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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 July 2016 (21.07.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/113322 A1

(51) International Patent Classification:
C07K 14/28 (2006.01) *C12N 1/20* (2006.01)
A61K 39/02 (2006.01) *C12N 9/14* (2006.01)

(21) International Application Number:
PCT/EP2016/050595

(22) International Filing Date:
14 January 2016 (14.01.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15305024.0 14 January 2015 (14.01.2015) 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, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, 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, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, 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))*

WO 2016/113322 A1

(54) Title: GENETICALLY MODIFIED VIBRIO CHOLERAE STRAINS AND USES THEREOF

(57) Abstract: The invention relates to a genetically modified *Vibrio cholerae* strain comprising i) a mutation suppressing the phage replication activity of Uvr D, ii) a mutation preventing the expression of functional HupA and HupB, and/or iii) a mutation preventing the expression of functional hupB, wherein when the strain comprises a mutation preventing the expression of functional HupB, the *V. cholera* strain preferably also expresses an RstR polypeptide and further comprises a distinct mutation consisting of an *attRS* deletion, a knockout mutation in *rstA* and/or a knockout mutation in *rstB*. Compositions comprising such a strain are also herein described as well as their uses typically for the prevention or treatment of a subject against a *Vibrio cholerae* infection.

GENETICALLY MODIFIED VIBRIO CHOLERAE STRAINS AND USES THEREOF

FIELD OF THE INVENTION

The invention relates to the field of medicine, in particular prevention and treatment of cholera. The invention more particularly relates to a genetically modified *Vibrio cholerae* strain comprising i) a mutation suppressing the phage replication activity of UvrD, ii) a mutation preventing the expression of functional HupA (HUα) and HupB (HUβ), and/or iii) a mutation preventing the expression of functional HupB, wherein when the strain comprises a mutation preventing the expression of functional HupB, the *V. cholerae* strain preferably also expresses an RstR polypeptide and further comprises a distinct mutation consisting of an *attRS* deletion, a knockout mutation in *rstA* and/or a knockout mutation in *rstB*.

The strain is resistant to infection by any filamentous phage expressing a HUH endonuclease selected from the *Rep_trans* protein family (PF02486), in particular a phage selected from CTXϕ, VGJϕ, VEJϕ and any variant or combination thereof. Compositions comprising such a strain are also herein described as well as their uses typically for the prevention or treatment of a subject against a *Vibrio cholerae* infection.

BACKGROUND

Cholera remains a major health problem in many part of the developing world, with an estimation of 2.8 million cases and 100 000 to 200 000 deaths each year [1]. In 2011, recognizing that cholera was not sufficiently addressed despite its prevalence in epidemic forms in both endemic and non endemic areas, the World Health Assembly called for a comprehensive approach to cholera control, including the development of oral cholera vaccines (http://www.who.int/wer).

The agent of the cholera, the Gram-negative *Vibrio cholerae* bacterium, is found in briny waters all over the world [2]. However, most *V. cholerae* strains are not pathogenic or only cause local outbreaks of gastroenteritis. Pathogenicity depends on the acquisition of several virulence factors, of which the cholera toxin (CT) and the toxin-coregulated pilus (TCP) are considered the most significant. CT causes a voluminous watery diarrhoea, which is responsible for the high rate of death associated with cholera and its epidemic propagation [3], while TCP is required for colonization of the small intestine [4]. The most promising live attenuated *V. cholerae* vaccine strains have been obtained by the deletion of one or both of the cholera toxin genes, ctxAB [5–8]. However, ctxAB are encoded in the genome of a lysogenic filamentous phage, CTXϕ [9]. Constant and rapid emergence of strains harbouring new ctxAB and/or new CTXϕ variants in environmental and clinical isolates suggest that CTXϕ infection events are frequent [10–12]. This raised safety concerns about the use of ctxAB- *V. cholerae* cells in a vaccine since they could promote the apparition of cholera symptoms in previously asymptomatic

individuals and participate in the spreading of CTXϕ in the environment when re-infected in the intestinal track (Figure 1A).

CTXϕ depends on TCP and the TolQRA cell division proteins to infect *V. cholerae* [9,13] (Figure 1B, (1)). Once delivered in the cytoplasm of the cell, the circular single-stranded DNA (ssDNA) genome of the phage can be converted into a double stranded DNA (dsDNA) replicative form by the host machinery, which permits genome amplification by rolling-circle replication (RCR) and the production of new phage particles [14,15] (Figure 1B, (2), (3) and (4)). RCR depends on a single phage-encoded protein, RstA (Figure 1B). The rest of the process is driven by the host machinery [16]. RstA is an HUH endonuclease [17]. It creates a 5′-phosphotyrosine intermediate and a free 3′-OH at a specific cleavage site of the replicative form of CTXϕ, ori(+), to prime replication (Figure 1B). RstA production is under the control of the host SOS response [18] (Figure 1B, (5)) and of a phage-encoded repressor, RstR [16] (Figure 1C). In addition to phage particle production, RCR participates in the vertical transmission of ctxAB in the lineage of infected cells (Figure 1B). However, vertical transmission is also assured by the integration of CTXϕ into the genome of its host [9] (Figure 1B). CTXϕ exploits a chromosomally encoded site-specific recombination (Xer) machinery for integration [19,20] (Figure 1B). The Xer machinery normally serves to resolve dimers of the circular chromosomes by the addition of a crossover at a specific site, *dif* [21,22]. In *V. cholerae*, as in most bacteria, it consists of two tyrosine recombinases, XerC and XerD. The attachment site of the phage, attPCTX, consists in the stem of a hairpin of its single stranded DNA genome [23,24] (Figure 1B). XerC catalyzes the formation of a Holliday Junction (HJ) between attPCTX and the *dif* site of one or the other of the two circular chromosomes of *V. cholerae* [23,24] (Figure 1B, (6)). Replication converts the HJ intermediate into product [23–25]. The process is facilitated by EndoIII, a host-encoded base excision repair enzyme, which inhibits XerC catalysis once the HJ has been formed [25] (Figure 1B, (7)). Nevertheless, the integration of non-replicative forms of CTXϕ is inefficient [25]. In contrast, the integration of replicative forms is very efficient and almost always leads to multiple tandem insertions, which suggests that it occurs after several rounds of amplification of the phage genome by RCR [25,26] (Figure 1B). Multiple tandem insertions are permitted because a functional *dif* site is re-created on the right side of the prophage [20] (Figure 1B). Tandem insertions are crucial for the life cycle of CTXϕ because dsDNA conversion masks the Xer recombination site on the left side of the prophage, which impedes excision [23] (Figure 1B). Production of new free copies of the phage genome then depends on a process analogous to RCR between tandem prophage copies [15] (Figure 1B, (8)).

The importance of RCR in the life cycle of CTXϕ makes it an attractive target to limit the risks for vaccine cells to re-acquire ctxAB and participate in their dispersion in the environment. Indeed, to inventor's knowledge, the best vaccine cell protection strategy proposed to date was based on the observation that production of RstR from a resident CTXϕ prophage provided immunity against secondary infections by blocking initial rounds of RCR [27] (Figure 1C). However, this strategy has

several limitations. First, several CTXϕ variants exist that harbour different RstR repressors and cross-immunity is not assured among them [28] (Figure 1C). Second, CTXϕ interacts with other Integrative Mobile Element exploiting Xer (IMEX). Two of them, the RS1 satellite phage and fs2, harbour an anti-repressor, RstC [29,30] (Figure 1C). Third, hybrid phage formation between CTXϕ and other IMEXs, such a VGJϕ, can circumvent both the requirement for TCP expression and repressor immunity [31–34] (Figure 1C). Finally, tandem CTXϕ genomes can be transduced by lytic phages, such as CP-T1 [35].

Host factors implicated in the replication of the *E. coli* filamentous phages are either essential, such as DNA polymerase III, or crucial to the proliferation of the cells, such as the Rep helicase [14,26]. However, marked differences in the life cycles of CTXϕ and of the proto-typical filamentous phages of enterobacteriaceae, including its ability to integrate into the genome of its host, the control exerted by the host SOS response on RstA production [18] (Figure 1B) and the requirement for a host-encoded protein for CTXϕ particle secretion [36] (Figure 1B), suggested that it might not be so for CTXϕ. Inventors screened for non-essential host factors involved in CTXϕ replication and found that the histone-like protein HU [37] was essential for CTXϕ replication because it was necessary for RstA to introduce a nick in the phage genome at ori(+). They further found that in place of Rep, CTXϕ exploited UvrD, a DNA helicase mainly involved in DNA repair [38]. Finally, they found that HU and UvrD were implicated in the replication of other Vibrio filamentous phages, such a VGJϕ.

SUMMARY OF THE INVENTION

Inventors herein describe for the first time a genetically modified *Vibrio cholerae* strain resistant to infection by a filamentous phage expressing a HUH endonuclease selected from the *Rep_trans* protein family (PF02486) such as a lysogenic filamentous phage selected from CTXϕ, VGJϕ, VEJϕ and any variant or combination thereof, in particular a genetically modified *Vibrio cholerae* strain resistant to infection by CTXϕ and any variant thereof.

The genetically modified *Vibrio cholerae* strain comprises i) a mutation suppressing the phage replication activity of UvrD, ii) a mutation preventing the expression of functional HupA and HupB, and/or iii) a mutation preventing the expression of functional HupB. When the strain comprises a mutation preventing the expression of functional HupB, the *V. cholera* strain advantageously also expresses an RstR polypeptide and/or comprises a distinct mutation consisting of an *attRS* deletion, a knockout mutation in *rstA* and/or a knockout mutation in *rstB*.

Herein described are strains for use as a pharmaceutical agent or as an adjuvant. These strains are typically for use for immunizing a subject. These strains are also typically for use in a composition, preferably a vaccine composition.

A particular composition is an immunogenic composition for use for the immunization of a subject against *Vibrio cholerae*, said composition comprising a genetically modified *Vibrio cholerae* strain as

herein described, optionally together with (into association with) a pharmaceutically acceptable carrier and/or any delivery system designed to deliver said strain in a viable state to the intestinal tract.

Also described are methods for the prevention or treatment of cholera or symptoms thereof in a subject, comprising administering to said subject an immunogenically effective dose of a strain or composition according to the invention, as well as kits for implementing such methods.

DETAILED DESCRIPTION

The *Vibrio cholerae* bacterium is the agent of cholera. The cholera toxin production, which is responsible for the deadly diarrhea associated with cholera epidemics, is allowed by the expression of the cholera toxin genes, which are harboured in the genome of an integrated filamentous phage, CTXϕ.

One of the major strategies to prevent Cholera epidemics in endemic and non-endemic regions of the world is the development of oral vaccines based on live attenuated Vibrio cholerae strains. The most promising of these strains have been obtained by deletion of the cholera toxin genes. However, vaccine cells can re-acquire these genes if they are re-infected by CTXϕ or by hybrid phages between CTXϕ and other vibrio phages, which raised safety concerns about their use.

Based on these observations, inventors have developed and herein describe new attenuated Vibrio cholera vaccine strains which are safe, i.e. which are not at risk of promoting the apparition of cholera symptoms in previously asymptomatic individuals or of participating in the spreading of CTXϕ in the environment, contrary to imperfect oral vaccines based on live attenuated Vibrio cholerae strains available before date.

More specifically, inventors herein describe the identification of non-essential host factors implicated in CTXϕ replication. They found that the histone-like HU protein and the UvrD helicase were both absolutely required for the replication of CTXϕ. They further found that they were also essential for the replication of VGJϕ, a representative member of a family of phages that can form hybrids with CTXϕ. Accordingly, they demonstrated that the disruption of the two subunits of HU and/or of UvrD prevented infection of the cells by CTXϕ and VGJϕ. In addition, they showed that it prevented the cells from producing CTXϕ particles. Taken together, these results demonstrate that HU- and/or UvrD- cells can be efficiently used to develop safe live attenuated Vibrio cholera vaccine strains.

Inventors herein provide stable genetically modified (mutant) *Vibrio cholerae* strains and stable compositions/formulations comprising such strains producing sustained and efficient immune response (protection) in vivo against cholera. This means that when use as a vaccine these strains are expected to confer substantially close to 100% efficacy in humans against subsequent infection with a *V. cholerae* strain whatever the strain's serotype or biotype.

A "stable" composition or formulation is one in which the biologically active material (the strain) therein essentially retains its physical stability, chemical stability, and/or biological activity upon storage. Stability can be measured at a selected temperature and humidity conditions for a selected time period. Trend analysis can be used to estimate an expected shelf life before a material has actually been in storage for that time period. For live bacteria, for example, stability may be defined as the time it takes to lose 1 log of CFU/g dry formulation under predefined conditions of temperature, humidity and time period.

The strains and compositions of the invention are far safer than vaccine strains available to date since, contrary to the last, they are resistant to infection by filamentous phage expressing a HUH endonuclease selected from the *Rep_trans* protein family (PF02486) such as a lysogenic filamentous phage selected from CTXϕ, VGJϕ, VEJϕ and any variant or combination thereof, in particular resistant to infection by CTXϕ and any variant thereof.

The herein described genetically modified strains comprise i) a mutation suppressing phage replication activity of UvrD, ii) a mutation preventing the expression of functional HupA (HUα) and HupB (HUβ), and/or iii) a mutation preventing the expression of functional HupB. When the strain comprises a mutation preventing the expression of functional HupB, the *V. cholera* strain preferably also expresses an RstR polypeptide and further comprises a distinct mutation consisting of an *attRS* deletion, a knockout mutation in *rstA* and/or a knockout mutation in *rstB*.

In the context of the invention:

- the ***hupA*** nucleic acid sequence consists in or is substantially identical to SEQ ID NO:1 and the ***hupB*** nucleic acid sequence consists in or is substantially identical to SEQ ID NO:2. *hupA* (also herein identified as VC0273) and *hupB* (also herein identified as VC1919) respectively encodes HUα and HUβ, the two subunits of the histone-like HU protein expressed in *V. cholerae*;
- the ***uvrd*** nucleic acid sequence consists in or is substantially identical to SEQ ID NO:3 (also herein identified as VC0190). *uvrd* encodes the *V. cholerae* UvrD helicase.
- the ***rstR*** nucleic acid sequence consists in or is substantially identical to SEQ ID NO:4 (also herein identified as *rstR* N16961) or SEQ ID NO:5 (also herein identified as *rstR* O395). The RstR polypeptide represses RstA, increases resistance to CTXϕ transduction and/or reduces CTXϕ transduction. By "CTXϕ transduction" is meant the ability of the CTXϕ to enter a *V. cholerae* bacterium and be retained therein, either as an extrachromosomal plasmid, or as exogenous DNA integrated into the *V. cholerae* genome. It will be understood that the RstR of the invention may be isolated from any filamentous phage that may be present in a *V. cholerae* cell. A preferred RstR polypeptide sequence consists in, or is substantially identical, to a sequence selected from SEQ ID NO: 4, SEQ ID NO: 5, any mutant thereof and any combination thereof.
- By "genomic attRS insertion site" or "**attRS**" (SEQ ID NO:6) is meant a site in the genome of *V. cholerae*, which allows the integration of CTXϕ.

- the ***rstA*** nucleic acid sequence consists in or is substantially identical to SEQ ID NO:7. *rstA* is an open reading frame (ORF) which is required in *V. cholerae* for CTXϕ replication.
- the ***rstB*** nucleic acid sequence consists in or is substantially identical to SEQ ID NO:8. *rstB* is an open reading frame (ORF) which is required for CTXϕ integration into the *V. cholerae* genome.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides. Preferably, such a sequence is at least 20%, 40%, 50%, 60%, 70%, more preferably 80%, and most preferably 90% or even 95% identical at the amino acid level or nucleic acid level to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs). These software programs match identical or similar sequences by assigning degrees of identity to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Within the context of the invention, a genetically modified *Vibrio cholerae* strain is either a natural variant strain which, contrary to the wildtype strain, has the property of resisting to infection by filamentous phage expressing a HUH endonuclease selected from the *Rep_trans* protein family (PF02486), or a recombinant strain having an artificially (e.g. recombinantly) engineered genome exhibiting said property.

The recombinant strain may be prepared starting from any known *Vibrio cholerae* strain, such as strains available from collections such as ATCC, CNCM, etc. or libraries, or may be cloned from their publicly available genomic sequences. Further *Vibrio cholerae* strains may also be isolated from infected animals and used to prepare genetically modified *Vibrio cholerae* strain of the invention. Examples of *Vibrio cholerae* strains are provided in the experimental part.

As explained previously, the genetically modified *Vibrio cholerae* strain comprises at least one mutation in a gene selected from *uvrD*, *hupA* and *hupB*, as herein above identified under options i), ii) and iii), and possibly a mutation in the *attRS* gene under option (iii).

The mutation is typically a (partial or total) gene deletion or a knock-out mutation. Preferred deletions are *uvrD* deletion and/or *hupA* and *hupB* deletion, optionally together with an *attRS* deletion.

By "*uvrD* deletion" is meant a live *V. cholerae* strain that does not express UvrD and that, as a consequence, cannot replicate CTXϕ.

