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(54) RECOMBINANT BACTERIUM AND USES THEREOF

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CPC ***A61K 39/12*** (2013.01); ***A61K 39/0011*** (2013.01)

USPC **424/200.1**; 435/252.3

(57) ABSTRACT

The present invention relates to a recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof. To this end, the present invention provides a recombinant bacterium comprising a nucleic acid encoding an antigen that is translocated to the cytosol of a host cell utilizing Type III secretion system. The recombinant bacterium is generally chosen from intracellular pathogens that reside in the phagosome and fail to induce rapid T cell activation. The translocated antigen may be a viral antigen, a bacterial antigen, or a tumour antigen. Methods of imparting immunity using the recombinant bacterium are also provided.

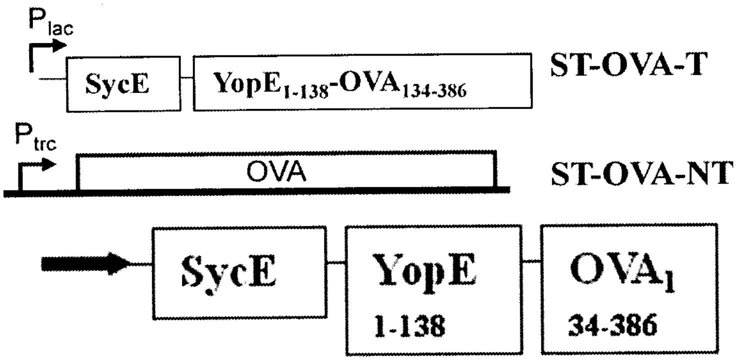
P_{lac}
SycE
$YopE_{1-138}$-$OVA_{134-386}$
ST-OVA-T
P_{trc}
OVA
ST-OVA-NT
SycE
YopE
1-138
OVA_1
34-386

FIG. 1A

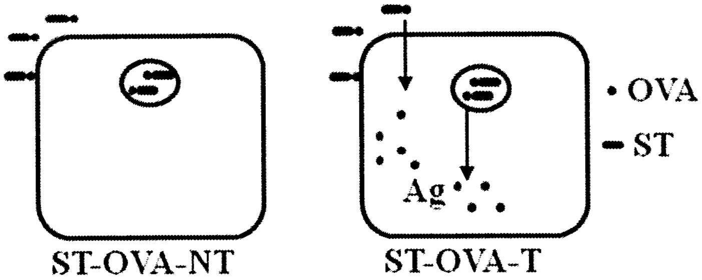
• OVA
▬ ST
Ag
ST-OVA-NT
ST-OVA-T

FIG. 1B

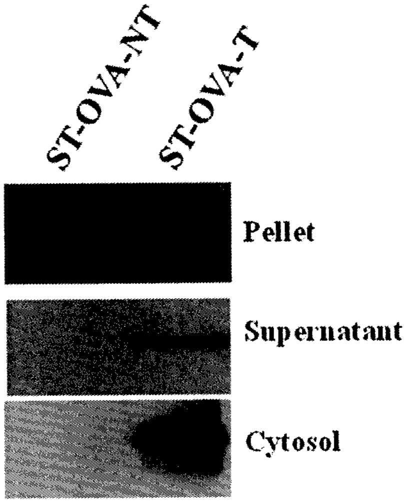
ST-OVA-NT
ST-OVA-T
Pellet
Supernatant
Cytosol

FIG. 1C

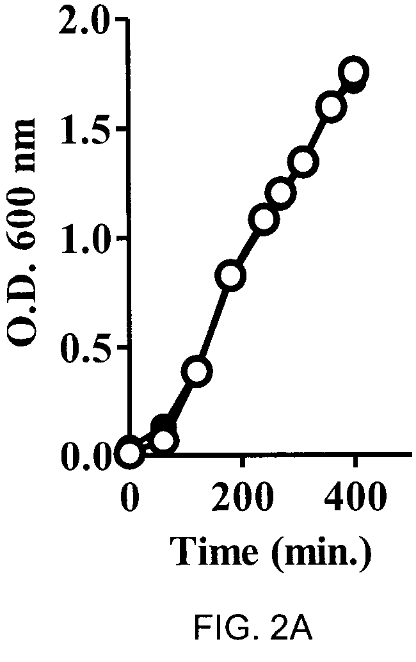
2.0
1.5
1.0
0.5
0.0
O.D. 600 nm
0
200
400
Time (min.)

FIG. 2A

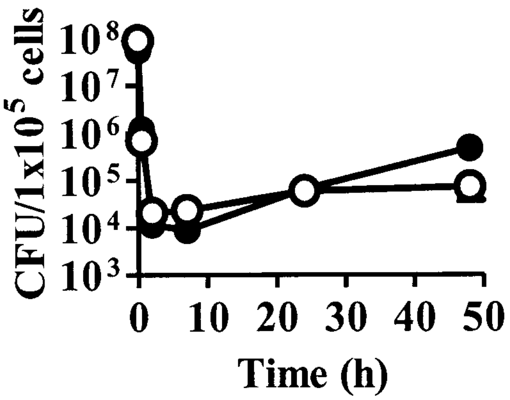
CFU/1x10^5 cells
10^8
10^7
10^6
10^5
10^4
10^3
0
10
20
30
40
50
Time (h)

FIG. 2B

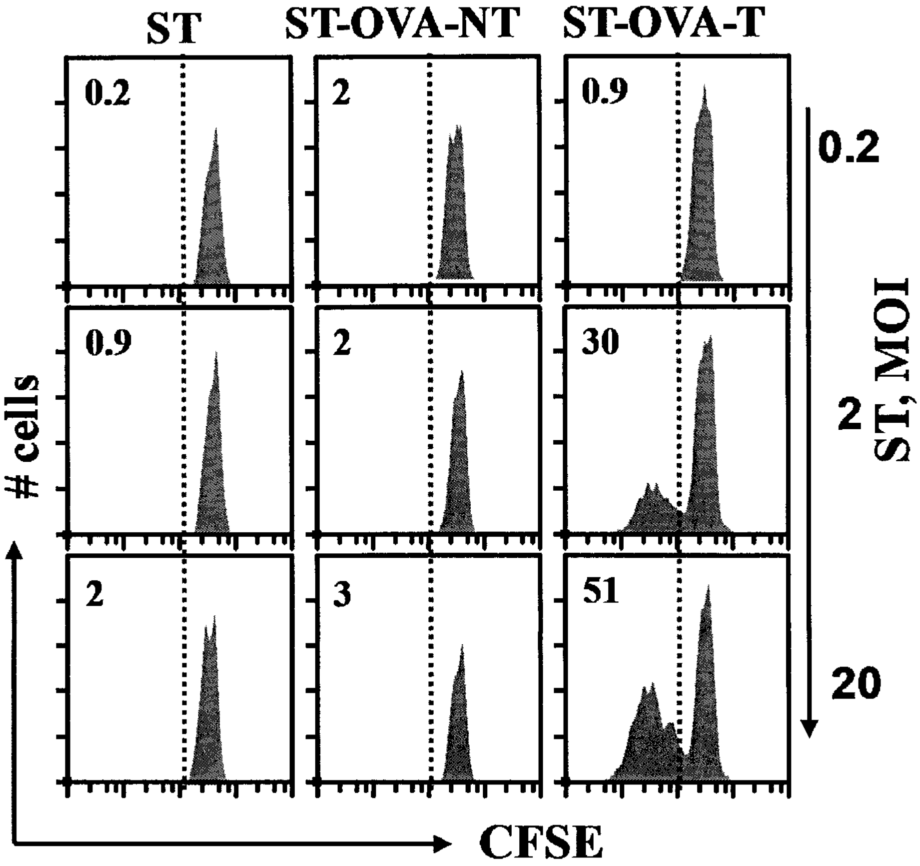
ST
ST-OVA-NT
ST-OVA-T
0.2
2
0.9
0.9
2
30
2
3
51
0.2
2
20
ST, MOI
cells
CFSE

FIG. 3A

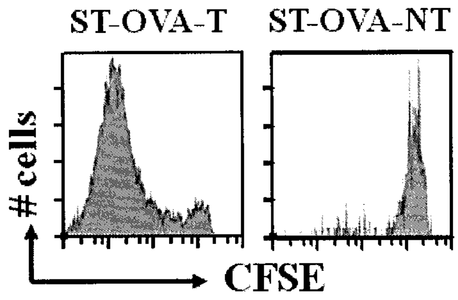

FIG. 3B

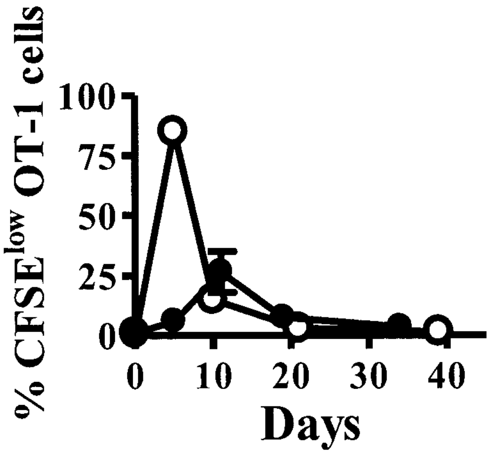

FIG. 3C

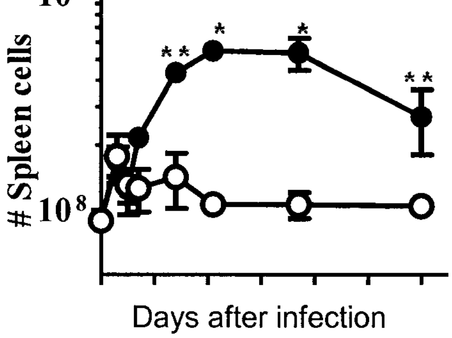

FIG. 4A

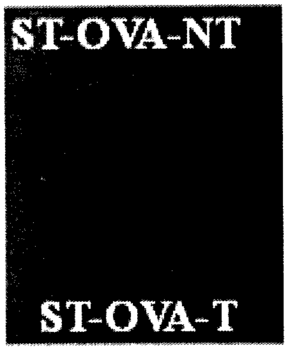

FIG. 4B

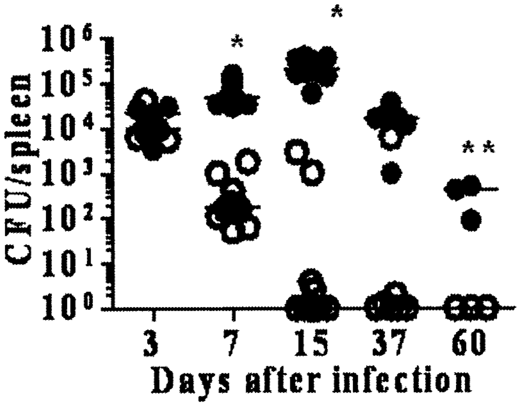

FIG. 4C

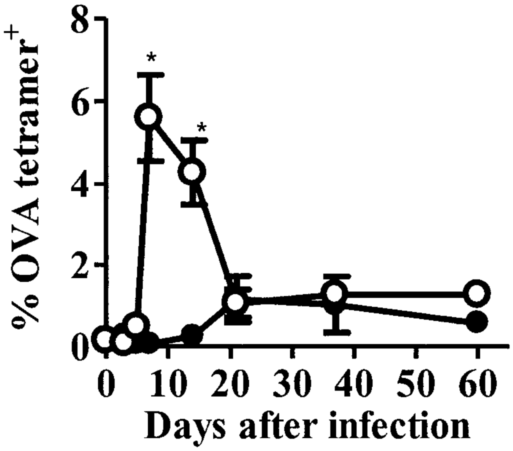

FIG. 4D

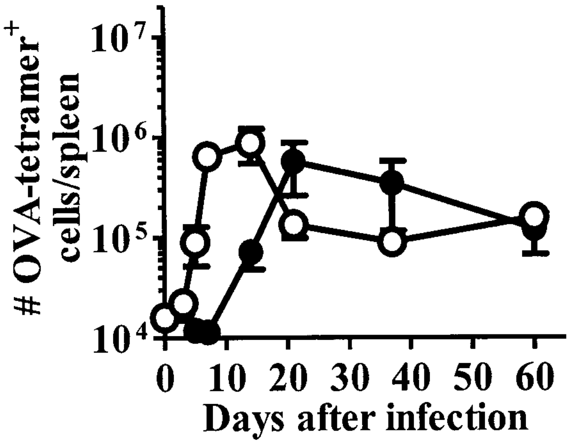
10^7
10^6
10^5
10^4
OVA-tetramer$^+$ cells/spleen
0
10
20
30
40
50
60
Days after infection

FIG. 4E

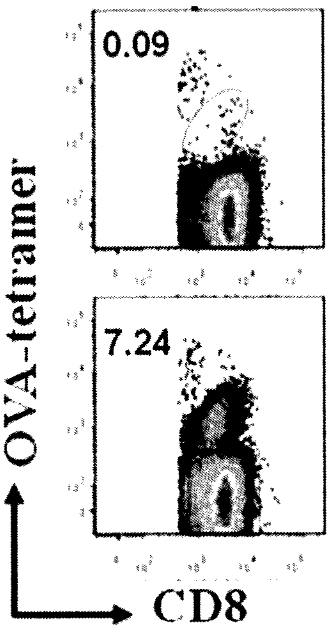
0.09
7.24
OVA-tetramer
CD8

FIG. 5A

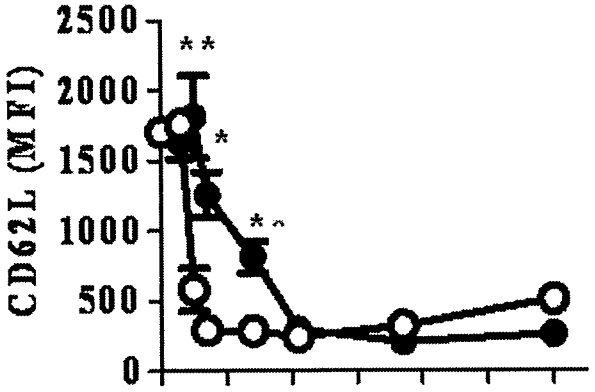
2500
2000
1500
1000
500
0
CD62L (MFI)
**
*
**

FIG. 5B

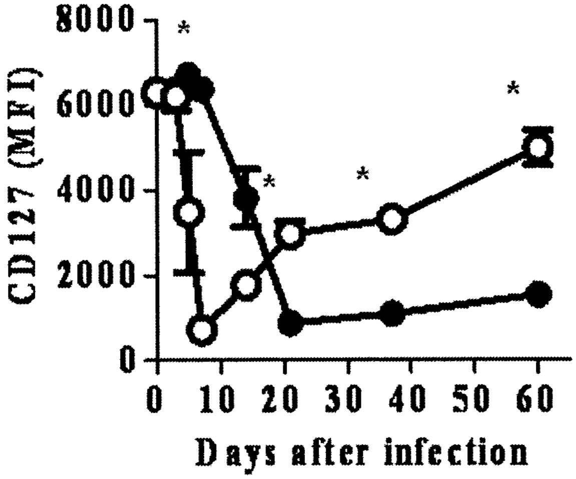
8000
6000
4000
2000
0
CD127 (MFI)
0
10
20
30
40
50
60
Days after infection
*
*
*
*

FIG. 5C

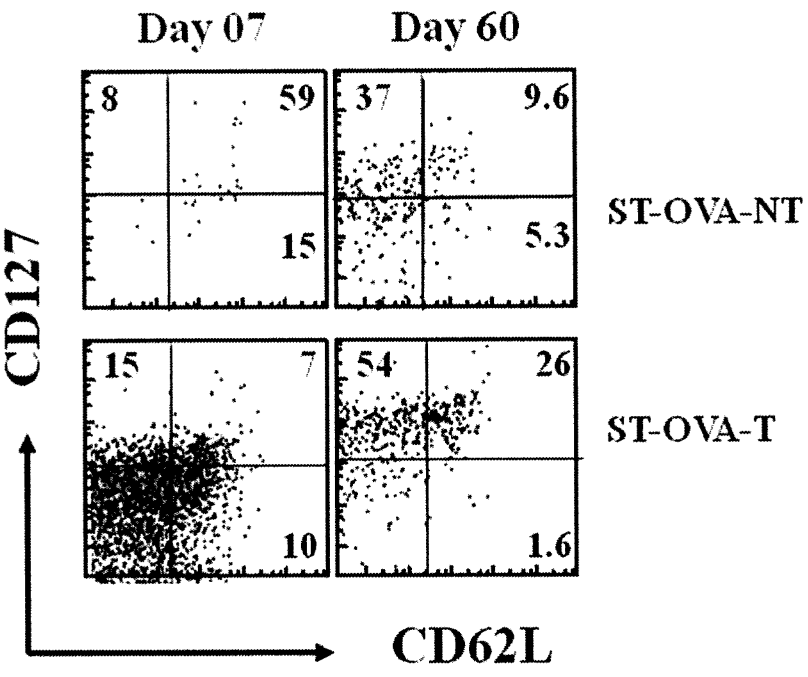
Day 07
Day 60
8
59
15
37
9.6
5.3
ST-OVA-NT
15
7
10
54
26
1.6
ST-OVA-T
CD127
CD62L

FIG. 5D

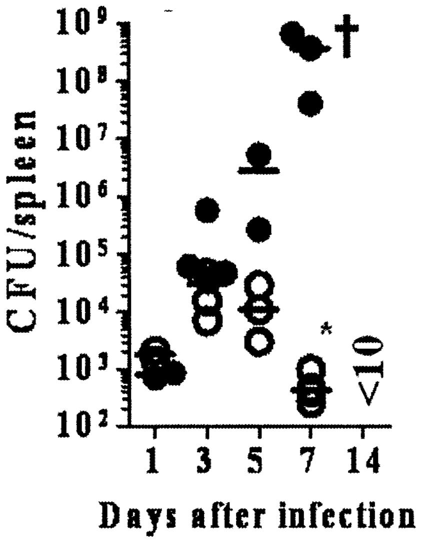
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
CFU/spleen
†
*
<10
1
3
5
7
14
Days after infection

