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(71) Applicant(s)
Absynth Biologics Limited

(72) Inventor(s)
Garcia Lara, Jorge;Foster, Simon

(74) Agent / Attorney
FB Rice, Level 23 44 Market Street, Sydney, NSW, 2000, AU

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(71) **Applicant** *(for all designated States except US)*: **ABSYNTH BIOLOGICS LIMITED** [GB/GB]; Leavygreave Road, Sheffield South Yorkshire S2 7DR (GB).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **GARCIA LARA, Jorge** [ES/GB]; Absynth Biologics Limited, Leavygreave Road, Sheffield South Yorkshire S2 7DR (GB). **FOSTER, Simon** [GB/GB]; Absynth Biologics Limited, Leavygreave Road, Sheffield South Yorkshire S2 7DR (GB).

(74) **Agent**: **SYMBIOSIS IP LIMITED**; Apollo House, Eboracum Way, Heworth Green, York Yorkshire YO31 7RE (GB).

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(54) **Title**: COMBINATION VACCINE

Table 3

	%Body Weight Loss			
	Control Sample	Experimental Samples		
Animal Number	**I** Adjuvant alone (Freunds)	**II** Antigen: rYdiE	**III** Antigen: rDivIB	**IV** Combination Antigen: rYdiE rDivIB
1	11.1	11.3	13.1	2.3
2	12.7	4.3	0.0	2.0
3	9.7	9.0	3.5	0.0
4	7.6	12.6	0.5	-4.3
5	9.4	1.0	7.1	-0.5
6	9.9	6.2	3.3	1.8
7	2.0	3.8	2.1	1.5
8	4.2	5.3	1.9	1.4
9	1.0	3.4	1.0	1.6
10	3.1	7.0	6.8	3.9
11	7.7	4.7	-2.5	-0.5

(57) **Abstract**: The disclosure relates to a composition comprising two or more immunogenic staphylococcal polypeptides and a mulitvalent vaccine composition comprising the immunogenic staphylococcal polypeptides.

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Combination Vaccine

The disclosure relates to a composition comprising two or more immunogenic staphylococcal polypeptides and a multivalent vaccine composition comprising the immunogenic staphylococcal polypeptides in the prevention or treatment of staphylococcal infections in humans and animals.

Vaccines protect against a wide variety of infectious diseases. Many modern vaccines are therefore made from protective antigens of the pathogen, which are isolated by molecular cloning and purified. These vaccines are known as 'subunit vaccines'. The development of subunit vaccines has been the focus of considerable research in recent years. The emergence of new pathogens and the growth of antibiotic resistance have created a need to develop new vaccines and to identify further candidate molecules useful in the development of subunit vaccines. Likewise the discovery of novel vaccine antigens from genomic and proteomic studies is enabling the development of new subunit vaccine candidates, particularly against bacterial pathogens. However, although subunit vaccines tend to avoid the side effects of killed or attenuated pathogen vaccines, their 'pure' status means that subunit vaccines do not always have adequate immunogenicity to confer protection.

An approach to improve the efficacy of vaccine compositions is to provide multivalent vaccines comprising dominant antigens that provoke both a B cell and T cell response thereby mounting a more rigorous immune response in the subject receiving the vaccine. A typical multivalent vaccine might be a whole cell vaccine comprising multiple antigenic molecules. For example the Bacillus Calmette Guerin ["BCG"] vaccine includes an attenuated *Mycobacterium bovis* strain that provokes protective immunity in humans. For many pathogens chemical or heat inactivation while it may give rise to vaccine immunogens that confer protective immunity also gives rise to side effects such as fever and injection site reactions. In the case of bacteria, inactivated organisms tend to be so toxic that side effects have limited the application of such crude vaccine immunogens and therefore vaccine development has lagged behind drug-development. Moreover, effective vaccine development using whole cell inactivated organisms suffers from problems of epitope masking, immunodominance, low antigen concentration and antigen redundancy.

Currently there is no effective vaccination procedure to prevent or treat *Staphylococcus aureus* infection. *S.aureus* is a bacterium whose normal habitat is the epithelial lining of the nose in about 20-40% of normal healthy people and is also commonly found on people's skin usually without causing harm. However, in certain circumstances, particularly when skin is damaged, this pathogen can cause infection. This is a particular problem in hospitals where patients may have surgical procedures and/or be taking immunosuppressive drugs. These patients are much more vulnerable to infection with *S.aureus* because of the treatment they have received. Antibiotic resistant strains of *S.aureus* have arisen since their wide spread use in controlling microbial infection. Methicillin resistant strains are prevalent and many of these resistant strains are also resistant to several other antibiotics.

S. aureus is therefore a major human pathogen capable of causing a wide range of diseases some of which are life threatening diseases including septicaemia, endocarditis, arthritis and toxic shock. This ability is determined by the versatility of the organism and its arsenal of components involved in virulence. At the onset of infection, and as it progresses, the needs and environment of the organism changes and this is mirrored by a corresponding alteration in the virulence determinants which *S. aureus* produces. At the beginning of infection it is important for the pathogen to adhere to host tissues and so a large repertoire of cell surface associated attachment proteins are made. The pathogen also has the ability to evade host defences by the production of factors that reduce phagocytosis or interfere with the ability of the cells to be recognised by circulating antibodies.

There is therefore a continuing need to identify staphylococcal antigens that are protective and can be used in multivalent vaccines. The combinations may be used in combination with non-protein immunogenic molecules such as polysaccharide antigens and anti-bacterial agents to provide a treatment regimen for control of staphylococcal infection. It is also within the scope of this disclosure to modify the treatment regimen to immunize subjects with a series of temporally separated administrations as an alternative to the administration of a single vaccine comprising multiple antigens.

This disclosure therefore relates to combination or multivalent immunogenic compositions and vaccines and their use in the prophylaxis and treatment of staphylococcal infections. We disclose polypeptides that individually are protective and are typically membrane spanning proteins that include an extracellular domain and are

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essential for staphylococcal cell growth. For example DivIB is an integral membrane protein comprising an intracellular domain, an intermembrane domain and an extracellular domain. DivIB and fragments thereof, provide protection from at least an *S.aureus* challenge in an animal model. The related gene DivIC is also an integral membrane protein the extracellular domain of which provokes protective immunity to staphylococcal infection. This disclosure also relates to antigens encoded by the genes *PheP, YdiE* and *FtsL* each of which have an extramembranous domain.

According to an aspect of the invention there is provided an immunogenic composition comprising two or more different polypeptides wherein said polypeptides are encoded by different staphylococcal genes selected from the group consisting of:

i) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 20;

ii) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 21;

iii) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 22;

iv) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 23;

v) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 24; or

vi) a modified staphylococcal polypeptide wherein said polypeptide is a staphylococcal polypeptide variant of the amino acid sequences presented in SEQ ID NO: 20, 21, 22, 23 or 24, wherein said sequences are modified by addition, deletion or substitution of one or more amino acid residues which modified polypeptides have retained or enhanced immunogenicity when compared to the polypeptide as represented in SEQ ID NO: 20, 21, 22, 23 or 24.

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In a further aspect of the invention there is provided an immunogenic or vaccine composition comprising:

i) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 21;

ii) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 22.

A modified staphylococcal polypeptide or variant staphylococcal polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations that may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and aspartic acid; c)

asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants that retain or enhance the immunogenecity and/or activity as the reference polypeptide from which it varies.

In one embodiment, the variant polypeptides have at least 80-89% sequence identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the full length amino acid sequences illustrated herein.

