

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

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Abogado
JUAN PABLO CADENA SARMIENTO

ASUNTO Radicación 08 099 559
 Trámite 011
 Evento 378
 Actuación 519
 Oficio 17 **1 06 9 0**
 Folios

Patente de Invención	☒	Patente de Modelo de Utilidad
Vía Nacional		
Vía PCT (Fase Nacional)	☒ Capítulo I	☒ Capítulo II
No. Solicitud Internacional	PCT/BE2006/000020	
Fecha de Presentación Internacional	20/Marzo/2006	

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 14 a 96	19/Septiembre/2008
Reivindicaciones:	Folios 97 a 101	19/Septiembre/2008

2. Objeto de la invención

Título de la solicitud: "Vacuna de Salmonella con virus vivos atenuada"

El problema planteado en esta solicitud consiste en la necesidad de suministrar cepas de la vacuna de Salmonella atenuadas con virus vivos, así como de cepas de la vacuna Salmonella atenuadas con virus vivos mejorados de bacterias que infectan especies veterinarias en general. Así mismo, existe la necesidad de cepas atenuadas con microbios vivos mejoradas similares a las cepas de la vacuna atenuada contra Salmonella que harían posible la diferenciación entre animales vacunados.

Para resolver este problema técnico la solicitud en estudio proporciona cepas de Salmonella entérica atenuadas con una mutación doble o triple. Además proporciona una vacuna con virus vivos atenuada contra la salmonelosis y procedimientos de prevención basados en la misma.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/108, A61K 39/112, A61P 31/04, C12N 1/20.

3. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 24 a 27 que hace referencia a un método de tratamiento no es patentable de acuerdo con el Art citado.

El objeto de las reivindicación 29 y 34 que hace referencia a un método de diagnostico no es patentable de acuerdo con el Art citado.

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 D 486)

El uso de la reivindicación 28 no es patentable de acuerdo con el Art 14.

5. De la Solicitud de Patente

El capítulo reivindicatorio no es claro debido a las siguientes razones:

1. La reivindicación independiente 1 se refiere a una cepa mutante atenuada de una bacteria, en este caso es necesario definir específicamente a que bacteria se está haciendo referencia, además se debe especificar el número de modificaciones que se están realizando en el genoma, y aclarar cuáles son las modificaciones realizadas y en qué posición se están llevando a cabo. Así mismo, se debe especificar de una manera clara a qué genes de movilidad y de supervivencia o proliferación se está refiriendo.
2. En las reivindicaciones 2, 6, 10 y 22 se está caracterizando la cepa mutante mediante un resultado a alcanzar, se recuerda que la invención debe ser definida por sus características técnicas en la reivindicación independiente.
3. En la reivindicación 3 se menciona que la cepa mutante es una de *Salmonella entérica* o de *Escherichia coli*, sin embargo para las invenciones con *E. coli* no se encuentra soporte en la descripción.
4. En la reivindicación 4 se debe definir el gen que codifica la flagelina y además la posición en la cual se está haciendo la mutación.
5. En la reivindicación 5 se está caracterizando la cepa mediante una mutación pero no se está diciendo el tipo de mutación, se solicita incluir dicha información y además definir claramente en cuál gen de los tres mencionados se está llevando a cabo la mutación. Los conectores y/o y o usados en este caso le restan claridad a la reivindicación.
6. La reivindicación 7 debe aclarar específicamente cuál es el gen implicado en la supervivencia, así como definir cuál es el gen constitutivo y el de virulencia.
7. En la reivindicación 8 se menciona que el gen constitutivo (se debe especificar cuál gen constitutivo) está inactivado pero no menciona de que tipo es la inactivación o que mutación tiene y en que posición.
8. La reivindicación 9 menciona que la cepa mutante tiene una mutación por eliminación, pero se debe especificar la posición de dicha mutación.
9. En la reivindicación 11 se menciona que la cepa mutante codifica y expresa un xenoantígeno, pero es necesario que se especifique a cuál xenoantígeno se está haciendo referencia y además la secuencia que fue añadida para que este pudiera ser expresado.

- 10. La reivindicación 13 debe definir las modificaciones genéticas que se están introduciendo y el fago mencionado.
- 11. Las características técnicas que se definen en las reivindicaciones 17, 18 y 19 se deben incluir como parte de la reivindicación independiente 1.
- 12. La reivindicación 20 debe definir claramente y sin ambigüedad a qué tipo de modificación se está refiriendo, así como especificar el gen en el cuál se esta llevando a cabo.
- 13. La reivindicación 21 se refiere a una vacuna pero es necesario que ésta se defina según sus características técnicas: componentes y proporciones de los mismos dentro de la composición.
- 14. La redacción de la reivindicación 23 no permite entender claramente a qué está haciendo referencia, se solicita redactar dicha reivindicación de tal forma que sea fácilmente entendida por el examinador.
- 15. Expresiones como: "por lo menos" (Reivindicación 1), "uno o más" (Reivindicaciones 1, 20), "se seleccionan de entre" (Reivindicación 20), y "cantidad farmacéuticamente eficaz" (Reivindicación 20) le restan claridad y concisión al capítulo reivindicatorio por cuanto son términos ambiguos y no permiten definir la invención.

Se requiere al señor solicitante realizar las correcciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación internacional: marzo 20 de 2006 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	INFECTION AND IMMUNITY, vol. 72, N° 4, Abril 2004, páginas: 2449-2451.	Salmonella flagelin is not a dominant protective antigen in oral immunization with attenuated live vaccine strains.	Abril/2004
D2	INFECTION AND IMMUNITY, vol. 71, N° 1, Enero 2003, páginas: 30-39.	Oral immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent <i>Salmonella enterica</i> serovar Typhimurium.	Enero/2003
D3	VACCINE, BUTTERWORTH SCIENTIFIC GUILDFORD, GB, vol. 15, N°6-7, Abril 1997, páginas 659-663	Protection in a gerbil model of amebiasis by oral immunization with Salmonella expressing the galactose/N-acetyl- D- galactosamine inhibitable lectin of Entamoeba histolytica.	Abril/1997
D4	US2004120962A1	Modulation of immune responses to foreign antigens expressed by recombinant attenuated bacterial vectors	24/Junio/2004

D1
Divulga cepas mutantes de S. Typhimurium a las cuales le fueron eliminados los genes ClpXP (Pág. 2449, 2da columna) y adicionalmente uno o más genes que codifican la flagelina.