By "*hupA* deletion" is meant a live *V. cholerae* strain that does not express HupA.

By "*hupB* deletion" is meant a live *V. cholerae* strain that does not express HupB.

By "*hupA* and *hupB* deletion" is meant a live *V. cholerae* strain that neither express HupA nor HupB and that, as a consequence, cannot replicate CTXϕ.

By "*attRS* deletion" is meant a live *V. cholerae* strain that cannot revert or otherwise regain the capacity to produce cholera toxin by integration of CTX at the attRS sites as a result of deletion of all copies of the attRS sequence(s). A *V. cholerae* strain carrying even a single copy of the *attRS* sequence can efficiently acquire a new copy of the CTX element through DNA transfer.

Methods of making *attRS* deletions and parental vaccine strains containing these mutations are described in Mekalanos, J. J., U.S. Patent No. 5,631,010, issued May 20, 1997, incorporated herein by reference.

The knock-out mutation is an alteration in the nucleic acid sequence that reduces the biological activity of the polypeptide normally encoded therefrom by at least 80% relative to the unmutated gene, for example 100%. When it concerns *uvrd* the knock-out mutation at least suppresses a phage replication activity of UvrD, preferably the activity of UvrD allowing the replication of any filamentous phage expressing a HUH endonuclease selected from the *Rep_trans* protein family (PF02486), preferably the CTX phage (CTXϕ) replication activity.

The knock-out mutation may, without limitation, be a deletion, an insertion, a frameshift mutation, for example a frameshift mutation that creates a stop codon, or a missense mutation.

Site-directed mutagenesis (SDM) is one of the methods of preference used to generate mutated genes in the context of the present invention. There are many techniques of SDM now known to the man of skill in the art, including oligonucleotide-directed mutagenesis using PCR as set out, for example by Sambrook et al., (1989) or using commercially available kits.

In a particular genetically modified *Vibrio cholerae* strain, the mutation is a knock-out mutation of *uvrD* affecting (preferably suppressing) only the replication activity of UvrD but conserving any other activity thereof such as its catalytic activity.

Genetically modified *Vibrio cholerae* strains of the invention optionally include additional mutations introduced to improve their safety and/or immunogenicity. Such additional mutations include, but are

not limited to a deletion or knockout mutation of MshA (SEQ ID NO: 9). By "*mshA* deletion" is meant a live *V. cholerae* strain that does not express MshA and by "*mshA* knockout mutation" is meant a live *V. cholerae* strain that does not express a functional MshA.

Herein described are examples of method of preparing the above described *V. cholerae* strains.
A particular method involves introducing a plasmid into a wild type *V. cholerae* which contains a fragment of *V. cholerae* DNA containing a mutation as previously described in the *uvrD*, *hupA* and/or *hupB* sequences. The *V. cholerae* DNA fragment present in the plasmid is capable of recombining with wild type *V. cholerae* DNA inside the organism to generate the mutant strain. The plasmid can be introduced using conjugation as described in Philippe N *et al.* [Philippe N, Alcaraz JP, Coursange E, Geiselmann J, Schneider D (2004) Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid 51(3): 246-255]. Another method involves the direct use of fragment of *V. cholerae* DNA containing a mutation as previously described in the *uvrD*, *hupA* and/or *hupB* sequences and natural transformation as described in Marvig RL *et al.* [Marvig RL, Blokesch M (2010) Natural transformation of Vibrio cholerae as a tool-optimizing the procedure. BMC Microbiol 10: 155].
Appropriate methods of producing *V. cholerae* strains comprising a mutation consisting of an attRS deletion, a knockout mutation in rstA and/or a knockout mutation in rstB are described in WO99/28442 which is incorporated herein by reference.

In a particular aspect, the *Vibrio cholerae* strain is for use as a pharmaceutical agent, for example as a live vaccine.
The terms "pharmaceutical agent" refers to any agent capable of preventing, of reducing the symptoms or of treating a *V. cholerae* infection, typically cholera.
The terms "Viability" and "live vaccine" with regard to the genetically modified strain of the invention refer to the ability for the bacteria to form a colony (CFU or Colony Forming Unit) on a nutrient media appropriate for the growth of the bacteria.

In another particular aspect, the *Vibrio cholerae* strain is for use as an adjuvant, for example as an adjuvant vaccine. The term "adjuvant" designates a product capable of enhancing effectiveness of an already immunogenic composition.
In a further particular aspect, the *Vibrio cholerae* strain is for use in a composition, for example in a pharmaceutical composition, typically in an immunogenic composition, for example in a therapeutic or prophylactic composition, preferably in a (live) vaccine composition (vaccine).

The term "vaccine" as used herein includes any composition, which may be used to cause, stimulate or amplify an immune response or immunological protection in an animal, typically a mammal,

preferably a human being against a pathogen. Particular examples of vaccines of the invention are composition able to cause or stimulate or amplify immunity against *V. cholerae*.

The term "immunization" includes the process of delivering an immunogen to a subject. Immunization may, for example, enable a continuing high level of antibody and/or cellular response in which T-lymphocytes can kill or suppress the pathogen in the immunized subject, which is directed against a pathogen or antigen to which the subject has been previously exposed.

In the context of the invention, the subject is an animal such as a mammal (for example an herbivore or a human being), a bird, or a fish. The subject may also be a crustacean such as shrimp or a mollusc such as oyster and mussel. A preferred mammal is a human being. Strains and/or compositions of the invention are preferably administered to a subject suffering or at risk of suffering of cholera. Desirably, the vaccine is administered to a subject who has not yet been exposed to *Vibrio cholerae*.

The herein described strains may be used alone or in combination with other genetically modified strains, viruses or antigens to generate improved therapeutic or prophylactic compositions, typically improved vaccines. The invention is particularly suited to produce human vaccines, particularly for vaccinating a human being against *Vibrio cholerae* infection, typically against cholera.

Compositions of the invention comprise an immunologically effective amount of a strain as described above optionally in association with a pharmaceutically acceptable vehicle or carrier and/or any delivery system designed to deliver said strain in a viable state to the intestinal tract of the subject.

A typical composition is an immunogenic composition for use for the immunization of a subject against *Vibrio cholerae* infection, typically against cholera, comprising a genetically modified *Vibrio cholerae* strain as herein described, optionally in association with a pharmaceutically acceptable carrier and/or any delivery system designed to deliver said strain in a viable state to the intestinal tract.

In practice, the exact amount required for an immunologically effective amount/dose may vary from subject to subject depending on factors such as the age and general condition of the subject, the nature of the formulation and the mode of administration. Appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation. For instance, methods are known in the art for determining or titrating suitable dosages of a vaccine to find minimal effective dosages based on the weight of the non-human animal subject, concentration of the vaccine and other typical factors. In a typical embodiment, the composition comprises a *Vibrio cholerae* strain unitary dose of between 10^5 and 10^{12} CFU/ml (colony-forming units per milliliter), typically 10^6 CFU/ml, for liquids, and their equivalent expressed in CFU/g (colony-forming units per gram) for solids.

The dosage of the vaccine, concentration of components therein and timing of administering the vaccine, which elicit a suitable immune response, can be determined by methods such as antibody titrations of sera, e.g., by ELISA and/or seroneutralization assay analysis and/or by vaccination challenge evaluation.

Vaccines may comprise other ingredients, known per se by one of ordinary skill in the art, such as pharmaceutically acceptable carrier or excipient, adjuvant, freeze drying stabilizer, diluent, wetting or emulsifying agent, pH buffering agent such as acetates, citrates or phosphates, chelating agent such as ethylenediaminetetraacetic acid, gelling or viscosity enhancing additives, agent for the adjustment of tonicity such as sodium chloride or dextrose, or preservative, depending on the route of administration.

"Pharmaceutically acceptable carriers" for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington 's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Examples of pharmaceutically acceptable carriers, excipients or diluents include, but are not limited to demineralised or distilled water; saline solution; vegetable based oils such as peanut oil, arachis oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oil, or coconut oil; silicone oils, including polysiloxanes, such as methyl polysiloxane, phenyl polysiloxane and methylphenyl polysolpoxane; volatile silicones; mineral oils such as light liquid paraffin oil, or heavy liquid paraffin oil; squalene; cellulose derivatives such as methyl cellulose, ethyl cellulose, carboxymethylcellulose, carboxymethylcellulose sodium salt, or hydroxypropyl methylcellulose; lower alkanols, for example ethanol or iso- propanol; lower aralkanols; lower polyalkylene glycols or lower alkylene glycols, for example polyethylene glycol, polypropylene glycol, ethylene glycol, propylene glycol, 1 ,3- butylene glycol or glycerin; fatty acid esters such as isopropyl palmitate, isopropyl myristate or ethyl oleate; polyvinylpyrrolidone; agar; carrageenan; gum tragacanth or gum acacia, and petroleum jelly. Typically, the carrier or carriers will form from 10% to 99.9% by weight of the composition and may be buffered by conventional methods using reagents known in the art, such as sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium hydrogen phosphate, potassium dihydrogen phosphate, a mixture thereof, and the like.

Examples of adjuvants include, but are not limited to:

(1) non-toxic mutants of heat labile toxin protein or pertussis toxin protein, such as described, for example in Tsuji et al, (1990); Harford et al., (1989) and Roberts et al., (1995);

(2) oil-in-water emulsion formulations with or without other specific immunostimulating agents such as muramyl peptides [for example N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-l-alanyl-d-isoglutamine (nor-MDP), N-acetylmuramyl-1-alanyl-d-isoglutaminyl-1-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.]; bacterial cell wall components such as formulations described in PCT Publ. No. WO 90/14837, including but not limited to MF59 (containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85

(optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA)); SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion; Ribi TM adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80; and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox TM);

(3) saponin adjuvants, such as Stimulon TM (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes);

(4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA);

(5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and

(6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Examples of freeze-drying stabilizer may be for example carbohydrates such as sorbitol, mannitol, starch, sucrose, dextran or glucose, proteins such as albumin or casein, and derivatives thereof.

The route of administration can be oral, sublingual, nasal, rectal, via mucosal administration, via a parenteral route (intradermal, intramuscular, subcutaneous, intravenous, or intraperitoneal). The vaccine of the invention can be conveniently administered orally, intranasally, transdermally, parenterally, rectally, etc. The parenteral route of administration includes, but is not limited to, intramuscular, intravenous, intraperitoneal routes and the like. In preferred embodiments, the pharmaceutical composition of the invention is administered orally.

The composition of the invention may be a solid formulation, in particular an oral delivery system, such as tablet, pill, capsule, granules, sachet of microgranules, powder, or a liquid formulation such as an aqueous solution, water-in-oil or oil-in-water emulsion, syrup, an elixir, or a preparation for enteral, parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration) such as sterile suspension or emulsion. Such formulations are known in the art and are typically prepared by mixture of the strain as pharmaceutical agent and other typical additives as described previously in the appropriate carrier or solvent systems.

The compositions provided herein are preferably in any form which allows for the composition to be administered to a patient by the oral route. In a particular embodiment, the pharmaceutical

composition is formulated so as to allow the active ingredients contained therein to be bioavailable at the intestinal site targeted upon administration of the composition to a subject.

For oral administration, an excipient and/or binder may be present. Examples are sucrose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose and ethyl cellulose. Coloring and/or flavoring agents may be present. A coating shell may be employed, applying common membranes used for microencapsulation and suitable for the microencapsulation of live bacteria include biodegradable synthetic "polymers" such as polylactide, polyglycolic acid, and polyanhydride. Established "polymers" for live encapsulation and enzyme encapsulation include alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroymethylacrylate-methyl methacrylate (HEMA-MMA), Multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitrile/sodium methallylsulfonate (AN-69), polyethylene glycol/poly pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD5/PDMS), poly N,N- dimethyl acrylamide (PDMAAm), Siliceous encapsulates and cellulose sulphate/sodium alginate/polymethylene-co-guanidine (CS/A/PMCG). Other materials that are useful include, without limitation, cellulose acetate phthalate, calcium alginate and k-carrageenan-Locust bean gum gel beads, gellan-xanthan beads, poly(lactide-co-glycolides), carrageenan, starch poly-anhydrides, starch polymethacrylates, polyamino acids, enteric coating polymers.

The composition of the invention is preferably formulated to resist digestion into the stomach in order for the active ingredients contained therein to be bioavailable at the site targeted upon administration of the composition to a patient, or in other words in order for the live genetically modified strain to reach the intestinal tract, in particular the small intestine.

In a particular embodiment, the strain or composition comprising the strain is encapsulated by a coating which is substantially insoluble at a pH of less than a range of between about 7.0 to about 8.0 and soluble in the pH range of about 7.0 to about 8.0. Thanks to such a formulation, the strain is not released until the pH is about 7 and there is essentially no loss of the strain through the digestive tract until the delivery systems reaches the small intestine.

Preferably, the coating comprises one or more compositions selected from the group consisting of poly(dl-lactide-co-glycolide, chitosan (Chi) stabilized with PVA (poly-vinylic alcohol), a lipid, an alginate, carboxymethylethylcellulose (CMEC), cellulose acetate trimellitiate (CAT), hydroxypropylmethyl cellulose phthalate (HPMCP), hydroxypropylmethyl cellulose, ethyl cellulose, color con, food glaze and mixtures of hydroxypropylmethyl cellulose and ethyl cellulose, polyvinyl acetate phthalate (PVAP), cellulose acetate phthalate (CAP), shellac, copolymers of methacrylic acid and ethyl acrylate, and copolymers of methacrylic acid and ethyl acrylate to which a monomer of methylacrylate has been added during polymerization.

In another particular embodiment, the strain or composition comprising the strain is encapsulated with a biodegradable first capsule that is coated with a first enteric coating solubilizing in a pH of about 6.2 to about 6.5, and with a second capsule sized to include the coated first capsule. The second capsule

typically comprises a biodegradable material and is coated with a second enteric coating that solubilizes in a pH of about 7 to 8. This second capsule releases the first capsule in the ileum and once released the first capsule is solubilized in the proximal colon at a pH of about 6.2 to about 6.5 with the release of the desirable strain or composition according to the invention. The second enteric coating is advantageously substantially insoluble at a pH of less than a range of between about 7.0 to about 8.0 and soluble in the pH range of about 7.0 to about 8.0.

The first and second enteric coatings typically comprise one or more compositions selected from the group consisting of copolymers of methacrylic acid and ethyl acrylate, and copolymers of methacrylic acid and ethyl acrylate to which a monomer of methylacrylate has been added during polymerization.

Compositions that will be administered to a patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of one or more compounds of the invention in oral form may hold a plurality of dosage units.

The vaccines of the invention can be administered via different protocol, for example as single doses or in repeated doses. The vaccines of the invention can be administered alone, or can be administered simultaneously or administered sequentially with one or more further compositions, such as for example other immunogenic or vaccine compositions. Where the compositions are administered at different times the administrations may be separate from one another or overlapping in time.

Strains of the invention and compositions comprising said strains can be co-administered with other therapeutic agents or drugs, foods, nutrients, vitamins, other beneficial substances, prebiotics that release in the distal small intestine at pH values between 7.0 and 8.0 in an amount sufficient to alleviate *V. cholerae* infection in a subject. Preferably, at least two coating are used to cover a tablet or capsule like form comprising the strain of the invention, wherein the outside coating is degraded in a pH environment of 5 to 6 and the inside coating is degraded in a pH environment of about 7 thereby dropping the strain in the intestinal tract, typically in the ileum area. In certain embodiments, microencapsulated live *V. cholerae* strains of the invention and formulations thereof are administered in conjunction with one or more antibiotic. The dosage formulation is preferably designed in these embodiments to completely separate the antibiotic from the bacteria, and testing is conducted to verify complete separation on a long term basis. Suitable antibiotics include, but are not limited to tetracycline, ciproflaxine, doxycycline, co-trimoxazole, ampicillin, kanamycin, streptomycin and trimethoprim-sulfamethoxazole.

The present invention also relates to methods of immunizing or inducing an immune response in a subject, typically to methods of preventing or treating *Vibrio cholerae* infection, *Vibrio cholerae* infection associated disease, cholera or symptoms thereof (typically diarrhea, nausea, vomiting, and dehydration) in a subject, comprising administering to said subject an immunogenically effective dose

of a strain or composition, typically immunogenic composition, according to the invention as described above.

A further aspect of the invention relates to methods of treating and/or preventing a *Vibrio cholerae* infection associated disease in a non-human animal subject, and to methods of immunizing or vaccinating a non-human animal subject, such as herbivore, bird, fish, crustacean or mollusc, typically a population thereof, against a *Vibrio cholerae* infection, comprising administering to said subject a genetically modified strain or composition as defined above. Preferably, a non-human animal subject is protected to an extent in which one to all of the adverse physiological symptoms or effects of *Vibrio cholerae* infections are significantly reduced, ameliorated or totally prevented.

In one embodiment, the compositions of the invention are administered to a non-human animal susceptible to or otherwise at risk for *Vibrio cholerae* infection to enhance the subject own immune response capabilities.

