

A&A Ref: **147628**

PUBLICATION PARTICULARS AND ABSTRACT
(Section 32(3)(a) - Regulations 22(1)(g) and 31)

21	01	PATENT APPLICATION NO	22	LODGING DATE	43	ACCEPTANCE DATE
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2003/1759

3 March 2003

22 6.04

51	INTERNATIONAL CLASSIFICATION	NOT FOR PUBLICATION
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A61K, A61P

CLASSIFIED BY: **ISA**

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EARLIEST PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
	33 GB	31 0021757.0	32 4 September 2000

NOTE: The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations

54	TITLE OF INVENTION
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Vaccine against microbial pathogens

57	ABSTRACT (NOT MORE THAN 150 WORDS)
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NUMBER OF SHEETS <i>50</i>

The sheet(s) containing the abstract is/are attached.

If no classification is furnished, Form P.9 should accompany this form.

~~The figure of the drawing to which the abstract refers is attached.~~

(67) Abstract: The present invention provides a vaccine comprising a microbial pathogen, wherein the microbial pathogen is subjected to a stress inducing stimuli. The stress inducing stimuli can be heat or osmotic stress, through preferably the microbial pathogen is genetically modified such that at least one repressor gene for a heat shock protein gene is inactivated, this allowing the constitutive expression of heat shock proteins. In particular the use of heat shock protein repressor mutant bacteria is shown to be effective for inducing immunity when comprised within vaccines of the present invention. The present invention further provides a method for producing a vaccine comprising stressed induced microbial pathogens and further the use of the heat shock protein repressor deletion mutant microbes as vaccine vectors which can be additionally allow the expression of heterologous antigen fragments.

1 **"VACCINE AGAINST MICROBIAL PATHOGENS"**

2

3 The present invention relates to a vaccine and a
4 method for producing a vaccine. More specifically,
5 there is provided a vaccine comprising a microbial
6 pathogen and a method of producing the same.

7

8 BACKGROUND OF THE INVENTION

9

10 An important component of any human immune response
11 is the presentation of antigens to T cells by
12 antigen presenting cells (APCs) such as macrophages,
13 B cells or dendritic cells. Peptide fragments of
14 foreign antigens are presented on the surface of the
15 macrophage in combination with major
16 histocompatibility complex (MHC) molecules, in
17 association with helper molecules, such as CD4 and
18 CD8 molecules. Such antigenic peptide fragments
19 presented in this way are recognised by the T cell

1 receptor of T cells. The interaction of the
2 antigenic peptide fragments with the T cell receptor
3 results in antigen-specific T cell proliferation,
4 and secretion of lymphokines by the T-cells. The
5 nature of the antigenic peptide fragment presented
6 by the APCs is critical in establishing immunity.

7
8 Heat shock proteins (hsps) form a family of highly
9 conserved proteins that are widely distributed
10 throughout the plant and animal kingdoms. On the
11 basis of their molecular weights, hsps are grouped
12 into six different families: small (hsp 20-30kDa);
13 hsp40; hsp60; hsp70; hsp90; and hsp100. Although
14 hsps were originally identified in cells subjected
15 to heat stress, they have been found to be
16 associated with many other forms of stress, such as
17 infections, and are thus also commonly referred to
18 as stress proteins (SPs).

19
20 Members of the mammalian hsp90 family include
21 cytosolic hsp90 (hsp83) and the endoplasmic
22 reticulum counterparts hsp90 (hsp83), hsp87, Grp94
23 (Erp99) and gp97, see for example Gething et al.
24 (1992) *Nature* 355:33-45. Members of the hsp70 family
25 include cytosolic hsp70 (p73) and hsp70 (p72), the
26 endoplasmic reticulum counterpart BiP (Grp78), and
27 the mitochondrial counterpart hsp70 (Grp75). Members
28 of the mammalian hsp60 family have only been
29 identified in the mitochondria.

30
31 Stress proteins are ubiquitously expressed within
32 cells. One of the roles of stress proteins is to

1 chaperone peptides from one cellular compartment to
2 another and to present peptides to the MHC molecules
3 for cell surface presentation to the immune system.
4 In the case of diseased cells, stress proteins also
5 chaperone viral or tumour-associated peptides to the
6 cell-surface, see Li and Sirivastave (1994) *Behring*
7 *Inst. Mitt*, 94: 37-47 and Suzue et al. (1997)
8 *Proc.Natl.Acad.Sci. USA* 94: 13146-51.

9
10 The chaperone function of stress proteins is
11 accomplished through the formation of complexes
12 between stress proteins and antigenic peptide
13 fragments, and between stress proteins and viral or
14 tumour-associated peptide fragments, in an ATP
15 dependent reaction. The peptide fragments complexed
16 with the stress proteins form antigenic complexes
17 (hspCs) which are captured by APCs to provide
18 antigenic peptide fragments.