D2
Divulga una cepa mutante de S. Typhimurium a la cual le fueron eliminados los genes ClpXP (Pág. 33, 1ra columna, párrafo 1) y adicionalmente unos genes que codifican la flagelina: FliC y FliB.

D3
Divulga cepa mutante de S. dublin a la cual le fue eliminado el gen fliC (Pág. 660, 2da columna, párrafo 1) y fue adicionalmente modificada con una porción de GalNAc lectina de E. histolytica.

D4
http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20040624&CC=US&NR=2004120962A1&KC=A1
Divulga una cepa mutante de S. Typhimurium a la cual le fue eliminado el gen fliC (Pág. 6, tabla 1, x8613) y adicionalmente modificada por eliminación del gen pop (gen de virulencia).

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

Las reivindicación 1 y sus dependientes 2-7, 12-13, 15, 20 y 21 consisten en una cepa mutante con las siguientes características esenciales:

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (D1):

Características esenciales	D1
Una cepa mutante atenuada de una bacteria en la que dicho mutante:	Cepa mutante de S. Typhimurium en la que las modificaciones consisten en:
Contiene por lo menos una primera modificación genética en uno o más genes de movilidad (gen que codifica la flagelina).	Eliminación de los genes: FliC y FliB en la cepa CS2056; Eliminación del gen: FliA en la cepa CS2062;
Contiene por lo menos una segunda modificación genética en uno o más genes implicados en la supervivencia o proliferación de la bacteria.	Y adicionalmente, la eliminación del gen: ClpXP en ambas cepas CS2056 y CS2062;

Las características **esenciales** de la cepa mutante de la reivindicación 1 habían sido divulgadas previamente en D1, el cual revela cepas mutantes de S. Typhimurium a las cuales le fueron eliminados los genes ClpXP (Pág. 2449, 2da columna) y adicionalmente uno o más genes que codifican la flagelina, cuyas características técnicas se muestran en el cuadro anterior, las cuales se traslapan con las divulgadas en la solicitud en estudio.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2-7, 12-13, 15, 20 y 21 no contienen características técnicas adicionales por lo tanto carecen de novedad.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (D2):

Características esenciales	D2
Una cepa mutante atenuada de una bacteria en la que dicho mutante:	Cepa mutante de S. Typhimurium en la que las modificaciones consisten en:
Contiene por lo menos una primera modificación genética en uno o más genes de movilidad (gen que codifica la flagelina).	Eliminación de los genes: FliC y FljB en la cepa CS2056;
Contiene por lo menos una segunda modificación genética en uno o más genes implicados en la supervivencia o proliferación de la bacteria.	Y adicionalmente, la eliminación del gen: ClpXP. (gen de supervivencia)

Las características **esenciales** de la cepa mutante de la reivindicación 1 habían sido divulgadas previamente en D2, el cual revela una cepa mutante de S. Typhimurium a la cual le fueron eliminados los genes ClpXP (Pág. 33, 1ra columna, párrafo 1) y adicionalmente unos genes que codifican la flagelina: FliC y FljB, cuyas características técnicas se muestran en el cuadro anterior, las cuales se traslapan con las divulgadas en la solicitud en estudio.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2-7, 12-13, 15, 20 y 21 no contienen características técnicas adicionales por lo tanto carecen de novedad.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (D3):

Características esenciales	D3
Una cepa mutante atenuada de una bacteria en la que dicho mutante:	Cepa mutante de S. dublin en la que las modificaciones consisten en:
Contiene por lo menos una primera modificación genética en uno o más genes de movilidad (gen que codifica la flagelina).	Modificación en el gen fliC (cepa SL5928)
Contiene por lo menos una segunda modificación genética en uno o más genes implicados en la supervivencia o proliferación de la bacteria.	Gen aro (gen de supervivencia). Y adicionalmente modificada con una porción de GalNAc lectina de E. histolytica

Las características **esenciales** de la cepa mutante de la reivindicación 1 habían sido divulgadas previamente en D3, que revela una cepa mutante de S. dublin a la cual le fue eliminado el gen fliC (Pág. 660, 2da columna, párrafo 1) y fue adicionalmente

modificada con una porción de GalNAc lectina de E. histolytica, cuyas características técnicas se muestran en el cuadro anterior, las cuales se traslapan con las divulgadas en la solicitud en estudio.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D3.

Las reivindicaciones dependientes 2-7, 11-13, 15, 20 y 21-23 no contienen características técnicas adicionales por lo tanto carecen de novedad.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (D4):

Características esenciales	D4
Una cepa mutante atenuada de una bacteria en la que dicho mutante:	Cepa mutante de S. Typhimurium en la que las modificaciones consisten en:
Contiene por lo menos una primera modificación genética en uno o más genes de movilidad (gen que codifica la flagelina).	Eliminación del gen fliC.
Contiene por lo menos una segunda modificación genética en uno o más genes implicados en la supervivencia o proliferación de la bacteria.	Eliminación del gen phoP (x8613).

Las características **esenciales** de la cepa mutante de la reivindicación 1 habían sido divulgadas previamente en D4, el cual revela una cepa mutante de S. Typhimurium a la cual le fue eliminado el gen fliC (Pág. 6, tabla 1, x8613) y adicionalmente modificada por eliminación del gen pop (gen de virulencia), cuyas características técnicas se muestran en el cuadro anterior, las cuales se traslapan con las divulgadas en la solicitud en estudio.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D4.

