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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 September 2013 (06.09.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/128274 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/IB2013/000392

(22) International Filing Date:
1 March 2013 (01.03.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
622/DEL/2012 2 March 2012 (02.03.2012) IN

(72) Inventor; and
(71) Applicant : **DATTA, Asis** [IN/IN]; National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, JNU Campus, New Delhi 110 067 (IN).

(72) Inventors: **GHOSH, Sumit**; National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, JNU Campus, New Delhi 110 067 (IN). **GHOSH, Swagata**; National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, JNU Campus, New Delhi 110 067 (IN). **KONGARA, Hanumantha Rao**; National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, JNU Campus, New Delhi 110 067 (IN). **KAMTHAN, Mohan**; National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, JNU Campus, New Delhi 110 067 (IN).

(74) Agents: **RAE, Konpal** et al.; B-6/10, Safdarjung Enclave, New Delhi 110 029 (IN).

(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, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, 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, 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, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: RECOMBINANT MICROORGANISMS AND USES THEREOF

(57) **Abstract**: The present invention provides recombinant microorganism incapable of utilizing the aminosugar, N-acetylglucosamine (GlcNAc). The recombinant microorganism show impaired colonization in the host as compared to a wild type microorganism strain. The present invention also provides compositions comprising the recombinant microorganism.

WO 2013/128274 A2

RECOMBINANT MICROORGANISMS AND USES THEREOF

FIELD OF INVENTION

The present invention relates to recombinant strains of microorganisms unable to utilize the amino sugar N-acetylglucosamine (GlcNAc) as a sole carbon source. This inability to utilize GlcNAc severely impairs the colonization property of the recombinants. The present invention also provides compositions comprising these recombinants for use in various areas.

BACKGROUND OF THE INVENTION

Successful pathogenesis involves the ability of a pathogen to adapt to diverse and often stressful conditions within the host. Pathogens have developed myriad ways of parallel metabolic pathways, complex regulatory systems and stress adaptive mechanisms, which are best suited to the variety of environmental conditions they encounter within the human host. The amino sugar N-acetylglucosamine (GlcNAc) plays an important role in a wide range of organisms from prokaryotes to eukaryotes. GlcNAc affects host colonization and biofilm formation in bacteria. GlcNAc is involved in cell cycle control and insulin signalling in mammals and the modification of proteins through attachment of GlcNAc at serine or threonine residues mediates functions of transcription factors like, p53 and c-myc (Alvarez FJ and Konopka JB, 2007, Molecular Biology of Cell, 18: 965-975). The GlcNAc catabolic pathway converts GlcNAc to fructose-6-phosphate through phosphorylation (hexokinase), deacetylation (deacetylase) and deamination (deaminase) reactions. The GlcNAc catabolic pathway is present in a vast range of organisms from prokaryotes to eukaryotes, suggesting the universal role of this catabolic pathway. GlcNAc catabolic pathway is present in bacterial pathogens, yeast ascomycetes, filamentous ascomycetes, basiodiomycetes and zygomycetes fungi. The *in silico* analysis of GlcNAc catabolic pathway in human pathogenic bacterium and plant pathogenic fungi revealed the presence of the catabolic pathway genes together in a cluster. This unique nature of GlcNAc catabolic pathway suggests important role of gene cluster in pathogenic microorganisms.

The gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, an acute dehydrating diarrhoeal disease, still endemic in many developing countries of the world. Pathogenesis of cholera involves ingestion of *Vibrio cholerae* through contaminated food or water followed by its migration, after crossing the gastric acid barrier of the stomach, to the upper intestine where it has to penetrate the mucous layer for attachment to the intestinal epithelium. The bacterial growth within the host is largely dependent on the host derived macromolecules including mucin. The oligonucleotides side chains of these macromolecules are rich in amino sugars for nitrogen and carbon. Hence, it is not surprising that *Vibrio cholerae* has an efficient system for the release, uptake and catabolism of these amino sugars. Rice (*Oryza sativa*) is the world's most important crop, with nearly half of the world's population relying on successful harvests. The rice blast disease, caused by *Magnaporthe grisea* is one of the most serious and recurrent problems affecting rice production worldwide (Veneault-Fourrey et al., 2006, Science, 312: 580-583). The fungus is widespread in at least 85 countries and can also infect barley, wheat and domestic grasses. In many parts of the world, it is resistant to fungicides and to genetic modifications created by plant breeders. Therefore, it is obligatory to comprehend this fungus critically to formulate more effective fungicides and create genetically modified crops that can resist the pathogen. Tuberculosis, caused by *Mycobacterium tuberculosis* is an infectious airborne disease, infecting approximately one third of the world's population. India and South East Asia contribute to 50% of global tuberculosis burden. Genes required for GlcNAc utilization are also identified in the genome of *Mycobacterium tuberculosis*. It is therefore very significant to control the virulence of this tuberculosis causing microorganism and stop its pathogenesis.

Numerous enzymes are involved in the catabolization of the amino sugar, GlcNAc. In *E.coli* this amino sugar utilization and its regulation has been studied in detail where *nagE-nagBACD* are present as a divergent operon. NagC is a transcriptional regulator that represses this operon in the absence of environmental supply of amino sugars. GlcNAc catabolization converts glucosamine-6-phosphate to fructose-6-phosphate.

In *V. cholerae,* enzymes involved in GlcNAc catabolization include ß-N-acetylglucosaminidase, GlcNAc specific transporter, encoded by *nagE,* N-acetylglucosamine-6-phosphate deacetylase encoded by nagA1and glucosamine-6-phosphate deaminase encoded by *nagB.* In *V. cholera, nagA* and *nagC* are co-transcribed and *nagE* is upstream of *nagAC* which is expressed in the opposite direction. In *V. cholerae*, *nagE-nagAC* exists as an operon but unlike *E.coli*, *nagB* is not present in the same operon. The region between *nagE* and *nagB* contains the cyclic AMP catabolic gene activator protein (CAP) binding site as well as NagC binding site (Plumbridge, J. (2001) DNA binding sites for Mlc and NagC proteins: regulation of *nagE*, encoding the N-acetylglucosamine transporter in *Escherichia coli. Nucleic Acids Res* 29: 506–514. Yamano, N., Oura, N., Wang, J., and Fujishima, S. (1997) Cloning and sequencing of the genes for N-acetylglucosamine use that construct divergent operons (*nagE-nagAC*) from *Vibrio cholerae* non-O1. *Biosci Biotechnol Biochem* 61: 1349–1353).

Presently, two variants of the oral vaccine for cholerae are in use, the WC-rBS and BivWC. WC-rBS, marketed as 'Dukoral', is a monovalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 plus additional recombinant cholera toxin B subunit. BivWC, marketed as 'Shanchol' and 'mORCVAX', is a bivalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 and *V. cholerae* O139. mORCVAX is available only in Vietnam. These oral vaccines provide protection in 52% of cases in the first year following vaccination and in 62% of cases in the second year.

There is a long felt need in the art for the inhibition and effective control of diseases caused by *Vibrio spp.*, especially *V. cholerae*, *Magnaporthe grisea* and *Mycobacterium tuberculosis*. Manipulation of pathogenic catabolic pathways vital for the sustenance of the pathogens in the host may prove to be an important method for the control and prevention of the pathogens.

US 8,039,008 describes *Vibrio cholerae* comprising a mutated transcriptional regulatory protein (ToxT) amino acid sequence, wherein the mutation results in a reduction in the expression of cholera toxin by the *Vibrio cholerae*.

US 6,203,799 describes *V. cholerae* vaccine strains which have a soft agar penetration-defective phenotype and lack a functional CtxA subunit. Further, methods for identifying new genes involved in *V. cholerae* motility and the cloning, identification, and sequencing of *V. cholerae* motB and fliC genes are disclosed.

US patent application 20120045475 describes a method for inhibiting or reducing colonization by a microbial pathogen in a subject or on a surface by administering to the subject or surface an effective amount of an agent that alters the expression of a polynucleotide selected from the group consisting of *rbmA*, *rbmB*, *rbmC*, *rbmD*, *rbmE*, *rbmF* and *bapl* or analogues or variants thereof.

Despite the availability of vaccines against cholera and tuberculosis, there is a dire requirement for effective prevention and control of diseases caused by *V. cholerae* and *M. tuberculosis*. In the present state of art, there is a lacuna in compositions that provide effective immunity against diseases mediated by these microorganisms. Further, there persists a need for the formulation of more effective fungicides and genetically modified crops that can resist the disease caused by *Magnaporthe grisea* pathogen.

SUMMARY OF THE INVENTION

An aspect of the present invention relates to a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWINGS

The following drawings form part of the present specification and are included to further illustrate aspects of the present invention. The invention may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

Figure 1A shows a hierarchical clustering of genes using an average linkage algorithm in *V. cholerae* El Tor strain CO-366 induced by Glucose or GlcNAc sugar. Vertical stripes represent genes, and columns show experimental samples at 60 minutes after induction. Log_2-based color scale is presented at the bottom of the panels (red, induced; green, repressed). Black asterisks represent genes of the classical catabolic cascade while the grey ones show the genes acquired by certain members of *Vibrionaceae* family. The occurrence of a second cluster of genes (VC1781- N-acetylmannosamine-6-phosphate 2-epimerase; VC1782- N-acetyl-mannosamine kinase/ ROK kinase and VC1783-*nagA2*) under the regulation of NagC is shown.

Figure 1B shows the GlcNAc catabolic gene transcripts in *Vibrio cholerae* El Tor strain CO366 (wild type) in response to GlcNAc by quantitative RT-PCR assay. Error bars represent the coefficient of variation (n=3). *RecA* is the endogenous control.

Figure 2A shows the organization of the genes involved in GlcNAc catabolism; hatched arrows represent the classical genes while open arrows represent the second cluster of the genes involved in GlcNAc catabolism.

Figure 2B shows the growth pattern of *V. cholerae* El Tor strain CO366 (wild type) and GlcNAc-defective mutants on M9-Glucose (0.5%) and M9-GlcNAc (0.5%) plates. In the GlcNAc media, Sector 2 shows spotty growth of the mutant SHNE while sectors 3 and 4 show no growth of mutants SHNB and SHNA1-A2, respectively.

Figure 3A shows the growth of *V. cholerae* El Tor strain CO366 (wild-type) and mutant strains in liquid M9-glucose. Error bars indicate co-efficient of variation.

Figure 3B show the growth of *V. cholerae* El Tor strain CO366 (wild-type) and mutant strains in M9-GlcNAc media. SHNB and SHNA1-A2 mutants showed completely abolished growth and SHNE mutant showed reduced growth in GlcNAc media. Error bars indicate co-efficient of variation.

Figure 3C shows the gene transcripts in *V. cholerae* El Tor strain CO366 (wild-type) and SHNE mutant strains in response to glucose or GlcNAc sugars. The names of the transcripts quantified by real-time-RT-PCR are indicated immediately below the bars.

Figure 4A shows gene transcripts in *V. cholerae* El Tor strain CO366 (wild type), SHNA1, SHNA2 and SHNA1-A2 mutant strains in response to GlcNAc sugar. Error bars indicate co-efficient of variation (n=3). A coordinated expression of GlcNAc catabolic genes, *nagA1* and *nagA2* is observed.

Figure 4B shows growth of SHNC mutant in liquid M9 media supplemented with non-fermentable carbon sources like glycerol and lactate. SHNC showed reduced growth on non-fermentable carbon sources like glycerol and lactate when compared with wild type.

Figure 5A shows competition index (CI) of *V. cholerae* El Tor strain CO366 (~1), SHNA1-A2 (~0.0001), SHNE (~0.1) and SHNB (0.001) mutants strains. Six mice were taken per group. Each point is the CI data obtained from an individual mouse. The SHNA1-A2 and SHNB are significantly attenuated compared with the *V. cholerae* El Tor strain CO366 (wild-type) strain ($P \leq 0.01$ by Student's two-tailed t-test).

Figure 5B shows the hierarchical clustering analysis of microarray expression data for genes found to be significantly regulated during growth of SHNC mutant in presence of glucose at 30 minutes time point. Each of the genes is shown as vertical colored stripe. The most intense red and green colors correspond to increased or decreased expression values of 5 fold or more, respectively. Genes of the classical GlcNAc catabolic cluster (VC0994, VC0995) along with GlcNAc binding protein (VCA0811) and chemotactic protein (VC0449) are shown with black asterisks.

Figure 6A shows virulence gene transcripts in *V. cholerae* El Tor strain CO366 (wild type), SHNA1-A2 and SHNE mutants in AKI medium, quantified by real-time-RT-PCR assay.

Figure 6B shows *hapR* gene transcripts in *V. cholerae* El Tor strain CO366 (wild type), SHNA1, SHNA2, SHNA1-A2, SHNB and SHNE mutants in AKI medium, quantified by RT-PCR assay.

Figure 7 shows growth assay for cells incubated at 37°C in 7H9 medium containing 2% glucose (ADC) or GlcNAc (ANC) in 7A.

Figure 7B shows the schematic pattern of hybridization for allelic exchange mutants.

Figure 8 shows disruption of GlcNAc catabolic pathway in *Magnaporthe grisea.*

Figure 8A shows map of *MgDAC* locus and gene replacement constructs.

Figure 8B and Figure 8C show the Southern blots confirming the disruption of *MgDAC* gene. Genomic DNA was digested with EcoRV and the blot was hybridized with gene replacement construct (B) and Hph gene probe (C). Lane 1, 2, 3, 5-mutants, lane 6- wt, lane 7-wt undigested, lane 4-shows incomplete digestion of DNA from one mutant.

Figure 8E and Figure 8D show mutants unable to utilize GlcNAc as sole carbon source (E) but grew normally in glucose (D).

Figure 9 shows functional GlcNAc catabolic pathway for rice blast fungus pathogenesis.

Figure 9A: Rice leaves were inoculated with 20 µl of wild type and mutant spore (104spore/ml). After inoculation leaves were kept under humid condition and photographed after 15 days of inoculation.

Figure 9B: Spray inoculation was done in 15 days old seedlings of two different rice varieties and photographed 12 days after inoculation.

Figure 9C: Disease lesions number in 5 cm length leaf were counted and represented as mean disease lesion density.

Figure 10 shows the map of gene replacement vector used for the generation of *M. grisea* mutants. The vector was digested with NotI and 3.4 kb fragment was selected for *M. grisea* transformation.

DETAILED DESCRIPTION OF THE INVENTION

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are fully explained in the literature and a person having ordinary skill in the art would be familiar with the techniques and would be able to perform them.

