DELIVERY OF BIOLOGICALLY ACTIVE POLYPEPTIDES

The present invention relates to the delivery of biologically active polypeptides *in vivo*. In particular, it relates to use of non-invasive bacteria, generally Gram-positive bacteria such as *Lactococcus*, in providing biologically active polypeptides in the body, especially at mucosa. In one aspect this relates to provision of an adjuvant effect by means of which an immune response raised to an antigen is enhanced. Nucleic acid constructs and host organism for these applications are also provided.

The limited number of adjuvants approved for use in human vaccines (owing to the toxicity or pathogenicity of the most active agents such as Freund's complete adjuvant) and the discovery during the past 20 or more years of numerous polypeptides involved in the proliferation, differentiation and activation of B cells and T cells has drawn attention to the possibility of using these factors (cytokines) to augment responses to vaccines, and to direct the immune response to a particular vaccine along desired pathways. The need for this approach has become even more apparent as recent immunological discoveries have emphasised that cell-mediated and antibody-mediated immune responses are to a large degree mutually exclusive responses. Whether antibody formation or effector T-cells and macrophages are activated is determined by which particular array of cytokines is elicited by any given antigen, pathogen or vaccine.

Most important is the functional activity of the types of helper T cells TH1, or TH2, which are involved in the response to any particular antigen or invading pathogen.

Since protective immunity to a pathogenic agent usually arises as a consequence either of antibody-formation (extracellular pathogens, soluble toxins or intracellular pathogens following their release into tissue fluids from dead, dying or productive cells) or of cell-mediated responses (intracellular pathogens) it is in principle highly advantageous to be able to direct immune responses to a vaccine towards either antibody formation or T-cell and macrophage activation. In order that the protective effects of vaccination should persist for as long as possible it is also important to be able to enhance the amplitude, duration and memory components of the immune response.

For these reasons numerous investigators have focused their attention on the possibility of harnessing one or more of the members of the cytokine network of signalling proteins as vaccine adjuvants. This approach may be even more significant when it is considered that the loss of helper T cells - and hence of their cytokine output - may be associated with the failure of individuals suffering from certain types of inherited or acquired immunodeficiencies to be able to respond to particular vaccines.

Although much attention has been paid to the use of cytokines for these purposes only limited success has been reported in harnessing cytokines as adjuvants. Considerable difficulty has been encountered in administering adjuvant cytokines by methods which would be appropriate for inclusion in a vaccine regimen. This difficulty may be exemplified by reference to studies of the use of IL-2 as an adjuvant.

IL-2 has attracted particular attention as a possible adjuvant because, although its principal source is thought to be T helper 1 cells, its major activities are believed to include involvement in wide ranging aspects of immune responses, such as T-cell proliferation, the synthesis of other cytokines, B-cell growth and immunoglobulin synthesis. Thus IL-2 is a T cell-derived cytokine which was first described as a T cell growth factor. It is now known to stimulate growth and differentiation of T cells, B cells, NK cells, monocytes, macrophages and oligodendrocytes. In general, adjuvant activity on the part of IL-2, which has been reported by many workers, has been found to depend on the use of multiple injections of the cytokine or its incorporation into liposomes or oily emulsions. To avoid this need, other workers have either co-expressed IL-2 with vaccine antigens in recombinant bacterial and viral vectors, or have engineered IL-2:antigen fusion proteins; the latter are claimed to provide marked enhancement of the

immunogenicity of the antigenic component of the fusion partner.

Other desirable characteristics of vaccines include the need to be as innocuous as possible, to act effectively following the administration of the smallest possible number of doses, and to be suitable for administration via mucosal surfaces (e.g. orally, intranasally, or intra-vaginally) thus obviating the need for hypodermic needles, and activating local, mucosal immune responses in addition to systemic immune responses. The capacity for continued proliferation of live, attenuated pathogens has resulted in numerous studies of the use of recombinant vaccine strains of viruses and bacteria (such as vaccine strains of pox viruses, or of salmonella and tubercle bacteria) as agents for the delivery of heterologous antigens.

We have previously developed systems for the expression of heterologous antigens in the non-pathogenic, non-colonising, non-invasive food-grade bacterium *Lactococcus lactis* (see UK patent GB-2278358B). We have shown previously that *Lactococcus lactis* is able to produce and secrete biologically active murine IL-2 when cultured *in vitro* (Steidler et al., *Applied and Environmental Microbiology*, April 1995, Vol. 61, No. 4, pp1627-1629). However, owing to the fact that *Lactococcus lactis* is non-invasive - it is indeed not a commensal bacterium nor otherwise normally associated with the colonisation of mucosal

surfaces in animals - it was not obvious that this bacterium could be successfully employed in a vaccination strategy which required the formation of an adjuvant cytokine *in vivo*. We have previously shown (GB-2278358B) that heterologous antigen can be fully antigenic when accumulated within the cytoplasm of *Lactococcus lactis* (from which it is presumed to leak *in vivo* as the cells are digested by phagocytic cells).

By the manipulation of the appropriate genetic elements we have provided nucleic acid constructs (here artificial operons - coordinately transcribed multigene units) for co-expression in *Lactococcus lactis* of an antigenic polypeptide (exemplified here using tetanus toxin fragment C - TTFC) and a biologically active cytokine polypeptide (exemplified here using Interleukin 2 and also Interleukin-6).

The IL-6 cytokine has been shown by other workers to have the capacity to augment murine antigen-specific antibody responses *in vivo* and *in vitro*, and we have also been able to prepare expression units for IL-6 in *L. lactis*. IL-6 is a multi-functional cytokine secreted by both lymphoid and non-lymphoid cells which is known to possess pleiotropic activities that play a central role in host defence. IL-6 can exert growth-inducing, growth-inhibitory and differentiation-inducing activities, depending on the target cells. These activities include differentiation and/or activation of T cells and macrophages, growth

promotion of B cells (seen as growth - promotion of B cell tumour lines *in vitro*), terminal differentiation (secretion of immunoglobulins) in B cells, and - acting systemically - elicitation of the hepatic acute-phase protein response. Most importantly for the purposes of mucosal immunisation IL-6 has been shown to induce high rate IgA secretion in IgA-committed B cells.