In a preferred embodiment of the invention said immunogenic composition comprises or consists essentially of 2, 3, 4 or 5 staphylococcal polypeptides.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, or antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO:20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO:23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided a composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 23 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21 and 22, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22 and 23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21 and 23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 23 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22, 23 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 22 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 22 and 23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 22, 23 and 24, or an antigenic fragment thereof.

In an alternative preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising 23 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22, and 23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22, 23 and 24, or an antigenic fragment thereof.

In an alternative preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 22, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 23 and 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 22, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 23, or an antigenic fragment thereof.

In a preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 22, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 24, or an antigenic fragment thereof.

In an alternative preferred embodiment of the invention there is provided an immunogenic composition comprising:

i) a polypeptide comprising SEQ ID NO: 23, or an antigenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 24, or an antigenic fragment thereof.

In a preferred embodiment of the invention said composition is a vaccine composition and includes at least one carrier and/or adjuvant.

Adjuvants (immune potentiators or immunomodulators) have been used for decades to improve the immune response to vaccine antigens. The incorporation of adjuvants into vaccine formulations is aimed at enhancing, accelerating and prolonging the specific immune response to vaccine antigens. Advantages of adjuvants include the enhancement of the immunogenicity of weaker antigens, the reduction of the antigen amount needed for a successful immunisation, the reduction of the frequency of booster immunisations needed and an improved immune response in elderly and immunocompromised vaccinees. Selectively, adjuvants can also be employed to optimise a desired immune response, e.g. with respect to immunoglobulin classes and induction of cytotoxic or helper T lymphocyte responses. In addition, certain adjuvants can be used to promote antibody responses at mucosal surfaces. Aluminium hydroxide and aluminium or calcium phosphate has been used routinely in human vaccines.

Adjuvants can be classified according to their source, mechanism of action and physical or chemical properties. The most commonly described adjuvant classes are gel-type, microbial, oil-emulsion and emulsifier-based, particulate, synthetic and cytokines. More than one adjuvant may be present in the final vaccine product according to the invention. They may be combined together with a single antigen or all antigens present in the vaccine, or each adjuvant may be combined with one particular antigen. The origin and nature of the adjuvants currently being used or developed is highly diverse. For example, aluminium based adjuvants consist of simple inorganic compounds and PLG is a polymeric carbohydrate. MDP is derived from bacterial cell walls; saponins are of plant origin, squalene is derived from shark liver and recombinant endogenous immunomodulators are derived from recombinant bacterial, yeast or mammalian cells. There are several adjuvants licensed for veterinary vaccines, such as mineral oil emulsions that are too reactive for human use. Similarly, complete Freund's adjuvant, although being one of the most powerful adjuvants known, is not suitable for human use.

A carrier is an immunogenic molecule which, when bound to a second molecule augments immune responses to the latter. The term carrier is construed in the following manner. A carrier is an immunogenic molecule which, when bound to a second molecule augments immune responses to the latter. Some antigens are not intrinsically immunogenic yet may be capable of generating antibody responses when associated with a foreign protein molecule such as keyhole-limpet haemocyanin or tetanus toxoid. Such antigens contain B-cell epitopes but no T cell epitopes. The protein moiety of such a conjugate (the "carrier" protein) provides T-cell epitopes which stimulate helper T-cells that in turn stimulate antigen-specific B-cells to differentiate into plasma cells and produce antibody against the antigen.

The vaccine compositions of the invention can be administered by any conventional route, including injection, intranasal spray by inhalation of for example an aerosol or nasal drops. The administration may be, for example, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or intradermally. The vaccine compositions of the invention are administered in effective amounts. An "effective amount" is that amount of a vaccine composition that alone or together with further doses, produces the desired response. In the case of treating a particular bacterial disease the desired response is providing protection when challenged by an infective agent.

In a preferred embodiment of the invention said vaccine composition is adapted for administration as a nasal spray.

In a preferred embodiment of the invention said vaccine composition is provided in an inhaler and delivered as an aerosol.

The amounts of vaccine will depend, of course, on the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used sufficient to provoke immunity; that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The doses of vaccine administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

In general, doses of vaccine are formulated and administered in effective immunizing doses according to any standard procedure in the art. Other protocols for the administration of the vaccine compositions will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration and the like vary from the foregoing. Administration of the vaccine compositions to mammals other than humans, (e.g. for testing purposes or veterinary therapeutic purposes), is carried out under substantially the same conditions as described above. A subject, as used herein, is a mammal, preferably a human, and including a non-human primate, cow, horse, pig, sheep or goat.

The ratio of antigens may be varied in pair wise fashion. The ratio of each antigen may be 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1 to optimize the response of the subject

to particular combinations of antigen. For example the ratio of DivIB and YdiE may be varied as described above.

In a preferred embodiment of the invention there is provided a vaccine composition according to the invention that includes at least one additional anti-bacterial agent.

In a preferred embodiment of the invention said agent is a second different vaccine and/or immunogenic agent (for example a bacterial polypeptide and/or polysaccharide antigen).

According to a further aspect of the invention there is provided a composition comprising a nucleic acid molecule[s] comprising or consisting of nucleotide sequences of two or more different staphylococcal genes and encoding immunogenic polypeptides selected from the group consisting of:

i) a nucleic acid molecule comprising or consisting of the nucleotide sequence as represented in SEQ ID NO: 1 or 6;

ii) a nucleic acid molecule comprising or consisting of the nucleotide sequence as represented in SEQ ID NO: 2 or 7;

iii) a nucleic acid molecule comprising or consisting of the nucleotide sequence as represented in SEQ ID NO: 3 or 8;

iv) a nucleic acid molecule comprising or consisting of the nucleotide sequence as represented in SEQ ID NO: 4 or 9;

v) a nucleic acid molecule comprising or consisting of the nucleotide sequence as represented in SEQ ID NO: 5 or 10;

vi) a nucleic acid molecule comprising or consisting of a nucleotide sequence wherein said sequence is degenerate as a result of the genetic code to the nucleotide sequence defined in i-v above; or

vii) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the sequence in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 and wherein said nucleic acid molecule encodes a staphylococcal antigenic polypeptide.

Hybridization of a nucleic acid molecule occurs when two complementary nucleic acid molecules undergo an amount of hydrogen bonding to each other. The stringency of hybridization can vary according to the environmental conditions surrounding the nucleic acids, the nature of the hybridization method, and the composition and length of the

nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001); and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes Part I, Chapter 2 (Elsevier, New York, 1993). The T_m is the temperature at which 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following is an exemplary set of hybridization conditions and is not limiting:

Very High Stringency (allows sequences that share at least 90% identity to hybridize)

Hybridization:	5x SSC at 65°C for 16 hours
Wash twice:	2x SSC at room temperature (RT) for 15 minutes each
Wash twice:	0.5x SSC at 65°C for 20 minutes each

High Stringency (allows sequences that share at least 80% identity to hybridize)

Hybridization:	5x-6x SSC at 65°C-70°C for 16-20 hours
Wash twice:	2x SSC at RT for 5-20 minutes each
Wash twice:	1x SSC at 55°C-70°C for 30 minutes each

Low Stringency (allows sequences that share at least 50% identity to hybridize)

Hybridization:	6x SSC at RT to 55°C for 16-20 hours
Wash at least twice:	2x-3x SSC at RT to 55°C for 20-30 minutes each.

In a preferred embodiment of the invention said composition is a vaccine composition and includes and includes at least one carrier and/or adjuvant.

The nucleic acid or DNA combination vaccines comprise nucleic acid molecules that encode antigenic polypeptides as herein disclosed. The specific combinations of polypeptide antigens as represented by amino acid SEQ ID can be substituted for the corresponding nucleotide SEQ ID as herein disclosed in the manufacture of DNA vaccines.