Definitions

For convenience, before further description of the present invention, certain terms employed in the specification, examples and appended claims are provided here. These definitions should be read in light of the remainder of the disclosure and understood as by a person of skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art.

The articles "a," "an" and "the" are used to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated element or step or group of element or steps but not the exclusion of any other element or step or group of element or steps. It is not intended to be construed as "consists of only."

The term "including" is used to mean "including but not limited to".

The term "nucleic acid" or "recombinant nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA).

The polynucleotides described in the present description include "genes" and nucleic acid molecules described include "vectors" or "plasmids." Accordingly, the term "gene", also called a "structural gene" refers to a polynucleotide that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed.

The term "primer" refers to a single-stranded oligonucleotide, the 3' end of which can be used as the initiation site for the DNA synthesis with a DNA polymerase. As used herein, the term "primer sequence" refers to the sequence of the primer or the complementary sequence.

The term "recombinant" means a cell or organism in which genetic recombination has occurred. It also includes a molecule (e.g., a nucleic acid or a polypeptide) that has been artificially or synthetically (i.e., non-naturally) altered by human intervention. The alteration can be performed on the molecule within, or removed from, its natural environment or state.

The term "mutant" and "mutation" may mean any detectable change in genetic material, e.g., DNA or any process, mechanism or result of such a change. This includes gene mutations, in which the structure (e.g., DNA sequence) of a gene is altered, any gene or

DNA arising from any mutation process and any expression product (e.g., RNA, protein or enzyme) expressed by a modified gene or DNA sequence.

The term "variant" may be use to indicate a modified or altered gene, DNA sequence, RNA, enzyme, cell etc, *i.e.*, any kind of mutant.

The terms "recombinant" and "mutant" are herein used interchangeably.The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence.

A "vector", "cloning vector" and "expression vector" is any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, that are "episomes", that is, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

"Transformation" refers to the process by which a recombinant DNA molecule is introduced into a host cell. Transformation (or transduction, or transfection), can be achieved by any one of a number of means including electroporation, microinjection, biolistics (or particle bombardment-mediated delivery), or *Agrobacterium*-mediated transformation.

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used

to encode a given amino acid sequence of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The disclosure includes such polypeptides with alternate amino acid sequences, and the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

The disclosure provides nucleic acid molecules in the form of recombinant DNA expression vectors or plasmids. Generally, such vectors can either replicate in the cytoplasm of the host microorganism or integrate into the chromosomal DNA of the host microorganism. In either case, the vector can be a stable vector (i.e., the vector remains present over many cell divisions, even if only with selective pressure) or a transient vector (i.e., the vector is gradually lost by host microorganisms with increasing numbers of cell divisions).

The disclosure provides DNA molecules in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) forms.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions, and methods are clearly within the scope of the invention, as described herein.

The inventors of the present invention hypothesized that the capacity of an organism, specifically a pathogen, to survive and multiply in the host cell is facilitated by its capability to utilize the host derived macromolecules like N-acetylglucosamine

(GlcNAc) sugars thereby leading to the establishment and persistence of infection by these microorganisms. The ability to utilize this aminosugar as carbon source is well conserved in several human pathogens.

The present invention relates to recombinant strains of microorganisms unable to utilize N-acetylglucosamine (GlcNAc). The GlcNAc catabolic pathway is present in a broad range of pathogens. The instant invention provides recombinants strains of microorganisms with impaired GlcNAc utilization and host colonization. The recombinant strains of the present invention show drastic reduction in virulence in the case of pathogens. The recombinant strains also show reduced or impaired property of colonization and additionally there is reduced colonization of the pathogenic recombinants of the present invention in host cells.

Specifically, the invention relates to recombinant microorganisms, specifically to *Vibrio cholerae, Mycobacterium tuberculosis* and *Magnaporthe grisea.* The present invention relates to recombinant strain of *Vibrio cholerae* which are unable to utilize the amino sugar, N-acetylglucosamine (GlcNAc) and possess an impaired ability to colonize. Recombinant strains of tuberculosis causing bacterium *Mycobacterium tuberculosis* with defect in N-acetylglucosamine utilization are also provided in the present invention. The present invention further relates to rice blast pathogen *Magnaporthe griseae* where deletion of GlcNAc utilization pathway in the pathogen leads to severely reduced virulence.

The present invention provides compositions comprising recombinant strains of microorganisms with compromised virulence. The present invention provides a composition for producing recombinant strains of a human pathogen compromised in virulence wherein the genes coding for the polypeptides having deacetylase and deaminase activities are mutated. Compositions comprising the recombinant microorganisms of the present invention can be used in developing fungicides or bactericides, as inhibitors of the GlcNAc catabolic enzymes and in inducing immunity in hosts as vaccine.

The present invention provides recombinant strains of *Vibrio cholerae* produced by recombinant DNA technology. The present invention further provides recombinant strains or mutants of *Vibrio cholerae* with atleast one mutated gene involved in the GlcNAc catabolic pathway. The present invention also provides a composition comprising recombinant strains of *Vibrio cholerae* with impaired GlcNAc utilization and reduced colonization in the host. Compositions comprising the mutant strains of *Vibrio cholerae* of the present invention can be used in myriad ways including vaccines for inducing immunity in hosts against the *Vibrio cholerae* pathogens and for effective prevention against *Vibrio cholerae* mediated diseases.

The recombinant strains of *Vibrio cholerae* of the present invention are created by disruptions in one or more of following genes: *nagA1* (N-acetylglucosamine-6-phosphate deacetylase/deacetylase I; mutant SHNA1), *nagA2* (N-acetylglucosamine-6-phosphate deacetylase II/*vc1783*; mutant SHNA2), *nagB* (glucosamine-6-phosphate deaminase/isomerase; mutant SHNB), *nagC* (N-acetylglucosamine specific repressor; mutant SHNC), *nagE* (GlcNAc transporter/PTS-transporter; mutant SHNE), *vc1781* (N-acetylmannosamine-6-phosphate 2-epimerase; mutant SHVC1781) and *vc1782* (N-acetylmannosamine kinase/ROK kinase; mutant SHVC1782).

The inventors of the present invention found unexpected and surprising results when microarray analysis of wild type *Vibrio cholerae* El Tor strain CO366 grown either in the presence of glucose or GlcNAc sugars revealed an up-regulation of GlcNAc catabolic genes (Figures 1A and 1B). Further analysis revealed that the GlcNAc catabolic genes are present in two distinct clusters (Figure 2A), where the second cluster of genes encompassing N-acetylmannosamine-6-phosphate 2-epimerase (cmr.jcvi.orgVC1781), N-acetylmannosamine kinase/ROK kinase (cmr.jcvi.org: VC1782) and N-acetylglucosamine 6-phosphate deacetylase 2 (*nagA2;* cmr.jcvi.org: VC1783) fall within the VPI-2 cluster known to be involved in sialic acid metabolism. The *nagA2* appears to be a homolog of the classical GlcNAc catabolic gene, *nagA1*.

In *Vibrio cholerae*, the GlcNAc catabolic pathway is highly specialized for the successful establishment of the pathogen in its preferred colonization site, during the critical early phase of infection. The bacterium uses the GlcNAc monosaccharide as a nutrient source to reach sufficient titers in the gut. A disruption in the GlcNAc catabolic cascade affects the capacity of *V. cholerae* to utilize the amino-sugar in the intestinal environment, as a result of which the organism loses its overall fitness to establish itself in a nutrient limited condition. The occurrence of more than one cluster of GlcNAc catabolic genes with similar functions within the genome of *Vibrio cholerae*, suggest an efficient catabolism of GlcNAc saccharide. *V. cholerae*, by acquiring two copies of deacetylase (*nagA1* and *nagA2*), GlcNAc kinase (PTS transporter/ VC0995) and ROK kinase), and simultaneously achieving a co-ordinated expression of the two copies of deacetylase genes *nagA1* and *nagA2*, is highly adapted for colonization in the host as a pathogen. Thus, producing mutants of *V. cholerae* which show impaired colonization in the host and impaired GlcNAc utilization thereby leading to reduced or compromised virulence is advantageous for the prevention and control of diseases caused by *V. cholerae*.

Without wishing to bind to a specific theory, the inventors believe that the N-acetylglucosamine specific repressor, NagC, performs a dual role. The classical GlcNAc catabolic genes are under its negative control while the genes belonging to the second cluster are positively regulated by it. In *V. cholerae,* NagC exerts a global regulation that allows cells to selectively assimilate a preferred compound among a mixture of several potential carbon sources (Figure 2A).

The recombinant strains of *Vibrio cholerae* as disclosed in the present invention, unable to utilize GlcNAc were created by at least one mutation in at least one of the genes, both classical and the second cluster, involved in the GlcNAc catabolic pathway, wherein the recombinant *V. cholerae* strain having mutation in the *nagA1* is designated as SHNA1, the recombinant *V. cholerae* strain having mutation in the *nagA2* is designated as SHNA2, the recombinant *V. cholerae* strain having mutation in the *nagB* is designated

as SHNB, the recombinant *V. cholerae* strain having mutation in the *nagC* is designated as SHNC, the recombinant *V. cholerae* strain having mutation in the *nagE* is designated as SHNE, the recombinant *V. cholerae* strain having mutation in the *vc1781* is designated as SHVC1781 and the recombinant *V. cholerae* strain having mutation in the *vc1782* gene is designated as SHVC1782.

The recombinant strains may be produced by site-directed mutagenesis in the desired genes. The mutations may be addition, substitution or deletion in the region of translational site of the desired genes *of Vibrio* species.

The recombinant strains were created by non-polar deletions in the gene of interest in the wild type strain *V. cholerae* El Tor strain CO366 (Example 2). In-frame deletions were carried out by the use of cross-over polymerase chain reaction (PCR) assays. The recombinant *V. cholerae* strain comprising mutations in more than one gene was also created. A recombinant *V. cholerae* strain comprising double mutation, *i.e.,* mutation in the *nagA1*and *nagA2* genes was created and designated as SHNA1-A2 (Example 3).

Differential growth response was observed for the recombinant *V. cholerae* strains in glucose and GlcNAc supplemented media. All the recombinant *V. cholerae* strains were able to grow on M9-glucose supplemented media but, on M9-GlcNAc supplemented media, the strains showed retarded growth or failed to grow at all (Figure 2B). Amongst the recombinant strains, SHNE showed reduced growth, SHNA1-A2 and SHNB showed complete arrest of growth in M9-GlcNAc supplemented media (Figures 3A, 3B and 3C).

Microarray analysis of the wild type *Vibrio cholerae* and SHNC mutant strains grown in the presence of glucose showed that *nagA1*, *nagE*, and *nagB* genes were upregulated in the SHNC recombinant. SHNC recombinant strains grown in the presence of GlcNAc showed a down regulation of *nagA2* and *ROK* kinase genes (VC1776-VC1784; Table 4).

The recombinant strains of *V. cholerae,* SHNA1-A2, comprising mutations in the *nagA1* and *nagA2* genes shows complete inhibition of growth on GlcNAc media

whereas a single mutant, SHNA1 or SHNA2 is able to grow on GlcNAc media. However, there is a difference in the growth rate of the mutants when compared to wild type, *V. cholerae* El Tor strain CO366 (Figure 3A and 3B). The transcript levels of *nagA2* is up-regulated by almost 13-fold in SHNA1 mutant in the presence of GlcNAc sugar compared to ~6-fold up-regulation in the wild type strain suggesting a coordinated regulation of both the copies of deacetylase genes in *V. cholerae* (Figure 4A). *NagC* mutants also showed decreased growth in liquid M9 media supplemented with non-fermentable carbon sources like glycerol and lactate (Figure 4B). Similar compromised growth was also seen on M9–glycerol agar plate study.

The recombinant strains of *V. cholerae* as disclosed in the present invention were unable to survive and multiply in the host cell because of the lost capacity to utilize the host derived macromolecules like GlcNAc sugars, lead to the impaired colonization of the strains in the host. The property of impaired colonization thereby leads to a decrease in the persistence of infection, making them ideal candidates for vaccine strains. The recombinant strains of *V. cholerae*, SHNE, SHNB and SHNA1-SHNA2, showed reduced intestinal colonization in *in-vivo* studies. The analysis with respect to the wild type *V. cholerae* revealed that the colonization efficiency of the recombinant SHNA1-A2 was nil; that of the recombinant SHNE strains was reduced by more than 10 folds and that of the SHNB recombinant strains was attenuated by more than 1000 folds (Figure 5A).

The recombinant *V. cholerae* strains with reduced colonization abilities and an inability to utilize GlcNAc have surprisingly no significant changes in their virulence or toxin gene transcript levels indicating them as ideal candidates for vaccines (Figures 6A and 6B). Nonetheless, these strains can be highly antigenic and have strong immunogenicity. When combined with mutations in the GlcNAc catabolic genes *nagE*, *nagB* and two copies of *nagA* genes, the GlcNAc-defective mutations result in strains which are excellent candidates for vaccines for the prevention of cholerae in humans.

However, these recombinant strains display increased constitutive expression of toxin, *Tcp* pili and *hapR* genes (Figures 6A and 6B). This increased expression may account for the enhanced immunogenicity of these strains. The present invention also provides recombinant strains of human pathogen *Mycobacterium tuberculosis* incapable of utilizing GlcNAc amino sugar. *Mycobacterium tuberculosis* H37Rv strain can utilize GlcNAc as a sole carbon source (Figure 7A). GlcNAc metabolic pathway recombinants of *Mycobacterium tuberculosis* H37Rv were created by disrupting *NagA* and *glmS* genes. The mutants failed to grow on a medium containing GlcNAc as sole carbon source but there was no change in the growth rate on medium containing glucose.

Deacetylase gene of *Magnaporthe grisea* was mutated through a one step gene replacement method. The mutant was checked for growth in media with glucose or GlcNAc as sole carbon source. Although the growth was unaffected in glucose media, all the *MgDAC* mutants were unable to grow in GlcNAc media. This confirmed the disruption of GlcNAc catabolic pathway (Figure 8A-E). The role of GlcNAc pathway during the host associated growth and development of *Magnaporthe grisea* was determined by performing an infection assay on detached leaves of 15 days old rice cultivar Pusa basmati-1. The mutant and wild type conidial suspension (104 spore/ml) was applied onto rice leaves and leaves were kept under humid condition (80-85% relative humidity and 27-28 °C temperature). Rice blast symptom was assayed 15 days after inoculation which revealed greatly loss of virulence in *MgDAC* mutants. Moreover, spray inoculation of 15 days old seedlings of rice cultivar pusa basmati-1 (PB-1) and pusa sugandh (PS) with equivalent mutant and wild type conidia revealed drastically reduced virulence in *MgDAC* mutant (Figure 9A-C).