To exemplify the present invention, operons for IL-2 and IL-6 co-expression were separately constructed in a constitutive expression vector (pTREX1, also known as pEX1) so that the transcription of the TTFC gene and the interleukin gene could be controlled by the activity of a lactococcal promoter element of previously defined activity (so-called P1). The constructs were prepared so that, following translation of the mRNA transcribed from the artificial operons, the TTFC antigen would accumulate intracellularly.

When preparations of these bacteria were administered intranasally to mice bacteria engineered to express either Interleukin-2 or Interleukin-6 elicited approximately 10x more anti-TTFC antibody than the constructs which expressed the TTFC alone. Thus, either of these interleukins possessed distinctive adjuvant activity in the experimental system.

It was not obvious from either the capacity of *Lactococcus lactis* to deliver a heterologous antigen or its ability to produce IL-2 *in vitro* that it would be an appropriate vehicle for a delivery of a cytokine *in*

vivo such that sufficient, active cytokine would be provided to provide an adjuvant effect. *Lactococcus lactis* is non-invasive and non-colonising, which means that when these bacteria are used to deliver an antigen to the immune system, e.g. via a mucosal surface, they are most likely to enter lymphoid tissue as a consequence of phagocytosis by the M (or microfold) cells which sample the contents of mucosal secretions adjacent to mucosal lymphoid tissue. Microparticulate antigens (e.g. tetanus toxoid incorporated into poly L-lactide microparticles) enter lymphoid tissue passively in this way, whereas pathogenic bacteria (or attenuated vaccines) such as species of *Listeria*, *Salmonella* and *Shigella* are able to invade cells and tissues by actively stimulating their uptake into mucosal epithelial cells, in addition to gaining entry via M cells. Since the activity of cytokines as adjuvants has been found previously to require multiple injections or sustained release delivery (Heath and Playfair (1992) *Vaccine* 7: 427-434), and since the cytokines will only be protected from proteolytic digestion within phagocytic cells while the *Lactococcus lactis* cells remain intact or viable, it is unexpected that *lactococcal* cells expressing cytokines should display marked adjuvant activity as demonstrated herein. This can perhaps be appreciated if it is understood that death and dissolution of the bacterial particles will favour antigen release, but prevent more

than very transient production of cytokines. Nevertheless, our findings indicate that the expression of IL-2 or IL-6 by *Lactococcus lactis* does have a marked adjuvant effect. Even if the expressor bacteria were to be administered by a parenteral rather than a mucosal route the same considerations would apply.

Thus, since *Lactococcus lactis* is not invasive - indeed it is not a commensal bacterium and it also depends for its nutrition on the provision of amino acids and peptides which are unlikely to be available *in vivo* - the demonstration that the cytokine-secreting strains of *L. lactis* are nevertheless able to augment antibody production is surprising. Hence these results demonstrate for the first time that recombinant strains of *Lactococcus lactis* can be used to synthesise and deliver biologically active molecules *in vivo*. Of particular interest is the fact that these results demonstrate the feasibility of augmenting the mucosal as well as the systemic immune response since IL-6 has been shown to be a cytokine able to induce a high rate of IgA secretion in IgA committed B cells.

The finding that *Lactococcus lactis* is able to sustain its biological activity on a mucous membrane for a sufficient length of time to deliver a biologically active dose of either of two different recombinant cytokines and thereby augment an immune response to a heterologous antigen demonstrates broad applicability for the delivery of polypeptides for

purposes other than adjuvant activity alone.

The capacity of *L. lactis* to produce and secrete polypeptides demonstrates that it is possible to utilise these bacteria for *in vivo* production and delivery of polypeptides which are known to be active at micromolar, nanomolar or picomolar concentrations. Since precise dosing of these polypeptides, and the need for the coincidental introduction of bacterial cells is of lesser concern for veterinary than human applications it is likely that this method for delivering recombinant polypeptides will be especially valuable in veterinary applications. However, even within human medicine, the fact that cytokine output can be constrained to the sites of deposition of harmless bacterial cells, and is available close to the antigen during the earliest phases of the immune response may favour its use in circumstances - such as adjuvant activity - where the biologically active polypeptide is best localised in order to avoid toxic systemic side effects.

Thus, the present invention provides:

(i) a method of delivering one or more biologically active polypeptides which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses said one or more polypeptides;

(ii) A method of delivering one or more antigens which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses said one or more antigens; and

(iii) A method of delivering one or more antigens and/or one or more biologically active polypeptides which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses both said one or more antigens and said one or more heterologous biologically active polypeptides.

The biologically active polypeptides can be either homologous to the bacterium or heterologous, derived from either eukaryotic sources or prokayotic sources, or their viruses.

According to another aspect of the present invention, there is provided a non-invasive or non-pathogenic bacterium expressing (i) one or more heterologous biologically active polypeptides and (ii) one ore more antigens.

"Biological activity" refers to ability to perform a biological function and with reference to a polypeptide implies that the polypeptide adopts a stable conformation ("folded form") which is the same

or closely analogous to its native configuration. When folded correctly or substantially correctly, for example with formation of proper folded units, α-helices, β-sheets, domains, disulphide bridges etc., a polypeptide should have the ability to perform its natural function. Generally, the unit of function in a polypeptide is a domain.

Mere ability to be bound by an antibody or other receptor, either with or without elicitation of an immune response, is passive and does not constitute "biological activity". Any antigen has the ability to be bound by an antibody but is not necessarily biologically active.

A "heterologous" polypeptide is one not native to the bacterium, i.e. not expressed by the bacterium in nature or prior to introduction into the bacterium, or an ancestor thereof, of encoding nucleic acid for the polypeptide.

