,22,28–30). Colibactin-producing *E. coli* are overrepresented in biopsies of patients with colorectal cancer (31,32) and they were shown to promote colorectal cancer in mouse models (31,33).

The ambivalence between the pathogenic and probiotic potential of EcN was uncovered when inventors showed that certain enzymes of the pks/clb island enable the synthesis of analgesic lipopeptides (34) and that the probiotic properties of EcN are related to the presence of the pathogenicity island (35). In the Olier et al. (2012) study, inventors inactivated the gene that encodes the phosphopantetheinyl transferase (PPTase) ClbA, which was thought to be specific for colibactin synthesis. However, more recent work has shown that ClbA has a pleiotropic effect and also modulates the synthesis of siderophores as well as that of the analgesic lipopeptides (34,36). As might be expected, use of a probiotic strain that produces a genotoxin is a public health concern. Inventors therefore attempted to clearly decouple the genotoxic from the probiotic activities. In this study, inventors used their knowledge of the biosynthetic pathway of colibactin and other secondary metabolites produced directly or indirectly by the pks/clb island to specifically abrogate the genotoxic activity of colibactin. Inventors examined the ability of mutants to inhibit the growth of pathogenic bacteria while still producing beneficiary secondary metabolites. Inventors successfully decoupled the probiotic activity from the genotoxic activity, consequently opening the way to optimize EcN. However, inventors were surprised to observe that the pks/clb island was even more intimately connected to EcN probiotic activity than we expected, and that there was a co-evolution of pathogenic and probiotic properties in bacteria. EcN is, to some extent, like the "miracle drug"

aspirin. Although, like aspirin, this bacterial strain has been used successfully for over a century, it is crucial to understand the method of action and to take into account the safety and potential side effects.

SUMMARY OF THE INVENTION:

The invention is based on the study of the mechanisms implicated in the probiotic properties of the *Escherichia coli* strain Nissle 1917 (EcN). In addition to the production of two siderophore-microcins (Mcc) responsible for its direct antibacterial effect, EcN synthesizes the genotoxin colibactin encoded by the *pks* island. Colibactin is a virulence factor and a putative pro-carcinogenic compound. Therefore, this study has allowed the inventors to decouple the probiotic activity of EcN from its genotoxic activity. They demonstrated that the pks-encoded ClbP, the enzyme that activates the genotoxin colibactin, is required for the siderophore-microcins activity of probiotic EcN, but interestingly, not its enzymatic domain that cleaves precolibactin to form active colibactin. This study has allowed the inventors to decouple the siderophore-microcins from the genotoxic activities by specifically targeting the peptidase domain of ClbP which opens the way to safe use of EcN. Indeed, ClbP peptidase activity mandatory for colibactin production, has no role in siderophore-microcin production, which provides a way to construct a non-genotoxic strain that retains its antibacterial activity. Furthermore, inventors demonstrate in an *in vivo* animal model infected by an intestinal bacterial pathogen (*S.* Typhimurium) that administration of an EcN modified strain with clbP gene encoding ClbP protein inactive for the peptidase domain (ie. ClbP mutated at position S95), is non-genotoxic (do not produce colibactin) but keeps the bacterial antagonist activity, and reduces colonization and virulence of the pathogen (figure 8) by maintaining the siderophore-microcin production.

Thus, in a first aspect, the present invention provides an *Escherichia coli* strain Nissle 1917 (EcN) bacterium carrying a gene encoding ClbP protein which is inactive for the peptidase domain, wherein the peptidase domain of ClbP protein having the amino acid sequence of SEQ ID NO:3 is involved in activation of the genotoxin colibactin.

A second object of the invention relates to an *Escherichia coli* strain Nissle 1917 (EcN) bacterium, as defined above for use as a drug.

A third object of the invention relates to an *Escherichia coli* strain Nissle 1917 (EcN) bacterium, as defined above for use in the treatment of gastro-intestinal disease.

DETAILED DESCRIPTION OF THE INVENTION:

Modified Escherichia coli strain Nissle 1917

In a first aspect, the present invention provides an *Escherichia coli* strain Nissle 1917 (EcN) bacterium carrying an gene encoding a ClbP protein which is inactive for the peptidase domain,

wherein the peptidase domain of ClbP protein having the amino acid sequence of SEQ ID NO:3 is involved in activation of the genotoxin colibactin.

The inventors have shown that the natural (wild-type) ClbP protein having the amino acid sequence of SEQ ID NO:1, the enzyme that activates the genotoxin colibactin, is also required for the siderophore-microcins activity of probiotic EcN, but interestingly, not its enzymatic domain that cleaves precolibactin to form active colibactin. In particular, the inventors have shown that, mutants EcN bacteria for the gene encoding the ClbP protein which is inactivate only for the peptidase domain according to the invention (ie. mutant S95A, S95R, K98T or ClbP-3H), these mutant maintain *in vitro* and *in vivo* its antibacterial activity (through siderophore-MCC production) but not mutant EcN bacteria with an inactivated gene encoding the whole ClbP protein (i.e. see figure 4 of Example section). These results evidence that ClbP peptidase activity mandatory for colibactin production, has no role in siderophore-microcin production, which provides a way to construct a non-genotoxic EcN strain that retains its antibacterial activity. Consequently, the mutants EcN bacteria, according to the invention, trigger a more safe and protective response, and thus constitute very promising new probiotic as demonstrated with the *in vivo* data using modified EcN strain according to the invention (see figure 8).

The term "*Escherichia coli* strain Nissle 1917" (also called EcN or DSM6601) means a probiotic *Escherichia coli* strain which was isolated during World War I by Alfred Nissle in a soldier who resisted a severe diarrhea outbreak (1,2). EcN was initially studied for its ability to fight bacterial gastrointestinal infections. It was demonstrated to impede intestinal colonization by *Salmonella* enterica serovar Typhimurium (3,4) and to exhibit an antibacterial activity against enterohemorrhagic *E. coli* strains (5). EcN is an excellent colonizer of the human gut, and exhibits beneficial effects in various intestinal dysfunctions such as acute diarrhea in infants and toddlers (6), chronic constipation (7), and abdominal pain in patients with irritable bowel syndrome (8). It has been widely used in the treatment of inflammatory bowel diseases (1) and has proven to be as effective as the gold standard mesalazine for the maintenance of remission in ulcerative colitis in children and adults (9).

Escherichia coli Nissle 1917 (EcN) is the active component of Mutaflor® (Ardeypharm GmbH, Herdecke, Germany), a probiotic drug licensed in several countries for the treatment of multiple intestinal disorders (10). EcN is known to harbour a genomic island, named pks, which carries a cluster of genes that enables the synthesis of hybrid peptide polyketides and especially a genotoxin called colibactin (21). Colibactin is a structurally uncharacterized PK-NRP that is thought to arise from a pro-drug called precolibactin, which has also not been fully structurally elucidated (Li, Z.R. et al. Nat Chem Biol 12, 773-5 (2016); Bode, H.B. Angew Chem Int Ed Engl 54, 10408-11 (2015)). This toxin is produced by a complex biosynthetic machinery involving the sequential action of proteins ClbA to ClbS (Taieb, F., et al EcoSal Plus 7(2016)). The core machinery consists of three polyketide synthases, three non-ribosomal peptide synthetases and two hybrids PKS-NRPS. The machinery also employs additional maturation proteins and efflux pump(s).

Colibactin is produced as a prodrug moiety that is exported in the periplasm by the efflux pump ClbM (23) and then hydrolyzed by the periplasmic membrane-bound ClbP protein with a peptidase activity, which releases the active colibactin (24,25). Colibactin is not only a bona fide virulence factor (26,27) but also a putative procarcinogenic compound.

The term "ClbP protein" means a peptidase encoded by the pks genomic island of *Escherichia coli* (and other *Enterobacteriaceae*). ClbP protein contains an N-terminal signal sequence that targets the protein to the inner membrane, a periplasmic peptidase domain containing the active site of protease (the AA residues forming the peptidase being 39-337), and three C-terminal transmembrane helices (the AA residues forming the three transmembrane helices being 390-412, 433-455, and 465-485). ClbP crystal structure and mutagenesis experiments revealed a serine-active site and original structural features, associated with a peptidase activity (24) ClbP allows the maturation of precolibactin in genotoxic colibactin through ClbP peptidase activity that removes the N-acyl-D-asparagine prodrug scaffold from precolibactin (24,25). S95 K98 and Y186 are key residues for ClbP peptidase activity, and mutants for these residues fail to cleave precolibactin to release mature active genotoxin (24,25).

The sequences of ClbP are indicated in the following Table 1:

ClbP	Sequence
Acid amino sequence of ClbP wild type (SEQ ID NO:_1)	MTIMEHVSIK TLYHLLCCML LFISAMCALA QEHEPIGAQD ERLSTLIHQR MQEAKVPALS VSVTIKGVRQ RFVYGVADVA SQKANTLDTV YELGSMSKAF TGLVVQILIQ EGRLRQGDDI

	ITYLPEMRLN YQGKPASLTV ADFLYHTSGL PFSTLARLEN PMPGSAVAQQ LRNENLLFAP GAKFSYASAN YDVLGAVIEN VTGKTFTEVI AERLTQPLGM SATVAVKGDE IIVNKASGYK LGFGKPVLFH APLARNHVPA AYIHSTLPDM EIWIDAWLHR KALPATLREA MSNSWRGNSD VPLAADNRIL YASGWFIDQN QGPYISHGGQ NPNFSSCIAL RPDQQIGIVA LANMNSNLIL QLCADIDNYL RIGKYADGAG DAITATDTLF VYLTLLLCFW GAVVVVRGAF RVYRATAHGP GKQQRLRLRV RDYIIALAVP GLVAAMLYVA PGILSPGLDW RFILVWGPSS VLAIPFGIIL LAFVLTLNHQ IKRILLHNKE WDDE
Nucleic sequence (ADNc) of ClbP wild type (SEQ ID NO:_2)	atgacaataatggaacacgttagcattaaaacattatatcatctcctgtgctgtatgctgctctttatt tccgctatgtgcgctttggcgcaagaacatgagcctatcggggcgcaagatgagcgcctgtcg acattaattcaccaacggatgcaggaggccaaggtcccagccctttccgtaagtgtgaccatta agggggtacgtcagcgatttgtctacggtgttgccgatgtggctagtcagaaagcgaatactcta gacacagtttacgagctgggatcgatgagtaaggcgtttaccggacttgtggtgcaaatactgat tcaggaaggcagactccggcaaggggatgatatcattacctatctgccggaaatgcgcttgaat tatcagggaaaacctgcttccctgaccgtggctgatttcctttatcatacatcaggattgccttttc aacactggctcggctggaaaaccctatgcctgggagcgctgtggcacagcaactgcgcaacg agaatctgctgtttgcgccgggtgcgaagtttagctatgcctccgccaattatgatgtgttgggcg cggtgattgaaaatgtgacgggaaaaacctttacagaggtcattgcggaacgactcacgcagc cgctgggcatgtcggcgactgtggcagttaaggggatgagattattgtcaacaaggcaagcg gctataaactgggattcggcaaacccgttctgtttcatgcgcctctggcccggaaccatgttcctg ccgcctatatccatagcactctgcctgatatggaaatatggatagacgcctggttgcacagaaag gctttgccggcaacgctgcgtgaggcgatgagtaacagttggcgtggtaatagtgatgttccgc ttgccgcagacaatcgtatcctctatgccagcggttggtttatcgaccagaatcaaggcccttaca tcagtcacggtgggcagaatccaaacttttcttcttgcattgcgttgcgaccggatcagcagattg gcattgttgcgctggcaaatatgaattcgaatctgatactacagctttgcgcggatatcgataatta tctgcgcattggcaaatatgctgacggcgctggtgatgcaattacagccaccgatacccttttcgt ctacctcacgttgttgctgtgtttttgggggggcggtggttgtagtgcgcggtgctttccgtgtttatc gcgcaacggcgcatggccctggaaaacagcagaggttacgtttacgcgtacgtgactatatcat cgccttggcggttcctgggctcgtggccgccatgctctatgtcgcaccgggtatactatctccag gacttgactggcgttttatcttggtatggggtccatcgagcgtgttggcgataccgttcggaattat

	cctgttagctttcgttctgacattaaatcatcaaattaaacgaattctattacacaacaaggagtgg gacgatgagtaa
Acid amino sequence of peptidase domain ClbP (SEQ ID NO:_3)	QD ERLSTLIHQR MQEAKVPALS VSVTIKGVRQ RFVYGVADVA SQKANTLDTV YELGSMSKAF TGLVVQILIQ EGRLRQGDDI ITYLPEMRLN YQGKPASLTV ADFLYHTSGL PFSTLARLEN PMPGSAVAQQ LRNENLLFAP GAKFSYASAN YDVLGAVIEN VTGKTFTEVI AERLTQPLGM SATVAVKGDE IIVNKASGYK LGFGKPVLFH APLARNHVPA AYIHSTLPDM EIWIDAWLHR KALPATLREA MSNSWRGNSD VPLAADNRIL YASGWFIDQN QGPYISHGGQ NPNFSSC

According to the invention, the term “a gene encoding a ClbP protein which is inactive for the peptidase domain”, means that it is meant a gene with mutation that encodes either a non-functional peptidase domain (such as ClbP S95, K98, Y186 mutants) or no peptidase domain at all ((such as ClbP-3H mutants). According to the invention, the inactivation of a specific domain of a gene can be carried out by the various methods known by the skilled person. Examples of methods for inactivating a gene are particularly the directed mutagenesis or the homologous recombination, as described in Conde-Alvarez R. et al., Cell Microbiol 2006 Aug;8(8):1322-35. A man skilled in the art, also knows as to design a specific “nuclease” or “endonuclease” (such as CRISPR) in order to introduce a genetic point mutations to inactivate the peptidase domain of ClbP protein or to genetically recombinate specifically the peptidase domain of ClbP protein in order to replace with an non active equivalent

A particular method for inactivating a specific domain of a gene according to the invention is also described in the experimental section (Gene mutagenesis performed by the lambda Red recombinase method (see 37)).

Thus typically, an EcN mutant according to the invention (i) has the capacity to have an antibacterial activity (through synthesis of siderophore-MCC: MccH47 and MccM); and ii) which is devoid of the capacity to activate the genotoxin colibactin (with an inactivate ClbP peptidase domain).

The skilled in the art can easily determine whether EcN bacterium according to the invention is biologically active. For example, the capacity to have an antibacterial activity can for example be determined by any routine test well known by the man skills in the art: antibiograms, Competitive growth assay, ...

The effect of antibacterial activity can be measured by monitoring reduction of the target bacterial strains (sensible to EcN such as *E. coli* strain LF82) present in a surface prior to and after application with the mutant EcN compositions according to the invention, using in vitro assays (Competitive growth assay). Both the producing and the target strains (ie. EcN or EcN mutants and LF82 respectively) are inoculated as previously described ((3) and also as described in Example + Figures 1 to 5).

The determination of a genotoxic effect induced by colibactin (for EcN mutant candidate) can be measured by monitoring the cellular senescence induced by colibactin with the associated cell enlargement called megalocytosis. As previously described (40), HeLa cells are infected for 4 hours with the EcN candidate. The genotoxicity of EcN and the EcN mutant (ie clbP-S95R) is confirmed by an In-Cell Western procedure, as previously described (36). In brief, HeLa cells are infected for 4 hours at a given multiplicity of infection (number of bacteria per cell at the onset of infection). Four hours after the end of infection cells are fixed, permeabilized and stained with rabbit monoclonal anti-gamma-H2AX followed by an infrared fluorescent secondary antibody.

As used herein, a "biologically active" EcN mutant according to the invention refers to an EcN mutant exhibiting all, of the biological activities of an EcN mutant, provided the biologically active mutant retains the wild-type EcN capacity of antibacterial activity but are devoid of the capacity to activate the genotoxin colibactin. The biologically active EcN mutant according to the invention may for example be characterized in that it is capable of having an antibacterial activity (through production of siderophore-MCC); and ii) is devoid of the capacity to activate the genotoxin colibactin (having an inactivate ClbP peptidase domain) (see Example and Figures 5).

In particular embodiment of the invention, the ClbP protein inactivate for the peptidase domain, is selected from the list consisting of

ClbP protein mutated at position S95,

ClbP protein mutated at position K98,

ClbP protein mutated at position Y186

ClbP protein without peptidase domain (SEQ ID N°3)

In the present invention the numbering of amino acids regarding mutations (punctual mutation or recombination of the peptidase domain) is according to the wild type ClbP protein sequence (SEQ ID NO: 1).

In a preferred embodiment the ClpP protein mutated at position S95, is preferably not substituted with an equivalent polar and non-charged amino acid of Serine. Accordingly, the

ClbP protein mutated at position S95 is preferably not substituted with Threonine, Asparagine or Glutamine.

In a preferred embodiment the ClpP protein mutated at position K98, is preferably not substituted with an equivalent positively charged amino acid of Lysine. Accordingly, ClbP protein mutated at position K98, is preferably not substituted with Arginine, Asparagine or Histidine.

In a preferred embodiment the ClbP protein mutated at position Y186, is preferably not substituted with an equivalent amino acid with hydrophobic side chain of Tyrosine. . Accordingly, ClbP protein mutated at position Y186, is preferably not substituted with alanine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine or Tryptophan.

In a preferred embodiment the ClbP protein without peptidase domain, the peptidase domain of ClbP is substituted or replace with a peptidic domain having a molecular weight between 15 and 150kDa, preferably between 25 and 100kDa, more preferably between 30 and 80kDa, even more preferably between 30 and 50kDa.

This substitution with a peptidic domain (equivalent in weight but devoid of any ClbP peptidase activity which consist to remove the *N*-acyl-*D*-asparagine prodrug scaffold from precolibactin to form colibactin (24,25)) allow to maintain the three-dimensional structure and organisation of the mutant ClbP protein and to maintain the antibacterial activity.

Example of peptidic domain which can used to replace the peptidase domain of ClbP is the mature form of the alkaline phosphatase PhoA encoded by the *Escherichia coli phoA* gene, or the mature form of the enzyme beta-lactamase, encoded by the *bla* gene, or the mature form of the enzyme β-galactosidase encoded by the *Escherichia coli lacZ* gene.

These examples of peptidic domain (alkaline phosphatase PhoA, enzyme beta-lactamase enzyme β-galactosidase) are the most commonly used, especially alkaline phosphatase PhoA (as in the present study) as reporter protein for gene fusion studies in prokaryotes, especially for transmembrane fusion protein having a periplasmic domain (see the review van Geest M. and. Lolkema JS. Microbiol Mol Biol Rev. 2000 Mar; 64(1): 13–33).