According to a further aspect of the invention there is provided a combination vaccine according to the invention for use in the protection or treatment of a subject animal to a staphylococcal infection or condition that results from a staphylococcal infection.

In a preferred embodiment of the invention said staphylococcal infection is caused by a staphylococcal species selected from the group consisting of: *S.epidermidis*, *S.aureus*, *S.hominis*, *S.haemolyticus*, *S.warneri*, *S.capitis*, *S.saccharolyticus*, *S.auricularis*, *S.simulans*, *S.saprophyticus*, *S.cohnii*, *S.xylosus*, *S.hyicus*, *S.caprae*, *S.gallinarum*, *S.intermedius*,

In a further preferred embodiment of the invention said staphylococcal species is *S. aureus* or *S.epidermidis*.

In a preferred embodiment of the invention said subject is a human.

In an alternative preferred embodiment of the invention said subject is a non-human animal, preferably a livestock animal, for example cattle.

In a preferred embodiment of the invention said live stock animal is vaccinated against bacterial mastitis caused by staphylococcal bacterial cells.

In a preferred embodiment of the invention said life stock animal is a caprine animal (e.g. sheep, goat).

In a preferred embodiment of the invention said life stock animal is a bovine animal (e.g. a cow).

Staphylococcal mastitis is a serious condition that affects live stock and can result in considerable expense with respect to controlling the disease through administration of antibiotics and in terms of lost milk yield. The vaccine according to the invention provides cost effective control of bacterial, in particular staphylococcal mastitis.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article

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is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will now be described by example only and with reference to the following Figures.

Table 3 and 4 illustrate the vaccination of a mouse model with a combination antigens of the extracellular domains of YdiE and DivIB compared to individual antigen vaccinations.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Materials and Methods

Construction of plasmids for the overexpression in E. coli of the extramembranous fragments of the S. aureus proteins

The PheP selected peptide was synthesized and conjugated through a cysteine at its C terminal to the carrier protein KLH to undertake as a chimeric protein used in vaccinations. The extramembranous fragments of YdiE, DivIB, DivIC and FtsL were PCR amplified from the chromosome of strain *S. aureus* SH1000 (*Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ:* sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. *J Bacteriol 2002,* 184:*5457-5467*) using oligonucleotide pairs indicated on Table 1 according to the following PCR reaction conditions: 1 initial denaturation cycle of 94^{0}C for 4 min; 30 amplification cycles of

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denaturation 94^{0}C for 30 seconds, annealing 45^{0}C for 30 seconds, and extension at 72^{0}C for up to 2.5 minutes; finally, ongoing amplification rounds were allow to complete at 72^{0}C for 4 min.

The restrictions sites engineered within the oligonucleotides are also indicated in Table 1 (underlined; NcoI or XhoI). The amplified fragments were digested with the corresponding restriction enzymes (NcoI for the 5' end, and XhoI for the 3' end) and cloned into the equivalent sites of the pET-21d(+) expression vector from Novagen (Cat.

No. 69743-3) and resulting in the overexpression plasmids indicated in Table 1 generating a T7-tagged (partial, at the N-terminal) and 6xHis-tagged (at the C-terminal end) form of the extramembranous fragments. In the SEQ IDs the T7- and His-tags are indicated in **bold**, and the extramembranous portion of the proteins of interest are underlined. The over expression plasmids were transferred into *E. coli* BL21 for over expression of the recombinant protein fragment.

The cloning of the PCR amplified fragment indicated above into the recipient pET21d(+) recipient plasmid vector at the NcoI and XhoI sites entailed the addition of hinge amino acids between the T7-tag and the extramembranous fragment, and between the latter and the His-tag. These amino acids are **neither bold** nor underlined in the SEQ IDs.

Over Expression of SEQ ID NO: 25-28

SEQ ID NOs 25 through 28 were over expressed from plasmids pGL597, pGL601, pALB26, and pALB27 in E. coli BL21 strain using Brain Heart Infusion Broth (CM0225, Oxoid, United Kingdom) in the presence of 100 µg/ml ampicillin and the Plac promoter gratuitous inducer IPTG (Isopropyl β-D-1-thiogalactopyranoside, 1mM) for 4 to 6 hours at 37^{0}C and vigorous shaking. Following harvesting of the cells by centrifugation (5,000xg for 15 minutes at 4^{0}C) and subsequent lysis with 1 mg/ml lysozyme in phosphate buffer (Buffer A; 0.1M pH7.2) containing 0.5M NaCl) for one hour and subsequent sonication (3 cycles of 10 second pulses in sonicating water bath) the soluble and insoluble forms of the proteins of interest were separated by centrifugation at 13,000xg for 10 minutes. The precipitate was then resuspended in Buffer A containing 8M urea by freeze/thawing (3 cycles of freezing at -80oC for 10 minutes and subsequent thawing to room temperature) and sonication (3 cycles of 10 second pulses in sonicating water bath), and subsequent centrifugation for 25 minutes at 18,000xg). The over expressed proteins of interest in the supernatant and the solubilised pellet were purified by initial specific binding (through their His-tag) to a nickel (NiSO4)-bound Sepharose chromatography column (Ni-Sepharose) and elution with an imidazole solution run through the column in the following stepwise manner: 5% for 5 minutes, 30% for 60 minutes, 35% for 60 minutes, 50% for 100 minutes and 55% for 100 minutes. Fractions from this stepwise elution were analysed in acrylamide denaturing gels with a 4% acrylamide/bis-acrilamide stacking layer and a 12% acrylamide/bis-acrylamide separating layer. The fractions containing the over expressed proteins of interest were

pooled and dyalized against sterile phosphate buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, per liter of distilled H_2O, pH 7.4).

All the proteins of interest were successfully over expressed from the indicated strains and under the indicated conditions. They were also subsequently extracted from the total cellular protein content of the over expressing E. coli strains with more than 95% purity. Examples of the purification obtained for each of the proteins are indicated below.

Evaluation of vaccination-mediated protection of Balb/C mice against infection by *S. aureus*

One week after the second boost each animal was infected with an i.v. (tail vein) injection of 100 microliters of endotoxin-free PBS containing $1.1x10^7$ ($\pm$ $0.5 x10^7$) cells of *S. aureus* strain Newman. The latter were prepared from cultures growing to early stationary phase in Brain Heart Infusion medium (BHI), which were then washed three times with the same volume of PBS.

After 10 to 14 days the animals were sacrificed according to Schedule 1 cervical dislocation. The pair of kidneys from each animal was extracted in aseptic conditions, and homogenized in sterile PBS. Serial dilutions of the kidney homogenates were carried out in PBS and plated on BHI agar plates. Plates containing between 10 to 150 staphylococcal colonies were counted and dilution corrected. The number of viable cells in the kidneys was inferred from the number of colony forming units (CFU) on the plates. Evaluation of the possible protection against infection conferred by vaccination with DivIB-2 was determined from difference in the number of *S. aureus* cells in the kidneys of animals vaccinated with KLH and those vaccinated with DivIB-2. The statistic significance of the difference was calculated using the Mann-Whitney test. A significantly higher ($p<0.05$) number of *S. aureus* in KLH vaccinated animals compared to the DivIB-2 vaccinated animals was concluded as protection.