The present invention also provides a container comprising an immunologically effective amount a strain or composition, typically vaccine, as described above.

The invention further provides kits, for example vaccination/immunization kits, comprising an optionally sterile container comprising an immunologically effective amount of the *Vibrio cholerae* strain or composition of the invention, means for administering said strain or composition to a subject, in particular a human being, and optionally an instruction manual including information for the administration of the immunologically effective amount the composition for treating and/or preventing infectious disease.

Another kit further comprises a rehydration solution, preferably a rehydration solution enriched with electrolytes.

The invention is particularly suited for the treatment (preventive or curative) of *Vibrio cholerae* infection and associated diseases, typically cholera.

Further aspects and advantages of the invention shall be disclosed in the following experimental section and figures, which illustrates the claimed invention.

LEGENDS OF THE FIGURES

Figure 1. Vaccine strains can re-acquire the capacity to produce the cholera toxin through their re-infection. **(A)** Scheme of the re-infection of a vaccine strain by CTXϕ in the intestinal track. The vaccine strain is depicted in blue and the pathogenic strain in red. Red points depict CTXϕ particles. **(B)** Schematic diagram showing key steps in the life cycle of CTXϕ. CTXϕ infection requires the host-

encoded toxin co-regulated pilus (TCP) and TolQRA proteins (1). After its release in the cytoplasm of its host, CTXϕ ssDNA is converted into a dsDNA by the host machineries (2). Rolling circle replication (RCR) of the phage depends on a single phage-encoded protein, RstA, and on the host machinery (3). New CTXϕ particle secretion depends on the host outer membrane protein EpsD (4). RstA production is under the control of the SOS response (5). Integration of CTXϕ depends on the host Xer machinery (6) and the accessory protein EndoIII (7). A process akin to RCR permits the production of free copies of CTXϕ ssDNA when the phage genome is integrated in tandem (8). **(C)** Scheme of the mechanism of action of phage immunity and its limitations for the protection of cholera vaccine strains. RstR production from a resident cholera vaccine genome represses RstA production and therefore provides immunity against secondary infections by a phage harbouring the same immunity region (CTX1). RS1 satellite phage-encoded RstC anti-repressor counteracts the activity of the resident RstR. Classical CTXϕ (CTX^{cl}) and other variants of CTXϕ (CTX2) contain a heterologous immunity regions with is not recognized by El Tor RstR. Hybrid CTX-VGJ phages escape RstR immunity by using the VGJϕ RCR module.

Figure 2. Screen for host factors implicated in CTXϕ replication. **(A)** Scheme of the conjugation of the pSC101-RS2 hybrid into a *lacZ*::*dif*1 reporter strain. **(B)** Schematic representation of the colonies obtained in different genetic backgrounds. *f*: relative frequency of formation of each type of depicted colony; *(i)*: colony formed upon direct integration; *(ii)*: colony obtained after RCR amplification of the phage DNA; *(iii)*: colony obtained when host factors implicated in integration are disrupted; (iv): colony obtained when host factors implicated in RCR are disrupted. **(C)** Scheme of the VC1919 region. Open triangles indicate the position of insertion of the transposon that impeded pSC101-RS2 RCR.

Figure 3. HU is essential for CTXϕ replication. **(A)** Relative colony-forming ability after conjugation with a replicative form of RS2 in the indicated strains. After 3 h of conjugation, serial dilutions were plated on chloramphenicol and incubated overnight at 42°C (left) and 37°C (right). Relative colony forming units (cfu) correspond to the ratio of the number of colonies obtained in the indicated strain over the mean number of colonies obtained in Δ*xerC* cells. Results are shown in a logarithmic scale and represent the mean and standard deviation of 3 independent experiments. The detection limit of the experiment is indicated by a dotted line. **(B)** Colonies obtained at 37°C were re-streaked on plate and incubated overnight at 42°C (Left) and 37°C (Right).

Figure 4. Impaired replication of CTXϕ in Δ*hupB* cells. **(A)** Q-PCR analysis of the number ssDNA and dsDNA copies of RS2 in the indicated strain*s*. The analysis was performed on the total DNA of cells that were grown under selection pressure at 30°C to an OD_{600nm} of 0.3. Data represent the mean of two independent experiments. The detection limit of the experiment is indicated by a dotted line. **(B)** Phage maintenance was measured in the indicated strain*s* after 5 h of growth in LB without selection. **(C)** Relative efficient of RS2 integration in *lacZ*::*dif*1, *lacZ*::*dif*1 Δ*hupA* and *lacZ*::*dif*1 Δ*hupB* cells. Integration was monitored after overnight growth in LB at 37°C. Data represents the

mean and standard deviation of 3 independent experiments. **(D)** Relative efficiency of integration of a non-replicative vector harbouring the *attP* of CTXϕ delivered by conjugation in *lacZ*::*dif*1, *lacZ*::*dif*1 Δ*hupA*, *lacZ*::*dif*1 Δ*hupB*, *lacZ*::*dif*1 Δ*hupAB* cells. Integration was monitored directly after conjugation. Data represents the mean and standard deviation of 3 independent experiments. **(E)** Relative production of CTXϕ particles by Δ*hupB* Δ*xerC* cells. Δ*xerC* recipient cells were incubated for 20' in the filtered supernatant Δ*xerC* and Δ*hupB* Δ*xerC* donor cells harbouring pCTX-Kn. Data represents the mean and standard deviation of 3 independent experiments. The detection limit of the experiment is indicated by a dotted line.

Figure 5. Replication of CTXϕ depends on UvrD. (A) Relative colony-forming ability after conjugation with RS2 in the indicated strain*s*. After 3 h of conjugation serial dilutions were plated on chloramphenicol and incubated overnight at 37°C. Relative colony forming units (cfu) correspond to the ratio of the number of colonies obtained in the indicated strain over the mean number of colonies obtained in Δ*xerC* cells. Results are shown in a logarithmic scale and represent the mean and standard deviation of 3 independent experiments. The detection limit of the experiment is indicated by a dotted line. **(B)** Complementation assay of Δ*xerC* Δ*uvrD* cells with a pBAD vector carrying the *V. cholerae uvrD* gene (pBAD-*uvrD*). RS2 was conjugated into Δ*xerC* and Δ*xerC* Δ*uvrD* cells harbouring pBAD or pBAD-*uvrD* and plated on LB supplemented with ampicillin, chloramphenicol and 0.2% of arabinose. Plates were incubated overnight at 37°C. **(C)** Relative colony-forming ability of *lacZ*::*dif*1, *lacZ*::*dif*1 Δ*rep* and *lacZ*::*dif*1 Δ*uvrD* cells after 3 h of conjugation with RS2. Serial dilutions were plated on chloramphenicol and incubated overnight at 37°C. Relative colony forming units (cfu) correspond to the ratio of the number of colonies obtained in the indicated strain over the mean number of colonies obtained in Δ*xerC* cells. Results are shown in a logarithmic scale and represent the mean and standard deviation of 3 independent experiments. The detection limit of the experiment is indicated by a dotted line. **(D)** Phenotype of colonies obtained after RS2 integration in *lacZ*::*dif*1 (Top) and *lacZ*::*dif*1 Δ*uvrD* (Bottom) cells.

Figure 6. Cleavage of CTXϕ *ori(+)* by RstA depends on HU. (A) Scheme depicting the primer extension assay used to monitor RstA cleavage. Red arrows in opposite direction depict potential loops in the *ori(+)* region. The *rstR* gene, the NotI site and the location of the primer used in the primer extension are shown. **(B)** RstA activity in the indicated strains. Top left. Electrophoresis of the products was performed with a 6% polyacrylamide/8M urea gel. Lane 1-4: dideoxy sequence ladder. Top right: schematic representation of *ori(+)* loop 2. Bottom: relative intensity of the primer extension profiles. Black triangle: position of the nick. **(C)** Schematic representing the formation of a dsDNA break when replication forks originating from ori^{101} encounter a nick created by RstA. Black and red stars depict Rep^{TS} and RstA proteins, respectively. **(D)** Relative colony-forming ability after conjugation. Column 1-3: pSC101-RS2 was conjugated in the indicated strains to Δ*xerC*, Δ*xerC* Δ*hupAB*, Δ*xerC* Δ*uvrD, and* Δ*xerC* Δ*hupAB* Δ*uvrD* cells, respectively; Column 5: pSC101-RS2 Δ*rstA* was conjugated in Δ*xerC* cells. After 3 h of conjugation, serial dilutions were plated on spectinomycin

and incubated overnight at 30°C. Relative colony forming units (cfu) correspond to the ratio of the number of colonies obtained in the indicated strain over the mean number of colonies obtained in Δ*xerC* cells. Results are shown in a logarithmic scale and represent the mean and standard deviation of 3 independent experiments. The detection limit of the experiment is indicated by a dotted line. **(E)** Q-PCR analysis of the number of pSC101-RS2 ssDNA and dsDNA copies in Δ*xerC*, Δ*xerC* Δ*hupAB* and Δ*xerC* Δ*hupAB* Δ*uvrD* cells, and of Δ*rstA* pSC101-RS2 ssDNA and dsDNA in Δ*xerC* cells. The analysis was performed on the total DNA of cells that were grown under selective pressure at 30°C to an OD_{600nm} of 0.3. Data represent the mean of two independent experiments.

Figure 7. HU and UvrD in RCR of other *V. cholerae* IMEXs. (A) Relative colony-forming ability of R6K-VGJ in the indicated strains. **(B)** Relative colony-forming ability of R6K-TLC in the indicated strains. Relative colony forming units (cfu) correspond to the ratio of the number of colonies obtained in the indicated strain over the mean number of colonies obtained in Δ*xerC* cells. Results are shown in a logarithmic scale and represent the mean and standard deviation of 3 independent experiments. The detection limit of the experiment is indicated by a dotted line.

Figure 8. Equal production of HUα and HUβ at 37°C and 42°C. Western blot analysis of His-tagged HUα and HUβ. *V. cholerae* containing a His-tagged version of HUα or HUβ was growth to an OD_{600nm} of 0.5. The cell lysates were loaded onto an SDS-PAGE gel. Proteins were transferred to a PVDF membrane and blocked with 5% milk in TBST for 1 hour. The membrane was probed with a 4x-His antibody.

Figure 9. HUα and HUβ bind DNA with equivalent affinity. *In vitro* HUα and HUβ binding assay on RS2 DNA. Black triangles depict increasing concentration of HU (1.5 ng, 7.5 ng, 15 ng, 75 ng, 150 ng, 750 ng, 1500 ng) in each lane.

Figure 10. Ectopic production of HUα or HUβ restores RS2 propagation in Δ*hupAB* cells. Complementation assay of Δ*hupAB* cells with a pUC18 vector carrying the *V. cholerae hupA* gene (pUC-*hupA*) *or hupB* gene (pUC-*hupB*). RS2 was conjugated into Δ*xerC* Δ*hupAB* + pUC18, Δ*xerC* Δ*hupAB* + pUC-*hupA* and Δ*xerC* Δ*hupAB* + pUC-*hupB* and then streaked on plate supplemented with ampicillin and chloramphenicol. Plates were incubated overnight at 37°C.

Figure 11. Deletion of the *rep* gene led to a severe growth defect in *V. cholerae*. Growth curve of wild type, Δ*uvrD* and Δ*rep V. cholerae* cells. The strains were grown in rich media LB at 37°C and the OD_{600nm} of each culture were measured over the course of time.

Figure 12. *V cholerae uvrD* is under the control of the SOS response. (A) Top: Scheme of the *E. coli* and *V. cholerae uvrD* promoter regions. The angled arrows depict the *uvrD* transcription start sites. Red boxes show LexA binding sites. Green boxes show predicted -35 and -10 core promoter elements. Bottom: Comparison of the sequence of *E. coli* and *V. cholerae* putative LexA binding sites in *uvrD* promoter. The mutated LexA binding site of *V. cholerae* is shown. **(B)** UV sensitivity of Δ*uvrD* cells. Cells were grown overnight on plates and then re-suspended in minimal media M9 for UV irradiation. Top: Control without UV exposition. Bottom: Cells irradiated up to UV doses of 25

J/m^2. (**C**) β-gal activity (Miller units) of strains harbouring a *lac*-gene transcriptional fusion to wild type or mutated *uvrD* promoters. Top: MV57 and MV57 Δ*recA*. Bottom; MV47 and MV47 *lexA*^{ind}.

Figure 13. *hupB* deletion limit CTXϕ horizontal transmission. (A) Relative susceptibility to CTXϕ infection. Donor: Δ*xerC* + CTX-Kn; Recipients: Δ*xerC*, Δ*xerC dif1*::El Tor RS2, Δ*xerC dif1::*Classical RS2 and Δ*xerC* Δ*hupB*. **(B)** Relative ability of CTXϕ production. Donors: Δ*xerC* + CTX-Kn, Δ*xerC dif1*::El Tor RS2 + CTX-Kn, Δ*xerC dif1::*Classical RS2 + CTX-Kn and Δ*xerC* Δ*hupB* + CTX-Kn .Donor strain was growth on LB media 5 hours. Filtered supernatant containing CTX-Kn particles was mixed the recipients strains which was growth in AKI media. After infection the strains were plated on LB supplemented with Kn. The number of CFU are shown in a logarithmic scale and represent the mean and standard deviation of 3 independent experiments.

EXAMPLES

Inventors herein describe the identification of non-essential host factors implicated in CTXϕ replication.

Rolling-circle replication (RCR) is central to the life cycle of CTXϕ because amplification of the phage genome permits its efficient integration into the genome and its packaging into new viral particles. A single phage-encoded HUH endonuclease initiates RCR of the proto-typical filamentous phages of *enterobacteriaceae* by introducing a nick at a specific position of the double stranded DNA form of the phage genome. The rest of the process is driven by host factors that are either essential or crucial for the replication of the host genome, such as the Rep SF1 helicase.

In contrast, inventors herein demonstrate that the histone-like HU protein of *V. cholerae* and the UvrD helicase were both absolutely required for the replication of CTXϕ. The histone-like HU protein of *V. cholerae* is in particular necessary for the introduction of a nick by the HUH endonuclease of CTXϕ. They further show that CTXϕ RCR depends on a SF1 helicase normally implicated in DNA repair, UvrD, rather than Rep. In addition to CTXϕ, they show that VGJϕ, a representative member of a second family of vibrio integrative filamentous phages that can form hybrids with CTXϕ, requires UvrD and HU for RCR while TLCϕ, a satellite phage depends on Rep and is independent from HU.

Accordingly, they demonstrated that the disruption of the two subunits of HU and/or of UvrD prevented infection of the cells by CTXϕ and VGJϕ. In addition, they showed that it prevented the cells from producing CTXϕ particles. Taken together, these results demonstrate that HU- and/or UvrD- cells are usable to develop safe live attenuated cholera vaccine.

Screening strategy

Inventors developed a colorimetric assay to monitor IMEX integration events in *V. cholerae* [24]. In brief, the *dif* site of the largest of the two chromosomes harboured by the *V. cholerae* N16961 El Tor

strain, *dif1*, was inserted in the coding region of the *Escherichia coli lacZ* gene in such a manner as not to perturb β-galactosidase production. The *lacZ::dif1* allele was inserted in place of the normal *dif1* site of a N16961 El Tor strain in which the endogenous *lacZ* gene was deleted (Figure 2A). This strain forms blue colonies on X-gal media. However, 100% of the colonies obtained after the delivery of a truncated form of the El Tor variant of CTXϕ, RS2, which is fully functional in replication and integration, were white or contained large white sectors around a blue star shaped centre on X-gal plates (Figure 2B, panel *(i)* and *(ii)*). Inventors previously used this property to search for non-essential host factors implicated in the integration of CTXϕ by transposition mutagenesis (Figure 2B, panel *(iii)*, [25]. During the course of this first screen, they noted that fully white colonies represented a very limited fraction of the total colonies, confirming the importance of ssDNA amplification by RCR for the integration process (Figure 2B, panel *(i)*). It suggested that the assay could be used in a second screen to identify non-essential host factors involved in RCR (Figure 2B, panel *(iv)*). To this end, they cloned RS2 on a pSC101 plasmid that harboured a spectinomycin resistance gene and that could be delivered by conjugation (Figure 2A). By using a temperature-sensitive version of the pSC101 origin of replication, they could distinguish if the absence of integration was due to the disruption of host factors implicated in RCR or in the integration process (Figure 2B, panel *(iii)* and *(iv)*). As a control, they verified that conjugation of the pSC101-RS2 hybrid in Δ*xerC* cells yielded fully blue colonies at 30°C and 42°C. They also verified that disruption of RstA, which abolishes RCR, led to fully blue colonies at 30°C that couldn't grow at 42 °C.

Inventors implemented the screen in two independent mariner transposition libraries of the *lacZ::dif1* reporter strain. Conjugants were selected on plates supplemented with spectinomycin and X-gal at 30°C. They screened over 40 000 clones, which allowed us to identify 6 fully blue clones that were thermo-sensitive. All of them carried a transposon insertion in the VC1919 ORF of the *V. cholerae* genome (Figure 2C). Sequence analysis revealed that they corresponded to at least three independent transposition insertion events (Figure 2C).