19
20 The complex association of stress proteins with
21 peptide fragments has also been observed in normal
22 tissues and is not therefore a tumour-specific
23 phenomenon, see Srivastava (1994) *Experimentia* 50:
24 1054-60. It is thought that the immunogenicity of
25 the members of the hsp70 family, including grp96,
26 reflects their normal role in the 'presentosome',
27 the postulated intracellular organelle functioning
28 in the loading of MHC Class I molecules required for
29 their cell surface expression. This step is
30 essential for their MHC-restricted recognition by
31 antigen-specific T-cells, see Srivastava et al.

1 (1998) *Immunity*, Singh-Jasuja et al. *J.Exp.Med*
2 (2000) 191, 1957.

3
4 Until recently, it has not been proposed to use
5 pathogen-derived endogenous hsp-peptide complexes
6 (hspCs) as vaccines, despite the fact that hsps from
7 pathogens have been used extensively as antigens and
8 adjuvants. PCT Application No GB00/03228 discloses
9 the use of pathogen-derived endogeneous hspCs and in
10 particular stress induced hspCs are shown to give
11 good protective immunity in vaccinated animals.

12
13 Members of the microbial hsp family include the Dna
14 J and Dna K families and the Gro-EL and Gro-ES
15 families. In prokaryotic microbes it appears that
16 these families are encoded in operons, with the
17 initial gene in the operon being a control gene
18 which suppresses the expression of the hsp genes
19 contained within the operon.

20
21 In *Streptomyces* and *Helicobacter*, expression of the
22 hspR gene suppresses the expression of Dna J and Dna
23 K. Deletion of the hspR gene therefore results in a
24 genetically modified microbe that constitutively
25 expresses hsps, see Bucca et al. (1997)
26 *Mol.Microbiol* 15:633-45. Homologous operons have
27 been identified in a number of recently sequenced
28 microbes, including other strains of *Streptomyces*
29 and *Mycobacterium tuberculosis* and the commonly used
30 related vaccine strain BCG.

31

1 Other repressor genes may also control the
2 expression of members of the hsp gene family and
3 that these may also be genetically engineered to
4 provide modified microbes that constitutively
5 expresses hspCs that may be utilised for the
6 production of vaccines and vaccine vectors. These
7 include, but are not limited to the transcriptional
8 control genes sigma and rho and the stress-gene
9 regulatory protein genes MerR and HmrR. It will also
10 be appreciated that the modified microbes containing
11 the constitutive hspCs may be used directly as
12 vaccines or as a source for the isolation of the
13 hspCs. Furthermore the expression of heterologous
14 gene(s) from other pathogen(s) in these microbes
15 will enable their use as vaccine vectors for the
16 simplified production of hspC-based vaccines for
17 these pathogens.

18
19 Whilst looking at the use of heat-induced
20 endogeneous microbial hsp-peptide complexes, the
21 extracted hsp-peptide complexes were compared to the
22 use of the stressed-induced microbe itself as a
23 vaccine. Surprisingly, the use of the microbe as a
24 vaccine gave significantly better immunity in
25 vaccinated animals than the use of the isolated hsp-
26 peptide complexes. Similar results were obtained
27 using genetically modified microbes that
28 constitutively produced hsps, indicating that hspCs
29 were formed *in situ* with endogenous microbial
30 polypeptides, including heterogenous genes expressed
31 as recombinant proteins in the microbe. The
32 genetically modified microbes could also be used as

1 an efficient source for the isolation of hspCs for
2 use in subunit and multi-subunit vaccines.

3
4 SUMMARY OF THE INVENTION

5
6 According to the present invention, there is
7 provided a vaccine comprising a microbial pathogen
8 as an immunogenic determinant, wherein the microbial
9 pathogen has been subjected to a stress inducing
10 stimuli.

11
12 Preferably the stress inducing stimuli results in
13 the expression of heat shock proteins by the
14 microbe.

15
16 Preferably the stress inducing stimuli is heat or
17 osmotic shock.

18
19 More preferably the stress inducing stimuli is the
20 genetic modification of the microbial pathogen such
21 that at least one repressor gene for a heat shock
22 protein gene is inactivated, thereby allowing the
23 expression of a heat shock protein gene.

24
25 Preferably the microbial pathogen is any pathogen
26 which is capable of inducing an infectious disease.

27
28 Preferably the microbial pathogen is a bacteria,
29 protozoa, fungi or a parasitic organism.

30
31 Preferably the genetic modification results in the
32 inactivation of the hspR repressor gene.

1 Alternatively the genetic modification results in
2 the inactivation of the stress gene regulatory
3 protein genes MerR or HmrR repressor genes, or the
4 transcriptional control genes sigma and rho.

5

6 Preferably the microbial pathogen is selected from
7 the group consisting of Mycobacteria, Salmonella,
8 Vibrio, Streptomyces, Helicobacter, Lactococcus and
9 Listeria.

10

11 Preferably the microbial pathogen is attenuated.

12

13 Preferably the vaccine further comprises an
14 adjuvant.

15

16 Preferably the adjuvant is selected from the group
17 consisting of; Freund's complete adjuvant, Freund's
18 incomplete adjuvant, Quil A, Detox, ISCOMs and
19 squalene.

