

## IMMUNISATION AGAINST *CHLAMYDIA TRACHOMATIS*

All documents cited herein are incorporated by reference in their entirety.

### TECHNICAL FIELD

This invention is in the field of immunisation against chlamydial infection, in particular against  
5 infection by *Chlamydia trachomatis*.

### BACKGROUND ART

*Chlamydiae* are obligate intracellular parasites of eukaryotic cells which are responsible for endemic sexually transmitted infections and various other disease syndromes. They occupy an exclusive eubacterial phylogenic branch, having no close relationship to any other known organisms – they are  
10 classified in their own order (*Chlamydiales*) which contains a single family (*Chlamydiaceae*) which in turn contains a single genus (*Chlamydia*, also referred to as *Chlamydophila*). A particular characteristic of the *Chlamydiae* is their unique life cycle, in which the bacterium alternates between two morphologically distinct forms: an extracellular infective form (elementary bodies, EB) and an intracellular non-infective form (reticulate bodies, RB). The life cycle is completed with the  
15 re-organization of RB into EB, which leave the disrupted host cell ready to infect further cells.

Four chlamydial species are currently known – *C.trachomatis*, *C.pneumoniae*, *C.pecorum* and *C.psittaci* {e.g. refs. 1, 2} – and genome sequences are available {refs. 3 to 9}.

The human serovariants (“serovars”) of *C.trachomatis* are divided into two biovariants (“biovars”). Serovars A-K elicit epithelial infections primarily in the ocular tissue (A-C) or urogenital tract (D-K). Serovars L1, L2 and L3 are the agents of invasive lymphogranuloma venereum (LGV).  
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Although chlamydial infection itself causes disease, it is thought that, in some patients, the severity of symptoms is due, in fact, to an aberrant host immune response. Failure to clear the infection results in persistent immune stimulation and, rather than helping the host, this results in chronic infection with severe consequences, including sterility and blindness {10}. In addition, the protection  
25 conferred by natural chlamydial infection, is usually incomplete, transient, and strain-specific.

Due to the serious nature of the disease, there is a desire to provide suitable vaccines. These may be useful (a) for immunisation against chlamydial infection or against chlamydia-induced disease (prophylactic vaccination) or (b) for the eradication of an established chronic chlamydial infection (therapeutic vaccination). Being an intracellular parasite, however, the bacterium can generally evade  
30 antibody-mediated immune responses.

Various antigenic proteins have been described for *C.trachomatis*, and the cell surface in particular has been the target of detailed research {eg. 1, 11}. These include, for instance, pgp3 {12, 13, 14}, MOMP {15}, Hsp60 (GroEL) {16} and Hsp70 (DnaK-like) {17}. Not all of these have proved to be effective vaccines, however, so it is an object of the invention to identify *C.trachomatis* antigens  
35 which elicit an immune response during natural infection, in order to provide antigens and immunogens suitable for use in vaccine development. It is a further object to identify antigens useful for diagnosis (e.g. immunodiagnosis) of *C.trachomatis*.

**DISCLOSURE OF THE INVENTION**

Reference 18 discloses various proteins from *C.pneumoniae* which were empirically verified as being immunoreactive, immunoaccessible and/or present in elementary bodies. These properties of the proteins were not derivable from the genomic sequence information. Reference 18 discloses that these proteins can be used in the treatment or prevention of infection due to *Chlamydia* bacteria, with *C.pneumoniae* being the main focus. The *C.pneumoniae* proteins can also be used for treating or preventing infection by other species of *Chlamydia*, due to inter-species cross-reactivity.

*C.pneumoniae* is closely related to *C.trachomatis*, as shown by whole genome comparisons {3,4,5}.

The present invention relates to *C.trachomatis* proteins (odd numbered SEQ IDs 1-261) which correspond to the *C.pneumoniae* proteins disclosed in reference 18. These proteins can be used in the treatment or prevention of infection due to *Chlamydia* bacteria, and in particular *C.trachomatis*. Particularly preferred proteins are those previously annotated as 'hypothetical protein' (see Table I herein) or those which were previously thought to have a cytoplasmic location.

***C.trachomatis* proteins**

The invention provides proteins comprising one or more of the odd-numbered amino acid sequences SEQ IDs 1-261.

It also provides proteins comprising sequences which share at least  $x\%$  sequence identity with one or more of the odd-numbered amino acid sequences SEQ IDs 1-261. Depending on the particular sequence,  $x$  is preferably 50% or more (e.g. 60%, 70%, 80%, 90%, 95%, 99% or more). These include mutants and allelic variants. Typically, 50% identity or more between two proteins is considered to be an indication of functional equivalence. Identity between proteins is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty*=12 and *gap extension penalty*=1.

The invention further provides proteins comprising fragments of the odd-numbered amino acid sequences SEQ IDs 1-261. The fragments should comprise at least  $n$  consecutive amino acids from the sequences and, depending on the particular sequence,  $n$  is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 75, 100, 150, 200 or more). Preferably the fragments comprise one or more epitope(s) from the sequence. Other preferred fragments omit a signal peptide.

The proteins of the invention can be prepared by various means e.g. by chemical synthesis (at least in part), by digesting longer polypeptides using proteases, by translation from RNA, by purification from cell culture (e.g. from recombinant expression or from *C.trachomatis* culture) etc. Heterologous expression in *E.coli* is a preferred preparative route.

The proteins of the invention can take various forms e.g. native, fusions, glycosylated, non-glycosylated, lipidated etc.).

Proteins of the invention are preferably prepared in substantially pure form (*ie.* substantially free from other *C.trachomatis* or host cell proteins).

Proteins of the invention may be attached to a solid support. They may comprise a detectable label (*e.g.* a radioactive or fluorescent label, or a biotin label).

- 5 Proteins of the invention are preferably *Chlamydial* proteins.

#### ***C.trachomatis* nucleic acids**

The invention provides proteins comprising one or more of the even-numbered nucleotide sequences SEQ IDs 2-262.

- 10 The invention also provides nucleic acid comprising sequences which share at least  $x\%$  sequence identity with the even-numbered nucleotide sequences SEQ IDs 2-262. Depending on the particular sequence,  $x$  is preferably 50% or more (*e.g.* 60%, 70%, 80%, 90%, 95%, 99% or more).

- 15 Furthermore, the invention provides nucleic acid which can hybridise to nucleic acid comprising the even-numbered nucleotide sequences SEQ IDs 2-262. Hybridisation reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridisation reaction of widely known and published in the art. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C, 55°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 x SSC, 1 x SSC, 0.1 x SSC, or de-ionized water. In some embodiments, the isolated nucleic acid of the invention selectively hybridises under low stringency conditions; in other embodiments it selectively hybridises under intermediate stringency conditions; in other embodiments, it selectively hybridises under high stringency conditions. An exemplary set of low stringency hybridisation conditions is 50°C and 10xSSC. An exemplary set of intermediate stringency hybridisation conditions is 55°C and 1xSSC. An exemplary set of high stringent hybridisation conditions is 68°C and 0.1 x SSC.

- 20 Nucleic acid comprising fragments of the even-numbered nucleotide sequences SEQ IDs 2-262 are also provided. These should comprise at least  $n$  consecutive nucleotides from the *C.trachomatis* sequences and, depending on the particular sequence,  $n$  is 7 or more (*e.g.* 10, 12, 14, 15, 18, 20, 25, 30, 35, 40, 50, 75, 100, 200, 300 or more).

According to a further aspect, the invention provides nucleic acid encoding the proteins and protein fragments of the invention.

The invention provides nucleic acid comprising sequences complementary to those described above (*e.g.* for antisense or probing purposes).

Nucleic acid of the invention can, of course, be prepared in many ways *e.g.* by chemical synthesis (at least in part), by digesting longer polynucleotides using restriction enzymes, from genomic or cDNA libraries, from the organism itself *etc.*

Nucleic acid of the invention can take various forms (*e.g.* single-stranded, double-stranded, linear, circular, vectors, primers, probes *etc.*).

Nucleic acids of the invention may be attached to a solid support (*e.g.* a bead, plate, filter, film, slide, resin, *etc.*). Nucleic acids of the invention may include a detectable label (*e.g.* a radioactive or fluorescent label, or a biotin label). This is particularly useful where the polynucleotide is to be used in nucleic acid detection techniques *e.g.* where the nucleic acid is a primer or as a probe for use in techniques such as PCR, LCR, TMA, NASBA, bDNA *etc.*

Nucleic acids of the invention are preferably *Chlamydial* nucleic acids.

The term “nucleic acid” includes DNA, RNA, DNA/RNA hybrids, and DNA or RNA analogs, such as those containing modified backbones or bases, and also peptide nucleic acids (PNA) *etc.*

Nucleic acids of the invention may be isolated and obtained in substantial purity, generally as other than an intact chromosome. Usually, the polynucleotides will be obtained substantially free of other naturally-occurring nucleic acid sequences, generally being at least about 50% (by weight) pure, usually at least about 90% pure.

Nucleic acids can be used, for example: to produce polypeptides; as probes for the detection of nucleic acid in biological samples; to generate additional copies of the polynucleotides; to generate ribozymes or antisense oligonucleotides; and as single-stranded DNA probes or as triple-strand forming oligonucleotides *etc.*

The invention provides vectors comprising nucleotide sequences of the invention (*e.g.* cloning or expression vectors) and host cells transformed therewith.

### **Compositions**

According to a further aspect, the invention provides compositions comprising protein and/or nucleic acid according to the invention. These compositions are preferably immunogenic compositions, such as vaccines, and are suitable for immunisation and vaccination purposes. Vaccines of the invention may be prophylactic or therapeutic, and will typically comprise an antigen which can induce antibodies capable of inhibiting (a) chlamydial adhesion, (b) chlamydial entry, and/or (c) successful replication within the host cell. The vaccines preferably induce any cell-mediated T-cell responses which are necessary for chlamydial clearance from the host.

The invention also provides nucleic acid or protein according to the invention for use as medicaments (*e.g.* as vaccines).

The invention also provides the use of nucleic acid or protein according to the invention in the manufacture of a medicament (e.g. a vaccine or an immunogenic composition) for treating or preventing infection due to a *Chlamydia*. This will generally be *C.trachomatis* but, due to inter-species cross-reactivity, it may also be *C.pneumoniae*, *C.pecorum* or *C.psittaci*. For prevention, the medicament preferably elicits an immune response which is specific to the EB form of *Chlamydia*; for treatment, the medicament preferably elicits an immune response which is specific to the RB form of *Chlamydia*.

The invention also provides the use of nucleic acid or protein according to the invention in the manufacture of a medicament (e.g. a vaccine or an immunogenic composition) for neutralizing *Chlamydia trachomatis* elementary bodies.

The invention also provides a method of treating (e.g. immunising) a patient (e.g. a human), comprising administering to the patient a therapeutically effective amount of nucleic acid or protein according to the invention.

The invention also provides a method of raising an immune response in a patient, comprising administering to the patient an immunologically effective amount of nucleic acid or protein according to the invention. The immune response may involve raising antibodies in the patient and/or raising a cellular immune response (e.g. a CTL response). The immune response may be specific for an EB or a RB protein, or to a protein which is expressed in the host cytoplasm. An antibody response is preferably specific to an EB, whereas a cellular immune response is preferably specific to a cytoplasmic protein or, preferably, to an RB protein.

The invention also provides a method of raising antibodies which recognise a protein of the invention, comprising the step of administering to a patient a *Chlamydia* elementary body or reticulate body. The antibodies are preferably specific to an EB.

The invention also provides a method of neutralizing *C.trachomatis* infectivity, comprising the step of administering to a patient a protein, nucleic acid or antibody of the invention. The method preferably neutralizes EB infectivity.

The invention also provides a method for detecting a *Chlamydia* EB or RB in a biological sample, comprising the step of contacting an antibody of the invention with the sample. The sample could be a blood sample, another bodily fluid, or a tissue sample. The method may be used to diagnose chlamydial infection.

Immunogenic compositions of the invention may also include one or more of the following antigens:

- a protein antigen from *Helicobacter pylori* such as VacA, CagA, NAP, HopX, HopY {e.g. WO98/04702} and/or urease.
- a protein antigen from *N.meningitidis* serogroup B, such as those in WO99/24578, WO99/36544, WO99/57280, WO00/22430, Tettelin *et al.* (2000) *Science* 287:1809-1815, Pizza

*et al.* (2000) *Science* 287:1816-1820 and WO96/29412, with protein '287' and derivatives being particularly preferred.

- an outer-membrane vesicle (OMV) preparation from *N.meningitidis* serogroup B, such as those disclosed in WO01/52885; Bjune *et al.* (1991) *Lancet* 338(8775):1093-1096; Fukasawa *et al.* (1999) *Vaccine* 17:2951-2958; Rosenqvist *et al.* (1998) *Dev. Biol. Stand.* 92:323-333 *etc.*
- a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in Costantino *et al.* (1992) *Vaccine* 10:691-698 from serogroup C {see also Costantino *et al.* (1999) *Vaccine* 17:1251-1263}.
- a saccharide antigen from *Streptococcus pneumoniae* {e.g. Watson (2000) *Pediatr Infect Dis J* 19:331-332; Rubin (2000) *Pediatr Clin North Am* 47:269-285, v; Jedrzejewski (2001) *Microbiol Mol Biol Rev* 65:187-207}.
- an antigen from hepatitis A virus, such as inactivated virus {e.g. Bell (2000) *Pediatr Infect Dis J* 19:1187-1188; Iwarson (1995) *APMIS* 103:321-326}.
- an antigen from hepatitis B virus, such as the surface and/or core antigens {e.g. Gerlich *et al.* (1990) *Vaccine* 8 Suppl:S63-68 & 79-80}.
- an antigen from hepatitis C virus {e.g. Hsu *et al.* (1999) *Clin Liver Dis* 3:901-915}.
- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 {e.g. Gustafsson *et al.* (1996) *N. Engl. J. Med.* 334:349-355; Rappuoli *et al.* (1991) *TIBTECH* 9:232-238}.
- a diphtheria antigen, such as a diphtheria toxoid {e.g. chapter 3 of *Vaccines* (1988) eds. Plotkin & Mortimer. ISBN 0-7216-1946-0} e.g. the CRM<sub>197</sub> mutant {e.g. Del Giudice *et al.* (1998) *Molecular Aspects of Medicine* 19:1-70}.
- a tetanus antigen, such as a tetanus toxoid {e.g. chapter 4 of Plotkin & Mortimer}.
- a saccharide antigen from *Haemophilus influenzae* B.
- an antigen from *N.gonorrhoeae* {e.g. WO99/24578, WO99/36544, WO99/57280}.
- an antigen from *Chlamydia pneumoniae* {e.g. PCT/IB01/01445; Kalman *et al.* (1999) *Nature Genetics* 21:385-389; Read *et al.* (2000) *Nucleic Acids Res* 28:1397-406; Shirai *et al.* (2000) *J. Infect. Dis.* 181(Suppl 3):S524-S527; WO99/27105; WO00/27994; WO00/37494}.
- an antigen from *Chlamydia trachomatis* {e.g. WO99/28475}.
- an antigen from *Porphyromonas gingivalis* {e.g. Ross *et al.* (2001) *Vaccine* 19:4135-4142}.
- polio antigen(s) {e.g. Sutter *et al.* (2000) *Pediatr Clin North Am* 47:287-308; Zimmerman & Spann (1999) *Am Fam Physician* 59:113-118, 125-126} such as IPV or OPV.
- rabies antigen(s) {e.g. Dreesen (1997) *Vaccine* 15 Suppl:S2-6} such as lyophilised inactivated virus {e.g. *MMWR Morb Mortal Wkly Rep* 1998 Jan 16;47(1):12, 19; RabAvert™}.