Vaccination: Generic protocol for polyvalent vaccines

Combination (or polyvalent) vaccines including variations of the antigens (conjugated selected PheP peptide, YdiE, DivIB, DivIC and FtsL) will follow an identical protocol with the following modifications. The vaccine priming and boost mixtures will contain rather than a single component 2 or more of the components. The total volume of mixed

vaccine used for priming and boosting injections will fluctuate in a range of 50-100 microliters per animal. Similarly the total amount in each of those injections may vary between 50-100 micrograms. The amount of each antigen to contribute to the total amount of vaccine in the priming or boosting mix will vary between 20% to 80% of the total.

The various combinations of antigens to be evaluated as a vaccine mix will be undertaken according to the matrix in Table 2. The combinations are grouped in 3 tiers. Depending on results from the 1st Tier of experiments the 2nd Tier of experiments would be undertaken accordingly, and depending on the results from the latter the 3rd Tier will be undertaken. In each Tier a vaccination experiment will contain an antigen in the Y axis, together with those ticked along the X axis, and labelled with the same colour. Each vaccination experiment is labelled with a different colour.

Example 1

The experimental sample consisted in a combination antigen of the extracellular domains of YdiE and DivIB. The amount of antigen administered to each mouse (Female Balb/C, approx. 5-6 weeks old) was 5ug of YdiE plus 50ug of DivIB. Those amounts were contained within 100 ul of eluent consisting on a 50:50 v:v of PBS (Phosphate Buffer Saline) and Complete Freund's Adjuvant (used for the vaccination priming) or Incomplete Freund's Adjuvant (used for the vaccination boost). Priming was undertaken day 0, Boost 1 at 14 days, and Boost 2 at 21 days. Subsequently, 7 days later, i.e., at day 28 the animals were infected with *Staphylococcus aureus* strain Newman. Each test group (control and experimental) had 10 animals. The bacterial dose administered to the animals (both, control and experimental) contained $4x10^6$ bacteria in 100ul of PBS. The infection period was run for 3 days, and the weight of the animals was monitored daily (we also extracted organs to evaluate bacterial loads in organs, Table 4). At that point the animals were sacrificed. The output of the experiment was calculated as the percentage body weight loss between day 3 and day 0 for every animal. The results of these experiments are shown in Table 3. Statistical analysis of data in Table 3: TEST: Non-parametric statistical hypothesis test – Mann-Whitney U

SELECTED OPTIONS: Two-tailed, unpaired, 95% confidence interval.

RESULTS: (two group comparison)

II and I: p=0.622

III and I: p=0.039*

IV and I: p=0.001*

II and III: p=0.061

II and IV: p=0.008*

III and IV: p=0.158

* comparisons with statistically significant difference between the groups

Statistical analysis Table 4:

TEST: Non-parametric statistical hypothesis test – Mann-Whitney U

SELECTED OPTIONS: Two-tailed, unpaired, 95% confidence interval.

RESULTS: (two group comparison)

II and I: p=0.2

III and I: p=0.105

IV and I: p=0.009*

II and III: p=0.378

II and IV: p=0.131

III and IV: p=0.504

* comparisons with statistically significant difference between the groups

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Claims

1 An immunogenic or vaccine composition comprising:

i) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 21; and

ii) a polypeptide, or immunogenic fragment thereof, comprising or consisting of the amino acid sequence as represented in SEQ ID NO: 22.

2. The immunogenic or vaccine composition according to claim 1 which comprises or consists essentially of 2, 3, 4 or 5 staphylococcal polypeptides.

3. The immunogenic or vaccine composition according to claim 1 or 2 wherein said composition further comprises SEQ ID NO: 23.

4. The immunogenic or vaccine composition according to claim 1 to 2 wherein said composition further comprises SEQ ID NO: 24.

5. The immunogenic or vaccine composition according to claim 1 or 2, comprising:

i) a polypeptide comprising SEQ ID NO: 21, or an immunogenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 22, 23 and 24, or an immunogenic fragment thereof.

6. The immunogenic or vaccine composition according to claim 1 or 2, comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an immunogenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21 and 22, or an immunogenic fragment thereof.

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7. The immunogenic or vaccine composition according to claim 1 or 2, comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an immunogenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 22 and 24, or an immunogenic fragment thereof.

8. The immunogenic or vaccine composition according to claim 1 or 2, comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an immunogenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 22 and 23, or an immunogenic fragment thereof.

9. The immunogenic or vaccine composition according to claim 1 or 2, comprising:

i) a polypeptide comprising SEQ ID NO: 20, or an immunogenic fragment thereof; and

ii) a polypeptide comprising SEQ ID NO: 21, 22, 23 and 24, or an immunogenic fragment thereof.

10. The immunogenic or vaccine composition according to any one of claims 1-9 further comprising at least one carrier and/or adjuvant.

11. The immunogenic or vaccine composition according to claim 10 wherein said composition comprises an adjuvant selected from the group consisting of aluminium hydroxide, aluminium phosphate or calcium phosphate.

12. The immunogenic or vaccine composition according to claim 11 wherein said adjuvant is aluminium phosphate.

13. The immunogenic or vaccine composition according to claim 11 or 12 wherein said adjuvant is formulated as a gel-type.

14. The immunogenic or vaccine composition according to any one of claims 10 to 13, wherein said composition is adapted for administration as a nasal spray.

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15. The immunogenic or vaccine composition according to claim 14 wherein composition is provided in an inhaler and delivered as an aerosol.

16. The immunogenic or vaccine composition according to any one of claims 10 to 15 which includes at least one additional anti-bacterial agent.

17. The immunogenic or vaccine composition according to claim 16 wherein said anti-bacterial agent is a second different vaccine and/or immunogenic agent.

18. A composition according to any one of claims 1 to 17 for use in the manufacture of a vaccine for the protection or treatment of a subject animal to a staphylococcal infection or condition that results from a staphylococcal infection.

19. The use according to claim 18 wherein said staphylococcal infection is caused by a staphylococcal species selected from the group consisting of: *S.epidermidis*, *S.aureus*, *S.hominis*, *S.haemolyticus, S.warneri, S.capitis, S.saccharolyticus, S.auricularis, S.simulans, S.saprophyticus*, *S.cohnii, S.xylosus*, *S.hyicus, S.caprae, S.gallinarum and S.intermedius*,

20. The use according to claim 18 or 19 wherein said subject is a human.

21. The use according to claim 18 or 19 wherein said subject is a non-human animal.

22. A method to treat a staphylococcus infection in an animal subject comprising administering an immunogenic or vaccine composition according to any one of claims 1 to 17.

23. The method according to claim 22 wherein said staphylococcal infection is caused by a staphylococcal species selected from the group consisting of: *S.epidermidis*, *S.aureus*, *S.hominis*, *S.haemolyticus, S.warneri, S.capitis, S.saccharolyticus, S.auricularis, S.simulans, S.saprophyticus*, *S.cohnii, S.xylosus*, *S.hyicus, S.caprae, S.gallinarum and S.intermedius*.

24. The method according to claim 22 or 23 wherein said subject is human.

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25. The method according to claim 22 or 23 wherein said subject is a non-human animal.