20

21 Preferably the vaccine is suitable for
22 administration by injection.

23

24 Alternatively the vaccine is suitable for oral
25 administration.

26

27 Preferably the vaccine is suitable for delivery by
28 means of a needle-less delivery format.

29

30 A further aspect of the present invention provides a
31 method of vaccinating an animal, characterised in
32 that said method comprises administering a

1 pharmaceutically acceptable quantity of a vaccine
2 composition according to the present invention
3 sufficient to elicit an immune response in the
4 animal.

5

6 Preferably the vaccine is administered as a
7 prophylactic vaccine.

8

9 Alternatively the vaccine is administered as a
10 therapeutic vaccine.

11

12 Preferably the vaccine composition is administered
13 by injection.

14

15 Alternatively the vaccine composition is
16 administered by needle-less delivery.

17

18 Alternatively the vaccine composition is
19 administered transdermally, by pulmonary delivery or
20 orally.

21

22 A yet further aspect of the present invention
23 provides a method of producing a vaccine
24 composition, comprising an immunogenic determinant,
25 characterised in that said method comprises the
26 steps of; subjecting microbial pathogens to stress
27 inducing stimuli; and using the stressed microbe in
28 the preparation of the vaccine composition as said
29 immunogenic determinant.

30

31 Preferably the stress inducing stimuli is heat or
32 osmotic shock.

1

2 More preferably the stress inducing stimuli is the
3 inactivation of a gene which represses the
4 expression of heat shock genes.

5

6 Preferably the repressed gene is the hspR gene.

7

8 Alternatively the repressed gene is the stress gene
9 regulatory protein gene MerR or HmrR, or the
10 transcriptional control genes sigma and rho.

11

12 Preferably the microbial pathogen cells are killed
13 prior to use in said vaccine.

14

15 Alternatively the microbial pathogen cells are dried
16 prior to use in said vaccine.

17

18 Preferably the vaccine composition is an aqueous
19 composition.

20

21 Alternatively the vaccine composition is a dry
22 composition or in a lyophilised composition.

23

24 The present invention further provides the use of a
25 pathogenic microbe which has been genetically
26 modified such that at least one repressor gene for a
27 heat shock protein is inactivated, thereby allowing
28 constitutive expression of heat shock proteins, as a
29 vaccine vector which further allows the expression
30 of a heterologous antigen.

31

1 Preferably the pathogenic microbe is a prokaryotic
2 organism, which is suitable for use as a vaccine
3 vector.

4

5 Preferably the prokaryotic organism is BCG and the
6 heterologous antigen fragment is the tetanus toxoid
7 fragment C.

8

9 Alternatively the prokaryotic organism is Salmonella
10 or Lactococcus, such that the vaccine can be
11 administered orally.

12

13 The present invention further provides the use of a
14 pathogenic microbe which has been genetically
15 modified such that at least one repressor gene for a
16 heat shock protein is inactivated, thereby allowing
17 constitutive expression of heat shock proteins, as a
18 source of heat shock protein - peptide complexes for
19 use in subunit and multi-subunit vaccines.

20

21 By "microbial pathogen" it is meant any pathogen
22 capable of inducing an infectious disease in an
23 animal, including particularly bacterial, protozoan,
24 fungal or parasitic organisms.

25

26 By "stress-inducing stimuli" it is meant any stimuli
27 that induces the production of SPs and in particular
28 hsp's in microbial pathogens, including heat or
29 osmotic stress. Such stress-inducing stimuli also
30 includes genetic changes designed to render
31 constitutive the expression of stress proteins and
32 in particular hsp's in microbial pathogens. These

1 genetic changes include the inactivation of
2 repressor (suppressor) genes that function in the
3 suppression of stress proteins and in particular the
4 suppression of the genes which express heat shock
5 proteins. This includes in particular the
6 inactivation of the hspR suppressor gene, the stress
7 gene regulatory protein genes MerR and HmrR, or the
8 transcriptional control genes sigma and rho.

9
10 It will be appreciated that such genetic changes are
11 readily achieved by molecular genetic means and
12 include insertional mutagenesis using phage or
13 transposon vectors, see for example Bucca et al.
14 *Mol.Microbiol.* (1997) 17:633. It will also be
15 appreciated that the presence of the genes for the
16 operons, suppressor genes and SPs and hsps in
17 microbial pathogens can be simply established by
18 recombinant DNA techniques, see *Current Techniques*
19 *in Molecular Biology*, (1999) Wiley Press.

20
21 Thus it should be appreciated that any microbe
22 expressing these genes can be genetically modified
23 to provide a vaccine according to this invention.
24 This includes existing microbial organisms used as
25 live or killed vaccines, such as *Mycobacteria*,
26 *Salmonella*, *Vibrio* and *Listeria* and in particular
27 attenuated varieties of these pathogens. In
28 particular, *Mycobacteria* mutants, which have been
29 genetically modified to delete the heat shock
30 protein repressor gene could be used in a vaccine
31 against tuberculosis, while *Salmonella* mutants which
32 have been genetically modified to delete the heat

1 shock protein repressor genes could be used in a
2 vaccine against typhoid. The use of such organisms
3 as vaccine vectors for the expression of
4 heterologous genes is further provided by the
5 present invention.