- measles, mumps and/or rubella antigens {e.g. chapters 9, 10 & 11 of Plotkin & Mortimer}.
- influenza antigen(s) {e.g. chapter 19 of Plotkin & Mortimer}, such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* {e.g. McMichael (2000) *Vaccine* 19 Suppl 1:S101-107}.
- 5 – an antigen from *Staphylococcus aureus* {e.g. Kuroda *et al.* (2001) *Lancet* 357(9264):1225-1240; see also pages 1218-1219}.
- an antigen from *Streptococcus agalactiae* {e.g. see WO02/34771}
- an antigen from *Streptococcus pyogenes* {e.g. see WO02/34771}

Where a saccharide or carbohydrate antigen is included, it is preferably conjugated to a carrier protein in order to enhance immunogenicity {e.g. Ramsay *et al.* (2001) *Lancet* 357(9251):195-196; Lindberg (1999) *Vaccine* 17 Suppl 2:S28-36; *Conjugate Vaccines* (eds. Cruse *et al.*) ISBN 3805549326, particularly vol. 10:48-114 *etc.*}. Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The CRM<sub>197</sub> diphtheria toxoid is particularly preferred. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein {e.g. EP-0372501}, synthetic peptides {e.g. EP-0378881, EP-0427347}, heat shock proteins {e.g. WO93/17712}, pertussis proteins {e.g. WO98/58668; EP-0471177}, protein D from *H.influenzae* {e.g. WO00/56360}, toxin A or B from *C.difficile* {e.g. WO00/61761}, *etc.* Any suitable conjugation reaction can be used, with any suitable linker where necessary.

Toxic protein antigens may be detoxified where necessary (e.g. detoxification of pertussis toxin by chemical and/or genetic means).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens.

Antigens are preferably adsorbed to an aluminium salt.

Antigens in the composition will typically be present at a concentration of at least 1 µg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

The invention also provides compositions comprising two or more proteins of the present invention.

## 30 Processes

The invention provides a process for producing proteins of the invention, comprising the step of culturing a host cell according to the invention under conditions which induce protein expression.

The invention provides a process for producing protein or nucleic acid of the invention, wherein the protein or nucleic acid is synthesised in part or in whole using chemical means.

The invention provides a process for detecting *C.trachomatis* in a sample, wherein the sample is contacted with an antibody which binds to a protein of the invention .

A summary of standard techniques and procedures which may be employed in order to perform the invention (e.g. to utilise the disclosed sequences for immunisation) follows. This summary is not a  
 5 limitation on the invention but, rather, gives examples that may be used, but are not required.

### General

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature e.g. Sambrook *Molecular Cloning; A Laboratory Manual*,  
 10 *Second Edition* (1989) and *Third Edition* (2001); *DNA Cloning, Volumes I and ii* (D.N Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed, 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Transcription and Translation* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.I. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning* (1984); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 &  
 15 155; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Mayer and Walker, eds. (1987), *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes, (1987) *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), and *Handbook of Experimental Immunology, Volumes I-IV* (D.M. Weir and C. C. Blackwell eds 1986).

20 Standard abbreviations for nucleotides and amino acids are used in this specification.

### Definitions

A composition containing X is "substantially free of" Y when at least 85% by weight of the total X+Y in the composition is X. Preferably, X comprises at least about 90% by weight of the total of X+Y in the composition, more preferably at least about 95% or even 99% by weight.

25 The term "comprising" means "including" as well as "consisting" e.g. a composition "comprising" X may consist exclusively of X or may include something additional to X, such as X+Y.

The term "heterologous" refers to two biological components that are not found together in nature. The components may be host cells, genes, or regulatory regions, such as promoters. Although the heterologous components are not found together in nature, they can function together, as when a promoter heterologous to a  
 30 gene is operably linked to the gene. Another example is where a Chlamydial sequence is heterologous to a mouse host cell. A further examples would be two epitopes from the same or different proteins which have been assembled in a single protein in an arrangement not found in nature.

An "origin of replication" is a polynucleotide sequence that initiates and regulates replication of polynucleotides, such as an expression vector. The origin of replication behaves as an autonomous unit of polynucleotide  
 35 replication within a cell, capable of replication under its own control. An origin of replication may be needed for a vector to replicate in a particular host cell. With certain origins of replication, an expression vector can be reproduced at a high copy number in the presence of the appropriate proteins within the cell. Examples of origins are the autonomously replicating sequences, which are effective in yeast; and the viral T-antigen, effective in COS-7 cells.

A "mutant" sequence is defined as DNA, RNA or amino acid sequence differing from but having sequence identity with the native or disclosed sequence. Depending on the particular sequence, the degree of sequence identity between the native or disclosed sequence and the mutant sequence is preferably greater than 50% (*e.g.* 60%, 70%, 80%, 90%, 95%, 99% or more, calculated using the Smith-Waterman algorithm as described above).

- 5 As used herein, an "allelic variant" of a nucleic acid molecule, or region, for which nucleic acid sequence is provided herein is a nucleic acid molecule, or region, that occurs essentially at the same locus in the genome of another or second isolate, and that, due to natural variation caused by, for example, mutation or recombination, has a similar but not identical nucleic acid sequence. A coding region allelic variant typically encodes a protein having similar activity to that of the protein encoded by the gene to which it is being compared. An allelic  
10 variant can also comprise an alteration in the 5' or 3' untranslated regions of the gene, such as in regulatory control regions (*e.g.* see US patent 5,753,235).

#### Expression systems

The Chlamydial nucleotide sequences can be expressed in a variety of different expression systems; for example those used with mammalian cells, baculoviruses, plants, bacteria, and yeast.

#### 15 i. Mammalian Systems

- Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream  
20 of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation {Sambrook et al. (1989) "Expression of Cloned Genes in Mammalian Cells." In *Molecular Cloning: A Laboratory Manual*, 2nd ed.}.

- 25 Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallothionein gene, also provide useful promoter sequences. Expression may be either constitutive or  
30 regulated (inducible), depending on the promoter can be induced with glucocorticoid in hormone-responsive cells.

- The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal  
35 RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter {Maniatis et al. (1987) *Science* 236:1237; Alberts et al. (1989) *Molecular Biology of the Cell*, 2nd ed.}. Enhancer elements derived from viruses may be particularly useful, because they usually have a broader host range. Examples include the SV40 early gene enhancer {Dijkema et al (1985) *EMBO J.* 4:761} and the  
40 enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus {Gorman et al.

(1982) *PNAS USA* 79:6777} and from human cytomegalovirus {Boshart et al. (1985) *Cell* 41:521}. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion {Sassone-Corsi and Borelli (1986) *Trends Genet.* 2:215; Maniatis et al. (1987) *Science* 236:1237}.

- 5 A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

- Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric  
10 DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a  
15 foreign protein in mammalian cells.

- Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation {Birnstiel et al. (1985) *Cell* 41:349; Proudfoot and Whitelaw (1988) "Termination and 3' end  
20 processing of eukaryotic RNA. In *Transcription and splicing* (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) *Trends Biochem. Sci.* 14:105}. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 {Sambrook et al (1989) "Expression of cloned genes in cultured mammalian cells." In *Molecular Cloning: A Laboratory Manual*}.

- 25 Usually, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those  
30 derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 {Gluzman (1981) *Cell* 23:175} or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replicaton systems, thus allowing it to be maintained, for example, in mammalian  
35 cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 {Kaufman et al. (1989) *Mol. Cell. Biol.* 9:946} and pHEBO {Shimizu et al. (1986) *Mol. Cell. Biol.* 6:1074}.

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated

transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of polynucleotide(s) in liposomes, direct microinjection of the DNA into nuclei.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.* Hep G2), and a number of other cell lines.

## ii. Baculovirus Systems

The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art. Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987) (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements; multiple genes, each with its own set of operably linked regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, *Virology* (1989) 17:31).

The plasmid usually also contains the polyhedrin polyadenylation signal (Miller et al. (1988) *Ann. Rev. Microbiol.*, 42:177) and a prokaryotic ampicillin-resistance (*amp*) gene and origin of replication for selection and propagation in *E.coli*.

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein, Friesen et al., (1986) "The Regulation of Baculovirus Gene Expression," in: *The Molecular Biology of Baculoviruses* (ed. Walter Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak et al., (1988), *J. Gen. Virol.* 69:765.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) *Gene*, 73:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human  $\alpha$ -interferon, Maeda et al., (1985), *Nature* 315:592; human gastrin-releasing peptide, Lebacqz-Verheyden et al., (1988), *Molec. Cell. Biol.* 8:3129; human IL-2, Smith et al., (1985) *Proc. Nat'l Acad. Sci. USA*, 82:8404; mouse IL-3, (Miyajima et al., (1987) *Gene* 58:273; and human glucocerebrosidase, Martin et al. (1988) *DNA*, 7:99, can also be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by *in vitro* incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus -- usually by co-transfection. The promoter and transcription termination sequence of the construct will usually comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith *supra*; Ju et al. (1987); Smith et al., *Mol. Cell. Biol.* (1983) 3:2156; and Luckow and Summers (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene.

Miller et al., (1989), *Bioessays* 4:91. The DNA sequence, when cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between ~1% and ~5%); thus, the majority of the virus produced after cotransfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 $\mu$ m in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies. "Current Protocols in Microbiology" Vol. 2 (Ausubel et al. eds) at 16.8 (Supp. 10, 1990); Summers & Smith, *supra*; Miller et al. (1989).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, *inter alia*: *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni* (WO 89/046699; Carbonell et al., (1985) *J. Virol.* 56:153; Wright (1986) *Nature* 321:718; Smith et al., (1983) *Mol. Cell. Biol.* 3:2156; and see generally, Fraser, et al. (1989) *In Vitro Cell. Dev. Biol.* 25:225).

Cells and cell culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression system; cell culture technology is generally known to those skilled in the art. See, e.g. Summers and Smith *supra*.

The modified insect cells may then be grown in an appropriate nutrient medium, which allows for stable maintenance of the plasmid(s) present in the modified insect host. Where the expression product gene is under inducible control, the host may be grown to high density, and expression induced. Alternatively, where expression is constitutive, the product will be continuously expressed into the medium and the nutrient medium must be continuously circulated, while removing the product of interest and augmenting depleted nutrients. The product may be purified by such techniques as chromatography, e.g. HPLC, affinity chromatography, ion exchange chromatography, etc.; electrophoresis; density gradient centrifugation; solvent extraction, or the like. As appropriate, the product may be further purified, as required, so as to remove substantially any insect proteins which are also secreted in the medium or result from lysis of insect cells, so as to provide a product which is at least substantially free of host debris, e.g. proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

### iii. Plant Systems

There are many plant cell culture and whole plant genetic expression systems known in the art. Exemplary plant cellular genetic expression systems include those described in patents, such as: US 5,693,506; US 5,659,122; and US 5,608,143. Additional examples of genetic expression in plant cell culture has been described by Zenk,  
 5 *Phytochemistry* 30:3861-3863 (1991). Descriptions of plant protein signal peptides may be found in addition to the references described above in Vaulcombe et al., *Mol. Gen. Genet.* 209:33-40 (1987); Chandler et al., *Plant Molecular Biology* 3:407-418 (1984); Rogers, *J. Biol. Chem.* 260:3731-3738 (1985); Rothstein et al., *Gene* 55:353-356 (1987); Whittier et al., *Nucleic Acids Research* 15:2515-2535 (1987); Wirsel et al., *Molecular Microbiology* 3:3-14 (1989); Yu et al., *Gene* 122:247-253 (1992). A description of the regulation of plant gene  
 10 expression by the phytohormone, gibberellic acid and secreted enzymes induced by gibberellic acid can be found in R.L. Jones and J. MacMillin, *Gibberellins*: in: *Advanced Plant Physiology*, Malcolm B. Wilkins, ed., 1984 Pitman Publishing Limited, London, pp. 21-52. References that describe other metabolically-regulated genes: Sheen, *Plant Cell*, 2:1027-1038(1990); Maas et al., *EMBO J.* 9:3447-3452 (1990); Benkel and Hickey, *Proc. Natl. Acad. Sci.* 84:1337-1339 (1987)

Typically, using techniques known in the art, a desired polynucleotide sequence is inserted into an expression cassette comprising genetic regulatory elements designed for operation in plants. The expression cassette is inserted into a desired expression vector with companion sequences upstream and downstream from the expression cassette suitable for expression in a plant host. The companion sequences will be of plasmid or viral origin and provide necessary characteristics to the vector to permit the vectors to move DNA from an original  
 20 cloning host, such as bacteria, to the desired plant host. The basic bacterial/plant vector construct will preferably provide a broad host range prokaryote replication origin; a prokaryote selectable marker; and, for *Agrobacterium* transformations, T DNA sequences for *Agrobacterium*-mediated transfer to plant chromosomes. Where the heterologous gene is not readily amenable to detection, the construct will preferably also have a selectable marker gene suitable for determining if a plant cell has been transformed. A general review of suitable markers,  
 25 for example for the members of the grass family, is found in Wilmink and Dons, 1993, *Plant Mol. Biol. Repr*, 11(2):165-185.

Sequences suitable for permitting integration of the heterologous sequence into the plant genome are also recommended. These might include transposon sequences and the like for homologous recombination as well as Ti sequences which permit random insertion of a heterologous expression cassette into a plant genome. Suitable  
 30 prokaryote selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art.

The nucleic acid molecules of the subject invention may be included into an expression cassette for expression of the protein(s) of interest. Usually, there will be only one expression cassette, although two or more are feasible. The recombinant expression cassette will contain in addition to the heterologous protein encoding  
 35 sequence the following elements, a promoter region, plant 5' untranslated sequences, initiation codon depending upon whether or not the structural gene comes equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

A heterologous coding sequence may be for any protein relating to the present invention. The sequence encoding  
 40 the protein of interest will encode a signal peptide which allows processing and translocation of the protein, as

appropriate, and will usually lack any sequence which might result in the binding of the desired protein of the invention to a membrane. Since, for the most part, the transcriptional initiation region will be for a gene which is expressed and translocated during germination, by employing the signal peptide which provides for translocation, one may also provide for translocation of the protein of interest. In this way, the protein(s) of interest will be translocated from the cells in which they are expressed and may be efficiently harvested. Typically secretion in seeds are across the aleurone or scutellar epithelium layer into the endosperm of the seed. While it is not required that the protein be secreted from the cells in which the protein is produced, this facilitates the isolation and purification of the recombinant protein.

Since the ultimate expression of the desired gene product will be in a eucaryotic cell it is desirable to determine whether any portion of the cloned gene contains sequences which will be processed out as introns by the host's splicosome machinery. If so, site-directed mutagenesis of the "intron" region may be conducted to prevent losing a portion of the genetic message as a false intron code, Reed and Maniatis, *Cell* 41:95-105, 1985.

The vector can be microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA. Crossway, *Mol. Gen. Genet*, 202:179-185, 1985. The genetic material may also be transferred into the plant cell by using polyethylene glycol, Krens, et al., *Nature*, 296, 72-74, 1982. Another method of introduction of nucleic acid segments is high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface, Klein, et al., *Nature*, 327, 70-73, 1987 and Knudsen and Muller, 1991, *Planta*, 185:330-336 teaching particle bombardment of barley endosperm to create transgenic barley. Yet another method of introduction would be fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies, Fraley, et al., *Proc. Natl. Acad. Sci. USA*, 79, 1859-1863, 1982.

The vector may also be introduced into the plant cells by electroporation. (Fromm et al., *Proc. Natl Acad. Sci. USA* 82:5824, 1985). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the gene construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.

All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are recovered which contain the transferred gene. It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to all major species of sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables. Some suitable plants include, for example, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersion*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hererocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, and *Datura*.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts containing copies of the heterologous gene is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced from the protoplast suspension. These embryos germinate as natural embryos to form plants. The culture media will

generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

In some plant cell culture systems, the desired protein of the invention may be excreted or alternatively, the protein may be extracted from the whole plant. Where the desired protein of the invention is secreted into the medium, it may be collected. Alternatively, the embryos and embryoless-half seeds or other plant tissue may be mechanically disrupted to release any secreted protein between cells and tissues. The mixture may be suspended in a buffer solution to retrieve soluble proteins. Conventional protein isolation and purification methods will be then used to purify the recombinant protein. Parameters of time, temperature pH, oxygen, and volumes will be adjusted through routine methods to optimize expression and recovery of heterologous protein.