In a preferred embodiment, the ClbP protein inactivate for the peptidase domain without peptidase domain, is ClbP protein with a peptidase domain substituted by alkaline phosphatase enzymatic domain of PhoA (SEQ ID N°11). The capacity to create gene fusion is well known by the man skills in the art. Regarding this ClbP fusion protein, only the mature part of PhoA, lacking its signal sequence, is fused behind C-terminal truncated parts of a membrane protein (see also the review van Geest M. and. Lolkema JS. Microbiol Mol Biol Rev. 2000 Mar; 64(1): 13–33). More precisely the PhoA domain is fused with the ClbP N-terminal signal sequence

which allows the translocation to periplasm, and the ClbP C-terminal sequence from amino-acid 390; the residues forming the three transmembrane helices being 390-412, 433-455, and 465-485 (see Example section).

As used herein, the term "amino acid" refers to natural or unnatural amino acids in their D and L stereoisomers for chiral amino acids. It is understood to refer to both amino acids and the corresponding amino acid residues, such as are present, for example, in peptidyl structure. Natural and unnatural amino acids are well known in the art. Common natural amino acids include, without limitation, alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), Lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val).

Amino acids are typically classified in one or more categories, including polar, hydrophobic, acidic, basic and aromatic, according to their side chains. Examples of polar amino acids include those having side chain functional groups such as hydroxyl, sulfhydryl, and amide, as well as the acidic and basic amino acids. Polar amino acids include, without limitation, asparagine, cysteine, glutamine, histidine, selenocysteine, serine, threonine, tryptophan and tyrosine. Examples of hydrophobic or non-polar amino acids include those residues having nonpolar aliphatic side chains, such as, without limitation, leucine, isoleucine, valine, glycine, alanine, proline, methionine and phenylalanine. Examples of basic amino acid residues include those having a basic side chain, such as an amino or guanidino group. Basic amino acid residues include, without limitation, arginine, homolysine and lysine. Examples of acidic amino acid residues include those having an acidic side chain functional group, such as a carboxy group. Acidic amino acid residues include, without limitation aspartic acid and glutamic acid. Aromatic amino acids include those having an aromatic side chain group. Examples of aromatic amino acids include, without limitation, biphenylalanine, histidine, 2-napthylalananine, pentafluorophenylalanine, phenylalanine, tryptophan and tyrosine. It is noted that some amino acids are classified in more than one group, for example, histidine, tryptophan and tyrosine are classified as both polar and aromatic amino acids. Amino acids may further be classified as non-charged, or charged (positively or negatively) amino acids. Examples of positively charged amino acids include without limitation lysine, arginine and histidine. Examples of negatively charged amino acids include without limitation glutamic acid and aspartic acid. Additional amino acids that are classified in each of the above groups are known to those of ordinary skill in the art.

"Equivalent amino acid" means an amino acid which may be substituted for another amino acid in the peptide compounds according to the invention without any appreciable loss of function. Equivalent amino acids will be recognized by those of ordinary skill in the art. Substitution of like amino acids is made on the basis of relative similarity of side chain substituents, for example regarding size, charge, hydrophilicity and hydrophobicity as described herein. The phrase "an equivalent amino acid " when used following a list of individual amino acids means an equivalent of one or more of the individual amino acids included in the list.

In a more particular embodiment of the invention, the ClbP protein inactivate for the peptidase domain, encoded by the gene is selected from the list consisting of

ClbP S95A mutant (SEQ ID N°4),

ClbP K98T mutant (SEQ ID N°6),

ClbP S95R mutant (SEQ ID N°8)

ClbP Y186G mutant (SEQ ID N°10)

ClbP protein with a peptidase domain substituted by alkaline phosphatase enzymatic domain of PhoA (SEQ ID N°11).

The sequences of mutated ClbP according to the invention are indicated in the following Table 2:

Mutant ClbP	Sequence
Acid amino sequence of ClbP S95A (SEQ ID NO:_4)	MTIMEHVSIK TLYHLLCCML LFISAMCALA QEHEPIGAQD ERLSTLIHQR MQEAKVPALS VSVTIKGVRQ RFVYGVADVA SQKANTLDTV YELGAMSKAF TGLVVQILIQ EGRLRQGDDI ITYLPEMRLN YQGKPASLTV ADFLYHTSGL PFSTLARLEN PMPGSAVAQQ LRNENLLFAP GAKFSYASAN YDVLGAVIEN VTGKTFTEVI AERLTQPLGM SATVAVKGDE IIVNKASGYK LGFGKPVLFH APLARNHVPA AYIHSTLPDM EIWIDAWLHR KALPATLREA MSNSWRGNSD VPLAADNRIL YASGWFIDQN QGPYISHGGQ NPNFSSCIAL RPDQQIGIVA LANMNSNLIL QLCADIDNYL RIGKYADGAG DAITATDTLF VYLTLLLCFW GAVVVVRGAF RVYRATAHGP GKQQRLRLRV RDYIIALAVP GLVAAMLYVA PGILSPGLDW RFILVWGPSS VLAIPFGIIL LAFVLTLNHQ IKRILLHNKE WDDE

Nucleic sequence (ADNc) of ClbP S95A (SEQ ID NO:_5)	Atgacaataatggaacacgttagcattaaaacattatatcatctcctgtgctgtatgctgctctttat ttccgctatgtgcgctttggcgcaagaacatgagcctatcggggcgcaagatgagcgcctgtcg acattaattcaccaacggatgcaggaggccaaggtcccagccctttccgtaagtgtgaccatta agggggtacgtcagcgatttgtctacggtgttgccgatgtggctagtcagaaagcgaatactcta gacacagtttacgagctgggagcgatgagtaaggcgtttaccggacttgtggtgcaaatactga ttcaggaaggcagactccggcaaggggatgatatcattacctatctgccggaaatgcgcttgaa ttatcagggaaaacctgcttccctgaccgtggctgatttcctttatcatacatcaggattgccttttc aacactggctcggctggaaaaccctatgcctgggagcgctgtggcacagcaactgcgcaacg agaatctgctgtttgcgccgggtgcgaagtttagctatgcctccgccaattatgatgtgttgggcg cggtgattgaaaatgtgacgggaaaaacctttacagaggtcattgcggaacgactcacgcagc cgctgggcatgtcggcgactgtggcagttaagggggatgagattattgtcaacaaggcaagcg gctataaactgggattcggcaaacccgttctgtttcatgcgcctctggcccggaaccatgttcctg ccgcctatatccatagcactctgcctgatatggaaatatggatagacgcctggttgcacagaaag gctttgccggcaacgctgcgtgaggcgatgagtaacagttggcgtggtaatagtgatgttccgc ttgccgcagacaatcgtatcctctatgccagcggttggtttatcgaccagaatcaaggcccttaca tcagtcacggtgggcagaatccaaacttttcttcttgcattgcgttgcgaccggatcagcagattg gcattgttgcgctggcaaatatgaattcgaatctgatactacagctttgcgcggatatcgataatta tctgcgcattggcaaatatgctgacggcgctggtgatgcaattacagccaccgatacccttttcgt ctacctcacgttgttgctgtgtttttgggggggcggtggttgtagtgcgcggtgctttccgtgtttatc gcgcaacggcgcatggccctggaaaacagcagaggttacgtttacgcgtacgtgactatatcat cgccttggcggttcctgggctcgtggccgccatgctctatgtcgcaccgggtatactatctccag gacttgactggcgttttatcttggtatggggtccatcgagcgtgttggcgataccgttcggaattat cctgttagctttcgttctgacattaaatcatcaaattaaacgaattctattacacaacaaggagtgg gacgatgagtaa
Acid amino sequence of ClbP K98T (SEQ ID NO:_6)	MTIMEHVSIK TLYHLLCCML LFISAMCALA QEHEPIGAQD ERLSTLIHQR MQEAKVPALS VSVTIKGVRQ RFVYGVADVA SQKANTLDTV YELGSMSTAF TGLVVQILIQ EGRLRQGDDI ITYLPEMRLN YQGKPASLTV ADFLYHTSGL PFSTLARLEN PMPGSAVAQQ LRNENLLFAP GAKFSYASAN YDVLGAVIEN VTGKTFTEVI AERLTQPLGM SATVAVKGDE IIVNKASGYK LGFGKPVLFH APLARNHVPA AYIHSTLPDM EIWIDAWLHR KALPATLREA MSNSWRGNSD VPLAADNRIL YASGWFIDQN QGPYISHGGQ NPNFSSCIAL RPDQQIGIVA LANMNSNLIL

	QLCADIDNYL RIGKYADGAG DAITATDTLF VYLTLLLCFW GAVVVVRGAF RVYRATAHGP GKQQRLRLRV RDYIIALAVP GLVAAMLYVA PGILSPGLDW RFILVWGPSS VLAIPFGIIL LAFVLTLNHQ IKRILLHNKE WDDE
Nucleic sequence (ADNc) of ClbP mutated at position S95 (SEQ ID NO:_7)	atgacaataatggaacacgttagcattaaaacattatatcatctcctgtgctgtatgctgctctttatt tccgctatgtgcgctttggcgcaagaacatgagcctatcggggcgcaagatgagcgcctgtcg acattaattcaccaacggatgcaggaggccaaggtcccagccctttccgtaagtgtgaccatta agggggtacgtcagcgatttgtctacggtgttgccgatgtggctagtcagaaagcgaatactcta gacacagtttacgagctgggagcgatgagtaaggcgtttaccggacttgtggtgcaaatactga ttcaggaaggcagactccggcaaggggatgatatcattacctatctgccggaaatgcgcttgaa ttatcagggaaaacctgcttccctgaccgtggctgatttcctttatcatacatcaggattgccttttc aacactggctcggctggaaaaccctatgcctgggagcgctgtggcacagcaactgcgcaacg agaatctgctgtttgcgccgggtgcgaagtttagctatgcctccgccaattatgatgtgttgggcg cggtgattgaaaatgtgacgggaaaaacctttacagaggtcattgcggaacgactcacgcagc cgctgggcatgtcggcgactgtggcagttaaggggatgagattattgtcaacaaggcaagcg gctataaactgggattcggcaaacccgttctgtttcatgcgcctctggcccggaaccatgttcctg ccgcctatatccatagcactctgcctgatatggaaatatggatagacgcctggttgcacagaaag gctttgccggcaacgctgcgtgaggcgatgagtaacagttggcgtggtaatagtgatgttccgc ttgccgcagacaatcgtatcctctatgccagcggttggtttatcgaccagaatcaaggcccttaca tcagtcacggtgggcagaatccaaacttttcttcttgcattgcgttgcgaccggatcagcagattg gcattgttgcgctggcaaatatgaattcgaatctgatactacagctttgcgcggatatcgataatta tctgcgcattggcaaatatgctgacggcgctggtgatgcaattacagccaccgatacccttttcgt ctacctcacgttgttgctgtgtttttgggggggcggtggttgtagtgcgcggtgctttccgtgtttatc gcgcaacggcgcatggccctggaaaacagcagaggttacgtttacgcgtacgtgactatatcat cgccttggcggttcctgggctcgtggccgccatgctctatgtcgcaccgggtatactatctccag gacttgactggcgttttatcttggtatggggtccatcgagcgtgttggcgataccgttcggaattat cctgttagctttcgttctgacattaaatcatcaaattaaacgaattctattacacaacaaggagtgg gacgatgagtaa
Acid amino sequence of ClbP S95R (SEQ ID NO:_8)	MTIMEHVSIK TLYHLLCCML LFISAMCALA QEHEPIGAQD ERLSTLIHQR MQEAKVPALS VSVTIKGVRQ RFVYGVADVA SQKANTLDTV YELG**R**MSKAF TGLVVQILIQ EGRLRQGDDI ITYLPEMRLN YQGKPASLTV ADFLYHTSGL PFSTLARLEN PMPGSAVAQQ LRNENLLFAP GAKFSYASAN YDVLGAVIEN

	VTGKTFTEVI AERLTQPLGM SATVAVKGDE IIVNKASGYK LGFGKPVLFH APLARNHVPA AYIHSTLPDM EIWIDAWLHR KALPATLREA MSNSWRGNSD VPLAADNRIL YASGWFIDQN QGPYISHGGQ NPNFSSCIAL RPDQQIGIVA LANMNSNLIL QLCADIDNYL RIGKYADGAG DAITATDTLF VYLTLLLCFW GAVVVVRGAF RVYRATAHGP GKQQRLRLRV RDYIIALAVP GLVAAMLYVA PGILSPGLDW RFILVWGPSS VLAIPFGIIL LAFVLTLNHQ IKRILLHNKE WDDE
Nucleic sequence (ADNc) of ClbP S95R (SEQ ID NO:_9)	atgacaataatggaacacgttagcattaaaacattatatcatctcctgtgctgtatgctgctctttatt tccgctatgtgcgctttggcgcaagaacatgagcctatcggggcgcaagatgagcgcctgtcg acattaattcaccaacggatgcaggaggccaaggtcccagccctttccgtaagtgtgaccatta agggggtacgtcagcgatttgtctacggtgttgccgatgtggctagtcagaaagcgaatactcta gacacagtttacgagctgggacggatgagtaaggcgtttaccggacttgtggtgcaaatactga ttcaggaaggcagactccggcaaggggatgatatcattacctatctgccggaaatgcgcttgaa ttatcagggaaaacctgcttccctgaccgtggctgatttcctttatcatacatcaggattgccttttc aacactggctcggctggaaaaccctatgcctgggagcgctgtggcacagcaactgcgcaacg agaatctgctgtttgcgccgggtgcgaagtttagctatgcctccgccaattatgatgtgttgggcg cggtgattgaaaatgtgacgggaaaaacctttacagaggtcattgcggaacgactcacgcagc cgctgggcatgtcggcgactgtggcagttaaggggatgagattattgtcaacaaggcaagcg gctataaactgggattcggcaaacccgttctgtttcatgcgcctctggcccggaaccatgttcctg ccgcctatatccatagcactctgcctgatatggaaatatggatagacgcctggttgcacagaaag gctttgccggcaacgctgcgtgaggcgatgagtaacagttggcgtggtaatagtgatgttccgc ttgccgcagacaatcgtatcctctatgccagcggttggtttatcgaccagaatcaaggcccttaca tcagtcacggtgggcagaatccaaacttttcttcttgcattgcgttgcgaccggatcagcagattg gcattgttgcgctggcaaatatgaattcgaatctgatactacagctttgcgcggatatcgataatta tctgcgcattggcaaatatgctgacggcgctggtgatgcaattacagccaccgatacccttttcgt ctacctcacgttgttgctgtgtttttgggggggcggtggttgtagtgcgcggtgctttccgtgtttatc gcgcaacggcgcatggccctggaaaacagcagaggttacgtttacgcgtacgtgactatatcat cgccttggcggttcctgggctcgtggccgccatgctctatgtcgcaccgggtatactatctccag gacttgactggcgttttatcttggtatggggtccatcgagcgtgttggcgataccgttcggaattat cctgttagctttcgttctgacattaaatcatcaaattaaacgaattctattacacaacaaggagtgg gacgatgagtaa

Acid amino sequence of ClbP Y186G (SEQ ID NO:_10)	MTIMEHVSIK TLYHLLCCML LFISAMCALA QEHEPIGAQD ERLSTLIHQR MQEAKVPALS VSVTIKGVRQ RFVYGVADVA SQKANTLDTV YELGSMSKAF TGLVVQILIQ EGRLRQGDDI ITYLPEMRLN YQGKPASLTV ADFLYHTSGL PFSTLARLEN PMPGSAVAQQ LRNENLLFAP GAKFSGASAN YDVLGAVIEN VTGKTFTEVI AERLTQPLGM SATVAVKGDE IIVNKASGYK LGFGKPVLFH APLARNHVPA AYIHSTLPDM EIWIDAWLHR KALPATLREA MSNSWRGNSD VPLAADNRIL YASGWFIDQN QGPYISHGGQ NPNFSSCIAL RPDQQIGIVA LANMNSNLIL QLCADIDNYL RIGKYADGAG DAITATDTLF VYLTLLLCFW GAVVVVRGAF RVYRATAHGP GKQQRLRLRV RDYIIALAVP GLVAAMLYVA PGILSPGLDW RFILVWGPSS VLAIPFGIIL LAFVLTLNHQ IKRILLHNKE WDDE
Acid amino sequence of ClbP-3H : ClbP protein with a peptidase domain substituted by alkaline phosphatase enzymatic domain of PhoA (fusion protein) (SEQ ID NO:_11)	MTIMEHVSIKTLYHLLCCMLLFISAMCALAQEHEPIGA**MPV LENRAAQGDITAPGGARRLTGDQTAALRDSLSDKPAKNI ILLIGDGMGDSEITAARNYAEGAGGFFKGIDALPLTGQY THYALNKKTGKPDYVTDSAASATAWSTGVKTYNGALGV DIHEKDHPTILEMAKAAGLATGNVSTAELQDATPAALVA HVTSRKCYGPSATSEKCPGNALEKGGKGSITEQLLNARA DVTLGGGAKTFAETATAGEWQGKTLREQAQARGYQLV SDAASLNSVTEANQQKPLLGLFADGNMPVRWLGPKATY HGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELLSKNE KGFFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRAL EFAKKEGNTLVIVTADHAHASQIVAPDTKAPGLTQALNT KDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHAANVV GLTDQTDLFYTMKAALGLK**IALRPDQQIGIVALANMNSNL ILQLCADIDNYLRIGKYADGAGDAITATDTLFVYLTLLLCFW GAVVVVRGAFRVYRATAHGPGKQQRLRLRVRDYIIALAVP GLVAAMLYVAPGILSPGLDWRFILVWGPSSVLAIPFGIILLAF VLTLNHQIKRILLHNKEWDDE
Nucleic sequence (ADNc) of ClbP-3H : ClbP protein with a	atgcctgttctggaaaaccgggctgctcagggcgatattactgcacccggcggtgctcgccgtt taacgggtgatcagactgccgctctgcgtgattctcttagcgataaacctgcaaaaaatattatttt gctgattggcgatgggatgggggactcggaaattactgccgcacgtaattatgccgaaggtgc

peptidase domain substituted by alkaline phosphatase enzymatic domain of PhoA (fusion protein) (SEQ ID NO:_12)	gggcggctttttaaaggtatagatgccttaccgcttaccgggcaatacactcactatgcgctgaa taaaaaaaccggcaaaccggactacgtcaccgactcggctgcatcagcaaccgcctggtcaa ccggtgtcaaaacctataacggcgcgctgggcgtcgatattcacgaaaaagatcacccaacga ttctggaaatggcaaaagccgcaggtctggcgaccggtaacgtttctaccgcagagttgcagg atgccacgcccgctgcgctggtggcacatgtgacctcgcgcaaatgctacggtccgagcgcg accagtgaaaaatgtccgggtaacgctctggaaaaaggcggaaaaggatcgattaccgaaca gctgcttaacgctcgtgccgacgttacgcttggcggcggcgcaaaaacctttgctgaaacggc aaccgctggtgaatggcagggaaaaacgctgcgtgaacaggcacaggcgcgtggttatcagt tggtgagcgatgctgcctcactgaattcggtgacggaagcgaatcagcaaaaacccctgcttg gcctgtttgctgacggcaatatgccagtgcgctggctaggaccgaaagcaacgtaccatggca atatcgataagcccgcagtcacctgtacgccaaatccgcaacgtaatgacagtgtaccaaccct ggcgcagatgaccgacaaagccattgaattgttgagtaaaaatgagaaaggctttttcctgcaa gttgaaggtgcgtcaatcgataaacaggatcatgctgcgaatccttgtgggcaaattggcgaga cggtcgatctcgatgaagccgtacaacgggcgctggaattcgctaaaaaggagggtaacacg ctggtcatagtcaccgctgatcacgcccacgccagccagattgttgcgccggataccaaagctc cgggcctcacccaggcgctaaataccaaagatggcgcagtgatggtgatgagttacgggaact ccgaagaggattcacaagaacataccggcagtcagttgcgtattgcggcgtatggcccgcatg ccgccaatgttgttggactgaccgaccagaccgatctcttctacaccatgaaagccgctctggg gctgaaa

Mutations (or substitution) in ClbP peptidase domain are indicated in bold in AA sequence. In the present invention the numbering of amino acids is according to the wild-type ClbP protein sequence (SEQ ID NO: 1).