FIG. 6A

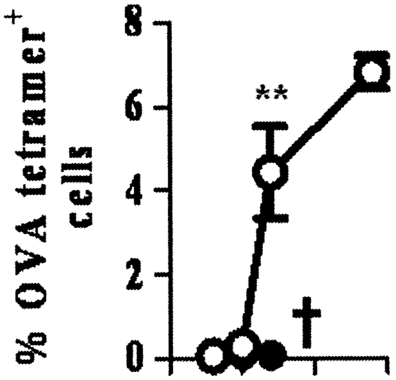
% OVA tetramer+ cells
8
6
4
2
0
**
†

FIG. 6B

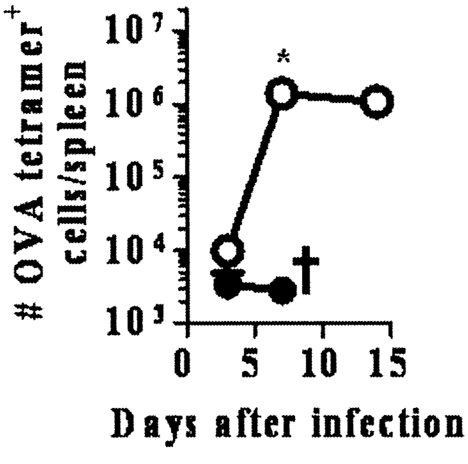
OVA tetramer+ cells/spleen
10^7
10^6
10^5
10^4
10^3
*
†
0
5
10
15
Days after infection

FIG. 6C

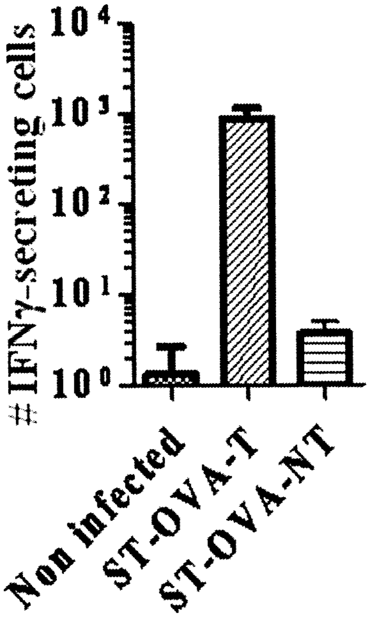

FIG. 6D

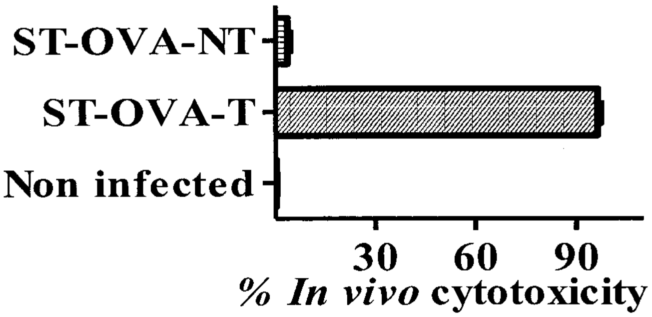

FIG. 6E

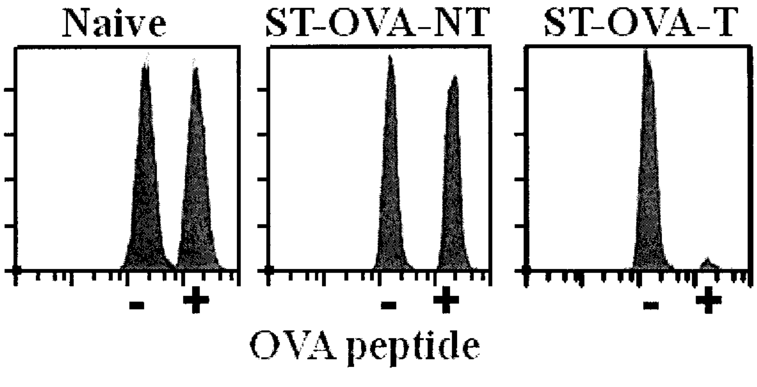

FIG. 6F

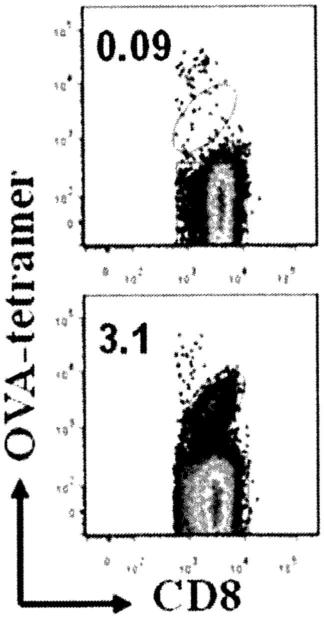
0.09
3.1
OVA-tetramer
CD8

FIG. 7A

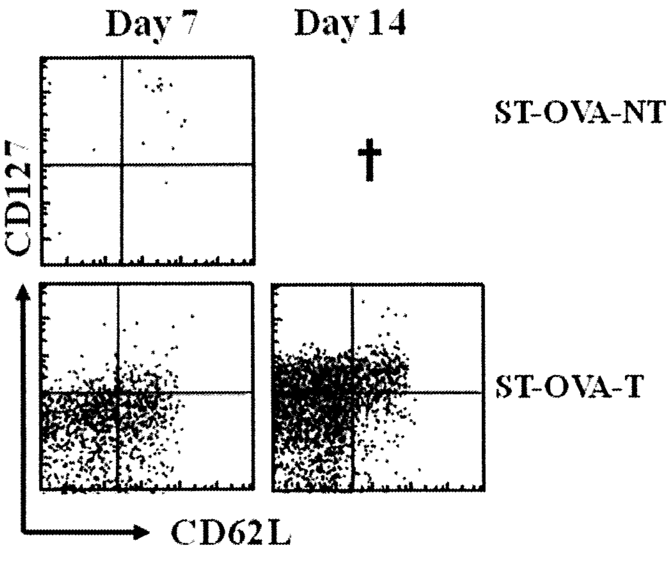
Day 7
Day 14
ST-OVA-NT
†
ST-OVA-T
CD127
CD62L