Las reivindicaciones dependientes 2-7, 11-13, 15, 20 y 21-23 no contienen características técnicas adicionales por lo tanto carecen de novedad.

8. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	INFECTION AND IMMUNITY, vol. 72, N° 4, Abril 2004, páginas: 2449-2451.	1-7, 12-13, 15, 20 y 21	Novedad
D2	INFECTION AND IMMUNITY, vol. 71, N° 1, Enero 2003,	1-7, 12-13, 15, 20 y 21	Novedad

	páginas: 30-39.		
D3	VACCINE, BUTTERWORTH SCIENTIFIC GUILDFORD, GB, vol. 15, N°6-7, Abril 1997, páginas 659- 663	1-7, 11-13, 15, 20 y 21-23	Novedad
D4	US2004120962A1	1-7, 11-13, 15, 20 y 21-23	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

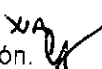
Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El ~~anterior~~ ^{120 JUN. 2015} requerimiento fue notificado por fijación en lista, de fecha

Realizado por: Ximena Andrade. 
Revisado por: Consuelo Leguizamón. 

Salmonella Flagellin Is Not a Dominant Protective Antigen in Oral Immunization with Attenuated Live Vaccine Strains

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Received 15 February 2006; Accepted 15 May 2006

We found that oral immunization with flagellin-defective mutant strains of *Salmonella enterica* serovar Typhimurium protected mice against oral challenge with virulent *Salmonella enterica* serovar Typhimurium. These data indicate that *Salmonella* flagellin is not a dominant protective antigen in oral immunization with attenuated live vaccine strains.

Salmonella enterica serovar Typhimurium is a major cause of salmonellosis in humans and animals. Oral immunization with attenuated live vaccine strains of *Salmonella enterica* serovar Typhimurium has been shown to be an effective method for controlling the spread of this pathogen in livestock and wildlife populations. The flagellin gene (*flaA*) is a major virulence factor of *Salmonella enterica* serovar Typhimurium, and its deletion results in a flagellin-defective mutant strain that is avirulent and noninfectious. In this study, we investigated the role of flagellin in the protective immunity induced by oral immunization with attenuated live vaccine strains of *Salmonella enterica* serovar Typhimurium. We found that oral immunization with flagellin-defective mutant strains of *Salmonella enterica* serovar Typhimurium protected mice against oral challenge with virulent *Salmonella enterica* serovar Typhimurium. These data indicate that *Salmonella* flagellin is not a dominant protective antigen in oral immunization with attenuated live vaccine strains.

INTRODUCTION *Salmonella enterica* serovar Typhimurium is a major cause of salmonellosis in humans and animals. Oral immunization with attenuated live vaccine strains of *Salmonella enterica* serovar Typhimurium has been shown to be an effective method for controlling the spread of this pathogen in livestock and wildlife populations. The flagellin gene (*flaA*) is a major virulence factor of *Salmonella enterica* serovar Typhimurium, and its deletion results in a flagellin-defective mutant strain that is avirulent and noninfectious. In this study, we investigated the role of flagellin in the protective immunity induced by oral immunization with attenuated live vaccine strains of *Salmonella enterica* serovar Typhimurium. We found that oral immunization with flagellin-defective mutant strains of *Salmonella enterica* serovar Typhimurium protected mice against oral challenge with virulent *Salmonella enterica* serovar Typhimurium. These data indicate that *Salmonella* flagellin is not a dominant protective antigen in oral immunization with attenuated live vaccine strains.

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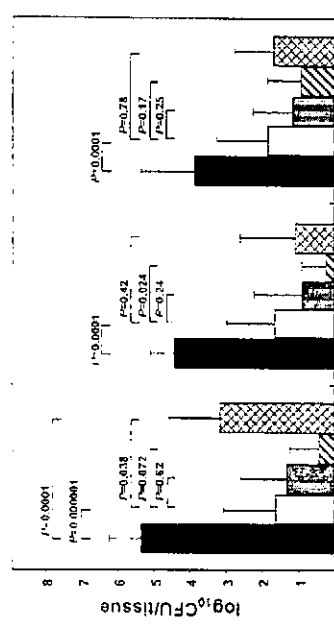


Fig. 1. Protection against oral challenge of *Salmonella enterica* serovar Typhimurium in mice immunized with flagellin-defective mutant strains of *Salmonella enterica* serovar Typhimurium. Mice were immunized with flagellin-defective mutant strains of *Salmonella enterica* serovar Typhimurium (CS2066, CS2067, CS2068, CS2069, CS2070, CS2071, CS2072, CS2073, CS2074, CS2075, CS2076, CS2077, CS2078, CS2079, CS2080, CS2081, CS2082, CS2083, CS2084, CS2085, CS2086, CS2087, CS2088, CS2089, CS2090, CS2091, CS2092, CS2093, CS2094, CS2095, CS2096, CS2097, CS2098, CS2099, CS2100) or wild-type strain (CS2101) and challenged with virulent *Salmonella enterica* serovar Typhimurium (CS2101). The y-axis represents log₁₀ CFU/tissue. The x-axis represents the tissues: spleen, MLN, and PP. The legend indicates: naive (white bar), CS2066 (black bar), CS2067 (hatched bar), CS2068 (white bar), CS2069 (black bar), CS2070 (hatched bar), CS2071 (white bar), CS2072 (black bar), CS2073 (hatched bar), CS2074 (white bar), CS2075 (black bar), CS2076 (hatched bar), CS2077 (white bar), CS2078 (black bar), CS2079 (hatched bar), CS2080 (white bar), CS2081 (black bar), CS2082 (hatched bar), CS2083 (white bar), CS2084 (black bar), CS2085 (hatched bar), CS2086 (white bar), CS2087 (black bar), CS2088 (hatched bar), CS2089 (white bar), CS2090 (black bar), CS2091 (hatched bar), CS2092 (white bar), CS2093 (black bar), CS2094 (hatched bar), CS2095 (white bar), CS2096 (black bar), CS2097 (hatched bar), CS2098 (white bar), CS2099 (black bar), CS2100 (hatched bar), CS2101 (white bar). Statistical significance is indicated by asterisks (*, **, ***) and p-values (p < 0.0001, p < 0.001, p < 0.01, p < 0.05).