A bacterium according to the present invention will in general be Gram-positive, and may in principle be any innocuous bacterium, for example *Listeria innocua*, *Staphylococcus* xylosus or a *Lactococcus*. *Lactococci*, in particular *Lactococcus lactis*, represent a preferred embodiment of the present invention. Such bacteria are non-colonising.

The skilled person will appreciate that the methods of the present invention could be used to deliver a range of biologically active polypeptides.

Examples of suitable polypeptides include ones which are capable of functioning locally or systemically, eg is a polypeptide capable of exerting endocrine activities affecting local or whole-body metabolism and/or the biologically active polypeptide(s) is/are one(s) which is/are capable of the regulation of the activities of cells belonging to the immunohaemopoeitic system and/or the one or more biologically active polypeptides is/are one(s) which is/are capable of affecting the viability, growth and differentiation of a variety of normal or neoplastic cells in the body or affecting the immune regulation or induction of acute phase inflammatory responses to injury and infection and/or the one or more biologically active polypeptides is/are one(s) which is/are capable of enhancing or inducing resistance to infection of cells and tissues mediated by chemokines acting on their target cell receptors, or the proliferation of epithelial cells or the promotion of wound healing and/or the one or more biologically active polypeptides modulates the expression or production of substances by cells in the body.

Specific examples of such polypeptides include insulin, growth hormone, prolactin, calcitonin, luteinising hormone, parathyroid hormone, somatostatin, thyroid stimulating hormone, vasoactive intestinal polypeptide, a structural group 1 cytokine adopting an antiparallel 4α helical bundle structure such as IL-2,

IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, GM-CSF, M-CSF, SCF, IFN-γ, EPO, G-CSF, LIF, OSM, CNTF, GH, PRL or IFNα/β, a structural group 2 cytokine which are often cell-surface asociated, form symetric homotrimers and the subunits take up the conformation of β-jelly roll described for certain viral coat proteins such as the TNF family of cytokines, eg TNFα, TNFβ, CD40, CD27 or FAS ligands, the IL-1 family of cytokines, the fibroblast growth factor family, the platelet derived growth factors, transforming growth factor β and nerve growth factors, a structural group 3 cytokine comprising short chain α/β molecules, which are produced as large transmembrane pre-cursor molecules which each contain at least one EGF domain in the extracellular region, eg the epidermal growth factor family of cytokines, the chemokines characterised by their possession of amino acid sequences grouped around conserved cysteine residues (the C-C or C-X-C chemokine subgroups) or the insulin related cytokines, a structural group 4 cytokine which exhibit mosaic structures such as the heregulins or neuregulins composed of different domains, eg EGF, immunoglobulin-like and kringle domains.

Alternatively, the biologically active polypeptide can be a receptor or antagonist for biologically active polypeptides as defined above.

The bacterium expresses the biologically active

polypeptide and the antigen from nucleic acid contained within it. The nucleic acid may comprise one or more nucleic acid constructs in which nucleic acid encoding the biologically active polypeptide and nucleic acid encoding the antigen are under control of appropriate regulatory sequences for expression in the bacterium.

Suitable vectors comprising nucleic acid for introduction into bacteria can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

In a preferred embodiment, the coding sequences for the biologically active polypeptide and the antigen are contained in an operon, i.e. a nucleic acid

construct for multi-cistronic expression. In an operon, transcription from the promoter results in a mRNA which comprises more than one coding sequence, each with its own suitably positioned ribosome binding site upstream. Thus, more than one polypeptide can be translated from a single mRNA. Use of an operon enables expression of the biologically active polypeptide and the antigen to be co-ordinated.

In an alternative embodiment, the coding sequences for the biologically active polypeptide and the antigen are part of the same nucleic acid vector, or separate vectors, and are individually under the regulatory control of separate promoters. The promoters may be the same or different.

A nucleic acid construct or vector comprising a coding sequence for a biologically active polypeptide and a coding sequence for an antigen wherein each coding sequence is under the control of a promoter for expression in a non-invasive bacterium (as disclosed - especially a non-commensal and/or non-colonising bacterium e.g. a *Lactococcus*), whether as an operon or not, is provided by a further aspect of the present invention.

A promoter employed in accordance with the present invention is preferably expressed constitutively in the bacterium. Use of a constitutive promoter avoids the need to supply an inducer or other regulatory signal for expression to take place.

Preferably, the promoter directs expression at a level at which the bacterial host cell remains viable, i.e. retains some metabolic activity, even if growth is not maintained. Advantageously then, such expression may be at a low level. For example, where the expression product accumulates intracellularly, the level of expression may lead to accumulation of the expression product at less than about 10% of cellular protein, preferably about or less than about 5%, for example about 1-3%. The promoter may be homologous to the bacterium employed, i.e. one found in that bacterium in nature. For example, a *Lactococcal* promoter may be used in a *Lactococcus*. A preferred promoter for use in *Lactococcus lactis* (or other *Lactococci*) is "P1" derived from the chromosome of *Lactococcus lactis* (Waterfield N.R.; Le Page, R.W.F.; Wilson P.W. and Wells J.M., *Gene* (in press)), the sequence of which is shown in the following (SEQ ID NO. 1):

GATTAAGTCA TCTTACCTCT TTTATTAGTT TTTTCTTATA ATCTAATGAT
AACATTTTTA TAATTAATCT ATAAACCATA TCCCTCTTTG GAATCAAAAT
TTATTATCTA CTCCTTTGTA GATATGTTAT AATACAAGTA TC

The nucleic acid construct or constructs may comprise a secretory signal sequence. Thus, in a preferred embodiment the nucleic acid encoding the biologically active polypeptide may provide for secretion of the biologically active polypeptide (by appropriately coupling a nucleic acid sequence encoding a single sequence to the nucleic acid sequence encoding

the polypeptide). Ability of a bacterium harbouring the nucleic acid to secrete the polypeptide may be tested *in vitro* in culture conditions which maintain viability of the organism.