Method for treating Gastro-intestinal disorder

A second object of the invention relates to the *Escherichia coli* strain Nissle 1917 (EcN) bacterium, as defined above for use as a drug.

A previously indicated EcN was initially studied for its ability to fight bacterial gastrointestinal infections. It was demonstrated to impede intestinal colonization by *Salmonella enterica* serovar Typhimurium (3,4) and to exhibit an antibacterial activity against enterohemorrhagic *E. coli* strains (5). EcN is an excellent colonizer of the human gut, and exhibits beneficial effects in various intestinal dysfunctions such as acute diarrhea in infants and toddlers (6), chronic constipation (7), and abdominal pain in patients with irritable bowel syndrome (8). It has been widely used in the treatment of inflammatory bowel diseases (1) and

has proven to be as effective as the gold standard mesalazine for the maintenance of remission in ulcerative colitis in children and adults (9). Furthermore, inventors demonstrate in an *in vivo* animal model infected by a bacterial intestinal pathogen (S. Typhimurium) that administration of an EcN modified strain with *clbP* gene encoding ClbP protein inactive for the peptidase domain (ie. ClbP mutated at position S95), is non-genotoxic (do not produce colibactin) but keeps the bacterial antagonist activity, and reduces colonization and virulence of the pathogen (see figure 8) by maintaining the siderophore-microcin production.

Accordingly, the third object of the invention relates to an *Escherichia coli* strain Nissle 1917 (EcN) bacterium, as defined above for in use the treatment of gastrointestinal disease.

Accordingly, another object of the present invention relates to a method of treating gastrointestinal disease in a subject thereof, the method comprising administering the subject a therapeutically effective amount of EcN bacterium of the invention.

Treatment may be for any purpose, including the therapeutic treatment of subjects suffering from pain, as well as the prophylactic treatment of subjects who do not suffer from pain (e.g., subjects identified as being at high risk for gastrointestinal disease). As used herein, the terms "treatment," "treat," and "treating" refer to reversing, alleviating, inhibiting the progress of a disease or disorder as described herein (i.e. gastro-intestinal disease), or delaying, eliminating or reducing the incidence or onset of a disorder or disease as described herein, as compared to that which would occur in the absence of the measure taken. The terms "prophylaxis" or "prophylactic use" and "prophylactic treatment" as used herein, refer to any medical or public health procedure whose purpose is to prevent the disease herein disclosed (i.e. gastro-intestinal disease). As used herein, the terms "prevent", "prevention" and "preventing" refer to the reduction in the risk of acquiring or developing a given condition (i.e. gastro-intestinal disease), or the reduction or inhibition of the recurrence or said condition (i.e. gastro-intestinal disease) in a subject who is not ill, but who has been or may be near a subject with the condition (i.e. gastro-intestinal disease)

In a specific *Escherichia coli* strain Nissle 1917 (EcN) bacterium may be used to treat gastrointestinal disease such as bacterial gastrointestinal infection, gut inflammatory disease and visceral pain.

There are a very large number of Bacteria that cause gastrointestinal disease (*E. coli, Salmonella, Shigella, Campylobacter, Clostridium*). Most of the time bacterial infections of the intestines result in diarrhea or dysentery, nausea, vomiting, and abdominal pain or cramping. If the bacterial infection is in the small intestine symptoms include watery diarrhea and/or vomiting. Bacterial infections in the large intestine usually result in dysentery (small fecal

volume, with mucus and many times blood). Some diseases follow certain predisposing conditions (antibiotic therapy: pseudomembranous colitis). Not all of these diseases follow infection but can occur following ingestion of preformed toxin (staphylococcal food poisoning). Usually symptoms (vomiting, diarrhea) of intoxication occur soon (few hours) after ingestion of the toxin. There are several ways of categorizing this set of diseases. Some categorize them based on location in the intestines (small vs. large intestine), others by how the disease was acquired (food vs. water vs. person to person), and still others categorize these diseases based on what the infectious agent does to the host (intoxication vs. gastroenteritis vs. noninflammatory diarrhea vs. inflammatory diarrhea vs. enteric fever). All of these means of categorizing these etiologies are used to help the physician narrow down the possible causes of the symptoms. GI tract infections are very common. Diarrhea is the most common cause of death in developing countries (2.5 million deaths/year). Pathogens causing diarrhea can be transmitted to humans in three basic ways: in food, in water, and person to person. Many of these infections are self-limiting and do not require treatment. Some can spread to other sites in the body and require treatment to prevent further damage. The trick is in knowing when to treat and how to treat patients.

In a particular embodiment bacterial gastrointestinal infections are *Salmonella* enterica serovar Typhimurium infection or enterohemorrhagic *E. coli* infection.

In a more particular embodiment bacterial gastrointestinal infections are *Salmonella* enterica infection.

In a specific embodiment *Escherichia coli* strain Nissle 1917 (EcN) bacterium may be used to treat gastrointestinal disease such as gut inflammatory diseases. Such gut inflammatory diseases are for instance Inflammatory Bowel Diseases (IBD) or Irritable Bowel Syndrome (IBS).

As used herein, the term “inflammatory bowel diseases (IBD)” is a group of inflammatory diseases of the colon and small intestine.

In particular embodiment Inflammatory Bowel Diseases (IBD), is selected from the group consisting of Crohn’s Disease, Ulcerative Colitis Celiac disease, Gluten hypersensitivity and Pouchitis.

As used herein, the term “Irritable Bowel Syndrome (IBS)” is a term for a variety of pathological conditions causing discomfort in the gastro-intestinal tract. It is a functional bowel disorder characterized by chronic abdominal pain, discomfort, bloating, and alteration of bowel habits in the absence of any organic cause. It also includes some forms of food-related visceral hypersensitivity, such as Gluten hypersensitivity (ie. Celiac disease).

Another type of gastrointestinal disease is also visceral pain which includes pain associated with inflammatory bowel disease (IBD). Visceral pain is pain associated with the viscera, which encompasses the organs of the abdominal cavity. These organs include spleen and part of the digestive system. Pain associated with the viscera can be divided into digestive visceral pain and non-digestive visceral pain. Commonly encountered gastrointestinal (Gl) disorders inducing pain include functional bowel disorder (FBD) and inflammatory bowel disease (IBD). These Gl disorders include a wide range of disease states that are currently only moderately controlled including in respect of FBD-, gastro-oesophageal reflux, dyspepsia, irritable bowel syndrome (IBS) and functional abdominal pain syndrome (FAPS) and –in respect of IBD- Crohn's disease, ileitis and ulcerative colitis, all of which regularly produce visceral pain.

In some embodiments the method of the present invention is particularly suitable for the treatment of visceral pain resulting from gastrointestinal disorders, including functional bowel disorder (FBD) and inflammatory bowel disease (IBD) gastro-oesophageal reflux, dyspepsia, irritable bowel syndrome (IBS) and functional abdominal pain syndrome (FAPS), and, in respect of IBD, Crohn's disease, ileitis, ulcerative colitis dysmenorrhea, cystitis and pancreatitis and pelvic pain.

In a specific embodiment, visceral pain is selected from the group consisting of Inflammatory Bowel Diseases (IBD) or Irritable Bowel Syndrome (IBS).

In some embodiments, the prophylactic methods of the invention are particularly suitable for subjects who are identified as at high risk for pain. Typically subject that are risk for pain include patient that will have a surgical operation.

Said EcN bacterium of the present invention can be used as a drug, in particular as probiotic.

The term "probiotic" has its general meaning in the art and refers to a live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (see : Clinical Infectious Diseases, Volume 46, Issue Supplement_2, 1 February 2008, Pages S58–S61, https://doi.org/10.1086/523341).

It will be understood that the daily dose of the compounds and the composition of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose for any particular patient will depend upon a variety of factors including the type and severity of the disorder to treat; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time and route of administration and the rate of

excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific bacterium employed and other factors well known in the medical arts. For example, within the skill of the art it is recommended to start the treatment with doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

The compound of the invention may be administered by any suitable route of administration. For example, the compound according to the invention it can be administered by oral (including buccal and sublingual), rectal, nasal, topical, pulmonary, vaginal, or parenteral (including intramuscular, intra-arterial, intrathecal, subcutaneous and intravenous).

In a preferred embodiment of the invention, the therapeutic composition containing the compound of the invention is administered intrarectally, topically or orally. A rectal administration preferably takes place in the form of a suppository, enema or foam. Intrarectal administration is particularly suitable for intestinal diseases which affect the lower intestinal sections, for example the colon.

Pharmaceutical composition

The EcN bacterium of the present invention, together with one or more conventional adjuvants, carriers, or diluents may be placed into the form of pharmaceutical compositions and unit dosages.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The pharmaceutical composition and unit dosage forms may comprise conventional ingredients in conventional proportions, with or without additional active compounds or principles, and the unit dosage forms may contain any suitable effective amount of the active ingredients commensurate with the intended daily dosage range to be employed. The pharmaceutical composition may be employed as solids, such as tablets or filled capsules, semisolids, powders, sustained release formulations, or liquids such as solutions, suspensions, emulsions, elixirs, or filled capsules for oral use; or in the form of suppositories for rectal administration; or in the form of sterile injectable solutions for parenteral uses. Formulations containing about one (1) milligram of active ingredient or, more broadly, about 0.01 to about

one hundred (100) milligrams, per tablet, are accordingly suitable representative unit dosage forms.

The compound of the present invention may be formulated in a wide variety of oral administration dosage forms. The pharmaceutical compositions and dosage forms may comprise compounds of the present invention or pharmaceutically acceptable salts thereof as the active component. The pharmaceutically acceptable carriers may be either solid or liquid. Solid form preparations include powders, tablets, pulls, capsules, cachets, suppositories, and dispersible granules. A solid carrier may be one or more substances which may also act as diluents, flavouring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. In powders, the carrier generally is a finely divided solid, which is a mixture with the finely divided active component. In tablets, the active component generally is mixed with the carrier having the necessary binding capacity in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain from about one (1) to about seventy (70) percent of the active compound. Suitable carriers include but are not limited to magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch gelatin, tragacanth, methylcellulose sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like.

The term “preparation” is intended to include the formulation of the active compound with an encapsulating material as carrier, providing a capsule in which the active component, with or without carriers, is surrounded by a carrier, which is in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pulls, cachets, and lozenges may be as solid forms suitable for oral administration.

Other forms suitable for oral administration include liquid form preparations including emulsions, syrups, elixirs, aqueous solutions, aqueous suspensions, or solid form preparations which are intended to be converted shortly before use to liquid form preparations. Emulsions may be prepared in solutions, for example, in aqueous propylene glycol solutions or may contain emulsifying agents, for example, such as lecithin, sorbitan monooleate, or acacia. Aqueous solutions can be prepared by dissolving the active component in water and adding suitable colorants, flavours, stabilizers, and thickening agents. Aqueous suspensions can be prepared by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents. Solid form preparations include solutions, suspensions, and emulsions, and may contain, in addition to the active component, colorants, flavours,

stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilising agents, and the like.

Alternatively, the active ingredient may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution for constitution before use with a suitable vehicle, e.g., sterile, pyrogen-free water.

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

FIGURES:

Figure 1. Role of the pks/clb island in EcN antibacterial activity on LF82. (**A**) Serial dilutions of 24-hour cocultures of *E. coli* LF82 (rifampicin resistant) with wild-type (WT) *E. coli* strain Nissle 1917 (EcN) or the mutant for the colibactin maturing peptidase ClbP, spotted (10 µL) on LB plate containing rifampicin and incubated overnight at 37°C. **(B)** Colony forming unit (CFU) counts of *E. coli* LF82 following a 24-hour co-culture in M63 medium with WT EcN, gene deletion for the phosphopantetheinyl transferase ClbA, the peptidase ClbP and the corresponding complemented mutant (pclbP), the polyketide synthases (PKS) ClbC and ClbO, the nonribosomal peptide synthases (NRPS) ClbH and ClbN, the hybrid PKS-NRPS ClbB, the putative amidase ClbL, the efflux pump ClbM, and the thioesterase ClbQ. LF82 was also cultured alone as a control (∅). The medians and individual results of independent experiments are shown. One-way ANOVA and Bonferroni post-tests in comparison with co-culture with WT; ★★★P<0.001.

Figure 2: Role of the microcin gene cluster in EcN antibacterial activity on LF82. Colony forming unit (CFU) counts of *E. coli* LF82 following a 24-hour co-culture in M63 medium with wild-type (WT) *E. coli* strain Nissle 1917 (EcN), EcN mutant for microcin M (MccM) precursor gene mcmA, for microcin H47 (MccH47) precursor gene mchB, for both mcmA mchB genes; EcN mutants and complemented strains for mchC mchD genes responsible for posttranslational modifications, and for mchE mchF genes that encode the MccM and MccH47 efflux pump. LF82 was also cultured alone as a control (∅). The medians and individual results of independent experiments are shown. One-way ANOVA and Bonferroni post-tests in comparison with co-culture with WT; ★★0.001<P<0.01; ★★★P<0.001..

Figure 3: Role of siderophores in EcN antibacterial activity on LF82. (**A**) Colony forming unit (CFU) counts of *E. coli* LF82 following a 24-hour co-culture in M63 medium with wild-type (WT) *E. coli* strain Nissle 1917 (EcN), EcN mutant for *entE* that encodes the

enterobactin synthase E, and the double mutant for the phosphopantetheinyl transferases ClbA and EntD; EcN mutant and complemented strain for the glucosyltransferase IroB, the cytoplasmic esterase IroD, the periplasmic esterase IroE, and the export protein IroC. LF82 was also cultured alone as a control (∅). The medians and individual results of independent experiments are shown. One way ANOVA and Bonferroni post-tests in comparison with co-culture with WT; ★★★P<0.001.

Figure 4: Role of ClbP catalytic and transmembrane domains in EcN antibacterial activity on LF82.

Colony forming unit (CFU) counts of *E. coli* LF82 following a 24-hour co-culture in M63 medium with wild-type (WT) E. coli strain Nissle 1917 (EcN), the clbP gene deletion and complemented mutant with a plasmid that encodes wild-type ClbP (pclbP), plasmids that encode ClbP with a mutation S95A or K98T in the catalytic site, and a plasmid that encodes a fusion between the alkaline phosphatase PhoA and the ClbP C-terminal sequence from amino-acid 390 (pclbP-3H). LF82 was also cultured alone as a control (∅). Medians and individual results of independent experiments are shown. One way ANOVA and Bonferroni post-tests in comparison with co-culture with WT; ★★★P<0.001.

Figure 5: A genomic point mutation that inactivates ClbP catalytic domain abrogates EcN genotoxicity but not the antibacterial activity on LF82. (A) HeLa cells were transiently infected with wild-type *E. coli* Nissle (WT), a genome edited mutant with a single chromosomal nucleotide change in the *clbP* gene that inactivates the catalytic site (clbP-S95R), and the genome edited mutant complemented with the plasmid pclbP. Cells were then fixed, permeabilized and stained with rabbit monoclonal anti-gamma-H2AX followed by an infrared fluorescent secondary antibody. DNA was counterstained with RedDot2. **(B)** HeLa cells were transiently infected with wild-type *E. coli* Nissle (photo B), a *clbP* gene deletion mutant (C), and the genome edited clbP-S95R mutant (D). These cells were then washed and incubated with gentamicin for 72 hours before staining with Giemsa. The control is shown in photo A. Bars represent 50 µm. **(C)** Colony forming unit (CFU) counts of *E. coli* LF82 following a 24-hour co-culture in M63 medium with wild-type (WT) *E. coli* strain Nissle 1917 (EcN), the *clbP* gene deletion mutant (ΔclbP), and the genome edited mutant with a single nucleotide change in the clbP gene that results in an S95R mutation in the catalytic site (clbP-S95R). LF82 was also cultured alone as a control (∅). Medians and individual results of independent experiments are shown. One way ANOVA and Bonferroni post-tests in comparison with co-culture with WT; ★★★P<0.001

Figure 6: Gene clusters involved in the production of microcins H47 and M (MccH47 and M) in *E. coli* strain Nissle (EcN) represented on a genomic map. The loci that encode enterobactin (ent), colibactin (pks), yersiniabactin (ybt) on EcN genomic island (GEI) IV, salmochelin (iro) and MccH47 (mch) and M (mcm) on GEI I are represented. The arrows represent the interplays between the different gene clusters involved in MccH47 and M production in EcN.

Figure 7: Model proposed for biosynthesis of siderophore-microcins H47 and M in *E. coli* Nissle. An enterobactin precursor is modified by the enterobactin glucosyltransferase IroB. This siderophore moiety is transferred onto the C-terminal extremity of the precursor peptide by the MchCD complex. The active form of the siderophore-microcin is the result of the leader peptide cleavage during its export by the specific efflux pump MchEF-TolC. The C-terminal domain of ClbP allows this final step of siderophore-microcin production, while its N-terminal enzymatic domain cleaves precolibactin to produce colibactin.