HU is essential for CTXϕ replication

In *E. coli*, HU is composed of two subunits, HUα and HUβ, which are encoded by *hupA* and *hupB,* respectively [37]. The major form of HU is a heterodimer of HUα and HUβ, but HUα homo-dimers and HUβ homo-dimers are also formed. VC1919 encodes for a homolog of the β subunit of *E. coli* HU, HUβ. A homolog of the α subunit of *E. coli* HU, HUα, is encoded by VC0273. Inventors engineered His-tag versions of the two gene products under their native promoters and showed that they were produced at the same level at 37°C and 42°C (Figure 8). They purified the recombinant proteins and showed that they bound DNA with similar affinities (Figure 9). These results suggested that VC0273 and VC1919 were the *V. cholerae* orthologs of *E. coli hupA* and *hupB*.

To confirm the results of their screen, they delivered a version of RS2 marked with a chloramphenicol resistance gene in a Δ*hupB* Δ*xerC* strain by conjugation. Because of the absence of XerC, RS2 cannot integrate in this strain and vertical transmission of chloramphenicol resistance to daughter cells

entirely depends on RS2 RCR. In agreement with the results of their screen, no colonies were obtained on selection plates at 42°C (Figure 3A). Colonies were obtained at 37°C (Figure 3A), but they failed to propagate when re-streaked at 42°C (Figure 3B). To further determine the potential role of HU in CTXϕ replication, they engineered a Δ*hupA* Δ*xerC* strain and a Δ*hupAB* Δ*xerC* strain. The deletion of *hupA* did not affect the maintenance of RS2 at 37°C and 42°C (Figure 3A and 3B). However, no colonies were obtained when RS2 was delivered in the Δ*hupAB* Δ*xerC* strain whether at 42°C or 37°C (Figure 3A and 3B). Ectopic production of HUα or HUβ in Δ*hupAB* Δ*xerC* cells restored colony formation at 37°C, excluding any polar effect of the two deletions (Figure 10). Taken together, these results suggested that HU was essential for CTXϕ replication, that HUα homo-dimers were sufficient to maintain the RF of the CTXϕ genome at 37°C but that HUβ homo-dimers and/or HUαβ hetero-dimers were absolutely required at 42°C.

The single deletion of *hupB* is sufficient to limit CTXϕ vertical and horizontal transmission

In order to gain a quantitative measure of the importance of HUα and HUβ in the CTXϕ replication process, inventors used quantitative PCR to monitor the number of RS2 ssDNA and dsDNA copies per genome equivalent in Δ*hupA* Δ*xerC* and Δ*hupB* Δ*xerC* cells that were grown under selection pressure at 37°C. The deletion of *hupA* had no visible effect on the relative number of RS2 copies, whether ssDNA or dsDNA (Figure 4A). In contrast, the deletion of *hupB* induced a 40% reduction in the number of RS2 copies per genome (Figure 4A). As the total number of RS2 copies per genome equivalent was now lower than 1, they suspected that the deletion of *hupB* would increase the instability of RS2 at 37°C even though it did not compromise colony formation on selection plates at this temperature. Indeed, a 100-fold reduction in the number of colony forming units was observed in Δ*hupB* Δ*xerC* cells compared to Δ*hupA* Δ*xerC* or Δ*xerC* cells after 5 hours of growth without selection pressure (Figure 4B). Because it limited the number of copies of the ssDNA CTXϕ genome, they further suspected that the deletion of *hupB* would also prevent RS2 integration. They observed a 5-fold reduction in the integration efficiency of RS2 in Δ*hupB lacZ::dif1* cells compared to *lacZ::dif1* cells (Figure 4C). A weaker, yet significant, decrease in RS2 integration was also observed in Δ*hupA lacZ::dif1* cells (Figure 4C). No decrease in the frequency of integration of a non-replicative plasmid harbouring $attP^{CTX}$ was observed in Δ*hupA*, Δ*hupB* and Δ*hupAB*, excluding any participation of HU in the integration process *per se* (Figure 4D). Finally, they suspected that the deletion of *hupB* might also prevent the production of phage particles by limiting the amount of ssDNA available for packaging. Indeed, a 1000-fold less phage particles were produced in Δ*hupB* Δ*xerC* cells than in Δ*xerC* cells (Figure 4E). Taken together, these results suggested that the deletion of *hupB* could by itself limit CTXϕ vertical transmission via lysogenic conversion and limit horizontal transmission via the production of new viral particles.

CTXϕ relies on UvrD for RCR

RCR of the proto-typical filamentous phages of *E. coli* depends on Rep, a helicase that is implicated in the replication of their host genome [39]. The *E. coli* Rep protein is not essential but its deletion leads

to a severe growth defect [40,41]. The genome of *V. cholerae* encodes for a homolog of *E. coli* Rep. Inventors found that it was not essential but that its deletion led to a severe growth defect, suggesting functional homology with *E. coli* Rep (Figure 11). However, the deletion of *V. cholerae* Rep impeded neither the maintenance of RS2 in Δ*xerC* cells (Figure 5A) nor its integration (Figure 5B), suggesting that it was not implicated in CTXϕ RCR.

Some RCR plasmids of Gram+ bacteria replicate in *E. coli* using the UvrD DNA helicase [42]. The *E. coli* UvrD protein plays essential roles in methyl-directed mismatch repair and nucleotide excision repair of DNA [43]. It is also involved in clearing and restarting stalled replication forks [44–46]. It is under the control of two promoters: one is constitutive while the other is governed by LexA, which leads to a 3 to 6-fold overproduction of UvrD during SOS [47,48] (Figure 12A). *E. coli* UvrD is not essential and its deletion does not affect cell proliferation under normal growth conditions. The genome of *V. cholerae* encodes a homolog of *E. coli* UvrD. Its deletion did not affect cell proliferation (Figure 11) but made them hyper sensitive to UV (Figure 12B). Inspection of the upstream region of the gene suggested the presence of two promoters, with a putative *lexA*-binding site overlapping the -10 box of one of them (Figure 12A). Correspondingly, introduction of a non-cleavable allele of *lexA* led to a 3-fold decrease in the expression of the gene (Figure 12C) while disruption of RecA or of the *lexA* box increased its expression (Figure 12D). Taken together, these results suggested that this gene was the functional homologue of *E. coli uvrD* and inventors wondered if its product was involved in CTXϕ RCR. Consistent with this view, deletion of *V. cholerae uvrD* impeded the maintenance of RS2 in Δ*xerC* cells (Figure 5A). Ectopic production of *V. cholerae* UvrD under an arabinose promoter on a plasmid restored colony formation, excluding any polar effect of the deletion (Figure 5B). The deletion of *V. cholerae uvrD* also led to over a 1000-fold drop in the frequency of integration of RS2 in $XerC^+$ cells (Figure 5C). The few colonies that were obtained were fully white or only displayed a pinpoint blue dot at their centre, further indicating that integration occurred immediately after entry into the cell (Figure 5D). Taken together, these results suggested that CTXϕ relied on the UvrD helicase for RCR.

Nicking of *ori(+)* depends on HU

There are three different steps in RCR: (i) addition of a nick at *ori(+)* to prime replication; (ii) displacement of the old (+) ssDNA copy of the genome and synthesis of a new one; (iii) termination of replication and re-circularization of the old (+) ssDNA genome copy. HU could be involved in any of these steps. By definition, UvrD was expected to be only involved in the second step. To investigate whether HU and UvrD were involved in the first step of RCR, total genomic DNA was extracted from *V. cholerae* cells 3 hours after conjugation of RS2 and the presence of a nick at *ori(+)* was revealed by primer extension (Figure 6A and B). In wild-type cells, they observed a strong signal consistent with the introduction of a nick between the guanine and the thymine bases of the apical loop of the second hairpin of CTXϕ *ori(+)* (Figure 6B). The position of the observed nick fitted with previous genetic analysis of the cleavage position of RstA [49]. Nick formation was entirely suppressed when HU was

deleted, suggesting that HU was essential for the activity of RstA (Figure 6B). In contrast, the deletion of UvrD did not affect nick formation, suggesting that UvrD was not implicated in RCR initiation.

One concern regarding to involvement of UvrD in RCR is that inventors did not recover any transposition event in the *uvrD* gene even though it is not essential in *V. cholerae*. However, they found that pSC101-RS2 is not able to propagate in Δ*uvrD* Δ*xerC V. cholerae* cells even at the permissive temperature (Figure 6D). They then hypothesized that replication forks originating from the pSC101 origin would generate fatal double strand breaks when they reached a nicked *ori*(+), which could explain why the pSC101-RS2 hybrid failed to propagate in Δ*uvrD* Δ*xerC* cells (Figure 6C). In agreement with this hypothesis, deletion of HU or inactivation of RstA restored the propagation of the pSC101-RS2 hybrid in Δ*uvrD* Δ*xerC* cells (Figure 6D). There was little or no production of RS2 ssDNA in such cells, further illustrating the importance of HU for RCR (Figure 6E).

Role of HU and UvrD in the RCR of other *V. cholerae* phages

Ecological interactions between CTXϕ and several other filamentous phages and their satellites drives the continuous and rapid emergence of new epidemic variants of *V. cholerae* [20,22]. Foremost among the phages implicated in those interactions are RS1, which encodes for an anti-repressor [30,50], VGJϕ, which participates in the horizontal spreading of CTXϕ via the formation of CTX-VGJϕ hybrids [31,32], and TLCϕ, which is almost always found integrated before CTXϕ prophages in clinical isolates and which can lead to their excision [51–53]. Inventors could predict that RS1 depended on HU and UvrD for replication, because it is essentially identical to RS2. To determine if VGJϕ and TLCJϕ might also depend on HU and UvrD, they conjugated a R6K suicide vector harbouring the replicative region of VGJϕ (R6K-VGJ) and a R6K suicide vector harbouring the replicative region of the satellite phage TLCϕ (R6K-TLC) in Δ*xerC* cells in which *hupA*, *hupB*, *uvrD* or *rep* were disrupted (Figure 7). No colonies were obtained when R6K-VGJ was conjugated in *hupA* or *hupB* mutants, suggesting that the HUαβ heterodimer was vital to VGJϕ RCR (Figure 7). R6K-VGJ also failed to be propagated in Δ*uvrD* cells, suggesting that UvrD was required for VGJϕ RCR (Figure 7). In contrast, Δ*hupAB* cells and Δ*uvrD* cells seemed to fully support TLCϕ replication (Figure 7). Finally, R6K-TLC was not maintained in Δ*rep* Δ*xerC* cells, suggesting that TLCϕ RCR depended on Rep (Figure 7).

Discussion

Inventors developed a strategy to identify non-essential *V. cholerae* host factors involved in CTXϕ replication. They thus found that contrary to the proto-typical filamentous phages so far studied, the histone-like HU protein was absolutely necessary for RstA to prime RCR of the phage genome (Figure 2, 3 and 6). In addition, they showed that CTXϕ exploited UvrD, a helicase normally implicated in DNA repair, rather than Rep, the helicase normally associated to replication (Figure 5). Finally, they

showed that a member of another family of vibrio filamentous phages, VGJϕ, also exploited HU and UvrD for RCR, demonstrating that CTXϕ is not an exception (Figure 7).

A role for HU in RCR

HU is a major component of the bacterial nucleoid, which binds dsDNA without any apparent specificity and with a low affinity but which recognizes with a higher affinity defined DNA structures and repair intermediates [54–56]. In *E. coli*, HU is involved in the initiation of chromosome replication [57–59]. However, it is not essential for survival: IHF, a protein belonging to the same family of DNA-binding proteins, can substitute for it initiation of replication at *oriC* [60]. Likewise, deletion of *hupAB* does not compromise cell viability in *V. cholerae,* possibly because its genome encode for a homolog of IHF.

As far as inventors know, no reports exist on the implication of HU in the life circle of any other filamentous phages than CTXϕ and VGJϕ. HU was shown to be essential for replication of Mini-F and Mini-P plasmids [61]. However, these plasmids replicate by a theta system. In this case, HU bind to the origin without sequence-specificity and help to melt the origin to initiate replication [62]. Interestingly, it was observed in *Salmonella typhimurium* that replication of a Mini-F plasmid was strongly affected in a Δ*hupB* mutant, totally deficient in a Δ*hupAB* double mutant, but only mildly affected in a Δ*hupA* mutant [63]. This is remarkably similar to what inventors have observed in the case of CTXϕ and a similar role of HU in the initiation of replication should not be discarded. More interestingly, however, it was reported that HU played an essential role in the replication of pKYM, a plasmid from the Gram$^-$ bacterium *Shigella sonei* [64]. A shared characteristic of proto-typical filamentous phages and of most RCR plasmids is a very simple (+) origin of replication: Ff coliphages contain an approximately 36bp replication origin [65]; the Gram$^+$ pC194 and pT181 plasmids harbour a small 55bp and 70bp origin, respectively [66,67]. None of these mobile elements require accessory proteins for the initiator protein nicking activity. In contrast, pKYM and CTXϕ (+) origins of replication are more complex. The (+) origin of replication of pKYM is 173bp long. It contains a core region corresponding to the RepK initiator binding-site and a downstream enhancer region. HU was shown to specifically recognize this enhancer region and assist in the binding of RepK [64]. CTXϕ *ori(+)* is 167bp long and contains several inverted repeat sequences upstream and downstream of the RstA cleavage site with the potential to form stem-loops [49]. It is therefore possible that HU helps CTXϕ replication by helping the binding of RstA and/or promoting its endonuclease activity. A weaker binding affinity and/or tighter control of the VGJϕ HUH endonuclease might explain why the two HU subunits are absolutely essential for this phage. Future biochemical work will need to clarify the exact mechanism of action of HU on RstA activity.

Implication of UvrD in RCR

Rep and UvrD are members of the SF1 family of helicases and share approximately 40% similarity [68]. They both unwind DNA in the 3' – 5' direction [69,70]. Despite the structural and functional similarities between Rep and UvrD, the physiological roles of the two helicases are well distinct. Rep

is constitutively expressed in *E. coli*, where it is implicated in chromosome replication: it directly interacts with the replicative helicase DnaB and helps remove nucleoproteins complex in front of replication forks [71,72]. Rep is also implicated in the restart of stalled replication forks [73]. As a result, a Δ*rep E. coli* mutants display a 50-60% reduction in their replication rate [40,41]. Nevertheless, Rep is not essential. On the contrary, UvrD is overexpressed during the SOS response in *E. coli* and its role seems to be mainly limited to DNA repair: its activity is involved in MutHLS-dependent mismatch DNA repair [74] and UvrABC-dependent nucleotide excision repair [75]. UvrD also helps dismantle RecA filaments from ssDNA, which prevents unwanted recombination [76]. Finally, UvrD can promote the movement of the replisome along protein-bound DNA and participate in the restart of replication forks [72]. Nevertheless, its deletion does not directly affect replication fork progression in *E. coli* [71]. Consistent with its role in replication fork progression, Rep was shown to be critical for phage RCR in *E. coli*, including ϕX174 and the Ff family of filamentous phages [39]. In contrast, inventors found that CTXϕ and VGJϕ both exploited UvrD for RCR. As far as they know, this is the first time that UvrD has been shown to participate in the replication of a phage genome. A single SF1 helicase, PcrA, is encoded in the genome of Gram+ bacteria instead of Rep and UvrD. RCR of plasmids from Gram+ bacteria relies on PcrA. However, some of them can replicate in *E. coli* using UvrD [42]. In addition, UvrD was shown to be implicated in the RCR of pKYM [77]. Together, these results suggest that RCR depends on an activity common to Rep and UvrD, raising the question as to why these two helicases are not interchangeable, similarly to PcrA and UvrD. Without wishing to be bound by a particular theory, inventors believe that exploitation of UvrD or Rep could be determined by the ability of the initiator protein to directly interact with one or the other of the two accessories helicase. In agreement with this hypothesis, the initiator protein of CTXϕ and VGJϕ share structural similarities with the initiator protein of the Gram+ plasmids that exploit UvrD to replicate in *E. coli* (pfam02486). In contrast, the initiator protein of TLCϕ shares sequence and structural similarities with the initiator protein of the *E. coli* proto-typical filamentous phages (pfam05144 and pfam05155).