Suitable secretory signal sequences include any of those with activity in Gram positive organisms such as *Bacillus*, Clostridium and *Lactobacillus*. Such sequences may include the α-amylase secretion leader of *Bacillus amyloliquefaciens* or the secretion leader of the Staphylokinase enzyme secreted by some strains of *Staphylococcus*, which is known to function in both Gram-positive and Gram-negative hosts (see "Gene Expression Using Bacillus", Rapoport (1990) *Current Opinion in Biotechnology* 1:21-27), or leader sequences from numerous other *Bacillus* enzymes or S-layer proteins (see pp341-344 of Harwood and Cutting, "Molecular Biological Methods for Bacillus", John Wiley & Co. 1990). For *Lactococcus* the leader sequence of the protein designated *Usp45* may be preferred (SEQ ID NO. 2):

ATG AAA AAA AAG ATT ATC TCA GCT ATT TTA ATG TCT ACA GTG
met lys lys lys ile ile ser ala ile leu met ser thr val

ATA CTT TCT GCT GCA GCC CCG TTG TCA GGT GTT TAC GCT
ile leu ser ala ala ala pro ley ser gly val tyr ala

However, it may be preferable that the antigen accumulates intracellularly. As discussed, preferably the level of accumulation should allow the bacterium to

remain viable, i.e. retain some metabolic activity, and may be less than about 10% of cellular protein, preferably about or less than about 5% of cellular protein.

The antigen may in principle be any peptide or polypeptide to which a receptor of the immune system, such as an antibody, can bind. In a preferred embodiment, the antigen is a bacterial toxoid form of a toxin or an antigenic fragment thereof. For good compatibility of expression in *Lactococcus*, which has a bias towards A/T usage over G/C in its coding sequences (60% A/T), the antigen may be one whose coding sequence is A/T rich (has a higher A/T content than G/C). For instance, the antigen may be a toxoid (or an antigenic fragment thereof), or another immunogenic component from *Clostridium* or *Pneumococcus* or other *Streptococcus* species. *Clostridial* coding sequences, for example, often have >70% A/T base pair content, as do genes from the important human malarial parasites belonging to the genus *Plasmodium*.

For use in enhancing an immune response to the antigen, i.e. antigenic peptide or polypeptide, as discussed herein, the biologically active polypeptide preferably has cytokine activity. Cytokines are discussed in "The Cytokine Facts Book", Callard and Gearing (1994), Academic Press. Preferred polypeptides with cytokine activity are interleukins, including Interleukin-2 (IL-2) and Interleukin 6 (IL-6). Many

cytokines contain a disulphide bridge and all are secreted from the cells which naturally produce them. The reducing nature of the cytoplasm of bacterial cells would be expected to prevent formation of disulphide bridges. It would not be obvious that a polypeptide which is naturally secreted, especially on which naturally contains a disulphide bridge, would be biologically active when retained in a bacterial cell.

Thus, in one embodiment, the biologically active polypeptide is one which is secreted from cells which naturally produce it.

The use of a cytokine to enhance an immune response to the antigen in accordance with the present invention is particularly apposite for antigens of low immunogenicity. Furthermore, application of an immunogen to a mucosal membrane generally elicits an IgA response. The ability of a vaccine to elicit a good (protective level) mucosal immune response is a highly desirable feature, since it is now known that sIgA antibodies play a vital role in protecting mucosal surfaces against infection. For example, sIgA which binds to the surface of the cholera bacillus has been shown to be capable of preventing experimental cholera in mice. sIgA which effectively neutralised HIV-1 may play an important role in protecting against infection with this virus, since once the virus has gained access to the body a lifelong infection is established. Methods for the reliable and long-lasting induction of

mucosal sIgA responses are therefore much sought after, since the great majority of human viruses and bacterial pathogens initiate infections by colonising mucosal surfaces.

Thus, antigens of low immunogenicity from a parasite against which an enhanced IgA response is beneficial may be employed particularly advantageously in the present invention, for instance the P28 immunogen (glutathione-S-transferase) of *Schistosoma mansoni*.

To generate a bacterium according to the present invention, nucleic acid is introduced into a bacterial host cell. Thus, a further aspect of the present invention provides a method comprising introducing nucleic acid as disclosed into a non-invasive bacterium, preferably a Gram-positive bacterium and most preferably a non-commensal, non-colonising bacterium (such as *Lactococcus*). The introduction may employ any available technique. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene. Growing the cells in culture under conditions for expression of the biologically active polypeptide and the antigen may be employed to verify that the bacteria contain the encoding nucleic acid and

are able to produce the encoded material.

In a further aspect, the present invention provides a method of delivering a biologically active dose of a polypeptide *in vivo*, the method comprising administering to an individual a non-invasive bacterium containing nucleic acid for expression of a biologically active polypeptide heterologous to the bacterium. As discussed *supra*, preferred bacteria include *Lactococci* such as *Lactococcus lactis* and a preferred route of administration may be by application to mucosa.

Although, it has previously been shown possible to express in such bacteria a heterologous polypeptide in a biologically active form, this has only ever been done *in vitro* in culture conditions which are optimised for bacterial viability and growth. *In* vivo, for instance on the mucosal membrane, the bacteria are in an environment which would not be expected to support their growth or viability. It is thus surprising that such bacteria are able to deliver a polypeptide in a dose (amount) which is sufficient for the biological activity of the polypeptide to result in a detectable biological effect.

In a preferred embodiment, the biologically active polypeptide has cytokine activity and the bacterium may also express an antigen. Interleukins such as IL-2 and IL-6 may advantageously be delivered.