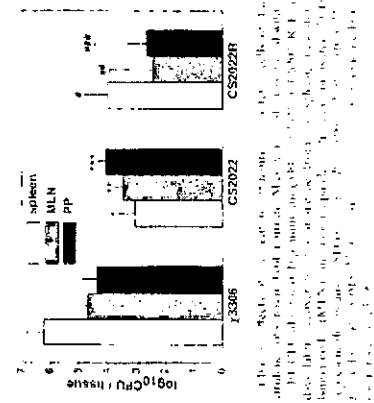


Fig. 3. Titres of *Y. enterocolitica* in the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP) of mice infected with T3386, CS2022 or CS2027. Mice were sacrificed 12 weeks after infection. Data are expressed as the mean $\pm$ SD of five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

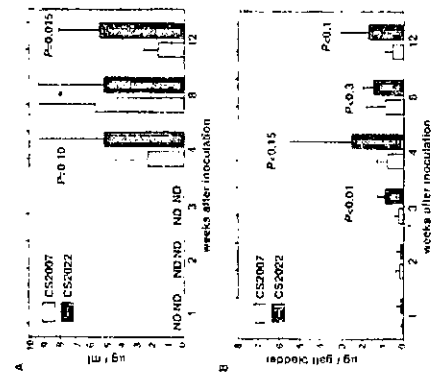


Fig. 4. Titres of IgG in the serum and Peyer's patches (PP) of mice infected with CS2007 or CS2022. Mice were sacrificed 12 weeks after infection. Data are expressed as the mean $\pm$ SD of five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

in each type of tissue 5 days after the challenge. As shown in Fig. 6, neither CS2007 nor CS2022 was detected in the spleen, mesenteric lymph nodes or Peyer's patches of mice 5 days after the challenge. It is noteworthy that the salmonellae were completely eliminated from the caecum of the CS2007 or CS2022-immunized mice. This result suggests that a single oral immunization of CS2007 or CS2022 should be effective in protecting against the colonization of wild-type serovar Typhimurium within the intestinal tract.

Challenge serovar Typhimurium may boost protective immunity. The above results show that oral vaccination with CS2007 or CS2022 induced protective immunity against *Salmonella* infection in mice. We considered both vaccine strains to have induced sufficient amounts of S IgA on the intestinal surfaces to protect against the colonization of wild-type serovar Typhimurium after a single oral immunization. We found it interesting that CS2007 or CS2022 persistently resided in caecal tissue by live challenge (Fig. 1) but that each vaccine strain was completely cleared from each sampled tissue of mice within 5 days after the challenge. To examine how salmonellae of vaccine strains were cleared in immune mice after the final challenge, we compared the antibody amount with and without the challenge. We hypothesized that the clearance of CS2007 or CS2022 after the challenge was the result of the stimulation of humoral and/or cell-mediated immunities caused by the final challenge. As shown in Fig. 4, a fivefold increase in the amount of S IgG in the serum was found in the CS2007 or

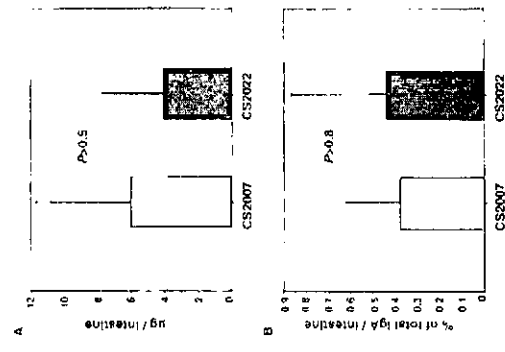


Fig. 5. Induced S IgG responses to the serovar Typhimurium LPS in wild-type mice immunized with CS2007 or CS2022. Mice were sacrificed 12 weeks after immunization. LPS-specific S IgG was measured in the serum and in the intestine. Data are expressed as the mean $\pm$ SD of five mice per group. * $P < 0.05$, ** $P < 0.01$.

CS2022-immunized mice 5 days after the challenge. In contrast, significant increases in the amount of LPS-specific S IgA in the intestine were observed in the CS2007- or CS2022-immunized mice (Fig. 7B). To demonstrate the consequences of introducing Th1 and Th2 cells on serum antibody responses, we measured the *Adjuvanted* LPS-specific IgG subclass responses. In naive mice, the Th1-type cytokine or IL-4/IL-13-type cytokine provides essential signals for antibody isotype switching to IgG2a and IgG3 or to IgE and IgG1 (4). As shown in Fig. 8, the amount of IgG2a included the majority of IgG subclasses in serum with or without the challenge of CS2007 or CS2022-immunized mice. In contrast, IgG1 was not elicited by an immunization with CS2007 or CS2022 and the subsequent challenge with the vaccine strain. In addition, we employed a quantitative RT-PCR to assess levels of the expression of cytokine and chemokine genes in the spleen homogenates of mice that were naive (unimmunized), vaccinated without challenge, or vaccinated with challenge (Fig. 9). It is generally thought that IFN- γ activates macrophages to phagocytose the intracellular killing of microbes by NK cells. The production of IFN- γ leads to the production of large amounts of nitric oxide, which plays a role in host defence in mice (3). Interestingly, the expression of IFN- γ and iNOS mRNA was significantly increased by the immunization and strongly stimulated by the subsequent challenge. On the other hand, the expression of IL-4 was not activated by the immunization, and it was hardly stimulated by the challenge. These results suggest that both

In the present study, we used a novel Peyer's patch (PP) challenge model to evaluate the protective efficacy of CS2007 or CS2022 against *Y. enterocolitica* infection. This model is more relevant to the natural infection of *Y. enterocolitica* in humans than the conventional oral challenge model.