FIG. 7B

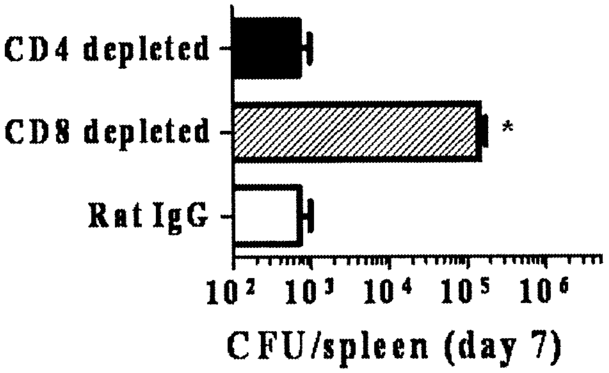

FIG. 8A

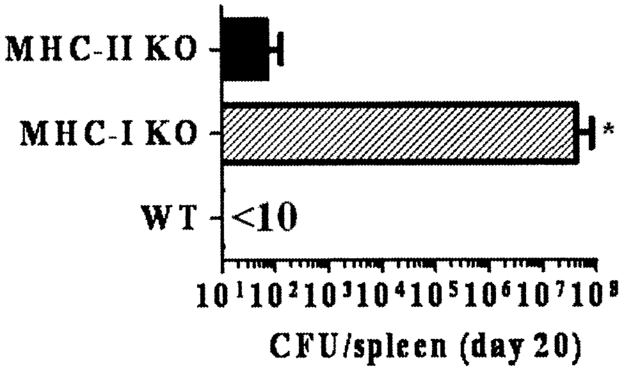

FIG. 8B

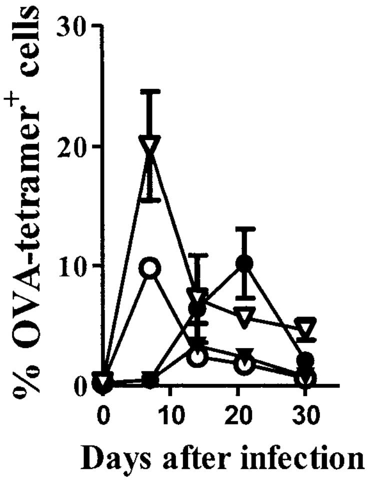

FIG. 9A

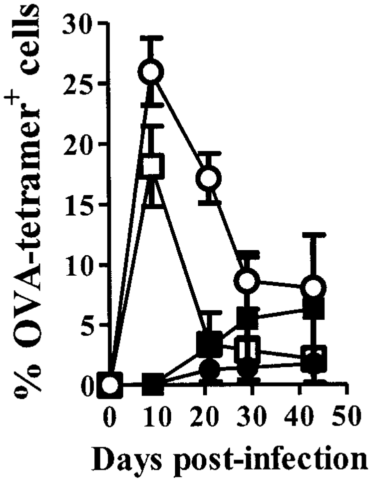

FIG. 9B

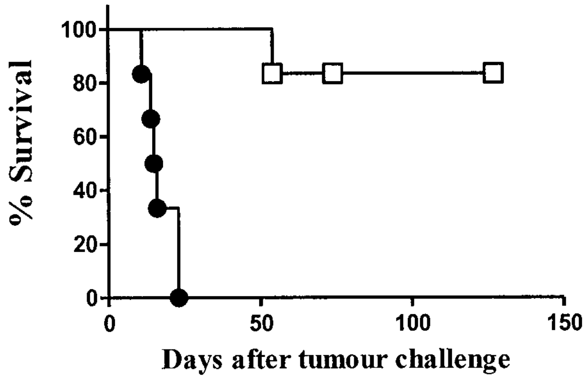

FIG. 10A

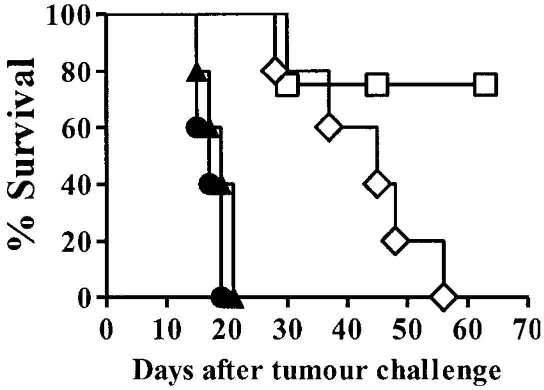

FIG. 10B

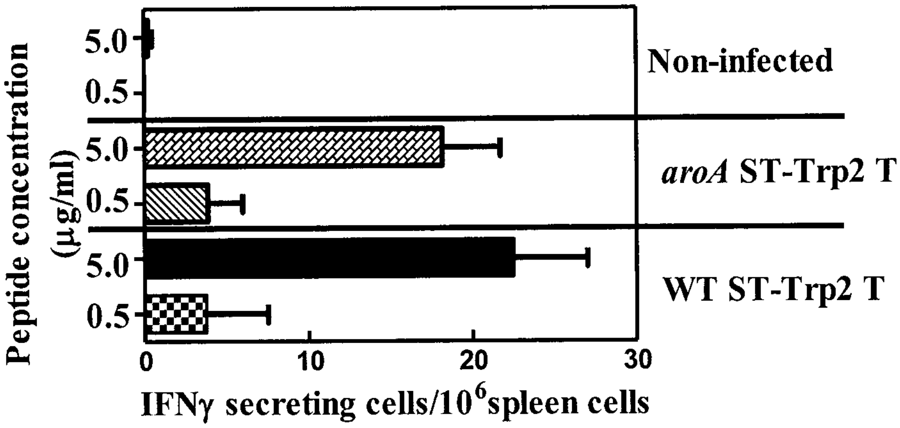

FIG. 11A

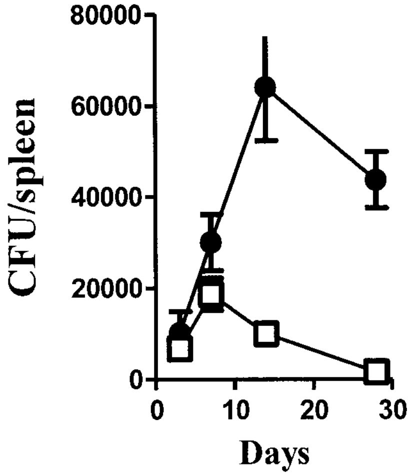

FIG. 11B

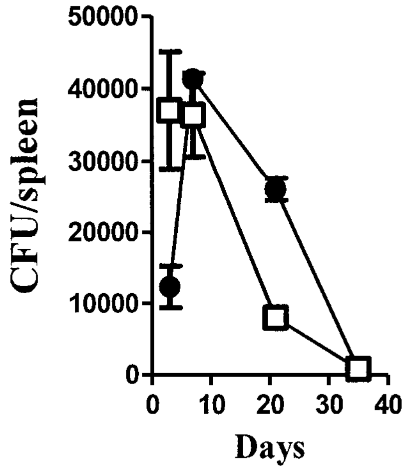

FIG. 11C

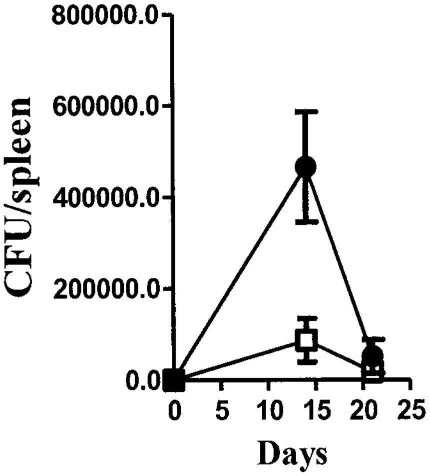

FIG. 12A

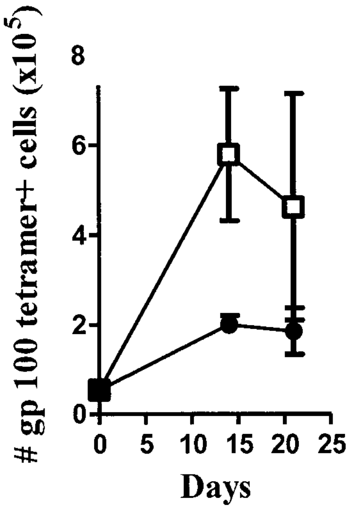

FIG. 12B

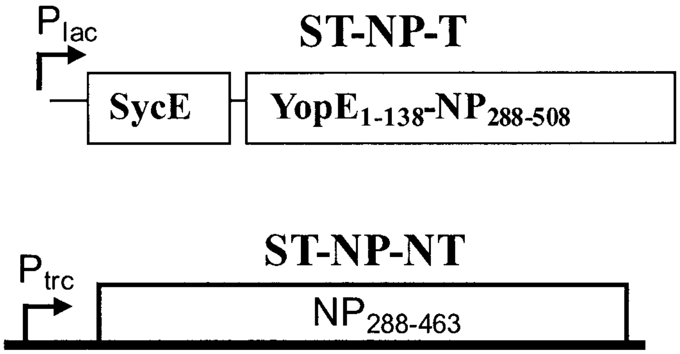

FIG. 13A

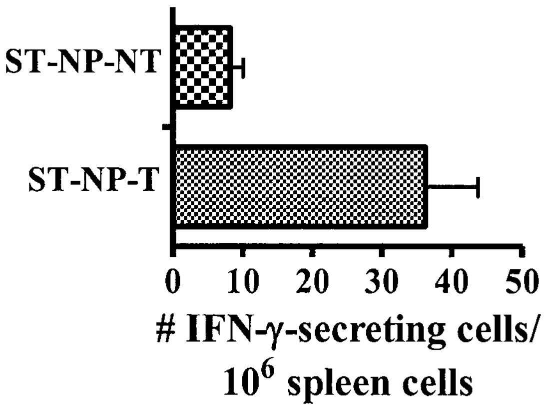

FIG. 13B

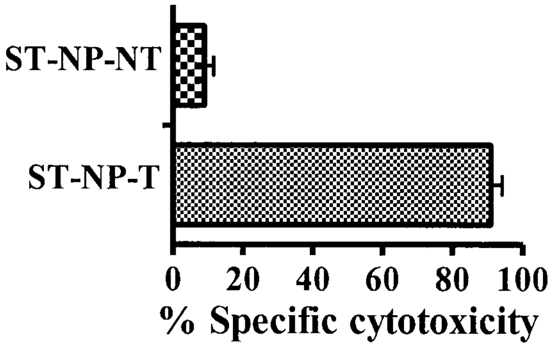

FIG. 13C

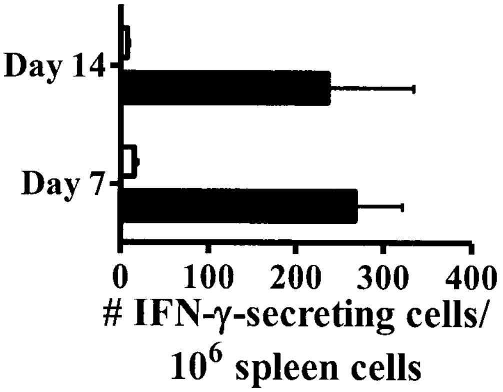

FIG. 13D

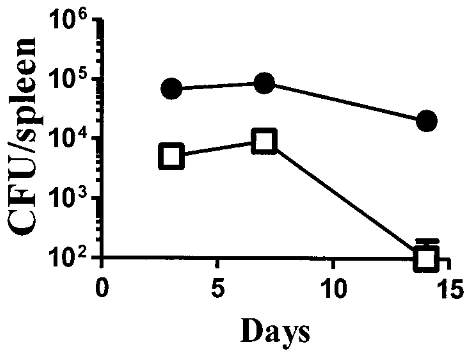

FIG. 13E

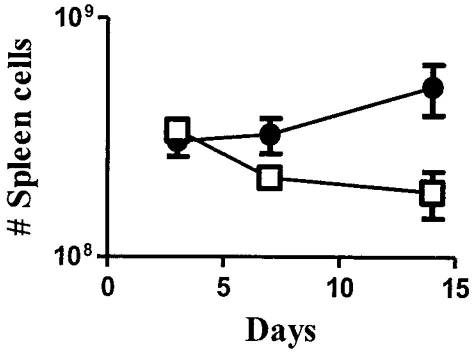

FIG. 13F

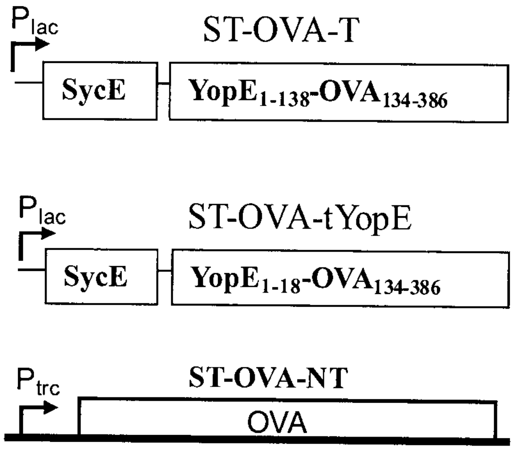

FIG. 14A

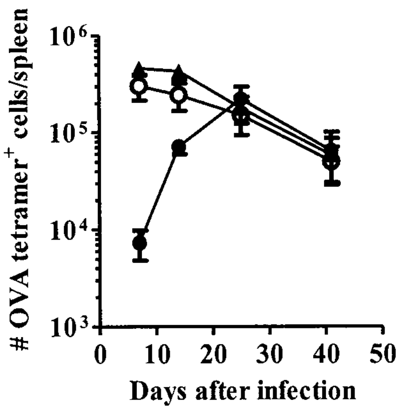

FIG. 14B

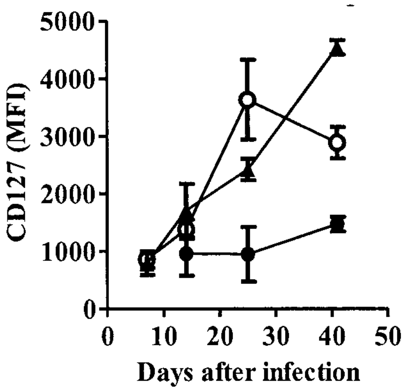

FIG. 14C

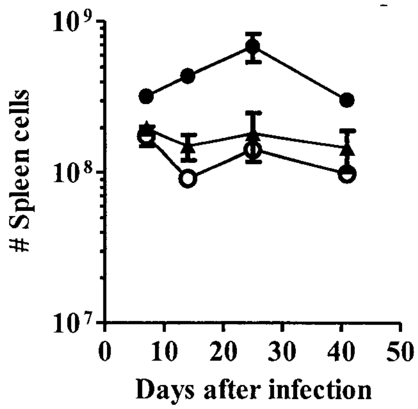

FIG. 14D

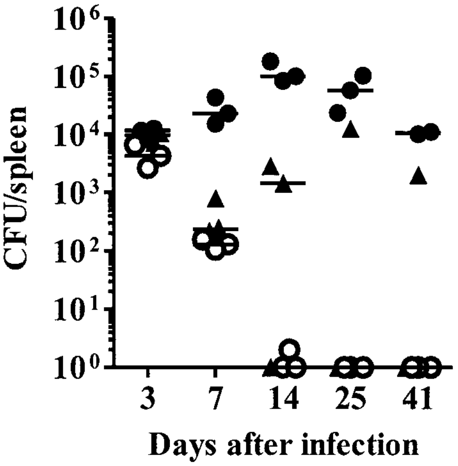
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU/spleen
3
7
14
25
41
Days after infection

FIG. 14E

RECOMBINANT BACTERIUM AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates to recombinant bacterium and uses thereof. More specifically, the invention relates to recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof.

BACKGROUND OF THE INVENTION

[0002] Various vaccine vectors or adjuvants that induce potent T cell responses are known in the art (Kaufmann and Hess, 1997). However, very few vaccine vectors exist that induce rapid and potent memory CD8+ cytolytic T cell responses, and that are safe and cost-effective (Raupach and Kaufmann, 2001). Unlike other T cells, CD8+ T cells uniquely provide immune-surveillance to the entire body because they recognize targets in the context of MHC class I molecules, which are present in every cell (Bevan, 1995). Furthermore, CD8+ T cells can eliminate infected cells or tumour cells rapidly. Thus, the induction of specific, potent CD8+ T cells is highly desirable for diseases that are caused by intracellular pathogens and tumours.

[0003] Intracellular pathogens induce CD8+ T cell responses; however, the responses are either highly attenuated or the organism itself is highly toxic. Generally, rapid proliferation of pathogens is countered by rapid presentation of antigen to $CD8^+$ T cells within the first few days of infection and activated $CD8^+$ T cells undergo profound expansion (>1000-fold) within the first week of infection, which results in resolution of infection (Kaech and Ahmed, 2001). Similarly, CD8+ T cells play a key role in mediating immune-surveillance against tumours (Smyth et al., 2000). While antibodies and helper T cells mainly promote clearance of extracelluar pathogens (Kaech et al., 2002), CD8+ T cells play a principal role in controlling intracellular pathogens and tumours. Thus, rapid induction of memory CD8+ T cells is essential for developing vaccines against tumours or intracellular pathogens.

[0004] While the CD8+ T cells play a key role against various diseases, their induction is highly tedious. Antigenic proteins injected into hosts in the absence or presence of adjuvants does not lead to the induction of CD8+ T cells (Moore et al., 1988). This is mainly because extracelluar proteins do not gain access to the cytoplasm (cytosol) of antigen-presenting cells (APC) (Rock, 1996). Rather, these extracellular proteins or vaccines are trafficked through specialized intracellular vesicles called phagosomes, which leads to the activation of helper T cells to aid antibody production. For induction of CD8+ T cell responses, the pathogen or the vaccine has to reside within the cytosol of an antigen-presenting cell (Bahjat et al., 2006).

[0005] Alternative routes of cross-presentation of non-cytosolic antigens to T cells have been suggested (Schaible et al., 2003; Houde et al., 2003; Yrlid and Wick, 2000), however the efficiency of these pathways in controlling pathogens isn't clear (Freigang et al., 2003). Dendritic cells may pick up antigen from dying APCs and present it to $CD8^+$ T cells (Albert et al., 1998). *Salmonella enterica serovar Typhimurium* (ST) nduces rapid death of macrophages and dendritic cells (Hersh et al., 1999; van der Velden et al., 2000) and it has been shown that cross-presentation of ST antigens occurs through dendritic cells (Yrlid and Wick, 2000). Phagosomes have themselves been considered to be competent at promoting cross-presentation (Houde et al., 2003). However, these mechanisms are of little protective value since rapid pathogen elimination is not observed. Cells that are cross-presenting ST antigens don't appear to serve as good targets for $CD8^+$ T cells to mediate their function. Thus, target cell accessibility seems to be the critical difference between direct and cross-presentation.

[0006] Subunit vaccines that consist of purified proteins admixed with adjuvants typically do not induce CD8+ T cell response due to residence of these entities within phagosomes of cells (Bahjat et al., 2006). However, some adjuvants induce CD8+ T cell responses most likely by the cross-presentation pathway (Krishnan et al., 2000). Subunit vaccines are difficult to mass-produce and are faced with numerous technical difficulties including batch to batch variability, quantitation of the antigen-adjuvant ratio, and extensively laborious procedures. To avoid this problem, live vaccines are preferred. However, live vaccines can be either over- or under-attenuated and it is difficult to find the right balance (Raupach and Kaufmann, 2001).

[0007] Typically, viral infections (such as Lyphochoriomeningitis virus, LCMV) lead to potent activation of CD8+ T cell responses due to their replication within the cytosol of infected cells (Kaech et al., 2002; Murali-Krishna et al., 1998). However, it is difficult to justify the use of viral vectors as a live vaccine due to the lack of availability of reagents to control the virus, particularly in immunocompromised hosts. Live bacteria can be considered as an alternative option for vaccine development since antibiotics can be used in case they are not controlled by the host. However, extracellular bacteria do not gain access to the cytosol of infected cells, hence fail to induce CD8+ T cell response (Bevan, 1995). On the other hand, intracellular bacteria induce CD8+ T cell response, albeit poor, despite residing within the phagosomes of infected cells, perhaps by cross-presentation (Kaufmann, 1993)—the caveat being that intracellular bacteria (e.g., *Salmonella, Mycobacteria, Leishmania*) that reside within the phagosomes of infected cells induce a chronic infection, implying that CD8+ T cells fail to eradicate them from the host (Kaufmann, 1993; Hess and Kaufmann, 1993).

[0008] There remains a need in the art for a safe, cost-effective method to induce rapid and potent memory CD8+ cytolytic T cell responses.

SUMMARY OF THE INVENTION

[0009] The present invention relates to recombinant bacterium and uses thereof. More specifically, the invention relates to recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof.

[0010] The present invention provides a recombinant bacterium, comprising a nucleic acid encoding an antigen that is translocated to the cytosol of a host cell. The bacterium may be *Salmonella, Mycobacteria, Brucella,* or *Leishmania.* In one example, the recombinant bacterium may be *Salmonella.*

[0011] The antigen expressed by the recombinant bacteria as just described may be a viral antigen, a bacterial antigen, or a tumour antigen. The antigen may be the nucleoprotein of LCMV, tyrosinase related protein 2 (TRP-2), MART-1, melanoma associated antigen 1 (MAGE1), gp100, or Her-2/neu or other viral or bacterial antigens.

[0012] The nucleic acid encoding the antigen may encode a fusion protein comprising the antigen and a translocation domain from a type III secretion system. For example, the translocation domain may be YopE, SopE, SptP, or a fragment thereof; in one specific example, the chaperone may be SycE or a fragment thereof (such as, but not limited to MKISSFISTSLPLPTSVS, SEQ ID NO:2). The fusion protein may optionally further comprise a chaperone. The chaperone may be derived from a type III secretion system. For example, the chaperone may be SycE or HSP70.

[0013] The nucleic acid may be comprised in a vector. The vector may be a pHR vector; in a specific example, the vector may be a modified pHR-241 vector. In the modified pHR-241 vector, the vector may be modified to remove the sequence of p60/M45, may be optionally further modified to remove the sequence of SycE.

[0014] Specific, non-limiting examples of fusion proteins encompassed by the present invention are those of SEQ ID NO:7 to SEQ ID NO:12.

[0015] The present invention also provides a method of imparting immunity against naturally-occurring bacterium in a subject, the method comprising administering the recombinant bacterium described above to said subject.

[0016] The present invention further provides a method of imparting immunity against tumours in a subject, the method comprising administering the recombinant bacterium described above to said subject. The recombinant bacterium may be administered by intravenous, oral, or subcutaneous routes of immunization.

[0017] The present invention also encompasses a use of the recombinant bacterium described herein as a vaccine.

[0018] Previously, it was known that pathogen-specific CD8+ T cells remain ineffective as long as the pathogen remained in the phagosome. For example, when conventional memory $CD8^+$ T cells against a given antigen were adoptively transferred to naïve hosts, they failed to respond rapidly in response to the same antigen expressed by ST infection (Luu et al., 2006). Presently, a recombinant ST that injects an antigen directly into the host cytosol has been developed. This results in profound $CD8^+$ T cell activation and consequent elimination of ST. It is also shown that when $CD8^+$ T cells are engaged in this manner, they undergo profound expansion which results in massive pathogen and tumour control as well as abridgment of pathogen chronicity. For example, as is evident in present FIG. **3**E, the numbers of OVA-specific $CD8^+$ T cells were similar at day 60 in ST-OVA-T versus ST-OVA-NT groups, but the burden was controlled only in the ST-OVA-T infected group, reiterating the notion that antigenic accessibility is the key to $CD8^+$ T cell functionality. This strategy works even with attenuated strains of *Salmonella.*

[0019] Notwithstanding the numerous genes that pathogens such as ST employ for virulence and chronicity (Jones and Falkow, 1996; Kaufmann et al., 2001), the present data provide novel insights into the incapacity of the immune system to efficiently control the bacterium, as well as reveal the power of the acquired immune system, wherein engagement of potent antigen-presentation early on can be sufficient to control an otherwise uncontrollable bacterium. The present results provide compelling evidence that modulation of the cell biology of antigen trafficking is a key avenue that is employed by various pathogens for immune evasion. Thus, a novel vaccine vector (*Salmonella*) is presently provided, wherein a key modification makes the bacterium generate rapid, potent CD8+ T cell response, resulting in self-destruction of the vaccine in vivo, making it highly efficacious, safe and cost-effective at the same time.

[0020] The use of OVA as an antigen is described herein as a proof of principle. Using a similar approach, other putative antigens from other pathogens (bacteria, virus) or tumours can be cloned into ST and these antigens can be translocated into the host cell cytosol for rapid and potent antigen-presentation using the YopE/SycE system. When a tumour-antigen is cloned into ST using the YopE/SycE system, potent and rapid anti-tumour CD8+ T cell response is generated which consequently results in rapid destruction of the bacterium.

[0021] Additional aspects and advantages of the present invention will be apparent in view of the following description. The detailed description and examples, while indicating preferred embodiments of the invention, are given by way of illustration only, as various changes and modifications within the scope of the invention will become apparent to those skilled in the art in light of the teachings of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] These and other features of the invention will now be described by way of example, with reference to the appended drawings, wherein:

[0023] FIG. **1**A shows a schematic of the fusion protein constructed, where an antigen (OVA) is fused to YopE, which is then incorporated into the plasmid pHR241 containing the SycE chaperone. FIG. **1**B shows a schematic of the antigen (OVA) translocation into the cytosol for ST-OVA-T, and the lack thereof for ST-OVA-NT. Ag: antigen; OVA: ovalbumin; ST: *Salmonella Typhimurium.* FIG. **1**C shows expression of OVA (by western blot) in the bacterial pellet, supernatant, and the cytosol of spleen cells of mice infected for 24 h with ST-OVA-NT and ST-OVA-T.

[0024] FIG. **2**A shows a graph representing the doubling times of the ST-OVA-NT (closed circles) and ST-OVA-T (open circles) bacteria in liquid culture, based on the measurement of OD at 600 nm. Based on these values, the bacteria were found to be similar. FIG. **2**B is a graph showing the ST burden in IC-21 macrophages ($H-2^b$) infected with ST-OVA-NT or ST-OVA-T (multiplicity of infection, MOI=10). No statistically significant difference was detected in the ability of ST-OVA-NT or ST-OVA-T to infect and replicate within macrophages ($p>0.05$). Results are representative of three independent experiments.

[0025] FIG. **3**A shows flow cytometry results of in vitro infection of IC-21 macrophages ($H-2^b$) with recombinant bacteria (ST-OVA-NT, ST-OVA-T, or ST). The reduction in CFSE intensity of OT-1 CD8+ T cells indicated that infection of macrophages with ST or ST-OVA-NT did not result in any detectable proliferation of OT-1 cells, and thus, a lack of antigen-presentation. Infection with ST-OVA-T resulted in strong dilution of CFSE expression, which is indicative of rapid and potent antigen-presentation. FIG. **3**B shows flow cytometry results of in vivo infection of B6.129F1 mice infected with ST-OVA-NT or ST-OVA-T (Day 5). In ST-OVA-T-infected mice, the majority of transferred OT-1 cells displayed reduced expression of CFSE while OT-1 cells in ST-OVA-NT-infected mice maintained high levels of CFSE expression. Results represent the mean of three mice±SD per group, and are representative of 2-3 independent experiments. FIG. **3**C is a graphical representation of the kinetic evaluation of in vivo antigen-presentation. ST-OVA-NT infected mice displayed muted and delayed activation of CFSE-labelled OT-1 cells. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

[0026] FIG. **4** shows the numbers of spleen cells (A), spleen size at Day 14 (B) and bacterial burden (C) in resistant (B6.129F1) mice infected with ST-OVA-T or ST-OVA-NT, as well as the percentage (D) and numbers (E) of OVA-specific CD8$^+$ T cells in the spleen. Results represent the mean of three to five mice ±SD per group and are representative of three independent experiments. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

[0027] FIG. **5**A shows the OVA-tetramer profile in the spleens of ST-OVA-T- or ST-OVA-NT-infected resistant (B6.129F1) mice at Day 7. The expression of CD62L (FIGS. **5**B, **5**D) and CD127 (FIGS. **5**C, **5**D) on OVA-tetramer+CD8+ T cells is also shown. Results are representative of three independent experiments. These results indicate early generation of memory CD8+ T cells in mice infected with ST-OVA-T. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

[0028] FIG. **6** shows the bacterial burdens (A) in spleen cells of susceptible (C57BL/6J) mice infected with ST-OVA-T or ST-OVA-NT, along with the percentage (B) and numbers (C) of OVA-specific CD8$^+$ T cells, as well as the frequency of OVA-specific CD8$^+$ T cells evaluated by ELISPOT assay (D). The specific killing of OVA-pulsed targets in naïve mice exposed to OVA-pulsed and control spleen cells is shown if FIGS. **6**E and F, indicating that ST-OVA-T infection results in rapid induction of antigen-specific CD8+ T cells that can efficiently kill antigen-bearing target cells. Results represent the mean of three to four mice±SD per group, and two independent experiments. ST-OVA-NT (closed circles); ST-OVA-T (open circles).

[0029] FIG. **7**A shows the OVA-tetramer profile in the spleens of susceptible (C57BL/6J) mice infected with ST-OVA-T or ST-OVA-NT at Day 7. FIG. **7**B shows the expression of CD62L versus CD127 on splenic OVA-tetramer$^+$ CD8$^+$ T cells in the ST-OVA-T versus ST-OVA-NT infected mice. CD8+ T cells generated with ST-OVA-T infection express high levels of CD127 and CD62L (memory markers). Results are representative of three independent experiments.