It will be appreciated that the methods of the

present invention and the use of a non-invasive or non-pathogenic bacterium as described herein provide a wide range of therapeutic methods which would enable the skilled person manipulate, for instance, the immume response of a subject. Thus, the present invention provides, in various other aspects:

(i) a method of regulating the survival, growth, differentiation, effector functions or susceptibility to infection of cells or tissues which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined herein;

(ii) a method of boosting an immune response against tumour cells or an infection colonising a mucosal surface or adjacent or distant tissue which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined herein;

(iii) a method of modulating the type of immune response (antibody versus cell-mediated) against a pathogenic infectious agent which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined herein;

(iv) a method of modulating the infiltration of normal tissues with inflammatory or tumour cells which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined herein;

(v) a method of controlling the rate of growth, rate of invasion or survival of tumour cells which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined herein;

(vi) a method of inducing apoptosis in tumour cells which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined herein;

(vii) a method of downregulating an immune response which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses a biologically active polypeptide; and

(viii) a method of treating an allergic autoimmune or other immune dysregulative disease state, which comprises administering to a subject a non-invasive

or non-pathogenic bacterium which expresses a biologically active polypeptide.

Alternatively stated, when a cytokine and an antigen are both expressed by a bacterium, an aspect of the present invention provides a method of enhancing an immune response to an antigen, the method comprising administering to an individual a non-invasive bacterium containing nucleic acid for expression of a polypeptide with cytokine activity and an antigen.

Enhancement of an immune response, such as an antibody response, preferably provides a level of immune response which is protective of the individual against subsequent challenge with the antigen in a pathogenic context. For example, if the antigen is a bacterial toxoid or a toxin fragment, the level of an antibody response to administration of a bacterium in accordance with the present invention may subsequently protect the individual against pathogenic consequences of challenge with the bacterial toxin, e.g. upon infection with bacteria which produce the toxin.

Administration of the bacterium by application to a mucosal surface may be advantageous in certain contexts by virtue of generating an enhanced immune response at the mucosal membrane (e.g. IgA response) in addition to a systemic response.

The bacterium may be applied in a nutrient medium, i.e. medium containing a substance or

substances which sustain (at least *in vitro*) metabolic activity in the bacterium. Such substances may sustain viability if not growth of the bacterium. Such substances may include an energy source such as glucose, amino acids and so on.

The individual to which the bacterium is administered may be human or animal, i.e. a non-human mammal. Administration may conveniently be nasal, and may be oral, vaginal or anal. In contexts where mucosal administration is not preferred, the bacterium may be administered by any other suitable means within the capacity of those skilled in the art, e.g. by parental routes (i/v, i/p, s/c, i/m).

In a therapeutic context, i.e. where the biological effect of delivery of the polypeptide to an individual is beneficial to that individual, administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. In a prophylactic context, the amount may be sufficient to reduce the deleterious effect on the individual of a subsequent pathogenic challenge, for instance by enhancing the immune response. The actual amount administered, and rate and time-course of administration, will depend on the aim of the administration, e.g. the biological effect sought in view of the nature and severity of the challenge, and

is the subject of routine optimisation. Prescription of treatment, including prophylactic vaccination, for example decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition comprising bacteria may be administered in accordance with the present invention alone or in combination with other treatments, either simultaneously or sequentially.

The present invention also provides a pharmaceutical composition comprising a bacterium as disclosed. Such a pharmaceutical composition is in one embodiment preferably suitable for application to a mucosal membrane.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to the bacterium, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration. For intravenous, cutaneous or subcutaneous injection, or injection at the site of an affliction, a parenterally acceptable aqueous solution may be employed which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill

in the art are well able to prepare suitable solutions. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required. As discussed, a pharmaceutical comprising a bacterium for administration in accordance with the present invention may comprise one or more nutrient substances, e.g. an energy source such as glucose, amino acids and so on.

In another aspect, the present invention provides a method of manufacture of a pharmaceutical comprising formulating bacteria as disclosed with a suitable carrier medium for administration to an individual. In one embodiment, the pharmaceutical is suitable for application to a mucosal membrane of an individual.

The present invention also provides a non-invasive bacterium expressing a heterologous biologically active polypeptide, and possibly also an antigen, for pharmaceutical use, i.e. use in a method of treatment of the human or animal body by surgery or therapy, including prophylaxis ("vaccination"). As disclosed, the bacterium may be Gram-positive, is preferably non-commensal and/or non-colonising and suitable examples include *Lactococcus*. The method preferably comprises administration to a mucosal membrane of an individual, e.g. to enhance an immune response in the individual.

A further aspect of the invention provides the use of any bacterium as disclosed in the manufacture of

a composition, i.e. a pharmaceutical composition or medicament, for administration to an individual. Such administration is preferably to a mucosal membrane of the individual and may be to enhance an immune response in the individual, e.g. to an antigen expressed by the bacterium.

Embodiments of each aspect of the present invention will be apparent from the disclosure and those skilled in the art will appreciate that modifications may be made. Further aspects and embodiments will be apparent. By way of experimental exemplification and not limitation, use of an embodiment of the present invention in achieving a protective level of immune response to an antigen will now be described in detail with reference to the figures.

Figure 1 shows a flow scheme of plasmid constructions. The resulting plasmid pTTI2 may be used to express TTFC and IL-2, and resulting plasmid pTTI6 may be used to express TTFC and IL-6, in an organism such as *Lactococcus lactis*.

Figure 2a shows the vector pEX1 (also called pTREX1) into which a gene, such as an operon construct comprising coding sequences for an antigen (e.g. TTFC) and a biologically active polypeptide (e.g. a cytokine such as IL-2 or IL-6), may be inserted at the multiple cloning site (MCS).

Figure 2b shows an expanded view of a region of

pEX1 (pTREX1) showing the P1 promoter, Shine-Dalgarno sequence (SD) and transcription terminator sequence operably positioned for expression of a gene (including a multi-(di-)cistronic coding sequence) when inserted at the gene MCS (multiple cloning site).