An embodiment of the present invention provides a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-

phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain.

In an embodiment of the present invention, there is provided a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and the mutation is selected from the group consisting of deletion, addition, and substitution.

In another embodiment of the present invention, there is provided a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and the mutation is selected from the group consisting of deletion, addition, and substitution, wherein the mutation is a non-polar deletion.

Another embodiment of the present invention provides a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase

(*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain and wherein, the host is selected from the group consisting of human, animal, and plant.

Another embodiment of the present invention provides a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and wherein the microorganism strain is selected from the group consisting of *Vibrio* species, *Mycobacterium* species, and *Magnaporthe* species.

Another embodiment of the present invention provides a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and wherein the microorganism strain is selected from the group consisting of *Vibrio* species, *Mycobacterium* species, and *Magnaporthe* species, wherein the *Vibrio* species is *Vibrio cholerae*.

In another embodiment of the present invention, there is provided a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and wherein the microorganism strain is selected from the group consisting of *Vibrio* species, *Mycobacterium* species, and *Magnaporthe* species wherein the *Vibrio* species is *Vibrio cholerae* E1 Tor strain CO366.

In still another embodiment of the present invention, there is provided a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and wherein the microorganism strain is selected from the group consisting of *Vibrio* species, *Mycobacterium* species, and *Magnaporthe* species, wherein the *Mycobacterium* species is *Mycobacterium tuberculosis*.

In yet another embodiment of the present invention, there is provided a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor

(*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and wherein the microorganism strain is selected from the group consisting of *Vibrio* species, *Mycobacterium* species, and *Magnaporthe* species, wherein the *Magnaporthe* species is *Magnaporthe grisea*.

In an embodiment of the present invention, there is provided a composition comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain.

In an embodiment of the present invention, there is provided a composition comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, and wherein the composition additionally comprises atleast one of a pharmaceutically acceptable carrier, a diluent, an adjuvant, and an additive.

In another embodiment of the present invention, there is provided a composition comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain

comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, wherein the composition additionally comprises atleast one of a pharmaceutically acceptable carrier, a diluent, an adjuvant, an additive and additionally comprises an agriculturally acceptable carrier.

In still another embodiment of the present invention, there is provided a composition comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, a pharmaceutically acceptable carrier, a diluent, an adjuvant, an additive, and an agriculturally acceptable carrier.

Another embodiment of the present invention provides a vaccine comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the

recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain.

Another embodiment of the present invention provides a vaccine comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, wherein the vaccines additionally comprises atleast one of a pharmaceutically acceptable carrier, a diluent, an adjuvant, and an additive.

In another embodiment of the present invention, there is provided a vaccine comprising a recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), and wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain, a pharmaceutically acceptable carrier, a diluent, an adjuvant, and an additive.

Although the subject matter has been described in considerable detail with reference to certain preferred embodiments thereof, other embodiments are possible. As such, the spirit and scope of the appended claims should not be limited to the description of the preferred embodiment contained therein.

EXAMPLES

The disclosure will now be illustrated with working examples, which is intended to illustrate the working of disclosure and not intended to take restrictively to imply any limitations on the scope of the present disclosure. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein.

Example 1

Generation of *Vibrio cholera* mutant

Bacterial strains, plasmids and culture conditions

V. cholerae El Tor strain CO366 was used as the wild type strain.

E. coli (DH5α) and *V. cholerae* strains were propagated at 37 °C in Luria Broth (LB) medium, containing ampicillin 75 µg/ml, polymixin B 20 Units/ml and streptomycin 30 µg/ml at 37°C. The suicide vector, pCVD442 was used for the molecular biology studies. pCVD442 is a suicide plasmid used for gene allele exchange in bacteria and is composed of the *mob*, *ori*, and *bla* regions in addition to the *sacB* gene and *ampicillin* resistant marker gene. *sacB* gene provides a conditionally lethal phenotype. The *sacB* locus encodes the enzyme levan sucrase, which is toxic for gram-negative organisms only in the presence of sucrose.

Luria Broth (LB) medium

The LB medium was prepared using 1% tryptone, 1% NaCl and 0-5% yeast extract (pH 8.0).

M9 medium

Na_2HPO_4 6g, KH_2PO_4 3g, NaCl 0.5g, NH_4Cl 1g and Casamino acid 2g were added per litre of distilled water. The pH of the media was adjusted to 7.4, autoclaved and cooled. 1M $MgCl_2$ 2 ml, 0.1ml of 1M $CaCl_2$ and 10ml of filter-sterilized 20% Glucose or GlcNAc were added to the media. For plate studies, 1.5% agar was also added to the media.

AKI media

AKI medium was prepared using 1.5% Bacto peptone, 0.4% yeast extract, 0.5% NaCl and 0.3% $NaHCO_3$. Studies on inducing virulence genes were carried out in AKI medium at 30°C.

Preparation of SHNA1 (*nagA1* - N-acetylglucosamine deacetylase/deacetylase I)

Construction of plasmid vector pCVD442-ΔNA1

A list of all primers and there sequences used for the creation of mutants of the present invention is provided in Table 1.

A PCR assay with primers NA1UF (SEQ ID NO: 1) and NA1UR (SEQ ID NO: 2) were used for amplification of the region 503 bp upstream of the putative translational start site of *nagA1* to obtain an amplified product of 503bp. Primers NA1DF (SEQ ID NO: 3) and NA1DR (SEQ ID NO: 4) were used for the amplification of the region 588 bp downstream of *nagA1* to obtain an amplified product of 588 bp.

For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2.

The PCR products of 503 bp and 588 bp, resulting from Ampli*Taq* polymerase PCR with primer sets NA1UF and NA1UR, and NA1DF and NA1DR, were purified by two passages over the QiaQuick PCR purification kit (Qiagen Inc).

Cross over PCR

The primers NA1UR and NA1DF have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream region of *NAGA1* after stop codon to the upstream region before the start codon of *NAGA1*. The upstream and downstream fragments were mixed in equimolar ratio and used for cross-over PCR with primers NA1UF and NA1DR to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

The cross-over PCR product of 1091 bp was purified by two passages over the QiaQuick PCR purification kit (Qiagen Inc). The amplified and purified product of crossover PCR assay was then ligated using NEB T4DNA-ligase to the counter-selectable plasmid pCVD442 digested with EcoRV to generate plasmid pCVD442-ΔNA1. The plasmid is counter selected using ampicillin and sucrose.

Non-polar deletion for generation of mutant *nagA1*

The plasmid pCVD442-ΔNA1 was introduced into *V. cholerae* O1 El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate. Four co-integrants, formed after conjugation of CO-366 and *E.coli* harbouring the plasmid pCVD442-ΔNA1, were purified by streaking, once under selection and then passaged once without selection, to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium (LB medium containing ampicillin and polymixin B). The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection. Best results were achieved when counter selection plates were incubated at room temperature for 2 days.

The non-polar deletion mutants of *nagA1* were confirmed by PCR assay. The strains were checked using the primers NA1UF and NA1DR. The sizes of the amplified products were 2.228 kb in wild type strain and 1.091 kb in the mutants. The size of the

ORF deleted in the mutants was 1137 bp. The non-polar deletion mutants of *nagA1* were denoted as SHNA1.

Preparation of SHNB (*nagB* - N-acetylglucosamine deaminase)

Construction of plasmid vector pCVD442-ΔNB

A PCR assay with primers NBUF (SEQ ID NO: 5) and NBUR (SEQ ID NO: 6) were used for amplification of the region 510 bp upstream of the putative translational start site of *nagB* to obtain an amplified product of 510 bp. Primers NBDF (SEQ ID NO: 7) and NBDR (SEQ ID NO: 8) were used for the amplification of the region 519 bp downstream of *nagA1* to obtain an amplified product of 519 bp.

For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2.

The PCR products of 510 bp and 519 bp, resulting from Ampli*Taq* polymerase PCR with primer sets, NBUF and NBUR, and NBDF and NBDR, were purified by two passages over the QiaQuick PCR purification kit.

Cross over PCR

The primers NBUR and NBDF have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream region of *nagB* after stop codon to the upstream region before the start codon of *nagB*. The upstream and downstream fragments were mixed in equimolar ratio and used for cross-over PCR with primers NBUF (SEQ ID NO: 5) and NBDR (SEQ ID NO: 8) to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

The cross-over PCR product of size 1029 bp was purified by two passages over the QiaQuick PCR purification kit. The crossover fragment was then ligated using NEB

T4DNA-ligase to the ampicillin and sucrose based counter-selectable plasmid pCVD442 digested with EcoRV to generate plasmid pCVD442-ΔNB.

Non-polar deletion for generation of mutant *nagB*

The plasmid pCVD442-ΔNB was introduced into *V. cholerae* O1 El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate.

Four co-integrants, formed after conjugation of *V. cholerae* O1 El Tor CO-366 and *E.coli* harbouring the plasmid pCVD442-ΔNB, were purified by streaking once under selection and then were passaged once without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection. Best results were achieved when counter-selection plates were incubated at room temperature for 2 days.

The non-polar deletion mutants of *nagB* were confirmed by PCR assay. The strains were checked using the primers NBUF and NBDR. The sizes of the amplified products were 2.830kb in wild type strain and 1.029kb in the mutants. The size of the ORF deleted in the mutants was 801 bp. The non-polar deletion mutants of *nagB* were denoted as SHNB.

Preparation of SHNA2 (*nagA2;* deacetylase II/VC1783)

Construction of plasmid vector pCVD442-ΔNA2

A PCR assay with primers NA2UF (SEQ ID NO: 9) and NA2UR (SEQ ID NO: 10) were used for amplification of the region 459 bp upstream of the putative translational start site of *nagA2* to obtain an amplified product of 459 bp. The primers NA2DF (SEQ ID NO: 11) and NA2DR (SEQ ID NO: 12) were used for the amplification of the region 486 bp downstream of *nagA2* to obtain an amplified product of 486 bp.

For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The

reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2.

The PCR products of 459 bp resulting from Ampli*Taq* polymerase PCR with primer sets NA2UF and NA2UR and product of 486 bp resulting from a PCR assay with the NA2DF and NA2DR primer sets were purified by two passages over the QiaQuick PCR purification kit (Qiagen).

Cross over PCR

The primers NA2UR (SEQ ID NO: 10) and NA2DF (SEQ ID NO: 11) have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream region of *nagA2* after stop codon to the upstream region before the start codon of *nagA2*. The amplified products (upstream and downstream fragments) were mixed in equimolar ratio and used for cross-over PCR with primers NA2UF and NA2DR to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

The PCR product size was 945 bp. The cross-over PCR product of 945 bp was purified by two passages over the QiaQuick PCR purification kit (Qiagen). The crossover fragment was then ligated using NEB T4DNA-ligase to the ampicillin and sucrose based counter-selectable plasmid, pCVD442 digested with EcoRV to generate the recombinant vector plasmid, pCVD442-ΔNA2.

Non-polar deletion for generation of mutant *nagA2*

The plasmid pCVD442-ΔNA2 was introduced into *V. cholerae* O1 El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate.

Four co-integrants, formed after conjugation of CO-366 and E.coli harbouring the plasmid pCVD442-ΔNA2, were purified by streaking one time under selection and then were passaged one time without selection to allow recombination to occur. Sixteen

independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection. Best results were achieved when counter-selection plates were incubated at room temperature for 2 days.

The non-polar deletion mutants of *nagA2* were confirmed by PCR assay. The strains were checked using the primers NA2UF and NA2DR. The sizes of the amplified products were 2.082 kb in wild type strain and 945 kb in the mutants. The size of the ORF deleted in the mutants was 1137 bp. The non-polar deletion mutants of *nagA2* were denoted as SHNA2.

Preparation of SHNC (*nagC;* N-acetylglucosamine specific repressor)

Construction of plasmid vector pCVD442-ΔNC

A PCR assay with primers NCUF (SEQ ID NO: 13) and NCUR (SEQ ID NO: 14) were used for the amplification of the region 616 bp upstream of the putative translational start site of *nagC* to obtain an amplified product of 616 bp. The primers NCDF (SEQ ID NO: 15) and NCDR (SEQ ID NO: 16) were used for the amplification of the region 432 bp downstream of *nagC* to obtain an amplified product of 432 bp.

For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2.

The PCR products of 616 bp and 432 bp resulting from Ampli*Taq* polymerase PCR with primer sets NCUF and NCUR and NCDF and NCDR, respectively, were purified by two passages over the QiaQuick PCR purification kit (Qiagen).

Cross over PCR

The primers NCUR (SEQ ID NO: 14) and NCDF (SEQ ID NO: 15) have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream

region of *nagC* after stop codon to the upstream region before the start codon of *nagC*. The amplified products or the upstream and downstream fragments were mixed in equimolar ratio and used for cross-over PCR with primers NCUF and NCDR to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

The crossover PCR assay yielded an amplified product of size 1048 bp which was purified using the QiaQuick PCR purification kit (Qiagen). The amplified product obtained from the croosover PCR assay was then ligated using NEB T4DNA-ligase into the ampicillin and sucrose based counter-selectable plasmid pCVD442, digested with EcoRV to generate the recombinant plasmid, pCVD442-ΔNC.

Non-polar deletion for generation of mutant *nagC*

The plasmid pCVD442-ΔNC was introduced into *V. cholerae* El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate.

Four co-integrants, formed after conjugation of *V. cholerae* El Tor strain CO-366 and *E.coli* harbouring the plasmid pCVD442-ΔNC, were purified by streaking one time under selection and then were passaged one time without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection. Best results were achieved when counter-selection plates were incubated at room temperature for 2 days.

The non-polar deletion mutants of *nagC* were confirmed by PCR assay. The strains were checked using the primers NCUF and NCDR. The sizes of the amplified products were 2.263 kb in wild type strain and 1.048 kb in the mutants. The size of the ORF deleted in the mutants was 1215 bp. The non-polar deletion mutants of *nagC* were denoted as SHNC.

Preparation of SHNE (*nagE;* GlcNAc transporter, PTS-transporter)

Construction of plasmid vector pCVD442-ΔNE

A PCR assay with primers NEUF (SEQ ID NO: 17) and NEUR (SEQ ID NO: 18) were used for the amplification of the region 505 bp upstream of the putative translational start site of *nagE* to obtain an amplified product of 505 bp. Primers NEDF (SEQ ID NO: 19) and NEDR (SEQ ID NO: 20) were used for the amplification of the region 577 bp downstream of *nagE* to obtain an amplified product of 577 bp.