[0030] FIG. **8**A shows the bacterial burden in spleens of C57BL/6J mice treated with anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43) or Rat IgG isotype antibodies following infection with ST-OVA-T. Results represent the mean of three to four mice ±SD per group. Anti-CD4 and anti-CD8 antibody treatment resulted in near complete elimination of CD4 and CD8+ T cells respectively. FIG. **8**B shows the bacterial burden in spleens of WT, MHC-I- or MHC-II-deficient mice following infection with ST-OVA-T. These results indicate that the control of bacterial burden in ST-OVA-T infected mice is mediate exclusively by CD8+ T cells. Results represent the mean of five mice±SD per group.

[0031] FIG. **9** shows the relative numbers of OVA-specific CD8$^+$ T cells in the spleen (FIG. **9**A) and peripheral blood (FIG. **9**B) of B6.129F1 mice infected with wild type (WT) or attenuated (ΔaroA) ST-OVA expressing non-translocated (NT) or translocated (T) OVA. Results represent the mean of five mice±SD per group. Results indicate that even attenuated strain of ST can induce potent and rapid CD8 T cell response when antigen is translocated to the cytosol of infected cells. WT-OVA-NT (closed circles); WT-OVA T (open circles); AroA-OVA-NT (closed inverted triangles); AroA-OVA-T (open inverted triangles).

[0032] FIG. **10**A is a graphical representation of the results of prophylactic vaccination with ST-OVA-T in C57BL/6J mice followed by subcutaneous challenge with B16-OVA tumor cells. This protocol resulted in potent protection against tumor challenge. Non-infected (closed circles); ST-OVA-T (open squares). FIG. **10**B shows a graph of results of therapeutic vaccination with ST-OVA-T in C57BL/6J mice after subcutaneous challenge with B16-OVA tumor cells. Mice receiving ST-OVA-T displayed the best protection against B16 melanoma cells. Protection induced by ST-OVA-T was far greater than that induced by ST-OVA-NT and the another recombinant bacterium, *Listeria* expressing OVA (LM-OVA). Results represent the mean of five mice±SD per group. Non-infected (full circles); ST-OVA-T (open squares); ST-OVA-NT (closed triangles); LM-OVA (open diamonds).

[0033] FIG. **11**A shows the frequency of CD8+ T cells against a tumour antigen (Trp-2) in the spleens of mice infected with wild-type (WT) or attenuated (aroA) ST-Trp2-T on Day 7. FIG. **11**B shows the bacterial burden in the spleens of mice at various time intervals post-infection with WT ST-Trp2-T (open squares) or ST-Trp2-NT (closed circles). FIG. **11**C shows the bacterial burden in the spleens of mice infected with aroA mutant of ST-Trp2-T (open squares) or NT (closed circles).

[0034] FIG. **12**A shows the bacterial burden in the spleens of mice infected with translocated or non-translocated aroA-ST expressing another tumour antigen (gp100). aroA-gp100-T (open squares) or aroA-gp100-NT (closed circles). FIG. **12**B shows the numbers of gp100-tetramer+ CD8+ T cells in the spleens of infected mice at various time intervals. aroA-ST-gp100-T (open squares); aroA-ST-gp100-NT (closed circles).

[0035] FIG. **13**A shows the schematic of the fusion constructs. FIG. **13**B shows the frequency of NP-specific CD8+ T cells in mice infected with ST-NP-T or ST-NP-NT at day 7 post-infection. FIG. **13**C shows the in vivo cytolytic activity of NP-specific CD8+ T cells on NP-pulsed target cells at day 7 post-infection. Cytolytic activity was evaluated after transferring naïve spleen cells (pulsed with media or NP peptide) into infected mice at day 7 and evaluated the killing of peptide-pulsed targets at 24 h post-transfer. FIG. **13**D shows the frequency of NP-specific CD8+ T cells in mice infected with aroA-NP-T (black bars) or aroA-NP-NT (white bars). FIG. **13**E shows the bacterial burden in the spleens at various time intervals. aroA-NP-T (open squares) or aroA-NP-NT (closed circles) FIG. **13**F shows the influence of antigenic translocation on the induction of inflammation in the spleen. aroA-NP-T (open squares); aroA-NP-NT (closed circles).

[0036] FIG. **14** shows that truncated YopE is equally effective at inducing CD8+ T cell response. FIG. **14**A shows the schematic representation of the full length (upper panel) and the truncated YopE (lower panel). FIG. **14**B shows the OVA-specific CD8+ T cell response in the spleens of mice infected with full YopE or truncated YopE. FIG. **14**C shows that both the full length and truncated YopE induce the rapid generation of OVA-specific CD8+ T cells expressing memory marker (CD127). FIG. **14**D shows the inflammation induced (numbers of spleen cells) in mice infected with full length or truncated YopE. FIG. **14**E shows the bacterial burden in the spleens of mice infected with full length or truncated YopE. ST-OVA-NT (closed circles); ST-OVA-T (open circles); ST-OVA-tYopE (closed triangles).

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present invention relates to recombinant bacterium and uses thereof. More specifically, the invention relates to recombinant bacterium expressing an antigen that is translocated to the cytosol of a host organism, and uses thereof.

[0038] The present invention provides a recombinant bacterium, comprising a nucleic acid encoding an antigen that is translocated to the cytosol of the host organism.

[0039] The bacterium may be any virulent or attenuated bacterium that resides in the phagosome of macrophages and/or dendritic cells and induces poor T cell activation. Such a bacterium may be, but is not limited to *Salmonella, Mycobacteria, Brucella, Leishmania,* and the like, which are all intracellular pathogens that reside in the phagosome and fail to induce rapid T cell activation, hence causing diseases that are not controlled by the immune system.

[0040] In one example, the virulent or attenuated bacterium may be *Salmonella.* Any suitable strain of *Salmonella* known in the art may be used; for example, and without wishing to be limiting in any manner, the virulent or attenuated bacterium may be *Salmonella enterica, serovar Typhimurium* (ST). ST is a highly virulent pathogen that induces gastroenteritis in humans, and typhoid-like disease in mice (Jones and Falkow, 1996). In susceptible C57BL/6J mice, which lack natural resistance-associated macrophage proteins (NRAMP), ST (strain SL1344) induces a systemic lethal infection even at doses as low as (10^2) (iv), and all mice die within 7 days of infection (Albaghdadi et al., 2009). In contrast, ST induces a chronic but non-lethal infection in resistant 129SvJ mice (which express NRAMP). F1 hybrids between susceptible and resistant mice (B6.129F1) also harbour a chronic, non fatal, infection (Luu et al., 2006). Genes that are involved in *Salmonella* invasion of epithelial cells are clustered at the *Salmonella* pathogenicity island-1 loci (SPI-1) (Bliska et al., 1993; Zhou and Galan, 2001; Galan and Curtiss, III, 1989; Hardt et al., 1998). They encode several factors, including a type III secretion system (TTSS) apparatus that exports specific proteins (effectors) into the host cell. Two major virulence loci allow *Salmonella* to survive inside cells (Jones and Falkow, 1996). The two-component regulatory system phoP-lphoQ, which controls >40 genes (Groisman et al., 1989; Miller et al., 1989), is involved in intracellular survival (Garvis et al., 2001). Another pathogenicity island (SPI-2) encodes a second TTSS, mediates resistance to intracellular killing, and is key to virulence (Hensel et al., 1995; Shea et al., 1996).

[0041] The CD8+ T cell response against ST is delayed, which fails to control the bacterium leading to a chronic infection (Albaghdadi et al., 2009). aroA mutant of ST was developed as a vaccine against *Salmonella* (Hoiseth and Stocker, 1981), which induces minimal inflammation and poor immunogenicity (Albaghdadi et al., 2009; Dudani et al., 2008). The virulent or attenuated bacterium of the present invention may be the aroA mutant of ST, comprising a vaccine vector modified such that the bacterium resides in the phagosome of infected cells, but translocates antigen to the cytosol. This modification allows rapid induction of CD8+ T cells; without wishing to be bound by theory, this may lead to the self-destruction of the vaccine. Phagosomal localization is considered a major impediment to T cell activation, and the antigenic translocation strategy described herein can be used for other intracellular bacterial vaccine vectors, including *Mycobacteria, Brucella* or *Leishmania.*

[0042] By the term "recombinant" it is meant that the bacterium has been genetically altered or engineered; such genetic engineering may be the inclusion of a recombinant (or artificial) nucleic acid or vector (comprising a nucleic acid) encoding a foreign protein that is an antigen.

[0043] The antigen may be any suitable protein or fragment thereof that is processed and presented efficiently by dendritic cells and/or macrophages resulting in efficient T cell activation. Without wishing to be limiting in any manner, the antigen or fragment thereof may be a nascent protein, a bacterial antigen, viral antigen, or a tumour antigen. For example, the antigen may be, but is not limited to tyrosinase related protein 2 (TRP-2), MART-1, melanoma associated antigen 1 (MAGE1), gp100, Her-2/neu or other proteins or fragments thereof known in the art. Other proteins may include, but are not limited to ovalbumin, hen egg lysozyme, and myelin basic protein, nuclear protein of LCMV. In a specific, non-limiting example, the antigens may be ovalbumin, TRP-2, gp-100, LCMV-NP, or fragments thereof.

[0044] Upon infection, the antigen is translocated into the cytosol of the host cell (for example macrophages and/or dendritic cells). The antigen may naturally translocate to the cytosol, or may be a recombinant protein engineered to do so. Thus, the antigen may be comprised in a fusion protein that further comprises a translocation domain from a type III secretion system; optionally, the fusion protein may further comprise a chaperone. As would be known to those of skill in the art, the fusion protein, also referred to herein as "fused proteins", comprising the antigen may be generated via recombinant methods well-known to those of skill in the art. The antigen and translocation domain, and the optional chaperone, may be joined directly or by a linker; appropriate linkers would be well-known to those of skill in the art.

[0045] By the term "translocation domain", it is meant a protein domain or fragment thereof that directs translocation of a protein from the phagosome to the cytosol of the host cell. The translocation domain may be any suitable translocation domain from known type III secretion systems of bacteria, which are well-known to those of skill in the art. For example, and without wishing to be limiting in any manner, the translocation domain may be YopE or a fragment thereof. YopE is a 23 kDa protein comprising a N-terminal secretion domain of approximately 11 amino acids and a translocation domain of at least 50 aa. In one specific, non-limiting example, the YopE translocation domain may comprise the sequence:

(SEQ ID NO: 1)
MKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTESPQGSSLASRI

TEKLSSMARSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQLAAETLPKYIQ

QLSSLDAETLQKNHDQFAT,

a fragment thereof (such as, but not limited to MKISS-FISTSLPLPTSVS, SEQ ID NO:2), or a sequence substantially identical thereto. Another suitable translocation domain may be the SptP protein of ST (Russmann et al., 1998); again, the SptP translocation domain could be the full length protein or a truncated version thereof. In one specific example, the SptP translocation domain may comprise the sequence:

(SEQ ID NO: 3)

MLKYEERKLNNLTLSSFSKVGVSNDARLYIAKENTDKAYVAPEKFSSKVLTWLGKMPLF

KNTEVVQKHTENIRVQDQKILQTFLHALTEKYGETAVNDALLMSRINMNKPLTQRLAVQI

TECVKAADEGFINLIKSKDNVGVRNAALVIKGGDTKVAEKNNDVGAESKQPLLDIALKGL

KRTLPQLEQMDGNSLRENFQEMASGNGPLRSLMTNLQNLNKIPEAKQLNDYVTTLTNI

QVGVARFSQWGTCGGEVERWVDKASTHELTQAVKKIHVIAKELKNVTAELEKIEAGAP

MPQTMSGPTLGLARFAVSSIPINQQTQVKLSDGMPVPVNTLTFDGKPVALAGSYPKNTP

DALEAHMKMLLEKECSCLVVLTSEDQMQAKQLPPYFRGSYTFGEVHTNSQKVSSASQ

GEAIDQYNMQLSCGEKRYTIPVLHVKNWPDHQPLPSTDQLEYLADRVKNSNQNGAPG

RSSSDKHLPMIHCLGGVGRTGTMAAALVLKDNPHSNLEQVRADFRDSRNNRMLEDAS

QFVQLKAMQAQLLMTTAS,

a fragment thereof, or a sequence substantially identical thereto. Yet another example of a suitable translocation domain is SopE, a type III secretion protein in *Salmonella* ST (Zhu et al., 2010). In a specific example, the SopE translocation domain may comprise the

sequence:

(SEQ ID NO: 4)

MTKITLSPQNFRIQKQETTLLKEKSTEKNSLAKSILAVKNHFIELRSKLSERFIS

HKNTESSATHFHRGSASEGRAVLTNKVVKDFMLQTLNDIDIRGSASKDPAYASQTREAI

LSAVYSKNKDQCCNLLISKGINIAPFLQEIGEAAKNAGLPGTTKNDVFTPSGAGANPFITP

LISSANSKYPRMFINQHQQASFKIYAEKIIMTEVAPLFNECAMPTPQQFQLILENIANKYIQ

NTP,

a fragment thereof, or a sequence substantially identical thereto.

[0046] The fusion protein may optionally comprise a chaperone. By the term "chaperone', it is meant a protein that assists in translocation of the immunodominant antigen. The chaperone protein may be any suitable protein known in the art, and must be compatible with translocation domain chosen. The chaperone may also be from a type III secretion system. For example, and without wishing to be limiting, the chaperone may be SycE. SycE is a YopE-specific chaperone that is required for YopE-mediated translocation of fused proteins to the cytosol (Russmann et al., 2001). SycE assists in translocation of the fused protein into the cytosol of infected cells through the type III secretion system of ST. In a specific, non-limiting example, the SycE chaperone may comprise the sequence:

(SEQ ID NO: 5)

MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE

TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS

SLISPPRSFS,

or a sequence substantially identical thereto. In another example, the SopE translocation domain has been used in combination with the chaperone protein heat shock protein 70 (Hsp70) to deliver an antigen to the cytosol (Zhu et al., 2010). In a specific, non-limiting example, the chaperone may comprise the sequence:

(SEQ ID NO: 6)
MGKIIGIDLGTTNSCVAIMDGTQARVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVT

NPQNTLFAIKRLIGRRFQDEEVQRDVSIMPYKIIGADNGDAWLDVKGQKMAPPQISAEVL

KKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYG

LDKEVGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDTRLINYLV

DEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKVT

RAKLESLVEDLVNRSIEPLKVALQDAGLSVSDINDVILVGGQTRMPMVQKKVAEFFGKE

PRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGIETMGGVMTPLITKNTTIPT

KHSQVFSTAEDNQSAVTIHVLQGERKRASDNKSLGQFNLDGINPAPRGMPQIEVTFDID

ADGILHVSAKDKNSGKEQKITIKASSGLNEEEIQKMVRDAEANAESDRKFEELVQTRNQ

GDHLLHSTRKQVEEAGDKLPADDKTAIESALNALETALKGEDKAAIEAKMQELAQVSQK

LMEIAQQQHAQQQAGSADASANNAKDDDVVDAEFEEVKDKK,

or a sequence substantially identical thereto. The inclusion of the chaperone is optional, as the translocation domain, or a fragment thereof, alone may be sufficient to cause translocation of the antigen to the cytosol; for example, and without wishing to be limiting, YopE alone, or an 18-amino acid fragment thereof (MKISSFISTSLPLPTSVS, SEQ ID NO:2) are presently shown to produce the desired effect. Similarly, expression of the endogenous *Salmonella* chaperone protein InvB is sufficient to mediate the translocation function of SopE (Lee and Galan, 2003).