Figure 3 shows the junction between the TTFC and Interleukin cistrons in the operon employed for expression.

Figure 4 shows TTFC-specific serum IgG titres of groups of six mice vaccinated intr-nasally with recombinant *Lactococcus lactis* expressing tetanus toxin fragment C (TTFC) with the murine cytokines IL-2 or IL-6.

All documents mentioned herein are incorporated by reference.

EXAMPLE 1

To acquire the simultaneous expression of TTFC and either mIL2 or mIL6, we have chosen for the construction of operons driving the two cistrons under investigation. We made use of vectors for constitutive expression. In general, we try to flank cistrons with an *XbaI* site immediately prior to the Shine-Dalgarno (SD) sequence and an *SpeI* site immediately after the stop codon. In this way, multiple cistrons can be easily exchanged and put in various combinations any desired array, since *XbaI* and *SpeI* yield the same

sticky ends. We have previously achieved the expression of mIL2 and mIL6 by means of the T7 promoter - T7 gene 10 ribosome binding site, so we chose to use the *XbaI* site present in the g10 ribosome binding site. For this arrangement we knew the SD sequence was well positioned. We choose to put the TTFC cistron in front of the interleukins.

Construction of plasmids

The construction of the plasmids is depicted in Figure 1. Plasmids carrying *mIL2* and *mIL6* were subjected to site directed mutagenesis to give extra *SpeI* sites immediately following the stop codons. The resulting plasmids were called pL2MIL2A and pL2MIL6A, respectively. A plasmid containing a fusion of the USP45 secretion leader and TTFC was used as the template for PCR amplification of the various TTFC sequences needed.

For operons driving intracellular TTFC production, the gene was amplified as a blunt - *SpeI/BamHI* fragment and cloned in the vector pTREX1, which was cut with *SphI*, blunted and recut with *BamHI*. The resulting plasmid was called pT1TT. From this plasmid, the 3' terminal 150 bp, *SpeI* TTFC fragment was isolated and cloned in the *XbaI* site of pL2MIL2A and pL2MIL6A. The resulting plasmids were called p3TTIL2 and p3TTIL6. We made use of a *KpnI* restriction site present in the 3' end of TTFC to reconstruct TTFC, and

thus obtain the desired operons, by ligating the *KpnI-SpeI* fragment from p3TTIL2 and p3TTIL6 with the appropriate *KpnI-PvuII* and *SpeI-PvuII* fragments from pT1TT. The resulting plasmids were called pTT12 and pTT16.

Expression of proteins

The expression of proteins was assayed by antibody detection. For this, colonies of the different strains under investigation were spotted on nitrocellulose membranes, placed on GM17 (difco) solid agar plates containing appropriate antibiotics. The plates were incubated overnight and blocked in PBS containing 2.5% skim milk powder. The filters were revealed with rabbit-anti-TTFC or rabbit anti MIL2. The experiment showed clear TTFC expression in all constructs which hold the TTFC gene. Moreover for pTTI2 and pTTAI2 the coexpression of IL2 and TTFC was detected. Since the junctions between TTFC units and *mil6* are identical to those between TTFC and *mil2* it may be presumed that IL6 was coexpressed with TTFC equally well.

Preparation of cells for immunisations

Bacterial strains for immunisations were grown from fresh overnight cultures which were back diluted at a ratio of 1ml of overnight culture into 15 ml fresh GM17 medium containing erythromycin at 5 ug/ml and

grown at 30°C. Cells were harvested at optical density at 600nm of between 0.5 and 1.0. Cells were washed in 1/10 of the original culture volume of 0.5% casamino acids, 0.2M sodium bicarbonate, 0.5% glucose before resuspending in 1/200th of the original culture volume and determination of bacterial cell concentration. Cells were then diluted in the above solution to give the required number of cells per immunisation.

Immunisation

Mice were lightly anaesthetised by inhalation using "Metofane". 10µl of the bacterial suspension, in a solution of 0.5% casein hydrolysate, 0.2M sodium bicarbonate and 0.5% glucose, were applied to each nostril in turn using an automatic pipette. The animals were observed closely for breathing difficulties until fully recovered from anaesthesia.

Results

Results are shown in Table 1 and Figure 4. Bacteria able to express either Interleukin-2 or Interleukin-6 elicited 10x more anti-TTFC antibody than bacteria expressing TTFC alone.

It is the rule for bacterial toxins that a protective effect is achieved once the antibody titre exceeds a threshold value. The levels of antibody titre found in the mice inoculated with bacteria containing pEX-TTFC/IL-2 and pEX-TTFC/IL-6 far exceeded

the threshold value for subsequent protection against tetanus toxin challenge (see Figure 4, titres at 35 days post vaccination).

Summary of the Experimental Exemplification

Artificial operons for the co-expression of an antigenic polypeptide (tetanus toxin fragment C-TTFC) and biologically active polypeptides (Interleukin 2; Interleukin-6) were separately constructed in a constitutive expression vector (pTREX1) so that the transcription of the TTFC gene and the interleukin gene could be controlled by the activity of a lactococcal promoter element of previously defined activity. The constructs were prepared so that, following translation of the mRNA transcribed from the artificial operons, the TTFC antigen would accumulate intracellularly. A secretion signal sequence was operably linked to the interleukin. When preparations of these bacteria were administered intranasally to mice, bacteria engineered to express either Interleukin-2 or Interleukin-6 elicited approximately 10x more anti-TTFC antibody than the constructs which expressed the TTFC alone. Thus, either of these interleukins possessed distinctive adjuvant activity in the experimental system.

Lactococcus lactis is not a commensal bacterium (unlike related species of lactobacilli, which inhabit the crops of chickens and are present in the enteric tracts of many mammals), and also depends for its

nutrition on the provision of amino acids and peptides which are unlikely to be available *in vivo*, so the demonstration that the cytokine-expressing strains of *L. lactis* are nevertheless able to augment antibody production is surprising. These results demonstrate for the first time that recombinant strains of a non-colonising, non-invasive bacterium such as *Lactococcus lactis* can be used to synthesise and deliver biologically active molecules *in vivo*.