#### iv. Bacterial Systems

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in *Escherichia coli* (*E.coli*) {Raibaud *et al.* (1984) *Annu. Rev. Genet.* 18:173}. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) {Chang *et al.* (1977) *Nature* 198:1056}, and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*) {Goeddel *et al.* (1980) *Nuc. Acids Res.* 8:4057; Yelverton *et al.* (1981) *Nucl. Acids Res.* 9:731; US patent 4,738,921; EP-A-0036776 and EP-A-0121775}. The g-laotamase (*bla*) promoter system {Weissmann (1981) "The cloning of interferon and other mistakes." In *Interferon 3* (ed. I. Gresser)}, bacteriophage lambda PL {Shimatake *et al.* (1981) *Nature* 292:128} and T5 {US patent 4,689,406} promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter {US patent 4,551,433}. For example, the *tac* promoter is a hybrid *trp-lac* promoter comprised of both *trp* promoter and *lac* operon sequences that is regulated by the *lac* repressor {Amann *et al.* (1983) *Gene* 25:167; de Boer *et al.*

(1983) *Proc. Natl. Acad. Sci.* 80:21}. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system {Studier *et al.* (1986) *J. Mol. Biol.* 189:113; Tabor *et al.* (1985) *Proc Natl. Acad. Sci.* 82:1074}. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E.coli* operator region (EPO-A-0 267 851).

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E.coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon {Shine *et al.* (1975) *Nature* 254:34}. The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' end of *E.coli* 16S rRNA {Steitz *et al.* (1979) "Genetic signals and nucleotide sequences in messenger RNA." In *Biological Regulation and Development: Gene Expression* (ed. R.F. Goldberger)}. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site {Sambrook *et al.* (1989) "Expression of cloned genes in *Escherichia coli*." In *Molecular Cloning: A Laboratory Manual*}.

A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* or *in vitro* incubation with a bacterial methionine N-terminal peptidase (EPO-A-0 219 237).

Fusion proteins provide an alternative to direct expression. Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor Xa) to cleave the bacteriophage protein from the foreign gene {Nagai *et al.* (1984) *Nature* 309:810}. Fusion proteins can also be made with sequences from the *lacZ* {Jia *et al.* (1987) *Gene* 60:197}, *trpE* {Allen *et al.* (1987) *J. Biotechnol.* 5:93; Makoff *et al.* (1989) *J. Gen. Microbiol.* 135:11}, and *Chey* {EP-A-0 324 647} genes. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (*e.g.* ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated {Miller *et al.* (1989) *Bio/Technology* 7:698}.

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein in bacteria {US patent 4,336,336}. The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites,

which can be cleaved either *in vivo* or *in vitro* encoded between the signal peptide fragment and the foreign gene.

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the *E.coli* outer membrane protein gene (*ompA*) {Masui *et al.* (1983), in: *Experimental Manipulation of Gene Expression*; Ghrayeb *et al.* (1984) *EMBO J.* 3:2437} and the *E.coli* alkaline phosphatase signal sequence (*phoA*) {Oka *et al.* (1985) *Proc. Natl. Acad. Sci.* 82:7212}. As an additional example, the signal sequence of the alpha-amylase gene from various *Bacillus* strains can be used to secrete heterologous proteins from *B. subtilis* {Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EP-A-0 244 042}.

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong promoters, such as the *trp* gene in *E.coli* as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (*e.g.* plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate into the *Bacillus* chromosome (EP-A- 0 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline {Davies *et al.* (1978) *Annu. Rev. Microbiol.* 32:469}. Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable market that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, *inter alia*, the following bacteria: *Bacillus subtilis* {Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EP-A-0 036 259 and EP-A-0 063 953; WO 84/04541}, *Escherichia coli* {Shimatake *et al.* (1981) *Nature* 292:128; Amann *et al.* (1985) *Gene* 40:183; Studier *et al.* (1986) *J. Mol. Biol.* 189:113; EP-A-0 036 776, EP-A-0 136 829 and EP-A-0 136 907}, *Streptococcus cremoris* {Powell *et al.* (1988) *Appl. Environ. Microbiol.* 54:655}; *Streptococcus lividans* {Powell *et al.* (1988) *Appl. Environ. Microbiol.* 54:655}, *Streptomyces lividans* {US patent 4,745,056}.

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with  $\text{CaCl}_2$  or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Transformation procedures usually vary with the bacterial species to be transformed. See *e.g.* {Masson *et al.* (1989) *FEMS Microbiol. Lett.* 60:273; Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EP-A-0 036 259 and EP-A-0 063 953; WO 84/04541, *Bacillus*}, {Miller *et al.* (1988) *Proc. Natl. Acad. Sci.* 85:856; Wang *et al.* (1990) *J. Bacteriol.* 172:949, *Campylobacter*}, {Cohen *et al.* (1973) *Proc. Natl. Acad. Sci.* 69:2110; Dower *et al.* (1988) *Nucleic Acids Res.* 16:6127; Kushner (1978) "An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids. In *Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering* (eds. H.W. Boyer and S. Nicosia); Mandel *et al.* (1970) *J. Mol. Biol.* 53:159; Taketo (1988) *Biochim. Biophys. Acta* 949:318; *Escherichia*}, {Chassy *et al.* (1987) *FEMS Microbiol. Lett.* 44:173 *Lactobacillus*}; {Fiedler *et al.* (1988) *Anal. Biochem* 170:38, *Pseudomonas*}; {Augustin *et al.* (1990) *FEMS Microbiol. Lett.* 66:203, *Staphylococcus*}, {Barany *et al.* (1980) *J. Bacteriol.* 144:698; Harlander (1987) "Transformation of *Streptococcus lactis* by electroporation, in: *Streptococcal Genetics* (ed. J. Ferretti and R. Curtiss III); Perry *et al.* (1981) *Infect. Immun.* 32:1295; Powell *et al.* (1988) *Appl. Environ. Microbiol.* 54:655; Somkuti *et al.* (1987) *Proc. 4th Eur. Cong. Biotechnology* 1:412, *Streptococcus*}.

#### v. Yeast Expression

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*e.g.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences. Examples include alcohol dehydrogenase (ADH) (EP-A-0 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO-A-0 329 203). The yeast *PHO5* gene, encoding acid phosphatase, also provides useful promoter sequences {Myanohara *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:1}.

In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (US Patent Nos. 4,876,197 and 4,880,734). Other  
5 examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, OR *PHO5* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EP-A-0 164 556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include, *inter alia*, {Cohen *et al.* (1980) *Proc. Natl. Acad. Sci. USA* 77:1078;  
10 Henikoff *et al.* (1981) *Nature* 283:835; Hollenberg *et al.* (1981) *Curr. Topics Microbiol. Immunol.* 96:119; Hollenberg *et al.* (1979) "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast *Saccharomyces cerevisiae*," in: *Plasmids of Medical, Environmental and Commercial Importance* (eds. K.N. Timmis and A. Puhler); Mercerau-Puigalon *et al.* (1980) *Gene* 11:163; Panthier *et al.* (1980) *Curr. Genet.* 2:109;}

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with  
15 the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast  
20 protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See *e.g.* EP-A-0 196 056. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin  
25 region that preferably retains a site for a processing enzyme (*e.g.* ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (*e.g.* WO88/024066).

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion  
30 in yeast of the foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the genes for invertase (EP-A-0012873; JPO 62,096,086) and A-factor (US patent 4,588,684). Alternatively, leaders  
35 of non-yeast origin exist, such as an interferon leader, that also provide for secretion in yeast (EP-A-0060057).

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated  
40 alpha-factor leaders (usually about 25 to about 50 amino acid residues) (US Patents 4,546,083 and 4,870,008; EP-A-0 324 274). Additional leaders employing an alpha-factor leader fragment that provides for secretion

include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (e.g. see WO 89/02463.)

Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes.

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g. plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a prokaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 {Botstein *et al.* (1979) *Gene* 8:17-24}, pCI/1 {Brake *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:4642-4646}, and YRp17 {Stinchcomb *et al.* (1982) *J. Mol. Biol.* 158:157}. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20. Enter a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See e.g. Brake *et al.*, *supra*.

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome {Orr-Weaver *et al.* (1983) *Methods in Enzymol.* 101:228-245}. An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector. See Orr-Weaver *et al.*, *supra*. One or more expression construct may integrate, possibly affecting levels of recombinant protein produced {Rine *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:6750}. The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as *ADE2*, *HIS4*, *LEU2*, *TRP1*, and *ALG7*, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418, respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of *CUP1* allows yeast to grow in the presence of copper ions {Butt *et al.* (1987) *Microbiol. Rev.* 51:351}.

Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, *inter alia*, the following yeasts: *Candida albicans* {Kurtz, *et al.* (1986) *Mol. Cell. Biol.* 6:142}, *Candida maltosa* {Kunze, *et al.* (1985) *J. Basic Microbiol.* 25:141}, *Hansenula polymorpha* {Gleeson, *et al.* (1986) *J. Gen. Microbiol.* 132:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet.* 202:302}, *Kluyveromyces fragilis* {Das, *et al.* (1984) *J. Bacteriol.* 158:1165}, *Kluyveromyces lactis* {De Louvencourt *et al.* (1983) *J. Bacteriol.* 154:737; Van den Berg *et al.* (1990) *Bio/Technology* 8:135}, *Pichia guilliermondii* {Kunze *et al.* (1985) *J. Basic Microbiol.* 25:141}, *Pichia pastoris* {Cregg, *et al.* (1985) *Mol. Cell. Biol.* 5:3376; US Patent Nos. 4,837,148 and 4,929,555}, *Saccharomyces cerevisiae* {Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:1929; Ito *et al.* (1983) *J. Bacteriol.* 153:163}, *Schizosaccharomyces pombe* {Beach and Nurse (1981) *Nature* 300:706}, and *Yarrowia lipolytica* {Davidow, *et al.* (1985) *Curr. Genet.* 10:380471 Gaillardin, *et al.* (1985) *Curr. Genet.* 10:49}.

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed. See *e.g.* {Kurtz *et al.* (1986) *Mol. Cell. Biol.* 6:142; Kunze *et al.* (1985) *J. Basic Microbiol.* 25:141; *Candida*}; {Gleeson *et al.* (1986) *J. Gen. Microbiol.* 132:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet.* 202:302; *Hansenula*}; {Das *et al.* (1984) *J. Bacteriol.* 158:1165; De Louvencourt *et al.* (1983) *J. Bacteriol.* 154:1165; Van den Berg *et al.* (1990) *Bio/Technology* 8:135; *Kluyveromyces*}; {Cregg *et al.* (1985) *Mol. Cell. Biol.* 5:3376; Kunze *et al.* (1985) *J. Basic Microbiol.* 25:141; US Patents 4,837,148 & 4,929,555; *Pichia*}; {Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:1929; Ito *et al.* (1983) *J. Bacteriol.* 153:163 *Saccharomyces*}; {Beach & Nurse (1981) *Nature* 300:706; *Schizosaccharomyces*}; {Davidow *et al.* (1985) *Curr. Genet.* 10:39; Gaillardin *et al.* (1985) *Curr. Genet.* 10:49; *Yarrowia*}.

#### Pharmaceutical Compositions

Pharmaceutical compositions can comprise polypeptides and/or nucleic acid of the invention. The pharmaceutical compositions will comprise a therapeutically effective amount of either polypeptides, antibodies, or polynucleotides of the claimed invention.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic or preventative effect. The effect can be detected by, for example, chemical markers or antigen levels. Therapeutic effects also include reduction in physical symptoms, such as decreased body temperature. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation and is within the judgement of the clinician.

For purposes of the present invention, an effective dose will be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

A pharmaceutical composition can also contain a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and

which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

- 5 Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

10 Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier.

15 Delivery Methods

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals; in particular, human subjects can be treated.

20 Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal or transcutaneous applications (*e.g.* see WO98/20734), needles, and gene guns or hypodermic sprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Vaccines

25 Vaccines according to the invention may either be prophylactic (*ie.* to prevent infection) or therapeutic (*ie.* to treat disease after infection).

30 Such vaccines comprise immunising antigen(s), immunogen(s), polypeptide(s), protein(s) or nucleic acid, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen or immunogen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, *H. pylori*, *etc.* pathogens.

35 Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, *etc.*; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59™ (WO 90/14837; Chapter 10 in *Vaccine design: the subunit and adjuvant approach*, eds. Powell & Newman, Plenum Press 1995), containing

5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi<sup>TM</sup> adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox<sup>TM</sup>); (3) saponin adjuvants, such as Stimulon<sup>TM</sup> (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*e.g.* gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), *etc.*; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59<sup>TM</sup> are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

The immunogenic compositions (*e.g.* the immunising antigen/immunogen/polypeptide/protein/ nucleic acid, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic or immunogenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (*e.g.* nonhuman primate, primate, *etc.*), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, *e.g.* by injection, either subcutaneously, intramuscularly, or transdermally/transcutaneously (*e.g.* WO98/20734). Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

As an alternative to protein-based vaccines, DNA vaccination may be employed {e.g. Robinson & Torres (1997) *Seminars in Immunology* 9:271-283; Donnelly *et al.* (1997) *Annu Rev Immunol* 15:617-648; see later herein}.

### Gene Delivery Vehicles

Gene therapy vehicles for delivery of constructs including a coding sequence of a therapeutic of the invention, to be delivered to the mammal for expression in the mammal, can be administered either locally or systemically. These constructs can utilize viral or non-viral vector approaches in *in vivo* or *ex vivo* modality. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence in vivo can be either constitutive or regulated.

The invention includes gene delivery vehicles capable of expressing the contemplated nucleic acid sequences.

The gene delivery vehicle is preferably a viral vector and, more preferably, a retroviral, adenoviral, adeno-associated viral (AAV), herpes viral, or alphavirus vector. The viral vector can also be an astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, or togavirus viral vector. See generally, Jolly (1994) *Cancer Gene Therapy* 1:51-64; Kimura (1994) *Human Gene Therapy* 5:845-852; Connelly (1995) *Human Gene Therapy* 6:185-193; and Kaplitt (1994) *Nature Genetics* 6:148-153.

Retroviral vectors are well known in the art and we contemplate that any retroviral gene therapy vector is employable in the invention, including B, C and D type retroviruses, xenotropic retroviruses (for example, NZB-X1, NZB-X2 and NZB9-1 (see O'Neill (1985) *J. Virol.* 53:160) polytropic retroviruses e.g. MCF and MCF-MLV (see Kelly (1983) *J. Virol.* 45:291), spumaviruses and lentiviruses. See RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985.

Portions of the retroviral gene therapy vector may be derived from different retroviruses. For example, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see US patent 5,591,624). Retrovirus vectors can be constructed for site-specific integration into host cell DNA by incorporation of a chimeric integrase enzyme into the retroviral particle (see WO96/37626). It is preferable that the recombinant viral vector is a replication defective recombinant virus.

Packaging cell lines suitable for use with the above-described retrovirus vectors are well known in the art, are readily prepared (see WO95/30763 and WO92/05266), and can be used to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Preferably, the packaging cell lines are made from human parent cells (e.g. HT1080 cells) or mink parent cell lines, which eliminates inactivation in human serum.

Preferred retroviruses for the construction of retroviral gene therapy vectors include Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe (1976) *J Virol* 19:19-25), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998) and Moloney Murine Leukemia Virus (ATCC No. VR-190). Such retroviruses may be obtained from

depositories or collections such as the American Type Culture Collection ("ATCC") in Rockville, Maryland or isolated from known sources using commonly available techniques.

Exemplary known retroviral gene therapy vectors employable in this invention include those described in patent applications GB2200651, EP0415731, EP0345242, EP0334301, WO89/02468; WO89/05349, WO89/09271, WO90/02806, WO90/07936, WO94/03622, WO93/25698, WO93/25234, WO93/11230, WO93/10218, WO91/02805, WO91/02825, WO95/07994, US 5,219,740, US 4,405,712, US 4,861,719, US 4,980,289, US 4,777,127, US 5,591,624. See also Vile (1993) *Cancer Res* 53:3860-3864; Vile (1993) *Cancer Res* 53:962-967; Ram (1993) *Cancer Res* 53 (1993) 83-88; Takamiya (1992) *J Neurosci Res* 33:493-503; Baba (1993) *J Neurosurg* 79:729-735; Mann (1983) *Cell* 33:153; Cane (1984) *Proc Natl Acad Sci* 81:6349; and Miller (1990) *Human Gene Therapy* 1.