[0047] In one specific example of the present invention, the recombinant bacterium comprises a nucleic acid encoding an antigen comprising a fusion protein comprising the sequence of SycE, YopE, and ovalbumin:

(SEQ ID NO: 7)
MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE

TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS

SLISPPRSFSMKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTES

PQGSSLASRITEKLSSMARSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQL

AAETLPKYIQQLSSLDAETLQKNHDQFATGSNFQTAADQARELINSRVESQTNGIIRNVL

QPSSVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRV

ASMASEKMKILELPFASGTMSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIK

VYLPRMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGR

EVVGSAEAGVDAASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP, a fusion protein comprising the sequence of SycE, a fragment of YopE, and ovalbumin:

(SEQ ID NO: 8)
MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE

TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS

SLISPPRSFSMKISSFISTSLPLPTSVSGSNFQTAADQARELINSRVESQTNGIIRNVLQPS

SVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASM

ASEKMKILELPFASGTMSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLP

RMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVV

GSAEAGVDAASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP,

a fusion protein comprising the sequence of a fragment of YopE and ovalbumin:

(SEQ ID NO: 9)
MKISSFISTSLPLPTSVSGSNFQTAADQARELINSRVESQTNGIIRNVLQPSSVDSQTAM

VLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKIL

ELPFASGTMSMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEK

YNLTSVLMAMGITDVFSSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGVD

AASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP,

a fusion protein comprising the sequence of SycE, YopE, and TRP-2:

(SEQ ID NO: 10)
MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE

TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS

SLISPPRSFSMKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTES

PQGSSLASRITEKLSSMARSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQL

AAETLPKYIQQLSSLDAETLQKNHDQFATMKISSFISTSLPLPTSVSGSSSVGEMSGRSV

SQQKSEQYANNLAGRTESPQGSSLASRITEKLSSMARSAIEFIKRMFSEGSHKPVVTPA

PTPAQMPSPTSFSDSIKQLAAETLPKYIQQLSSLDAETLQKNHDQFATGSGILLRARAQF

PRVCMTLDGVLNKECCPPLGPEATNICGFLEGRGQCAEVQTDTRPWSGPYILRNQDD

REQWPRKFFNRTCKCTGNFAGYNCGGCKFGWTGPDCNRKKPAILRRNIHSLTAQERE

QFLGALDLAKKSIHPDYVITTQHWLGLLGPNGTQPQIANFSVYDFFVWLHYYSVRDTLL

GPGRPYKAIDFSHQGPAFVTWH,

a fusion protein comprising the sequence of SycE, YopE, and gp100:

(SEQ ID NO: 11)
MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE

TLLSNIFSQDILKPILSWDEVGGHPVLWNRQPLNSLDNNSLYTQLEMLVQGAERLQTSS

LISPPRSFSMKISSFISTSLPLPASVSGSSSVGEMSGRSVSQQKSDQYANNLAGRTESP

QGSSLASRIIERLSSMAHSVIGFIQRMFSEGSHKPVVTPALTPAQMPSPTSFSDSIKQLA

AETLPKYMQQLSSLDAETLQKNHDQFATGSGKNTMDLVLKRCLLHLAVIGALLAVGATK

-continued

VPRNQDWLGVSRQLRTKAWNRQLYPEWTEAQRLDCWRGGQVSLKVSNDGPTLIGAN

ASFSIALNFPGSQKVLPDGQVIWVNNTIINGSQVWGGQPVYPQETDDACIFPDGGPCPS

GSWSQKRSFVYVWKTWGQYWQVLGGPVSGLSIGTGRAMLGTHTMEVTVYHRRGSR

SYVPLAHSSSAFTITDQVPFSVSVSQLRALDGGNKHFLRNQPLTFALQLHDPSGYLAEA

DLSYTWDFGDSSGTLISRALVVTHTYLEPGPVTAQVVLQAAIPLT,

a fusion protein comprising the nuclear protein of SycE, YopE, and LCMV-NP:

(SEQ ID NO: 12)
MYSFEQAITQLFQQLSLSIPDTIEPVIGVKVGEFACHITEHPVGQILMFTLPSLDNNNEKE

TLLSHNIFSQDILKPILSWDEVGGHPVLWNRQPLNNLDNNSLYTQLEMLVQGAERLQTS

SLISPPRSFSMKISSFISTSLPLPTSVSGSSSVGEMSGRSVSQQKSEQYANNLAGRTES

PQGSSLASRITEKLSSMAHSAIEFIKRMFSEGSHKPVVTPAPTPAQMPSPTSFSDSIKQL

AAETLPKYMQQLSSLDAETLQKNHDQFATGSFVSDQVGDRNPYENILYKVCLSGEGWP

YIACRTSIVGRAWENTTIDLTSEKPAVNSPRPAPGAAGPPQVGLSYSQTMLLKDLMGGI

DPNAPTWIDIEGRFNDPVEIAIFQPQNGQFIHFYREPVDQKQFKQDSKYSHGMDLADLF

NAQPGLTSSVIGALPQGMVLSCQGSDDIRKLLDSQNRKDIKLIDVEMTREASREYEDKV

WDKYGWLCKMHTGIVRD,

or a sequence substantially identical thereto. The fusion protein further comprises the sequence of the antigen of interest.

[0048] A substantially identical sequence may comprise one or more conservative amino acid mutations. It is known in the art that one or more conservative amino acid mutations to a reference sequence may yield a mutant peptide with no substantial change in physiological, chemical, or functional properties compared to the reference sequence; in such a case, the reference and mutant sequences would be considered "substantially identical" polypeptides. Conservative amino acid mutation may include addition, deletion, or substitution of an amino acid; a conservative amino acid substitution is defined herein as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge, or polarity).

[0049] In a non-limiting example, a conservative mutation may be an amino acid substitution. Such a conservative amino acid substitution may substitute a basic, neutral, hydrophobic, or acidic amino acid for another of the same group. By the term "basic amino acid" it is meant hydrophilic amino acids having a side chain pK value of greater than 7, which are typically positively charged at physiological pH. Basic amino acids include histidine (His or H), arginine (Arg or R), and lysine (Lys or K). By the term "neutral amino acid" (also "polar amino acid"), it is meant hydrophilic amino acids having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Polar amino acids include serine (Ser or S), threonine (Thr or T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), and glutamine (Gin or Q). The term "hydrophobic amino acid" (also "non-polar amino acid") is meant to include amino acids exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg (1984). Hydrophobic amino acids include proline (Pro or P), isoleucine (Ile or I), phenylalanine (Phe or F), valine (Val or V), leucine (Leu or L), tryptophan (Trp or W), methionine (Met or M), alanine (Ala or A), and glycine (Gly or G). "Acidic amino acid" refers to hydrophilic amino acids having a side chain pK value of less than 7, which are typically negatively charged at physiological pH. Acidic amino acids include glutamate (Glu or E), and aspartate (Asp or D).

[0050] Sequence identity is used to evaluate the similarity of two sequences; it is determined by calculating the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residue positions. Any known method may be used to calculate sequence identity; for example, computer software is available to calculate sequence identity. Without wishing to be limiting, sequence identity can be calculated by software such as NCBI BLAST2 service maintained by the Swiss Institute of Bioinformatics (and as found at http://ca.expasy.org/tools/blast/), BLAST-P, Blast-N, or FASTA-N, or any other appropriate software that is known in the art.

[0051] The substantially identical sequences of the present invention may be at least 70%, 80%, 90%, or 95% identical; in another example, the substantially identical sequences may be at least 70, 71, 72, 73, 74, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identical at the amino acid level to sequences described herein. Importantly, the substantially identical sequences retain the activity and specificity of the reference sequence.

[0052] The present invention also encompasses nucleic acids encoding the antigen as described above, as well as vectors comprising the nucleic acid.

[0053] Thus, the recombinant bacterium of the present invention may comprise a nucleic acid encoding the antigen as described above, or may comprise a vector (also referred to herein as"plasmid") comprising such nucleic acid that is fused with the nucleic acid of a translocation domain and optionally the nucleic acid of a chaperone; for example, and without wishing to be limiting in any manner, the vector in which antigen is translocated to the cytosol of infected cells may be a modified pHR plasmid. The modified pHR construct uses the type III secretion protein to generate fusion proteins that are transported out of the phagosome and through the host bacterial type III secretion system for direct cytosolic antigen presentation. The pHR constructs may comprise sequences encoding a translocation domain and optionally a chaperone protein to aid in proper trafficking of the downstream fusion protein. In a specific, non-limiting example, the vector may be a modified pHR-241 plasmid (Russmann et al., 2001); more specifically, the pHR-241 comprising the sequence of SycE-YopE-p60/M45 fusion protein (Russmann et al., 2000) modified to remove the sequence of p60/M45. In another example, the pHR241 vector is modified to comprise YopE or a fragment of YopE (for example, but not limited to MKISSFISTSLPLPTSVS, SEQ ID NO:2) with the sequence for the SycE and p60/M45 proteins removed. Replacement of the antigenic sequence by appropriate restriction enzymes and subsequent ligation of other antigens would result in development of the desired CD8+ T cell response against said antigens upon vaccination. Furthermore, the recombinant bacterium that harbors this plasmid need not be a highly virulent bacterium; for example, attenuated *Salmonella* is presently shown to be effective at inducing the desired response. The response can be accentuated further by vaccination with higher doses of the attenuated strain.

[0054] The recombinant bacterium as described above may be utilized to impart immunity against other naturally-occurring and virulent bacteria. This may be accomplished by administering an effective amount of the recombinant bacterium of the present invention to a subject, and allowing a CD8$^+$ T cell response to be mounted. Similarly, the recombinant bacterium may be utilized to impart immunity against tumors in a subject, by administering an effective amount of the recombinant bacterium of the present invention to said subject. In both methods, the recombinant bacterium may be administered through intravenous, oral or subcutaneous routes of immunization. This approach avoids the unwanted side-effects of persisting bacteria and undesirable toxicity/inflammation associated with live vaccines. Thus, higher doses of the vaccine can be used for improved efficacy. Because the recombinant bacterium of the present invention is eliminated after a few weeks, there is little concern regarding toxicity. Furthermore, *Salmonella* when given orally induces a mucosal CD8+ T cell response (Jones and Falkow, 1996). Thus, the modified bacterium can be administered through the oral route for induction of the desired CD8+ T cell response.

[0055] The recombinant bacterium as described above may also be utilized as a vaccine; the vaccine may protect against other naturally-occurring and virulent bacteria, other bacterial pathogens, viral pathogens, or tumors. When the antigen is a tumour-antigen, the tumour-antigen will be translocated to the host cell cytosol, resulting in rapid activation of tumor-specific CD8$^+$ T cells, which will translate to better tumour control by tumor-specific CD8+ T cells.

[0056] A recombinant ST that injects an antigen directly into the host cytosol has presently been developed. This results in profound CD8$^+$ T cell activation and consequent elimination of ST. It is also shown that when CD8$^+$ T cells are engaged in this manner, they undergo profound expansion which results in massive pathogen and tumour control as well as abridgment of pathogen chronicity. The present data provide novel insights into the incapacity of the immune system to efficiently control the bacterium, as well as reveal the power of the acquired immune system, wherein engagement of potent antigen-presentation early on may be sufficient to control an otherwise uncontrollable bacterium. The present results provide compelling evidence that modulation of the cell biology of antigen trafficking is a key avenue that is employed by various pathogens for immune evasion. The recombinant bacterium described herein may be used as a novel vaccine, wherein a key modification makes the bacterium generate rapid, potent CD8+ T cell response, resulting in self-destruction of the vaccine in vivo, making it highly efficacious, safe and cost-effective.

[0057] The utility of the recombinant bacterium described herein is demonstrated using OVA, TRP-2, and gp-100 as antigens. Using a similar approach, other putative antigens from other pathogens (bacteria, virus) or tumours can be cloned into the recombinant bacterium; these antigens can then be translocated into the host cell cytosol for rapid and potent antigen-presentation using the a translocation domain/chaperone system.

[0058] The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only and should not be used to limit the scope of the present invention in any manner.

EXAMPLE 1

Preparation of Recombinant Bacteria

[0059] Recombinant bacteria comprising *Salmonella enterica, serovar Typhimurium* (ST) expressing ovalbumin (OVA) were prepared. Construct ST-OVA-NT, which does not translocate antigen to the cytosol, was prepared as previously described (Luu et al., 2006). A recombinant construct, ST-OVA-T, that produces an OVA fusion protein that is translocated to the cytosol; FIG. **1**A shows a schematic of the fusion protein, where OVA is fused to YopE and SycE. YopE is a 23 kDa protein comprising a N-terminal secretion domain (~11 aa) and a translocation domain (at least 50 aa); the latter domain provides the binding site for the YopE-specific chaperone (SycE) that is required for YopE-mediated translocation of fused proteins to the cytosol (R). SycE is a chaperone necessary for translocation of the fused protein into the cytosol of infected cells through the type III secretion system of ST. A schematic of both ST-OVA-NT and ST-OVA-T constructs and their proposed actions are shown in FIG. **1**B.

[0060] Plasmid pHR-OVA was constructed by the modification of the plasmid pHR-241 (Russmann et al., 2001), which contains the sequence of the fusion protein SycE-YopE-p60/M45 (Russmann et al., 2000). In a first step, the genes of p60/M45 were removed by cutting plasmid pHR-241 with BamHI and KpnI. Then, the pKK-OVA plasmid was purified from the recombinant ST-OVA-NT bacteria by mid prep kit (Invitrogen, US) according to the manufacturer's instructions. The OVA gene was PCR-amplified using the plasmid pKK-OVA as a template (forward primer BamHI 5'-CGGGATCCAACTTTCAAACAGCTG-3' (SEQ ID NO:13) and reverse primer KpnI 5'-GGGGTACCTTAAGGGGAAACACATC-3' (SEQ ID NO:14). Subsequently, the OVA gene was inserted between the BamHI-KpnI sites of the cut pHR-241 palsmid, creating new plasmid pHR-OVA. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94° C., 5 min; 25 cycles of 94° C., 30 s to 58° C., 1 min to 72° C., 1 min; followed by a 7 min extension time at 72° C. The amplified insert was ligated into the intended vector then sequenced to verify the accuracy of the amplified cDNA. The pHR-OVA plasmid was then transfected into the highly virulent ST (strain SL1344). 50 μL of electrocompetent *Salmonella* (WT or aroA) were mixed with -20 ng plasmid DNA and pulsed in a Bio-Rad micropulser using one pulse of 2.5 kV. Immediately afterwards, 1 mL of SOC recovery medium was added to the bacteria and they were allowed to recover with shaking at 37° C. The bacteria were then plated on LB agar plates with ampicillin for the selection of individual clones.

EXAMPLE 2

Detection of Antigen

[0061] ST-OVA-NT and ST-OVA-T constructs of Example 1 were grown and expression and translocation of ovalbumin was evaluated. Pellet and supernatant of ST-OVA-NT and ST-OVA-T growing in liquid cultures were tested for the presence of OVA.

[0062] C57BL/6J mice were injected intravenously with 10^6 ST-OVA-NT or ST-OVA-T reconstituted in 200 microlitres normal saline. Two days later, spleens were obtained from infected mice; spleen cells were isolated and lysed with Triton X-100 in the presence of protease inhibitor, phenylmethylsulfonyl fluoride. The soluble lysate containing cytosolic proteins was tested for OVA expression by western blotting. Samples were normalized for cell number and were loaded on SDS-10% polyacrylamide gels. SDS-PAGE was performed and proteins were transferred to membranes, which were then blocked with 5% skim milk powder in PBS-Tween. OVA expression was detected using a 1/10,000 dilution of polyclonal anti-OVA antibody (Sigma-Aldrich), followed by incubation with HRP-conjugated goat anti-rabbit Ab (1/5,000 dilution in PBS-Tween) from Roche Applied Science. Immuno-reactive bands were detected with enhanced chemiluminescence substrate (Roche Applied Bioscience). Results show that OVA-expression by ST-OVA-NT and ST-OVA-T (from ~5×10^6) in the bacterial pellets was similar (FIG. **1**C). However, OVA could only be detected in the supernatant of ST-OVA-T cultures. Expression of OVA was detectable in the cytosol of spleen cells from mice infected with ST-OVA-T- but not ST-OVA-NT (FIG. **1**C).

EXAMPLE 3

Proliferation of ST-OVA-T and ST-OVA-NT

[0063] The ability of ST-OVA to proliferate extra- and intra-cellularly was also analyzed.

[0064] Liquid cultures of ST-OVA-NT and ST-OVA-T were set up in flasks to enumerate extracellular proliferation. At various time intervals (eg, 60 min., 120 min., 240 min., etc), aliquots were removed for measurement of OD at 600 nm. Both ST-OVA-NT and ST-OVA-T displayed similar proliferation and doubling time (FIG. **2**A).

[0065] The influence of antigenic translocation on the ability of ST-OVA to proliferate within the intracellular compartment was evaluated. IC-21 macrophages (H-2^b) (5×10^4/well) were infected with ST-OVA-NT or ST-OVA-T (MOI=10). After 30 min, cells were washed and cultured in media containing gentamicin (50 pg/ml) to remove extracellular bacteria. After 2 h, cells were washed again and cultured in media containing reduced levels of gentamicin (10 μg/m1). At various time intervals cells were lysed and bacterial burden in the cells determined. No statistically significant difference was detected in the ability of ST-OVA-NT or ST-OVA-T to infect and replicate within macrophages ($p>0.05$). Results are shown in FIG. **2**B and are representative of three independent experiments. Thus, the ability of ST-OVA to infect and survive within macrophages in vitro was not influenced by antigenic translocation.

EXAMPLE 4

Translocation and Antigen Presentation

[0066] It was previously reported that ST-OVA-NT infection does not induce a detectable $CD8^+$ T cell response within the first week of infection (Luu et al., 2006), due to delayed presentation of antigen to $CD8^+$ T cells (Albaghdadi et al., 2009). Therefore, it was evaluated whether translocation of antigen to the cytosol would result in rapid antigen-presentation.

[0067] In vitro antigen-presentation was performed as previously described (Albaghdadi et al., 2009). IC-21 macrophages (H-2^b) cells (10^5/well) were infected with different MOI of ST (Albaghdadi et al., 2009), ST-OVA-NT (Example 1), or ST-OVA-T (Example 1) for 30 min. Extracellular bacteria were removed after incubation in medium containing gentamicin (50 μg/ml). At 2 h, cells were cultured in media containing lower levels of gentamicin (10 μg/ml) and incubated with CFSE-labelled OT-1 ($CD45.1^+45.2^-$) TCR transgenic cells (10^6/well). After 4 days of culture, cells were harvested, stained with anti-CD45.1 and anti-CD8 antibodies, and the reduction in CFSE intensity of OT-1 $CD8^+$ T cells was evaluated by flow cytometry.

[0068] Infection of macrophages with ST or ST-OVA-NT did not result in any detectable proliferation of OT-1 cells, indicating lack of antigen-presentation (FIG. **3**A). Interestingly, infection with ST-OVA-T, even at reduced doses, resulted in strong dilution of CFSE expression, which is indicative of rapid and potent antigen-presentation in vitro (FIG. **3**A).

[0069] In vivo antigen-presentation was done as previously described (Albaghdadi et al., 2009). B6129F1 mice were infected with the recombinant bacteria of Example 1, followed by adoptive transfer of CFSE labelled OT-1 cells. B6.129F1 mice were used because B6 parents are highly susceptible and die within the first week of infection (Albaghdadi et al., 2009). Briefly, B6129F1 mice were generated in house by mating 129x1SvJ female mice with C57BL/6J male mice; mice were obtained from The Jackson Laboratory and were maintained at the Institute for Biological Sciences (National Research Council of Canada, Ottawa, Canada) in accordance with the guidelines of the Canadian Council on Animal Care. For immunization, frozen stocks of ST-OVA-NT or ST-OVA-T (Example 1) were thawed and diluted in 0.9% NaCl; mice were inoculated (iv) with 10^3 organisms suspended in 200 μl. At various time intervals, CFSE-labelled OT-1 cells were injected (5×10^6, iv). Four days after the transfer of OT-1 cells, spleens were isolated from recipient mice and spleen cells were stained with OVA-tetramer and anti-CD8 antibody. Reduction in the expression of CFSE intensity was evaluated by flow cytometry, as described above.