TABLE 1 (overleaf)

In the table, "TT/9" is used to indicate inoculation with bacteria expressing TTFC at a dose of $1x10^9$ bacteria, "TT/8" at a dose of $1x10^8$ bacteria, and so on. "TT IL-2/9" and "TT IL-6/9" indicate inoculation with bacteria expressing TTFC and IL-2, and TTFC and IL-6, respectively, at a dose of $1x10^9$ bacteria, "TT IL-2/8" at a dose of $1x10^8$ bacteria and so on. The figures given are ELISA titres for individual mice.

Table 1 IMMUNE RESPONSE DATA - DAY 35

End-point titres Bleed 3 Nasal vaccinations data

	TT/9	TT/8	TT/7	TT/6
	10000	50	50	50
	11000	60	50	50
	10000	50	50	75
	9000	50	50	50
	4500	50	50	70
	600	110	55	250
Mean	7516.7	61.7	50.8	90.8
sd	4089.2	24.0	2.0	78.8

	TT IL-2/9	TT IL-2/8	TT IL-2/7	TT IL-2/6
	14000	50	50	50
	30000	50	50	150
	100000	50	50	50
	100000	105	150	50
	120000	50	80	50
	100000	100	50	50
Mean	77333.0	67.5	71.7	66.7
sd	43848.0	27.2	40.2	40.8

	TT IL-6/9	TT IL-6/8	TT IL-6/7	TT IL-6/6
	80000	200	50	50
	170000	300	50	50
	190000	200	100	50
	100000	10000	50	50
	50000	750	50	50
	80000	260	400	50
Mean	111670.0	1951.7	116.7	50.0
sd	55648.0	3948.3	140.2	0.0

CONTROLS

	pEX1/9	pEX1/8	pEX1/7	pEX1/6	Naive
	75	75	55	75	60
	75	50	75	55	60
	50	55	75	75	55
	55	50	55	50	55
	75	55	75	50	50
	60	55	70	50	60
Mean	65.0	56.7	67.5	59.2	56.7
sd	11.4	0.9	1.0	12.4	4.1

CLAIMS:

1. A method of delivering one or more biologically active polypeptides which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses said one or more polypeptides.

2. A method of delivering one or more antigens which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses said one or more antigens.

3. A method of delivering one or more antigens and/or one or more biologically active polypeptides which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses both said one or more antigens and said one or more heterologous biologically active polypeptides.

4. A method as claimed in claim 1 or claim 3 wherein the one or more biologically active polypeptides is/are heterologous to the bacterium.

5. A method as claimed in claim 4 wherein at least one of the heterologous polypeptides is derived from a eukaryote or its virus.

6. A method as claimed in claim 4 wherein at least one of the heterologous polypeptides is derived from a prokaryote or its virus or from a virus homologous to the bacterial species.

7. A method as claimed in any one of claims 1 to 6 wherein the bacterium is a Gram-positive bacterium.

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8. A method as claimed in claim 7 wherein the Gram-positive bacterium is *Listeria innocua*, *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Streptococcus gordoni*, a *Lactococcus* species or a *Lactobacillus* species.

9. A method as claimed in claim 8 wherein the Gram-positive bacterium is *Lactococcus lactis*.

10. A method as claimed in claim 7 wherein the bacterium is an attenuated strain of a Gram-positive pathogenic bacterium.

11. A method as claimed in claim 10 wherein the bacterium is *Listeria monocytogenes*.

12. A method as claimed in any one of claims 5 to 11 wherein the one or more biologically active polypeptides is/are one(s) which is/are capable of functioning locally or systemically, eg is a polypeptide capable of exerting endocrine activities affecting local or whole-body metabolism.

13. A method as claimed in any one of claims 5 to 11 wherein the one or more biologically active polypeptides is/are one(s) which is/are capable of the regulation of the activities of cells belonging to the immunohaemopoeitic system.

14. A method as claimed in any one of claims 5 to 11 wherein the one or more biologically active polypeptides is/are one(s) which is/are capable of affecting the viability, growth and differentiation of a variety of normal or neoplastic cells in the body or

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affecting the immune regulation or induction of acute phase inflammatory responses to injury and infection.

15. A method as claimed in any one of claims 5 to 11 wherein the one or more biologically active polypeptides is/are one(s) which is/are capable of enhancing or inducing resistance to infection of cells and tissues mediated by chemokines acting on their target cell receptors, or the proliferation of epithelial cells or the promotion of wound healing.

16. A method as claimed in any one of claims 5 to 11 wherein the one or more biologically active polypeptides modulates the expression or production of substances by cells in the body.

17. A method as claimed in any one of claims 12 to 16 wherein the one or more biologically active polypeptides is/are insulin, growth hormone, prolactin, calcitonin, luteinising hormone, parathyroid hormone, somatostatin, thyroid stimulating hormone or vasoactive intestinal polypeptide.

18. A method as claimed in any one of claims 12 to 16 wherein the one or more biologically active polypeptides is/are a structural group 1 cytokine adopting an antiparallel 4α helical bundle structure such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, GM-CSF, M-CSF, SCF, IFN-γ, EPO, G-CSF, LIF, OSM, CNTF, GH, PRL or IFNα/β.

19. A method as claimed in any one of claims 12 to 16 wherein the one or more biologically active polypeptides is/are a structural group 2 cytokine which

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are often cell-surface asociated, form symetric homotrimers and the subunits take up the conformation of β-jelly roll described for certain viral coat proteins such as the TNF family of cytokines, eg TNFα, TNFβ, CD40, CD27 or FAS ligands, the IL-1 family of cytokines, the fibroblast growth factor family, the platelet derived growth factors, transforming growth factor β and nerve growth factors.