Considerations for the biosafety of live-attenuated vaccine cells

The possibility for *ctxAB*$^-$ *V. cholerae* cells to be re-infected by CTXϕ raised safety concerns about their use in a live attenuated vaccine (Figure 1A). Several possibilities exist to limit the risk of re-acquisition of the genes and their further spreading. A simple way to block the delivery of the genome of CTXϕ could be to delete the production of its receptors at the cell surface, TCP and TolQRA. However, TCP is essential for intestinal colonization and hence immunogenicity [4]. TolQRA is part of the cell division machinery and is critical for the outer membrane stability of Gram$^-$ bacteria and their resistance to extra-cytoplasmic stress [78–82]. A simple way to limit further spreading of CTXϕ particles could be to block their secretion by deleting EspD [36]. However, EspD appears to be essential in *V. cholerae* [36]. As a result, the only valid vaccine cell protection strategy proposed to date was based on the constitutive expression of RstR repressors, with each repressor providing

immunity against secondary infections by phages encoding the same repressor [27] (Figure 13A). However, this strategy is limited to known CTXϕ repressor variants. In addition, it can be circumvented by the production of anti-repressors, by hybrid phage formation and by general transduction of the genome of CTXϕ by lytic phages. Finally, production of RstR does not affect the efficiency of the RCR process once it has been established, which permits production of new phage particles and further spreading of CTXϕ (Figure 13B). Thus, it was imperative to search for new comprehensive strategies to prevent the re-acquisition and the environmental spreading of *ctxAB*. Here, inventors showed that the deletion of *hupB* impedes *ctxAB* re-acquisition by CTX-VGJϕ hybrid infection and dramatically reduces CTXϕ production when its genome has been acquired by other horizontal transfer mechanisms (Figure 13B). They demonstrate that the deletion of *hupB* considerably increases the safety of RstR-producing vaccine cells. Moreover, they found that HU and UvrD were both essential for CTXϕ and VGJϕ replication, that their deletion compromised the ability of CTXϕ to integrate into the genome of its host and blocked the secretion of CTXϕ particles. HU is not essential for the proliferation of *V. cholerae*. Therefore, the deletion of *hupA* and *hupB* is a second promising strategy for the development of safe live attenuated cholera vaccines. UvrD participates in DNA mismatch repair, many genes of which have been shown to be important for colon colonization [83]. However, in the case the deletion of *uvrD* affects colon colonization, mutating it in such a way as to compromise its role in RCR without affecting its DNA repair activities offers a third strategy for the development of safe live attenuated cholera vaccines.

Materials and methods

Strains, plasmids and oligonucleotides

Strains, plasmids and oligonucleotides used in this study are described in Table 1, 2 and 3, respectively.

Table 1. Strains used in this study

Strains	Genotipe/phenotipes	References
N16061	V. cholerae O1 El Tor strain, Str	Heidelberg et al., Nature, 2000
EMV01	N16061 lacZEc::dif1, hapA	This study
EMV02	EMV01 hupA::Kmr	This study
EMV03	EMV01 hupB::Zeor	This study
EMV04	EMV01 hupA::Kmr hupB::Zeor	This study
EMV05	EMV01 xerC::rifr	This study
EMV06	EMV02 xerC::rifr	This study
EMV07	EMV03 xerC::rifr	This study
EMV08	EMV04 xerC::rifr	This study

EMV18	EMV01 uvrD::zeor	This study
EMV22	EMV01 rep::zeor	This study
EMV37	EMV18 xerC::rifr	This study
EMV38	EMV22 xerC::rifr	This study
EMV68	EMV01 dif1::pBS66 xerC::rifr	This study
EMV69	EMV01 dif1::pBS22 xerC::rifr	This study

All *V. cholerae* strains were constructed by natural transformation. Engineered strains were confirmed by PCR and sequencing. Bacterial strains were grown on Luria-Bertani (LB) agar. Antibiotics were used at the following concentrations: ampicillin (Amp), 100 µg/mL; spectinomycin (Sp), 100 µg/mL; chloramphenicol (Cm), 34 µg/mL for *E. coli* and 3 µg/mL for *V. cholerae*; kanamycin (Kn), 50 µg/mL; Zeocin (Zeo), 100 µg/mL for *E. coli* and 1 µg/mL for *V. cholerae* and rifampicin (Rif), 100 µg/mL for *E. coli* and 2 µg/mL for *V. cholerae*. 0.2% arabinose was used to induce UvrD production from the pBAD24 vector.

Table 2. Plasmids used in this study

	Description	References
pSW23T	pSW23::oriTRP4; oriVR6Kγ; Cmr	Demarre et al. Res Microbiol. 2005
pBS22	pSW23T harboring the replication and integration machinery RS2 of the CTX classical phage of V. cholerae 569B strain; Cmr	Das et al., PNAS, 2010
pBS66	pSW23T harboring the replication and integration machinery RS2 of the CTX El Tor phage of V. cholerae N16061 strain; Cmr	Das et al., PNAS, 2011
pBS73	pSW23T harboring the replication and integration machinery of VGJ phage; Cmr	This study
pBS90	pSW23T harboring the replication and integration machinery of TLC satellite phage; Cmr	Midonet et al., PNAS, 2014
pSW-Short-CT	AttP from CTX El Tor phage cloned into pSW23T	Val et al., Mol Cell, 2005
pMEV245	pDS132 carrying an arr2 cassette flanked by the upstream and downstream region of V. cholerae xerC; Cmr ,Rifr	Das et al., PNAS, 2010
pFX524	pSC101 with a repAts; Ampr	This study

pEM011	pFX524 harboring the replication and integration machinery RS2 of the CTX El Tor phage of V. cholerae N16061 strain; Cmr, Specr	This study
pEM017	pEM011 digested EcoRV, PmlI and circularized to delete RstA	This study
pEM019	pUC18 harboring hupB gene flanked by the upstream and downstream regions	This study
pEM020	pUC18 harboring hupA gene flanked by the upstream and downstream regions	This study
pEM021	pUC18 carrying an Zeor cassette flanked by the upstream and downstream regions of hupB; Apr	This study
pEM022	pUC18 carrying an Kmr cassette flanked by the upstream and downstream regions of hupA; Apr	This study
pEM029	pUC18 harboring uvrD gene flanked by the upstream and downstream regions	This study
pEM037	pUC18 carrying an Zeor cassette flanked by the upstream and downstream regions of uvrD; Apr	This study
pEM039	pUC18 harboring rep gene flanked by the upstream and downstream regions	This study
pEM044	pUC18 carrying an zeor cassette flanked by the upstream and downstream regions of uvrD; Apr	This study
pEM062	pBAD24 harboring uvrD gene cloned in front of arabinose inducible promotor	This study

Table 3. Oligonucleotides used in this study

	Sequence
768	TAAAGTGGCGCTTACGCTTGG
769	GAAGCTGCGGTACAGAAGCTC
1269	GACATTCTACCAAGAGCATC
2247	TTGCGCTTTCAGTCCATCAG
2248	GTGGTCTGGAGCGCGAAATC
2251	CACGGATCCCGTCATAACTTGCGTTACTG

2252	CACGCATGCACTCGGCAATACCGTATTAG
2249	CACCTCGAGTCACTGTGATTCCCCTTTGG
2250	CACAGATCTCTCTGAAAGACGCTTGCAAC
2253	CACTCTAGATCATTTAGGTTTCCCTTCTC
2254	CACGTCGACAGACGCGATCAAGTAATTGC
2425	TCACGGTACCTGCGTGGCAGCTTCTATCTC
2426	TCACTCTAGATTGGCTGCTTACCACGTCTG
2427	TCACGGATCCTTGAGACCGTCGAGCAATAG
2441	TCACGAATTCTCAACCGGATTGGCGGTCAC
2442	TCACTCTAGAAACCCACGACAACCGGAATC
2443	TCACAGATCTCCGCTTCATCTTGTCTTGGG
2690	ACCCTACTCCCTCAATTTGG
2444	TCACAGATCTCAAAGGCCAAGCGCATATCG
2701	ACACCATGGCCAGTGGCGAAGTCATGATC
2702	CACGCATGCCTTACTTACTTGTCATCGTCGTCCTTGTAGTCTCTAGACACCTTCTCTAATCTCG
2704	GTCTTCGTATGTCGCCTTGG
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
ARB2	GGCCACGCGTCGACTAGTAC
ARB6	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
1314	GTCATCGTCATCCTTGTAATCG
846	AAATGATCACGAGATAGGGTTGAGTGTTG

Mariner transposon-mutagenesis, screening and mutant characterization

A mariner transposon-mutagenesis bank of a *V. cholerae* reporter strain was created as described [25]. The bank was conjugated with a spectinomycin resistant (SpecR) derivative of RS2 El Tor containing a thermosensitive (TS) origin of replication (pSC101-RS2). Individual colonies were selected on X-Gal, IPTG and spectinomycin plates after 48 h of growth at 30°C. Fully blue colonies were selected and re-streaked in parallel at 30°C and 42°C. TS clones were cured from pSC101-RS2 by overnight growth in the absence of antibiotic and their phenotype was corroborated by re-conjugation with the same plasmid. The insertion was mapped by direct sequencing of the DNA flanking the point of insertion of the mariner transposons, which was amplified by arbitrary-random PCR [84].

Conjugation assay.

E. coli β2163 meso-diaminopimelic acid (DAP) auxotroph donors and *V. cholerae* recipients were grown to 0.3 at OD_{600nm}. Bacteria were pelleted by centrifugation, re-suspended in 50 µL and mixed at a 1:10 ratio, dropped onto sterile filter paper on top of an LB-agar plate supplemented with DAP and

incubated for 3 h. Conjugants were selected for the plasmid antibiotic resistance and DAP prototrophy. To monitor integration, conjugants were spread on plates containing X-gal and incubated at 37°C overnight. Conjugants carrying a TS origin of replication were re-covered at 30°C.

Assay of CTXϕ infection efficiency and phage production

Strains harbouring kanamycin-marked CTXϕ were used as donors. Eighty microliters of filtered supernatant containing CTX-Kn particles was mixed with 20 µl of recipients strains that had been grown in AKI media to induce TCP expression [85]. The mix was incubated 20 min at 37°C to allow infection and then plated on LB to determine the number of potential recipients and LB supplemented with kanamycin to determine the number of infected cells. The frequency of infection was determined by the ration of Kn^R cells and the total number of recipients.

Q-PCR analysis

Total DNA was purified using the GenElute^{tm} Bacterial Genomic DNA Kit from Sigma. Samples were analysed using a LightCycler FastStart DNA masterSYBR Green I system from Roche. Reactions were run in triplicate using a LightCycler 480 instrument (Roche). Primer 2690 and 2704, which amplify a specific 150 bp fragment inside *rstA* gene, were used for phage DNA quantification. Data were normalized with the bacterial chromosome using primers 768 and 769, which amplify a 150 bp fragment within the *matP* gene. For single strand DNA quantification, total DNA was digested 3 hours with ScaI to remove phage dsDNA. There is a cleavage site for ScaI within the phage fragment used for the analysis. Relative copy number of ssDNA was calculated as follows: $2 \times e_1^{Cp\_digested}/e_2^{Cp\_chromosome}$, in which e represents the amplification efficiency of the primers pairs used. A factor of 2 was used to normalize the ssDNA of the phage with the dsDNA of the chromosome. The analysis was run out in parallel without prior digestion, which permitted to calculate the relative copy number of dsDNA as follows: $(e_1^{Cp\_undigested}-e_1^{Cp\_digested})/e_2^{Cp\_chromosome}$.

SDS-page and western blot

Bacterial lysates were electrophoresed on 12% SDS-page gel. HUα or HUβ with a C-terminal 6xHis tag were analysed by western blot with a primary anti-4His mouse monoclonal antibody (Invitrogen) and a secondary anti-mouse IgG antibody coupled to peroxidase (Pierce). ECL Western Blotting Substrate (Pierce) was used to detect the reaction on a LAS-3000 Luminescent Analyser (Fujifilm).

Sequencing gel and nick detection

For nick detection, pBS66 was conjugated to the strain of interest and then total DNA was purified directly from the conjugation assay. After digestion with NotI, inventors performed a primer extension reaction using as a primer the 1269 oligonucleotide that had been labelled with γ-[32P] ATP. The sequence ladder was prepared using pBS66 purified from *E. coli*, in which CTXϕ does not replicate, and the fmol DNA Cycle Sequencing System (Promega).

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CLAIMS

1. A genetically modified *Vibrio cholerae* strain comprising i) a mutation suppressing the phage replication activity of UvrD, ii) a mutation preventing the expression of functional HupA (HUα) and HupB (HUβ), and/or iii) a mutation preventing the expression of functional HupB, wherein when the strain comprises a mutation preventing the expression of functional HupB, the *V. cholera* strain preferably also expresses an RstR polypeptide and further comprises a distinct mutation consisting of an *attRS* deletion, a knockout mutation in *rstA* and/or a knockout mutation in *rstB*.

2. The strain according to claim 1, wherein the mutation is a gene deletion or a knock-out mutation.

3. The strain according to claim 2, wherein the mutation is *UvrD* deletion and/or *hupA* and *hupB* deletion.

4. The strain according to claim 2, wherein the mutation is a knock-out mutation of *UvrD*.

5. The strain according to anyone of claims 1 to 4 for use as a pharmaceutical agent.

6. The strain according to anyone of claims 1 to 4 for use as an adjuvant.

7. The strain according to anyone of claims 1 to 4 for use in a vaccine composition.

8. An immunogenic composition for use for the immunization of a subject against *Vibrio cholerae* infection comprising a genetically modified *Vibrio cholerae* strain according to anyone of claims 1 to 7, optionally together with a pharmaceutically acceptable carrier and/or any delivery system designed to deliver said strain in a viable state to the intestinal tract.

9. The immunogenic composition according to claim 8, wherein said immunogenic composition is a live vaccine.

10. Kit comprising an immunologically effective amount of the *Vibrio cholerae* strain according to anyone of claims 1 to 4 or a composition according to claim 8 or 9, and means for administering said strain or composition to a subject.

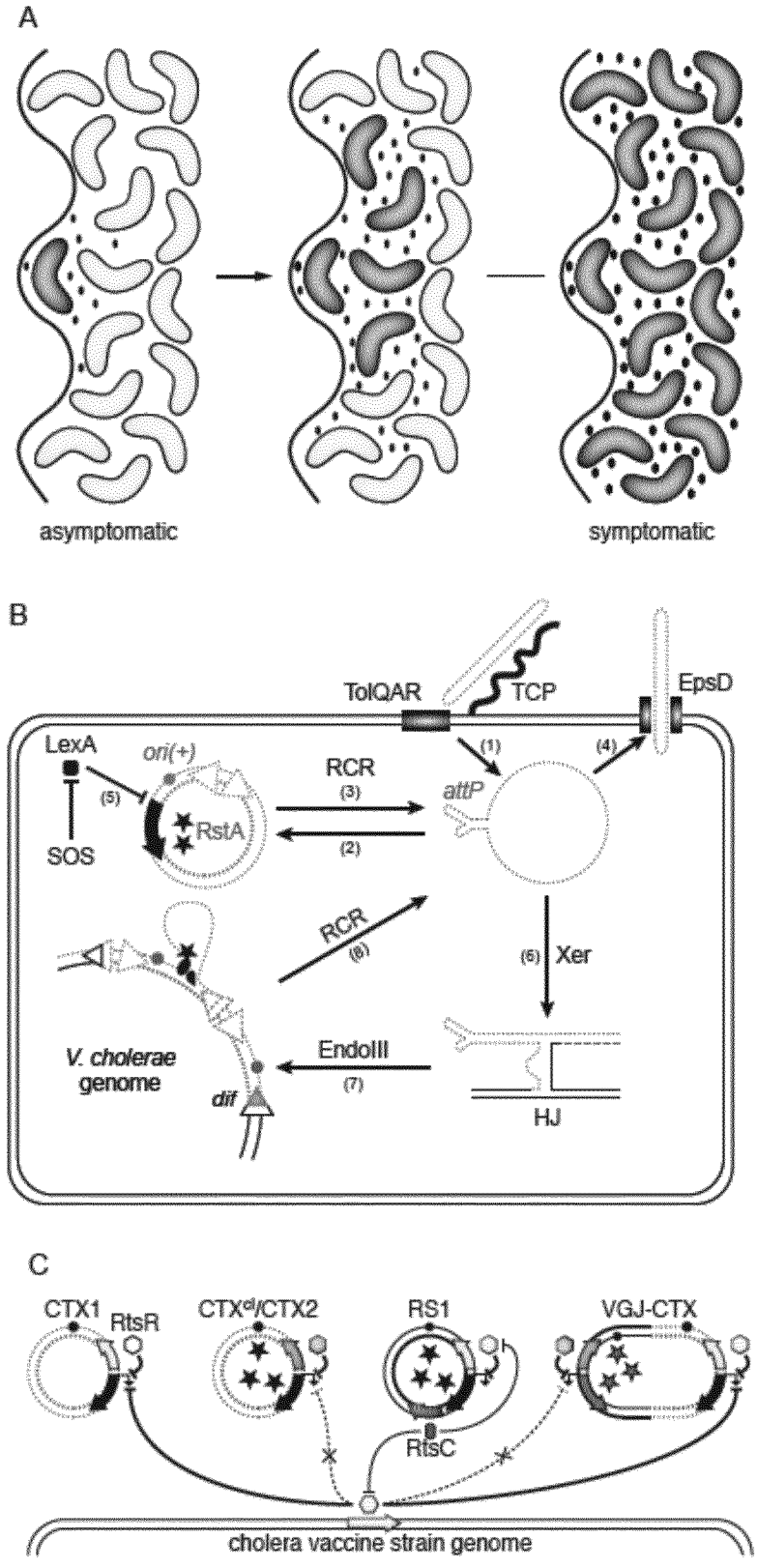

A
asymptomatic
symptomatic
B
TolQAR
TCP
EpsD
LexA
ori(+)
(5)
SOS
RstA
RCR
(3)
(2)
attP
(1)
(4)
RCR
(8)
(5) Xer
V. cholerae
genome
dif
EndoIII
(7)
HJ
C
CTX1
RtsR
CTXd/CTX2
RS1
VGJ-CTX
RtsC
cholera vaccine strain genome