26. The method according to claim 25 wherein said non-human animal is a livestock animal.

SEQ 1 (complete *pheP* sequence)

ATGGAAGATAATAAAATGAACCGTAGTCTTAACTCAAGACACATTTCCATGATTGCTATAGGTGGTGCAATTGGG
ACTGGTCTATTTGTAGCTACTGGTAATATCATTTCTCAAGCTGGTCCTGGAGGCGCTATACTCGCTTATCTTGTT
ATTGGTGTCATGCTATATTTCTTAATGTCATCAATTGGAGAGTTGGCAACATTTTATCCAGTATCAGGTTCATTC
AGCTCTTATTCAACACGCTTTATTGACTCATCTCTTGGCTTTACCATGGGATGGTTGTATTGGGCATTGTGGTCA
TTAGTTACAAGTGTTGATGTCATAGTAGCGTCAAATGTGCTTTATTTCTGGGACACATTTAAATTTTTCCACCCC
ATTACTTGGAGCTTAATCTTTATTACAATTTTACTATTATTAAACATTTTTTCTGTAAAATCATTTGGAGAAACT
GAGTTTTGGTTATCATTGATTAAAGTGTTAACAATTATCGTATTCGTTATTTTTGGCTTTTTAATGATTTTCGGT
ATCTTAGGTGGTCATACATATGGATTTGAAAACTATACAAAAGGCCAAGCACCGTTTGTTGGTGGTATCTCTGGT
TTCTTAGGCGTATTATTAGTCGCCGGATTTTCGGTTGGTGGTACAGAAGTAGTAGCAGTAACTGCTGGTGAATCA
GATGACCCTAAAAAGTCTATGCCTAAGGCAATTAAACAAGTATTTTGGCGTATTCTTTTATTCTATGTCTTATCA
ATTGCAGTAATTGGTGCAATTATTCCGTACACAGATCCATCATTATTAAGAGCAAGTAGTTCAATAAGTCAAAGC
CCATTTACAATTGTATTCGATAGAGTAGGCATAGCCTTTGCAGCATCAGTAATCAACGCGGTTATTTTAACTTCA
TTATTATCCGCTGCAAATTCAGGTGTTTATACAACAGGCAGAATGTTGTATTCCTTAAGTTCAGACAAAAAAGCA
CCCCAATTTTTAAGTAAATTAAACAAGACAACTAAGTTACCTTTAAGAGCATTATTAACTACTTATGCAGTCGTT
GTTATTGTTATTATTTATGCAAACTTTAATTCAAATGCCGTTTTTAATTTACTTGAAATTATTGGTTCAATGATT
ATAGTTGTTTGGGGATCAAGCATTTGGTCACAAATACGATTGCGACAAGCTATTAAAAAACAAGGTCAAGACCCT
AATAAGGTCCTACCATATAAAGCACCTTTTTATCCATTAGGACCAATCATTGTCATCACTACACTATTATTCTTG
CTATTTGGTGGCTCAGTTGAATATATTTTAAAAGATCAATGGTTAAATGCTTTTAAAAACTTTTTACCTTTAATC
ATTCTAGCGTTGATTTACTTTATTCATAAAATCATTCACAAAACAAAATTTGTAAAGCTAGAAACAATTAATTTA
AAACCACACGATTATGACAATCAAAAATAA

SEQ 2 (complete *ydiE/gcp* sequence)

ATGACTAAAGATATATTAATACTAGCTGTTGAAACAAGTTGTGATGAAACAAGCGTTAGTGTTATAAAAAATGGC
AGAGATATTTTATCAAATACAGTTTTAAGTCAGATTGAAAGTCATAAACGATTTGGCGGTGTCGTTCCCGAAGTG
GCAAGTAGACATCACGTTGAAGGTATAACAGCAACAATAAACGAGGCTCTAGGGGATGCCGATGTATCAATAGAA
GATATTGATGCCATAGCGGTTACAGAAGGCCCTGGACTAATTGGTGCGTTACTAATAGGTGTTAATGCAGCCAAA
GCATTGGCATTTGCTTACGATAAGCCACTTATTCCTGTTCATCATATTGCAGGACATATATATGCTAATCACATA
GAAGAGCCATTAACATTCCCGCTAATTGCACTTATTGTTTCAGGTGGACATACTGAATTAGTTTATATGAAGGAT
CATTTATCATTTGAAGTCATTGGTGAAACACGAGATGACGCAGTAGGTGAGGCTTATGATAAAGTGGCACGAACA
ATTGGTTTAAATTATCCAGGTGGTCCACAAGTTGATCGGTTGGCTGCTGAAGGTGAAGATACTTATTCATTCCCT
CGTGTTTGGTTGGATAAAGATAGTTATGATTTTAGTTTTAGTGGGTTGAAAAGTGCCGTAATCAATCAACTTCAC
AATCAACGACAAAAAAATATTCCAATCATTGAAGCTAACGTAGCAACGAGCTTTCAAAACAGTGTTGTAGAGGTG
CTCACGTTTAAAGCTATTCAAGCTTGTAAAGAATATGGTGTTCAGCGATTAATTGTTGCTGGTGGCGTGGCGAGT
AATAAAGGATTACGTCAATCTTTAGCGGATCAATGCAAAGTCAATGACATTCAATTAACTATCCCAAGTCCTAAA
TTATGCACAGATAATGCTGCAATGATAGGCGTTGCCGGCCACTATTTGTATCAGCAAGGTCGATTTGCTGATTTA
GCATTAAATGGGCACAGCAATATAGATTTAGAAGAGTATTCTGCAGAATAA

SEQ 3 (complete *divIB* sequence)

ATGGATGATAAAACGAAGAACGATCAACAAGAATCAAATGAAGATAAAGATGAATTAGAATTATTTACGAGGAAT
ACATCTAAGAAAAGACGGCAAAGAAAAAGATCAAAGGCTACACATTTTTCTAATCAAAATAAAGATGATACATCT
CAACAAGCTGATTTTGATGAAGAAATTTACTTGATAAATAAAGACTTCAAAAAAGAAGAAAGCAATGATAAAAAT
AATGATTCTGCTTCTAGTCATGCGAATGATAATAATATCGATGATTCTACAGACTCTAATATTGAAAATGAGGAT
TATAGATATAATCAAGAAATTGACGACCAAAATGAATCGAATGTAATTTCAGTCGACAACGAACAACCTCAATCA
GCTCCTAAAGAACAAAATAGCGACTCGATTGATGAGGAAACAGTAACGAAAAAAGAACGAAAAAGTAAAGTAACA
CAATTAAAGCCATTAACACTTGAAGAAAAGCGGAAGTTAAGACGTAAGCGACAAAAGCGAATCCAATACAGTGTT
ATTACAATATTGGTATTGTTGATTGCTGTTATATTAATTTACATGTTTTCACCACTTAGTAAAATTGCGCATGTA
AATATAAATGGAAATAATCACGTTAGTACTTCAAAGATAAACAAAGTTTTAGGTGTTAAAAATGATTCAAGGATG
TATACGTTTAGTAAAAAAAATGCTATTAATGATCTCGAAGAGAATCCATTAATCAAAAGTGTTGAGATACACAAG
CAATTACCAAACACATTAAACGTAGATATCACAGAAAATGAAATTATTGCTTTAGTGAAATATAAAGGTAAATAT
TTACCTTTATTAGAAAATGGTAAATTGCTTAAAGGTTCAAATGATGTCAAAATTAATGATGCACCTGTCATGGAT
GGTTTCAAAGGTACAAAAGAAGATGATATGATTAAGGCGTTATCTGAAATGACACCTGAAGTTAGACGATATATT
GCCGAAGTGACATACGCCCCAAGTAAAAACAAACAAAGCAGAATTGAATTGTTTACGACAGATGGACTTCAAGTA
ATCGGTGATATTTCGACGATATCTAAGAAAATGAAATATTATCCGCAGATGTCACAATCATTATCAAGGGATAGT

TCGGGTAAACTAAAAACACGAGGCTATATTGATTTATCAGTCGGTGCTTCATTTATCCCATACCGTGGAAACACG
TCTAGTCAATCAGAAAGCGATAAAAATGTGACTAAATCATCTCAAGAGGAAAATCAAGCAAAAGAAGAATTACAA
AGCGTTTTAAACAAAATTAACAAACAATCAAGTAAGAATAATTAA