Figure 8:.A *clbP* deletion, but not a genomic point mutation that inactivates ClbP catalytic domain, impairs EcN protection against the enteric pathogen Salmonella Typhimurium in mice.

C57BL/6 female mice were treated with 20 mg streptomycin per os, then 24 h later infected orally with 10^9 S. Typhimurium (STm) in PBS or co-administered with 10^9 S. Typhimurium and 10^9 EcN wild-type, ΔclbP or clbPS95R strains. (A) The mice were monitored for clinical signs (weight loss, diarrhea, signs of abdominal pain) daily during 4 days. Each point corresponds to the mean clinical score +/- SEM of 10 to 15 animals per group in three independent experiment. The animals were scored blindly (without knowledge of the infecting bacteria) in the last two of the three experiments. Two way ANOVA with Bonferonni post-test compared to STm + EcN, a: $p<0.05$, c: $p<0.001$. (B) The fecal shedding of STm was examined by enumeration of the feces collected at day 2 and 4 after infection. The median and individual result are shown. One way ANOVA of log-transformed CFU counts compared to STm + PBS, a: $p<0.05$ (C) Fecal counts of STm and EcN were used to determine the competitive index (CFU STm / CFU EcN). One way ANOVA compared to STm + EcN clbPS95R, a: $p<0.05$.

Figure 9: Detection of N-acyl-Asn-GABAOH in EcN wild-type, ΔclbN and clbPS95R strains.

EcN wild-type, ΔclbN or clbPS95R strains were grown 8 hours in DMEM Hepes then N-acyl-Asn-GABAOH was quantified in bacterial pellets by high-performance liquid chromatography (Agilent 1290 Infinity) coupled to a triple quadrupole mass spectrometer (G6460 Agilent). The concentration C is shown as picogram per 1E+8 CFU).

EXAMPLE:

Material & Methods

Bacterial strains, mutants and plasmids

The bacterial strains and plasmids used in this study are listed in Table 3. Gene mutagenesis was performed by the lambda Red recombinase method (37). The double mutants were constructed sequentially. The mutations and deletion of FRT cassettes were verified by PCR using primers upstream and downstream of the target genes.

The fusion between ClbP N-terminal signal sequence, the alkaline phosphatase PhoA, and the three transmembrane helices of ClbP were constructed using the HiFi DNA assembly kit (New England Biolabs, Ipswich, MA, USA) with primers overlapping between each fragment. The constructions were verified by PCR and confirmed by sequencing. The blue-stained colony-forming units on LB plates with 40 mg/L of 5-bromo-4-chloro-3-indolyl phosphate revealed the presence of the PhoA alkaline phosphatase domain in the periplasm as previously reported (38).

To construct plasmids pmchEF and pmchCD, the genes were PCR-amplified and cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA).

To construct plasmid pIroB, the iroB gene was PCR-amplified with EcN genomic DNA as a template and primers IRSDNG7 and IRSDNG8, digested by EcoRI and BamHI and ligated into pBbA5a-RFP (obtained from Addgene) digested to remove the rfp gene.

The strain EcN clbP-S95R chromosomal isogenic mutant was constructed using a genome editing technique (39). EcN was transformed with pORTMAGE, then grown in LB at 30°C and 300 rpm to reach OD600=0.5. An initial mutagenesis cycle was started by inducing the expression of Lambda recombinases and the dominant negative mutLE32K allele at 42°C for 15 minutes at 250 rpm. The culture was then cooled to 0°C, washed in water and electroporated with 50 µM of oligonucleotide IRSDNG26 that includes the S95A mutation in the clbP gene sequence. In a control experiment, the lacZ gene was targeted by a specific mutagenic oligonucleotide. Following recovery in LB at 30°C and 300 rpm for 1 hour, two other mutagenesis cycles were performed, and the bacteria were finally plated on MacConkey agar without any antibiotic. Approximately 33% of the isolates were LacZ negative in the control experiment. Sixty candidate clbP-S95R mutants were tested for loss of genotoxicity and megalocytosis phenotype in infected HeLa cells as previously described (40). Non genotoxic mutants that had lost the pORTMAGE plasmid were selected, and were finally verified for removal of a ClaI restriction site by S95A mutation in the PCR amplified clbP sequence.

Determination of the genotoxic effect induced by colibactin

The cellular senescence induced by colibactin was with the associated cell enlargement called megalocytosis and was determined for every EcN mutant constructed in this study in the Mcc gene cluster, in the iroA locus, and for the clbP-S95R mutant. As previously described (40), HeLa cells (ATCC, CCL-2) were infected for 4 hours. The cells were then washed and incubated with gentamicin for 72 hours before staining with Giemsa. The genotoxicity of EcN and the clbP-S95R chromosomal mutant was confirmed by an In-Cell Western procedure, as previously described (36). In brief, HeLa cells were infected in 96-well plates for 4 hours at a given multiplicity of infection (number of bacteria per cell at the onset of infection). Four hours after the end of infection cells were fixed, permeabilized and stained with rabbit monoclonal anti-gamma-H2AX (Cell Signaling, 20E4, 1:200) followed by an infrared fluorescent secondary antibody. DNA was counterstained with RedDot2 (Biotum). Fluorescence was recorded with an Odyssey infrared imaging system (Li-Cor).

Competitive growth assay

Strains were grown in lysogeny broth (LB Lennox, Invitrogen) overnight at 37°C with shaking at 240 rpm. Rifampicin, streptomycin, kanamycin, carbenicillin or chloramphenicol was added as required to the medium.

The media used for co-culture experiments were either M63 minimal medium with final concentrations of 15 mM ammonium sulfate, 1 mM magnesium sulfate heptahydrate, 100 mM monopotassic phosphate, 2.5 g/L glucose, 1 mg/L thiamine, and 1 g/L Bacto tryptone (BD Biosciences, Le Pont de Claix, France), or Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Invitrogen) supplemented with 25 mM Hepes, 10% (v/v) Fetal Calf Serum (FCS, Eurobio, Courtaboeuf, France), and 1% (v/v) Non Essential Amino Acids (NEAA, Invitrogen).

500 µL of each overnight culture were cultured in 9.5 mL of co-culture medium and incubated for 2h at 37°C with shaking at 240 rpm. Both the producing and the target strains (EcN and LF82 respectively) were inoculated from these 2-hour-cultures at 106 CFU/mL in 10 mL of co-culture medium as previously described (3) and incubated for 24h at 37°C with shaking at 240 rpm. For CFU numeration, the culture broth was serial-diluted in PBS and plated on selective LB agar plates containing the antibiotic required (e.g. rifampicin for LF82 (41)). In the total results section, only the growth of the target strains (mainly LF82) is reported. As a control, the growth of the competitive strains (mainly EcN and EcN mutants) was systematically checked (data not shown).

Animal infections

The animal infections were performed following the European directives for the protection of animal used for scientific purposes (2010/63/EU). The protocol was approved by

a local ethic committee (number of protocol: 2019041710292271). Female C57BL/6 (Janvier) were housed in ventilated cages, 5 animals per cage, with *ad libitum* access to food and water.

The animals were administered by oral gavage 20 mg of streptomycin, then 24h later, infected *per os* with 10^9 *S.* Typhimurium strain IR715 (nalidixic acid resistant) or co-administered with 10^9 *S.* Typhimurium and 10^9 EcN, EcN Δ*clbP* or EcN *clbP*S95R (with the *rpsL*K42R allele to confer resistance to streptomycin).

Fecal shedding of *S.* Typhimurium and EcN was determined by homogenization of feces in PBS, serial dilution and plating on LB agar plates supplemented with nalidixic acid or streptomycin.

The severity of the salmonellosis was evaluated by daily scoring of weight loss, signs of abdominal pain, fever and diarrhea.

The experiment was terminated at 4 days after infection to avoid lethality.

The experiment was repeated three times with five animals per group, and the clinical score was scored blindly (without knowledge of the infecting bacteria) in two out of the three independent experiments.

Bioinformatic analysis

Genes involved in MccH47 and MccM synthesis were searched using BLASTn and the CA58 Mcc gene cluster as a reference: mchB and mcmA which encode precursor proteins, the immunity genes mchI and mcmI, genes mchE and mchF which encode a specific efflux pump, and genes mcmK and mcmL (and their respective homologs in the E. coli H47 Mcc gene cluster, mchS1 and mchA) responsible for posttranslational modifications. A query cover > 80%, an identity > 90%, and an E value < 1e 40 were chosen as cutoff values for significance. The genes clbB and clbP, as respective markers for the 5' and 3' regions of the pks island, were identified using the same method, and so were genes iroN and iroB as markers for the 5' and 3' regions of the salmochelin gene cluster (iroA locus). Phylogroups were determined in silico based on the presence/absence of 4 genes: arpA, chuA, yjaA, and tspE4.C2 (and trpA to distinguish the A and C phylogroups) (42). The phylogenetic tree was constructed with the rpoC sequence. The sequences were collected using PATRIC 3.5.8 (43), aligned by multiple sequence comparison by log expectation (MUSCLE) with the MEGA7.0.26 software (44), and the phylogenetic tree was constructed according to the maximum likelihood method with MEGA7.0.26.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism 7.0a (GraphPad, San Diego, CA, USA). P values were calculated using one-way ANOVA followed by Bonferroni post-

tests. CFU/ml were log-transformed for the analyses. P values < 0.05 were considered significant and are denoted by ★, P<0.01 is denoted by ★★, and P<0.001 by ★★★.

Results

EcN antibacterial activity requires ClbP but not the other components of the colibactin synthesis pathway

In order to specifically decouple the genotoxic activity from the probiotic activity, we tested the antibacterial activity of the EcN mutant deleted for ClbP that allows the maturation of precolibactin in genotoxic colibactin (24,25). We compared it to the pleiotropic ClbA mutant coding for a PPTase (27,35,36). We performed co-culture experiments with the wild-type EcN, the EcN Δ*clbA* and Δ*clbP* mutants, and the Crohn's disease-associated *E. coli* strain LF82 which have been previously shown to be susceptible to EcN (45,46). CFU showed that the EcN strain strongly inhibited LF82 growth. EcN antibacterial activity on LF82 was not altered in a ΔclbA mutant but was completely lost in a ΔclbP mutant (Fig 1). A kinetic experiment indicated that EcN inhibitory activity on LF82 started 6 hours post-inoculation and reached its maximum 8 hours post-inoculation, at the beginning of the stationary phase (data not shown). LF82 growth was not altered at any time by the ΔclbP mutant, further proving the ClbP-dependence of the EcN antibacterial effect. This EcN ClbP-dependent inhibitory activity was also observed with other pathogenic strains of *E. coli* (JJ186 and NRG857c) and closely related bacteria species, such as *Salmonella enterica* subsp. *enterica* Typhimurium, and *Enterobacter aerogenes* (data not shown).

To further determine whether other components of the colibactin synthesis pathway besides ClbP are required for EcN antibacterial activity, the inhibitory effect of mutants for the PKS ClbC and ClbO, the NRPS ClbH and ClbN, the hybrid PKS-NRPS ClbB, the putative amidase ClbL, the efflux pump ClbM, and the thioesterase ClbQ were assessed against LF82. EcN antibacterial activity against LF82 was not altered in any of these mutants (Fig 1). These results confirm that colibactin itself or the cleavage product N-myristoyl-D-asparagine is not essential for EcN antibacterial activity against LF82 (and other Gram-negative bacteria, data not shown). Therefore, the probiotic activities of EcN are clearly associated with the presence of the pks/clb island and ClbP but not colibactin is involved in EcN inhibitory activity.

EcN ClbP-dependent antibacterial activity requires MccH47 and MccM

Previous studies have associated EcN antibacterial activity with MccH47 and MccM (4,11,14,15). Therefore, we performed co-culture experiments with LF82, EcN and mutants in MccH47 and MccM production systems. EcN antibacterial activity against LF82 was not

affected by the deletion of the MccM precursor gene mcmA alone or the MccH47 precursor gene mchB alone (Fig 2). In contrast, deletions of both mcmA and mchB almost completely abrogated the inhibitory effect of EcN on LF82. Similarly, deletion of the MccM and MccH47 efflux pump encoding genes mchE and mchF resulted in a loss of antibacterial activity (Fig 2). The trans-complementation of mchE and mchF increased EcN inhibitory activity compared to the wild-type EcN strain (Fig 2), probably because of an increase in Mcc export following overexpression of the MchE-MchF efflux pump. None of these mutations in the Mcc production system affected the ability of EcN to produce active colibactin (data not shown).

To further confirm the role of MccH47 and MccM in EcN antibacterial activity, plasmids that encode MccH47 or MccM immunity genes were transformed in LF82, and the resulting resistance of the strains was assessed against EcN (data not shown). EcN ΔmchB mutant antibacterial activity was almost completely abrogated on LF82 that carries the MccM immunity gene mcmI (data not shown). A similar result was obtained with the ΔmcmA mutant and LF82 that carries MccH47 immunity gene mchI (data not shown). Overall, these results confirmed that the EcN ClbP-dependent inhibitory activity against LF82 is due to MccH47 and MccM.

EcN ClbP-dependent antibacterial activity is due to the production of siderophore-Mcc

MccH47 and MccM can be modified posttranslationally by the linkage of a catechol siderophore to form a “siderophore-Mcc” (13). Therefore, we hypothesized that the ClbP-dependent antibacterial activity might be dependent on these modified forms of microcins. In fact, EcN antibacterial activity against LF82 was strongly reduced in a ΔentE mutant deprived of the enzyme 2,3-dihydroxybenzoate-AMP ligase essential for siderophore enterobactin production (47). Similar results were obtained with the EcN ΔclbA ΔentD double mutant which was unable to produce enterobactin (36) (Fig 3).

The two genes responsible for enterobactin glycosylation and esterification (mcmL and mcmK) are missing from the EcN Mcc gene cluster (18,48). As a result, whether MccH47 and MccM are siderophore-Mcc or unmodified Mcc is still being debated (13). Considering that EcN carries the McmL and McmK homologs, glucosyltransferase IroB and esterase IroD respectively (13), we investigated the interplay between the Mcc and the salmochelin production systems. The antibacterial activity of EcN mutants for genes that encode the glucosyltransferase IroB, the cytoplasmic esterase IroD, the periplasmic esterase IroE, and the export protein IroC (49,50) was compared to the activity of the wild-type EcN strain. Only iroB deletion led to a significant decrease in EcN antibacterial activity (Fig 3). Complementation of

the ΔiroB mutant fully restored the antibacterial activity. None of these mutations in the iroA locus affected EcN ability to cause megalocytosis linked with the colibactin genotoxic effect (data not shown). These results suggest that IroB could be responsible for enterobactin glycosylation, which enables the linkage of Mcc precursor proteins to the siderophore-derived moiety in the absence of McmL.

MchC and MchD are respective homologous to MceJ and MceI of *K. pneumoniae* strain E492 (13). These proteins form a complex responsible for the linkage of glycosylated enterobactin derivatives to MccE492 the precursor peptide MceA (51). The EcN mutant for mchC and mchD lost the antibacterial effect against LF82, whereas complementation restored the initial phenotype (Fig 2). These results indicate that the posttranslational modification of MccH47 and MccM with an enterobactin-derived moiety is required for EcN antibacterial activity. In short, EcN ClbP-dependent antibacterial activity is due to siderophore-Mcc.

The ClbP transmembrane domain, rather than the periplasmic peptidase catalytic site, is required for the antibacterial activity of EcN

To further elucidate the role of ClbP in siderophore-Mcc production, we examined whether ClbP catalytic activity is required for EcN antibacterial activity. S95 and K98 are key residues for ClbP peptidase activity, and mutants for these residues fail to cleave precolibactin to release mature active genotoxin (24,25). Co-culture experiments were performed with LF82 and the EcN ΔclbP mutant complemented with plasmids that encode the wild-type ClbP protein, or the ClbP protein that harbors the substitutions S95A or K98T. EcN ΔclbP mutants complemented with ClbP S95A or K98T demonstrated antibacterial activities similar to those of the wild-type ClbP protein (Fig 4), whereas they lost their ability to cause megalocytosis linked with the colibactin genotoxic effect (data not shown).

To exclude the role of another putative catalytic site of ClbP enzymatic domain, this enzymatic domain was replaced by alkaline phosphatase enzymatic domain of PhoA, as previously reported (38). The PhoA domain was fused with the ClbP N-terminal signal sequence which allows the translocation to periplasm, and the ClbP C-terminal sequence from amino-acid 390; the residues forming the three transmembrane helices being 390-412, 433-455, and 465-485 (24). An EcN ΔclbP mutant transformed with a plasmid bearing this fusion demonstrated a similar inhibitory activity against LF82 as the EcN WT strain (Fig 4), whereas it did not cause megalocytosis (data not shown). Therefore, the C-terminal domain of ClbP that comprises the three transmembrane helices is essential for EcN antibacterial activity, as opposed to the ClbP periplasmic peptidase domain which is crucial only for genotoxic activity.

To confirm this observation, and as a proof of the concept that a non-genotoxic EcN probiotic strain could be engineered, we used genome editing to construct an EcN mutant strain that exhibits a single nucleotide mutation in the chromosomic *clbP* gene, which leads to an S95R mutation in the ClbP catalytic site at the amino-acid level. This mutant did not produce colibactin and is not genotoxic but still exhibited an antibacterial activity towards LF82 that is similar to that of the wild-type genotoxic EcN strain (Fig 5).

An EcN strain with a point mutation in the *clbP* gene is non-genotoxic but keeps the antagonist activity, and reduces S. Typhimurium intestinal colonization and virulence

The EcN probiotic is well known to offer protection against enteric pathogens such as *Salmonella*, by competing for iron and producing the siderophore-microcins (3,4). Thus, we examined whether the EcN wild-type, Δ*clbP* and *clbP*-S95R mutants reduces *S.* Typhimurium intestinal colonization and pathogenesis using an *in vivo* model. We utilized C57BL/6 mice treated with streptomycin (to ensure a high colonization) then 24 h later infected with *S.* Typhimurium alone, or co-administered with *S.* Typhimurium and each EcN strain (3,4,66). The mice were monitored for clinical signs (weight loss, diarrhea, signs of abdominal pain) and the bacterial colonization was examined by enumeration of the feces, during 4 days (the point where the experiment must be arrested because of the lethality). When administered alone, *S.* Typhimurium readily colonized the intestine and this was associated with a high clinical score linked to a strong enteric salmonellosis (Fig 8). In animals co-administered with the wild-type EcN, there was a marked reduction in the clinical scores and in *S.* Typhimurium fecal colonization (Fig 8A-B). By day 2 following infection, EcN significantly outcompeted *S.* Typhimurium (Fig 8C). In contrast, animals co-administered with the EcN Δ*clbP* mutant exhibited higher clinical scores and reduced antagonism of *S.* Typhimurium colonization, demonstrating the role of ClbP in EcN beneficial effect during acute *Salmonella* colitis. The EcN *clbP*-S95R strain reduced substantially the fecal shedding and outcompeted *S.* Typhimurium, and diminished the clinical scores, similarly to the wild-type EcN (Fig 8).