FIGURE 1

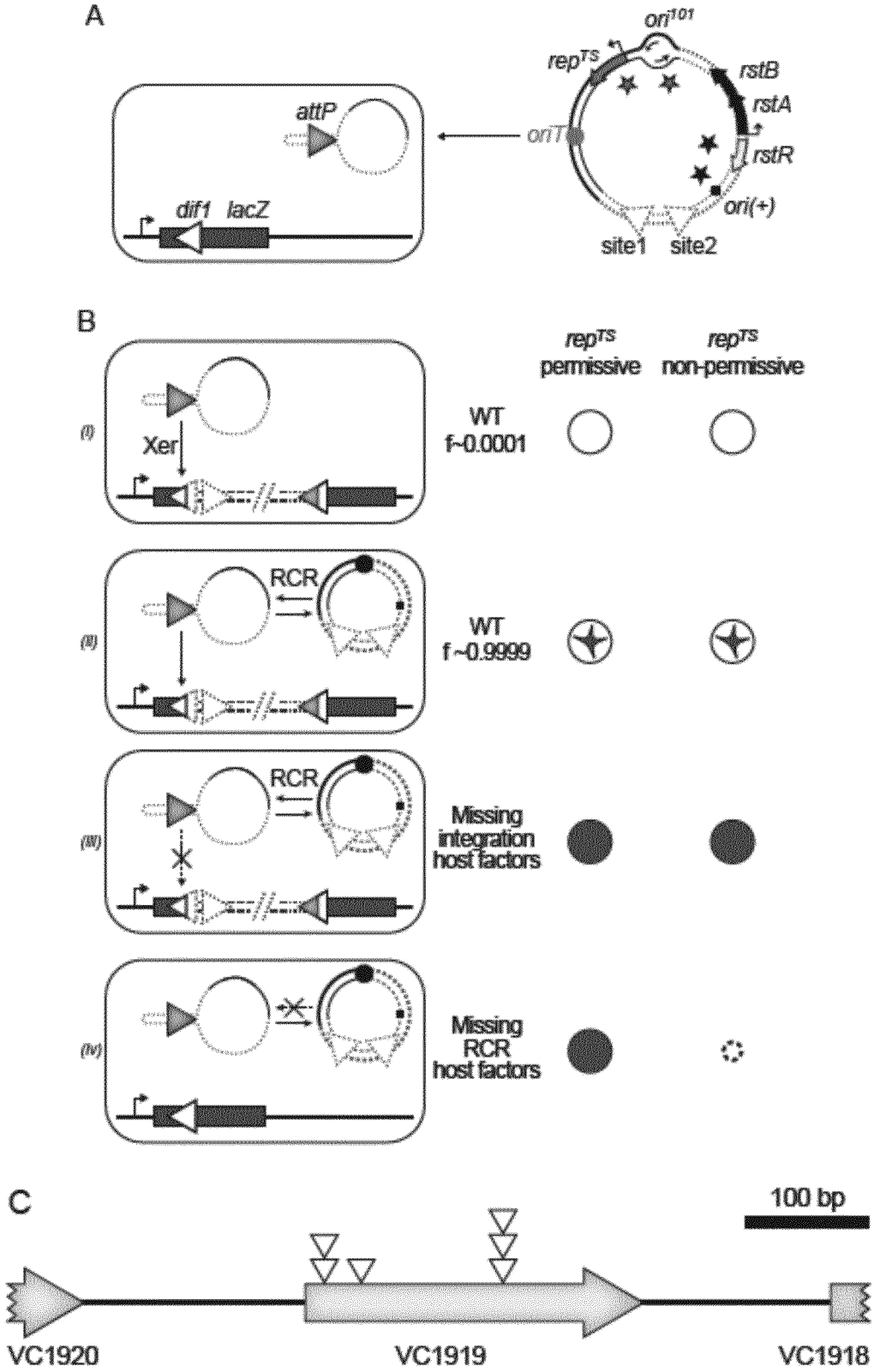

A
ori101
repTS
rstB
rstA
rstR
oriT
ori(+)
site1
site2
attP
dif1
lacZ
B
repTS permissive
repTS non-permissive
(I)
Xer
WT f~0.0001
(II)
RCR
WT f ~0.9999
(III)
RCR
Missing integration host factors
(IV)
Missing RCR host factors
C
100 bp
VC1920
VC1919
VC1918

FIGURE 2

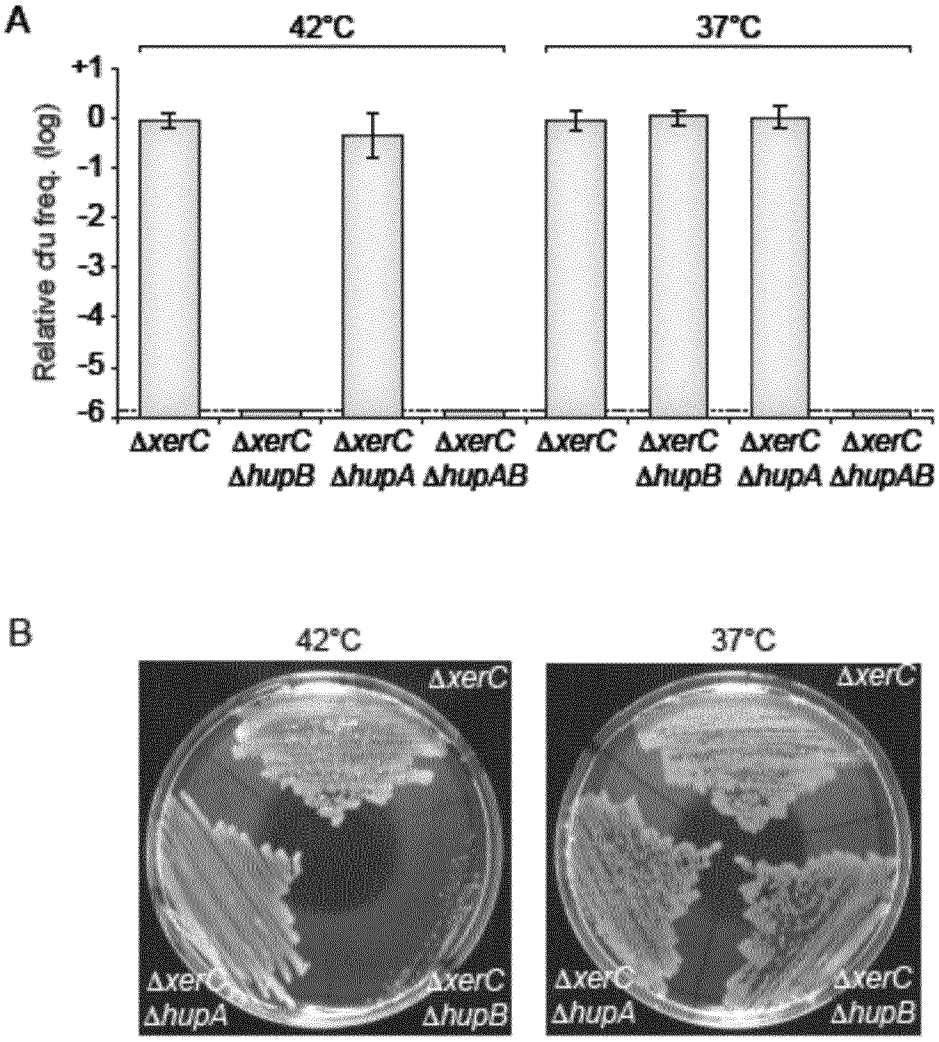

A
42°C
37°C
Relative cfu freq. (log)
+1
0
-1
-2
-3
-4
-5
-6
ΔxerC
ΔxerC ΔhupB
ΔxerC ΔhupA
ΔxerC ΔhupAB
ΔxerC
ΔxerC ΔhupB
ΔxerC ΔhupA
ΔxerC ΔhupAB
B
42°C
37°C
ΔxerC
ΔxerC ΔhupA
ΔxerC ΔhupB
ΔxerC
ΔxerC ΔhupA
ΔxerC ΔhupB

FIGURE 3

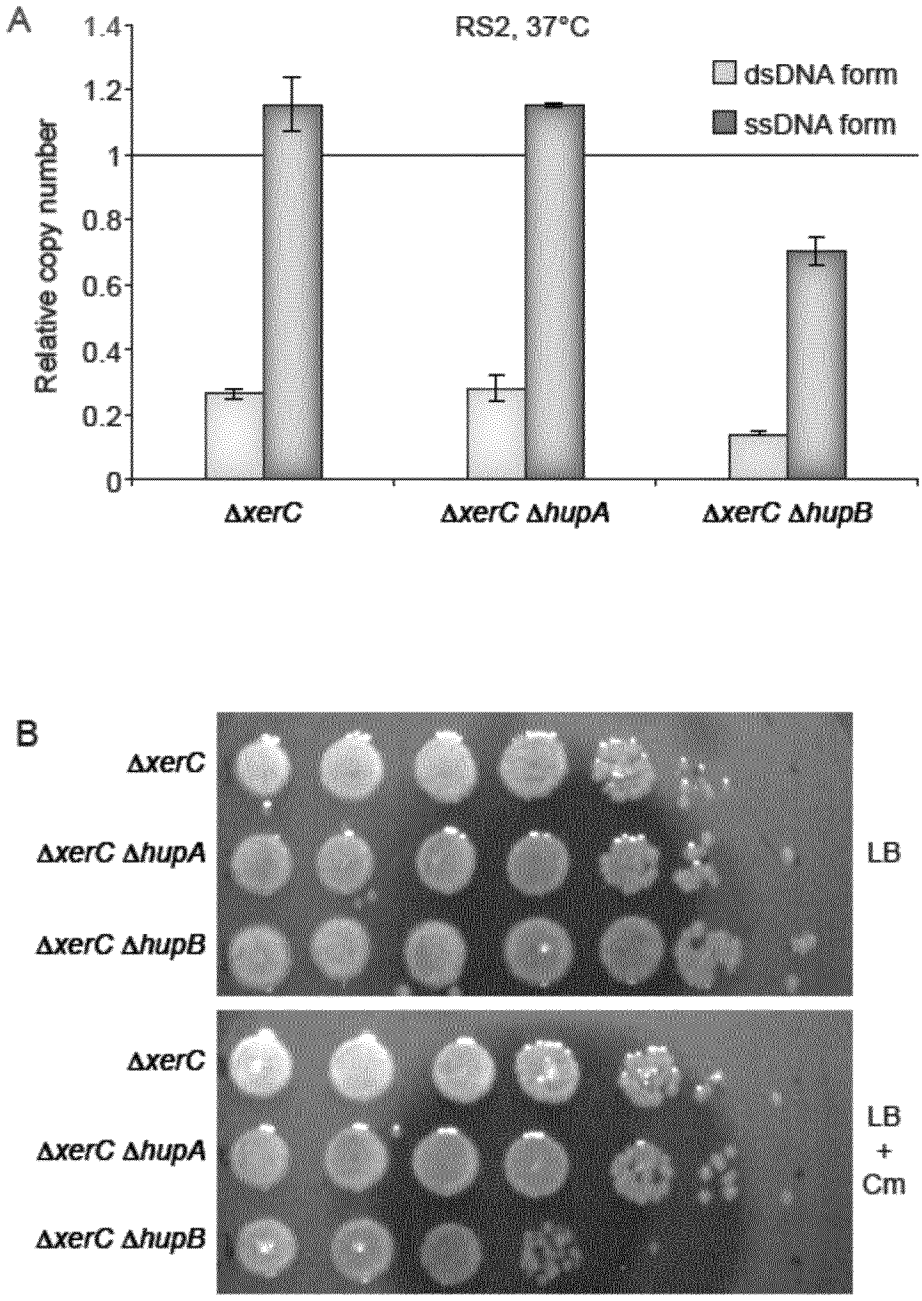

A
RS2, 37°C
dsDNA form
ssDNA form
Relative copy number
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ΔxerC
ΔxerC ΔhupA
ΔxerC ΔhupB
B
ΔxerC
ΔxerC ΔhupA
ΔxerC ΔhupB
LB
ΔxerC
ΔxerC ΔhupA
ΔxerC ΔhupB
LB + Cm

FIGURE 4

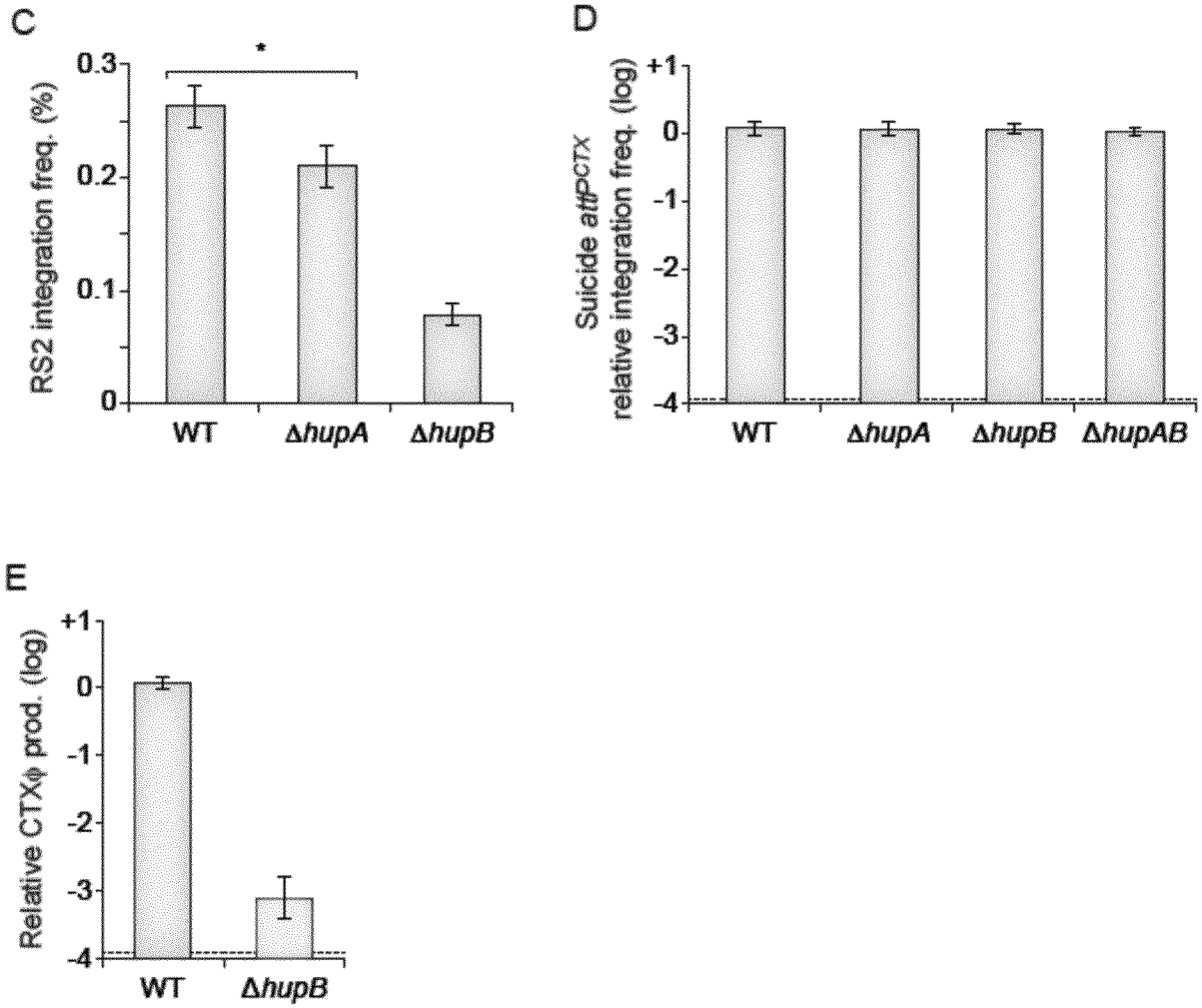


FIGURE 4 (Following)