A PCR assay with Ampli*Taq* polymerase and the primer set NEUF (SEQ ID NO: 17) and NEUR (SEQ ID NO: 18) resulted in an amplified product of size 505 bp (upstream fragment). Another PCR assay with Ampli*Taq* polymerase and the primer set NEDF (SEQ ID NO: 19) and NEDR (SEQ ID NO: 20) resulted in an amplified product of size 577 bp (downstream fragment). For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2. The amplified products were further purified by two passages over the QiaQuick PCR purification kit (Qiagen).

Crossover PCR assay

The primers NEUR (SEQ ID NO: 18) and NEDF (SEQ ID NO: 19) have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream region of *nagE* after stop codon to the upstream region before the start codon of *nagE.* The amplified products (upstream and downstream fragments) were mixed in equimolar ratio and used for a cross-over PCR assay with the primers NEUF and NEDR to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

An amplified product of size 1082 bp was obtained and purified by two passages over the QiaQuick PCR purification kit (Qiagen). The amplified product obtained by the crossover PCR assay was then ligated using NEB T4DNA-ligase into the ampicillin and sucrose based counter-selectable plasmid pCVD442, digested with EcoRV to generate the recombinant plasmid, pCVD442-ΔNE.

Non-polar deletion for generation of mutant *nagE*

The plasmid pCVD442-ΔNE was introduced into *V. cholerae* O1 El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate.

Four co-integrants, formed after conjugation of *V. cholerae* O1 El Tor strain CO-366 and *E.coli* harbouring the plasmid pCVD442-ΔNE, were purified by streaking one time under selection and then were passaged one time without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection.

The non-polar deletion mutants of *nagE* were confirmed by PCR assay. The strains were checked using the primers NEUF and NEDR. The sizes of the amplified products were 2.653 kb in the wild type strain and 1.082 kb in the mutant strain. The size of the ORF deleted in the mutants was 1572 bp. The non-polar deletion mutants of *nagE* were denoted as SHNE.

Preparation of SHVC1781 (N-acetylmannosamine-6-phosphate 2-epimerase; *vc1781*)

Construction of plasmid vector pCVD442-Δ1781

A PCR assay with primers 1781UF (SEQ ID NO: 21) and 1781UR (SEQ ID NO: 22) were used for amplification of the region 474 bp upstream of the putative translational start site of *vc1781* to obtain an amplified product of 474 bp. The primers 1781DF (SEQ

ID NO: 23) and 1781DR (SEQ ID NO: 24) were used for the amplification of the region 475 bp downstream of *vc1781* to obtain an amplified product of 475 bp.

For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2.

The amplified products (upstream and downstream fragments) were purified by two passages over the QiaQuick PCR purification kit (Qiagen).

Cross-over PCR assay

The primers 1781UR (SEQ ID NO: 22) and 1781DF (SEQ ID NO: 23) have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream region of *vc1781*after stop codon to the upstream region before the start codon of *vc1781.* The upstream and downstream fragments were mixed in equimolar ratio and used for cross-over PCR with primers 1781UR (SEQ ID NO: 22) and 1781DF (SEQ ID NO: 23) to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

The cross-over PCR assay resulted in an amplified product of size 949 bp. The amplified product was purified by two passages over the QiaQuick PCR purification kit (Qiagen). The amplified and purified product of the cross-over PCR assay was then ligated using NEB T4DNA-ligase to the ampicillin and sucrose based counter-selectable plasmid pCVD442. The plasmid pCVD442 is digested with the restriction enzyme, EcoRV, to generate the recombinant plasmid pCVD442-Δ1781.

Non-polar deletion for generation of mutant VC1781

The plasmid pCVD442-Δ1781 was introduced into *V. cholerae* O1 El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate. Four co-integrants,

formed after conjugation of *V. cholerae* O1 El Tor strain CO-366 and *E.coli* harbouring the plasmid pCVD442-Δ1781, were purified by streaking one time under selection and then were passaged one time without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection.

The non-polar deletion mutants of *vc1781* were confirmed by PCR assay. The strains were checked using the primers 1781UF and1781DR. The sizes of the amplified products were 1.672 kb in the wild type strain and 949 bp in the mutants. The size of the ORF deleted in the mutants was 723 bp. The non-polar deletion mutants of *vc1781* were denoted as SHVC1781.

Preparation of SHVC1782 (N-acetylmannosamine kinase/ROK kinase; VC1782)

Construction of plasmid vector pCVD442-Δ1782

A PCR assay with primers 1782UF (SEQ ID NO: 25) and 1782UR (SEQ ID NO: 26) were used for amplification of the region 379 bp upstream of the putative translational start site of *vc1782* to obtain an amplified product of 379 bp. The primers 1782DF (SEQ ID NO: 27) and 1782DR (SEQ ID NO: 28) were used for the amplification of the region 433 bp downstream of *vc1782* gene of the *V. cholerae* El Tor strain CO366, to obtain an amplified product of 433 bp.

A PCR assay comprising Ampli*Taq* polymerase PCR with primer pair 1782UF and 1782UR yielded amplified products of size 379 bp (upstream fragment) and with primer pair 1782DF and 1782DR yielded amplified products of size 433 bp (downstream fragment). For each set of PCR analysis, the reaction volume comprised the specific primers, genomic DNA, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The reaction volume was 25µl. PCR parameters for amplifying upstream and downstream fragment are provided in Table 2. The amplified products were

purified by two passages over the QiaQuick PCR purification kit (Qiagen Inc.) and used for the cross-over PCR assay.

Cross-over PCR assay

The primers 1782UR (SEQ ID NO: 26) and 1782DF (SEQ ID NO: 27) have 23 bp of overlapping nucleotide sequence, such that the crossover PCR brings the downstream region of *vc1782* after stop codon to the upstream region before the start codon of *vc1782*.

The amplified upstream and downstream fragments were mixed in equimolar ratio and used for cross-over PCR with primers 1782UR (SEQ ID NO: 26) and 1782DF (SEQ ID NO: 27) to delete the ORF. For the cross-over PCR analysis, the reaction volume of 25 µl comprised the crossover primers, 10x PCR buffer with $Mgcl_2$, dNTPs mix and *Taq* DNA polymerase. The PCR parameters for the cross-over PCR assay is provided in Table 3.

The cross-over PCR assay yielded an amplified product of size 812 bp and was purified by two passages over the QiaQuick PCR purification kit (Qiagen Inc). The amplified and purified product of the cross-over PCR assay was then ligated using NEB T4DNA-ligase into the ampicillin and sucrose based counter-selectable plasmid pCVD442 and digested with *EcoRV* to generate the recombinant plasmid pCVD442-Δ1782.

Non-polar deletion for generation of mutant VC1782

The plasmid pCVD442-Δ1782 was introduced into *V. cholerae* O1 El Tor strain, CO 366 by bi-parental mating or conjugation on an LB agar plate. Four co-integrants, formed after conjugation of *V. cholerae* El Tor strain CO-366 and *E.coli* harbouring the plasmid pCVD442-Δ1782, were purified by streaking once under selection followed by passaging without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for

sucrose-based selection. Best results were achieved when counter-selection plates were incubated at room temperature for 2 days.

The non-polar deletion mutants of *vc1782* were confirmed by PCR assay. The strains were checked using the primers 1782UF and 1782DR. The sizes of the amplified products were 1.676 kb in wild type strain and 812 bp in the mutants. The size of the ORF deleted in the mutants was 864 bp. The non-polar deletion mutants of *vc1782* were denoted as SHVC1782.

Preparation of SHNA1-A2 (double mutant of deacetylase I *nagA1* and deacetylase II *nagA2*)

The mutant of *V. cholerae*, SHNA1 was used as the background strain. The plasmid pCVD442-ΔNA2 (of Example 2) was introduced into *V. cholerae* SHNA1 strain, by bi-parental mating or conjugation on an LB agar plate. Four co-integrants, formed after conjugation of SHNA1 and *E.coli* harbouring the plasmid pCVD442-ΔNA2, were purified by streaking once under selection and then passaged once without selection to allow recombination to occur. Sixteen independent colonies were then streaked on the counter-selection medium - the LB medium containing ampicillin and polymixin B. The ampicillin and polymixin B resistant colonies were next streaked on LB medium without NaCl but containing 6% sucrose for sucrose-based selection. Best results were achieved when counter-selection plates were incubated at room temperature for 2 days.

Colony lysis PCR assay using the primers NA2UF (SEQ ID NO: 9) and NA2DR (SEQ ID NO: 12) was carried out to check for gain of mutation. The single mutated strain gave an amplified product of 2.082 kb while the double mutated strain gave an amplified product of size 945 bp. The non-polar deletion mutants of *nagA1-nagA2* were denoted as SHNA1-A2.

Colonization property of the recombinant strains of *Vibrio cholerae*

In-vitro competition assay

Wild type *V. cholerae* El Tor strain CO 366 strains were made *lacZ* negative. The mutant strains of SHNE, SHNB, SHNA1-A2 were each mixed with the wild-type *V. cholerae* in a ratio of 1:1 based on their optical densities and plated on LB streptomycin plates supplemented with 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal) and incubated at 37^0C, overnight. The viable colony forming units (CFU) were counted after the incubation period using the formula:

Competitive Index (CI) = CI of output / CI of input

CI of input or output = CFU of mutant / CFU of wild type

The *in vitro* competition index was almost 1 for the wild type and the three mutants of *V. cholerae*

In-vivo Infant mouse colonization assay

To test the in-vivo colonization properties of the mutant strains, a mouse intestinal competition assay was carried out. Wild type *V. cholerae* El Tor strain CO 366 strains were made *lacZ* negative. 3-5 days old suckling mice were orogastrically challenged with a 100µl mixture of wild type and mutant *V. cholerae* SHNA1-A2 strain mixed in a ratio of 1:1 based on their optical densities.

20 hours post challenge, the mice were sacrificed and their small intestines dissected, homogenized and plated on LB streptomycin plates supplemented with X-gal and incubated overnight at 37°C.

Viable CFU were counted for the *in vivo* competitive index. The competitive index was found to be 0.0001. Figure 5A shows the results of the infant colonization studies for the mutants SHNB, SHNE and SHNA1-A2. The recovery rate of the mutant and wild type strains is given in Table 4.

Estimation of virulence gene transcripts in recombinant strains of *Vibrio cholerae*

RNA Extraction

Total RNA was isolated using TRIZOL Reagent following the standard protocol (Invitrogen, Carlsbad, CA). To the cell lysate 1 ml TRIZOL reagent was added and mixed well. After a 5-min incubation at 25°C, chloroform 200 µl was added and the tubes were vigorously vortexed for 15 s and incubated at room temperature for 2 min. The upper aqueous RNA-containing phase was collected following centrifugation at 12,000 x g for 10 min at 4°C, into a fresh tube and then precipitated into pellet with isopropyl alcohol (~350 µl) by incubating for 10 min at room temperature and centrifuging 12,000 x g for 10 min at 4°C. The RNA pellet was washed once with 1 ml 75% EtOH by centrifugation at 7500x g for 10 min at 4°C. The RNA pellet was dried in a vacuum concentrator (Concentrator Plus, Eppendorf) for 3 min and RNA was resuspended in RNase free water (Ambion, AM9939). RNA concentration and purity was determined at an optical density ratio of 260/280 using the Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the integrity of total RNA was verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip (Agilent Technologies). RNA was stored at −80°C until further use.

RNA Quality Control

Total RNA integrity was assessed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, Palo Alto, CA) following the manufacturer's protocol. Total RNA purity was assessed by the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Rockland, USA). Total RNA with OD260/OD280>1.8 and OD260/OD230 ≥ 1.3 was used for microarray experiments. RNA was considered as good quality when the rRNA 28S/18S ratios were greater than or equal to 1.5, with the rRNA contribution being 30% or more and an RNA integrity number (RIN) was ≥7.0.

Estimation of gene expression

Total RNA from the wild type *V. cholerae* El Tor strain CO366 and the mutants (SHNB, SHNE, SHNA1-A2) was used for the study. 500 ng of DNase I (Invitrogen)-treated RNA was used for single-stranded cDNA synthesis in 10 µl of reaction mixture using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). For

qRT-PCR assays, the SYBR green PCR master mix was used. RT-PCR assays were carried out on an ABI Prism 7000 real-time PCR apparatus (Applied Biosystems).

The comparative *CT* method ($2^{-\Delta\Delta C}{}_T$) was used to determine the relative gene expression (Schmittgen, T. D., and Livak, K. J. (2008) Analyzing real-time PCR data by the comparative CTmethod *Nat. Protoc.* **3,** 1101–1108).

Control reactions without reverse transcriptase were carried out for each cDNA preparation and ascertained that no amplification was obtained as judged by high *CT* (Murad, A. M., Lee, P. R., Broadbent, I. D., Barelle, C. J., and Brown, A. J.(2000) CIp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16,** 325–327), values and gel analysis. The qRT-PCR analyses revealed that while the expression of *toxR, toxT* and *tcpA* increased almost 1.5, 2 and ≥3 folds respectively in all the mutants, the levels of *tcpP*, *tcpH*, *ctxA*, *ctxB* and remained unaltered or decreased slightly (Figure 6A and 6B).

Detection of Cholera Toxin (CT) using GM_1 ELISA

The wild type *V. cholerae* El Tor strain CO366 and the mutant strains, SHNA1-A2, SHNB and SHNE, were grown in AKI media at 30^0C, overnight (OD 3.0) under constant shaking condition and an average of two independent assays was considered. Each assay was performed in duplicate. 20 µl cell free culture supernatants were added to wells of Microtitre plates coated with GM_1 (monosialoganglioside). The plates were subsequently treated with 1:100 diluted rabbit anti-CT antisera, anti-rabbit Ig peroxidase conjugate and developed with substrate solution containing 1mg/ml O-phenelynediamine dihydrochloride and 0.12% H_2O_2. For each set known amount of purified CT were used to generate a standard curve from which the amount of CT in the test samples were calculated. CT produced was expressed as µg ml^{-1} per unit of optical density at 540 nm of bacterial cell suspension. As a negative control, *E. coli* was used. Average of two independent experiments was taken.

GM_1 ganglioside enzyme-linked Cholera Toxin (CT) assays showed that the production of CT was not significantly altered in the mutants when compared with the wild type (Table 6).