Altogether, these results show that it is possible to decouple the genotoxic activity of EcN from its probiotic (antibacterial) activity, but also that the biosynthetic pathways of colibactin and siderophore-microcins are more entangled than initially thought.

The ClbP dependent antibacterial activity is observed in a subset of *E. coli* strains that carry a truncated Mcc gene cluster and the pks island

Comparative genomic analyses have shown that EcN is closely related to *E. coli* pyelonephritis strain CFT073 and the asymptomatic bacteriuria strain ABU83972 (18). These three strains, as well as the reference strain ATCC®25922, carry the pks island, the iroA locus,

and a truncated Mcc gene cluster deprived of genes mcmL/mchA and mcmK/mchS1. Therefore, we assessed whether the siderophore-Mcc antibacterial effect of these strains was ClbP-dependent, as observed in EcN. The inhibitory effect of two sets of E. coli strains was tested in co-culture experiments against LF82, as well as their respective ΔclbP mutants: i) strains similar to EcN that carry both a truncated Mcc gene cluster and the pks island: strains CFT073, ABU83972, and ATCC®25922; and ii) strains that carry the pks island but which are deprived of Mcc encoding genes: the human commensal strain M1/5, the meningitis-causing strain SP15, the murine commensal strain NC101, and the laboratory strain MG1655 that hosts a bacterial artificial chromosome (BAC) bearing the pks island. The three wild-type strains that carry both a truncated Mcc gene cluster and the pks island exhibited a marked inhibitory effect as observed in EcN (data not shown). The inhibitory effect of all three corresponding ΔclbP mutant strains was significantly reduced, whereas ClbP complementation restored the initial phenotype (data not shown). In contrast, in strains carrying only the pks island, there was no significant difference in LF82 growth whether it was cultivated with the wild-type strains or the ΔclbP mutants (data not shown). Cumulatively, these results show that the peptidase ClbP is involved in MccH47 and MccM antibacterial activity in E. coli strains that carry both the pks island and a truncated form of the Mcc gene cluster. Our results also show that this association is present in both pathogenic strains and probiotic strains.

Distribution of pks, salmochelin and the MccH47 and MccM gene clusters in an E. coli population

We demonstrated that strains of E. coli that carry a truncated Mcc gene cluster exhibit a siderophore-Mcc-dependent antibacterial activity (data not shown). This antibacterial activity requires ClbP from the biosynthetic pathway that produces the genotoxin colibactin and IroB from the biosynthetic pathway that produces the siderophore salmochelin. Consequently, we checked this association between the pks island, the iro locus and the Mcc island in E. coli strains with genomes available in GenBank. Interestingly, all strains that lacked the mcmL and mcmK genes responsible for posttranslational modifications belonged to the B2 phylogroup and carried the pks island and iroA (data not shown), except for strain 1105 deprived of pks island. Conversely, the strains that carry mcmL/mchA and mcmK/mchS1 belonged to B1, C or D phylogroups and lacked the pks island. These particular associations of genetic determinants led to the hypothesis that the truncated island is present almost exclusively in strains that carry pks and the iroA locus. It suggests that this interplay between colibactin, salmochelin, and the siderophore-Mcc biosynthetic pathways is due to a co-selection in strains that is either pathogenic or probiotic.

Production of beneficial compounds likely involved in the probiotic activity of Nissle 1917 by the Nissle clbPS95R strain

Other metabolites than colibactin that are synthesized by the enzymes encoded on the pks island might have a role on the probiotic properties of Nissle 1917. It was recently shown that the metabolite C12AsnGABAOH is produced by Nissle 1917 wild-type (but not by a clbN mutant, indicating that the pks-encoded machinery has a role in its production) (34). C12-Asn-GABAOH was shown to inhibit nociceptors activation in neurons. Nociceptor neurons in the intestinal tract play an important role in protecting against enteropathogens and intestinal homeostasis, as they regulate M cell density and inflammation. Thus, it is important that we ensure that a non-genotoxic modified strain retain production of C12AsnGABAOH with a role in the anti-inflammatory property of Nissle.

We have quantified by HPLC-QQQ (34) N-acyl-Asn-GABAOH in bacterial cultures of Nissle 1917 wild type, Nissle clbN mutant and Nissle clbPS95R. The Nissle clbPS95R still produces the beneficial GABAOH lipopeptide similarly to the wild type strain (Fig. 9).

Genetic stability of Nissle clbPS95R

To verify that the Nissle clbPS95R strain is genetically stable, we sequenced its genome DNA before and after oral gavage of a mouse (together with a pathogenic Salmonella (67)) and reisolation from the feces. The genomes were compared to that of the wild-type strain, which was also sequenced. The Nissle clbPS95R strain shown only 2 bp change (out of 5441200 bp) compared to the wild-type. No difference was found between Nissle clbPS95R before and after passage through the mouse intestine (data not shown). Thus, the Nissle clbPS95R appear genetically stable.

Discussion

Since Fleming discovered penicillin in 1928, antibiotics have contributed to the increase in human life expectancy. Many infections which were previously fatal became curable. Unfortunately, the overuse and misuse of antibiotics, in parallel with the lack of new antibacterial drugs enabled multi-resistant bacteria to emerge and spread (52). According to the World Health Organization (WHO), this phenomenon “poses a substantial threat to morbidity and mortality worldwide” (53). The trend is especially worrying for Gram-negative bacteria. For instance, the number of deaths attributable to 3rd generation cephalosporin-resistant or carbapenem-resistant E. coli increased by more than 4 times in Europe between 2007 and 2015 (54). Of the antibiotics that are currently being developed for intravenous administration, only a small proportion (15 out of 44) demonstrates some activity against Gram-negative bacteria, and all these molecules are derived from known antibiotic classes. Consequently, the WHO

established that research and development of new antibiotics against Gram-negative bacteria was a "critical priority" (53).

In the search for new antimicrobials, microcins seem a promising alternative to "conventional" antibiotics. In fact, many microcins exhibit potent narrow-spectrum antimicrobial activity, whereas antibiotics can eliminate beneficial bacteria, alter the microbiota and promote the selection of resistant strains (55,56). A major challenge in using microcins is their delivery in sufficient quantities to the site of infection, especially after oral administration because they are often degraded in the upper digestive tract (57,58). Engineered probiotic bacteria were consequently proposed as in situ producers of microcins to fight against enteropathogens (59) or to reduce colonization by multi-resistant bacteria (60).

EcN has been used as a probiotic for over a century, with numerous therapeutic benefits described. However, serious concerns about the safety of EcN administration have emerged over the years. EcN was reported to be responsible for severe sepsis in an infant (61) and its genome was shown to have the pathogenicity island pks (21,35), which codes for colibactin, a bona fide virulence factor for E. coli strains responsible for extraintestinal infections (26,27). In addition, the carriage of colibactin producing E. coli could also be deleterious to gut homeostasis. In adult rats, it increased intestinal epithelial permeability, led to signs of genotoxic damages in intestinal cells, such as crypt fission, and increased cell proliferation (28). In mice predisposed to colorectal cancer, pks-positive E. coli increased the size and the number of tumors (31,62). In human beings, several studies reported that pks-positive E. coli were over-represented in colorectal cancer biopsies compared to controls (31,32,63). On a whole, these studies suggest that colibactin-producing bacteria could promote tumorigenesis. Therefore, our goal was to understand the interplay between the production of the genotoxin colibactin and the beneficial effects related to the pks island in the probiotic activity of EcN. Consequently, we attempted to disarm EcN while keeping its probiotic properties.

In a previous attempt, our team constructed a non-genotoxic EcN PPTase ClbA mutant, which also lost its probiotic activity (35). Subsequently, it was discovered that the PPTase ClbA contributes to the synthesis of enterobactin (and therefore salmochelin) and yersiniabactin (36). In this study, we demonstrated that there is collaboration between the salmochelin (iroB) and the Mcc gene clusters, both of which are located on EcN genomic island I, and the pks island (clbP) (Fig 6). The interweaving is so strong between these determinants, that a single protein, ClbP is involved both in colibactin and Mcc production. Up until now, ClbP had only been described as a peptidase that removes the N-acyl-D-asparagine prodrug scaffold from precolibactin (24,25). Although the complete C-terminal domain with the three transmembrane

helices is required for the bioactivity of ClbP, the catalytic activity is performed by the N-terminal periplasmic domain (25,38). In this study, we demonstrated that the C-terminal domain of ClbP, deprived of the known enzymatic function, is necessary for EcN antibacterial activity due to MccH47 and MccM. It suggests that the ClbP C-terminal transmembrane domain could facilitate the export of the MccH47 and MccM of EcN through the MchE-MchF efflux pump (Fig 7).

Using both functional and bioinformatic analyses, we demonstrated interplay between siderophore-Mcc, salmochelin, and colibactin assembly lines. Strikingly, two groups of E. coli strains emerged. On one hand, all strains that carry a "truncated" MccH47 and MccM gene cluster (i.e. strains such as EcN lacking mcmL/mchA and mcmK/mchS1) are B2 strains that also bear the pks island and the iroA locus. It should be noted that isolates from urine were over-represented in this group of strains (CFT073, clones D i14 and D i2, UPEC 26-1, and ABU 83972). On the other hand, the pks island and the iroA locus are absent in the non-B2 strains that carry a "complete" MccH47 and MccM gene cluster. All these strains were isolated from stools (except ACN002 for which the origin is unknown). Therefore, we can hypothesize that these strains with a "complete" Mcc gene cluster are specialized in Mcc production in order to survive in the competitive intestinal environment, which is their exclusive niche. In contrast, extraintestinal pathogenic *E. coli* (ExPEC) must be efficient gut colonizers in order to emerge from the intestinal niche and infect other body sites (such as the urinary tract) to which they must subsequently adapt. That is why it has been suggested that ExPEC are "generalists" rather than specialized strains (64). The strains we examined in our study fit this model. They can express various virulence factors depending on their environment: MccH47 and M, siderophores and analgesic lipopeptides derived from the colibactin pathway, for instance. To be able to produce so many virulence or fitness factors with a genome of limited size (65), the elements of the assembly lines that produce these determinants must be versatile and intervene in several apparently independent metabolic pathways.

In conclusion, we discovered that the pks island is even more intimately connected to EcN probiotic activity than expected. This entanglement reflects the co-evolution of probiotic and pathogenic determinants to adapt to various environments. Decoupling the probiotic from the genotoxic activities by specifically targeting the enzymatic domain of ClbP opens the way to safe use of EcN.

Table 3. Strains and plasmids used in this study.

Strain or plasmid	Genotype or phenotype	Source or reference
E. coli Nissle (EcN)	Probiotic strain; colibactin genotoxin producer; enterobactin and salmochellin siderophores producer; microcins H47 and M producer	DSM 6601, Mutaflor®
EcN WT	EcN mutant in *rpsl*, Str^R	[1,2]
EcN Δ*clbA*	*clbA* mutant of strain EcN WT, Str^R, Kan^R	[1]
EcN Δ*clbB*	*clbB* mutant of strain EcN WT, Str^R, Kan^R	[3]
EcN Δ*clbC*	*clbC* mutant of strain EcN, Str^R, Chl^R	[3]
EcN Δ*clbH*	*clbH* mutant of strain EcN WT, Str^R, Kan^R	This study
EcN Δ*clbL*	*clbL* mutant of strain EcN WT, Str^R, Kan^R	This study
EcN Δ*clbM*	*clbM* mutant of strain EcN WT, Str^R, Kan^R	This study
EcN Δ*clbN*	*clbN* mutant of strain EcN WT, Str^R, Kan^R	[3]
EcN Δ*clbO*	*clbO* mutant of strain EcN WT, Str^R, Kan^R	This study
EcN Δ*clbP*	*clbP* mutant of strain EcN WT, Str^R, Kan^R	[3]
EcN Δ*clbP* p*clbP*	EcN Δ*clbP* mutant complemented with pBRSKp*clbP*, Str^R, Kan^R, $Carb^R$	This study
EcN Δ*clbP* pclbP-S95A	EcN Δ*clbP* mutant complemented with pclbP-S95A, Str^R, Kan^R, $Carb^R$	This study
EcN Δ*clbP* pclbP-K98T	EcN Δ*clbP* mutant complemented with pclbP-K98T, Str^R, Kan^R, $Carb^R$	This study
EcN Δ*clbP* pclbP-3H	EcN Δ*clbP* mutant complemented with pclbP-3H	This study
EcN *clbP*-S95R	EcN *clbP*-S95R chromosomal isogenic mutant	This study
EcN Δ*clbQ*	*clbQ* mutant of strain EcN WT, Str^R, Kan^R	This study
EcN Δ*mcmA*	*mcmA* mutant of EcN WT, Str^R, Kan^R	This study
EcN Δ*mchB*	*mchB* mutant of EcN WT, Str^R, Kan^R	This study
EcN Δ*mcmA*Δ*mchB*	*mcmA mchB* mutant of EcN WT, Str^R, Kan^R	This study

EcN Δ*mchCD*	*mchC mchD* mutant of EcN WT, Str^R, Kan^R	This study
EcN Δ*mchCD*::FRT	*mchCD*::FRT mutant of strain EcN WT, Str^R	This study
EcN Δ*mchCD* p*mchCD*	EcN Δ*mchCD*::FRT complemented with TopoXL *mchCD,* Str^R, Kan^R	This study
EcN Δ*mchEF*	*mchE mchF* mutant of EcN WT, Str^R, Kan^R	This study
EcN Δ*mchEF*::FRT	*mchEF*::FRT mutant of strain EcN WT, Str^R	This study
EcN Δ*mchEF* p*mchEF*	EcN Δ*mchEF*::FRT complemented with TopoXL *mchEF,* Str^R, Kan^R	This study
EcN Δ*entE*	*entE* mutant of EcN WT, Str^R, Chl^R	This study
EcN Δ*entD*	*entE* mutant of EcN WT, Str^R, Chl^R	This study
EcN Δ*entD*Δ*clbA*	*clbA, entE* mutant of EcN WT, Str^R, Kan^R	This study
EcN Δ*iroB*	*iroB* mutant of EcN WT, Str^R, Chl^R	This study
EcN Δ*iroB* p*iroB*	EcN Δ*iroB* mutant complemented with pASK75 iroB	This study
EcN Δ*iroC*	*iroC* mutant of EcN WT, Str^R, Kan^R	This study
EcN Δ*iroD*	*iroD* mutant of EcN WT, Str^R, Chl^R	This study
EcN Δ*iroE*	*iroE* mutant of EcN WT, Str^R, Kan^R	This study
E. coli LF82	Strain isolated from an ileal biopsy of a patient with Crohn's disease; adherent-invasive *E. coli,* Rif^R	[4]
LF82 pMcMi	LF82 carrying pMcMi, Rif^R, $Carb^R$	This study
LF82 pMcHi	LF82 carrying pMcHi, Rif^R, $Carb^R$	This study
E. coli M1/5	Commensal *E. coli* strain isolated from feces of a healthy adult; B2 phylogenetic group; colibactin genotoxin producer; aerobactin, enterobactin and yersiniabactin siderophores producer	[5]
M1/5 Δ*clbP*	*clbP* mutant of strain M1/5, Kan^R	[6]
E. coli SP15	Strain isolated from spinal fluid of a neonate with meningitis;	[7]

	O18:K1 serotype; colibactin genotoxin producer; aerobactin, enterobactin, salmochelins and yersiniabactin siderophores producer	
SP15 Δ*clbP*	*clbP* mutant of strain SP15, Kan^R	This study
E. coli NC101	Non-pathogenic murine *E. Coli* strain; colibactin genotoxin producer;	[8,9]
NC101 Δ*clbP*	*clbP* mutant of strain NC101, Kan^R	[10]
E. coli MG1655 bac*pks*	Enterobactin siderophore producer *E. coli* strain carrying a bacterial artificial chromosome bearing the entire *pk*s island, Chl^R	[11]
MG1655 bac*pks* Δ*clbP*	*clbP* mutant of strain MG1655 bac*pks*, Chl^R	This study
E. coli CFT073	Strain isolated from a patient with pyelonephritis, colibactin genotoxin producer; enterobactin siderophores producer; microcins H47 and M producer	[12]
CFT073 Δ*clbP*	*clbP* mutant of strain CFT073, Kan^R	This study
CFT Δ*clbP* p*clbP*	CFT073 Δ*clbP* mutant complemented with pBRSKp*clbP*, Kan^R, $Carb^R$	This study
E. coli ABU83972	Strain isolated from a patient with asymptomatic bacteriuria, colibactin genotoxin producer; enterobactin siderophores producer; microcins H47 and M producer	[13,14]
ABU83972 Δ*clbP*	*clbP* mutant of strain ABU83972, Kan^R	This study
ABU83972 Δ*clbP* p*clbP*	ABU83972 Δ*clbP* mutant complemented with pBRSKp*clbP*, Kan^R, $Carb^R$	This study

E. coli ATCC®25922	Strain isolated from a patient in Seattle (1946), colibactin genotoxin producer; enterobactin siderophores producer; microcins H47 and M producer	DSM1103
ATCC®25922 Δ*clbP*	*clbP* mutant of strain ATCC®25922, Kan^R	This study
ATCC®25922 Δ*clbP* p*clbP*	ATCC®25922 Δ*clbP* mutant complemented with pBRSKp*clbP*, Kan^R, $Carb^R$	This study
E. coli ST131 isolate JJ1886	Strain isolated in the USA (2007) from a patient with fatal urosepsis, Str^R, Kan^R, $Carb^R$, Chl^R	[15]
E. coli NRG857c	Strain isolated from the ileum of a Crohn's Disease patient, $Carb^R$, Chl^R	[16]
Salmonella enterica serovar Typhimurium IR715	Nal^R derivative of *S. enterica* serovar Typhimurium ATCC14028	[17]
Enterobacter aerogenes ATCC®13048	Strain isolated from sputum in the USA (Center for Disease Control and Prevention)	ATCC®13048
Klebsiella oxytoca ATCC®13182	Strain isolated from a pharyngeal tonsil	ATCC®13182
p*clbP*	pBRSK encoding *clbP* sequence	[18]
p*clbP*-S95A	pBRSK encoding the mutant S95A of ClbP (pOB902), $Carb^R$	[18]
p*clbP*-K98T	pBRSK encoding the mutant K98T of ClbP (pOB903), $Carb^R$	[18]
p*clbP*-3H	pASK74 carrying the fusion between ClbP N-terminal signal sequence, the alkaline phosphatase PhoA, and the 3 transmembrane helices of ClbP, $Carb^R$	This study
p*mchCD*	pCR XL-TOPO vector encoding *mchC* and *mchD* from EcN, Kan^R	This study
p*mchEF*	pCR XL-TOPO vector encoding *mchE* and *mchF* from EcN, Kan^R	This study

pMcMi	Carrying *mcmI* from MccM gene cluster, $Carb^R$	F. Moreno, unpublished data [19]
pMcHi	Carrying *mchI* from MccH47 gene cluster, ChlR	F. Moreno, unpublished data, [19]

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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enzyme-bound (2,3-dihydroxybenzoyl)adenylate product. Biochemistry. 1989 Aug 22;28(17):6827–35.