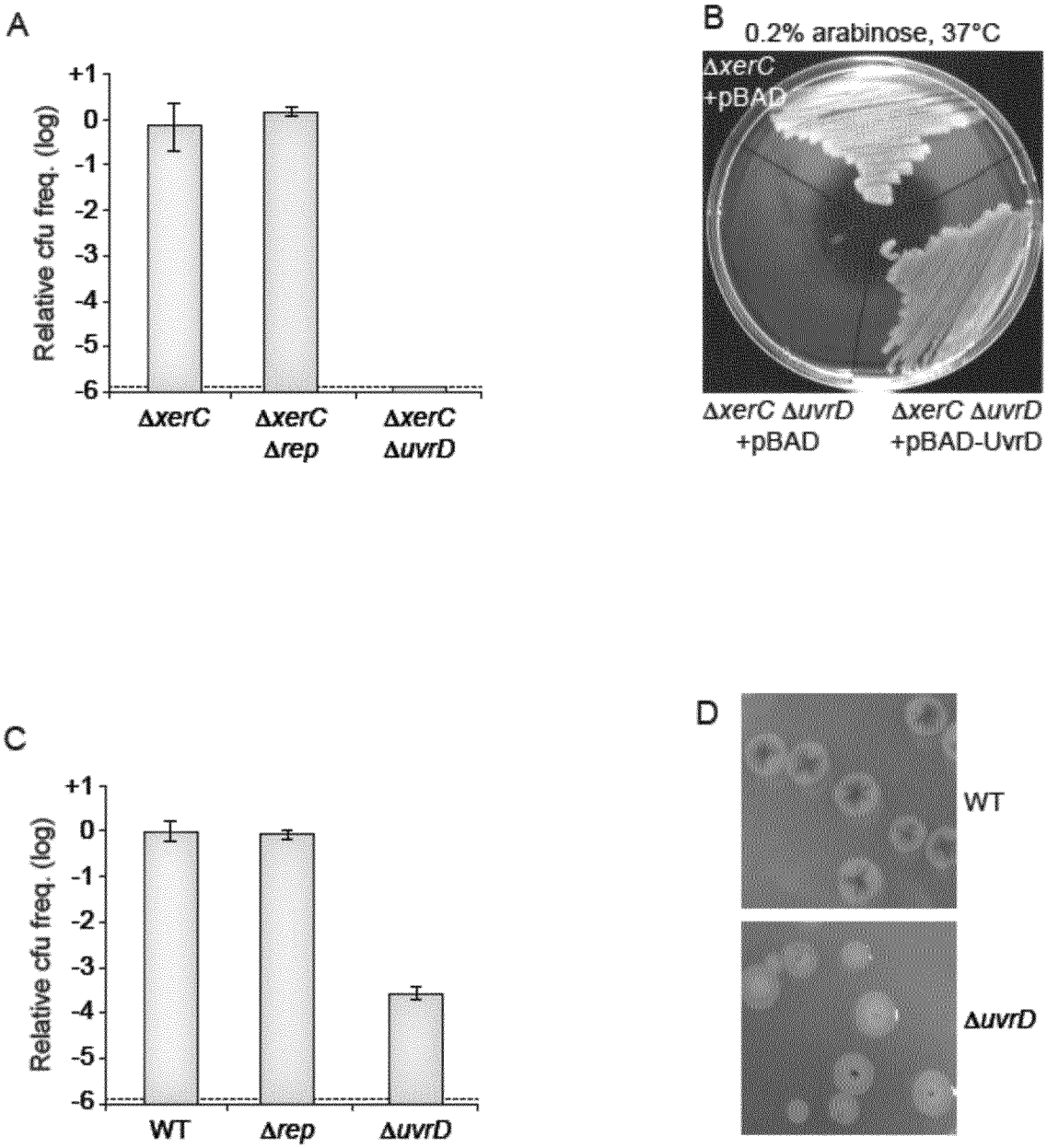

A
Relative cfu freq. (log)
+1
0
-1
-2
-3
-4
-5
-6
ΔxerC
ΔxerC Δrep
ΔxerC ΔuvrD
B
0.2% arabinose, 37°C
ΔxerC +pBAD
ΔxerC ΔuvrD +pBAD
ΔxerC ΔuvrD +pBAD-UvrD
C
Relative cfu freq. (log)
+1
0
-1
-2
-3
-4
-5
-6
WT
Δrep
ΔuvrD
D
WT
ΔuvrD

FIGURE 5

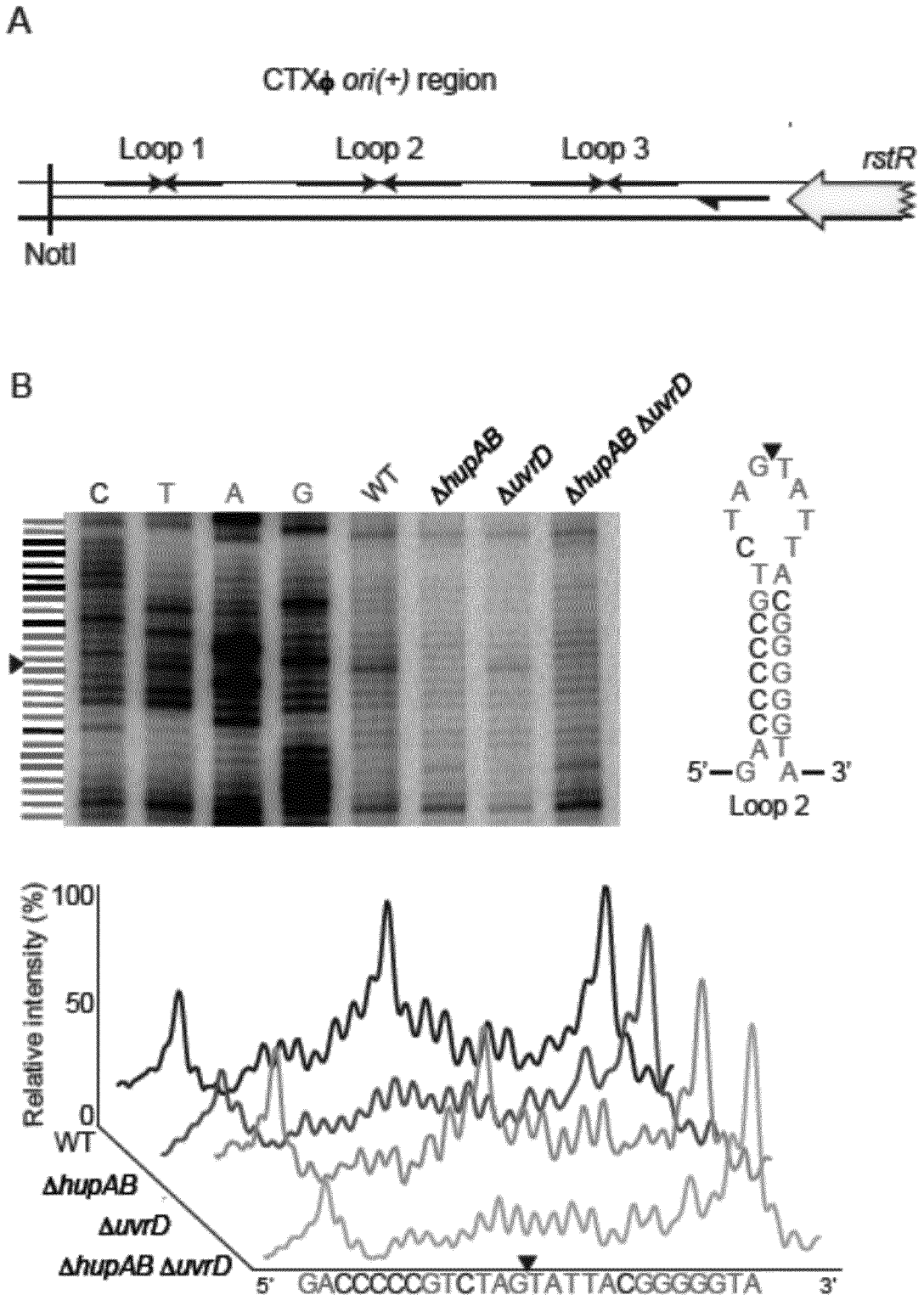
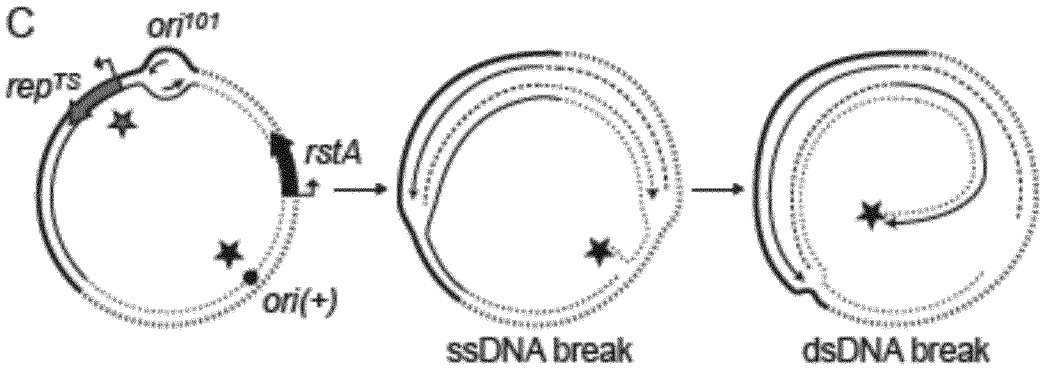
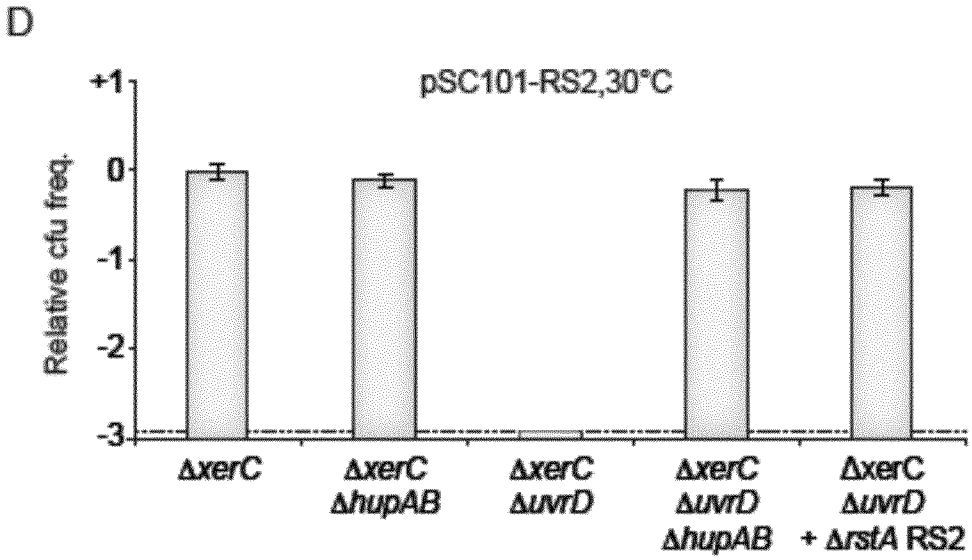

A
CTXϕ ori(+) region
Loop 1
Loop 2
Loop 3
rstR
NotI
B
C T A G WT ΔhupAB ΔuvrD ΔhupAB ΔuvrD
5'—G A—3'
Loop 2
Relative intensity (%)
100
50
0
WT
ΔhupAB
ΔuvrD
ΔhupAB ΔuvrD
5' GACCCCCGTCTAGTATTACGGGGGTA 3'

FIGURE 6





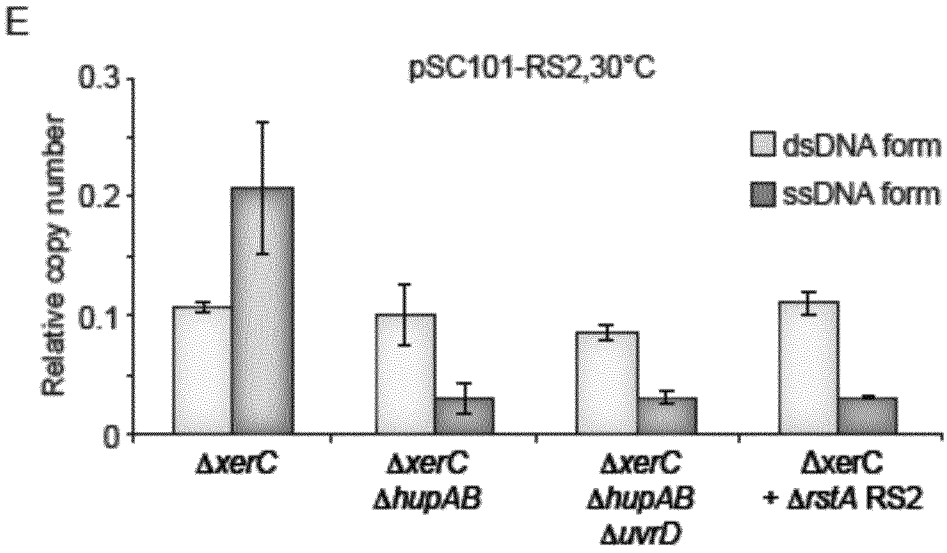


FIGURE 6 (Following)

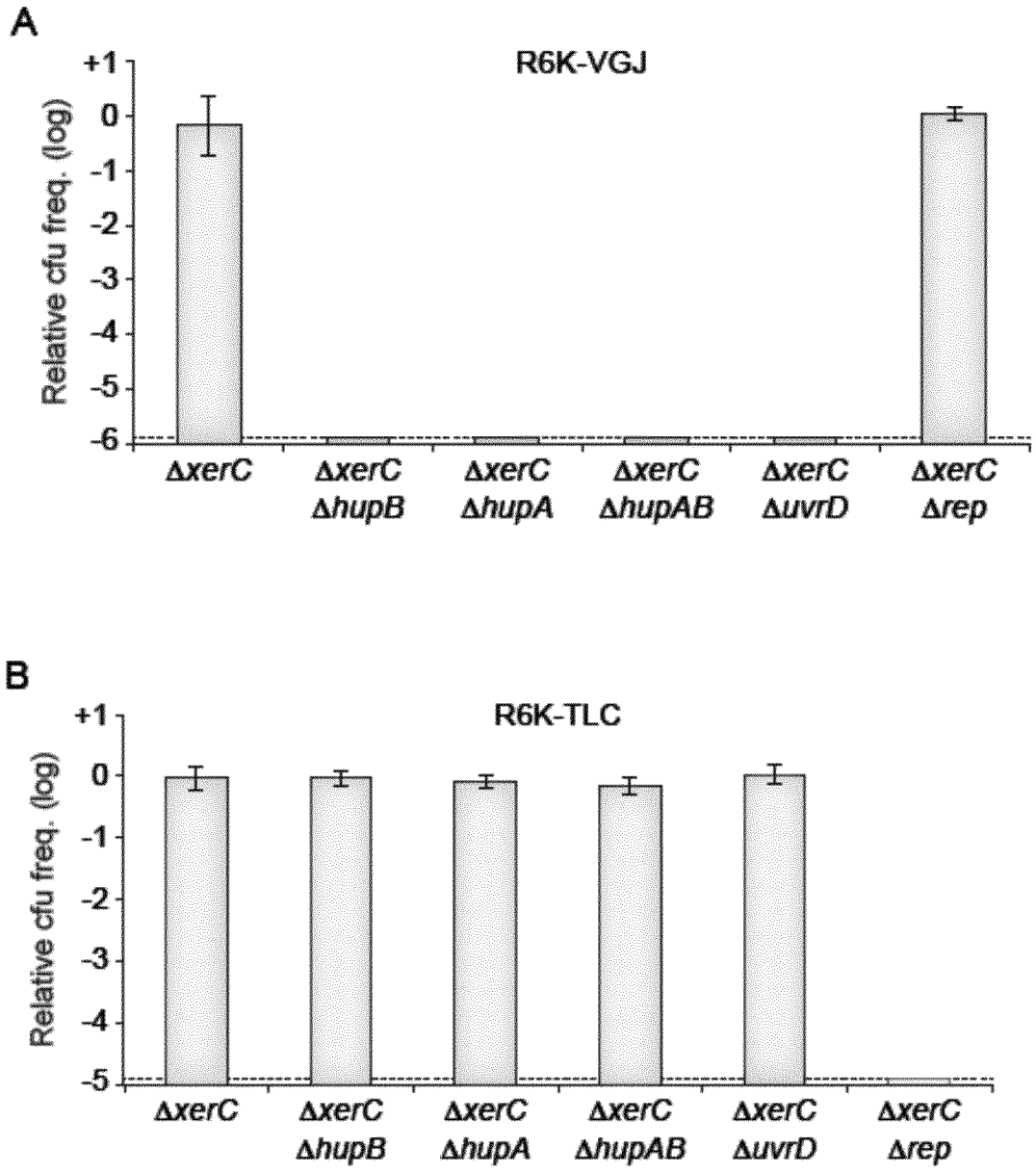

A
R6K-VGJ
Relative cfu freq. (log)
+1
0
-1
-2
-3
-4
-5
-6
ΔxerC
ΔxerC ΔhupB
ΔxerC ΔhupA
ΔxerC ΔhupAB
ΔxerC ΔuvrD
ΔxerC Δrep
B
R6K-TLC
Relative cfu freq. (log)
+1
0
-1
-2
-3
-4
-5
ΔxerC
ΔxerC ΔhupB
ΔxerC ΔhupA
ΔxerC ΔhupAB
ΔxerC ΔuvrD
ΔxerC Δrep