[0070] Results are shown in FIGS. **3**B and **3**C; results represent the mean of three mice±SD per group, and are representative of two-three independent experiments. At day 5 of infection, the majority of transferred OT-1 cells displayed reduced expression of CFSE in mice infected with ST-OVA-T (FIG. **3**B). In contrast, OT-1 cells in ST-OVA-NT-infected mice maintained high levels of CFSE expression. When in vivo antigen-presentation was evaluated kinetically, ST-OVA-NT infected mice displayed muted and delayed activation of CFSE-labelled OT-1 cells (FIG. **3**C). Interestingly, the massive antigen-presentation that was induced early on in ST-OVA-T infected mice was subsequently reduced to baseline levels as the pathogen was cleared.

EXAMPLE 5

Antigen Translocation and $CD8^+$ T Cell Response

[0071] The question of whether the induction of rapid antigen-presentation in vitro and in vivo by antigenic translocation to the cytosol would result in the development of a rapid $CD8^+$ T cell response in vivo and whether this had any influence on pathogen control was examined.

[0072] B6.129F1 mice were infected (10^3, iv) with ST-OVA-T or ST-OVA-NT without any adoptive transfer of OT-1 cells. At various time intervals, the numbers of spleen cells, spleen size and bacterial burden were evaluated. OVA-specific CD8+ T cell response was enumerated by Flow cytometry. Briefly, aliquots of spleen cells (5×10^6) were incubated in 80 μl of PBS plus 1% BSA (PBS-BSA) with anti-CD16/32 at 4° C. After 10 min., cells were stained with H-$2K^b OVA_{257-264}$ tetramer-PE (Beckman Coulter, US) and various antibodies (anti-CD8 PerCP-Cy5, anti-CD62L APC-Cy7, and anti-CD127 (PE-Cy7) for 30 min. All antibodies were obtained from BD Biosciences. Cells were washed with PBS, fixed in 0.5% formaldehyde and acquired on a BD Biosciences FACSCanto analyzer.

[0073] Results are shown in FIG. **4**; these results represent the mean of three to five mice±SD per group and are representative of three independent experiments. Infection of mice with ST-OVA-T resulted in the development of a rapid and potent OVA-specific $CD8^+$ T cell response as evaluated by staining with OVA-tetramers (FIGS. **4**D, **4**E; FIG. **5**A); these mice displayed reduced spleen cell numbers and size (FIGS. **4**A, **4**B). At day 3 of infection, similar bacterial burdens were noted in mice that received ST-OVA-T or ST-OVA-NT (FIG. **4**C). However, at subsequent time intervals, the burden of ST-OVA-T were enormously controlled which was reduced to non-detectable levels by day 30. In contrast, ST-OVA-NT burden was maintained at high levels and the burden was detectable even at day 60 (FIG. **4**C). Interestingly, at day 60, while both groups of mice had similar numbers of OVA-$tetramer^+$ cells (FIG. **4**E), the ST-OVA-T group of mice had controlled the burden whereas the ST-OVA-NT group of mice failed to control it (FIG. **4**C); this suggests that direct antigen-presentation in case of ST-OVA-T makes the targets susceptible.

[0074] Phenotypic analysis of OVA-specific $CD8^+$ T cells induced against ST-OVA-T versus ST-OVA-NT was also performed. FIG. **5**A shows the OVA-tetramer profile in the spleens of infected mice, and the expression (MFI) of CD62L (FIGS. **5**B, **5**D) and CD127 (FIGS. **5**C, **5**D) on OVA-$tetramer^+CD8^+$ T cells. In contrast to ST-OVA-NT, OVA-specific $CD8^+$ T cells induced against ST-OVA-T displayed rapid activation (CD62L down-regulation) and rapid progression to the memory state (CD127 up-regulation) (FIG. **5**B-D). Taken together, these results clearly indicate that antigenic translocation to the cytosol in the context of ST infection accelerates the kinetics and increases the potency of antigen-presentation, $CD8^+$ T cell differentiation, and memory development. Thus, the differentiation of $CD8^+$ T cells that is noted with ST-OVA-T infection mirrors the one that is induced against the potent pathogen, LM.

EXAMPLE 6

Rapid $CD8^+$ T Cells Response and Survival of Susceptible Mice

[0075] Given the results noted with antigenic translocation in resistant mice (Example 5), determination of whether the rapid induction of $CD8^+$ T cells would influence the survival of susceptible C57BL/6J mice was undertaken.

[0076] C57BL/6J mice were infected (10^3, iv) with ST-OVA-T or ST-OVA-NT. At different time points (day 1, 3, 5, 7 and 14) after infection, spleens were removed and the bacterial burdens were enumerated. Spleen cells were stained with OVA-tetramers and antibodies against CD8, CD62L and CD127. The percentage and numbers of OVA-specific $CD8^+$ T cells were determined, as was the expression of CD62L versus CD127 on OVA-$tetramer^+CD8^+$ T cells.

[0077] Results are shown in FIG. **6** and represent the mean of three to four mice±SD per group; results are representative of two independent experiments. At days 1 and 3, similar bacterial burdens were noted in ST-OVA-NT- and ST-OIVA-T-infected groups (FIG. **6**A). At later time periods, while the bacterial burden in ST-OVA-NT-infected mice continued to increase exponentially to lethal levels, the burden in ST-OVA-T-infected mice was rapidly controlled and became undetectable after day 14. Abridgment of bacterial burden in ST-OVA-T-infected mice correlated to the early emergence of potent OVA-specific $CD8^+$ T cell response, as detected by OVA-tetramer staining (FIG. **6**B-C) that peaked at day 7.

EXAMPLE 7

Antigen Translocation Induces Functional $CD8^+$ T Cells

[0078] Two functional assays were carried out to determine whether the CD8+ T cells that were induced by antigenic translocation would result in induction of CD8+ T cells that mediate appropriate functions.

[0079] Enumeration of IFN-γ secreting cells was performed by ELISPOT assay as reported previously at day 7 of infection (Dudani et al., 2002). ST-OVA-T-infected mice mounted a profound $CD8^+$ T cell response (FIG. **6**D), indicative of IFN-γ production. In contrast, infection of susceptible mice with ST-OVA-NT did not result in any detectable IFN-γ-secreting $CD8^+$ T cells.

[0080] The ability of stimulated $CD8^+$ T cells to kill target cells specifically was enumerated as another functional readout. To do this, CFSE-labelled, OVA-pulsed and control spleen cells from naïve mice were transferred to ST-OVA-T- and ST-OVA-NT-infected mice on day 7, and the specific killing of OVA-pulsed targets was evaluated. In vivo cytolytic activity of $CD8^+$ T cells was performed according to previously published reports (Luu et al., 2006; Barber et al., 2003).

OVA-specific CD8+ T cells that were induced at day 7 in ST-OVA-T-infected mice displayed rapid, potent and specific cytolytic activity towards OVA-pulsed target cells (FIG. **6**E-F). In contrast, ST-OVA-NT-infected mice displayed little cytolytic activity, as expected (Luu et al., 2006). Thus, the kinetics of CD8+ T cells response in ST-OVA-T infected susceptible C57BL/6J was similar to that observed in resistant B6.129 F1 mice, as was their phenotype.

[0081] FIG. **7** shows results of phenotypic analysis of OVA-specific CD8$^+$ T cells induced against ST-OVA-T versus ST-OVA-NT. Similar to the profile in resistant mice, OVA-specific CD8$^+$ T cells induced against ST-OVA-T in susceptible mice displayed rapid down-regulation of CD62L and rapid transition to the memory phenotype (increased CD127 up-regulation; FIG. **7**).

EXAMPLE 8

Control of Bacterial Growth

[0082] While the data of Example 6 indicated that rapid emergence of functional CD8$^+$ T cells by antigenic translocation can control ST burden rapidly, it was still correlative. In order to test if the rapid emergence of CD8$^+$ T cells are responsible for elimination of bacteria during ST-OVA-T infection, C57BL/6J mice were treated with anti-CD8 or anti-CD4 antibody or isotype control then infected with ST-OVA-T to eliminate those cells specifically. C57BL/6J mice were treated with (100 μg/injection) anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), or Rat IgG isotype antibodies on days −3, 0 and 3 after infection with 10^3 ST-OVA-T. At day 7 after infection, spleens were harvested and the bacterial burden evaluated. At day 7 after infection, anti-CD8 antibody treated mice had a >100-fold higher ST-OVA-T burden (FIG. **8**A), suggesting that when CD8$^+$ T cells are depleted, ST-OVA-T cannot be controlled by the host. Depletion of CD4$^+$ T cells had no effect on the bacterial burden.

[0083] The importance of CD8$^+$ T cells in controlling bacterial burden was further confirmed by infecting WT, MHC-I or MHC-II-deficient C57BL/6J mice with ST-OVA-T. Since MHC class I deficient mice lack CD8$^+$ T cells they should be susceptible to infection. Twenty days after infection, MHC-I deficient mice were moribund, displaying very high bacterial loads (FIG. **8**B) whereas control mice had undetectable burden, and MHC class II-deficient hosts (lacking CD4$^+$ T cells) showed only a minor effect. MHC-I deficient mice were sick due to high bacterial loads, while MHC-II-deficient and WT mice were healthy. Taken together, these results indicate that antigenic translocation to cytosol in the context of ST infection results in a rapid emergence of a potent CD8$^+$ T cell response which is sufficient to control the burden.

EXAMPLE 9

Translocation of Antigen in Attenuated Strain of *Salmonella*

[0084] In order to design vaccines, attenuated strains of bacteria are often used to avoid undesirable toxicity that occurs with highly virulent bacteria. It was therefore determined whether translocation of OVA in a highly attenuated strain of ST (AaroA) would induce rapid activation of CD8$^+$ T cells.

[0085] B6.129F1 mice were infected with 10^3 (virulent) wild type (WT; SL1344)) or 10^5 attenuated (avirulent; ΔaroA) ST-OVA expressing non-translocated or non-translocated OVA. At various time intervals (day 7, 14, 21 and 30), spleens and peripheral blood were collected and the relative change in the numbers of OVA-specific CD8$^+$ T cells enumerated after staining with OVA-tetramers and anti-CD8 antibodies as described in Example 5.

[0086] Results in FIG. **9** represent the mean of five mice±SD per group. Translocation of OVA by avirulent ST also resulted in rapid and profound induction of OVA-specific CD8$^+$ T cell response in the spleen (FIG. **9**A) and peripheral blood (FIG. **9**B). Thus, these results indicate that antigenic translocation works equally well for virulent and avirulent bacteria.

EXAMPLE 10

Translocation of Antigen and Tumour Control

[0087] It was also investigated whether antigenic translocation would result in effective protection upon tumour challenge.

[0088] C57BL/6J mice were infected with 10^3 ST-OVA-T; non-infected (naïve) mice served as controls. On day 60, mice were challenged subcutaneously in the lower dorsal region with 10^6 B16 melanoma cells carrying the OVA gene (B16-OVA). Survival of mice was measured subsequently. As shown in FIG. **10**A, prophylactic vaccination with ST-OVA-T resulted in potent protection against tumour challenge. Protection in a therapeutic model, where mice were first challenged with tumours and then vaccinated with immunogens, was also tested. B6.129F1 mice were challenged first with 10^6 B16-OVA tumour cells subcutaneously in the lower dorsal region. Three days later, mice were vaccinated with ST-OVA-NT or ST-OVA-T. Non-infected mice served as negative controls and LM-OVA infected mice served as positive controls. At various time intervals subsequently, survival of mice was monitored. Mice receiving ST-OVA-T displayed the best protection against B16 melanoma cells (FIG. **10**B). Protection induced by ST-OVA-T was far greater than that induced by ST-OVA-NT and LM-OVA. Results represent the mean of five mice±SD per group.

EXAMPLE 11

CD8+ T Cell Response Against Tumor-Antigens

[0089] The use of OVA as an immunodominant antigen is described herein as a proof of principle. Using a similar approach, other putative antigens from other pathogens (bacteria, virus) or tumours can be cloned into ST and these antigens can be translocated into the host cell cytosol for rapid and potent antigen-presentation using the YopE/SycE system.

[0090] The gene for the tumour-antigen (Trp-2) (Schumacher and Restifo, 2009) was cloned into the WT or aroA mutant of ST, which translocates antigen to the cytosol. PCR was done using pCDNA3-Trp2 as template using the following primers:

```
Forward primer:
                                (SEQ ID NO: 15)
TAGGATCCGGAATTCTGCTCAGAG,
and

Reverse primer:
                                (SEQ ID NO: 16)
AGATGGTACCTTTAGTGCCACGTG.
```

[0091] The PCR product and pHR-OVA were digested with BamHI and KpnI and ligated. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94° C., 5 min; 25 cycles of 94° C., 30 s to 58° C., 1 min to 72° C., 1 min; followed by a 7 min extension time at 72° C. The amplified insert was ligated into the intended vector, then sequenced to verify the accuracy of the amplified cDNA. The PCR product was digested with BglII and KpnI; pHR-241 was digested with BamHI and KpnI and the digested products were ligated. pHR-Trp2 plasmid was then transfected into the highly virulent ST (SL1344) or aroA mutant of ST. 50 μL of electrocompetent *Salmonella* (WT or aroA) were mixed with ~20 ng plasmid DNA and pulsed in a Bio-Rad micropulser using one pulse of 2.5 kV. Immediately afterwards, 1 mL of SOC recovery medium was added to the bacteria and they were allowed to recover shaking at 37° C. The bacteria were then plated on LB agar plates with ampicillin for the selection of individual clones.

[0092] The gene for gp100 tumour-antigen (Rosenberg et al., 2008) was cloned into a pHR or pKK plasmid. PCR was done using pCDNA3-gp100 as template with the following primers:

```
Forward primer:
                                 (SEQ ID NO: 17)
GAAGATCTGGGAAGAACACAATGG,
and

Reverse primer:
                                 (SEQ ID NO: 18)
GGGGTACCTTAGGTGAGAGGAATGG.
```

[0093] The PCR product was digested with BglII and KpnI; pHR-241 was digested with BamHI and KpnI and the digested products were ligated. Infection of B6.129F1 mice with these recombinant nucleic acids resulted in the induction of CD8+ T cell response against Trp-2 (FIG. **11**A). This was associated with accelerated control of the bacterium (FIG. **11**B,C). Similarly, infection of mice with the gp100 expressing aroA-ST resulted in accelerated control of the bacterium (FIG. **12**A) and induction of a better CD8+ T cell response against gp100 (FIG. **12**B).

EXAMPLE 12

CD8+ T Cell Response to a Viral Antigen

[0094] The immunodominant epitope recognized to stimulate a CD8$^+$ T cell response from LCMV nucleoprotein (NP) in C57Bl/6 mice was also used as an antigen and cloned into ST, and its effect on T cell response in mice was evaluated.

[0095] LCMV-NP was encoded over amino acids 396-404 (FQPQNGQFI) of the protein (Basler et al., 2004). cDNA encoding amino acids 288-463 of the NP protein was cloned into plasmid pKK to generate pKK-NP (FIG. **13**), using PCT methods as described in Example 1 and 11. Again, DH5α clones were selected using ampicillin. In this case, NcoI and HindIII restriction sites were added to the oligonucleotides used for amplification of the insert sequence. The oligonucleotide sequences used for the cDNA amplification were:

```
                                 (SEQ ID NO: 19)
5' TACCATGGCATTTGTTTCAGACCAAGT 3'
and

                                 (SEQ ID NO: 20)
5' TAAAGCTTCTAGTCCCTTACTATTCCAG 3'.
```

[0096] The final insert in the pKK plasmid was truncated prior to the end of the amplified insert due to the presence of an internal HindIII restriction site, ending at codon 461. After confirmation of the sequence, this plasmid was also transferred into ST wild type and STΔAro using a standard electroporation protocol (as described below and in Examples 1 and 11). cDNA encoding amino acids 288-461 of the NP protein was similarly cloned into the plasmid, pKK, to generate pKK-NP (FIG. **13**). Again, DH5α clones were selected using ampicillin. In this case, NcoI and HindIII restriction sites were added to the oligonucleotides used for amplification of the insert sequence. The oligonucleotide sequences used for the cDNA amplification are:

```
                                 (SEQ ID NO: 21)
5' TACCATGGCATttgtttcagaccaaGT 3'
and

                                 (SEQ ID NO: 22)
5' TAAAGCTTCTAGTCCCTTACTATTCCAG 3'.
```

[0097] The final insert in the pKK plasmid was truncated prior to the end of the amplified insert due to the presence of an internal HindIII restriction site, ending at codon 461. After confirmation of the sequence, this plasmid was also transferred into ST wild type and STΔAro using a standard electroporation protocol. Briefly, 50 μL of electrocompetent *Salmonella* (WT or aroA) were mixed with ~20 ng plasmid DNA and pulsed in a Bio-Rad micropulser using one pulse of 2.5 kV. Immediately afterwards, 1 mL of SOC recovery medium was added to the bacteria and they were allowed to recover shaking at 37° C. The bacteria were then plated on LB agar plates with ampicillin for the selection of individual clones. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94° C., 5 min; 25 cycles of 94° C., 30 s to 58° C., 1 min to 72° C., 1 min; followed by a 7 min extension time at 72° C. The amplified insert was ligated into the intended vector then sequenced to verify the accuracy of the amplified cDNA.

[0098] B6.129F1 mice were infected intravenously with 10^3 recombinant ST expressing NP. Both virulent (FIG. **13**B, C) and avirulent (FIG. **13**D) ST induced profound NP-specific CD8+ T cell response when NP was translocated to the cytosol. Furthermore, antigenic translocation resulted in decreased bacterial burden (FIG. **13**E) and control of vaccine induced inflammation (FIG. **13**F).