SEQ 4 (complete *divIC* sequence)

ATGAAAAATAAAGTAGAACATATAGAAAATCAGTACACGTCGCAAGAGAACAAGAAAAAACAACGTCAAAAAATG
AAAATGCGTGTTGTTCGTAGGCGTATTACAGTATTTGCGGGCGTATTACTTGCGATAATTGTTGTTTTATCAATC
TTGCTTGTTGTCCAAAAACATCGCAATGATATTGATGCACAGGAGCGAAAAGCGAAAGAAGCACAGTTTCAAAAG
CAACAAAATGAAGAAATTGCGTTAAAAGAAAAGTTGAATAATCTGAATGACAAAGATTACATTGAAAAAATTGCG
CGTGATGATTATTACTTAAGCAACAAAGGTGAAGTGATTTTTAGGTTGCCAGAAGACAAAGATTCGTCTAGCTCA
AAATCTTCGAAAAAATAA

SEQ 5 (complete *ftsL* sequence)

ATGGCTGTAGAAAAAGTGTACCAACCATATGACGAACAAGTTTATAATAGTATACCGAAGCAACAACCACAAACT
AAGCCCGAAAAGAAGACTGTTTCGAGAAAAGTGGTTGTACAATTAACTAAATTTGAAAAAGTTTTATACATAACT
TTGATTACTGTAATTGCTATGTTAAGTATTTATATGCTATCTTTAAAAATGGATGCGTATGATACGCGAGGAAAG
ATTGCAGATTTAGATTATAAAATAGATAAACAATCAAGTGAAAACAGTGCTTTACAATCTGAAATCAAAAAGAAT
TCTTCTTATGAACGCATATACGAAAAGGCTAAGAAACAGGGGATGAGCCTTGAGAACGATAATGTAAAGGTAGTG
CGTAGTAATGGCGAAGCAAAAAATTAA

SEQ 6 (selected *pheP* peptide DNA sequence)

CTTTATTTCTGGGACACATTTAAATTTTTCCACCCCATTACT

SEQ 7 (extramembranous *ydiE/gcp* sequence

GTTCATCATATTGCAGGACATATATATGCTAATCACATAGAAGAGCCATTAACATTCCCGCTAATTGCACTTATT
GTTTCAGGTGGACATACTGAATTAGTTTATATGAAGGATCATTTATCATTTGAAGTCATTGGTGAAACACGAGAT
GACGCAGTAGGTGAGGCTTATGATAAAGTGGCACGAACAATTGGTTTAAATTATCCAGGTGGTCCACAAGTTGAT
CGGTTGGCTGCTGAAGGTGAAGATACTTATTCATTCCCTCGTGTTTGGTTGGATAAAGATAGTTATGATTTTAGT
TTTAGTGGGTTGAAAAGTGCCGTAATCAATCAACTTCACAATCAACGACAAAAAAATATTCCAATCATTGAAGCT
AACGTAGCAACGAGCTTTCAAAACAGTGTTGTAGAGGTGCTCACGTTTAAAGCTATTCAAGCTTGTAAAGAATAT
GGTGTTCAGCGATTAATTGTTGCTGGTGGCGTGGCGAGTAATAAAGGATTACGTCAATCTTTAGCGGATCAATGC
AAAGTCAATGACATTCAATTAACTATCCCAAGTCCTAAATTATGCACAGATAATGCTGCAATGATAGGCGTTGCC
GGCCACTATTTGTATCAGCAAGGTCGATTTGCTGATTTAGCATTAAATGGGCACAGCAATATAGATTTAGAAGAG
TATTCTGCAGAA

SEQ 8 (extramembranous divIB sequence, also named in preceding patent DivIB-1))

CCACTTAGTAAAATTGCGCATGTAAATATAAATGGAAATAATCACGTTAGTACTTCAAAGATAAACAAAGTTTTA
GGTGTTAAAAATGATTCGAGGATGTATACGTTTAGTAAAAAAAATGCTATTAATGATCTCGAAGAGGATCCATTA
ATCAAAAGTGTTGAGATACACAAGCAATTACCAAACACATTAAACGTAGATATCACAGAAAATGAAATTATTGCT
TTAGTGAAATATAAAGGTAAATATTTACCTTTATTAGAAAATGGTAAATTGCTTAAAGGTTCAAATGATGTCAAA
ATTAATGATGCACCTGTCATGGATGGTTTCAAAGGTACAAAAGAAGATGATATGATTAAGGCGTTATCTGAAATG
ACACCTGAAGTTAGACGATATATTGCCGAAGTGACATACGCCCCAAGTAAAAACAAACAAAGCAGAATTGAATTG
TTTACGACAGATGGACTTCAAGTAATCGGTGATATTTCGACGATATCTAAGAAAATGAAATATTATCCGCAGATG
TCACAATCATTATCAAGGGATAGTTCGGGTAAACTAAAAACAAGAGGCTATATTGATTTATCAGTCGGTGCTTCA
TTTATCCCATACCGTGGAAACACGTCTAGTCAATCAGAAAGCGATAAAAATGTGACTAAATCATCTCAAGAGGAA
AATCAAGCAAAAGAAGAATTACAAAGCGTTTTAAACAAAATTAACAAACAATCAAGTAAGAATAAT

SEQ 9 (extramembranous divIC sequence)

AAACATCGCAATGATATTGATGCACAGGAGCGAAAAGCGAAAGAAGCACAGTTTCAAAAGCAACAAAATGAAGAA
ATTGCGTTAAAAGAAAAGTTGAATAATCTGAATGACAAAGATTACATTGAAAAAATTGCGCGTGATGATTATTAC
TTAAGCAACAAAGGTGAAGTGATTTTTAGGTTGCCAGAAGACAAAGATTCGTCTAGCTCAAAATCTTCGAAAAAA

SEQ 10 (extramembranous *ftsL* sequence)

AAAATGGATGCGTATGATACGCGAGGAAAGATTGCAGATTTAGATTATAAAATAGATAAACAATCAAGTGAAAAC
AGTGCTTTACAATCTGAAATCAAAAAGAATTCTTCTTATGAACGCATATACGAAAAGGCTAAGAAACAGGGGATG
AGCCTTGAGAACGATAATGTAAAGGTAGTGCGTAGTAATGGCGAAGCAAAAAAT

SEQ 11 (T7-tag / intermediate bases / *ydiE* extramembranous / intermediate bases / His-tag sequence)

ATGGCTGTTCATCATATTGCAGGACATATATATGCTAATCACATAGAAGAGCCATTAACATTCCCGCTAATTGCA
CTTATTGTTTCAGGTGGACATACTGAATTAGTTTATATGAAGGATCATTTATCATTTGAAGTCATTGGTGAAACA
CGAGATGACGCAGTAGGTGAGGCTTATGATAAAGTGGCACGAACAATTGGTTTAAATTATCCAGGTGGTCCACAA
GTTGATCGGTTGGCTGCTGAAGGTGAAGATACTTATTCATTCCCTCGTGTTTGGTTGGATAAAGATAGTTATGAT
TTTAGTTTTAGTGGGTTGAAAAGTGCCGTAATCAATCAACTTCACAATCAACGACAAAAAAATATTCCAATCATT
GAAGCTAACGTAGCAACGAGCTTTCAAAACAGTGTTGTAGAGGTGCTCACGTTTAAAGCTATTCAAGCTTGTAAA
GAATATGGTGTTCAGCGATTAATTGTTGCTGGTGGCGTGGCGAGTAATAAAGGATTACGTCAATCTTTAGCGGAT
CAATGCAAAGTCAATGACATTCAATTAACTATCCCAAGTCCTAAATTATGCACAGATAATGCTGCAATGATAGGC
GTTGCCGGCCACTATTTGTATCAGCAAGGTCGATTTGCTGATTTAGCATTAAATGGGCACAGCAATATAGATTTA
GAAGAGTATTCTGCAGAACTCGAGCACCACCACCACCACCACTGA