Expression analysis of genes regulated by *nagC*

The mutant SHNC and wild type *Vibrio cholerae* O1 El Tor strain CO366, were grown to log phase in M9-Glucose medium supplemented with amino-acids till early exponential phase at 30°C (n=2 each strain), washed and induced in M9-Glucose (0.5%) or GlcNAc medium (0.5%) at 30°C for 1 hour. Custom Vibrio Cholera 8x15k array slides were used (AMADID: 22386). The probes were designed using the annotated genes in TIGR (cmr.jcvi.org) using N16961 (*Vibrio cholerae* O1 El Tor) as the reference strain. Probes were spotted in triplicates. NCBI protein coding sequence information was also taken into consideration.

Labelling and Microarray Hybridization

The RNA samples for gene expression were labelled using Agilent Quick Amp Kit PLUS (Part number: 5190-0444). 500 ng each of the samples were incubated with reverse trancription mix at 42°C and converted to double stranded cDNA primed by oligodT with a T7 polymerase promoter. The cleaned up double stranded cDNA were used as template for aRNA generation. aRNA was generated by *in vitro* transcription and the dyes Cy3 CTP(Agilent) and Cy5 CTP(Agilent) were incorporated during this step. The cDNA synthesis and *in vitro* transcription steps were carried out at 40°C. Labelled aRNA was cleaned up and quality assessed for yields and specific activity.

Hybridization and Scanning

The labelled aRNA samples were hybridized on to a Vibrio Cholera Gene Expression Array 8X15K. 300 ng of Cy3 labelled and 300 ng of Cy5 labelled samples were fragmented and hybridized. Fragmentation of labelled aRNA and hybridization were done using the Gene Expression Hybridization kit of Agilent (Part Number 5188-5242). Hybridization was carried out in Agilent's Surehyb Chambers at 65°C for 16 hours. The

hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327) and scanned using the Agilent Microarray Scanner G Model G2565BA at 5 micron resolution.

Hierarchical clustering analysis of microarray expression data for genes of the wild type *Vibrio cholerae* and SHNC mutant grown in the presence of glucose, showed that VC0994 (*nagA1*/Deacaetylasel), VC0995(*nagE*), and VCA1025 (*nagB*/Deaminase) were upregulated in the SHNC mutant (Figure 5B). When SHNC mutant was grown in presence of GlcNAc, surprisingly there was a down regulation of genes *viz.* VC1776-VC1784 which included *nagA2* and *ROK* kinase, due to the positive regulatory effect of NagC, as seen in Table 5. This transcriptome data indicates a more general role of NagC, and in particular, the significant role of NagC in the core intermediary metabolism as in gluconeogenesis, fatty acid metabolism, glycolysis, sialic acid degradation, *etc*.

Table 1: Primers used for the creation of mutants of the present invention

S. No	Primer name	SEQ ID NO	Primer Sequence	Product size
1.	NA1UF	SEQ ID NO: 1	5'TTACCTAACTTTTGCGCATAT 3'	503 bp
2	NA1UR	SEQ ID NO: 2	5'ACCAATCTGTCCGCCATTCATTAA ATCAGCTAATCCTCTTGTC 3'	
3	NA1DF	SEQ ID NO: 3	5'TAATGAATGGCGGACAGATTGGT 3'	588 bp
4	NA1DR	SEQ ID NO: 4	5'TACCACGAACGTCGTTACCCA 3'	
5	NBUF	SEQ ID NO: 5	5'GTTACCACGCATGAAGAT 3'	510 bp
6	NBUR	SEQ ID NO: 6	5'GTTTTTATTAGCTTGATTGAGATGT ATTGCCCTTAGATTTGAAT 3'	
7	NBDF	SEQ ID NO: 7	5'ATCTCAATCAAGCTAATAAAAAC 3'	519 bp

8	NBDR	SEQ ID NO: 8	5'CCGTGCTGCTCACGGTAA 3'	
9	NA2UF	SEQ ID NO: 9	5'ATCATTGATGGCAAGCTTCAC 3'	459 bp
10	NA2UR	SEQ ID NO: 10	5'AGGCATGTTTGATCGATAGCCGTT TACTCCTTAAACTGAAATG 3'	
11	NA2DF	SEQ ID NO: 11	5'GCTATCGATCAAACATGCC 3'	486 bp
12	NA2DR	SEQ ID NO: 12	5'GCTTGTCGCCATACCGAAC 3'	
13	NCUF	SEQ ID NO: 13	5'CTGAACATATTGAGAAGCTGG 3'	616 bp
14	NCUR	SEQ ID NO: 14	5'TTGCGTAAGCTTAACTAAAAAGCT ATCAATTCTGCTCGTATTG 3'	616 bp
15	NCDF	SEQ ID NO: 15	5'GCTTTTTAGTTAAGCTTACGCAA 3'	432 bp
16	NCDR	SEQ ID NO: 16	5'ATGAGTTTATCAAAAGAAAG 3'	
17	NEUF	SEQ ID NO: 17	5'GCCTGTGTAGATTTTGCAG 3'	505 bp
18	NEUR	SEQ ID NO: 18	5'AGGCTAGGGTTTAAACTCGACTTA AGTTCCCCCTATAGGAT 3'	
19	NEDF	SEQ ID NO: 19	5'TCGAGTTTAAACCCTAGCCTGA 3'	577 bp
20	NEDR	SEQ ID NO: 20	5'CGTATTCATACAACTTGTCAAAA 3'	
21	1781UF	SEQ ID NO: 21	5'AGCATAAGTTATATCGAGATC 3'	475 bp
22	1781UR	SEQ ID NO: 22	5'TCCGCCGATATCGATTTTTCTTTTC TAAAAACG 3'	
23	1781DF	SEQ ID NO: 23	5'CCATCGATATCGGCGGAAC 3'	474 bp
24	1781DR	SEQ ID NO: 24	5'ACCTTCAATGGCCACCGAC 3'	
25	1782UF	SEQ ID NO: 25	5'TCGAATCACTCCTTTTGTTTC 3'	379 bp
26	1782UR	SEQ ID NO: 26	5'ATTGCCTTTAATGCCATCGTTTCCT TTCTCCCGCAGCTT 3'	
27	1782DF	SEQ ID NO: 27	5'ACGATGGCATTAAAGGCAATT 3'	433 bp

28	1782DR	SEQ ID NO: 28	5'ATAGAACAGATCTGGGTTATG 3'	

Table 2: Run protocol for conventional PCR assay

S. No.	Description	Temperature	Time	Cycles
1.	Initial Denaturation	94°C	5 mins	
2	Denaturation	94°C	2 mins	29 Cycles
3	Annealing	55°C	45 secs	
4	Extension	72°C	45 secs	
5	Final extension	72°C	10 mins	

Table 3: Run protocol for Cross over PCR assay

S. No.	Description	Temperature	Time	Cycles
1.	Initial Denaturation	94°C	5 mins	
2	Denaturation	94°C	2 mins	5 Cycles
3	Annealing	50°C	45 secs	
4	Extension	72°C	1 min	
5	Denaturation	94°C	2 mins	29 cycles
6	Annealing	55°C	45 secs	
7	Extension	72°C	1 min	
8	Final extension	72°C	10 mins	

Table 4: Recovery rate of wild type and SHNA1-A2 mutant strains of *V.cholerae* in mice small intestinal homogenates. White colonies represent wild type, blue colonies represent the mutant. (TNTC: Too numerous to count)

Tube dilution	Plating dilution	Average CFU/ml
10^{-3}	10^{-4}	Set1: TNTC Set2: TNTC Set3: TNTC
10^{-4}	10^{-5}	Set1: White=477; Blue=0 Set2: White=438; Blue=2 Set3: White:464; Blue=1
10^{-5}	10^{-6}	Set1: White=53; Blue=Nil Set2: White=59; Blue=Nil Set3: White=48; Blue=Nil

Table 5: List of genes down-regulated in *nagC* mutant in the presence of GlcNAc

Gene	Fold change	Biological function
VC0620	3.25	ABC-transporter, periplasmic peptide binding protein (Putative GlcNAc transporter)
VC0298	2.89	Acetyl Co-A synthase
VC1781	3.21	Epimerase (Putative ManNAc-6-p 2-epimerase)
VC1782	2.82	ROK kinase (Putative ManNAc kinase)
VC1783	2.76	GlcNAc-6-phosphate deacetylase 2 (Nag A2)
VC0972	2.65	Porin, putative
VC2698	2.62	Alanine/aspartate metabolism
VC1776	2.41	N-acetylneuraminic acid lyase (nanA)
VC1779	4.05	TripartoteATP-independent periplasmic transporter
VC1778	2.45	TRAP transporter
VC1777	2.42	TRAP transporter
VC2738	2.00	Gluconeogenesis
VC1741	2.14	TetR family transcriptional regulator
VC2704	2.13	Hypothetical protein
VC2758	2.13	FadB (fattyacid metabolism)
VC2759	2.08	FadR (fattyacid metabolism)
VC0730	2.02	Copper homeostasis (cutC)
VCA0052	2.0	Hypothetical protein
VC1784	1.99	Neuraminidase (nanH)

VC0728	1.99	Conserved hypothetical protein
VC1774	1.6	Conserved hypothetical protein

Table 6: CT production by *V. cholerae* El Tor strain CO366 and its recombinant strains, SHNA1-A2 and SHNB, grown in AKI medium at 30°C for 16 h and assayed by GM_1 ELISA method.

Strains	**Amount of CT (ng/ ml /opacity unit at 540 nm) produced**	
	Set I	**Set II**
Wild type *V. cholerae*	83.2	136.5
SHNA1-A2 mutant	111.0	95.4
SHNB mutant	81.0	72.5
E. coli (DH5α)	<0.001	<0.001

Example 2

Generation of *Mycobacterium* mutant

Mycobacterium strains H37Rv and CDC1551 were taken. The mutants were created by disrupting *NagA* and *glmS* genes using a two step procedure with a suicide-sacB vector for allelic exchange. Kan cassette conferring kanamycin resistance was cloned into pJetNagA and pJetglmS on a single BamHI and XhoI site respectively (Figure 7B). The nagA::Km and glms::Km were extracted from the resulting vectors and ligated into SmaI cut pXYL4, an *E. coli* vector. This construct was used for allelic exchange. Virulence assay with the generated mutants showed that impaired ability to utilize N-acetylglucosamine resulted in a significant reduction of virulence in mouse model.

Mouse colonization study

Aliquots of *M. tuberculosis* strain H37Rv and its mutant strains were grown in modified 7H10 liquid medium (7H10 agar formulation with agar and malachite green omitted)

supplemented with 10% OADC for 1 week on a 37°C rotary shaker. Media for the mutant strains was supplemented with 50 µg of hygromycin per ml. At the end of the incubation period, culture growth was measured and diluted to yield a final concentration of 1 Klett unit/ml. The inoculum size was determined by titration in triplicate on Middlebrook 7H10 (Difco Laboratories, Detroit, Mich.) agar plates with 10% OADC enrichment. Female C57BL/6J mice were housed in micro isolator cages (Lab Products, Maywood, N.J.) and maintained with water and Prolab RMH 3000 rodent chow (Purina, St. Louis, Mo.) in a biosafety level 3 animal facility. Mice were randomly assigned to groups on day 1 post infection (four mice) and day 20 post infection (four mice). Mice anesthetized with telazol and xylazine were infected intranasally with 20 µl of a suspension containing 10^{-4} CFU. At the designated time points, mice were euthanized by CO_2 asphyxiation, and their right lungs were sterilely removed. Lungs were homogenized in a 1-ml volume contained in an aerosol-resistant grinding assembly (Idea Works Laboratory Devices, Syracuse, N.Y.). Aliquots of the homogenate were serially diluted and titrated on 7H10 agar plates. Agar plates were incubated at 37°C in ambient air for 4 weeks, and viable colonies were enumerated.

Example 3

Generation of *Magnaporthe grisea* mutant

The *Magnaporthe griseae* deacetylase *(MgDAC)* genomic sequences (~2.9kb) including 5' and 3' upstream region was PCR amplified and cloned into pGEM-T Easy vector. This vector (pGdac) was then digested with *XhoI* to delete ~900bp internal region of the gene and ligated to *Hph* gene derived from pCB1003 after *SalI* cleavage. The resulting vector, named pGRdac was digested with *NotI* to release ~3.4kb gene replacement construct used for protoplast transformation (Figure 10). Protoplasts were generated from *M. grisea* mycelia with lysing enzyme (Sigma). Putative transformants were selected on hygromycinB (Sigma) containing medium. Genomic DNA was isolated from transformed colonies and digested with *EcoRV* for confirmation of *MgDAC* disruption through southern hybridization. Mutants were then checked for growth on

media with 20mM glucose or 20mM GlcNAc as sole carbon source. *M. grisea* mutant and wild type strains were inoculated separately on to oat meal agar plates and incubated at 22°C for 14 days under constant illumination of white light. Conidia (Spore) were detached from mycelia by scraping agar surface with a glass rod after adding 0.2% gelatin solution. Conidia were separated from mycelia after passing though two layers of cheese cloth. Spore concentration was adjusted to 10^4spore/ml in 0.2% gelatin. 15 days old rice seedlings were sprayed separately with equivalent mutant and wild type conidia (10^4spore/ml). After spray plants were covered with plastic bag to maintain high humidity and placed in a humid chamber (80-85% relative humidity and 27-28°C temperature) set for 14 hours/10 hours of day/night cycles. The plastic bag was removed after 24 hours.