6
7 The term "vaccine" is used herein to denote to any
8 composition containing an immunogenic determinant
9 which stimulates the immune system such that it can
10 better respond to subsequent infections. It will be
11 appreciated that a vaccine usually contains an
12 immunogenic determinant and an adjuvant, the
13 adjuvant serving to non-specifically enhance the
14 immune response to that immunogenic determinant.
15 Suitable adjuvants are readily apparent to the
16 person skilled in the art, and include; Freund's
17 complete adjuvant, Freund's incomplete adjuvant,
18 Quil A, Detox, ISCOMs or squalene.

19
20 However, it will be appreciated that the vaccine of
21 the present invention may also be effective without
22 an adjuvant.

23
24 For the non-genetic induction of SPs, the optimum
25 conditions for inducing the SPs can readily be
26 determined by simple trial and error with the effect
27 of a change of stimuli being assessed using
28 conventional techniques, such as *in vivo* testing on
29 animals, or by other techniques, for example those
30 described in 'Current Protocols in Immunology',
31 Wiley Interscience, 1997. Other such conditions are

1 described in PCT Application No GB00/03228 and
2 citations referred to therein.

3
4 The invention also provides a method for exposing an
5 animal to a vaccine of the invention by
6 administering a pharmaceutically acceptable quantity
7 of the vaccine of the invention, optionally in
8 combination with an adjuvant, sufficient to elicit
9 an immune response in the animal.

10

11 The animal is typically a human. However, the
12 invention can also be applied to the treatment of
13 other mammals such as horses, cattle, goats, sheep
14 or swine, and to the treatment of birds, notably
15 poultry such as chicken or turkeys. Preferably the
16 microbial pathogen selected for use in a particular
17 vaccine of the present invention causes disease or
18 infection in the species of animal to which the
19 vaccine is administered to, or a closely related
20 species. The vaccines of this invention may be used
21 as both prophylactic or therapeutic vaccines though
22 it will be appreciated that they will be
23 particularly useful as prophylactic vaccines due to
24 their economy of production.

25

26 The vaccine compositions of the present invention
27 may be administered by any suitable means, such as
28 orally, by inhalation, transdermally or by injection
29 and in any suitable carrier medium. However, it is
30 preferred to administer the vaccine as an aqueous
31 composition by injection using any suitable needle
32 or needle-less technique.

1

2 It will be appreciated that the vaccine of the
3 invention may be applied as an initial treatment
4 followed by one or more subsequent "booster"
5 treatments at the same or a different dosage rate at
6 an interval of from 1 to 26 weeks between each
7 treatment to provide prolonged immunisation against
8 the pathogen.

9

10 The present invention will now be described, by way
11 of example, with reference to the accompanying
12 figures, wherein;

13

14 Figure 1 shows the nucleotide sequence of the
15 hspR gene of *M. tuberculosis*, and

16

17 Figure 2 shows a list of identified suppressor
18 genes which have homology to hspR.

19

20 **Example 1: Preparation of heat-induced microbes.**

21

22 *M.vaccae* strain NCTC11659 was grown to saturation in
23 Sauton's media, diluted into fresh media and grown
24 overnight to give a log phase culture which was then
25 heat-shocked at 42°C for 3 hours or at 39°C for 5
26 hours and cultured overnight. The cells were then
27 washed in media, followed by a wash in saline and
28 either lyophilised in individual vaccine aliquots or
29 used directly for the immunisation of test animals.
30 For comparison isolated endogeneous SP-peptide
31 complexes were prepared as described in PCT
32 Application No GB00/03228. Essentially, washed

1 stress-induced cells are then re-suspended in
2 homogenisation buffer such as PBS with 0.5% Tween
3 and cells are then disrupted by freeze-thaw cycles
4 or using cell disruptor (e.g. bead beater, French
5 press). The cell lysate is then treated by
6 centrifugation, typically 3-5000g for 5 minutes, to
7 remove the nuclei and cell debris, followed by a
8 high speed centrifugation step, typically 100,000g
9 for 15-30 minutes. The supernatant thus obtained is
10 processed to give an SP/antigenic peptide fragment
11 complex suitable for use in a vaccine. This can be
12 done simply by ammonium sulphate precipitation which
13 uses a 20-70% ammonium sulphate cut. Specifically,
14 20% (w/w) ammonium sulphate is added at 4°C, the
15 precipitate is discarded, followed by the addition
16 of more ammonium sulphate to bring the concentration
17 to 70% w/w. The protein precipitate is harvested by
18 centrifugation and then dialysed into an appropriate
19 physiological, injectable buffer, such as saline, to
20 remove the ammonium sulphate before use. The SP
21 complexes may be used at any suitable concentration
22 to provide the immunogenic determinant in the
23 vaccine composition.