DISCUSSION

In this study, we demonstrated that serovar Typhimurium could colonize the Peyer's patches of mice infected with CS2007 or CS2022. However, the colonization of CS2007 or CS2022 in the Peyer's patches was significantly reduced compared to the wild-type strain. This result suggests that CS2007 or CS2022 may have induced a protective immune response in the Peyer's patches. In fact, the amount of S IgG in the serum and the amount of S IgA in the intestine were significantly increased in the CS2007- or CS2022-immunized mice. This result suggests that CS2007 or CS2022 may have induced a protective immune response in the Peyer's patches. In fact, the amount of S IgG in the serum and the amount of S IgA in the intestine were significantly increased in the CS2007- or CS2022-immunized mice. This result suggests that CS2007 or CS2022 may have induced a protective immune response in the Peyer's patches.

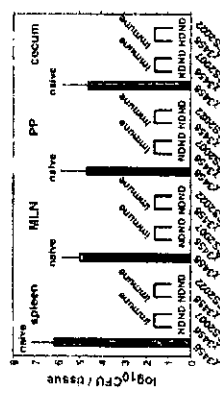


Fig. 6. Titres of *Y. enterocolitica* in the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP) of mice infected with CS2007 or CS2022. Mice were sacrificed 12 weeks after infection. Data are expressed as the mean $\pm$ SD of five mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

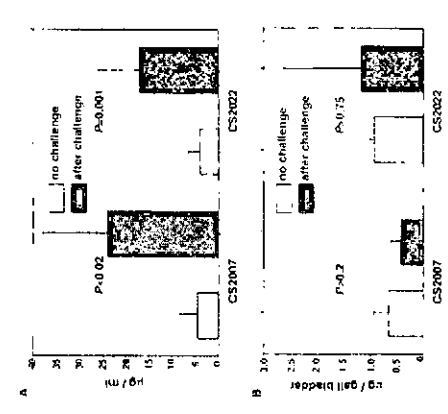


FIG 3. Cecal colonization of the cecum. Top: mean log10 CFU in the cecum of mice challenged with CS2007 or CS2022. Bottom: mean log10 CFU in the cecum of mice challenged with CS2007 or CS2022. *P < 0.05, **P < 0.01, ***P < 0.001.

The revertant of CS2022 from the cecum of 1 mouse out of 10 mice tested at week 24 after oral vaccination. Two distinct possibilities as to the nature of a revertant of CS2022 (CS2022R) were considered: first, a suppressor may represent a revertant that reduces the *hlyA* expression. Second, a dominant point mutation of *hlyA* or *hlyB* may represent a revertant that removes the binding of *hlyA* and *hlyB*. While that was happening, CS2022R as well as CS2022 did not have mucous colonies suggesting that the stabilization of *hlyA* was not induced by the *hlyB* protein disruption or that the stabilized *hlyA* was

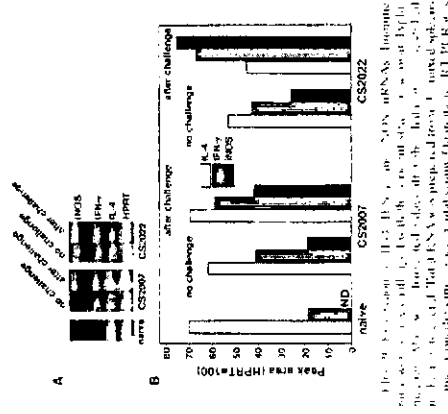


FIG 4. Cecal colonization of the cecum. Top: mean log10 CFU in the cecum of mice challenged with CS2007 or CS2022. Bottom: mean log10 CFU in the cecum of mice challenged with CS2007 or CS2022. *P < 0.05, **P < 0.01, ***P < 0.001.

not stable, but as a positive regulator of constant acid synthesis in both strains. It thus appears unlikely that the inability to synthesize colonies and clumps for restoration of the virulence of CS2022. In addition, in serum of Typh, a capsular polysaccharide (A antigen) synthesis is induced in a *hlyA*-independent manner [3]. The point is that an attenuation of virulence of CS2007 or CS2022 was clearly independent of its type of challenge or mucous phenotype, respectively (Fig. 2 and 3). Therefore, even if CS2007 or CS2022 loses the ability to synthesize flagella or gains the ability of normal bacterial cell division in a host, respectively, the revertant strain would not restore the virulence.

If we now consider CS2022, we noticed the infection in mice longer than did CS2007 (Fig. 1). In other words, it was not easy to clean the bacteria of CS2022, and it was somewhat easier to clean the bacteria of CS2007 in mice. This could be due to the cell volume of CS2022 being approximately 50-fold larger than that of the wild-type strain. Therefore, immunization with CS2022 might result in more severe Typhimurium LPS-specific IgG1 production in serum and S-IgA production in bile than would immunization with CS2007 (Fig. 4). However, there was no difference between CS2007 and CS2022 in the amounts of the intestinal washes containing S-IgA (Fig. 5) and the amount of IgG1 in sera after the challenge (Fig. 7) in the immune mice. Nevertheless, this both is sufficient to completely protect from acute challenge of virulent serovar Typhimurium and colonized CS2007 or CS2022 in even fewer than 5 days after the challenge (Fig. 6). We believe that the S-IgA on the mucosal wall is induced by vaccination mainly prevented the infection by the virulent strain and that simultaneously the

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Protection in a gerbil model of amebiasis by oral immunization with *Salmonella* expressing the galactose/ N-acetyl D-galactosamine, inhibitable lectin of *Entamoeba histolytica*

Barbara J. Mann*, Burr V. Burkholder and Lauren Ashley Lockhart

Infection with the enteric parasite Entamoeba histolytica can result in colitis and dysentery as well as abscesses at extra-intestinal sites. An effective vaccine must be able to protect against both mucosal and systemic disease. In this study an attenuated Salmonella strain that expressed a portion of the GalNAc lectin of E. histolytica was used to orally immunize gerbils. Animals were challenged by intraperitoneal injection of amebic trophozoites. A significant decrease in size of amebic liver abscesses was observed in orally immunized animals. Oral immunization with a Salmonella-based vaccine was as effective as systemic immunization for protection against systemic challenge. © 1997 Elsevier Science Ltd.