SEQ ID NO: 1 Forward primer sequence for amplification of upstream fragment of putative translation site of *nagA1* gene of *Vibrio cholerae*

TTACCTAACTTTTGCGCATAT

SEQ ID NO: 2 Reverse primer sequence for amplification of upstream fragment of putative translation site of *nagA1* gene of *Vibrio cholerae*

ACCAATCTGTCCGCCATTCATTAAATCAGCTAATCCTCTTGTC

SEQ ID NO: 3 Forward primer sequence for amplification of downstream fragment of putative translation site of *nagA1* gene of *Vibrio cholerae*

TAATGAATGGCGGACAGATTGGT

SEQ ID NO: 4 Reverse primer sequence for amplification of downstream fragment of putative translation site of *nagA1* gene of *Vibrio cholerae*

TACCACGAACGTCGTTACCCA

SEQ ID NO: 5 Forward primer sequence for amplification of upstream fragment of putative translation site of *nagB* gene of *Vibrio cholerae*

GTTACCACGCATGAAGAT

SEQ ID NO: 6 Reverse primer sequence for amplification of upstream fragment of putative translation site of *nagB* gene of *Vibrio cholerae*

GTTTTTATTAGCTTGATTGAGATGTATTGCCCTTAGATTTGAAT

SEQ ID NO: 7 Forward primer sequence for amplification of downstream fragment of putative translation site of *nagB* gene of *Vibrio cholerae*

ATCTCAATCAAGCTAATAAAAAC

SEQ ID NO: 8 Reverse primer sequence for amplification of downstream fragment of putative translation site of *nagB* gene of *Vibrio cholerae*

CCGTGCTGCTCACGGTAA

SEQ ID NO: 9 Forward primer sequence for amplification of upstream fragment of putative translation site of *nagA2* gene of *Vibrio cholerae*

ATCATTGATGGCAAGCTTCAC

SEQ ID NO: 10 Reverse primer sequence for amplification of upstream fragment of putative translation site of *nagA2* gene of *Vibrio cholerae*

AGGCATGTTTGATCGATAGCCGTTTACTCCTTAAACTGAAATG

SEQ ID NO: 11 Forward primer sequence for amplification of downstream fragment of putative translation site of *nagA2* gene of *Vibrio cholerae*

GCTATCGATCAAACATGCC

SEQ ID NO: 12 Reverse primer sequence for amplification of downstream fragment of putative translation site of *nagA2* gene of *Vibrio cholerae*

GCTTGTCGCCATACCGAAC

SEQ ID NO: 13 Forward primer sequence for amplification of upstream fragment of putative translation site of *nagC* gene of *Vibrio cholerae*

CTGAACATATTGAGAAGCTGG

SEQ ID NO: 14 Reverse primer sequence for amplification of upstream fragment of putative translation site of *nagC* gene of *Vibrio cholerae*

TTGCGTAAGCTTAACTAAAAAGCTATCAATTCTGCTCGTATTG

SEQ ID NO: 15 Forward primer sequence for amplification of downstream fragment of putative translation site of *nagC* gene of *Vibrio cholerae*

GCTTTTTAGTTAAGCTTACGCAA

SEQ ID NO: 16 Reverse primer sequence for amplification of downstream fragment of putative translation site of *nagC* gene of *Vibrio cholerae*

ATGAGTTTATCAAAAGAAAG

SEQ ID NO: 17 Forward primer sequence for amplification of upstream fragment of putative translation site of *nagE* gene of *Vibrio cholerae*

GCCTGTGTAGATTTTGCAG

SEQ ID NO: 18 Reverse primer sequence for amplification of upstream fragment of putative translation site of *nagE* gene of *Vibrio cholerae*

AGGCTAGGGTTTAAACTCGACTTAAGTTCCCCCTATAGGAT

SEQ ID NO: 19 Forward primer sequence for amplification of downstream fragment of putative translation site of *nagE* gene of *Vibrio cholerae*

TCGAGTTTAAACCCTAGCCTGA

SEQ ID NO: 20 Reverse primer sequence for amplification of downstream fragment of putative translation site of *nagE* gene of *Vibrio cholerae*

CGTATTCATACAACTTGTCAAAA

SEQ ID NO: 21 Forward primer sequence for amplification of upstream fragment of putative translation site of *vc1781* gene of *Vibrio cholerae*

AGCATAAGTTATATCGAGATC

SEQ ID NO: 22 Reverse primer sequence for amplification of upstream fragment of putative translation site of *vc1781* gene of *Vibrio cholerae*

TCCGCCGATATCGATTTTTCTTTTCTAAAAACG

SEQ ID NO: 23 Forward primer sequence for amplification of downstream fragment of putative translation site of *vc1781* gene of *Vibrio cholerae*

CCATCGATATCGGCGGAAC

SEQ ID NO: 24 Reverse primer sequence for amplification of downstream fragment of putative translation site of *vc1781* gene of *Vibrio cholerae*

ACCTTCAATGGCCACCGAC

SEQ ID NO: 25 Forward primer sequence for amplification of upstream fragment of putative translation site of *vc1782* gene of *Vibrio cholerae*

TCGAATCACTCCTTTTGTTTC

SEQ ID NO: 26 Reverse primer sequence for amplification of upstream fragment of putative translation site of *vc1782* gene of *Vibrio cholerae*

ATTGCCTTTAATGCCATCGTTTCCTTTCTCCCGCAGCTT

SEQ ID NO: 27 Forward primer sequence for amplification of downstream fragment of putative translation site of *vc1782* gene of *Vibrio cholerae*

ACGATGGCATTAAAGGCAATT

SEQ ID NO: 28 Reverse primer sequence for amplification of downstream fragment of putative translation site of *vc1782* gene of *Vibrio cholerae*

ATAGAACAGATCTGGGTTATG

I/We claim:

1. A recombinant microorganism strain incapable of utilizing N-acetylglucosamine, wherein genome of the recombinant microorganism strain comprises at-least one mutation in at-least one of the genes selected from the group consisting of N-acetylglucosamine-6-phosphate deacetylase (*nagA1*), N-acetylglucosamine-6-phosphate deacetylase II (*nagA2*), glucosamine 6-phosphate deaminase (*nagB*), N-acetylglucosamine specific repressor (*nagC*), and N-acetylglucosamine specific transporter (*nagE*), wherein the recombinant microorganism strain shows impaired colonization in a host as compared to a wild type microorganism strain.

2. The recombinant microorganism strain as claimed in claim 1, wherein the mutation is selected from the group consisting of deletion, addition, and substitution.

3. The recombinant microorganism strain as claimed in claim 2, wherein the mutation is a non-polar deletion.

4. The recombinant microorganism strain as claimed in claim 1, wherein the host is selected from the group consisting of human, animal, and plant.

5. The recombinant microorganism strain as claimed in claim 1, wherein the microorganism strain is selected from the group consisting of *Vibrio* species, *Mycobacterium* species, and *Magnaporthe* species.

6. The recombinant microorganism strain as claimed in claim 5, wherein the *Vibrio* species is *Vibrio cholerae.*

7. The recombinant microorganism strain as claimed in claim 6, wherein the *Vibrio cholerae* is *Vibrio cholerae* E1 Tor strain CO366.

8. The recombinant microorganism strain as claimed in claim 5, wherein the *Mycobacterium* species is *Mycobacterium tuberculosis*.

9. The recombinant microorganism strain as claimed in claim 5, wherein the *Magnaporthe* species is *Magnaporthe grisea.*

10. A composition comprising the recombinant microorganism strain as claimed in claim 1.

11. The composition as claimed in claim 10, wherein the composition additionally comprises atleast one of a pharmaceutically acceptable carrier, a diluent, an adjuvant, and an additive.

12. The composition as claimed in claim 11, wherein the composition additionally comprises an agriculturally acceptable carrier.

13. A composition comprising the recombinant microorganism strain as claimed in claim 1, a pharmaceutically acceptable carrier, a diluent, an adjuvant, an additive, and an agriculturally acceptable carrier.

14. A vaccine comprising the recombinant microorganism strain as claimed in claim 1.

15. The vaccine as claimed in claim 14, wherein the vaccines additionally comprises atleast one of a pharmaceutically acceptable carrier, a diluent, an adjuvant, and an additive.

16. A vaccine comprising the recombinant microorganism strain as claimed in claim 1, a pharmaceutically acceptable carrier, a diluent, an adjuvant, and an additive.

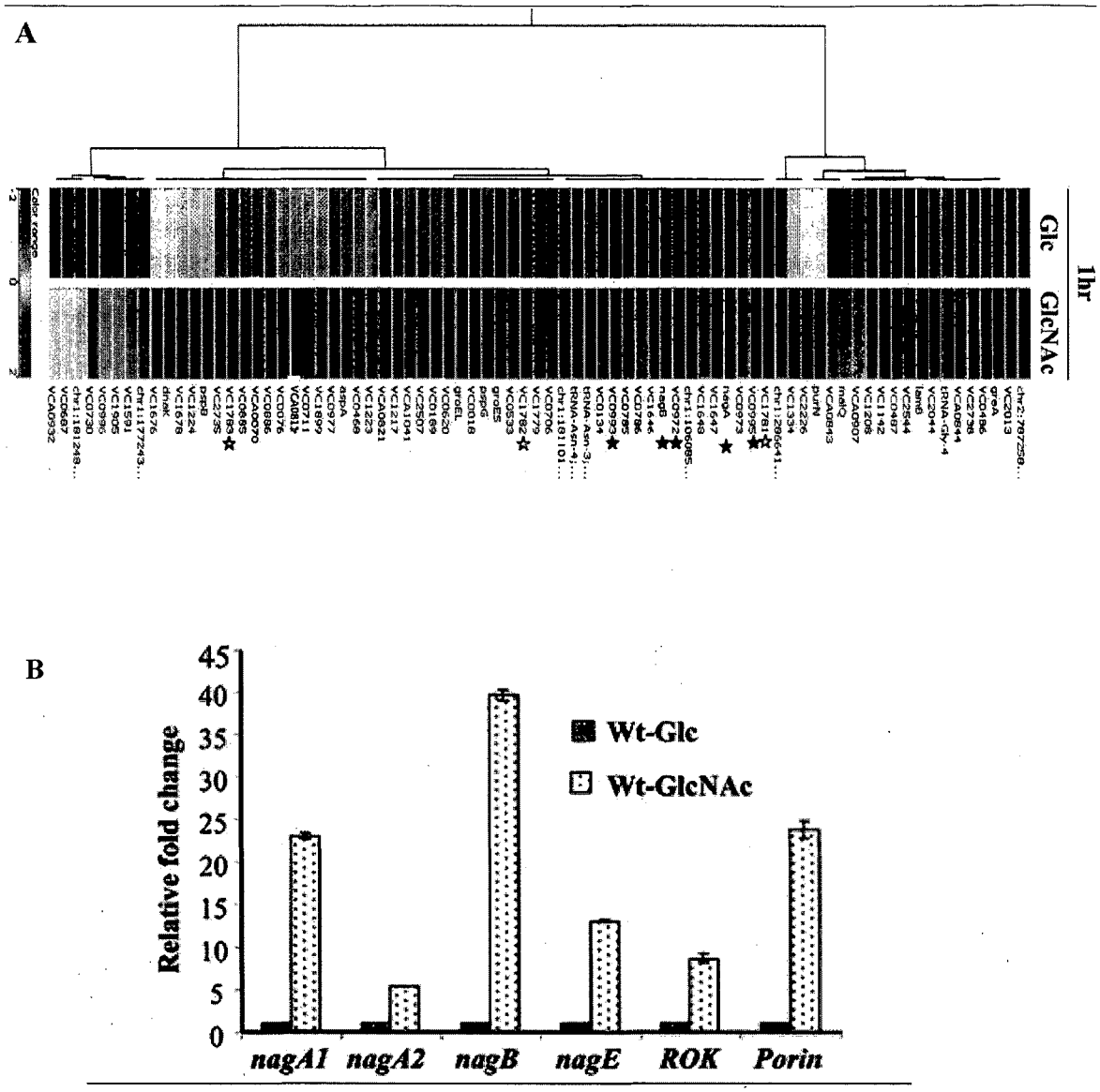
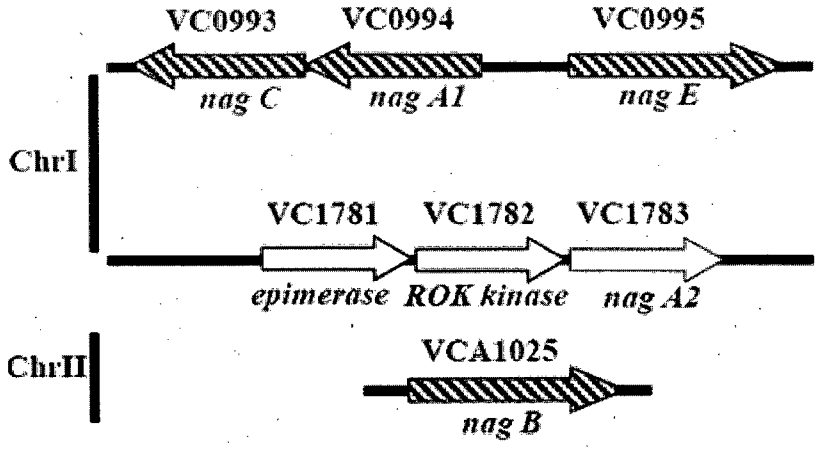

A
Glc
GlcNAc
1hr
B
Relative fold change
45
40
35
30
25
20
15
10
5
0
Wt-Glc
Wt-GlcNAc
nagA1
nagA2
nagB
nagE
ROK
Porin

FIGURE 1

A


VC0993
VC0994
VC0995
nag C
nag A1
nag E
ChrI
VC1781
VC1782
VC1783
epimerase
ROK kinase
nag A2
ChrII
VCA1025
nag B

B

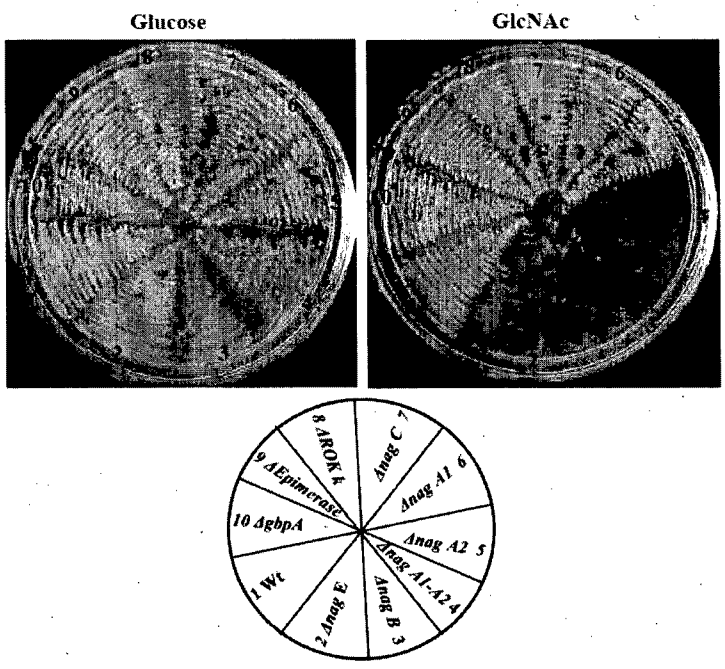
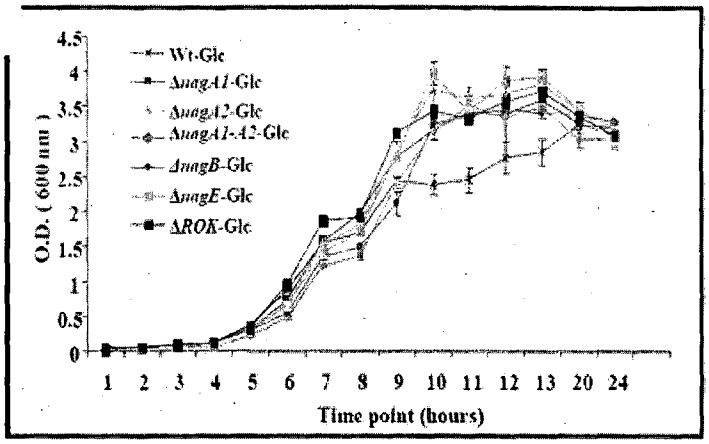
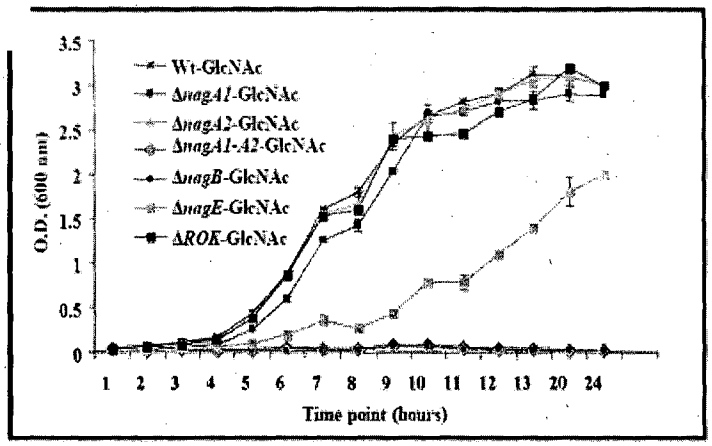