Human adenoviral gene therapy vectors are also known in the art and employable in this invention. See, for example, Berkner (1988) *Biotechniques* 6:616 and Rosenfeld (1991) *Science* 252:431, and WO93/07283, WO93/06223, and WO93/07282. Exemplary known adenoviral gene therapy vectors employable in this invention include those described in the above referenced documents and in WO94/12649, WO93/03769, WO93/19191, WO94/28938, WO95/11984, WO95/00655, WO95/27071, WO95/29993, WO95/34671, WO96/05320, WO94/08026, WO94/11506, WO93/06223, WO94/24299, WO95/14102, WO95/24297, WO95/02697, WO94/28152, WO94/24299, WO95/09241, WO95/25807, WO95/05835, WO94/18922 and WO95/09654. Alternatively, administration of DNA linked to killed adenovirus as described in Curiel (1992) *Hum. Gene Ther.* 3:147-154 may be employed. The gene delivery vehicles of the invention also include adenovirus associated virus (AAV) vectors. Leading and preferred examples of such vectors for use in this invention are the AAV-2 based vectors disclosed in Srivastava, WO93/09239. Most preferred AAV vectors comprise the two AAV inverted terminal repeats in which the native D-sequences are modified by substitution of nucleotides, such that at least 5 native nucleotides and up to 18 native nucleotides, preferably at least 10 native nucleotides up to 18 native nucleotides, most preferably 10 native nucleotides are retained and the remaining nucleotides of the D-sequence are deleted or replaced with non-native nucleotides. The native D-sequences of the AAV inverted terminal repeats are sequences of 20 consecutive nucleotides in each AAV inverted terminal repeat (*ie.* there is one sequence at each end) which are not involved in HP formation. The non-native replacement nucleotide may be any nucleotide other than the nucleotide found in the native D-sequence in the same position. Other employable exemplary AAV vectors are pWP-19, pWN-1, both of which are disclosed in Nahreini (1993) *Gene* 124:257-262. Another example of such an AAV vector is psub201 (see Samulski (1987) *J. Virol.* 61:3096). Another exemplary AAV vector is the Double-D ITR vector. Construction of the Double-D ITR vector is disclosed in US Patent 5,478,745. Still other vectors are those disclosed in Carter US Patent 4,797,368 and Muzyczka US Patent 5,139,941, Chartejee US Patent 5,474,935, and Kotin WO94/288157. Yet a further example of an AAV vector employable in this invention is SSV9AFABTKneo, which contains the AFP enhancer and albumin promoter and directs expression predominantly in the liver. Its structure and construction are disclosed in Su (1996) *Human Gene Therapy* 7:463-470. Additional AAV gene therapy vectors are described in US 5,354,678, US 5,173,414, US 5,139,941, and US 5,252,479.

The gene therapy vectors of the invention also include herpes vectors. Leading and preferred examples are herpes simplex virus vectors containing a sequence encoding a thymidine kinase polypeptide such as those disclosed in US 5,288,641 and EP0176170 (Roizman). Additional exemplary herpes simplex virus vectors

include HFEM/ICP6-LacZ disclosed in WO95/04139 (Wistar), pHSVlac described in Geller (1988) *Science* 241:1667-1669 and in WO90/09441 & WO92/07945, HSV Us3::pgC-lacZ described in Fink (1992) *Human Gene Therapy* 3:11-19 and HSV 7134, 2 RH 105 and GAL4 described in EP 0453242 (Breakefield), and those deposited with ATCC as accession numbers ATCC VR-977 and ATCC VR-260.

5 Also contemplated are alpha virus gene therapy vectors that can be employed in this invention. Preferred alpha virus vectors are Sindbis viruses vectors. Togaviruses, Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described in US patents 5,091,309, 5,217,879, and WO92/10578. More particularly, those alpha virus vectors described in  
10 US Serial No. 08/405,627, filed March 15, 1995, WO94/21792, WO92/10578, WO95/07994, US 5,091,309 and US 5,217,879 are employable. Such alpha viruses may be obtained from depositories or collections such as the ATCC in Rockville, Maryland or isolated from known sources using commonly available techniques. Preferably, alphavirus vectors with reduced cytotoxicity are used (see USSN 08/679640).

DNA vector systems such as eukaryotic layered expression systems are also useful for expressing the nucleic  
15 acids of the invention. See WO95/07994 for a detailed description of eukaryotic layered expression systems. Preferably, the eukaryotic layered expression systems of the invention are derived from alphavirus vectors and most preferably from Sindbis viral vectors.

Other viral vectors suitable for use in the present invention include those derived from poliovirus, for example ATCC VR-58 and those described in Evans, *Nature* 339 (1989) 385 and Sabin (1973) *J. Biol. Standardization*  
20 1:115; rhinovirus, for example ATCC VR-1110 and those described in Arnold (1990) *J Cell Biochem* L401; pox viruses such as canary pox virus or vaccinia virus, for example ATCC VR-111 and ATCC VR-2010 and those described in Fisher-Hoch (1989) *Proc Natl Acad Sci* 86:317; Flexner (1989) *Ann NY Acad Sci* 569:86, Flexner (1990) *Vaccine* 8:17; in US 4,603,112 and US 4,769,330 and WO89/01973; SV40 virus, for example ATCC VR-305 and those described in Mulligan (1979) *Nature* 277:108 and Madzak (1992) *J Gen Virol* 73:1533;  
25 influenza virus, for example ATCC VR-797 and recombinant influenza viruses made employing reverse genetics techniques as described in US 5,166,057 and in Enami (1990) *Proc Natl Acad Sci* 87:3802-3805; Enami & Palese (1991) *J Virol* 65:2711-2713 and Luytjes (1989) *Cell* 59:110, (see also McMichael (1983) *NEJ Med* 309:13, and Yap (1978) *Nature* 273:238 and *Nature* (1979) 277:108); human immunodeficiency virus as described in EP-0386882 and in Buchschacher (1992) *J. Virol.* 66:2731; measles virus, for example ATCC  
30 VR-67 and VR-1247 and those described in EP-0440219; Aura virus, for example ATCC VR-368; Bebaru virus, for example ATCC VR-600 and ATCC VR-1240; Cabassou virus, for example ATCC VR-922; Chikungunya virus, for example ATCC VR-64 and ATCC VR-1241; Fort Morgan Virus, for example ATCC VR-924; Getah virus, for example ATCC VR-369 and ATCC VR-1243; Kyzylagach virus, for example ATCC VR-927; Mayaro virus, for example ATCC VR-66; Mucambo virus, for example ATCC VR-580 and ATCC VR-1244; Ndumu  
35 virus, for example ATCC VR-371; Pixuna virus, for example ATCC VR-372 and ATCC VR-1245; Tonate virus, for example ATCC VR-925; Trinita virus, for example ATCC VR-469; Una virus, for example ATCC VR-374; Whataroa virus, for example ATCC VR-926; Y-62-33 virus, for example ATCC VR-375; O'Nyong virus, Eastern encephalitis virus, for example ATCC VR-65 and ATCC VR-1242; Western encephalitis virus, for example ATCC VR-70, ATCC VR-1251, ATCC VR-622 and ATCC VR-1252; and coronavirus, for  
40 example ATCC VR-740 and those described in Hamre (1966) *Proc Soc Exp Biol Med* 121:190.

Delivery of the compositions of this invention into cells is not limited to the above mentioned viral vectors. Other delivery methods and media may be employed such as, for example, nucleic acid expression vectors, polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example see US Serial No. 08/366,787, filed December 30, 1994 and Curiel (1992) *Hum Gene Ther* 3:147-154 ligand linked DNA, for example see Wu (1989) *J Biol Chem* 264:16985-16987, eucaryotic cell delivery vehicles cells, for example see US Serial No. 08/240,030, filed May 9, 1994, and US Serial No. 08/404,796, deposition of photopolymerized hydrogel materials, hand-held gene transfer particle gun, as described in US Patent 5,149,655, ionizing radiation as described in US 5,206,152 and in WO92/11033, nucleic charge neutralization or fusion with cell membranes. Additional approaches are described in Philip (1994) *Mol Cell Biol* 14:2411-2618 and in Woffendin (1994) *Proc Natl Acad Sci* 91:1581-1585.

Particle mediated gene transfer may be employed, for example see US Serial No. 60/023,867. Briefly, the sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu & Wu (1987) *J. Biol. Chem.* 262:4429-4432, insulin as described in Hucked (1990) *Biochem Pharmacol* 40:253-263, galactose as described in Plank (1992) *Bioconjugate Chem* 3:533-539, lactose or transferrin.

Naked DNA may also be employed. Exemplary naked DNA introduction methods are described in WO90/11092 and US 5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated latex beads are efficiently transported into cells after endocytosis initiation by the beads. The method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

Liposomes that can act as gene delivery vehicles are described in US 5,422,120, WO95/13796, WO94/23697, WO91/14445 and EP-524,968. As described in USSN. 60/023,867, on non-viral delivery, the nucleic acid sequences encoding a polypeptide can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, insulin, galactose, lactose, or transferrin. Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters. Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in Woffendin *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91(24):11581-11585. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand-held gene transfer particle gun, as described in US 5,149,655; use of ionizing radiation for activating transferred gene, as described in US 5,206,152 and WO92/11033

Exemplary liposome and polycationic gene delivery vehicles are those described in US 5,422,120 and 4,762,915; in WO 95/13796; WO94/23697; and WO91/14445; in EP-0524968; and in Stryer, *Biochemistry*, pages 236-240 (1975) W.H. Freeman, San Francisco; Szoka (1980) *Biochem Biophys Acta* 600:1; Bayer (1979) *Biochem Biophys Acta* 550:464; Rivnay (1987) *Meth Enzymol* 149:119; Wang (1987) *Proc Natl Acad Sci* 84:7851; Plant (1989) *Anal Biochem* 176:420.

A polynucleotide composition can comprises therapeutically effective amount of a gene therapy vehicle, as the term is defined above. For purposes of the present invention, an effective dose will be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

#### Delivery Methods

- 5 Once formulated, the polynucleotide compositions of the invention can be administered (1) directly to the subject; (2) delivered *ex vivo*, to cells derived from the subject; or (3) *in vitro* for recombinant protein expression. The subjects to be treated can be mammals or birds. Also, human subjects can be treated.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The  
10 compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal or transcutaneous applications (*e.g.* see WO98/20734), needles, and gene guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known in the art and described in *e.g.* WO93/14778. Examples of cells useful in *ex vivo* applications include, for example, stem cells,  
15 particularly hematopoietic, lymph cells, macrophages, dendritic cells, or tumor cells.

Generally, delivery of nucleic acids for both *ex vivo* and *in vitro* applications can be accomplished by the following procedures, for example, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well known in the art.

#### 20 Polynucleotide and polypeptide pharmaceutical compositions

In addition to the pharmaceutically acceptable carriers and salts described above, the following additional agents can be used with polynucleotide and/or polypeptide compositions.

#### A.Polypeptides

One example are polypeptides which include, without limitation: asioloorosomucoid (ASOR); transferrin;  
25 asialoglycoproteins; antibodies; antibody fragments; ferritin; interleukins; interferons, granulocyte, macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor and erythropoietin. Viral antigens, such as envelope proteins, can also be used. Also, proteins from other invasive organisms, such as the 17 amino acid peptide from the circumsporozoite protein of plasmodium falciparum known as RII.

#### 30 B.Hormones, Vitamins, etc.

Other groups that can be included are, for example: hormones, steroids, androgens, estrogens, thyroid hormone, or vitamins, folic acid.

#### C.Polyalkylenes, Polysaccharides, etc.

Also, polyalkylene glycol can be included with the desired polynucleotides/polypeptides. In a preferred  
35 embodiment, the polyalkylene glycol is polyethylene glycol. In addition, mono-, di-, or polysaccharides can be included. In a preferred embodiment of this aspect, the polysaccharide is dextran or DEAE-dextran. Also, chitosan and poly(lactide-co-glycolide)

#### D.Lipids, and Liposomes

The desired polynucleotide/polypeptide can also be encapsulated in lipids or packaged in liposomes prior to delivery to the subject or to cells derived therefrom.

Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed polynucleotide to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight (1991) *Biochim. Biophys. Acta.* 1097:1-17; Straubinger (1983) *Meth. Enzymol.* 101:512-527.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-7416); mRNA (Malone (1989) *Proc. Natl. Acad. Sci. USA* 86:6077-6081); and purified transcription factors (Debs (1990) *J. Biol. Chem.* 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N{1-2,3-dioleoyloxy}propyl}-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner *supra*). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, *e.g.* Szoka (1978) *Proc. Natl. Acad. Sci. USA* 75:4194-4198; WO90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See *e.g.* Straubinger (1983) *Meth. Immunol.* 101:512-527; Szoka (1978) *Proc. Natl. Acad. Sci. USA* 75:4194-4198; Papahadjopoulos (1975) *Biochim. Biophys. Acta* 394:483; Wilson (1979) *Cell* 17:77; Deamer & Bangham (1976) *Biochim. Biophys. Acta* 443:629; Ostro (1977) *Biochem. Biophys. Res. Commun.* 76:836; Fraley (1979) *Proc. Natl. Acad. Sci. USA* 76:3348; Enoch & Strittmatter (1979) *Proc. Natl. Acad. Sci. USA* 76:145; Fraley (1980) *J. Biol. Chem.* (1980) 255:10431; Szoka & Papahadjopoulos (1978) *Proc. Natl. Acad. Sci. USA* 75:145; and Schaefer-Ridder (1982) *Science* 215:166.

#### E.Lipoproteins

In addition, lipoproteins can be included with the polynucleotide/polypeptide to be delivered. Examples of lipoproteins to be utilized include: chylomicrons, HDL, IDL, LDL, and VLDL. Mutants, fragments, or fusions of these proteins can also be used. Also, modifications of naturally occurring lipoproteins can be used, such as acetylated LDL. These lipoproteins can target the delivery of polynucleotides to cells expressing lipoprotein

receptors. Preferably, if lipoproteins are including with the polynucleotide to be delivered, no other targeting ligand is included in the composition.

Naturally occurring lipoproteins comprise a lipid and a protein portion. The protein portion are known as apoproteins. At the present, apoproteins A, B, C, D, and E have been isolated and identified. At least two of these contain several proteins, designated by Roman numerals, AI, AII, AIV; CI, CII, CIII.

A lipoprotein can comprise more than one apoprotein. For example, naturally occurring chylomicrons comprises of A, B, C, & E, over time these lipoproteins lose A and acquire C and E apoproteins. VLDL comprises A, B, C, & E apoproteins, LDL comprises apoprotein B; HDL comprises apoproteins A, C, & E.

The amino acid of these apoproteins are known and are described in, for example, Breslow (1985) *Annu Rev Biochem* 54:699; Law (1986) *Adv. Exp Med. Biol.* 151:162; Chen (1986) *J Biol Chem* 261:12918; Kane (1980) *Proc Natl Acad Sci USA* 77:2465; and Utermann (1984) *Hum Genet* 65:232.

Lipoproteins contain a variety of lipids including, triglycerides, cholesterol (free and esters), and phospholipids. The composition of the lipids varies in naturally occurring lipoproteins. For example, chylomicrons comprise mainly triglycerides. A more detailed description of the lipid content of naturally occurring lipoproteins can be found, for example, in *Meth. Enzymol.* 128 (1986). The composition of the lipids are chosen to aid in conformation of the apoprotein for receptor binding activity. The composition of lipids can also be chosen to facilitate hydrophobic interaction and association with the polynucleotide binding molecule.

Naturally occurring lipoproteins can be isolated from serum by ultracentrifugation, for instance. Such methods are described in *Meth. Enzymol. (supra)*; Pitas (1980) *J. Biochem.* 255:5454-5460 and Mahey (1979) *J Clin Invest* 64:743-750. Lipoproteins can also be produced by *in vitro* or recombinant methods by expression of the apoprotein genes in a desired host cell. See, for example, Atkinson (1986) *Annu Rev Biophys Chem* 15:403 and Radding (1958) *Biochim Biophys Acta* 30: 443. Lipoproteins can also be purchased from commercial suppliers, such as Biomedical Technologies, Inc., Stoughton, Massachusetts, USA. Further description of lipoproteins can be found in Zuckermann *et al.* PCT/US97/14465.