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CLAIMS:

1. An *Escherichia coli* strain Nissle 1917 (EcN) bacterium carrying a gene encoding a ClbP protein which is inactive for the peptidase domain,
wherein the peptidase domain of ClbP protein having the amino acid sequence of SEQ ID NO:3 is involved in activation of the genotoxin colibactin, and
wherein said EcN bacterium (i) has the capacity to have an antibacterial activity; and ii) is devoid of the capacity to activate the genotoxin colibactin.

2. The EcN bacterium according to claim 1 wherein the ClbP protein inactivate for the peptidase domain, is selected from the list consisting of :
ClbP protein mutated at position S95,
ClbP protein mutated at position K98,
ClbP protein mutated at position Y186,
ClbP protein without peptidase domain.

3. The EcN bacterium according to claim 2, wherein the ClbP protein mutated at position S95, is preferably not substituted with an equivalent polar and non-charged amino acid of Serine.

4. The EcN bacterium according to claim 2, wherein the ClbP protein mutated at position K98, is preferably not substituted with an equivalent positively charged amino acid of Lysine.

5. The EcN bacterium according to claim 2, wherein the ClbP protein mutated at position Y186, is preferably not substituted with an equivalent amino acid with hydrophobic side chain of Tyrosine.

6. The EcN bacterium according to claim 2 to 5 wherein the ClbP protein inactivate for the peptidase domain, is selected from the list consisting of
ClbP S95A mutant (SEQ ID N°4),
ClbP K98T mutant (SEQ ID N°6),
ClbP S95R mutant (SEQ ID N°8),
ClbP Y186G mutant (SEQ ID N°10),

ClbP protein with a peptidase domain substituted by alkaline phosphatase enzymatic domain of PhoA (SEQ ID N°11).

7. An *Escherichia coli* strain Nissle 1917 (EcN) bacterium, according to anyone of claim 1 to 6 for use as a drug.

8. The EcN bacterium for use according to claim 7 for use as a probiotic.

9. An *Escherichia coli* strain Nissle 1917 (EcN) bacterium, according to anyone of claim 1 to 6 for use in the treatment of gastro-intestinal disease.

10. The EcN bacterium for use according to claim 9 bacterial wherein the gastro-intestinal disease is selected from the list consisting of bacterial gastrointestinal infections, gut inflammatory disease and visceral pain.

11. The EcN bacterium for use according to claim 10, wherein the bacterial gastrointestinal infections is *Salmonella enterica* serovar Enteritidis or Typhimurium infection or enterohemorrhagic *E. coli* infections.

12. The EcN bacterium for use according to claim 10, wherein the gut inflammatory disease is Inflammatory Bowel Diseases (IBD) or Irritable Bowel Syndrome (IBS).

13. The EcN bacterium for use according to claim 12, wherein the Inflammatory Bowel Diseases (IBD) is selected from the list consisting of Crohn's Disease, Ulcerative Colitis, Celiac disease, Gluten hypersensitivity and Pouchitis.

14. The EcN bacterium for use according to claim 10, wherein visceral pain is pain associated with inflammatory bowel disease (IBD) or with Irritable Bowel Syndrome (IBS).

15. A method for treating gastro-intestinal disease in a subject thereof comprising administering to said subject a therapeutically effective amount of an EcN bacterium according to anyone of claim 1 to 6.

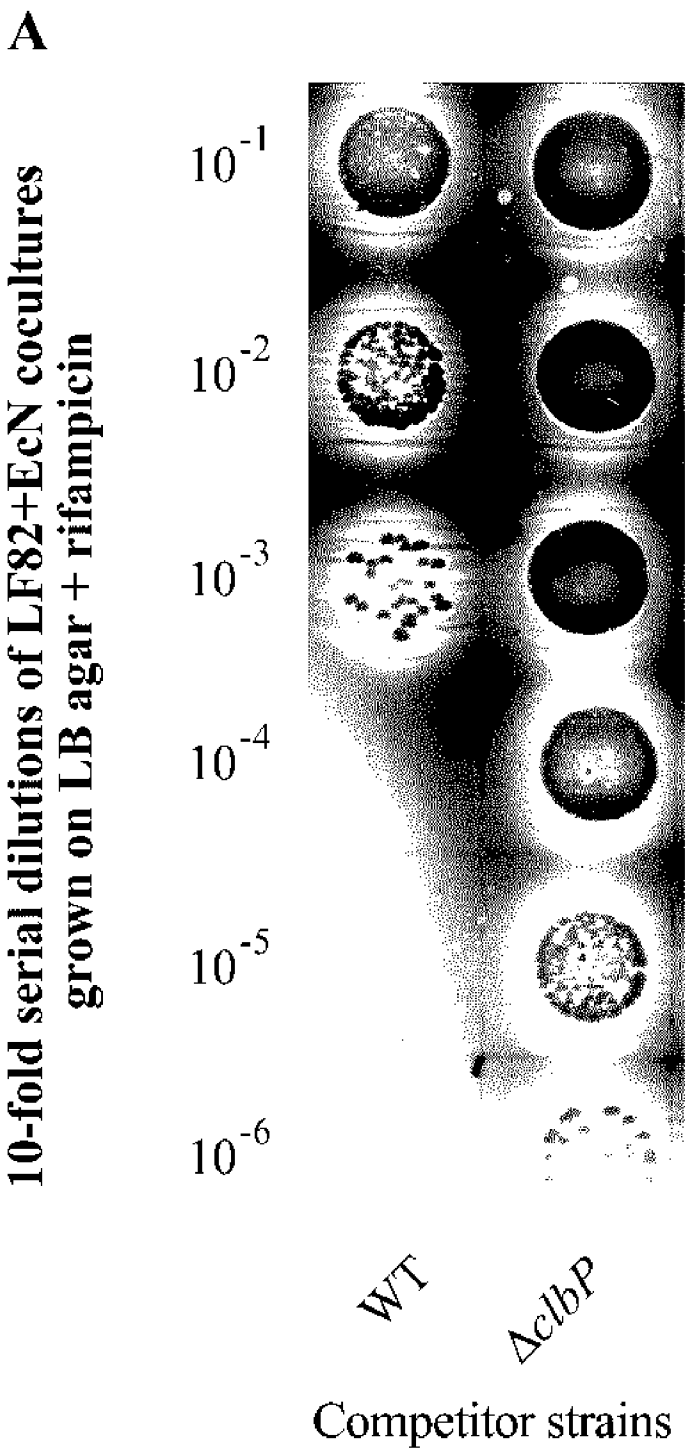
A
10-fold serial dilutions of LF82+EcN cocultures grown on LB agar + rifampicin
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
WT
ΔclbP
Competitor strains

Figure 1A

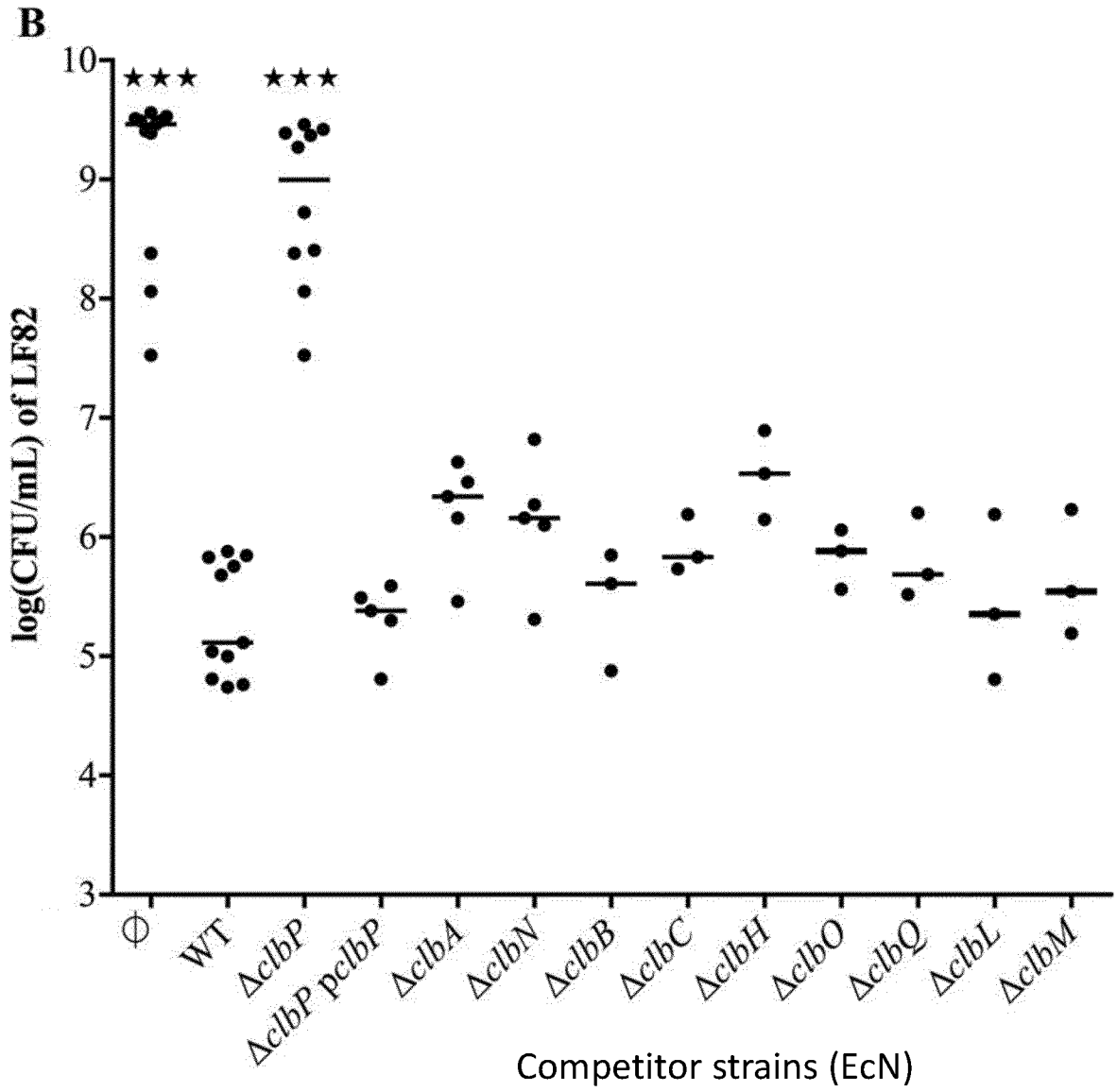
B
log(CFU/mL) of LF82
10
9
8
7
6
5
4
3
★★★
★★★
∅
WT
ΔclbP
ΔclbP pclbP
ΔclbA
ΔclbN
ΔclbB
ΔclbC
ΔclbH
ΔclbO
ΔclbQ
ΔclbL
ΔclbM
Competitor strains (EcN)

Figure 1B

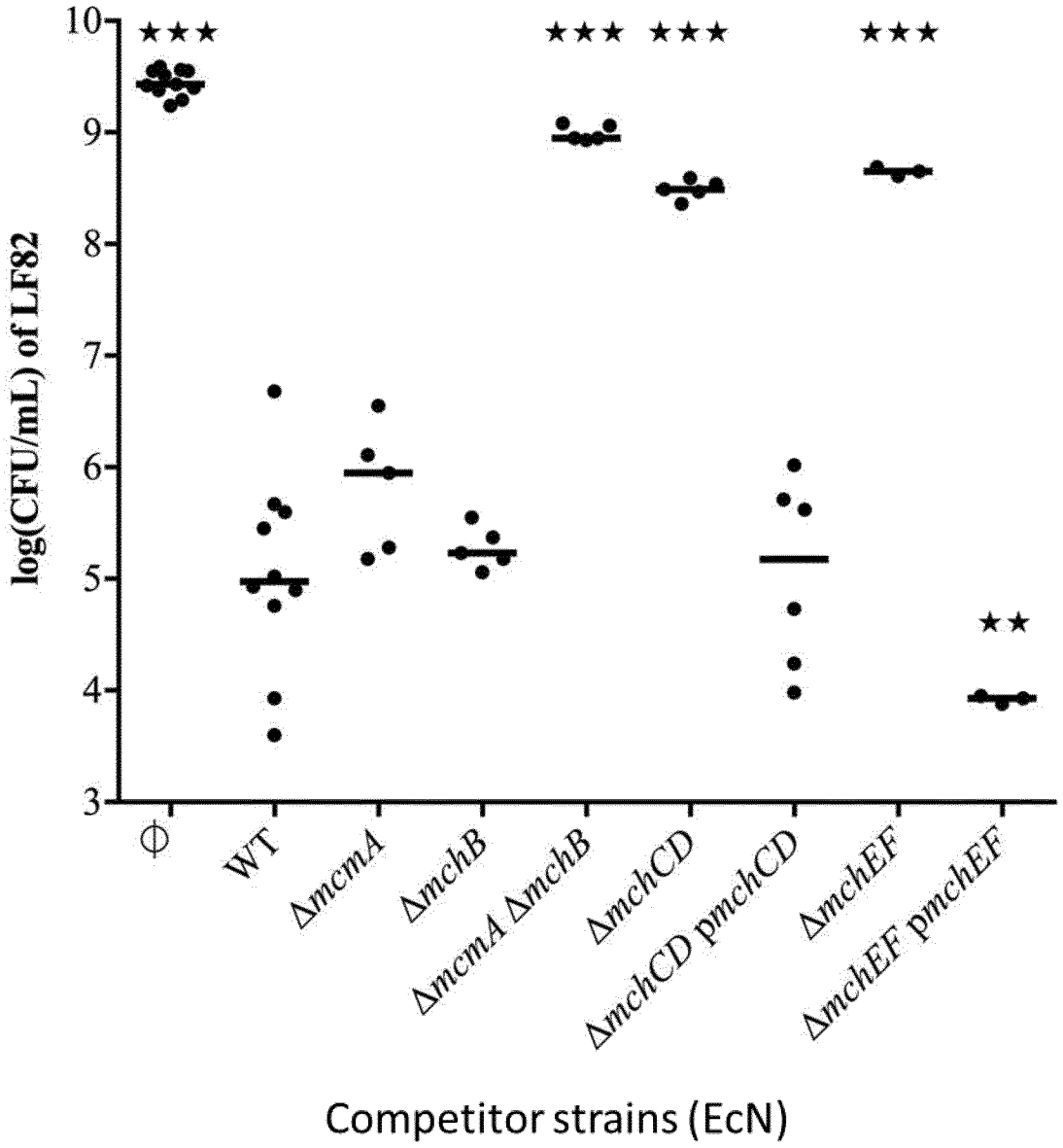
log(CFU/mL) of LF82
10
9
8
7
6
5
4
3
★★★
★★★
★★★
★★★
★★
∅
WT
ΔmcmA
ΔmchB
ΔmcmA ΔmchB
ΔmchCD
ΔmchCD pmchCD
ΔmchEF
ΔmchEF pmchEF
Competitor strains (EcN)

Figure 2

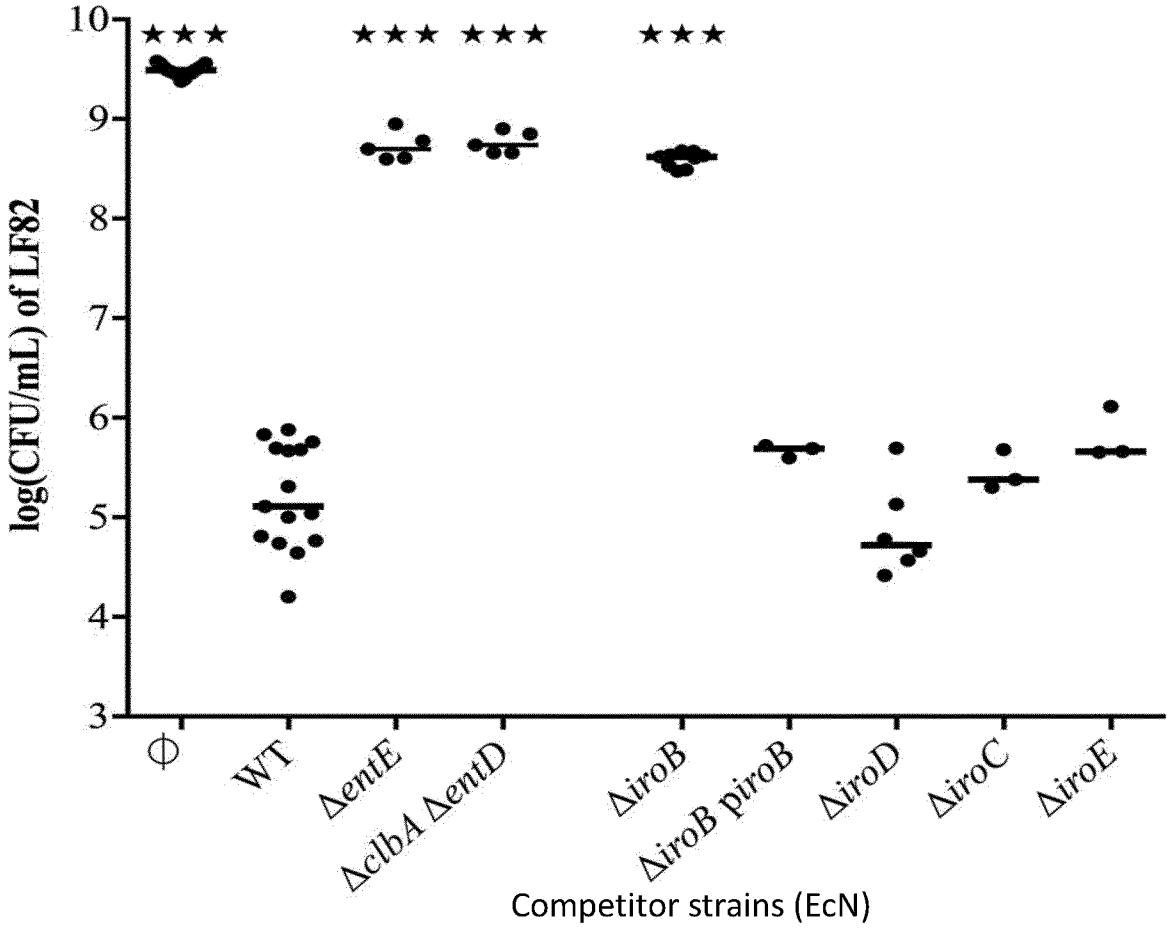
log(CFU/mL) of LF82
10
9
8
7
6
5
4
3
★★★
★★★
★★★
★★★
∅
WT
ΔentE
ΔclbA ΔentD
ΔiroB
ΔiroB piroB
ΔiroD
ΔiroC
ΔiroE
Competitor strains (EcN)