24

25 It is preferred that the amount of the induced
26 stress protein-peptide complex is in the range of
27 10-600 μ g, more preferably 10-100 μ g, and most
28 preferably 25 μ g per kg of animal body weight.

29

30 In order to determine the immunogenicity of the SP
31 complexes, T cell proliferation assays may be used.
32 Suitable assays include the mixed-lymphocyte

1 reaction (MLR), assayed by tritiated thymidine
2 uptake, and cytotoxicity assays to determine the
3 release of ^{51}Cr from target cells, see 'Current
4 Protocols in Immunology', Wiley Interscience, 1997.
5 For Mycobacteria, MLRs can also be assayed for the
6 induction of cytokine production such as the
7 production of interferon gamma using the commercial
8 kit (CSL Ltd). Alternatively, antibody production
9 may be examined, using standard immunoassays or
10 plaque-lysis assays, or assessed by intrauterine
11 protection of a foetus, see 'Current Protocols in
12 Immunology'.

13
14 Mice were immunised with either heat-stressed
15 organisms or the endogenous SP-peptide complexes
16 isolated from the heat stressed organisms in
17 phosphate buffered saline without any added adjuvant
18 in either the primary or booster vaccinations.

19
20 Immunisation with whole stress-induced organisms and
21 in particular lyophilised organisms gave
22 significantly better immunity than that induced by
23 isolated endogeneous SP-peptide complexes including
24 increased IFN-gamma and antibody production.

25

26 **Example 2: Preparation and use of constitutive hsp**
27 **mutants.**

28

29 Constitutive hsp producing microbes can be
30 constructed through the knockout of transcriptional
31 regulator suppressor genes such as hspR, MerR
32 (mercuric resistance operon regulatory protein),

1 HmrR (heavy metal regulatory protein) and their
2 homologues or the transcriptional control genes rho
3 and sigma. Such genes are readily identified by
4 screening of genome sequence databases with typical
5 test sequences. An example of such a typical test
6 sequence is the hspR gene of *M. tuberculosis* (shown
7 in Figure 1). An example of a list of identified
8 suppressors which have homology to the hspR gene is
9 shown in Figure 2.

10

11 *M.bovis* strains constitutively producing hspCs were
12 constructed by genetically engineering the *M. bovis*
13 strain BCG to yield deletion mutants for the hspR
14 gene. The hspR gene was deleted by homologous
15 recombination using a suicide vector carrying a
16 large fragment of the hspR gene and a kanamycin
17 selection marker. The hspR gene fragment was cloned
18 by PCR from BCG genomic DNA using primers derived
19 from the *M. tuberculosis* hspR sequence shown in
20 Figure 1. Such an approach should be widely
21 applicable to the production of similar mutants from
22 any prokaryote carrying the relevant suppressor
23 gene.

24

25 BCG hspR deletion mutants were used to produce hspCs
26 and the protein yields obtained from these were
27 comparable to those obtained from heat shocked wild
28 type strains. Such an approach should again be
29 widely applicable to the production of hspCs from
30 any prokaryote carrying the relevant suppressor
31 gene. These would provide a valuable resource for
32 the manufacture of hspC-based vaccines. Thus hspCs

1 produced from BCG hspR deletion mutants induced
2 protective immunity against aerosol challenge by *M.*
3 *tuberculosis* comparable to that induced by hspCs
4 obtained from heat shocked wild type BCG strains.
5
6 BCG hspR deletion mutants can be also be used as
7 vaccine vectors to express heterologous antigens.
8 For example, mice immunised with a BCG hspR deletion
9 mutant expressing the tetanus toxoid (TT) fragment C
10 showed the production of anti-TT antibodies as
11 detected by Western blotting. Such an approach
12 should be widely applicable to the production of
13 similar vectors for the expression of heterologous
14 vaccine antigens using mutants from any prokaryote
15 suitable for use as a vaccine vector. For example
16 the use of *Salmonella* or *Lactococcus* mutants would
17 enable the production of vectors targeted for
18 mucosal delivery of vaccines. Vaccines developed on
19 this principle would be particularly advantageous in
20 that they could be administered orally.
21

1 **Claims**

2

3 1. A vaccine comprising a microbial pathogen as an
4 immunogenic determinant, wherein the microbial
5 pathogen has been subjected to a stress inducing
6 stimuli.

7

8 2. A vaccine as claimed in claim 1 wherein the
9 stress inducing stimuli results in the
10 expression of heat shock proteins by the
11 microbe.

12

13 3. A vaccine as claimed in claim 1 or claim 2,
14 wherein the stress inducing stimuli is heat or
15 osmotic shock.

16

17 4. A vaccine as claimed in claims 1 to 3, wherein
18 the stress inducing stimuli is the genetic
19 modification of the microbial pathogen such that
20 at least one repressor gene for a heat shock
21 protein gene is inactivated, thereby allowing
22 the expression of a heat shock protein gene.