EXAMPLE 13

Use of Truncated YopE as a Means to Induce Potent CD8+ T Cell Response

[0099] To determine whether the full length YopE was needed for induction of a better CD8+ T cell response, or whether a truncated version of this protein would be sufficient, the gene for OVA was fused with truncated YopE (first eighteen amino acids only), which does not carry the C-terminal domain for binding to the SycE chaperon (FIG. **14**A). PCR was done using pHR-OVA as template with the following primers:

```
Forward primer:
                                    (SEQ ID NO: 23)
GTGTCAAAGTTGGGGAATTCGC,
and

Reverse primer:
                                    (SEQ ID NO: 24)
CTGCTGGATCCTGACACTGATG.
```

[0100] The PCR product and pHR-OVA were digested with EcoRI and BamHI and ligated. PCR amplification of the inserts was performed with Taq polymerase using the following cycling parameters: 94° C., 5 min; 25 cycles of 94° C., 30 s to 58° C., 1 min to 72° C., 1 min; followed by a 7 min extension time at 72° C. The amplified insert was ligated into the intended vector, then sequenced to verify the accuracy of the amplified cDNA.

[0101] B6.129F1 mice were infected with ST-OVA-NT, ST-OVA-T (carrying full length YopE), and ST-OVA-tYopE (carrying truncated YopE). As is clear from results shown in FIG. **14**B, the fusion of the desired antigen with the first eighteen amino acids of YopE is sufficient to induce rapid CD8+ T cell response. CD8+ T cells induced by the truncated YopE differentiated rapidly into memory cells (FIG. **14**C), which lead to curtailment of inflammation (FIG. **14**D) and bacterial burden (FIG. **14**E).

[0102] The embodiments and examples described herein are illustrative and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments, including alternatives, modifications and equivalents, are intended by the inventors to be encompassed by the claims. Furthermore, the discussed combination of features might not be necessary for the inventive solution.

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[0103] All patents, patent applications and publications referred to herein and throughout the application are hereby incorporated by reference.

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Gly Ser His Lys Pro Val Val Thr Pro Ala Pro Thr Pro Ala Gln Met
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Pro Ser Pro Thr Ser Phe Ser Asp Ser Ile Lys Gln Leu Ala Ala Glu
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Val Leu Thr Trp Leu Gly Lys Met Pro Leu Phe Lys Asn Thr Glu Val
    50                  55                  60

Val Gln Lys His Thr Glu Asn Ile Arg Val Gln Asp Gln Lys Ile Leu
65                  70                  75                  80

Gln Thr Phe Leu His Ala Leu Thr Glu Lys Tyr Gly Glu Thr Ala Val
                85                  90                  95

Asn Asp Ala Leu Leu Met Ser Arg Ile Asn Met Asn Lys Pro Leu Thr
            100                 105                 110

Gln Arg Leu Ala Val Gln Ile Thr Glu Cys Val Lys Ala Ala Asp Glu
        115                 120                 125

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        195                 200                 205

Val Ala Pro Leu Phe Asn Glu Cys Ala Met Pro Thr Pro Gln Gln Phe
    210                 215                 220

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225                 230                 235                 240


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            20                  25                  30

Glu Phe Ala Cys His Ile Thr Glu His Pro Val Gly Gln Ile Leu Met
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Phe Thr Leu Pro Ser Leu Asp Asn Asn Asn Glu Lys Glu Thr Leu Leu
    50                  55                  60

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65                  70                  75                  80
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                85                  90                  95

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            100                 105                 110

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        115                 120                 125

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    130                 135                 140

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                165                 170                 175

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Tyr Asp Leu Gly Gly Gly Thr Phe Asp Ile Ser Ile Ile Glu Ile Asp
        195                 200                 205

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    210                 215                 220

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225                 230                 235                 240

Val Asp Glu Phe Lys Lys Asp Gln Gly Ile Asp Leu Arg Asn Asp Pro
                245                 250                 255

Leu Ala Met Gln Arg Leu Lys Glu Ala Ala Glu Lys Ala Lys Ile Glu
            260                 265                 270

Leu Ser Ser Ala Gln Gln Thr Asp Val Asn Leu Pro Tyr Ile Thr Ala
        275                 280                 285

Asp Ala Thr Gly Pro Lys His Met Asn Ile Lys Val Thr Arg Ala Lys
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    290                 295                 300

Leu Glu Ser Leu Val Glu Asp Leu Val Asn Arg Ser Ile Glu Pro Leu
305                 310                 315                 320

Lys Val Ala Leu Gln Asp Ala Gly Leu Ser Val Ser Asp Ile Asn Asp
                325                 330                 335

Val Ile Leu Val Gly Gly Gln Thr Arg Met Pro Met Val Gln Lys Lys
            340                 345                 350

Val Ala Glu Phe Phe Gly Lys Glu Pro Arg Lys Asp Val Asn Pro Asp
        355                 360                 365

Glu Ala Val Ala Ile Gly Ala Ala Val Gln Gly Gly Val Leu Thr Gly
    370                 375                 380

Asp Val Lys Asp Val Leu Leu Leu Asp Val Thr Pro Leu Ser Leu Gly
385                 390                 395                 400

Ile Glu Thr Met Gly Gly Val Met Thr Pro Leu Ile Thr Lys Asn Thr
                405                 410                 415

Thr Ile Pro Thr Lys His Ser Gln Val Phe Ser Thr Ala Glu Asp Asn
            420                 425                 430

Gln Ser Ala Val Thr Ile His Val Leu Gln Gly Glu Arg Lys Arg Ala
        435                 440                 445

Ser Asp Asn Lys Ser Leu Gly Gln Phe Asn Leu Asp Gly Ile Asn Pro
    450                 455                 460

Ala Pro Arg Gly Met Pro Gln Ile Glu Val Thr Phe Asp Ile Asp Ala
465                 470                 475                 480

Asp Gly Ile Leu His Val Ser Ala Lys Asp Lys Asn Ser Gly Lys Glu
                485                 490                 495

Gln Lys Ile Thr Ile Lys Ala Ser Ser Gly Leu Asn Glu Glu Glu Ile
            500                 505                 510

Gln Lys Met Val Arg Asp Ala Glu Ala Asn Ala Glu Ser Asp Arg Lys
        515                 520                 525

Phe Glu Glu Leu Val Gln Thr Arg Asn Gln Gly Asp His Leu Leu His
    530                 535                 540

Ser Thr Arg Lys Gln Val Glu Glu Ala Gly Asp Lys Leu Pro Ala Asp
545                 550                 555                 560

Asp Lys Thr Ala Ile Glu Ser Ala Leu Asn Ala Leu Glu Thr Ala Leu
                565                 570                 575

Lys Gly Glu Asp Lys Ala Ala Ile Glu Ala Lys Met Gln Glu Leu Ala
            580                 585                 590

Gln Val Ser Gln Lys Leu Met Glu Ile Ala Gln Gln Gln His Ala Gln
        595                 600                 605

Gln Gln Ala Gly Ser Ala Asp Ala Ser Ala Asn Asn Ala Lys Asp Asp
    610                 615                 620

Asp Val Val Asp Ala Glu Phe Glu Glu Val Lys Asp Lys Lys
625                 630                 635


<210> SEQ ID NO 7
<211> LENGTH: 523
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic SycE-YopE-OVA

<400> SEQUENCE: 7

Met Tyr Ser Phe Glu Gln Ala Ile Thr Gln Leu Phe Gln Gln Leu Ser
1               5                   10                  15
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Leu Ser Ile Pro Asp Thr Ile Glu Pro Val Ile Gly Val Lys Val Gly
            20                  25                  30

Glu Phe Ala Cys His Ile Thr Glu His Pro Val Gly Gln Ile Leu Met
        35                  40                  45

Phe Thr Leu Pro Ser Leu Asp Asn Asn Asn Glu Lys Glu Thr Leu Leu
    50                  55                  60

Ser His Asn Ile Phe Ser Gln Asp Ile Leu Lys Pro Ile Leu Ser Trp
65                  70                  75                  80

Asp Glu Val Gly Gly His Pro Val Leu Trp Asn Arg Gln Pro Leu Asn
                85                  90                  95

Asn Leu Asp Asn Asn Ser Leu Tyr Thr Gln Leu Glu Met Leu Val Gln
            100                 105                 110

Gly Ala Glu Arg Leu Gln Thr Ser Ser Leu Ile Ser Pro Pro Arg Ser
        115                 120                 125

Phe Ser Met Lys Ile Ser Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro
    130                 135                 140

Thr Ser Val Ser Gly Ser Ser Ser Val Gly Glu Met Ser Gly Arg Ser
145                 150                 155                 160

Val Ser Gln Gln Lys Ser Glu Gln Tyr Ala Asn Asn Leu Ala Gly Arg
                165                 170                 175

Thr Glu Ser Pro Gln Gly Ser Ser Leu Ala Ser Arg Ile Thr Glu Lys
            180                 185                 190

Leu Ser Ser Met Ala Arg Ser Ala Ile Glu Phe Ile Lys Arg Met Phe
        195                 200                 205

Ser Glu Gly Ser His Lys Pro Val Val Thr Pro Ala Pro Thr Pro Ala
    210                 215                 220

Gln Met Pro Ser Pro Thr Ser Phe Ser Asp Ser Ile Lys Gln Leu Ala
225                 230                 235                 240

Ala Glu Thr Leu Pro Lys Tyr Ile Gln Gln Leu Ser Ser Leu Asp Ala
                245                 250                 255

Glu Thr Leu Gln Lys Asn His Asp Gln Phe Ala Thr Gly Ser Asn Phe
            260                 265                 270

Gln Thr Ala Ala Asp Gln Ala Arg Glu Leu Ile Asn Ser Arg Val Glu
        275                 280                 285

Ser Gln Thr Asn Gly Ile Ile Arg Asn Val Leu Gln Pro Ser Ser Val
    290                 295                 300

Asp Ser Gln Thr Ala Met Val Leu Val Asn Ala Ile Val Phe Lys Gly
305                 310                 315                 320

Leu Trp Glu Lys Ala Phe Lys Asp Glu Asp Thr Gln Ala Met Pro Phe
                325                 330                 335

Arg Val Thr Glu Gln Glu Ser Lys Pro Val Gln Met Met Tyr Gln Ile
            340                 345                 350

Gly Leu Phe Arg Val Ala Ser Met Ala Ser Glu Lys Met Lys Ile Leu
        355                 360                 365

Glu Leu Pro Phe Ala Ser Gly Thr Met Ser Met Leu Val Leu Leu Pro
    370                 375                 380

Asp Glu Val Ser Gly Leu Glu Gln Leu Glu Ser Ile Ile Asn Phe Glu
385                 390                 395                 400

Lys Leu Thr Glu Trp Thr Ser Ser Asn Val Met Glu Glu Arg Lys Ile
                405                 410                 415

Lys Val Tyr Leu Pro Arg Met Lys Met Glu Glu Lys Tyr Asn Leu Thr
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            420                 425                 430

Ser Val Leu Met Ala Met Gly Ile Thr Asp Val Phe Ser Ser Ser Ala
        435                 440                 445

Asn Leu Ser Gly Ile Ser Ser Ala Glu Ser Leu Lys Ile Ser Gln Ala
    450                 455                 460

Val His Ala Ala His Ala Glu Ile Asn Glu Ala Gly Arg Glu Val Val
465                 470                 475                 480

Gly Ser Ala Glu Ala Gly Val Asp Ala Ala Ser Val Ser Glu Glu Phe
                485                 490                 495

Arg Ala Asp His Pro Phe Leu Phe Cys Ile Lys His Ile Ala Thr Asn
            500                 505                 510

Ala Val Leu Phe Phe Gly Arg Cys Val Ser Pro
        515                 520


<210> SEQ ID NO 8
<211> LENGTH: 403
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic SycE-YopE(18)-OVA

<400> SEQUENCE: 8

Met Tyr Ser Phe Glu Gln Ala Ile Thr Gln Leu Phe Gln Gln Leu Ser
1               5                   10                  15

Leu Ser Ile Pro Asp Thr Ile Glu Pro Val Ile Gly Val Lys Val Gly
            20                  25                  30

Glu Phe Ala Cys His Ile Thr Glu His Pro Val Gly Gln Ile Leu Met
        35                  40                  45

Phe Thr Leu Pro Ser Leu Asp Asn Asn Asn Glu Lys Glu Thr Leu Leu
    50                  55                  60

Ser His Asn Ile Phe Ser Gln Asp Ile Leu Lys Pro Ile Leu Ser Trp
65                  70                  75                  80

Asp Glu Val Gly Gly His Pro Val Leu Trp Asn Arg Gln Pro Leu Asn
                85                  90                  95

Asn Leu Asp Asn Asn Ser Leu Tyr Thr Gln Leu Glu Met Leu Val Gln
            100                 105                 110

Gly Ala Glu Arg Leu Gln Thr Ser Ser Leu Ile Ser Pro Pro Arg Ser
        115                 120                 125

Phe Ser Met Lys Ile Ser Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro
    130                 135                 140

Thr Ser Val Ser Gly Ser Asn Phe Gln Thr Ala Ala Asp Gln Ala Arg
145                 150                 155                 160

Glu Leu Ile Asn Ser Arg Val Glu Ser Gln Thr Asn Gly Ile Ile Arg
                165                 170                 175

Asn Val Leu Gln Pro Ser Ser Val Asp Ser Gln Thr Ala Met Val Leu
            180                 185                 190

Val Asn Ala Ile Val Phe Lys Gly Leu Trp Glu Lys Ala Phe Lys Asp
        195                 200                 205

Glu Asp Thr Gln Ala Met Pro Phe Arg Val Thr Glu Gln Glu Ser Lys
    210                 215                 220

Pro Val Gln Met Met Tyr Gln Ile Gly Leu Phe Arg Val Ala Ser Met
225                 230                 235                 240

Ala Ser Glu Lys Met Lys Ile Leu Glu Leu Pro Phe Ala Ser Gly Thr
                245                 250                 255
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Met Ser Met Leu Val Leu Leu Pro Asp Glu Val Ser Gly Leu Glu Gln
            260                 265                 270

Leu Glu Ser Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr Ser Ser
        275                 280                 285

Asn Val Met Glu Glu Arg Lys Ile Lys Val Tyr Leu Pro Arg Met Lys
    290                 295                 300

Met Glu Glu Lys Tyr Asn Leu Thr Ser Val Leu Met Ala Met Gly Ile
305                 310                 315                 320

Thr Asp Val Phe Ser Ser Ser Ala Asn Leu Ser Gly Ile Ser Ser Ala
                325                 330                 335

Glu Ser Leu Lys Ile Ser Gln Ala Val His Ala Ala His Ala Glu Ile
            340                 345                 350

Asn Glu Ala Gly Arg Glu Val Val Gly Ser Ala Glu Ala Gly Val Asp
        355                 360                 365

Ala Ala Ser Val Ser Glu Glu Phe Arg Ala Asp His Pro Phe Leu Phe
    370                 375                 380

Cys Ile Lys His Ile Ala Thr Asn Ala Val Leu Phe Phe Gly Arg Cys
385                 390                 395                 400

Val Ser Pro


<210> SEQ ID NO 9
<211> LENGTH: 273
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic YopE(18)-OVA

<400> SEQUENCE: 9

Met Lys Ile Ser Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro Thr Ser
1               5                   10                  15

Val Ser Gly Ser Asn Phe Gln Thr Ala Ala Asp Gln Ala Arg Glu Leu
            20                  25                  30

Ile Asn Ser Arg Val Glu Ser Gln Thr Asn Gly Ile Ile Arg Asn Val
        35                  40                  45

Leu Gln Pro Ser Ser Val Asp Ser Gln Thr Ala Met Val Leu Val Asn
    50                  55                  60

Ala Ile Val Phe Lys Gly Leu Trp Glu Lys Ala Phe Lys Asp Glu Asp
65                  70                  75                  80

Thr Gln Ala Met Pro Phe Arg Val Thr Glu Gln Glu Ser Lys Pro Val
                85                  90                  95

Gln Met Met Tyr Gln Ile Gly Leu Phe Arg Val Ala Ser Met Ala Ser
            100                 105                 110

Glu Lys Met Lys Ile Leu Glu Leu Pro Phe Ala Ser Gly Thr Met Ser
        115                 120                 125

Met Leu Val Leu Leu Pro Asp Glu Val Ser Gly Leu Glu Gln Leu Glu
    130                 135                 140

Ser Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr Ser Ser Asn Val
145                 150                 155                 160

Met Glu Glu Arg Lys Ile Lys Val Tyr Leu Pro Arg Met Lys Met Glu
                165                 170                 175

Glu Lys Tyr Asn Leu Thr Ser Val Leu Met Ala Met Gly Ile Thr Asp
            180                 185                 190

Val Phe Ser Ser Ser Ala Asn Leu Ser Gly Ile Ser Ser Ala Glu Ser
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        195                 200                 205

Leu Lys Ile Ser Gln Ala Val His Ala Ala His Ala Glu Ile Asn Glu
    210                 215                 220

Ala Gly Arg Glu Val Val Gly Ser Ala Glu Ala Gly Val Asp Ala Ala
225                 230                 235                 240

Ser Val Ser Glu Glu Phe Arg Ala Asp His Pro Phe Leu Phe Cys Ile
                245                 250                 255

Lys His Ile Ala Thr Asn Ala Val Leu Phe Phe Gly Arg Cys Val Ser
            260                 265                 270

Pro


<210> SEQ ID NO 10
<211> LENGTH: 613
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic SycE-YopE-Trp2

<400> SEQUENCE: 10

Met Tyr Ser Phe Glu Gln Ala Ile Thr Gln Leu Phe Gln Gln Leu Ser
1               5                   10                  15

Leu Ser Ile Pro Asp Thr Ile Glu Pro Val Ile Gly Val Lys Val Gly
            20                  25                  30

Glu Phe Ala Cys His Ile Thr Glu His Pro Val Gly Gln Ile Leu Met
        35                  40                  45