Glucose
GlcNAc
8 ΔROK k
Δnag C 7
9 ΔEpimerase
Δnag A1 6
10 ΔgbpA
Δnag A2 5
1 Wt
Δnag A1-A2 4
2 Δnag E
Δnag B 3

FIGURE 2

A


Wt-Glc
ΔnagA1-Glc
ΔnagA2-Glc
ΔnagA1-A2-Glc
ΔnagB-Glc
ΔnagE-Glc
ΔROK-Glc
O.D. (600 nm)
Time point (hours)

B


Wt-GlcNAc
ΔnagA1-GlcNAc
ΔnagA2-GlcNAc
ΔnagA1-A2-GlcNAc
ΔnagB-GlcNAc
ΔnagE-GlcNAc
ΔROK-GlcNAc
O.D. (600 nm)
Time point (hours)

C

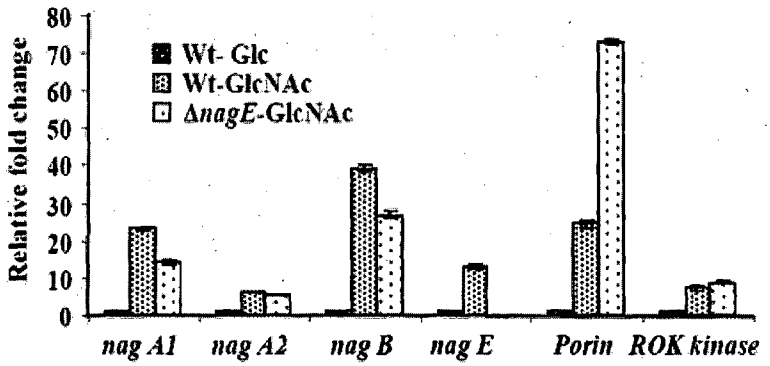
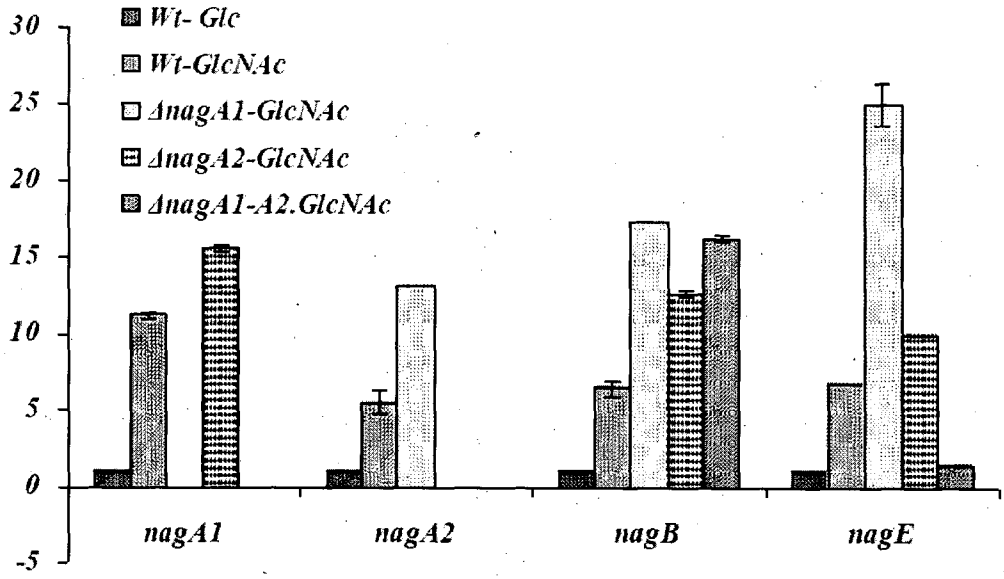

Wt- Glc
Wt-GlcNAc
ΔnagE-GlcNAc
Relative fold change
nag A1
nag A2
nag B
nag E
Porin
ROK kinase

FIGURE 3

A


30
25
20
15
10
5
0
-5
Wt- Glc
Wt-GlcNAc
ΔnagA1-GlcNAc
ΔnagA2-GlcNAc
ΔnagA1-A2.GlcNAc
nagA1
nagA2
nagB
nagE

B

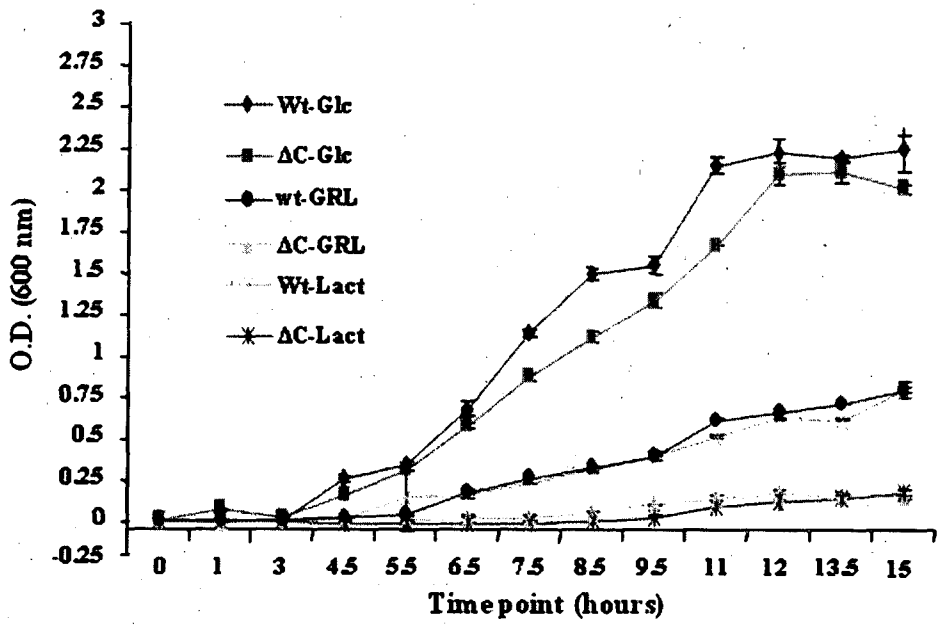

O.D. (600 nm)
3
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
-0.25
Wt-Glc
ΔC-Glc
wt-GRL
ΔC-GRL
Wt-Lact
ΔC-Lact
0 1 3 4.5 5.5 6.5 7.5 8.5 9.5 11 12 13.5 15
Timepoint (hours)

FIGURE 4

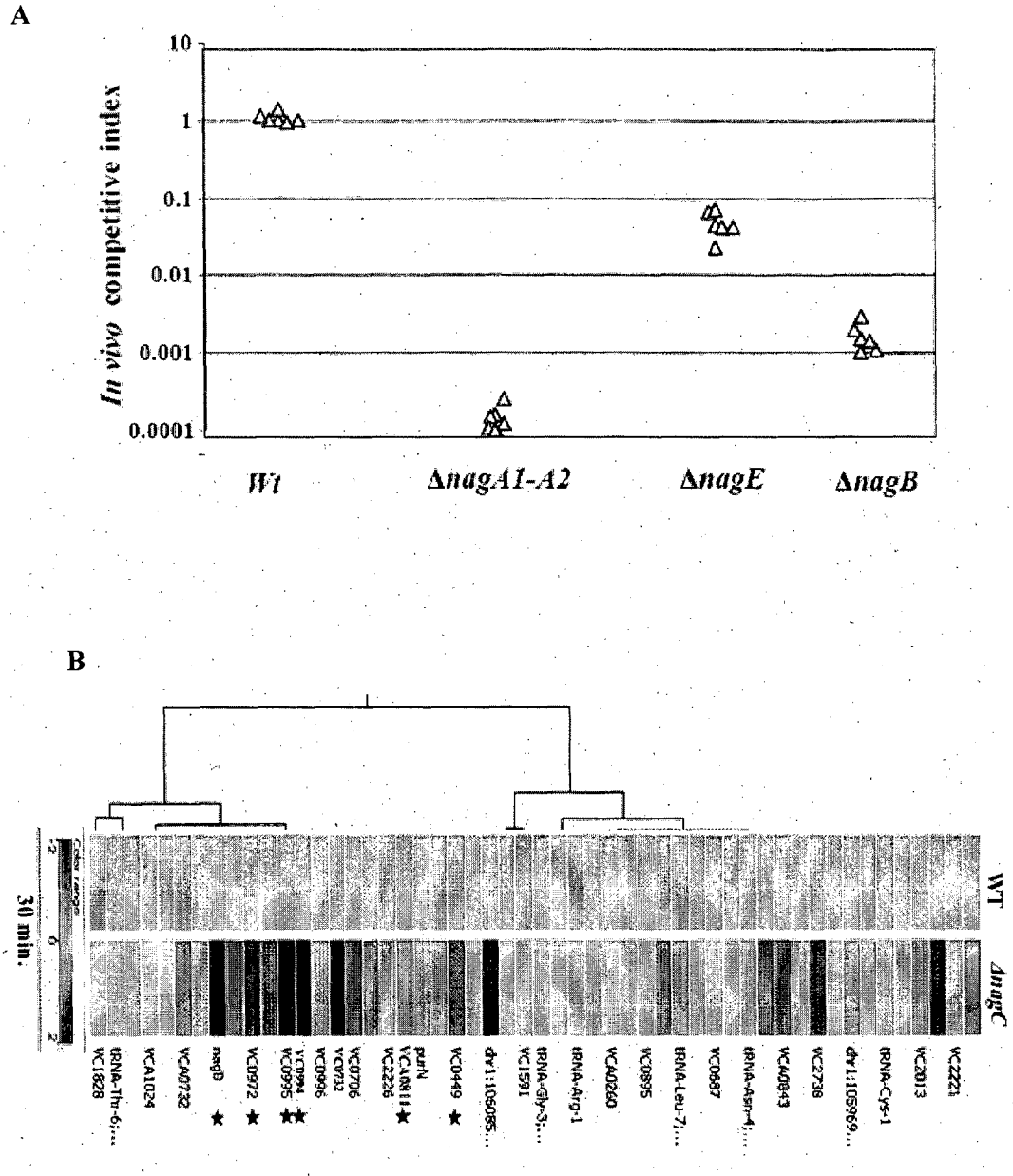

A
10
1
0.1
0.01
0.001
0.0001
In vivo competitive index
Wt
ΔnagA1-A2
ΔnagE
ΔnagB
B
30 min.
WT
ΔnagC
tRNA-Thr-6;...
VCA1024
VCA0732
nagB
VC0972
VC0995
VC0994
VC0996
VC0732
VC0706
VC2226
purN
VCA0811
VC0449
dnr1:105085...
VC1591
tRNA-Gly-3;...
tRNA-Arg-1
VCA0260
VC0895
tRNA-Leu-7;...
VC0687
tRNA-Asn-4;...
VCA0843
VC2736
dnr1:105969...
tRNA-Cys-1
VC2013
VC2221