23

24 5. A vaccine as claimed in any of the preceding
25 claims, wherein the microbial pathogen is any
26 pathogen which is capable of inducing an
27 infectious disease.

28

29 6. A vaccine as claimed in any of the preceding
30 claims, wherein the microbial pathogen is a
31 bacteria, protozoa, fungi or a parasitic
32 organism.

1

2 7. A vaccine as claimed in claims 4 to 6, wherein
3 the genetic modification results in the
4 inactivation of the hspR repressor gene.

5

6 8. A vaccine as claimed in claims 4 to 6, wherein
7 the genetic modification results in the
8 inactivation of the stress gene regulatory
9 protein genes MerR or HmrR.

10

11 9. A vaccine as claimed in claims 4 to 6, wherein
12 the genetic modification results in the
13 inactivation of the transcription control genes
14 rho or sigma.

15

16 10. A vaccine as claimed in any of the preceding
17 claims wherein the microbial pathogen is
18 selected from the group consisting of
19 Mycobacteria, Salmonella, Vibrio, Listeria,
20 Streptomyces, Helicobacter and Lactococcus.

21

22 11. A vaccine as claimed in any of the preceding
23 claims wherein the microbial pathogen is
24 attenuated.

25

26 12. A vaccine as claimed in any of the preceding
27 claims, wherein the vaccine further comprises an
28 adjuvant.

29

30 13. A vaccine as claimed in claim 12, wherein the
31 adjuvant is selected from the group consisting
32 of; Freund's complete adjuvant, Freund's

1 incomplete adjuvant, Quil A, Detox, ISCOMs
2 and squalene.

3

4 14. A vaccine as claimed in any of the preceding
5 claims, wherein the vaccine is suitable for
6 administration by injection.

7

8 15. A vaccine as claimed in claims 1 to 13
9 wherein the vaccine is suitable for oral
10 administration.

11

12 16. A vaccine as claimed in claim 14, wherein the
13 vaccine is suitable for delivery by means of
14 a needle-less delivery format.

15

16 17. A method of vaccinating an animal,
17 characterised in that said method comprises
18 administering a pharmaceutically acceptable
19 quantity of a vaccine composition as claimed
20 in any of claims 1 to 13, sufficient to
21 elicit an immune response in the animal.

22

23 18. A method as claimed in claim 17 wherein said
24 vaccine is administered as a prophylactic
25 vaccine.

26

27 19. A method as claimed in claim 17, wherein the
28 vaccine composition is administered by
29 injection.

30

31 20. A method as claimed in claim 17, wherein the

1 vaccine composition is administered by
2 needle-less delivery.

3

4 21. A method as claimed in claim 17, wherein the
5 vaccine composition is administered
6 transdermally.

7

8 22. A method as claimed in claim 17, wherein the
9 vaccine composition is administered by
10 pulmonary delivery.

11

12 23. A method as claimed in claim 17, wherein the
13 vaccine composition is administered orally.

14

15 24. A method of producing a vaccine composition,
16 comprising an immunogenic determinant,
17 characterised in that said method comprises
18 the steps of:

19 subjecting microbial pathogens to
20 stress inducing stimuli; and

21 using the stressed microbe in the
22 preparation of the vaccine composition as
23 said immunogenic determinant.

24

25 25. A method as claimed in claim 24,
26 characterised in that the stress inducing
27 stimuli is heat or osmotic shock.

28

29 26. A method as claimed in claim 24,
30 characterised in that the stress inducing
31 stimuli is the inactivation of a gene which

1 represses the expression of heat-shock genes.

2

3 27. A method as claimed in claim 26,
4 characterised in that the repressor gene is
5 the hspR gene.

6

7 28. A method as claimed in claim 26,
8 characterised in that the repressor gene is
9 MerR or HmrR.

10

11 29. A method as claimed in any one of claims 24
12 to 28, characterised in that the microbial
13 pathogen cells are killed prior to use in
14 said vaccine.

15

16 30. A method as claimed in any of claims 24 to
17 29, characterised in that the microbial
18 pathogen cells are dried prior to use in said
19 vaccine.

20

21 31. A vaccine composition as defined in any one
22 of claims 24 to 30, characterised in that the
23 composition is an aqueous composition.

24

25 32. A vaccine composition as defined in any one
26 of claims 24 to 30, characterised in that the
27 composition is a dry composition.

28

29 33. A vaccine composition as defined in any one
30 of claims 24 to 30, characterised in that the
31 composition is a lyophilised composition.