Keywords: *Salmonella*; *Entamoeba histolytica*; vaccine

An effective vaccine against an enteric pathogen must be able to stimulate the gut-associated lymphoid tissues (GALT). Since systemic immunization may not adequately stimulate protective responses by the GALT, several types of oral vaccines that would be more effective in stimulating mucosal responses are under development. These include non-viable carriers such as microspheres, liposomes, adjuvants like cholera toxin B subunit, and viable carriers such as attenuated bacteria and viruses.

Salmonella vectors have been demonstrated to be an effective means of delivering foreign antigens to the GALT¹⁻⁴. *Salmonella* colonizes and invades Peyer's patches which are part of the GALT and can also invade across the intestinal epithelium and cause infection in the spleen and liver. Cellular and humoral responses to foreign antigens expressed in *Salmonella* have been detected after oral immunization with recombinant attenuated strains^{5,6}. An oral live attenuated *Salmonella typhi* vaccine for typhoid fever is currently in use for humans⁷.

One disease candidate for the development of a protective oral vaccine is amebiasis. Amebiasis is caused by the enteric parasite *Entamoeba histolytica*. The primary manifestations of symptomatic infection are dysentery

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and colitis and in about 10% of cases amebic liver abscess. Infection with *E. histolytica* has been estimated to cause up to 50,000 deaths each year⁸. The development of an effective vaccine could theoretically result in the complete elimination of this organism as an agent of disease since humans are the primary reservoir and there are no other animal or insect intermediaries in the life cycle.

Central to the pathogenesis of *E. histolytica* is the adherence of amebic trophozoites to intestinal mucosa. A galactose/N-acetyl-D-galactosamine-inhibitable (GalNAc) lectin mediates adherence and contact dependent cytotoxicity *in vitro* to a variety of target cells including the physiologically relevant targets of human colonic mucosa and colonic epithelium^{9,10}. The GalNAc lectin is a 260 kDa heterodimeric molecule consisting of 170 and 353 kDa subunits^{11,12}.

The GalNAc lectin has been shown to be a protective antigen in a gerbil model of amebic liver abscess^{13,14}. Immunization with a portion of the extracellular cysteine-rich region of the 170 kDa subunit appears to be as protective as immunization with the native protein^{15,16}. The portion of the cysteine-rich region used in these vaccine trials has been shown to contain epitopes recognized by adherence-inhibitory monoclonal antibodies¹⁷ and also has been implicated as the region of the molecule responsible for TNF- α and nitric oxide production by activated macrophages^{18,19}.

The purpose of this study was to determine if oral immunization with an attenuated strain of *Salmonella*, which expressed a portion of the 170 kDa subunit of the

GalNAc lectin, could protect against liver abscess formation in the gerbil model of amebiasis. A decrease in the size of amebic liver abscesses and the number of animals infected was observed in gerbils orally immunized with GalNAc lectin-expressed in *Salmonella*. Interestingly, gerbils orally immunized with a *Salmonella*-based vaccine was as effective as systemic immunization with the GalNAc lectin for protection against a systemic challenge.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

Salmonella dublin live vaccine strain SL5928, *ura-1/uvr* β /Ct1, Tn10 and *S. typhimurium* LB5000 *hsdSR121* *lee-1121*, were kindly donated by R.A.D. Stocker, Stanford University²⁰. The host strain for pGEX vectors (Pharmacia Biotech, Uppsala, Sweden) was *Escherichia coli* MC1061. JM109 was the host strain used to express proteins generated from pRSET vectors (Lifetech, San Diego, CA). All bacterial strains were grown in Luria Broth (LB). The concentration of antibiotic, when added, was 50 μ g ml⁻¹ for ampicillin, or 40 μ g ml⁻¹ for spectinomycin. Plasmid pADE171, which encodes spectinomycin resistance²¹, was kindly supplied by Eric Elsinghorst, Naval Medical Research Institute.

Expression and purification of recombinant proteins

Recombinant lectin proteins encoded by pGEX plus plasmids were induced and expressed according to the procedure described by Smith and Johnson²². Induction and expression of recombinant proteins encoded by pRSET plasmids was carried out according to the manufacturer's instructions.

Recombinant proteins from induced bacterial cultures were separated by electrophoresis on 10% SDS polyacrylamide gel electrophoresis (PAGE) overnight at 4°C. Gels were briefly stained with 0.5% Coomassie Blue in acetic acid/isopropanol/water (1:1:6) and the recombinant protein band excised. The protein was eluted and concentrated from the gel using an Amicon centrifugal (Haverly, MA). Protein concentration was determined using BCA protein assay system (Pierce, Rockford, IL). The eluted protein was incubated with anti-lectin antibodies on immunoblots to confirm identity.