FIGURE 5

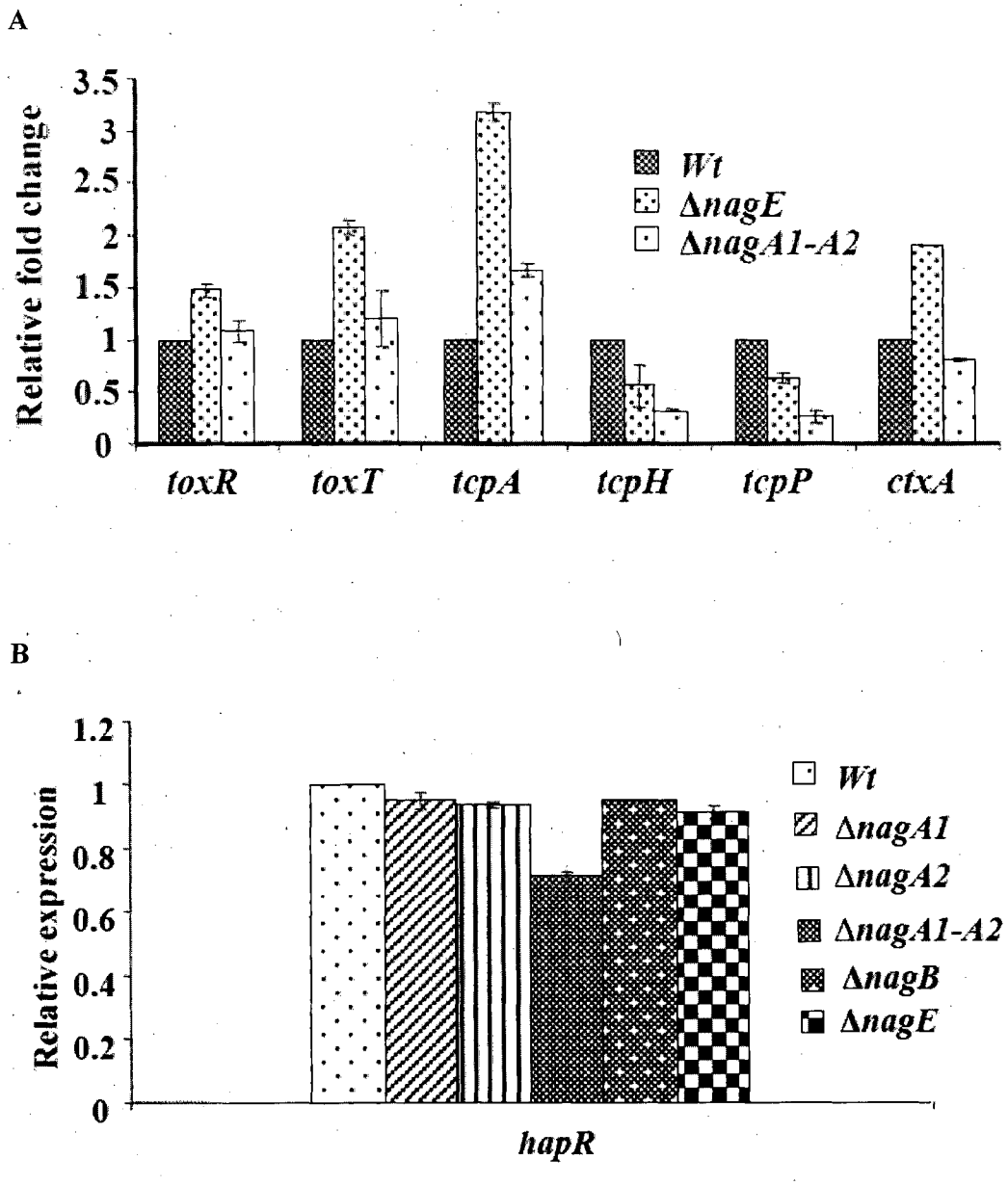
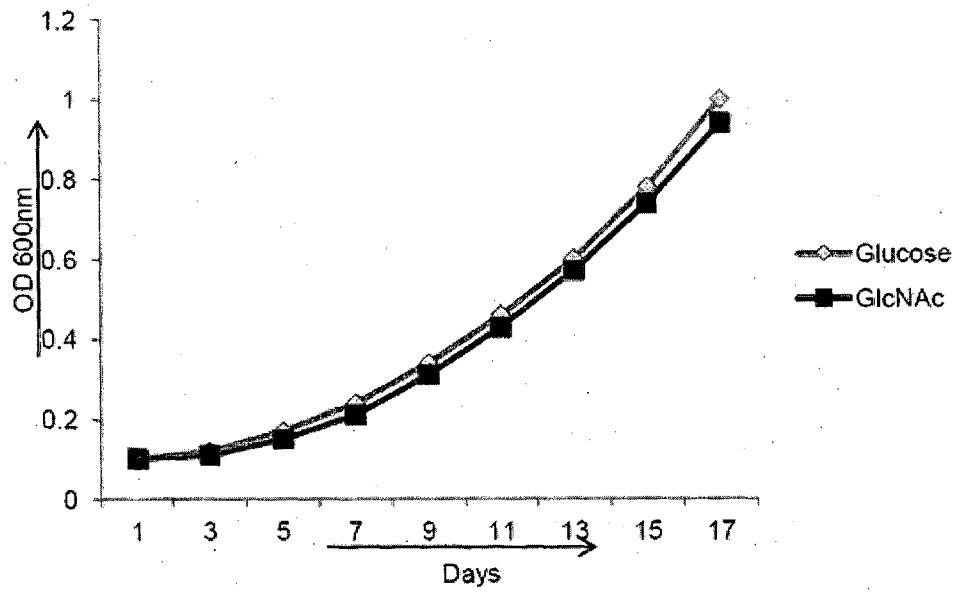

A
Relative fold change
3.5
3
2.5
2
1.5
1
0.5
0
Wt
ΔnagE
ΔnagA1-A2
toxR
toxT
tcpA
tcpH
tcpP
ctxA
B
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Wt
ΔnagA1
ΔnagA2
ΔnagA1-A2
ΔnagB
ΔnagE
hapR

FIGURE 6

A


1.2
1
0.8
0.6
0.4
0.2
0
OD 600nm
1
3
5
7
9
11
13
15
17
Days
Glucose
GlcNAc

B

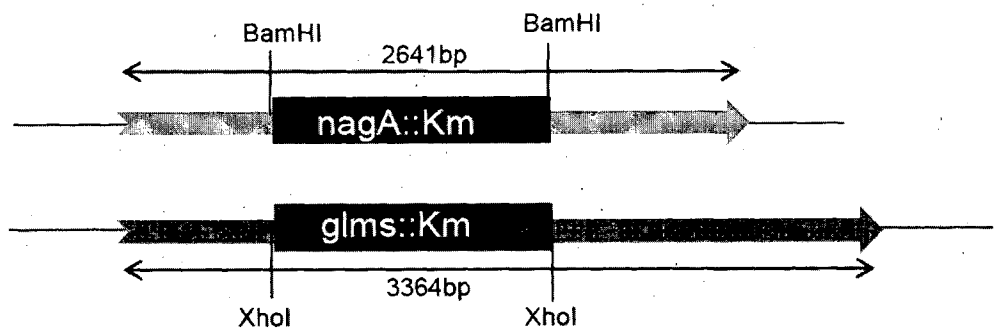

BamHI
BamHI
2641bp
nagA::Km
glms::Km
3364bp
XhoI
XhoI

FIGURE 7

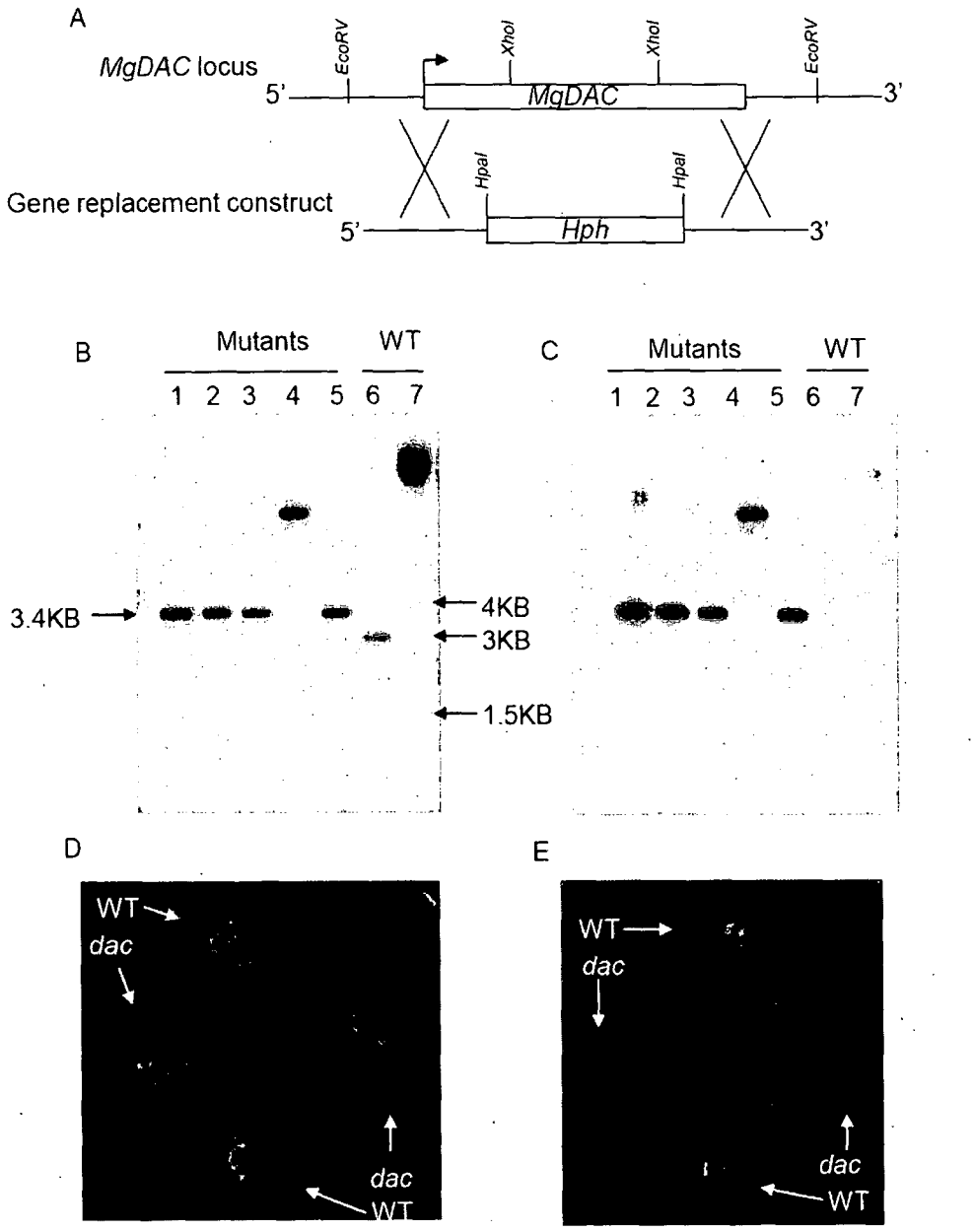
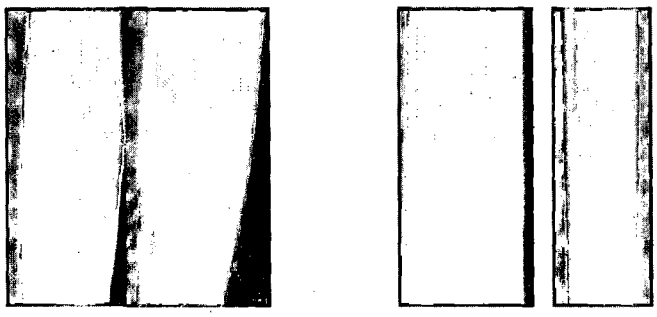
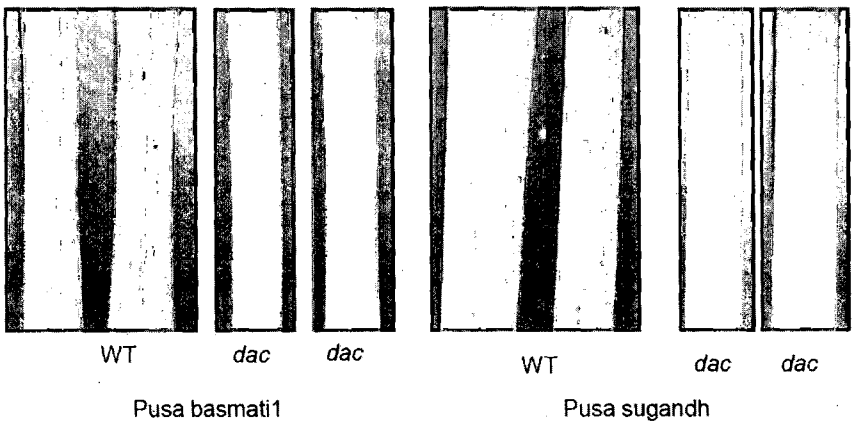

A
MgDAC locus
EcoRV
XhoI
XhoI
EcoRV
5'
MgDAC
3'
Gene replacement construct
HpaI
HpaI
5'
Hph
3'
B
Mutants
WT
1 2 3 4 5 6 7
3.4KB
4KB
3KB
1.5KB
C
Mutants
WT
1 2 3 4 5 6 7
D
WT
dac
dac
WT
E
WT
dac
dac
WT

FIGURE 8

A


WT
dac
dac
Pusa basmati1

B


WT
dac
dac
WT
dac
dac
Pusa basmati1
Pusa sugandh

C

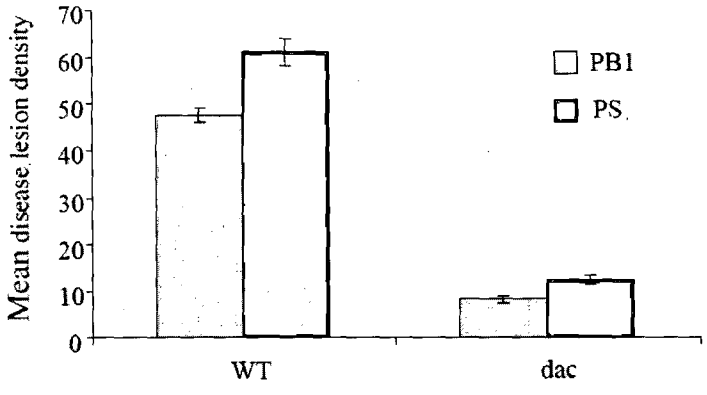

Mean disease lesion density
70
60
50
40
30
20
10
0
PB1
PS
WT
dac

FIGURE 9

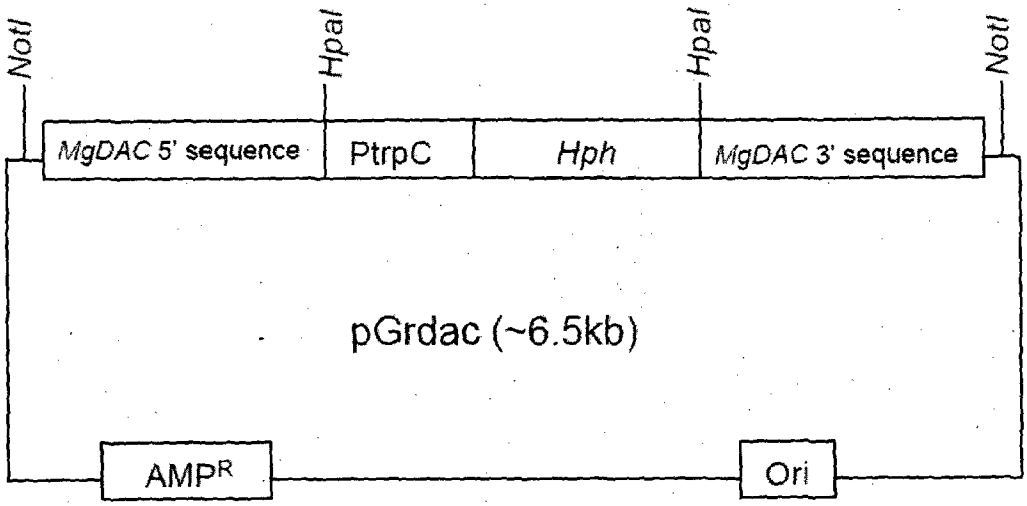

NotI
HpaI
HpaI
NotI
MgDAC 5' sequence
PtrpC
Hph
MgDAC 3' sequence
pGrdac (~6.5kb)
AMPR
Ori

FIGURE 10