- 1 34. Use of a pathogenic microbe which has been
2 genetically modified such that at least one
3 repressor gene for a heat shock protein is
4 inactivated, thereby allowing constitutive
5 expression of heat shock proteins, as a
6 vaccine vector which further allows the
7 expression of a heterologous antigen.
8
- 9 35. Use of pathogenic microbe as claimed in claim
10 34 wherein the pathogenic microbe is a
11 prokaryotic organism, which is suitable for
12 use as a vaccine vector.
13
- 14 36. Use of pathogenic microbe as claimed in claim
15 35 wherein the prokaryotic organism is BCG
16 and the heterologous antigen fragment is the
17 tetanus toxoid fragment C.
18
- 19 37. Use of pathogenic microbe as claimed in claim
20 35 wherein the prokaryotic organism is
21 Salmonella or Lactococcus, such that the
22 vaccine can be administered orally.
23
- 24 38. Use of a pathogenic microbe which has been
25 genetically modified such that at least one
26 repressor gene for a heat shock protein is
27 inactivated, thereby allowing constitutive
28 expression of heat shock proteins, as a
29 source of heat shock protein-peptide
30 complexes for use in subunit and multi-
31 subunit vaccines.

- 1 39. Use of a microbial pathogen in the
2 manufacture of a vaccine composition as
3 claimed in any of claims 1 to 13, for
4 vaccinating an animal.
5
- 6 40. Use as claimed in claim 39 wherein said
7 vaccine is administrable as a prophylactic
8 vaccine.
9
- 10 41. Use as claimed in claim 39 wherein said
11 vaccine is administrable as a therapeutic
12 vaccine.
13
- 14 42. Use as claimed in claim 39, wherein the
15 vaccine composition is administrable by
16 injection.
17
- 18 43. Use as claimed in claim 39, wherein the
19 vaccine composition is administrable by
20 needle-less delivery.
21
- 22 44. Use as claimed in claim 39, wherein the
23 vaccine composition is administrable
24 transdermally.
25
- 26 45. Use as claimed in claim 39, wherein the
27 vaccine composition is administrable by
28 pulmonary delivery.
29
- 30 46. Use as claimed in claim 39, wherein the
31 vaccine composition is administrable orally.

- 1 47. A vaccine for use in a method of vaccinating
2 an animal, said vaccine comprising a vaccine
3 composition as claimed in any of claims 1 to
4 13, and said method comprises administering a
5 pharmaceutically acceptable quantity of said
6 vaccine.
7
- 8 48. A vaccine for use in a method of vaccination
9 as claimed in claim 47 wherein said vaccine
10 is administrable as a prophylactic vaccine.
11
- 12 49. A vaccine for use in a method of vaccination
13 as claimed in claim 47 wherein said vaccine
14 is administrable as a therapeutic vaccine.
15
- 16 50. A vaccine for use in a method of vaccination
17 as claimed in claim 47, wherein said vaccine
18 is administrable by injection.
19
- 20 51. A vaccine for use in a method of vaccination
21 as claimed in claim 47, wherein said vaccine
22 is administrable by needle-less delivery.
23
- 24 52. A vaccine for use in a method of vaccination
25 as claimed in claim 47, wherein said vaccine
26 is administrable transdermally.
27
- 28 53. A vaccine for use in a method of vaccination
29 as claimed in claim 47, wherein said vaccine
30 is administrable by pulmonary delivery.
31

- 1 54. A vaccine for use in a method of vaccination
2 as claimed in claim 47, wherein said vaccine
3 is administrable orally.
4
- 5 55. A vaccine as claimed in claim 1,
6 substantially as herein described and
7 illustrated.
8
- 9 56. A vaccine composition as claimed in any one
10 of claims 31 to 33, substantially as herein
11 described and illustrated.
12
- 13 57. A method as claimed in claim 17,
14 substantially as herein described and
15 illustrated.
16
- 17 58. A method as claimed in claim 24,
18 substantially as herein described and
19 illustrated.
20
- 21 59. Use as claimed in claim 34, or claim 38, or
22 claim 39, substantially as herein described
23 and illustrated.
24
- 25 60. A vaccine for use in a method of treatment as
26 claimed in claim 47, substantially as herein
27 described and illustrated.
28
- 29 61. A new vaccine, a new non-therapeutic method
30 of treatment, a new method of producing a
31 vaccine, a new use of a pathogenic microbe,

1 or a vaccine for a new use in a method of
2 treatment, substantially as herein described.
3

1/2

Figure 1

hspR: 378 bp - *M. tuberculosis* -
 atggcgagaacccaaaggacggggaatcccggacgtttttgatctcggtagccgccgagctag
 ccggcatgcatgcacagaccctgcgtagctacgatcgtcttgggttggtcagcccgcggcgac
 ctccggtggcgggcgccgctattccctgcatgacgtcgagttgctgcgccaggtgcagcacctc
 tcgcaggacgagggggtcaacttgccggcatcaagcgattattgaactgaccagtcaggtcg
 aggcgctgcagtcaggttgcaagagatggctgaggagttggcgggtgtgcgtgccaccagcg
 ccgcgaggtcgcggtggtgccgaagagcaccgcctggtcgtctggaaaccgcgccg