#### F. Polycationic Agents

Polycationic agents can be included, with or without lipoprotein, in a composition with the desired polynucleotide/polypeptide to be delivered.

Polycationic agents, typically, exhibit a net positive charge at physiological relevant pH and are capable of neutralizing the electrical charge of nucleic acids to facilitate delivery to a desired location. These agents have both *in vitro*, *ex vivo*, and *in vivo* applications. Polycationic agents can be used to deliver nucleic acids to a living subject either intramuscularly, subcutaneously, etc.

The following are examples of useful polypeptides as polycationic agents: polylysine, polyarginine, polyornithine, and protamine. Other examples include histones, protamines, human serum albumin, DNA binding proteins, non-histone chromosomal proteins, coat proteins from DNA viruses, such as (X174, transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic acid condensing agents. Briefly, transcriptional factors such as C/CEBP, c-jun, c-fos, AP-1, AP-2, AP-3, CPF, Prot-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences.

Organic polycationic agents include: spermine, spermidine, and putrescine.

The dimensions and of the physical properties of a polycationic agent can be extrapolated from the list above, to construct other polypeptide polycationic agents or to produce synthetic polycationic agents.

Synthetic polycationic agents which are useful include, for example, DEAE-dextran, polybrene. Lipofectin™, and lipofectAMINE™ are monomers that form polycationic complexes when combined with polynucleotides/polypeptides.

#### Nucleic Acid Hybridisation

“Hybridization” refers to the association of two nucleic acid sequences to one another by hydrogen bonding. Typically, one sequence will be fixed to a solid support and the other will be free in solution. Then, the two sequences will be placed in contact with one another under conditions that favor hydrogen bonding. Factors that affect this bonding include: the type and volume of solvent; reaction temperature; time of hybridization; agitation; agents to block the non-specific attachment of the liquid phase sequence to the solid support (Denhardt's reagent or BLOTTO); concentration of the sequences; use of compounds to increase the rate of association of sequences (dextran sulfate or polyethylene glycol); and the stringency of the washing conditions following hybridization. See Sambrook *et al.* {*supra*} vol.2, chapt.9, pp.9.47 to 9.57.

“Stringency” refers to conditions in a hybridization reaction that favor association of very similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 120 to 200°C below the calculated T<sub>m</sub> of the hybrid under study. The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the sequence of interest and then washed under conditions of different stringencies. See Sambrook *et al.* at page 9.50.

Variables to consider when performing, for example, a Southern blot are (1) the complexity of the DNA being blotted and (2) the homology between the probe and the sequences being detected. The total amount of the fragment(s) to be studied can vary a magnitude of 10, from 0.1 to 1 µg for a plasmid or phage digest to 10<sup>-9</sup> to 10<sup>-8</sup> g for a single copy gene in a highly complex eukaryotic genome. For lower complexity polynucleotides, substantially shorter blotting, hybridization, and exposure times, a smaller amount of starting polynucleotides, and lower specific activity of probes can be used. For example, a single-copy yeast gene can be detected with an exposure time of only 1 hour starting with 1 µg of yeast DNA, blotting for two hours, and hybridizing for 4-8 hours with a probe of 10<sup>8</sup> cpm/µg. For a single-copy mammalian gene a conservative approach would start with 10 µg of DNA, blot overnight, and hybridize overnight in the presence of 10% dextran sulfate using a probe of greater than 10<sup>8</sup> cpm/µg, resulting in an exposure time of ~24 hours.

Several factors can affect the melting temperature (T<sub>m</sub>) of a DNA-DNA hybrid between the probe and the fragment of interest, and consequently, the appropriate conditions for hybridization and washing. In many cases the probe is not 100% homologous to the fragment. Other commonly encountered variables include the length and total G+C content of the hybridizing sequences and the ionic strength and formamide content of the hybridization buffer. The effects of all of these factors can be approximated by a single equation:

$$T_m = 81 + 16.6(\log_{10} C_i) + 0.4\{\%(G + C)\} - 0.6(\%\text{formamide}) - 600/n - 1.5(\%\text{mismatch}).$$

where C<sub>i</sub> is the salt concentration (monovalent ions) and *n* is the length of the hybrid in base pairs (slightly modified from Meinkoth & Wahl (1984) *Anal. Biochem.* 138: 267-284).

In designing a hybridization experiment, some factors affecting nucleic acid hybridization can be conveniently altered. The temperature of the hybridization and washes and the salt concentration during the washes are the simplest to adjust. As the temperature of the hybridization increases (*ie.* stringency), it becomes less likely for hybridization to occur between strands that are nonhomologous, and as a result, background decreases. If the radiolabelled probe is not completely homologous with the immobilized fragment (as is frequently the case in gene family and interspecies hybridization experiments), the hybridization temperature must be reduced, and background will increase. The temperature of the washes affects the intensity of the hybridizing band and the degree of background in a similar manner. The stringency of the washes is also increased with decreasing salt concentrations.

In general, convenient hybridization temperatures in the presence of 50% formamide are 42°C for a probe with is 95% to 100% homologous to the target fragment, 37°C for 90% to 95% homology, and 32°C for 85% to 90% homology. For lower homologies, formamide content should be lowered and temperature adjusted accordingly, using the equation above. If the homology between the probe and the target fragment are not known, the simplest approach is to start with both hybridization and wash conditions which are nonstringent. If non-specific bands or high background are observed after autoradiography, the filter can be washed at high stringency and reexposed. If the time required for exposure makes this approach impractical, several hybridization and/or washing stringencies should be tested in parallel.

#### Nucleic Acid Probe Assays

Methods such as PCR, branched DNA probe assays, or blotting techniques utilizing nucleic acid probes according to the invention can determine the presence of cDNA or mRNA. A probe is said to “hybridize” with a sequence of the invention if it can form a duplex or double stranded complex, which is stable enough to be detected.

The nucleic acid probes will hybridize to the Chlamydial nucleotide sequences of the invention (including both sense and antisense strands). Though many different nucleotide sequences will encode the amino acid sequence, the native Chlamydial sequence is preferred because it is the actual sequence present in cells. mRNA represents a coding sequence and so a probe should be complementary to the coding sequence; single-stranded cDNA is complementary to mRNA, and so a cDNA probe should be complementary to the non-coding sequence.

The probe sequence need not be identical to the Chlamydial sequence (or its complement) — some variation in the sequence and length can lead to increased assay sensitivity if the nucleic acid probe can form a duplex with target nucleotides, which can be detected. Also, the nucleic acid probe can include additional nucleotides to stabilize the formed duplex. Additional Chlamydial sequence may also be helpful as a label to detect the formed duplex. For example, a non-complementary nucleotide sequence may be attached to the 5' end of the probe, with the remainder of the probe sequence being complementary to a Chlamydial sequence. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the a Chlamydial sequence in order to hybridize therewith and thereby form a duplex which can be detected.

The exact length and sequence of the probe will depend on the hybridization conditions, such as temperature, salt condition and the like. For example, for diagnostic applications, depending on the complexity of the analyte sequence, the nucleic acid probe typically contains at least 10-20 nucleotides, preferably 15-25, and more

preferably  $\geq 30$  nucleotides, although it may be shorter than this. Short primers generally require cooler temperatures to form sufficiently stable hybrid complexes with the template.

Probes may be produced by synthetic procedures, such as the triester method of Matteucci *et al.* {*J. Am. Chem. Soc.* (1981) 103:3185}, or according to Urdea *et al.* {*Proc. Natl. Acad. Sci. USA* (1983) 80: 7461}, or using commercially available automated oligonucleotide synthesizers.

The chemical nature of the probe can be selected according to preference. For certain applications, DNA or RNA are appropriate. For other applications, modifications may be incorporated *e.g.* backbone modifications, such as phosphorothioates or methylphosphonates, can be used to increase *in vivo* half-life, alter RNA affinity, increase nuclease resistance *etc.* {*e.g.* see Agrawal & Iyer (1995) *Curr Opin Biotechnol* 6:12-19; Agrawal (1996) *TIBTECH* 14:376-387}; analogues such as peptide nucleic acids may also be used {*e.g.* see Corey (1997) *TIBTECH* 15:224-229; Buchardt *et al.* (1993) *TIBTECH* 11:384-386}.

Alternatively, the polymerase chain reaction (PCR) is another well-known means for detecting small amounts of target nucleic acids. The assay is described in: Mullis *et al.* {*Meth. Enzymol.* (1987) 155: 335-350}; US patents 4,683,195 & 4,683,202. Two 'primers' hybridize with the target nucleic acids and are used to prime the reaction. The primers can comprise sequence that does not hybridize to the sequence of the amplification target (or its complement) to aid with duplex stability or, for example, to incorporate a convenient restriction site. Typically, such sequence will flank the desired Chlamydial sequence.

A thermostable polymerase creates copies of target nucleic acids from the primers using the original target nucleic acids as a template. After a threshold amount of target nucleic acids are generated by the polymerase, they can be detected by more traditional methods, such as Southern blots. When using the Southern blot method, the labelled probe will hybridize to the Chlamydial sequence (or its complement).

Also, mRNA or cDNA can be detected by traditional blotting techniques described in Sambrook *et al* {*supra*}. mRNA, or cDNA generated from mRNA using a polymerase enzyme, can be purified and separated using gel electrophoresis. The nucleic acids on the gel are then blotted onto a solid support, such as nitrocellulose. The solid support is exposed to a labelled probe and then washed to remove any unhybridized probe. Next, the duplexes containing the labelled probe are detected. Typically, the probe is labelled with a radioactive moiety.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 to 44 show data from examples 1 to 44. Where a figure is of a gel, lane 1 is at the left of the figure.

For Western Blots, two samples were tested for each protein. The left lane in a pair used membrane strips stained with pre-immune sera whilst the right lane used membrane strips stained with immune sera. In the Western blots in figures 1 to 5, 35B, 37B, 38B and 39, markers are at 66, 45, 30, 20.1 and 14.4 kDa. In the Western blots in figures 6 to 16, 20B, 23C, 24D, 27E, 38A, 40, 41, 42 and 43 markers are at 172.6, 111.4, 79.6, 61.3, 49.0, 36.4, 24.7, 19.2 and 13.1 kDa.

In the Western Blots in figures 1 to 5, lanes 2 and 3 show control sera raised against GST-fusion control antigens. In the Western blots in figures 1 to 5, lanes 4 and 5 contain control sera raised against His-tagged control antigens.

Low molecular weight markers are run in lane 1 of the purification gels.

## MODES FOR CARRYING OUT THE INVENTION

Table I gives the names of *C.pneumoniae* proteins from reference 18, the GenBank accession numbers and titles for those proteins, the GenBank accession numbers and titles for the corresponding *C.trachomatis* proteins of the invention, and SEQ ID numbers (SEQ IDs 1-262, with odd numbers being amino acid sequences and even numbers being nucleotide sequences) for these *C.trachomatis* proteins. These can be expressed and used in the same ways as described in reference 18 for the corresponding *C.pneumoniae* proteins. The *C.trachomatis* proteins are useful for diagnostic and immunogenic purposes. These properties are not evident from the sequence alone.

Various tests can be used to assess the *in vivo* immunogenicity of the proteins of the invention. For example, the proteins can be expressed recombinantly and used to screen patient sera by immunoblot. A positive reaction between the protein and patient serum indicates that the patient has previously mounted an immune response to the protein in question *i.e.* the protein is an immunogen. This method can also be used to identify immunodominant proteins.

The recombinant protein can also be conveniently used to prepare antibodies *e.g.* in a mouse. These can be used for direct confirmation that a protein is located on the cell-surface of *C.trachomatis* (*e.g.* by using the antibodies in a Western Blot against intact Chlamydia). Labelled antibody (*e.g.* fluorescent labelling for FACS) can be incubated with intact bacteria and the presence of label on the bacterial surface confirms the location of the protein. FACS figures show a scatter profile of the *Chlamydia* preparation used in the assay, the peak shift obtained when antibodies against the recombinant antigen bind to the *Chlamydia* cells (open area = control sample; filled area = antibody-reacted sample), quantitative Kolmogorov-Smirnov (K-S) statistical analysis, and output of the FACS analysis software.

### Example 1

CT242 (SEQ ID 57 and SEQ ID 58) was expressed in *E.coli*. The recombinant product was purified both as a GST-fusion protein (Figure 1A; lanes 4 and 5, chromatography fractions 1 and 2, expected molecular weight 42.4 kDa) and as a His-tagged fusion protein (Figure 1B; lanes 2-4, chromatography fractions 1, 2 and 3, expected molecular weight 16.4 kDa).

The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 1C: His-tagged: lanes 12 and 13; GST-fusion: lanes 20 and 21). Lane 12 shows membrane strips stained with pre-immune sera for His-tagged CT242 whilst lane 13 shows membrane strips stained with immune sera for His-tagged CT242. Lane 20 shows membrane strips stained with preimmune sera for GST-fusion CT242 whilst lane 21 shows membrane strips stained with immune sera for GST-fusion CT242.

These experiments show that CT242 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

**Example 2**

CT045 (SEQ ID 71 and SEQ ID 72) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 2A; lanes 4-6, chromatography fractions 1, 2 and 3, expected molecular weight 55.8 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 2B, lanes 8 and 9) and for FACS analysis (Figure 2C, K-S value 16.81).

These experiments show that CT045 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

**Example 3**

CT381 (SEQ ID 105 and SEQ ID 106) was expressed in *E.coli*. The recombinant product was purified both as a GST-fusion protein (Figure 3A; lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 52.7 kDa) and as a His-tagged fusion protein (Figure 3A; lanes 7-9, chromatography fractions 1, 2 and 3, expected molecular weight 26.7 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 3B: His-tagged: lanes 6 and 7; GST-fusion: lanes 16 and 17) and for FACS analysis (Figure 3C: GST-tagged, K-S value 35.98; Figure 3D: His-tagged, K-S value 32.54).

These experiments show that CT381 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

**Example 4**

CT396 (SEQ ID 107 and SEQ ID 108) was expressed in *E.coli*. The recombinant product was purified both as a GST-fusion protein (Figure 4A; lanes 6 and 7, chromatography fractions 1 and 2, expected molecular weight 99.5 kDa) and as a His-tagged fusion protein (Figure 4B; lanes 5-7, chromatography fractions 1, 2 and 3, expected molecular weight 73.5 kDa). The recombinant His-tagged protein was used to immunise mice, whose sera were used in a Western blot (Figure 4C, lanes 14 and 15). The recombinant His-tagged protein and GST-fusion protein were also used for FACS analysis (Figure 4D: His-tagged, K-S value 34.50; Figure 4E: GST-fusion, K-S value 32.76).

These experiments show that CT396 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

**Example 5**

CT398 (SEQ ID 111 and SEQ ID 112) was expressed in *E.coli*. The recombinant product was purified both as a GST-fusion protein (Figure 5A; lanes 8 and 9, chromatography fractions 1 and 2, expected molecular weight 54.8 kDa) and as a His-tagged fusion protein (Figure 5B; lanes 8-10, chromatography fractions 1, 2 and 3, expected molecular weight 28.8 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 5C: His-tagged: lanes

10 and 11; GST-fusion: lanes 18 and 19) and for FACS analysis (Figure 5D: GST-fusion, K-S value 31.24; Figure 5E: His-tagged, K-S value 26.10).

These experiments show that CT398 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### 5 Example 6

CT089 (SEQ ID 61 and SEQ ID 62) was expressed in *E.coli*. The recombinant product was purified both as a GST-fusion protein (Figure 6C: lane 2, chromatography fraction 1, expected molecular weight 70.8 kDa) and as a His-tagged fusion protein (Figure 6C: lanes 3, 4 and 5, chromatography fractions 1, 2 and 3, expected molecular weight 44.8 kDa). The recombinant proteins were used to  
10 immunise mice, whose sera were used in a Western blot (Figure 6A: GST-fusion: lanes 14 and 15; His-tagged: lanes 16 and 17) and for FACS analysis (Figure 6B: His-tagged, K-S value 26.59).

These experiments show that CT089 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 7

15 CT443 (SEQ ID 125 and SEQ ID 126) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 7A: lanes 10 and 11) and for FACS analysis (Figure 7B: K-S value 21.28).