SEQ 12 (T7-tag / intermediate bases / *divIB* extramembranous / intermediate bases / His-tag sequence)

ATGGCTCCACTTAGTAAAATTGCGCATGTAAATATAAATGGAAATAATCACGTTAGTACTTCAAAGATAAACAAA
GTTTTAGGTGTTAAAAATGATTCGAGGATGTATACGTTTAGTAAAAAAAATGCTATTAATGATCTCGAAGAGGAT
CCATTAATCAAAAGTGTTGAGATACACAAGCAATTACCAAACACATTAAACGTAGATATCACAGAAAATGAAATT
ATTGCTTTAGTGAAATATAAAGGTAAATATTTACCTTTATTAGAAAATGGTAAATTGCTTAAAGGTTCAAATGAT
GTCAAAATTAATGATGCACCTGTCATGGATGGTTTCAAAGGTACAAAAGAAGATGATATGATTAAGGCGTTATCT
GAAATGACACCTGAAGTTAGACGATATATTGCCGAAGTGACATACGCCCCAAGTAAAAACAAACAAAGCAGAATT
GAATTGTTTACGACAGATGGACTTCAAGTAATCGGTGATATTTCGACGATATCTAAGAAAATGAAATATTATCCG
CAGATGTCACAATCATTATCAAGGGATAGTTCGGGTAAACTAAAAACAAGAGGCTATATTGATTTATCAGTCGGT
GCTTCATTTATCCCATACCGTGGAAACACGTCTAGTCAATCAGAAAGCGATAAAAATGTGACTAAATCATCTCAA
GAGGAAAATCAAGCAAAAGAAGAATTACAAAGCGTTTTAAACAAAATTAACAAACAATCAAGTAAGAATAATCTC
GAGCACCACCACCACCACCACTGA

SEQ 13 (T7-tag / intermediate bases / *divIC* extramembranous / intermediate bases / His-tag sequence)

ATGGCTAAACATCGCAATGATATTGATGCACAGGAGCGAAAAGCGAAAGAAGCACAGTTTCAAAAGCAACAAAAT
GAAGAAATTGCGTTAAAAGAAAAGTTGAATAATCTGAATGACAAAGATTACATTGAAAAAATTGCGCGTGATGAT
TATTACTTAAGCAACAAAGGTGAAGTGATTTTTAGGTTGCCAGAAGACAAAGATTCGTCTAGCTCAAAATCTTCG
AAAAAACTCGAGCACCACCACCACCACCAC

SEQ 14 (T7-tag / intermediate bases / *ftsL* extramembranous / intermediate bases / His-tag sequence)

ATGGCTAAAATGGATGCGTATGATACGCGAGGAAAGATTGCAGATTTAGATTATAAAATAGATAAACAATCAAGT
GAAAACAGTGCTTTACAATCTGAAATCAAAAAGAATTCTTCTTATGAACGCATATACGAAAAGGCTAAGAAACAG
GGGATGAGCCTTGAGAACGATAATGTAAAGGTAGTGCGTAGTAATGGCGAAGCAAAAAATCTCGAGCACCACCAC
CACCACCAC

SEQ 15 (complete PheP sequence)

MEDNKMNRSLNSRHISMIAIGGAIGTGLFVATGNIISQAGPGGAILAYLVIGVMLYFLMSSIGELATF
YPVSGSFSSYSTRFIDSSLGFTMGWLYWALWSLVTSVDVIVASNVLYFWDTFKFFHPITWSLIFITIL
LLLNIFSVKSFGETEFWLSLIKVLTIIVFVIFGFLMIFGILGGHTYGFENYTKGQAPFVGGISGFLGV
LLVAGFSVGGTEVVAVTAGESDDPKKSMPKAIKQVFWRILLFYVLSIAVIGAIIPYTDPSLLRASSSI
SQSPFTIVFDRVGIAFAASVINAVILTSLLSAANSGVYTTGRMLYSLSSDKKAPQFLSKLNKTTKLPL
RALLTTYAVVVIVIIYANFNSNAVFNLLEIIGSMIIVVWGSSIWSQIRLRQAIKKQGQDPNKVLPYKA
PFYPLGPIIVITTLLFLLFGGSVEYILKDQWLNAFKNFLPLIILALIYFIHKIIHKTKFVKLETINLK
PHDYDNQK

SEQ 16 (complete YdiE sequence)

MTKDILILAVETSCDETSVSVIKNGRDILSNTVLSQIESHKRFGGVVPEVASRHHVEGITATINEALG
DADVSIEDIDAIAVTEGPGLIGALLIGVNAAKALAFAYDKPLIPVHHIAGHIYANHIEEPLTFPLIAL
IVSGGHTELVYMKDHLSFEVIGETRDDAVGEAYDKVARTIGLNYPGGPQVDRLAAEGEDTYSFPRVWL
DKDSYDFSFSGLKSAVINQLHNQRQKNIPIIEANVATSFQNSVVEVLTFKAIQACKEYGVQRLIVAGG
VASNKGLRQSLADQCKVNDIQLTIPSPKLCTDNAAMIGVAGHYLYQQGRFADLALNGHSNIDLEEYSA
E

SEQ 17 (complete DivIB sequence)

MDDKTKNDQQESNEDKDELELFTRNTSKKRRQRKRSKATHFSNQNKDDTSQQADFDEEIYLINKDFKKEESNDKN
NDSASSHANDNNIDDSTDSNIENEDYRYNQEIDDQNESNVISVDNEQPQSAPKEQNSDSIDEETVTKKERKSKVT
QLKPLTLEEKRKLRRKRQKRIQYSVITILVLLIAVILIYMFSPLSKIAHVNINGNNHVSTSKINKVLGVKNDSRM
YTFSKKNAINDLEENPLIKSVEIHKQLPNTLNVDITENEIIALVKYKGKYLPLLENGKLLKGSNDVKINDAPVMD
GFKGTKEDDMIKALSEMTPEVRRYIAEVTYAPSKNKQSRIELFTTDGLQVIGDISTISKKMKYYPQMSQSLSRDS
SGKLKTRGYIDLSVGASFIPYRGNTSSQSESDKNVTKSSQEENQAKEELQSVLNKINKQSSKNN

SEQ 18 (complete DivIC sequence)

MKNKVEHIENQYTSQENKKKQRQKMKMRVVRRRITVFAGVLLAIIVVLSILLVVQKHRNDIDAQERKAKEAQFQK
QQNEEIALKEKLNNLNDKDYIEKIARDDYYLSNKGEVIFRLPEDKDSSSSKSSKK

SEQ 19 (complete FtsL sequence)

MAVEKVYQPYDEQVYNSIPKQQPQTKPEKKTVSRKVVVQLTKFEKVLYITLITVIAMLSIYMLSLKMDAYDTRGK
IADLDYKIDKQSSENSALQSEIKKNSSYERIYEKAKKQGMSLENDNVKVVRSNGEAKN

SEQ 20 (selected extramembranous PheP sequence)

LYFWDTFKFFHPIT

SEQ 21 (extramembranous YdiE sequence)