Phe Thr Leu Pro Ser Leu Asp Asn Asn Asn Glu Lys Glu Thr Leu Leu
    50                  55                  60

Ser His Asn Ile Phe Ser Gln Asp Ile Leu Lys Pro Ile Leu Ser Trp
65                  70                  75                  80

Asp Glu Val Gly Gly His Pro Val Leu Trp Asn Arg Gln Pro Leu Asn
                85                  90                  95

Asn Leu Asp Asn Asn Ser Leu Tyr Thr Gln Leu Glu Met Leu Val Gln
            100                 105                 110

Gly Ala Glu Arg Leu Gln Thr Ser Ser Leu Ile Ser Pro Pro Arg Ser
        115                 120                 125

Phe Ser Met Lys Ile Ser Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro
    130                 135                 140

Thr Ser Val Ser Gly Ser Ser Ser Val Gly Glu Met Ser Gly Arg Ser
145                 150                 155                 160

Val Ser Gln Gln Lys Ser Glu Gln Tyr Ala Asn Asn Leu Ala Gly Arg
                165                 170                 175

Thr Glu Ser Pro Gln Gly Ser Ser Leu Ala Ser Arg Ile Thr Glu Lys
            180                 185                 190

Leu Ser Ser Met Ala Arg Ser Ala Ile Glu Phe Ile Lys Arg Met Phe
        195                 200                 205

Ser Glu Gly Ser His Lys Pro Val Val Thr Pro Ala Pro Thr Pro Ala
    210                 215                 220

Gln Met Pro Ser Pro Thr Ser Phe Ser Asp Ser Ile Lys Gln Leu Ala
225                 230                 235                 240

Ala Glu Thr Leu Pro Lys Tyr Ile Gln Gln Leu Ser Ser Leu Asp Ala
                245                 250                 255

Glu Thr Leu Gln Lys Asn His Asp Gln Phe Ala Thr Met Lys Ile Ser
            260                 265                 270
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```
Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro Thr Ser Val Ser Gly Ser
        275                 280                 285

Ser Ser Val Gly Glu Met Ser Gly Arg Ser Val Ser Gln Gln Lys Ser
    290                 295                 300

Glu Gln Tyr Ala Asn Asn Leu Ala Gly Arg Thr Glu Ser Pro Gln Gly
305                 310                 315                 320

Ser Ser Leu Ala Ser Arg Ile Thr Glu Lys Leu Ser Ser Met Ala Arg
                325                 330                 335

Ser Ala Ile Glu Phe Ile Lys Arg Met Phe Ser Glu Gly Ser His Lys
            340                 345                 350

Pro Val Val Thr Pro Ala Pro Thr Pro Ala Gln Met Pro Ser Pro Thr
        355                 360                 365

Ser Phe Ser Asp Ser Ile Lys Gln Leu Ala Ala Glu Thr Leu Pro Lys
    370                 375                 380

Tyr Ile Gln Gln Leu Ser Ser Leu Asp Ala Glu Thr Leu Gln Lys Asn
385                 390                 395                 400

His Asp Gln Phe Ala Thr Gly Ser Gly Ile Leu Leu Arg Ala Arg Ala
                405                 410                 415

Gln Phe Pro Arg Val Cys Met Thr Leu Asp Gly Val Leu Asn Lys Glu
            420                 425                 430

Cys Cys Pro Pro Leu Gly Pro Glu Ala Thr Asn Ile Cys Gly Phe Leu
        435                 440                 445

Glu Gly Arg Gly Gln Cys Ala Glu Val Gln Thr Asp Thr Arg Pro Trp
    450                 455                 460

Ser Gly Pro Tyr Ile Leu Arg Asn Gln Asp Asp Arg Glu Gln Trp Pro
465                 470                 475                 480

Arg Lys Phe Phe Asn Arg Thr Cys Lys Cys Thr Gly Asn Phe Ala Gly
                485                 490                 495

Tyr Asn Cys Gly Gly Cys Lys Phe Gly Trp Thr Gly Pro Asp Cys Asn
            500                 505                 510

Arg Lys Lys Pro Ala Ile Leu Arg Arg Asn Ile His Ser Leu Thr Ala
        515                 520                 525

Gln Glu Arg Glu Gln Phe Leu Gly Ala Leu Asp Leu Ala Lys Lys Ser
    530                 535                 540

Ile His Pro Asp Tyr Val Ile Thr Thr Gln His Trp Leu Gly Leu Leu
545                 550                 555                 560

Gly Pro Asn Gly Thr Gln Pro Gln Ile Ala Asn Phe Ser Val Tyr Asp
                565                 570                 575

Phe Phe Val Trp Leu His Tyr Tyr Ser Val Arg Asp Thr Leu Leu Gly
            580                 585                 590

Pro Gly Arg Pro Tyr Lys Ala Ile Asp Phe Ser His Gln Gly Pro Ala
        595                 600                 605

Phe Val Thr Trp His
    610


<210> SEQ ID NO 11
<211> LENGTH: 572
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic SycE-YopE-gp100

<400> SEQUENCE: 11

Met Tyr Ser Phe Glu Gln Ala Ile Thr Gln Leu Phe Gln Gln Leu Ser
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1               5                   10                  15

Leu Ser Ile Pro Asp Thr Ile Glu Pro Val Ile Gly Val Lys Val Gly
            20                  25                  30

Glu Phe Ala Cys His Ile Thr Glu His Pro Val Gly Gln Ile Leu Met
        35                  40                  45

Phe Thr Leu Pro Ser Leu Asp Asn Asn Asn Glu Lys Glu Thr Leu Leu
    50                  55                  60

Ser Asn Ile Phe Ser Gln Asp Ile Leu Lys Pro Ile Leu Ser Trp Asp
65                  70                  75                  80

Glu Val Gly Gly His Pro Val Leu Trp Asn Arg Gln Pro Leu Asn Ser
                85                  90                  95

Leu Asp Asn Asn Ser Leu Tyr Thr Gln Leu Glu Met Leu Val Gln Gly
            100                 105                 110

Ala Glu Arg Leu Gln Thr Ser Ser Leu Ile Ser Pro Pro Arg Ser Phe
        115                 120                 125

Ser Met Lys Ile Ser Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro Ala
    130                 135                 140

Ser Val Ser Gly Ser Ser Ser Val Gly Glu Met Ser Gly Arg Ser Val
145                 150                 155                 160

Ser Gln Gln Lys Ser Asp Gln Tyr Ala Asn Asn Leu Ala Gly Arg Thr
                165                 170                 175

Glu Ser Pro Gln Gly Ser Ser Leu Ala Ser Arg Ile Ile Glu Arg Leu
            180                 185                 190

Ser Ser Met Ala His Ser Val Ile Gly Phe Ile Gln Arg Met Phe Ser
        195                 200                 205

Glu Gly Ser His Lys Pro Val Val Thr Pro Ala Leu Thr Pro Ala Gln
    210                 215                 220

Met Pro Ser Pro Thr Ser Phe Ser Asp Ser Ile Lys Gln Leu Ala Ala
225                 230                 235                 240

Glu Thr Leu Pro Lys Tyr Met Gln Gln Leu Ser Ser Leu Asp Ala Glu
                245                 250                 255

Thr Leu Gln Lys Asn His Asp Gln Phe Ala Thr Gly Ser Gly Lys Asn
            260                 265                 270

Thr Met Asp Leu Val Leu Lys Arg Cys Leu Leu His Leu Ala Val Ile
        275                 280                 285

Gly Ala Leu Leu Ala Val Gly Ala Thr Lys Val Pro Arg Asn Gln Asp
    290                 295                 300

Trp Leu Gly Val Ser Arg Gln Leu Arg Thr Lys Ala Trp Asn Arg Gln
305                 310                 315                 320

Leu Tyr Pro Glu Trp Thr Glu Ala Gln Arg Leu Asp Cys Trp Arg Gly
                325                 330                 335

Gly Gln Val Ser Leu Lys Val Ser Asn Asp Gly Pro Thr Leu Ile Gly
            340                 345                 350

Ala Asn Ala Ser Phe Ser Ile Ala Leu Asn Phe Pro Gly Ser Gln Lys
        355                 360                 365

Val Leu Pro Asp Gly Gln Val Ile Trp Val Asn Asn Thr Ile Ile Asn
    370                 375                 380

Gly Ser Gln Val Trp Gly Gly Gln Pro Val Tyr Pro Gln Glu Thr Asp
385                 390                 395                 400

Asp Ala Cys Ile Phe Pro Asp Gly Gly Pro Cys Pro Ser Gly Ser Trp
                405                 410                 415
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```
Ser Gln Lys Arg Ser Phe Val Tyr Val Trp Lys Thr Trp Gly Gln Tyr
            420                 425                 430

Trp Gln Val Leu Gly Gly Pro Val Ser Gly Leu Ser Ile Gly Thr Gly
        435                 440                 445

Arg Ala Met Leu Gly Thr His Thr Met Glu Val Thr Val Tyr His Arg
    450                 455                 460

Arg Gly Ser Arg Ser Tyr Val Pro Leu Ala His Ser Ser Ser Ala Phe
465                 470                 475                 480

Thr Ile Thr Asp Gln Val Pro Phe Ser Val Ser Val Ser Gln Leu Arg
                485                 490                 495

Ala Leu Asp Gly Gly Asn Lys His Phe Leu Arg Asn Gln Pro Leu Thr
            500                 505                 510

Phe Ala Leu Gln Leu His Asp Pro Ser Gly Tyr Leu Ala Glu Ala Asp
        515                 520                 525

Leu Ser Tyr Thr Trp Asp Phe Gly Asp Ser Ser Gly Thr Leu Ile Ser
    530                 535                 540

Arg Ala Leu Val Val Thr His Thr Tyr Leu Glu Pro Gly Pro Val Thr
545                 550                 555                 560

Ala Gln Val Val Leu Gln Ala Ala Ile Pro Leu Thr
                565                 570


<210> SEQ ID NO 12
<211> LENGTH: 491
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic SycE-YopE-LCMV-NP

<400> SEQUENCE: 12

Met Tyr Ser Phe Glu Gln Ala Ile Thr Gln Leu Phe Gln Gln Leu Ser
1               5                   10                  15

Leu Ser Ile Pro Asp Thr Ile Glu Pro Val Ile Gly Val Lys Val Gly
            20                  25                  30

Glu Phe Ala Cys His Ile Thr Glu His Pro Val Gly Gln Ile Leu Met
        35                  40                  45

Phe Thr Leu Pro Ser Leu Asp Asn Asn Asn Glu Lys Glu Thr Leu Leu
    50                  55                  60

Ser His Asn Ile Phe Ser Gln Asp Ile Leu Lys Pro Ile Leu Ser Trp
65                  70                  75                  80

Asp Glu Val Gly Gly His Pro Val Leu Trp Asn Arg Gln Pro Leu Asn
                85                  90                  95

Asn Leu Asp Asn Asn Ser Leu Tyr Thr Gln Leu Glu Met Leu Val Gln
            100                 105                 110

Gly Ala Glu Arg Leu Gln Thr Ser Ser Leu Ile Ser Pro Pro Arg Ser
        115                 120                 125

Phe Ser Met Lys Ile Ser Ser Phe Ile Ser Thr Ser Leu Pro Leu Pro
    130                 135                 140

Thr Ser Val Ser Gly Ser Ser Ser Val Gly Glu Met Ser Gly Arg Ser
145                 150                 155                 160

Val Ser Gln Gln Lys Ser Glu Gln Tyr Ala Asn Asn Leu Ala Gly Arg
                165                 170                 175

Thr Glu Ser Pro Gln Gly Ser Ser Leu Ala Ser Arg Ile Thr Glu Lys
            180                 185                 190

Leu Ser Ser Met Ala His Ser Ala Ile Glu Phe Ile Lys Arg Met Phe
```

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```
        195                 200                 205

Ser Glu Gly Ser His Lys Pro Val Val Thr Pro Ala Pro Thr Pro Ala
    210                 215                 220

Gln Met Pro Ser Pro Thr Ser Phe Ser Asp Ser Ile Lys Gln Leu Ala
225                 230                 235                 240

Ala Glu Thr Leu Pro Lys Tyr Met Gln Gln Leu Ser Ser Leu Asp Ala
                245                 250                 255

Glu Thr Leu Gln Lys Asn His Asp Gln Phe Ala Thr Gly Ser Phe Val
            260                 265                 270

Ser Asp Gln Val Gly Asp Arg Asn Pro Tyr Glu Asn Ile Leu Tyr Lys
        275                 280                 285

Val Cys Leu Ser Gly Glu Gly Trp Pro Tyr Ile Ala Cys Arg Thr Ser
    290                 295                 300

Ile Val Gly Arg Ala Trp Glu Asn Thr Thr Ile Asp Leu Thr Ser Glu
305                 310                 315                 320

Lys Pro Ala Val Asn Ser Pro Arg Pro Ala Pro Gly Ala Ala Gly Pro
                325                 330                 335

Pro Gln Val Gly Leu Ser Tyr Ser Gln Thr Met Leu Leu Lys Asp Leu
            340                 345                 350

Met Gly Gly Ile Asp Pro Asn Ala Pro Thr Trp Ile Asp Ile Glu Gly
        355                 360                 365

Arg Phe Asn Asp Pro Val Glu Ile Ala Ile Phe Gln Pro Gln Asn Gly
    370                 375                 380

Gln Phe Ile His Phe Tyr Arg Glu Pro Val Asp Gln Lys Gln Phe Lys
385                 390                 395                 400

Gln Asp Ser Lys Tyr Ser His Gly Met Asp Leu Ala Asp Leu Phe Asn
                405                 410                 415

Ala Gln Pro Gly Leu Thr Ser Ser Val Ile Gly Ala Leu Pro Gln Gly
            420                 425                 430

Met Val Leu Ser Cys Gln Gly Ser Asp Asp Ile Arg Lys Leu Leu Asp
        435                 440                 445

Ser Gln Asn Arg Lys Asp Ile Lys Leu Ile Asp Val Glu Met Thr Arg
    450                 455                 460

Glu Ala Ser Arg Glu Tyr Glu Asp Lys Val Trp Asp Lys Tyr Gly Trp
465                 470                 475                 480

Leu Cys Lys Met His Thr Gly Ile Val Arg Asp
                485                 490


<210> SEQ ID NO 13
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Forward Primer

<400> SEQUENCE: 13

cgggatccaa ctttcaaaca gctg                                            24


<210> SEQ ID NO 14
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Reverse primer

<400> SEQUENCE: 14
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```
ggggtacctt aaggggaaac acatc                                       25


<210> SEQ ID NO 15
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Forward primer

<400> SEQUENCE: 15

taggatccgg aattctgctc agag                                        24


<210> SEQ ID NO 16
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Reverse primer

<400> SEQUENCE: 16

agatggtacc tttagtgcca cgtg                                        24


<210> SEQ ID NO 17
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Forward primer

<400> SEQUENCE: 17

gaagatctgg gaagaacaca atgg                                        24


<210> SEQ ID NO 18
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Reverse primer

<400> SEQUENCE: 18

ggggtacctt aggtgagagg aatgg                                       25


<210> SEQ ID NO 19
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Primer

<400> SEQUENCE: 19

taccatggca tttgtttcag accaagt                                     27


<210> SEQ ID NO 20
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Primer

<400> SEQUENCE: 20

taaagcttct agtcccttac tattccag                                    28


<210> SEQ ID NO 21
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```
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Primer

<400> SEQUENCE: 21

taccatggca tttgtttcag accaagt                                          27


<210> SEQ ID NO 22
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Primer

<400> SEQUENCE: 22

taaagcttct agtcccttac tattccag                                         28


<210> SEQ ID NO 23
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Forward primer

<400> SEQUENCE: 23

gtgtcaaagt tggggaattc gc                                               22


<210> SEQ ID NO 24
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Reverse primer

<400> SEQUENCE: 24

ctgctggatc ctgacactga tg                                               22
```

1. A recombinant bacterium, comprising a nucleic acid encoding an antigen that is translocated to the cytosol of a host cell.

2. The recombinant bacterium of claim **1**, wherein the bacterium is an intracellular bacterium such as *Salmonella, Mycobacteria, Brucella,* or *Leishmania.*

3. The recombinant bacterium of claim **1**, wherein the bacterium is *Salmonella.*

4. The recombinant bacterium of claim **1**, wherein the antigen is a viral antigen, a bacterial antigen, or a tumour antigen.

5. The recombinant bacterium of claim **1**, wherein the antigen is a fusion protein comprising an antigen and a translocation domain from a type III secretion system.

6. The recombinant bacterium of claim **5**, wherein the translocation protein is YopE, SopE, SptP, or a fragment thereof.

7. The recombinant bacterium of claim **5**, wherein the fusion protein further comprises a chaperone.

8. The recombinant bacterium of claim **7**, wherein the chaperone is derived from type III secretion systems.

9. The recombinant bacterium of claims **8**, wherein the chaperone is SycE or HSP70.

10. The recombinant bacterium of claim **1**, wherein the nucleic acid is comprised in a vector.

11. The recombinant bacterium of claim **10**, wherein the vector is a pHR vector.

12. The recombinant bacterium of claim **10**, wherein the vector is pHR-241.

13. The recombinant bacterium of claim **1**, wherein the antigen is tyrosinase related protein 2 (TRP-2), MART-1, melanoma associated antigen 1 (MAGE1), or Her-2/neu, gp100, or other viral or bacterial antigens.

14. A method of imparting immunity against naturally-occurring bacterium or virus in a subject, the method comprising administering the recombinant bacterium of claim **1** to said subject.

15. A method of imparting immunity against tumors in a subject, the method comprising administering the recombinant bacterium of claim **13** to said subject.

16. The method of claim **14**, wherein the recombinant bacterium is administered through intravenous, oral or subcutaneous routes of immunization.

17. The use of the recombinant bacterium of claim **1** as a vaccine.

18. The method of claim **15**, wherein the recombinant bacterium is administered through intravenous, oral or subcutaneous routes of immunization.

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