Immunoblotting

Proteins, separated by electrophoresis on 10% SDS-PAGE, were electrophoretically transferred to a polyvinylidene difluoride (Immobilon-P[®] membrane (Millipore Corp., Bedford, MA) at 4°C for 2 h at 0.5 mA in 20% methanol 25 mM Tris base-192 mM glycine. After transfer, the membranes were blocked in 3% bovine serum albumin in blot wash buffer (HBW) (50 mM Tris base, 200 mM NaCl, 0.1% Tween 20) at 4°C overnight. Membranes were incubated with pooled protein A-purified gerbil sera (50 μ g ml⁻¹) or rabbit anti-lectin polyclonal antibody for 1 h at room temperature, washed, then incubated with protein A-conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Bound protein A-HRP was visualized using chemiluminescent detection as recommended by the manufacturer (LumiGLO, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

Construction of a *Salmonella* strain expressing fragment A of the GalNAc lectin

A pAD171-based plasmid encoding fragment A, containing the *pro* promoter and fragment A of the *hgtII* gene, was first amplified by PCR using pGEX-A as a template²³. The sequence of the two primers used corresponded to pGEX2T sequences located upstream of the *pro* promoter or downstream of the 3' end of fragment A, and included extra sequences that specified a *PstI* site. The sequence of the sense primer was GGGCGGC TGAAGACGCAATTAAATGAGT and the sequence of the anti-sense primer was GGGCGGCTGC AGCTTACAGACAACTGTGTA. PCR was performed using the conditions recommended by the manufacturer (Perkin Elmer, Cetus, Norwalk, CT) and incubated for 30 cycles at 94°C for 1 min, 48°C for 1 min, 72°C for 3 min. The reaction was preceded by a 5 min incubation at 94°C, and terminated with an incubation at 72°C for 15 min. The PCR fragment was digested with *PstI* and ligated to *PstI*-digested pAD171. The recombinant plasmid was first selected in *Es. coli* strain MC1061. The purified plasmid was then introduced into *S. typhimurium* strain LB5000 so that the plasmid DNA would be properly modified for introduction into *Salmonella* strains. Plasmid DNA isolated from LB5000 was introduced into *S. dublin* strain SL5928 by electroporation. Expression of the lectin fragment encoded by pADE-A in SL5928 was verified by Western blots of bacterial lysates and anti-170 kDa GalNAc lectin subunit antibody.

Immunization and challenge protocol

For intraperitoneal (i.p.) immunizations animals were injected with 30 μ g of electro-eluted fragments of the 170 kDa subunit or glutathione S-transferase (GST) in complete Freund's adjuvant. Four weeks later animals received booster immunizations with 30 μ g of antigen in incomplete Freund's. For oral immunization animals were fed 10⁸ 10¹⁰ bacteria on days 1, 5, and 7 by oral gavage. Six weeks after the first immunization the animals were challenged by direct intraperitoneal injection with 5 $\times$ 10⁵ amebic trophozoites^{23,24}. Fourteen days post-challenge the gerbils were killed and any liver abscess was dissected away from the remaining liver tissue and weighed. Each abscess was cultured for *E. histolytica* in TYI medium²⁵. Results were analyzed using Student's *t*-test.

RESULTS

Intraperitoneal immunization with purified 170 kDa polypeptides

To test the ability of different regions of the 170 kDa subunit to elicit protection against amebic liver abscess formation portions of the 170 kDa subunit, fragment A (containing amino acids 482-1138 of the cysteine-rich region) and fragment C (amino acids 2-996) were expressed in *Es. coli* and purified as described in Materials and Methods. Gerbils were immunized by i.p. injection with the purified recombinant lectin polypeptides, then challenged by intraperitoneal injection of amebic trophozoites and analyzed for the presence and size of an amebic liver abscess 14 days later. The results of three

Table 1 Vaccine trials with i.p. immunization purified bacterin preparations

Immunogen	Abscess weight (g)*	
Experiment 1 Control (CS1) Fragment A	4.70±1.71 (n=1) 2.75±1.98 (n=2)	(100%) [†] (100%) [†] P=0.11*
Experiment 2 Control (CS2) Fragment A Fragment AC	1.44±1.64 (n=1) 0.18±0.10 (n=2) 0.29±0.29 (n=3)	(71%) (100%) (100%) P<0.000 P<0.018
Experiment 3 Control Fragment AC Fragment AC A	1.95±2.82 (n=10) 0.029±0.083 (n=5)	(100%) (55%) P<0.002
Total Control Fragment A	2.74±2.47 (n=24) 0.78±1.44 (n=25)	(95.8%) (80%) P=0.011

*Mean S.D.: number of animals; [†]wt were calculated to compare the differences in liver abscess size of bacterin-treated animals with control animals; Values in parentheses are percentages of animals with abscesses

independent trials are presented in Table 1. In Experiment 1, 3 animals were immunized with a polyhistidine fusion version of fragment A that was encoded by an PRSET vector containing fragment A. In Experiment 2 animals A and C, however, there was no significant additional decrease in amebic liver abscess size compared to animals immunized with fragment A alone. Since the addition of fragment C did not enhance protection, further studies with *Salmonella* expression were confined to fragment A.

Construction of *Salmonella* expressing the GalNAc lectin

The first step in testing the effectiveness of an oral anti-amebic *Salmonella* vaccine was to create a stable recombinant strain expressing fragment A of the 170 kDa subunit. Initially a pGEX plasmid containing the DNA encoding fragment A was inserted into plasmid vector pADE171¹¹, creating pADE-A. This plasmid was introduced into *S. dublin* strain, SL5928 creating strain SL5928-A. The expression of fragment A in SL5928-A was verified by Western blots of bacterial lysates using anti-170 kDa subunit antibody (Figure 1). A strain carrying the parent plasmid, pADE171, was also created and designated SL5928-171.

To test the stability of the fragment A expression in SL5928-A, *in vitro* overnight culture, grown in the presence of antibiotic, was diluted 1:500 in LB without antibiotic and grown for an additional 24 h. This dilution procedure was repeated for five consecutive days using the previous day's culture. The percentage of spectinomycin resistant bacteria was determined by plating the bacteria on LB and LB plus spectinomycin plates each day. The percentage of spectinomycin resistant bacteria dropped to 60% on day 2 and by day 6 it had dropped to 23%. Fragment A protein was detectable by immunoblotting in cultures grown up to four days without antibiotic (data not shown).