Figure 2

GI PROTEIN DESCRIPTION

13094040 heat shock regulator [*Mycobacterium leprae*]
 987631 heat shock protein [*Streptomyces coelicolor*]
 1850936 HspR [*Streptomyces albus*]
 6458658 transcriptional regulator, MerR family [*Deinococcus radiodurans*]
 6968663 putative heat shock transcriptional regulator [*Campylobacter jejuni*]
 2314167 putative heat shock protein (hspR) [*Helicobacter pylori* 26695]
 4154930 putative TRANSCRIPTIONAL REGULATOR [*Helicobacter pylori* J99]
 2983291 transcriptional regulator (MerR family) [*Aquifex aeolicus*]
 216395 GlnR protein [*Bacillus subtilis*]
 80358 glutamine synthetase transcription repressor glnR [validated] - *Bacillus subtilis*
 577593 For protein sequence: merR (Hg resistance) proteins. [*Myxococcus xanthus*]
 80075 hypothetical 15K protein (glnA 5' region) - *Bacillus cereus*
 7635981 putative MerR-family transcriptional regulator. [*Streptomyces coelicolor* A3(2)]
 2120400 hypothetical protein 11 - *Myxococcus xanthus* (fragment)
 15140739 putative protein, merR and LacI family [*Sinorhizobium meliloti*]
 468509 glutamine synthetase repressor [*Staphylococcus aureus*]
 12722376 unknown [*Pasteurella multocida*]
 13472246 probable transcriptional regulator [*Mesorhizobium loti*]
 2808790 putative transcriptional regulator [*Streptomyces coelicolor* A3(2)]
 13622913 putative transcriptional regulator [*Streptococcus pyogenes* M1 GAS]
 4062747 Hypothetical transcriptional regulator in metG-dld intergenic region. [*Escherichia coli*]
 1787409 putative transcriptional regulator [*Escherichia coli* K12]
 2116754 YfmP [*Bacillus subtilis*]
 1405480 scgR [*Bacillus subtilis*]
 13476954 hypothetical protein [*Mesorhizobium loti*]
 595259 Nola [*Bradyrhizobium elkanii*]
 15156241 AGR_C_2207p [*Agrobacterium tumefaciens*]
 9657661 transcriptional regulator, putative [*Vibrio cholerae*]

Figure 2: Cont'd/...

1075010 merR homolog HI0293 - *Haemophilus influenzae* (strain Rd KW20)
6093208 GlnR protein [*Lactobacillus rhamnosus*]
10174111 transcriptional regulator [*Bacillus halodurans*]
15073910 PUTATIVE TRANSCRIPTION REGULATOR PROTEIN [*Sinorhizobium meliloti*]
988279 Nola [*Bradyrhizobium* sp.]
12060165 ORF1 [*Streptomyces coelicolor*]
11493190 putative merR-family transcriptional regulator [*Streptomyces coelicolor*]
9950914 probable transcriptional regulator [*Pseudomonas aeruginosa*]
98171 hypothetical protein 1 (mer operon) - *Bacillus* sp
6562779 putative merR-family transcriptional regulator [*Streptomyces coelicolor*]
3413184 regulatory protein [*Exiguobacterium* sp.]
4633809 heavy metal regulator HmrR [*Rhizobium leguminosarum* bv. *viciae*]
11071705 regulatory protein [*Bacillus licheniformis*]
1006583 merR [*Synechocystis* sp. PCC 6803]
577592 For protein sequence: merR (Hg resistance) proteins. [*Myxococcus xanthus*]
7321278 hypothetical protein SCD31.13c [*Streptomyces coelicolor* A3(2)]
7649594 hypothetical protein [*Streptomyces coelicolor* A3(2)]
11071711 regulatory protein [*Bacillus macroides*]
15156225 AGR_C_2183p [*Agrobacterium tumefaciens*]
98175 mercuric resistance operon regulatory protein - *Bacillus* sp
6179679 regulator of pmrA [*Streptococcus pneumoniae*]
12723097 transcriptional regulator [*Lactococcus lactis* subsp. *lactis*]
12642170 nodulation transcriptional activator protein Nola [*Bradyrhizobium* sp. WM9]
9657439 transcriptional regulator, MerR family [*Vibrio cholerae*]
4680351 heavy metal regulator HmrR [*Sinorhizobium meliloti*]
15140905 putative transcriptional regulator, merR family protein [*Sinorhizobium meliloti*]
6941979 putative MerR-family transcriptional regulator [*Streptomyces coelicolor*]
7688302 putative MerR-family transcriptional regulator. [*Streptomyces coelicolor* A3(2)]
2894244 hypothetical protein Rv3334 [*Mycobacterium tuberculosis* H37Rv]
48151 merR2 [*Acidithiobacillus ferrooxidans*]
2815330 SC10A5.22, unknown, [*Streptomyces coelicolor* A3(2)]
1074017 probable DNA-binding protein homolog - *Haemophilus influenzae* (strain Rd KW20)
13622897 putative transcriptional activator regulator protein [*Streptococcus pyogenes* M1 GAS]
12517921 putative transcriptional regulator [*Escherichia coli* O157:H7 EDL933]