20. A method as claimed in any one of claims 12 to 16 wherein the one or more biologically active polypeptides is/are a structural group 3 cytokine comprising short chain α/β molecules, which are produced as large transmembrane pre-cursor molecules which each contain at least one EGF domain in the extracellular region, eg the epidermal growth factor family of cytokines, the chemokines characterised by their possession of amino acid sequences grouped around conserved cysteine residues (the C-C or C-X-C chemokine subgroups) or the insulin related cytokines.

21. A method as claimed in any one of claims 12 to 16 wherein the one or more biologically active polypeptides is/are a structural group 4 cytokine which exhibit mosaic structures such as the heregulins or neuregulins composed of different domains, eg EGF, immunoglobulin-like and kringle domains.

22. A method as claimed in any one of claims 12 to 16 wherein the one or more biologically active polypeptides is/are a receptor or antagonist for biologically active polypeptides as defined in any one of claims 10 to 19.

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23. A method of regulating the survival, growth, differentiation, effector functions or susceptibility to infection of cells or tissues which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11.

24. A method of boosting an immune response against tumour cells or an infection colonising a mucosal surface or adjacent or distant tissue which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11.

25. A method of modulating the type of immune response (antibody versus cell-mediated) against a pathogenic infectious agent which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11.

26. A method of modulating the infiltration of normal tissues with inflammatory or tumour cells which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11.

27. A method of controlling the rate of growth, rate of invasion or survival of tumour cells which comprises administering to a subject a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11.

28. A method of inducing apoptosis in tumour cells which comprises administering to a subject a non-

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invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11.

29. A method as claimed in any one of claims 23 to 28 modified by any one or more of the features of claims 12 to 22

30. A method of downregulating an immune response which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses a biologically active polypeptide.

31. A method of treating an allergic autoimmune or other immune dysregulative disease state, which comprises administering to a subject a non-invasive or non-pathogenic bacterium which expresses a biologically active polypeptide.

32. A method as claimed in claim 30 or claim 31 modified by any one or more of the features of claims 7 to 22.

33. A pharmaceutical formulation comprising a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11, optionally together with one or more pharmaceutically acceptable excipients, adjuvants, carriers,or the like.

34. A pharmaceutical formulation as claimed in claim 33 which is a vaccine formulation.

35. A pharmaceutical formulation as claimed in claim 33 or claim 34 modified by any one or more of the features of claims 12 to 22.

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36. A method of producing a pharmaceutical formulation as defined in any one of claims 33 to 35 which comprises the step of admixing one or more non-invasive or non-pathogenic bacteria as defined in any one of claims 1 to 11 with one or more pharmaceutically acceptable carriers.

37. Nucleic acid comprising one or more coding sequences for one or more biologically active polypeptides and one or more coding sequences for one or more antigens wherein each coding sequence is under the control of a promoter for expression in a non-invasive or non-pathogenic bacterium.

38. Nucleic acid as claimed in claim 37 modified by one or more of the features of claims 7 to 22.

39. Nucleic acid as claimed in claim 37 or claim 38 which comprises one or more nucleic acid constructs in which the nucleic acid encoding the one or more biologically active polypeptides and/or the nucleic acid encoding the one or more antigens are under the control of appropriate regulatory sequences.

40. Nucleic acid as claimed in claim 39 wherein the appropriate regulatory sequences are selected from promoter sequences, terminator fragments, enhancer sequences and marker genes.

41. Nucleic acid as claimed in claim 39 or claim 40 wherein the one or more nucleic acid constructs comprise an artificial operon capable of generating a polycistronic RNA transcript.

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42. Nucleic acid as claimed in any one of claims 37 to 41 wherein the promoter is a *Lactococcal* promoter for use in *Lactococcus lactis*.

43. Nucleic acid as claimed in any one of claims 37 to 42 which further comprises a secretory signal sequence, upstream of the coding sequence(s) for the one or more biologically active polypeptides.

44. Nucleic acid as claimed in claim 43 wherein the secretory signal sequence is the α-amylase secretion leader of *Bacillus amyloliquefaciens*, the secretion leader of the Staphylokinase enzyme, leader sequences for other *Bacillus* enzymes or S-layer proteins or the leader sequence of the *Lactococcal* protein *Usp*45.

45. Nucleic acid as claimed in any one of claims 37 to 44 wherein the antigen(s) is/are antigen(s) capable of eliciting a protective immune response.

46. Nucleic acid as claimed in any one of claims 37 to 44 wherein the antigen(s) is/are ones where a protective immune response is accelerated, amplified or rendered of longer duration in the presence of one or more coexpressed biologically active polypeptides as defined in any one of claims 12 to 22.

47. The use of nucleic acid as defined in any one of claims 37 to 46 in the transformation of a non-invasive or non-pathogenic bacterium, eg *Lactococcus lactis*.

48. A method of generating a bacterium as defined in any one of claims 1 to 11 comprising the step of introducing into a non-invasive or non-pathogenic

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bacterial host cell nucleic acid as defined in any one of claims 37 to 46.

49. A non-invasive or non-pathogenic bacterium expressing (i) one or more heterologous biologically active polypeptides and (ii) one or more antigens.

50. A non-invasive or non-pathogenic bacterium as claimed in claim 49 modified by any one or more of the features of claims 7 to 22.

51. A non-invasive or non-pathogenic bacterium as claimed in claim 49 or claim 50 which comprises nucleic acid as defined in any one claims 37 to 46.

52. A non-invasive or non-pathogenic bacterium as defined in any one of claims 49 to 51 for use in medicine.

53. The use of a non-invasive or non-pathogenic bacterium as defined in any one of claims 1 to 11 or one which comprises nucleic acid as defined in any one of claims 37 to 46 in the manufacture of an agent for the delivery of one or more biologically active polypeptides and/or one or more antigens.

54. The use as claimed in claim 53 modified by any one or more of the features of claims 23 to 32.

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