20 These experiments show that CT443 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 8

CT541 (SEQ ID 149 and SEQ ID 150) was expressed in *E.coli*. The recombinant product was purified as a GST-fusion protein (Figure 8C: lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 51.6 kDa). The recombinant protein was used to immunise mice, whose  
25 sera were used in a Western blot (Figure 8A: lanes 6 and 7) and for FACS analysis (Figure 8B: K-S value 9.94).

These experiments show that CT541 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 9

30 CT547 (SEQ ID 151 and SEQ ID 152) was expressed in *E.coli*. The recombinant product was purified both as a GST-fusion protein (Figure 9D: lanes 4 and 5, chromatography fractions 1 and 2, expected molecular weight 58.3 kDa) and as a His-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 9A: His-tagged: lanes

20 and 21) and for FACS analysis (Figure 9B: GST-fusion, K-S values 14.60 and 15.57; Figure 9C: His-tagged, K-S value 28.21).

These experiments show that CT547 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### 5 Example 10

CT587 (SEQ ID 189 and SEQ ID 190) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 10C: lanes 5, 6 and 7, chromatography fractions 1, 2 and 3, expected molecular weight 47.5 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 10A: lanes 12 and 13) and for FACS analysis (Figure 10B: His-tagged, K-S value 20.85).

These experiments show that CT587 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 11

CT266 (SEQ ID 77 and SEQ ID 78) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 11C: lanes 11 and 12, chromatography fractions 1 and 2, expected molecular weight 44.1 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 11A: lanes 4 and 5) and for FACS analysis (Figure 11B: K-S value 21.29).

These experiments show that CT266 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 12

CT444 (SEQ ID 127 and SEQ ID 128) was expressed in *E.coli*. The recombinant product was purified as a GST-fusion protein (Figure 12B: lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 87.3 kDa) and as a His-tagged fusion protein (Figure 12C: lanes 3 and 4, chromatography fractions 2 and 3, expected molecular weight 9.0 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 12A: lanes 16 and 17) and for FACS analysis (Figure 12D: GST-tagged: K-S value 14.98; Figure 12E: His-tagged: K-S value 13.28).

These experiments show that CT444 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 13

CT559 (SEQ ID 199 and SEQ ID 200) was expressed in *E.coli*. The recombinant product was purified as a His-tagged protein (Figure 13C: lanes 2, 3 and 4, chromatography fractions 1, 2 and 3).

expected molecular weight 34.9 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 13A: lanes 6 and 7) and for FACS analysis (Figure 13B: K-S value 23.21).

These experiments show that CT559 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 14

CT681 (SEQ ID 155 and SEQ ID 156) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 14C: lanes 5 and 6, chromatography fractions 1 and 2, expected molecular weight 41.8 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 14A: lanes 10 and 11) and for FACS analysis (Figure 14B: K-S value 34.66).

These experiments show that CT681 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 15

CT713 (SEQ ID 201 and SEQ ID 202) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 15B: lanes 4, 5 and 6; chromatography fractions 1, 2 and 3, expected molecular weight 35.4 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 15A: lanes 12 and 13) and for FACS analysis (Figure 15C: K-S value 25.82).

These experiments show that CT713 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 16

CT823 (SEQ ID 229 and SEQ ID 230) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 16C: lanes 7, 8 and 9, chromatography fractions 1, 2 and 3, expected molecular weight 53.9 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 16A: lanes 14 and 15) and for FACS analysis (Figure 16B: K-S value 26.62).

These experiments show that CT823 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 17

CT114 (SEQ ID 243 and SEQ ID 244) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 17; lanes 6 and 7, chromatography fractions 1 and 2, expected molecular weight 48.5 kDa).

**Example 18**

CT198 (SEQ ID 43 and SEQ ID 44) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 18A; lane 6, chromatography fraction 1, expected molecular weight 56.3 kDa). The His-tagged recombinant protein was used to immunise mice, whose sera were used for FACS analysis (Figure 18B).

These experiments show that CT198 is present in only part of an EB heterogeneous population (as chlamydial preparations usually are). Where it is present, it is a surface-exposed and immunoaccessible protein. These properties are not evident from the sequence alone.

**Example 19**

CT241 (SEQ ID 55 and SEQ ID 56) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 19: lane 4, chromatography fraction 3, expected molecular weight 85.3 kDa).

**Example 20**

CT350 (SEQ ID 27 and SEQ ID 28) was expressed in *E.coli*. The recombinant product was purified both as a His-tagged fusion protein (Figure 20A: lanes 2, 3 and 4, chromatography fractions 1, 2 and 3, expected molecular weight 61.3 kDa) and as a GST-tagged fusion protein. (Figure 20A: lanes 7, 8 and 9, chromatography fractions 1, 2 and 3, expected molecular weight 87.3 kDa). The recombinant proteins were used to immunise mice, whose sera were used in a Western blot (Figure 20B: His-tagged, lanes 4 and 5; GST-tagged, lanes 8 and 9).

**Example 21**

CT351 (SEQ ID 25 and SEQ ID 26) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 21: lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 76.1 kDa)

**Example 22**

CT391 (SEQ ID 251 and SEQ ID 252) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 22: lanes 8 and 9, chromatography fractions 1 and 2, expected molecular weight 32.6 kDa).

**Example 23**

CT077 (SEQ ID 65 and SEQ ID 66) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein (Figure 23: lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 59.7 kDa) and as a His-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 23C: lanes 6 and 7) and for FACS analysis (Figure 23B, His-tagged: K-S value 9.17).

These experiments show that CT077 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 24

CT181 (SEQ ID 245 and SEQ ID 246) was expressed in *E.coli*. The recombinant product was purified both as a GST-tagged fusion protein (Figure 24A: lane 4, chromatography fraction 1, expected molecular weight 50.1 kDa) and a His-tagged fusion protein (Figure 24B: lanes 2, 3 and 4, chromatography fractions 1, 2 and 3, expected molecular weight 32.0 kDa). The GST-tagged recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 24D, lanes 4 and 5 (indicated by arrow)) and for FACS analysis (Figure 24C, K-S value 7.62).

These experiments show that CT181 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### Example 25

CT589 (SEQ ID 185 and SEQ ID 186) was expressed in *E.coli*. The recombinant product was purified both as a GST-tagged fusion protein (Figure 25A: lanes 4 and 5, chromatography fractions 1 and 2, expected molecular weight 89.4 kDa) and as a His-tagged fusion protein (Figure 25B: lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 63.4 kDa). The His-tagged recombinant protein was used to immunise mice, whose sera were used for FACS analysis (Figure 25C).

These experiments show that CT589 is present in only part of an EB heterogeneous population (as chlamydial preparations usually are). Where it is present, it is a surface-exposed and immunoaccessible protein. These properties are not evident from the sequence alone.

#### Example 26

CT597 (SEQ ID 179 and SEQ ID 180) was expressed in *E.coli*. The recombinant product was purified both as a GST-tagged fusion protein (Figure 26A: lanes 5 and 6, chromatography fractions 1 and 2, expected molecular weight 36.0 kDa) and as a His-tagged fusion protein (Figure 26B: lanes 2, 3 and 4, chromatography fractions 1, 2 and 3, expected molecular weight 10.3 kDa).

#### Example 27

CT623 (SEQ ID 163 and SEQ ID 164) was expressed in *E.coli*. The recombinant product was purified both as a GST-tagged fusion protein (Figure 27A: lanes 3 and 4, chromatography fractions 1 and 2, expected molecular weight 71.8 kDa) and as a His-tagged fusion protein (Figure 27B: lanes 2, 3 and 4, chromatography fractions 1, 2 and 3, expected molecular weight 45.8 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western Blot (Figure 27E: GST-tagged, lane 4 (indicated by arrow); His-tagged, lane 13 (indicated by arrow)) and for

FACS analysis (Figure 27C: GST-tagged: K-S value 15.89; Figure 27D: His-tagged: K-S value 20.27).

These experiments show that CT623 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### 5    **Example 28**

CT700 (SEQ ID 261 and SEQ ID 262) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein (Figure 28A: lanes 5, 6 and 7, chromatography fractions 1, 2 and 3, expected molecular weight 73.7 kDa). The recombinant protein was used to immunise mice, whose sera were used for FACS analysis (Figure 28B: K-S value 8.72).

10    These experiments show that CT700 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### **Example 29**

CT761 (SEQ ID 217 and SEQ ID 218) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein (Figure 29A: lanes 6 and 7, chromatography fractions 1 and  
15    2, expected molecular weight 63.9 kDa). The recombinant protein was used to immunise mice, whose sera were used for FACS analysis (Figure 29B, K-S value 11.45).

These experiments show that CT761 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

#### **Example 30**

20    CT415 (SEQ ID 117 and SEQ ID 118) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein (Figure 30: lanes 3 and 4, chromatography fractions 1 and 2, expected molecular weight 55.4 kDa).

#### **Example 31**

CT454 (SEQ ID 253 and SEQ ID 254) was expressed in *E.coli*. The recombinant product was  
25    purified as a His-tagged fusion protein (Figure 31: lanes 2 and 3, chromatography fractions 1 and 2, expected molecular weight 56.2 kDa).

#### **Example 32**

CT467 (SEQ ID 129 and SEQ ID 130) was expressed in *E.coli*. The recombinant product was  
30    purified as a GST-tagged fusion protein (Figure 32: lanes 3 and 4, chromatography fractions 1 and 2, expected molecular weight 65.6 kDa).

**Example 33**

CT551 (SEQ ID 257 and SEQ ID 258) was expressed in *E.coli*. The recombinant product was purified both as a His-tagged fusion protein (Figure 33A: lanes 5, 6 and 7, chromatography fractions 1, 2 and 3, expected molecular weight 34.1 kDa) and as a GST-tagged fusion protein (Figure 33B: lanes 4 and 5, chromatography fractions 1 and 2, expected molecular weight 60.1 kDa).

**Example 34**

CT567 (SEQ ID 195 and SEQ ID 196) was expressed in *E.coli*. The recombinant product was purified both as a GST-tagged fusion protein (Figure 34A: lanes 8 and 9, chromatography fractions 1 and 2, expected molecular weight 44.0 kDa) and as a His-tagged fusion protein (Figure 34B: lanes 7 and 8, chromatography fractions 1 and 2, expected molecular weight 18.3 kDa).

**Example 35**

CT569 (SEQ ID 193 and SEQ ID 194) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 35A: lanes 2, 3 and 4, chromatography fractions 1, 2 and 3, expected molecular weight 11.2 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 35B: lanes 8 and 9, indicated with an arrow).

**Example 36**

CT647 (SEQ ID 169 and SEQ ID 170) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein (Figure 36: lanes 6 and 7, chromatography fractions 1 and 2, expected molecular weight 45.7 kDa).

**Example 37**

CT600 (SEQ ID 173 and SEQ ID 174) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein (Figure 37A: lanes 5, 6 and 7, chromatography fractions 1, 2 and 3, expected molecular weight 19.5 kDa). The recombinant protein was used to immunise mice, whose sera were used in a Western Blot (Figure 37B, lanes 10 and 11, indicated by arrow) and for FACS analysis (Figure 37C, K-S value 10.46).

These experiments show that CT600 is a surface-exposed and immunoaccessible protein, and that it is a useful immunogen. These properties are not evident from the sequence alone.

**Example 38**

CT279 (SEQ ID 247 and SEQ ID 248) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein and as a His-tagged fusion protein. The recombinant His-tagged protein and the recombinant GST-tagged protein were used to immunise mice, whose sera were used in Western blots (Figure 38A: His-tagged: lane 5 (indicated by an arrow); Figure 38B: GST-tagged: lanes 12 and 13 (indicated by an arrow)).

**Example 39**

CT560 (SEQ ID 259 and SEQ ID 260) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein. The recombinant His-tagged protein was used to immunise mice, whose sera were used in a Western blot (Figure 39: lanes 6 and 7 (indicated by an arrow)).

**5 Example 40**

CT389 (SEQ ID 249 and SEQ ID 250) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 40: lanes 16 and 17 (indicated by an arrow)).

**Example 41**

10 CT456 (SEQ ID 255 and SEQ ID 256) was expressed in *E.coli*. The recombinant product was purified as a GST-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 41: lanes 2 and 3 (indicated by an arrow)).

**Example 42**

15 CT622 (SEQ ID 161 and SEQ ID 162) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 42: lane 9 (indicated by an arrow)).

**Example 43**

20 CT759 (SEQ ID 213 and SEQ ID 214) was expressed in *E.coli*. The recombinant product was purified as a His-tagged fusion protein. The recombinant protein was used to immunise mice, whose sera were used in a Western blot (Figure 43: lanes 8 and 9 (indicated by an arrow)).

**Example 44**

*In vitro* neutralization assays, which show the ability of sera obtained from mice that have been immunised with the different recombinant proteins of the present invention to inhibit *C. trachomatis* infectivity for eukaryotic cells in culture, were performed using LLCMK2 (Rhesus monkey kidney epithelial) cells. Serial four-fold dilutions of mouse polyclonal sera were prepared in SP (Sucrose-Phosphate) buffer. Mouse antisera to whole EBs were used as a positive control, and preimmune sera and SP buffer alone were used as negative controls. Purified EBs from *C. trachomatis* (serovar D) were diluted in SP buffer to contain  $3 \times 10^5$  IFU/ml, and 10µl of this suspension were added to each serum dilution in a final volume of 100µl. Antibody-EB interaction was allowed to proceed for 30 min at 37°C. Then 100µl of reaction mix from each sample were added on top of PBS-washed LLCMK2 cell monolayers, in a 96-well microtiter plate, and centrifuged at 805xg for 1 hour at 37°C. All sera and controls were examined in duplicated samples. After removal of the excess inoculum, the cells were rinsed once with PBS, replenished with 200µl of DMEM medium supplemented with

20%FCS and 1µg/ml cycloheximide, and incubated at 37°C for 48 hours. The cells were fixed with methanol and the typical cytoplasmic inclusions generated by the ongoing intracellular chlamydial infection were stained with an anti-Chlamydia fluorescein-conjugated monoclonal antibody (Meridian Diagnostics). At adequate dilutions and EB to host cell ratios, the number of inclusions observed is considered to be equal to the number of viable chlamydiae which were initially capable of successfully establishing a host cell infection (these are named Inclusion Forming Units, IFU). Fluorescein-labelled inclusions were counted in four microscopical fields per well at a magnification of 40X. The inhibition of infectivity due to antibody interaction was calculated as percentage reduction in mean IFU as compared to the SP control (buffer only). According to common practice, the sera were labelled as “neutralizing” if they could cause a 50% or greater reduction in infectivity, however, considering the complexity of the whole screening assay (for instance, a change of host cell, or chlamydial isolate, or a variation in the environmental conditions in the preparation of the infectious inoculum), sera capable of inhibiting EB infectivity to a lower extent should also be considered as vaccine candidates for further study. Figure 44A shows an example of a result obtained from a neutralisation-positive serum whilst Figure 44B shows an example of a result obtained from a neutralisation-negative serum.

*In vitro* neutralization assays were carried out using sera obtained from mice immunised with the recombinant proteins mentioned in Example 1 to 10, 13-22, 24-26 and 29-37. The results are presented in Table II. These results indicate that CT045, CT242, CT381, CT396, CT398, CT467, CT547, CT587 and CT681 are all particularly good candidates for vaccines to prevent infection by *C. trachomatis*. These properties are not evident from the sequences alone.

In further experiments, the sera raised against *C. trachomatis* were tested against *C. pneumoniae* EBs for cross-neutralization activity. The procedure was as described above, but purified EBs from *C. pneumoniae* were diluted in SP buffer to contain  $3 \times 10^6$  IFU/ml, and 10µl of this suspension were added to each serum dilution in a final volume of 100µl. Sera obtained using CT242 and CT467 were able to cross-neutralise *C. pneumoniae* EBs.

It will be appreciated that the invention has been described by way of example only and that modifications may be made whilst remaining within the spirit and scope of the invention.

**REFERENCES** (the contents of which are hereby incorporated in full)

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- {13} EP-A-0499681
- {14} International patent application WO95/28487
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- {16} Cerrone *et al.* (1991) *Infect Immun* 59(1):79-90.
- {17} Raulston *et al.* (1993) *J. Biol. Chem.* 268:23139-23147.
- {18} International patent application WO02/02606.