Figure 3

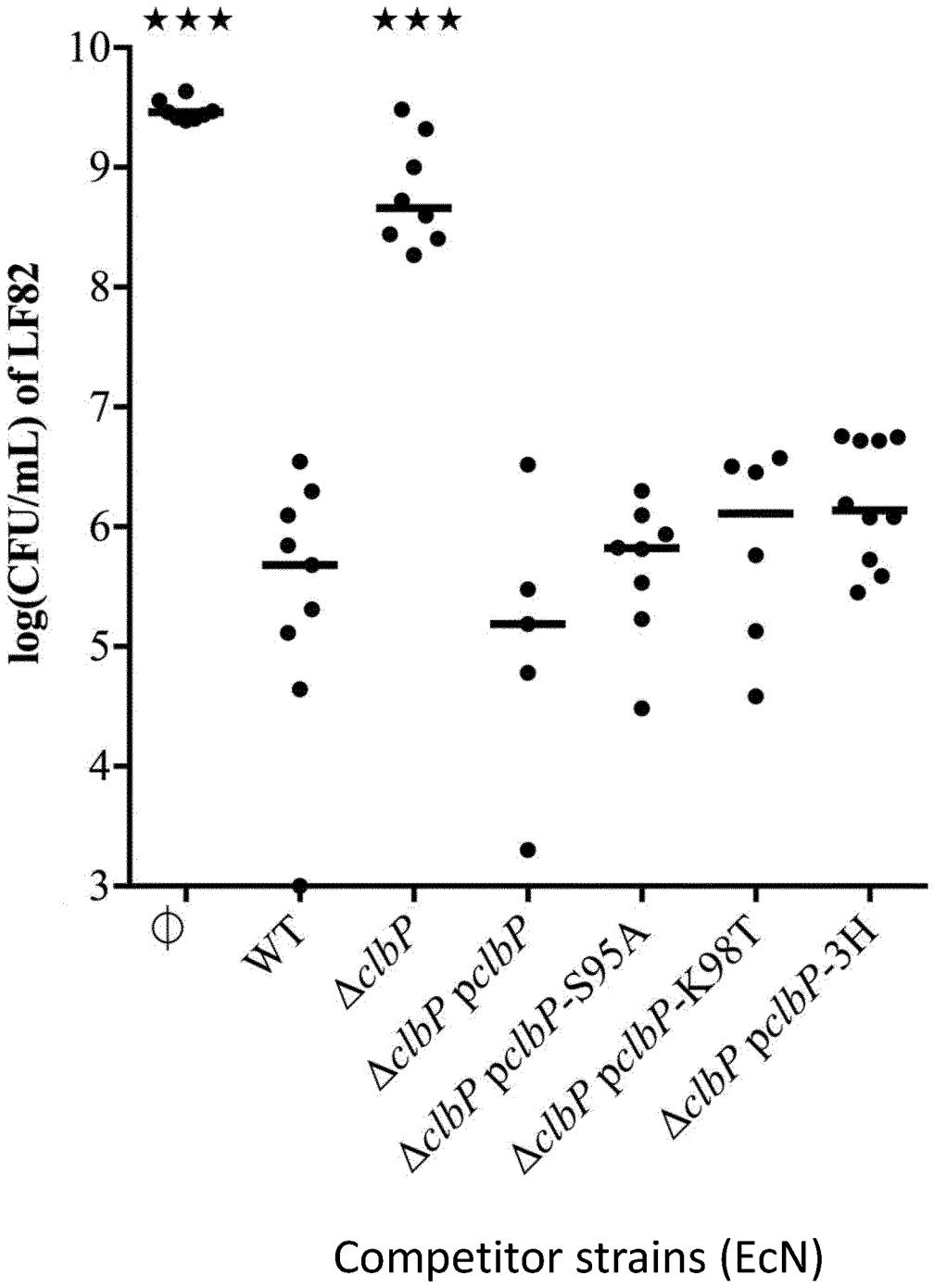
★★★
★★★
10
9
8
7
6
5
4
3
log(CFU/mL) of LF82
∅
WT
ΔclbP
ΔclbP pclbP
ΔclbP pclbP-S95A
ΔclbP pclbP-K98T
ΔclbP pclbP-3H
Competitor strains (EcN)

Figure 4

A

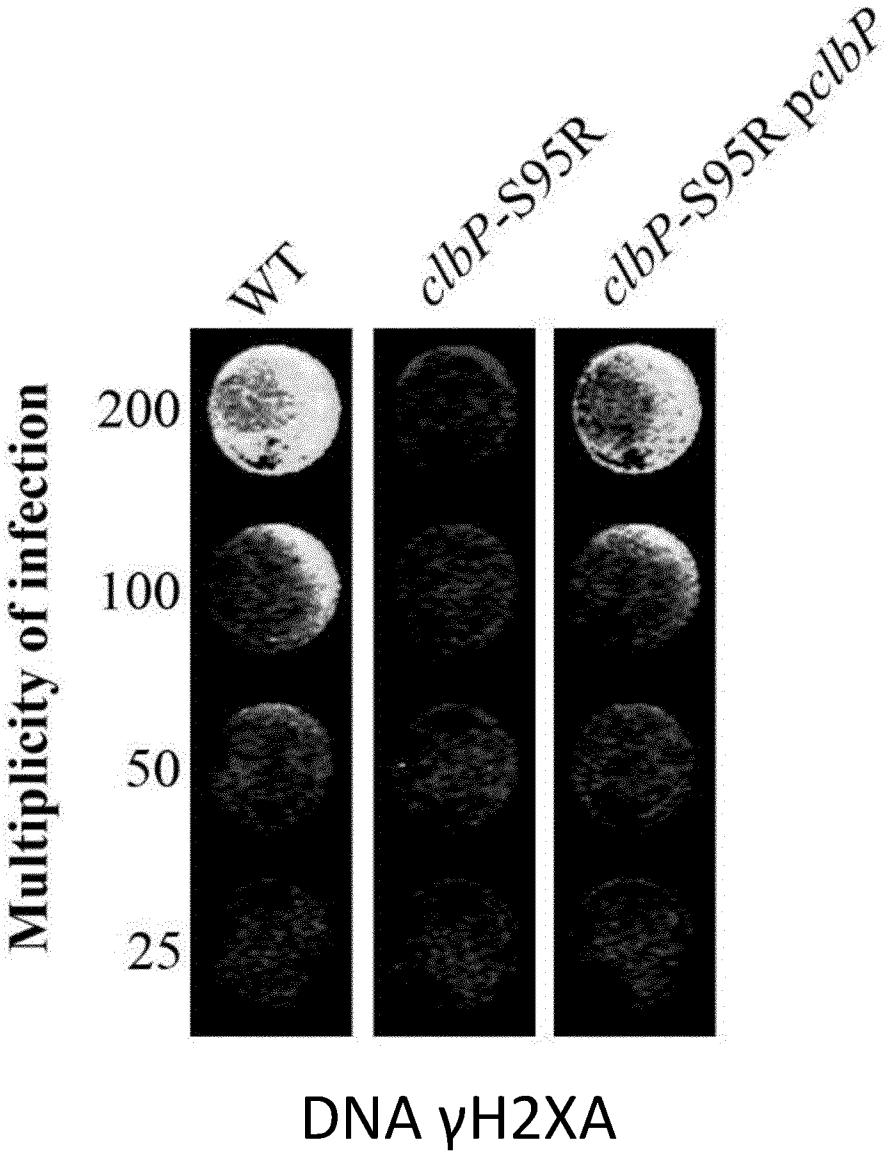
WT
clbP-S95R
clbP-S95R pclbP
Multiplicity of infection
200
100
50
25
DNA γH2XA

Figure 5A

Figure 5B

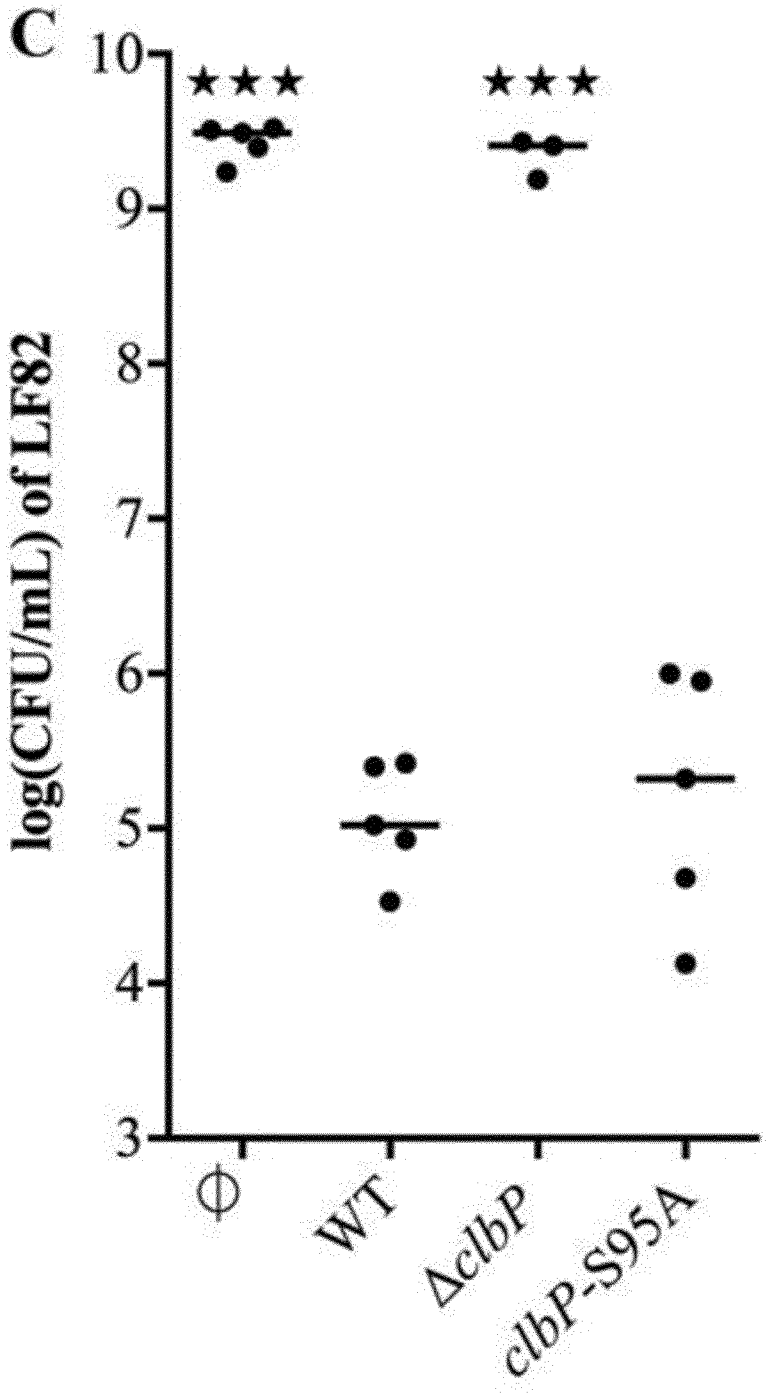

Figure 5C

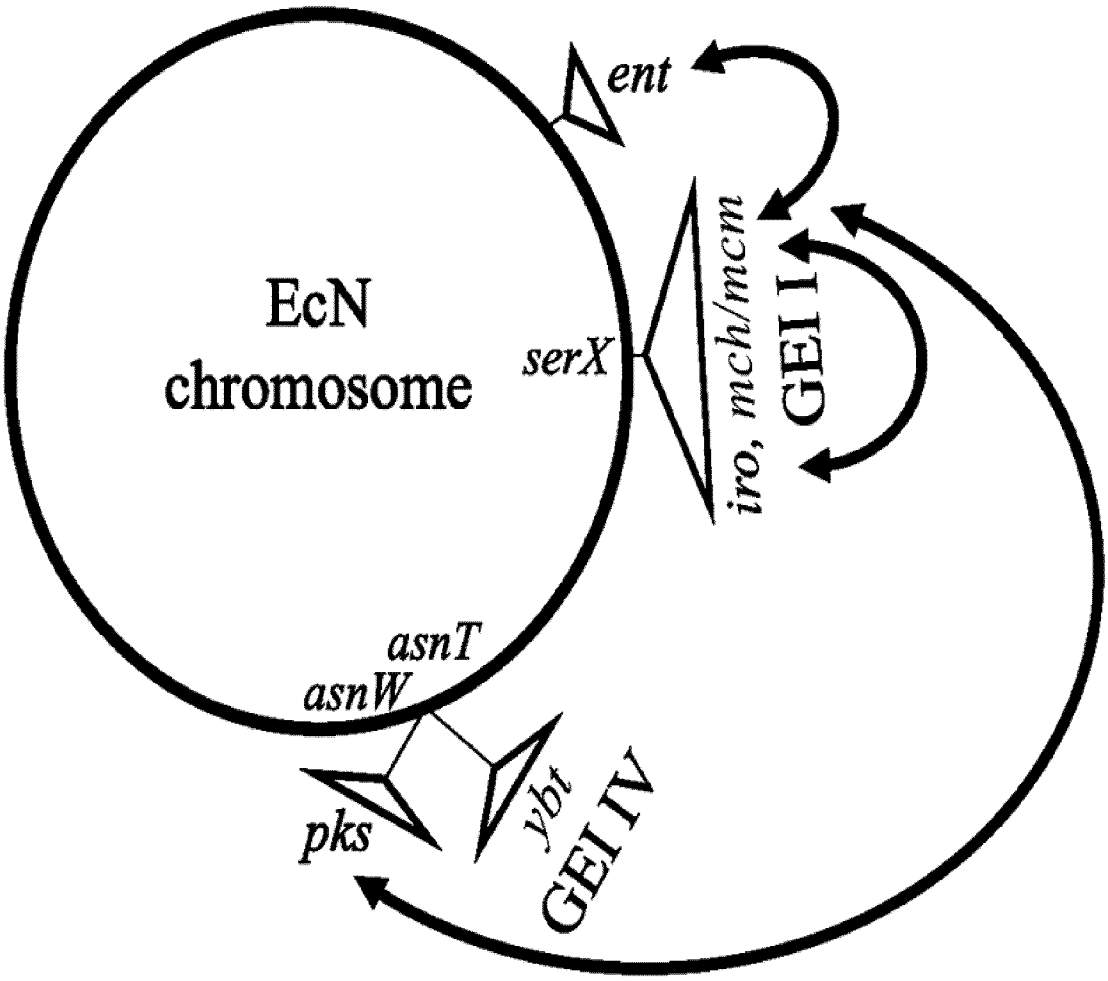
ent
EcN
chromosome
serX
iro, mch/mcm
GEI I
asnT
asnW
pks
ybt
GEI IV

Figure 6

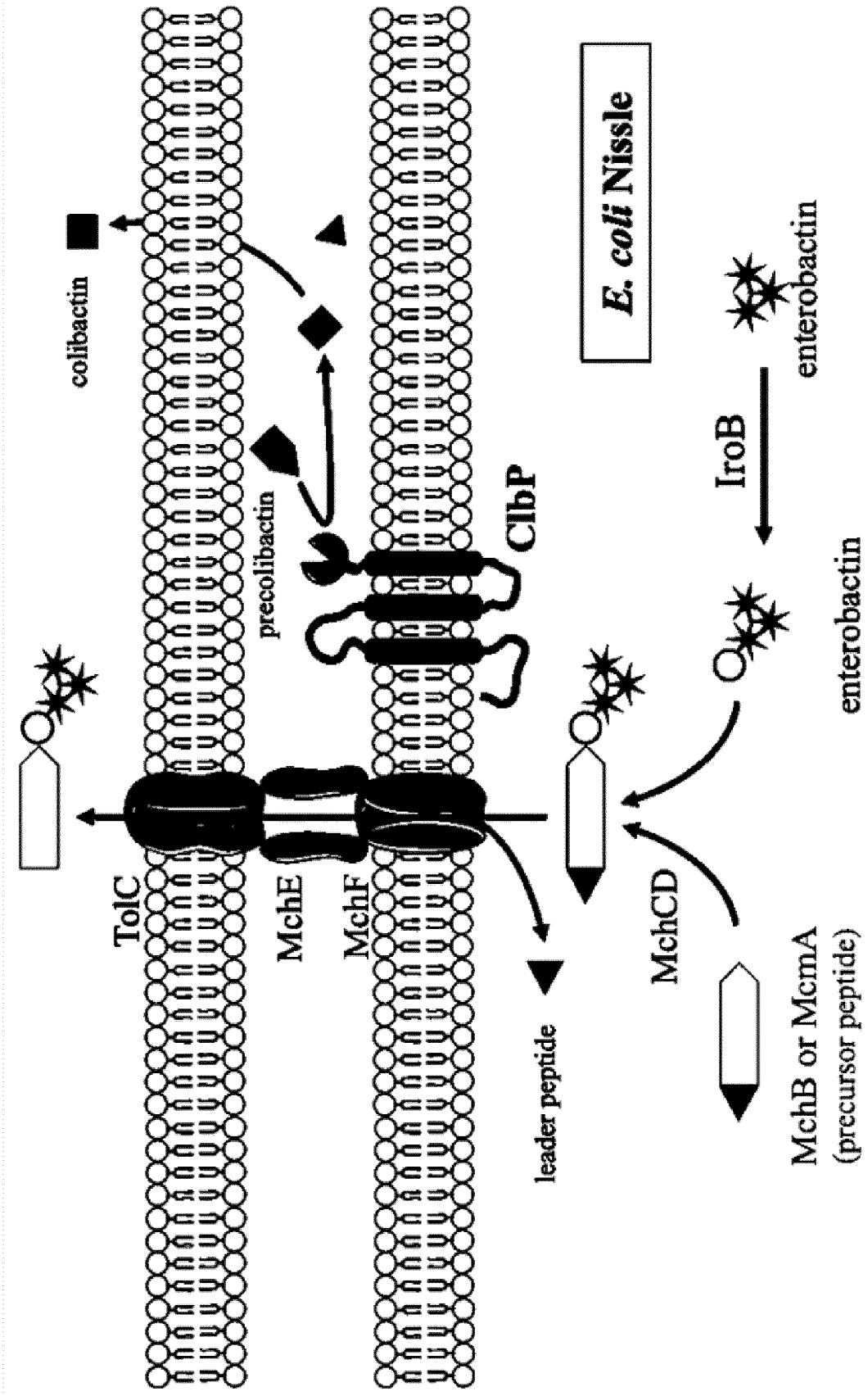
colibactin
precolibactin
ClbP
TolC
MchE
MchF
leader peptide
E. coli Nissle
IroB
enterobactin
enterobactin
MchCD
MchB or McmA
(precursor peptide)