FIGURE 7

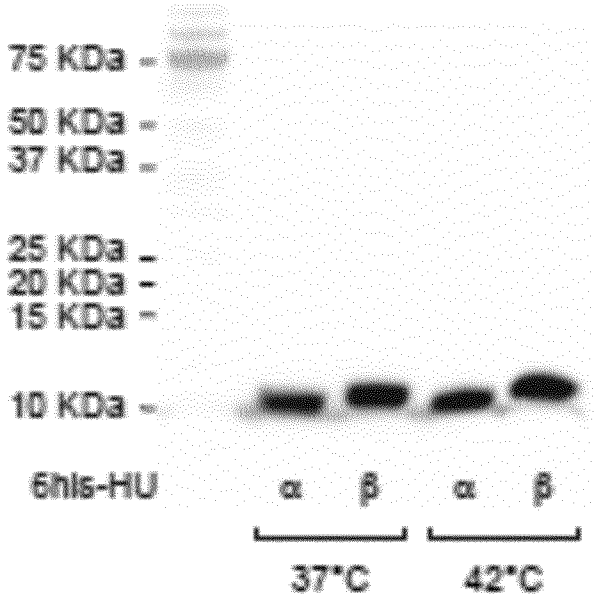

75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
6his-HU
α
β
α
β
37°C
42°C

FIGURE 8

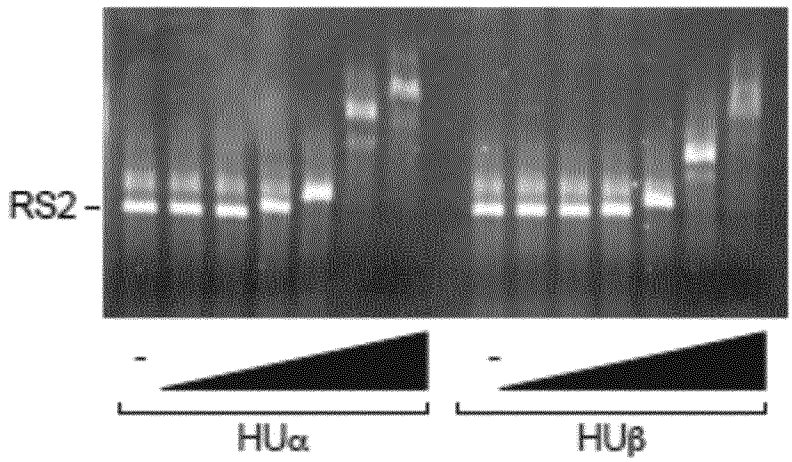


FIGURE 9

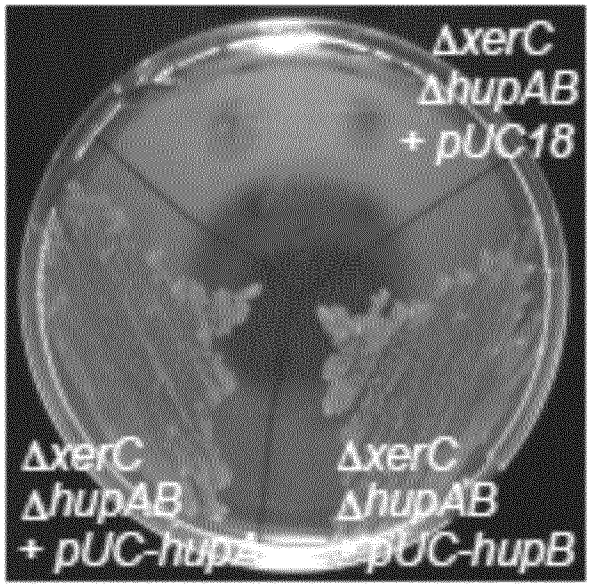


FIGURE 10

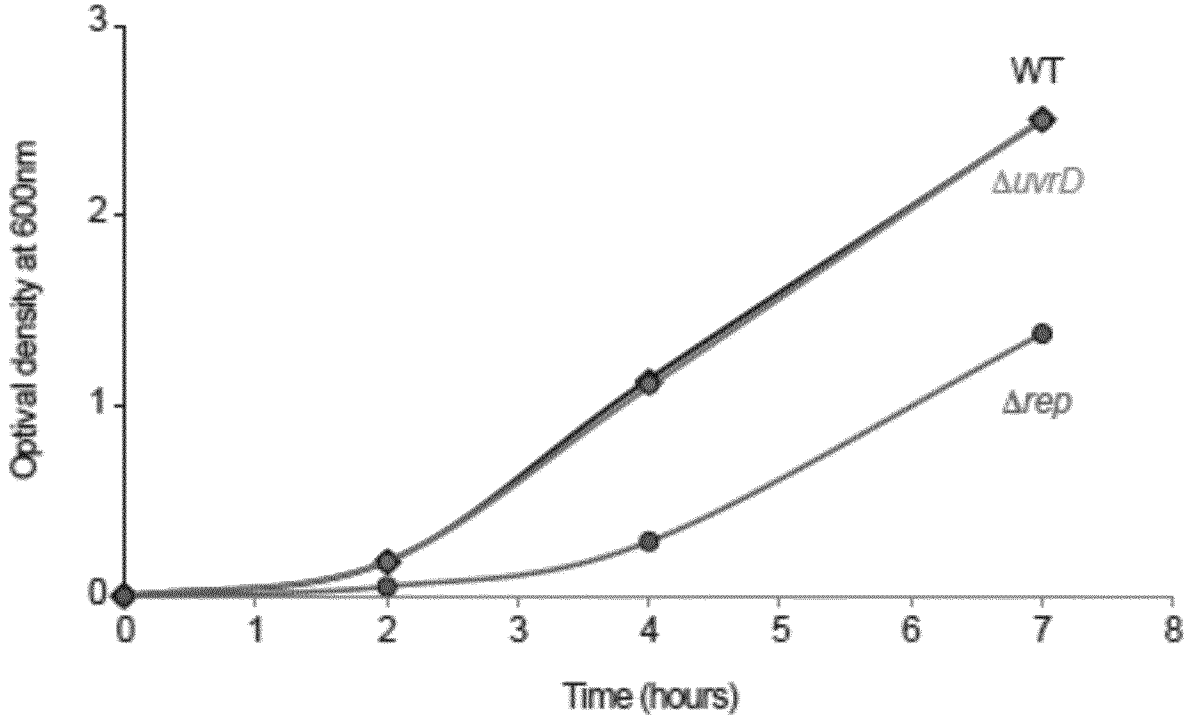

Optival density at 600nm
Time (hours)
WT
ΔuvrD
Δrep
0
1
2
3
4
5
6
7
8

FIGURE 11

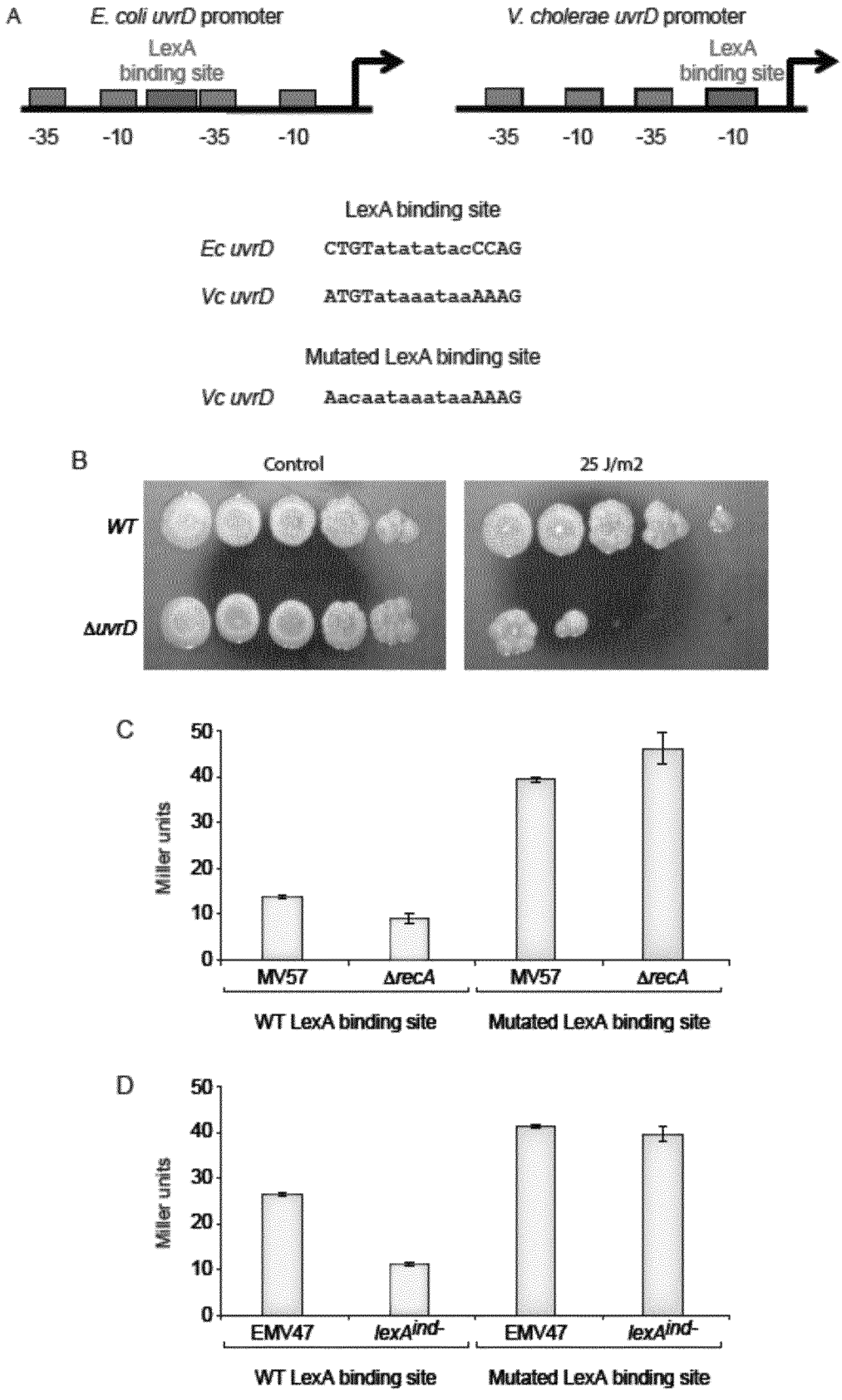

A
E. coli uvrD promoter
LexA binding site
-35
-10
-35
-10
V. cholerae uvrD promoter
LexA binding site
-35
-10
-35
-10
LexA binding site
Ec uvrD CTGTatatatacCCAG
Vc uvrD ATGTataaataaAAAG
Mutated LexA binding site
Vc uvrD AacaataaataaAAAG
B
Control
25 J/m2
WT
ΔuvrD
C
Miller units
50
40
30
20
10
0
MV57
ΔrecA
MV57
ΔrecA
WT LexA binding site
Mutated LexA binding site
D
Miller units
50
40
30
20
10
0
EMV47
lexA ind-
EMV47
lexA ind-
WT LexA binding site
Mutated LexA binding site

FIGURE 12

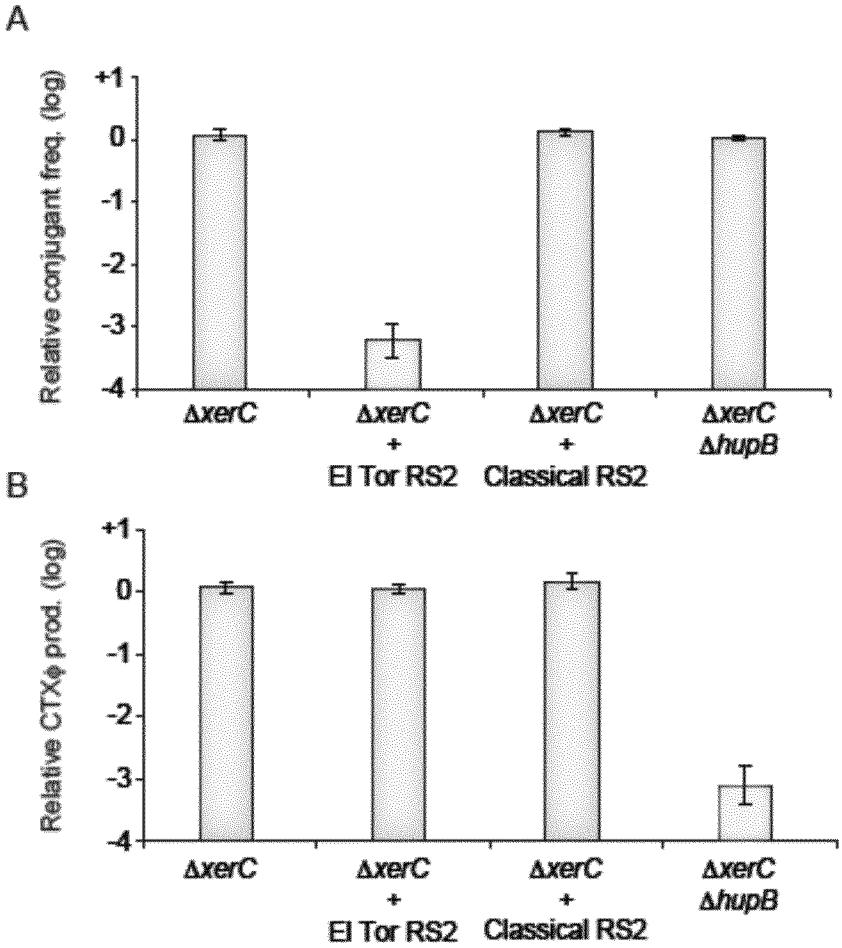

A
Relative conjugant freq. (log)
+1
0
-1
-2
-3
-4
ΔxerC
ΔxerC + El Tor RS2
ΔxerC + Classical RS2
ΔxerC ΔhupB
B
Relative CTXφ prod. (log)
+1
0
-1
-2
-3
-4
ΔxerC
ΔxerC + El Tor RS2
ΔxerC + Classical RS2
ΔxerC ΔhupB

FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/050595

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/050595

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/28 A61K39/02 C12N1/20 C12N9/14
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)
C07K A61K 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, FSTA, 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	WO 2012/142684 A1 (UNICAMP [BR]; BROCCHI MARCELO [BR]; PIVETTA LUCIANE BENEDITA DUARTE [B) 26 October 2012 (2012-10-26) the whole document claims 1-3 -----	1-3,5-10
X	JOHN F HEIDELBERG ET AL: "DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae.", NATURE, vol. 406, no. 6795, 3 August 2000 (2000-08-03), pages 477-483, XP055083642, ISSN: 0028-0836, DOI: 10.1038/35020000 the whole document ----- -/--	1-4

[X] Further documents are listed in the continuation of Box C.

[X] 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
8 April 2016	15/04/2016

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	van de Kamp, Mart

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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.
A	WO 99/28442 A1 (HARVARD COLLEGE [US]; NEW ENGLAND MEDICAL CENTER INC [US]; MEKALANOS J) 10 June 1999 (1999-06-10) cited in the application the whole document	1-10
A	KIMSEY H H ET AL: "CTX[phi] immunity: Application in the development of cholera vaccines", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 95, no. 12, 9 June 1998 (1998-06-09), pages 7035-7039, XP055198169, ISSN: 0027-8424, DOI: 10.1073/pnas.95.12.7035 cited in the application the whole document	1-10
A	LIANG W ET AL: "Construction and evaluation of a safe, live, oral Vibrio cholerae vaccine candidate, IEM108", INFECTION AND IMMUNITY, vol. 71, no. 10, 1 October 2003 (2003-10-01), pages 5498-5504, XP002343355, ISSN: 0019-9567, DOI: 10.1128/IAI.71.10.5498-5504.2003 cited in the application the whole document	1-10
A	YAN ET AL: "A Vibrio cholerae serogroup O1 vaccine candidate against CTX<ET>PHI infection", VACCINE, vol. 25, no. 20, 24 April 2007 (2007-04-24), pages 4046-4055, XP022046887, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2007.02.043 the whole document	1-10
A	EP 1 614 426 A1 (CNIC CT NAC INVESTIGACIONES [CU]) 11 January 2006 (2006-01-11) the whole document	1-10
A	BHABATOSH DAS: "Mechanistic insights into filamentous phage integration in Vibrio cholerae", FRONTIERS IN MICROBIOLOGY, vol. 5, 28 November 2014 (2014-11-28), XP055198332, DOI: 10.3389/fmicb.2014.00650 the whole document	1-10

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/050595

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	MARTÍNEZ E ET AL: "CTX[phi] Replication Depends on the Histone-Like HU Protein and the UvrD Helicase", PLOS GENETICS, vol. 11, no. 5, 20 May 2015 (2015-05-20), page e1005256, XP055198033, DOI: 10.1371/journal.pgen.1005256 the whole document -----	1-10

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/050595

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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