VHHIAGHIYANHIEEPLTFPLIALIVSGGHTELVYMKDHLSFEVIGETRDDAVGEAYDKVARTIGLNYPGGPQVD
RLAAEGEDTYSFPRVWLDKDSYDFSFSGLKSAVINQLHNQRQKNIPIIEANVATSFQNSVVEVLTFKAIQACKEY
GVQRLIVAGGVASNKGLRQSLADQCKVNDIQLTIPSPKLCTDNAAMIGVAGHYLYQQGRFADLALNGHSNIDLEE
YSAE

SEQ 22 (extramembranous DivIB sequence, also named in preceding patent DivIB-1)

PLSKIAHVNINGNNHVSTSKINKVLGVKNDSRMYTFSKKNAINDLEEDPLIKSVEIHKQLPNTLNVDITENEIIA
LVKYKGKYLPLLENGKLLKGSNDVKINDAPVMDGFKGTKEDDMIKALSEMTPEVRRYIAEVTYAPSKNKQSRIEL
FTTDGLQVIGDISTISKKMKYYPQMSQSLSRDSSGKLKTRGYIDLSVGASFIPYRGNTSSQSESDKNVTKSSQEE
NQAKEELQSVLNKINKQSSKNN

SEQ 23 (extramembranous DivIC sequence)

KHRNDIDAQERKAKEAQFQKQQNEEIALKEKLNNLNDKDYIEKIARDDYYLSNKGEVIFRLPEDKDSSSSKSSKK

SEQ 24 (extramembranous *FtsL* sequence)

KMDAYDTRGKIADLDYKIDKQSSENSALQSEIKKNSSYERIYEKAKKQGMSLENDNVKVVRSNGEAKN

SEQ 25 (T7-tag / intermediate bases / YdiE extramembranous / intermediate bases / His-tag sequence)

MAVHHIAGHIYANHIEEPLTFPLIALIVSGGHTELVYMKDHLSFEVIGETRDDAVGEAYDKVARTIGLNYPGGPQ
VDRLAAEGEDTYSFPRVWLDKDSYDFSFSGLKSAVINQLHNQRQKNIPIIEANVATSFQNSVVEVLTFKAIQACK
EYGVQRLIVAGGVASNKGLRQSLADQCKVNDIQLTIPSPKLCTDNAAMIGVAGHYLYQQGRFADLALNGHSNIDL
EEYSAELE**HHHHHH**

SEQ 26 (T7-tag / intermediate bases / DivIB extramembranous / intermediate bases / His-tag sequence; also termed DivB-2 in a preceding patent)

MAPLSKIAHVNINGNNHVSTSKINKVLGVKNDSRMYTFSKKNAINDLEEDPLIKSVEIHKQLPNTLNVDITENEI
IALVKYKGKYLPLLENGKLLKGSNDVKINDAPVMDGFKGTKEDDMIKALSEMTPEVRRYIAEVTYAPSKNKQSRI
ELFTTDGLQVIGDISTISKKMKYYPQMSQSLSRDSSGKLKTRGYIDLSVGASFIPYRGNTSSQSESDKNVTKSSQ
EENQAKEELQSVLNKINKQSSKNNLE**HHHHHH**

SEQ 27 (T7-tag / intermediate bases / DivIC extramembranous / intermediate bases / His-tag sequence)

MAKHRNDIDAQERKAKEAQFQKQQNEEIALKEKLNNLNDKDYIEKIARDDYYLSNKGEVIFRLPEDKDSSSSKSS
KKLE**HHHHHH**

SEQ 28 (T7-tag / intermediate bases / ftsL extramembranous / intermediate bases / His-tag sequence)

MAKMDAYDTRGKIADLDYKIDKQSSENSALQSEIKKNSSYERIYEKAKKQGMSLENDNVKVVRSNGEAKNLE**HHH
HHH**

Table 1 – Oligonucleotide sequences

Progenitor gene name	Code - complete gene progenitor sequence	Code - fragment sequence amplified from progenitor	Oligonucleotide name	Oligonucleotide sequence code	Oligonucleotide sequence	Name of the resulting over-expression plasmids
ydiE/gcp	Sequence 2	Sequence 21	5'GLUSh318B	**Sequence 29**	ATAATACCATGGCTGTTCATCATATTGCAGGAC	pGL597
			3'GLUSh318B	**Sequence 30**	ATAATACTCGAGTTCTGCAGAATACTCTTCTAAATC	
divIB	Sequence 3	Sequence 22	5'GLUSh341C	**Sequence 31**	ATAATACCATGGCTCCACTTAGTAAAATTGCGCATG	pGL601
			3'GLUSh341C	**Sequence 32**	ATAATACTCGAGATTATTCTTACTTGATTGTTTG	
divIC	Sequence 4	Sequence 23	ALB21	**Sequence 33**	ATAATACCATGGCTAAACATCGCAATGATATTGAT	pALB26
			ALB22	**Sequence 34**	ATAATTCTCGAGTTTTTTCGAAGATTTTGAGCT	
ftsL	*Sequence* 5	*Sequence* 24	*ALB19*	**Sequence 35**	*ATAATACCATGGCTAAAATGGATGCGTATGATACG*	pALB27
			ALB20	**Sequence 36**	ATAATACTCGAGATTTTTTGCTTCGCCATTACT	

CCATGG : NcoI
CTCGAG: XhoI

Table 2 – Multivalent vaccine experiments: Vaccine combinations

	Antigen											
	Tier 1				Tier 2				Tier 3			
	YdiE	DivIB	DivIC	FtsL SEQ 5 SEQ10 SEQ19 SEQ24	YdiE	DivIB	DivIC	FtsL	YdiE	DivIB	DivIC	FtsL
PheP SEQ 1 SEQ 6 SEQ 15 SEQ 20	✓	✓	✓	✓								
	✓	✓										
							✓	✓				
									✓			
											✓	
												✓
YdiE SEQ 2 SEQ 7 SEQ 16 SEQ 21		✓										
						✓	✓	✓				
							✓	✓				
											✓	
												✓
DivIB SEQ 3 SEQ 8 SEQ 17 SEQ 22			✓	✓								
							✓					
								✓				
DivIC SEQ 4 SEQ 9 SEQ 18 SEQ 23								✓				

Table 3

	%Body Weight Loss			
	Control Sample	Experimental Samples		
Animal Number	**I** Adjuvant alone (Freunds)	**II** Antigen: rYdiE	**III** Antigen: rDivIB	**IV** Combination Antigen: rYdiE rDivIB
1	11.1	11.3	13.1	2.3
2	12.7	4.3	0.0	2.0
3	9.7	9.0	3.5	0.0
4	7.6	12.6	0.5	-4.3
5	9.4	1.0	7.1	-0.5
6	9.9	6.2	3.3	1.8
7	2.0	3.8	2.1	1.5
8	4.2	5.3	1.9	1.4
9	1.0	3.4	1.0	1.6
10	3.1	7.0	6.8	3.9
11	7.7	4.7	-2.5	-0.5

Table 4

	Log10 CFUs in Kidneys per animal			
	Control Sample	Experimental Samples		
Animal Number	**I** Adjuvant alone (Freunds)	**II** Antigen: rYdiE	**III** Antigen: rDivIB	**IV** Combination Antigen: rYdiE rDivIB
1	7.82	6.98	5.6	6.04
2	7.73	6.5	6.5	6.12
3	7.7	5.61	4.64	5.97
4	6.98	7.61	5.99	5.61
5	6.74	5.09	6.54	5.38
6	7.32	6.38	6.03	5.88
7	6.5	6.6	6.41	6.74
8	6.2	6.75	6.66	5.96
9	5.61	6.72	7.88	6.98
10	6.06	6.63	6.63	6.68
11	7.04	6.45		4.74