Figure 7

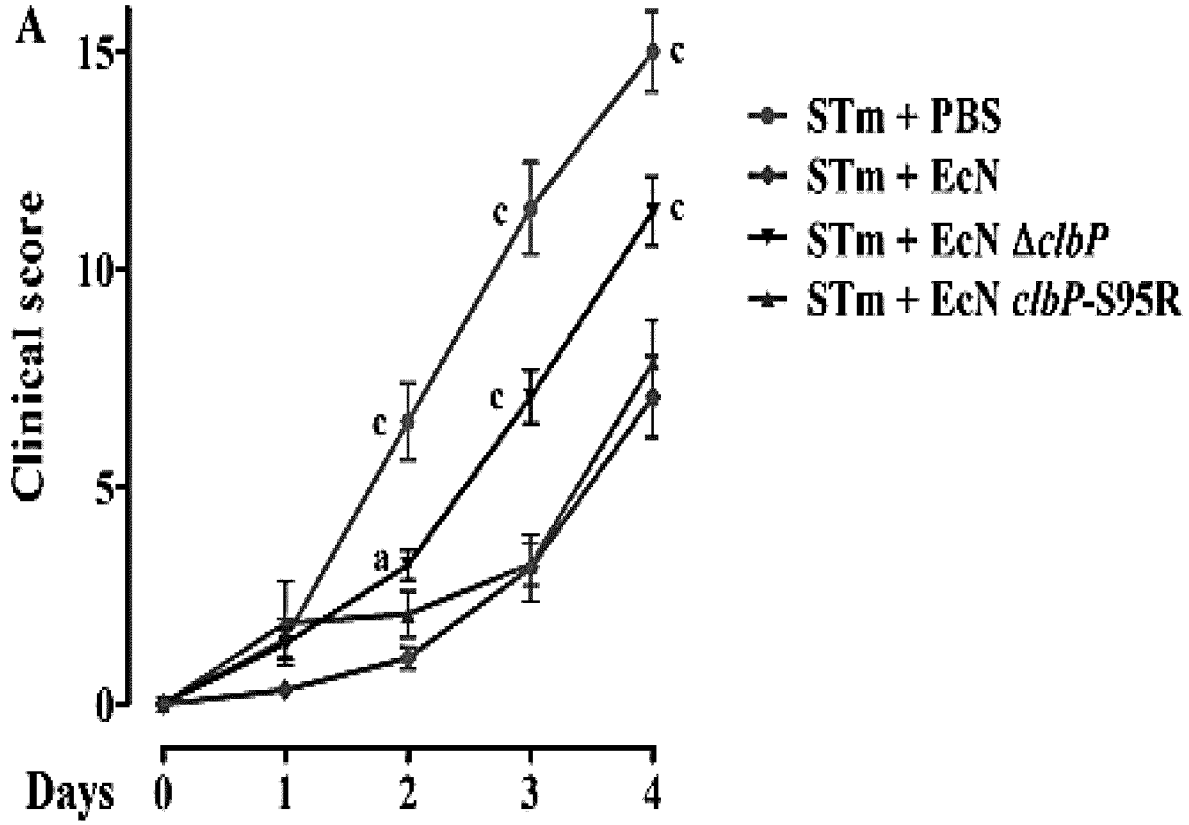
A
Clinical score
15
10
5
0
Days
0
1
2
3
4
c
c
c
c
a
STm + PBS
STm + EcN
STm + EcN ΔclbP
STm + EcN clbP-S95R

Figure 8A

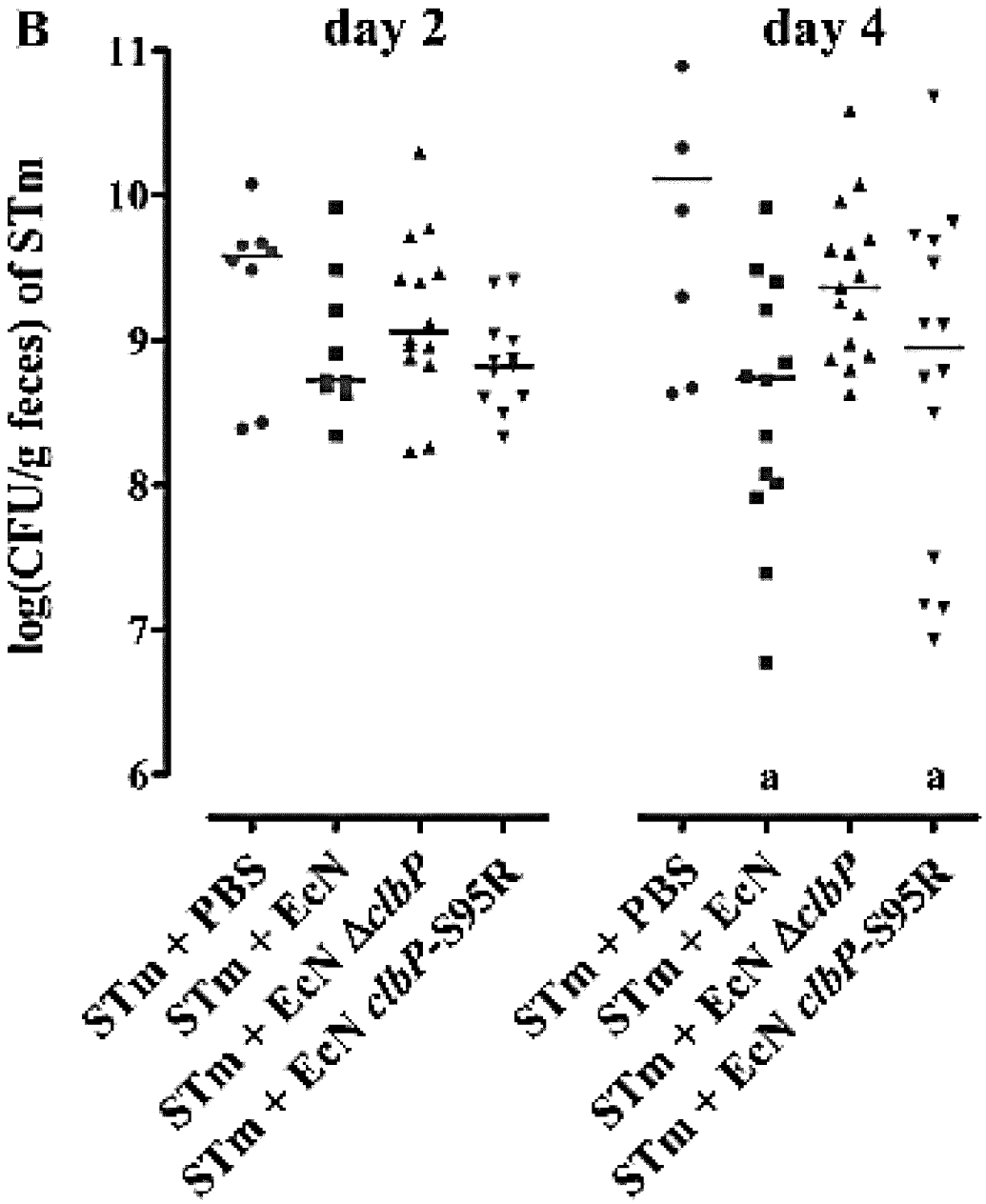
B
day 2
day 4
log(CFU/g feces) of STm
11
10
9
8
7
6
a
a
STm + PBS
STm + EcN
STm + EcN ΔclbP
STm + EcN clbP-S95R
STm + PBS
STm + EcN
STm + EcN ΔclbP
STm + EcN clbP-S95R

Figure 8B

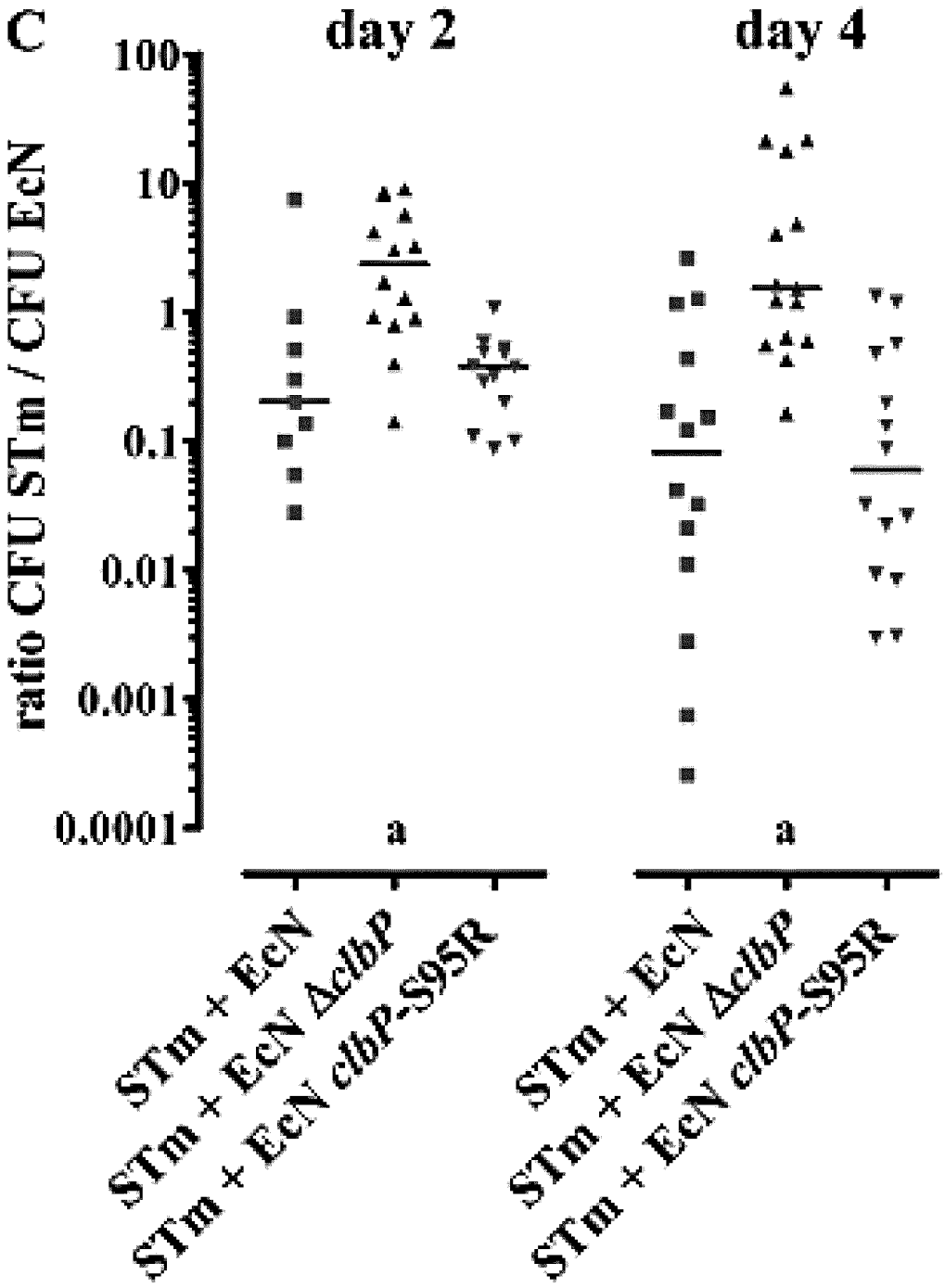
C
day 2
day 4
ratio CFU STm / CFU EcN
100
10
1
0.1
0.01
0.001
0.0001
a
a
STm + EcN
STm + EcN ΔclbP
STm + EcN clbP-S95R
STm + EcN
STm + EcN ΔclbP
STm + EcN clbP-S95R

Figure 8C

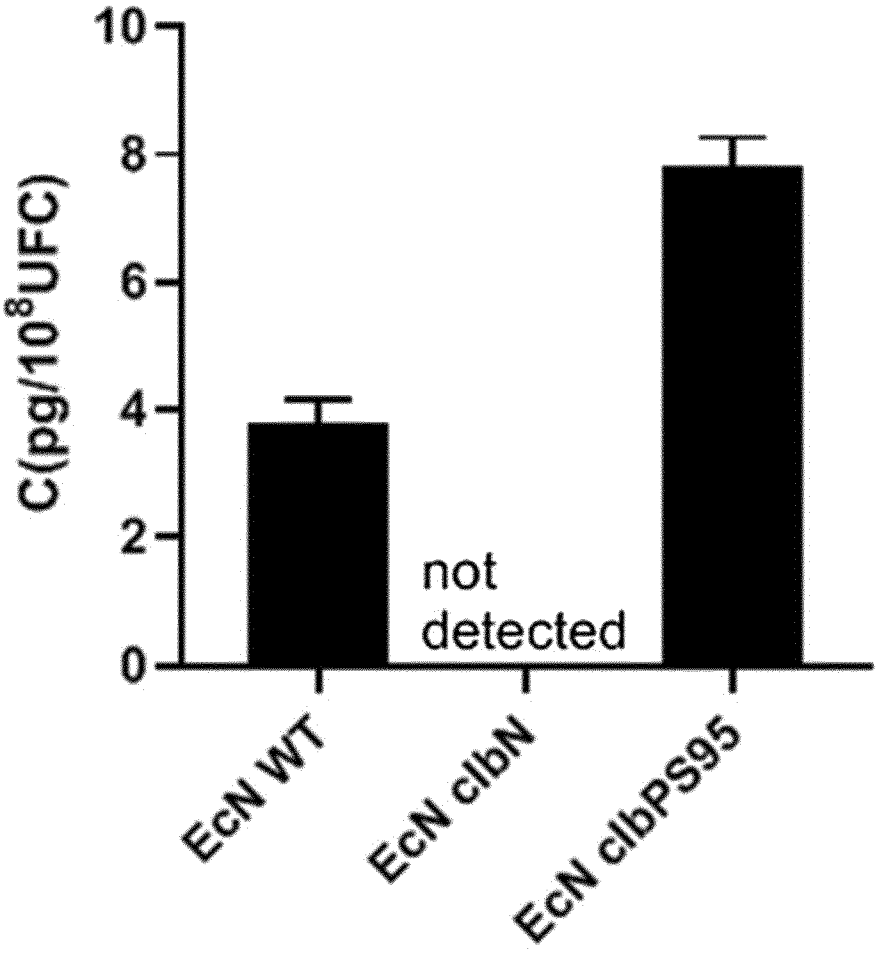

Figure 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/069124

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/741 C12N1/20
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12R C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, 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	XIAOYING BIAN ET AL: "Two more pieces of the colibactin genotoxin puzzle from Escherichia coli show incorporation of an unusual 1-aminocyclopropanecarboxylic acid moiety", CHEMICAL SCIENCE, vol. 6, no. 5, 1 January 2015 (2015-01-01) , pages 3154-3160, XP055649878, United Kingdom ISSN: 2041-6520, DOI: 10.1039/C5SC00101C figure 1 ----- -/--	1-15

[X] 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 application or patent 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	Date of mailing of the international search report
21 September 2020	01/10/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Herrmann, Klaus

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMILY P. BALSKUS: "Colibactin: understanding an elusive gut bacterial genotoxin", NATURAL PRODUCT REPORTS, vol. 32, no. 11, 1 January 2015 (2015-01-01), pages 1534-1540, XP055649467, GB ISSN: 0265-0568, DOI: 10.1039/C5NP00091B page 1536, right-hand column, paragraph 1 -----	1-15
Y	D. DUBOIS ET AL: "ClbP Is a Prototype of a Peptidase Subgroup Involved in Biosynthesis of Nonribosomal Peptides", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 286, no. 41, 14 October 2011 (2011-10-14), pages 35562-35570, XP055064059, ISSN: 0021-9258, DOI: 10.1074/jbc.M111.221960 cited in the application page 35563, left-hand column, paragraph 3 -----	1-15
Y	CAROLYN A. BROTHERTON ET AL: "A Prodrug Resistance Mechanism Is Involved in Colibactin Biosynthesis and Cytotoxicity", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 135, no. 9, 20 February 2013 (2013-02-20), pages 3359-3362, XP055649733, ISSN: 0002-7863, DOI: 10.1021/ja312154m page 3361, right-hand column, paragraph 3 page 3359, left-hand column, lines 1-3 -----	1-15
Y	NOUGAYRÈDE JEAN-PHILIPPE ET AL: "Escherichia coli induces DNA double-strand breaks in eukaryotic cells", SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, vol. 313, no. 5788, 11 August 2006 (2006-08-11), pages 848-851, XP002423942, ISSN: 0036-8075, DOI: 10.1126/SCIENCE.1127059 page 850, right-hand column, lines 2-7 -----	1-15
T	CLÉMENCE MASSIP ET AL: "Deciphering the interplay between the genotoxic and probiotic activities of Escherichia coli Nissle 1917", PLOS PATHOGENS, vol. 15, no. 9, 23 September 2019 (2019-09-23), page e1008029, XP055649469, DOI: 10.1371/journal.ppat.1008029 -----	

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