TABLE I

| Ref. 18 | <i>C. pneumoniae</i> accession number & annotation                              | <i>C. trachomatis</i> accession number & annotation                               | SEQ IDs |
|---------|---------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|---------|
| cp0010  | gi 4376729 gb AAD18590.1 Polymorphic Outer Membrane Protein G Family            | gi 3329346 gb AAC68469.1 Putative Outer Membrane Protein G                        | 1, 2    |
| cp0014  | gi 4376729 gb AAD18590.1 Polymorphic Outer Membrane Protein G Family            | gi 3329346 gb AAC68469.1 Putative Outer Membrane Protein G                        | 3, 4    |
| cp0015  | gi 4376731 gb AAD18591.1 Polymorphic Outer Membrane Protein G/I Family          | gi 3329346 gb AAC68469.1 Putative Outer Membrane Protein G                        | 5, 6    |
| cp0016  | gi 4376731 gb AAD18591.1 Polymorphic Outer Membrane Protein G/I Family          | gi 3329350 gb AAC68472.1 Putative Outer Membrane Protein I                        | 7, 8    |
| cp0017  | gi 4376731 gb AAD18591.1 Polymorphic Outer Membrane Protein G/I Family          | gi 3329346 gb AAC68469.1 Putative Outer Membrane Protein G                        | 9, 10   |
| cp0018  | gi 4376733 gb AAD18593.1 Polymorphic Outer Membrane Protein G Family            | gi 3328840 gb AAC68009.1 Putative outer membrane protein A                        | 11, 12  |
| cp0019  | gi 4376731 gb AAD18591.1 Polymorphic Outer Membrane Protein G/I Family          | gi 3329346 gb AAC68469.1 Putative Outer Membrane Protein G                        | 13, 14  |
| cp0468  | gi 4376754 gb AAD18611.1 Polymorphic Outer Membrane Protein (Frame-shift with C | gi 3329344 gb AAC68467.1 Putative Outer Membrane Protein E                        | 15, 16  |
| cp6260  | gi 4376260 gb AAD18163.1 Polymorphic Outer Membrane Protein G Family            | gi 3329346 gb AAC68469.1 Putative Outer Membrane Protein G                        | 17, 18  |
| cp6262  | gi 4376262 gb AAD18165.1 hypothetical protein                                   | gi 3328765 gb AAC67940.1 hypothetical protein                                     | 19, 20  |
| cp6269  | gi 4376269 gb AAD18171.1 hypothetical protein                                   | gi 3328825 gb AAC67995.1 hypothetical protein                                     | 21, 22  |
| cp6270  | gi 4376270 gb AAD18172.1 Polymorphic Outer Membrane Protein G Family            | gi 3329350 gb AAC68472.1 Putative Outer Membrane Protein I                        | 23, 24  |
| cp6272  | gi 4376272 gb AAD18173.1 Predicted OMP {leader peptide: outer membrane}         | gi 3328772 gb AAC67946.1 hypothetical protein                                     | 25, 26  |
| cp6273  | gi 4376273 gb AAD18174.1 Predicted OMP {leader peptide}                         | gi 3328771 gb AAC67945.1 hypothetical protein                                     | 27, 28  |
| cp6296  | gi 4376296 gb AAD18195.1 hypothetical protein                                   | gi 3328520 gb AAC67712.1 Ribulose-P Epimerase                                     | 29, 30  |
| cp6362  | gi 4376362 gb AAD18254.1 YbbP family hypothetical protein                       | gi 3328401 gb AAC67602.1 hypothetical protein                                     | 31, 32  |
| cp6372  | gi 4376372 gb AAD18263.1 Signal Peptidase I                                     | gi 3328410 gb AAC67610.1 Signal Peptidase I                                       | 33, 34  |
| cp6397  | gi 4376397 gb AAD18286.1 CHLPS hypothetical protein                             | gi 3328506 gb AAC67700.1 CHLPS hypothetical protein                               | 35, 36  |
| cp6402  | gi 4376402 gb AAD18290.1 ACR family                                             | gi 3328505 gb AAC67699.1 ACR family                                               | 37, 38  |
| cp6419  | gi 4376419 gb AAD18305.1 CT149 hypothetical protein                             | gi 3328551 gb AAC67740.1 possible hydrolase                                       | 39, 40  |
| cp6446  | gi 4376446 gb AAD18330.1 hypothetical protein                                   | gi 3329261 gb AAC68390.1 hypothetical protein                                     | 41, 42  |
| cp6466  | gi 4376466 gb AAD18348.1 Oligopeptide Binding Protein                           | gi 3328604 gb AAC67790.1 Oligopeptide Binding Protein                             | 43, 44  |
| cp6467  | gi 4376467 gb AAD18349.1 Oligopeptide Binding Protein                           | gi 3328604 gb AAC67790.1 Oligopeptide Binding Protein                             | 45, 46  |
| cp6468  | gi 4376468 gb AAD18350.1 Oligopeptide Binding Protein                           | gi 3328539 gb AAC67730.1 Oligopeptide Binding Protein                             | 47, 48  |
| cp6469  | gi 4376469 gb AAD18351.1 Oligopeptide Binding Protein                           | gi 3328579 gb AAC67766.1 Oligopeptide binding protein permease                    | 49, 50  |
| cp6520  | gi 4376520 gb AAD18398.1 Polysaccharide Hydrolase-Invasin Repeat Family         | gi 3328526 gb AAC67718.1 predicted polysaccharide hydrolase-invasin repeat family | 51, 52  |
| cp6567  | gi 4376567 gb AAD18441.1 Inclusion Membrane Protein C                           | gi 3328642 gb AAC67825.1 Inclusion Membrane Protein C                             | 53, 54  |
| cp6576  | gi 4376576 gb AAD18449.1 Omp85 Analog                                           | gi 3328651 gb AAC67834.1 Omp85 Analog                                             | 55, 56  |
| cp6577  | gi 4376577 gb AAD18450.1 (OmpH-Like Outer Membrane Protein)                     | gi 3328652 gb AAC67835.1 (OmpH-Like Outer Membrane Protein)                       | 57, 58  |
| cp6601  | gi 4376601 gb AAD18472.1 Low Calcium Response D                                 | gi 3328486 gb AAC67881.1 Low Calcium Response D                                   | 59, 60  |
| cp6602  | gi 4376602 gb AAD18473.1 Low Calcium Response E                                 | gi 3328485 gb AAC67880.1 Low Calcium Response E                                   | 61, 62  |
| cp6607  | gi 4376607 gb AAD18478.1 Phospholipase D Superfamily                            | gi 3328479 gb AAC67675.1 Phospholipase D Superfamily {leader (33) peptide}        | 63, 64  |
| cp6615  | gi 4376615 gb AAD18485.1 YolL hypothetical protein                              | gi 3328472 gb AAC67868.1 hypothetical protein                                     | 65, 66  |
| cp6624  | gi 4376624 gb AAD18493.1 Solute Protein Binding Family                          | gi 3328461 gb AAC67658.1 Solute Protein Binding Family                            | 67, 68  |
| cp6639  | gi 4376639 gb AAD18507.1 Flagellar Secretion Protein                            | gi 3328453 gb AAC67651.1 Flagellar Secretion Protein                              | 69, 70  |

|        |                                                                                  |                                                                                 |          |
|--------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------|----------|
| cp6664 | gi 4376664 gb AAD18529.1  Leucyl Aminopeptidase A                                | gi 3328437 gb AAC67636.1  Leucyl Aminopeptidase A                               | 71, 72   |
| cp6672 | gi 4376672 gb AAD18537.1  CBS Domain protein (Hemolysin Homolog)                 | gi 3328667 gb AAC67849.1  Hypothetical protein containing CBS domains           | 73, 74   |
| cp6679 | gi 4376679 gb AAD18543.1  CT253 hypothetical protein                             | gi 3328664 gb AAC67846.1  hypothetical protein                                  | 75, 76   |
| cp6696 | gi 4376696 gb AAD18559.1  CT266 hypothetical protein                             | gi 3328678 gb AAC67859.1  hypothetical protein                                  | 77, 78   |
| cp6717 | gi 4376717 gb AAD18579.1  Phospholipase D superfamily                            | gi 3328698 gb AAC67877.1  Phospholipase D superfamily                           | 79, 80   |
| cp6727 | gi 4376727 gb AAD18588.1  Polymorphic Outer Membrane Protein G/I Family          | gi 3329345 gb AAC68469.1  Putative Outer Membrane Protein G                     | 81, 82   |
| cp6728 | gi 4376728 gb AAD18589.1  Polymorphic Outer Membrane Protein G Family            | gi 3329346 gb AAC68469.1  Putative Outer Membrane Protein G                     | 83, 84   |
| cp6729 | gi 4376729 gb AAD18590.1  Polymorphic Outer Membrane Protein G Family            | gi 3329350 gb AAC68472.1  Putative Outer Membrane Protein I                     | 85, 86   |
| cp6731 | gi 4376731 gb AAD18591.1  Polymorphic Outer Membrane Protein G/I Family          | gi 3329350 gb AAC68472.1  Putative Outer Membrane Protein I                     | 87, 88   |
| cp6733 | gi 4376733 gb AAD18593.1  Polymorphic Outer Membrane Protein G Family            | gi 3328840 gb AAC68009.1  Putative outer membrane protein A                     | 89, 90   |
| cp6735 | gi 4376735 gb AAD18594.1  Polymorphic Outer Membrane Protein (truncated) A/I Fam | gi 3328840 gb AAC68009.1  Putative outer membrane protein A                     | 91, 92   |
| cp6736 | gi 4376736 gb AAD18595.1  Polymorphic Outer Membrane Protein G Family            | gi 3329346 gb AAC68469.1  Putative Outer Membrane Protein G                     | 93, 94   |
| cp6737 | gi 4376737 gb AAD18596.1  Polymorphic Outer Membrane Protein H Family            | gi 3329347 gb AAC68470.1  Putative Outer Membrane Protein H                     | 95, 96   |
| cp6751 | gi 4376751 gb AAD18608.1  Polymorphic Outer Membrane Protein E Family            | gi 3329344 gb AAC68467.1  Putative Outer Membrane Protein E                     | 97, 98   |
| cp6752 | gi 4376752 gb AAD18609.1  Polymorphic Outer Membrane Protein E Family            | gi 3329344 gb AAC68467.1  Putative Outer Membrane Protein E                     | 99, 100  |
| cp6753 | gi 4376753 gb AAD18610.1  Polymorphic Outer Membrane Protein E/F Family          | gi 3329344 gb AAC68467.1  Putative Outer Membrane Protein E                     | 101, 102 |
| cp6757 | gi 4376757 gb AAD18613.1  hypothetical protein                                   | gi 3328701 gb AAC67880.1  PP-loop superfamily ATPase                            | 103, 104 |
| cp6767 | gi 4376767 gb AAD18622.1  Arginine Periplasmic Binding Protein                   | gi 3328806 gb AAC67977.1  Arginine Binding Protein                              | 105, 106 |
| cp6790 | gi 4376790 gb AAD18643.1  Heat Shock Protein-70                                  | gi 3328822 gb AAC67993.1  HSP-70                                                | 107, 108 |
| cp6802 | gi 4376802 gb AAD18654.1  CT427 hypothetical protein                             | gi 3328857 gb AAC68024.1  hypothetical protein                                  | 109, 110 |
| cp6814 | gi 4376814 gb AAD18665.1  CT398 hypothetical protein                             | gi 3328825 gb AAC67995.1  hypothetical protein                                  | 111, 112 |
| cp6829 | gi 4376829 gb AAD18679.1  polymorphic membrane protein A Family                  | gi 3328840 gb AAC68009.1  Putative outer membrane protein A                     | 113, 114 |
| cp6830 | gi 4376830 gb AAD18680.1  polymorphic membrane protein B Family                  | gi 3328841 gb AAC68010.1  Putative outer membrane protein B                     | 115, 116 |
| cp6832 | gi 4376832 gb AAD18681.1  Solute binding protein                                 | gi 3328844 gb AAC68012.1  Solute-binding protein                                | 117, 118 |
| cp6834 | gi 4376834 gb AAD18683.1  (Metal Transport Protein)                              | gi 3328846 gb AAC68014.1  (Metal Transport Protein)                             | 119, 120 |
| cp6847 | gi 4376847 gb AAD18695.1  Tail-Specific Protease                                 | gi 3328872 gb AAC68040.1  Tail-Specific Protease                                | 121, 122 |
| cp6848 | gi 4376848 gb AAD18696.1  15 kDa Cysteine-Rich Protein                           | gi 3328873 gb AAC68041.1  15kDa Cysteine-Rich Protein                           | 123, 124 |
| cp6849 | gi 4376849 gb AAD18697.1  60 kDa Cysteine-Rich OMP                               | gi 3328874 gb AAC68042.1  60kDa Cysteine-Rich OMP                               | 125, 126 |
| cp6850 | gi 4376850 gb AAD18698.1  9 kDa-Cysteine-Rich Lipoprotein                        | gi 3328876 gb AAC68043.1  9kDa-Cysteine-Rich Lipoprotein                        | 127, 128 |
| cp6878 | gi 4376878 gb AAD18723.1  2-Component Sensor                                     | gi 3328901 gb AAC68067.1  2-component regulatory system-sensor histidine kinase | 129, 130 |
| cp6879 | gi 4376879 gb AAD18724.1  similarity to CHLPS InCA                               | gi 3328451 gb AAC67649.1  hypothetical protein                                  | 131, 132 |
| cp6884 | gi 4376884 gb AAD18729.1  CT471 hypothetical protein                             | gi 3329905 gb AAC68071.1  hypothetical protein                                  | 133, 134 |
| cp6886 | gi 4376886 gb AAD18731.1  YidD family                                            | gi 3329908 gb AAC68073.1  hypothetical protein                                  | 135, 136 |
| cp6890 | gi 4376890 gb AAD18734.1  CT478 hypothetical protein                             | gi 3328911 gb AAC68076.1  hypothetical protein                                  | 137, 138 |
| cp6892 | gi 4376892 gb AAD18736.1  Oligopeptide Permease                                  | gi 3328913 gb AAC68078.1  Oligopeptide Permease                                 | 139, 140 |
| cp6894 | gi 4376894 gb AAD18738.1  Oligopeptide Binding Lipoprotein                       | gi 3328915 gb AAC68080.1  oligopeptide Binding Lipoprotein                      | 141, 142 |
| cp6900 | gi 4376900 gb AAD18743.1  Glutamine Binding Protein                              | gi 3328922 gb AAC68086.1  Glutamine Binding Protein                             | 143, 144 |
| cp6909 | gi 4376909 gb AAD18752.1  Protease                                               | gi 6578107 gb AAC68094.2  Protease                                              | 145, 146 |

|        |                                                                         |                                                                         |          |
|--------|-------------------------------------------------------------------------|-------------------------------------------------------------------------|----------|
| cp6952 | gi 4376952 gb AAD18792.1  Apolipoprotein N-Acetyltransferase            | gi 3328972 gb AAC68136.1  Apolipoprotein N-Acetyltransferase            | 147, 148 |
| cp6960 | gi 4376960 gb AAD18800.1  FKBP-type peptidyl-prolyl cis-trans isomerase | gi 3328979 gb AAC68143.1  FKBP-type peptidyl-prolyl cis-trans isomerase | 149, 150 |
| cp6968 | gi 4376968 gb AAD18807.1  CT547 hypothetical protein                    | gi 3328986 gb AAC68149.1  hypothetical protein                          | 151, 152 |
| cp6969 | gi 4376969 gb AAD18808.1  CT548 hypothetical protein                    | gi 3328987 gb AAC68150.1  hypothetical protein                          | 153, 154 |
| cp6998 | gi 4376998 gb AAD18834.1  Major Outer Membrane Protein                  | gi 3329133 gb AAC68276.1  Major Outer Membrane Protein                  | 155, 156 |
| cp7005 | gi 4377005 gb AAD18841.1  YopC/Gen Secretion Protein D                  | gi 3329125 gb AAC68269.1  probable Yop proteins translocation protein   | 157, 158 |
| cp7015 | gi 4377015 gb AAD18851.1  FHA domain; (homology to adenylate cyclase)   | gi 3329115 gb AAC68259.1  (FHA domain; homology to adenylate cyclase)   | 159, 160 |
| cp7033 | gi 4377033 gb AAD18867.1  CHLPN 76 kDa Homolog. 1 (CT622)               | gi 3329069 gb AAC68226.1  CHLPN 76kDa Homolog                           | 161, 162 |
| cp7034 | gi 4377034 gb AAD18868.1  CHLPN 76 kDa Homolog. 2 (CT623)               | gi 6578109 gb AAC68227.2  CHLPN 76kDa Homolog                           | 163, 164 |
| cp7035 | gi 4377035 gb AAD18869.1  Integral Membrane Protein                     | gi 3329071 gb AAC68228.1  Integral Membrane Protein                     | 165, 166 |
| cp7072 | gi 4377072 gb AAD18902.1  CT648 hypothetical protein                    | gi 3329097 gb AAC68825.1  hypothetical protein                          | 167, 168 |
| cp7073 | gi 4377073 gb AAD18903.1  CT647 hypothetical protein                    | gi 3329096 gb AAC68824.1  hypothetical protein                          | 168, 170 |
| cp7085 | gi 4377085 gb AAD18914.1  CT605 hypothetical protein                    | gi 3329050 gb AAC68208.1  hypothetical protein                          | 171, 172 |
| cp7090 | gi 4377090 gb AAD18919.1  Peptidoglycan-Associated Lipoprotein          | gi 3329044 gb AAC68202.1  Peptidoglycan-Associated Lipoprotein          | 173, 174 |
| cp7091 | gi 4377091 gb AAD18920.1  macromolecule transporter                     | gi 3329043 gb AAC68201.1  component of a macromolecule transport system | 175, 176 |
| cp7092 | gi 4377092 gb AAD18921.1  CT598 hypothetical protein                    | gi 3329042 gb AAC68200.1  hypothetical protein                          | 177, 178 |
| cp7093 | gi 4377093 gb AAD18922.1  Biopolymer Transport Protein                  | gi 3329041 gb AAC68199.1  Biopolymer Transport Protein                  | 179, 180 |
| cp7094 | gi 4377094 gb AAD18923.1  Macromolecule transporter                     | gi 3329040 gb AAC68198.1  polysaccharide transporter                    | 181, 182 |
| cp7101 | gi 4377101 gb AAD18929.1  CT590 hypothetical protein                    | gi 3329033 gb AAC68192.1  hypothetical protein                          | 183, 184 |
| cp7102 | gi 4377102 gb AAD18930.1  CT589 hypothetical protein                    | gi 3329032 gb AAC68191.1  hypothetical protein                          | 185, 186 |
| cp7106 | gi 4377106 gb AAD18933.1  hypothetical protein                          | gi 3328796 gb AAC67968.1  hypothetical protein                          | 187, 188 |
| cp7111 | gi 4377111 gb AAD18938.1  Enolase                                       | gi 3329030 gb AAC68189.1  Enolase                                       | 189, 190 |
| cp7127 | gi 4377127 gb AAD18953.1  General Secretion Protein D                   | gi 3329013 gb AAC68174.1  Gen. Secretion Protein D                      | 191, 192 |
| cp7130 | gi 4377130 gb AAD18956.1  predicted OMP {leader peptide}                | gi 3329010 gb AAC68171.1  predicted OMP                                 | 193, 194 |
| cp7132 | gi 4377132 gb AAD18958.1  CT567 hypothetical protein                    | gi 3329008 gb AAC68169.1  hypothetical protein                          | 195, 196 |
| cp7133 | gi 4377133 gb AAD18959.1  CT566 hypothetical protein                    | gi 3329007 gb AAC68168.1  hypothetical protein                          | 197, 198 |
| Cp7140 | gi 4377140 gb AAD18965.1  Yop Translocation J                           | gi 3329000 gb AAC68161.1  Yop proteins translocation lipoprotein J      | 199, 200 |
| Cp7170 | gi 4377170 gb AAD18992.1  Outer Membrane Protein B                      | gi 3329169 gb AAC68308.1  Outer Membrane Protein Analog                 | 201, 202 |
| Cp7177 | gi 4377177 gb AAD18998.1  Flagellar M-Ring Protein                      | gi 3329175 gb AAC68314.1  Flagellar M-Ring Protein                      | 203, 204 |
| Cp7182 | gi 4377182 gb AAD19003.1  CT724 hypothetical protein                    | gi 3329181 gb AAC68319.1  hypothetical protein                          | 205, 206 |
| Cp7184 | gi 4377184 gb AAD19005.1  Rod Shape Protein                             | gi 3329183 gb AAC68321.1  Rod Shape Protein                             | 207, 208 |
| Cp7193 | gi 4377193 gb AAD19013.1  CT734 hypothetical protein                    | gi 3329192 gb AAC68329.1  hypothetical protein                          | 209, 210 |
| Cp7206 | gi 4377206 gb AAD19025.1  CHL TR possible phosphoprotein                | gi 3329204 gb AAC68339.1  CHL TR possible phosphoprotein                | 211, 212 |
| Cp7222 | gi 4377222 gb AAD19040.1  Muramidase (invasin repeat family)            | gi 3329221 gb AAC68354.1  Muramidase (invasin repeat family)            | 213, 214 |
| Cp7223 | gi 4377223 gb AAD19041.1  Cell Division Protein FtsW                    | gi 3329222 gb AAC68355.1  Cell Division Protein FtsW                    | 215, 216 |
| Cp7224 | gi 4377224 gb AAD19042.1  Peptidoglycan Transferase                     | gi 3329223 gb AAC68356.1  Peptidoglycan Transferase                     | 217, 218 |
| Cp7225 | gi 4377225 gb AAD19043.1  Muramate-Ala Ligase & D-Ala-D-Ala Ligase      | gi 3329224 gb AAC68357.1  UDP-N-acetylmuramate-alanine ligase           | 219, 220 |
| Cp7248 | gi 4377248 gb AAD19064.1  Thioredoxin Disulfide Isomerase               | gi 3329244 gb AAC68375.1  Thioredoxin Disulfide Isomerase               | 221, 222 |

|        |                                                                                 |                                                                                     |          |
|--------|---------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|----------|
| Cp7261 | gi 4377261 gb AAD19076.1 CT788 hypothetical protein -{leader peptide-periplasmi | gi 3329253 gb AAC68383.1 {leader (60) peptide-periplasmic}                          | 223, 224 |
| Cp7280 | gi 4377280 gb AAD19093.1 Insulinase family/Protease III                         | gi 3329273 gb AAC68402.1 Insulinase family/Protease III                             | 225, 226 |
| Cp7287 | gi 4377287 gb AAD19099.1 Putative Outer Membrane Protein D Family               | gi 3329279 gb AAC68408.1 Putative Outer Membrane Protein D                          | 227, 228 |
| Cp7306 | gi 4377306 gb AAD19116.1 DO Serine Protease                                     | gi 3329293 gb AAC68420.1 DO Serine Protease                                         | 229, 230 |
| Cp7342 | gi 4377342 gb AAD19149.1 ABC transporter permease                               | gi 3329327 gb AAC68451.1 ABC transporter permease — pyrimidine biosynthesis protein | 231, 232 |
| Cp7347 | gi 4377347 gb AAD19153.1 CT858 hypothetical protein                             | gi 6578118 gb AAC68456.2 predicted Protease containing IRBP and DHR domains         | 233, 234 |
| Cp7353 | gi 4377353 gb AAD19159.1 CT863 hypothetical protein                             | gi 3329337 gb AAC68461.1 hypothetical protein                                       | 235, 236 |
| Cp7367 | gi 4377367 gb AAD19171.1 Predicted OMP                                          | gi 3328795 gb AAC67967.1 hypothetical protein                                       | 237, 238 |
| Cp7408 | gi 4377408 gb AAD19209.1 hypothetical protein                                   | gi 3328795 gb AAC67967.1 hypothetical protein                                       | 239, 240 |
| Cp7409 | gi 4377409 gb AAD19210.1 Predicted Outer Membrane Protein (CT371)               | gi 3328795 gb AAC67967.1 hypothetical protein                                       | 241, 242 |
|        | gi 4376411 gb                                                                   | gi 3328512 gb AAC67705.1 hypothetical protein                                       | 243, 244 |
|        | gi 4376508 gb                                                                   | gi 3328585 gb AAC67772.1 hypothetical protein                                       | 245, 246 |
|        | gi 4376710 gb                                                                   | gi 3328692 gb AAC67872.1 NADH (Ubiquinone) Oxidoreductase, Gamma                    | 247, 248 |
|        | gi 4376777 gb                                                                   | gi 3328815 gb AAC67986.1 hypothetical protein                                       | 249, 250 |
|        | gi 4376782 gb                                                                   | gi 3328817 gb AAC67988.1 hypothetical protein                                       | 251, 252 |
|        | gi 4376863 gb                                                                   | gi 3328887 gb AAC68054.1 Arginyl tRNA transferase                                   | 253, 254 |
|        | gi 4376866 gb                                                                   | gi 3328889 gb AAC68056.1 hypothetical protein                                       | 255, 256 |
|        | gi 4376972 gb                                                                   | gi 3328991 gb AAC68153.1 D-Ala-D-Ala Carboxypeptidase                               | 257, 258 |
|        | gi 4377139 gb                                                                   | gi 3329001 gb AAC68162.1 hypothetical protein                                       | 259, 260 |
|        | gi 4377154 gb                                                                   | gi 3329154 gb AAC68295.1 hypothetical protein                                       | 261, 262 |

TABLE II

| CT     | Fusion type | 50% neutralization titer | % neutralization of EB infectivity for LLCMK2 cell cultures at specified serum dilutions |       |       |        |
|--------|-------------|--------------------------|------------------------------------------------------------------------------------------|-------|-------|--------|
|        |             |                          | 1/40                                                                                     | 1/160 | 1/640 | 1/2560 |
| CT045  | HIS         | 1:160                    | 32                                                                                       | 50    | 18    |        |
| CT089  | HIS         |                          | 44                                                                                       | 37    | 0     |        |
|        | GST         |                          | 6                                                                                        | 25    | 37    |        |
| CT114  | HIS         |                          | 0                                                                                        | 18    |       |        |
| CT181  | GST         |                          | 19                                                                                       | 0     |       |        |
| CT198  | HIS         |                          | 19                                                                                       | 0     | 13    |        |
| CT241  | HIS         |                          | 5                                                                                        | 42    |       |        |
| CT242  | HIS         | 1:100                    | 58                                                                                       | 45    | 0     |        |
|        | GST         |                          | 46                                                                                       | 24    | 40    |        |
| CT350  | GST         |                          | 0                                                                                        | 39    |       |        |
| CT351  | HIS         |                          | 1                                                                                        | 5     |       |        |
| CT381  | HIS         | 1:450                    | 67                                                                                       | 56    | 48    |        |
|        | GST         |                          | 24                                                                                       | 0     | 0     |        |
| CT391  | HIS         |                          | 0                                                                                        | 14    |       |        |
| CT396  | HIS         | 1:300                    | 55                                                                                       | 62    | 35    |        |
|        | GST         |                          | 8                                                                                        | 33    | 25    |        |
| CT398  | HIS         | 1:640                    | 57                                                                                       | 45    | 50    |        |
|        | GST         | 1:>640                   | 68                                                                                       | 60    | 60    |        |
| CT415  | GST         |                          | 21                                                                                       | 21    |       |        |
| CT427  | HIS         |                          | 25                                                                                       | 13    | 13    |        |
| CT443  | HIS         |                          | 25                                                                                       | 25    |       |        |
| CT454  | HIS         |                          | 16                                                                                       | 4     |       |        |
| CT467  | GST         | 1:1100                   | 65                                                                                       | 67    | 62    | 48     |
| CT541  | GST         |                          | 10                                                                                       | 24    | 13    |        |
| CT547  | HIS         | 1:40                     | 50                                                                                       | 13    | 18    |        |
|        | GST         |                          | 0                                                                                        | 0     | 18    |        |
| CT551  | HIS         |                          | 5                                                                                        | 11    |       |        |
| CT559  | HIS         |                          | 20                                                                                       | 23    |       |        |
| CT567  |             |                          | 0                                                                                        | 26    |       |        |
| CT569  | HIS         |                          | 0                                                                                        | 5     |       |        |
| CT587  | HIS         | 1:1200                   | 51                                                                                       | 61    | 56    | 42     |
| CT589  | HIS         |                          | 37                                                                                       | 21    |       |        |
|        | GST         |                          | 0                                                                                        | 33    |       |        |
| CT597  | GST         |                          | 0                                                                                        | 4     |       |        |
| CT600  | HIS         |                          | 0                                                                                        | 11    |       |        |
| CT647  | GST         |                          | 15                                                                                       | 0     |       |        |
| CT681  | HIS         | 1:160                    | 95                                                                                       | 53    |       |        |
| CT713  | HIS         |                          | 10                                                                                       | 10    |       |        |
| CT761  | GST         |                          | 0                                                                                        | 16    |       |        |
| CT823  | HIS         |                          | 5                                                                                        | 23    |       |        |
| NN-GST |             |                          | 0                                                                                        | 0     | 0     |        |
| NN-HIS |             |                          | 0                                                                                        | 0     | 0     |        |

## CLAIMS

1. The use of a protein in the manufacture of a medicament for the treatment or prevention of infection due to *Chlamydia trachomatis*, wherein the protein is (a) a protein comprising an amino acid sequence selected from the group consisting of SEQ IDs 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259 and 261, (b) a protein comprising an amino acid sequence having 50% or greater sequence identity to the amino acid sequence of (a), or (c) a protein comprising a fragment of an amino acid sequence of (a).
2. The use of a nucleic acid in the manufacture of a medicament for the treatment or prevention of infection due to *Chlamydia trachomatis*, wherein the nucleic acid is (a) a nucleic acid comprising a nucleotide sequence selected from the group consisting of SEQ IDs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260 and 262.
3. & 242, (b) a nucleic acid comprising a nucleotide sequence having 50% or greater sequence identity to the nucleotide sequence of (a), or (c) a nucleic acid comprising a fragment of an nucleotide sequence of (a).
4. The use of claim 1 or claim 2, wherein infection is treated or prevented by the medicament eliciting an immune response which is specific to a *Chlamydia* elementary body.
5. The use of a protein as defined in claim 1, or a nucleic acid as defined in claim 2, in the manufacture of a medicament for neutralizing *Chlamydia trachomatis* elementary bodies.
6. An immunogenic composition comprising a protein and an adjuvant, wherein the protein is (a) a protein comprising an amino acid sequence selected from the group consisting of SEQ IDs 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219,

221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259 and 261, (b) a protein comprising an amino acid sequence having 50% or greater sequence identity to the amino acid sequence of (a), or (c) a protein comprising a fragment of an amino acid sequence of (a).

- 5 7. An immunogenic composition comprising a nucleic acid and an adjuvant, wherein the nucleic acid is (a) a nucleic acid comprising a nucleotide sequence selected from the group consisting of SEQ IDs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 10 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260 and 262, (b) a nucleic acid comprising a nucleotide sequence having 50% or greater sequence identity to the nucleotide sequence of (a), or (c) a nucleic acid comprising a 15 fragment of an nucleotide sequence of (a).
8. The composition of claim 5 or claim 6, for use as a pharmaceutical.
9. A method of neutralizing *C.trachomatis* infectivity in a patient, comprising the step of administering to the patient the composition of claim 5 or claim 6, or an antibody which recognises a protein as defined in claim 1.
- 20 10. A method of immunising a patient against *Chlamydia trachomatis*, comprising administering to the patient the composition of claim 5 or claim 6.
11. A method of raising antibodies specific for *Chlamydia trachomatis* elementary bodies, comprising administering to the patient the composition of claim 5 or claim 6.
12. A method of raising antibodies which recognise a protein as defined in claim 1, comprising the 25 step of administering to a patient a *Chlamydia trachomatis* elementary body.
13. A method for detecting a *Chlamydia trachomatis* elementary body in a biological sample, comprising the step of contacting the sample with an antibody which recognizes a protein as defined in claim 1.