In vivo stability of the expression of fragment A in SL5928-A was tested by examining the bacteria isolated

Table 2 Vaccine trials with oral immunization with attenuated *Salmonella* expressing fragment A of the 170 kDa subunit

Immunogen	Abscess weight (g)*	
Experiment 4 5928-171 5928-A	0.56±0.32 (n=5) 0.086±0.132 (n=5)	(80%) [†] (50%) P=0.016
Experiment 5 5928-171 5928-A	0.50±0.62 (n=8) 0.045±0.048 (n=8)	(87.5%) (56.2%) P=0.059
Experiment 6 5928-171 5928-A	0.52±0.51 (n=13) 0.061±0.10 (n=13)	(94.6%) (80.3%) P=0.001

*Mean S.D.: n=number of animals; Values in parentheses are percentages of animals with abscess; [†]wt were calculated to compare the differences in liver abscess size of animals fed lectin expressing bacteria and those fed control bacteria

1 2 3 4 5 6



Figure 2 Western blot of pooled gerbil antisera from vaccine trial No. 5 with reduced native GalNAc lectin. Lane 1, pre-challenge sera from SL5928-A immunized animals; lane 2, post-challenge sera from SL5928-A immunized animals; lane 3, pre-challenge sera from SL5928-171 immunized animals; lane 4, post-challenge sera from SL5928-171 immunized animals; lane 5, rabbit anti-GalNAc lectin antibody; lane 6, no primary antibody incubated with protein A-HRP only

chemiluminescent substrate. No anti-GalNAc lectin antibody could be detected in pre-challenge sera from animals orally immunized with SL5928-A (Figure 2, lane 1). In addition no anti-GalNAc lectin antibody could be detected in pre-challenge sera from SL5928-A immunized animals by ELISA (data not shown). Anti-GalNAc lectin antibody reactivity was observed in post-challenge sera from both SL5928-171 and SL5928-A immunized animals. It should be noted that pre-challenge sera from SL5928-A immunized animals did recognize total *Salmonella* proteins on Western blots whereas pre-immune gerbil sera did not (data not shown).

DISCUSSION

This study has demonstrated that oral administration of attenuated *Salmonella*, expressing a portion of the

GalNAc lectin, can provide protection against amebic liver abscess formation in the gerbil model of amebiasis. A *S. dublin* strain of bacteria was created that expressed a portion of the cysteine-rich region of the GalNAc lectin designated fragment A. Protection against amebic liver abscess formation was observed in animals that had been orally immunized with fragment A-expressing bacteria. An oral vaccine for amebiasis has the potential to be more effective than a systemic vaccine since the primary site of protection against infection, with *E. histolytica* is at the intestinal mucosal surface. Even though we did not study the mucosal immune response via the mucosal route it was effective in preventing systemic disease.

Previous vaccine trials have demonstrated that systemic immunization with the GalNAc lectin confers protection in the gerbil model of amebiasis. Putri and Raudon first identified the GalNAc lectin as a protective antigen by i.p. immunization with native heterodimeric protein that had been purified using monoclonal antibody affinity chromatography¹². Protection was observed in 67% of immunized animals. Zhang and Stanley have reported that i.p. immunization with a portion of the cysteine-rich region of the 170 kDa subunit conferred protection against liver abscess formation in gerbils with a vaccine efficacy of 81%. In a similar study Soong *et al.* observed a significant number of gerbils protected against liver abscess formation with i.p. immunization with the cysteine-rich region. Their vaccine efficacy was 71%. In this study there was no significant difference in abscess size in immunized uninfected animals when compared to controls¹³. The cysteine-rich region used by Zhang and Stanley corresponds to amino acids 649-1201. Soong *et al.* used amino acids 767-1138, while amino acids 483-1118 of the cysteine-rich region were used in this study¹⁴. The lack of complete protection observed in these experiments and the inability to detect antibody may be due to the low level of infectivity of the bacteria in the spleen and liver and/or instability of the plasmid. The plasmid appeared to be somewhat unstable in culture and the number of spectinomycin resistant bacteria recovered from the liver and spleen was low. However, all of the spectinomycin resistant bacteria isolated from the spleen and liver that were tested still expressed fragment A, indicating that the plasmid was not substantially rearranged.

The cysteine-rich region of the 170 kDa subunit has been shown to be the immunologically reactive portion of the protein. Six different monoclonal antibodies that recognize functionally distinct epitopes located within a portion of the cysteine-rich region containing amino acids 597-1138 have been shown to neutralize the virulence traits of adherence, contact-dependent cytotoxicity, and amebic resistance to lysis by complement components C5b-9¹⁵. This region has also been implicated in stimulating cell mediated immune responses. The GalNAc lectin has been shown to stimulate TNF- α in naive macrophages and to stimulate nitric oxide production and amebicidal activity by activated macrophages *in vitro*¹⁶. This stimulatory activity was blocked by some of the monoclonal antibodies that recognize amino acids 597-1138 of the cysteine-rich region. The cysteine-rich region and peptides derived from this region have also been shown to cause proliferation of lymphocytes from patients recovered from amebic disease¹⁷.

Anti-lectin antibodies were not detected by Western blots of reduced GalNAc lectin in pre-challenge immune sera from gerbils orally immunized with *Salmonella* expressing fragment A. The lack of an antibody response to antigens introduced by oral immunization with recombinant strains of *Salmonella* has been observed for several other antigens.¹⁻⁴ These results suggest that a humoral response was not primarily responsible for protection in this model system. Previous vaccine trials have found no correlation between pre-challenge antibody titer and protection.^{4,11} However, our inability to detect anti-GalNAc lectin antibody could have been due to the protein A purification of the gerbil sera. Protein A purification was found to be necessary in order to reduce the background and visualize a specific signal. Protein A preferentially binds to specific antibody isotypes and isotype binding can also vary depending upon the animal species. If the response was primarily of antibody isotype that did not bind well to protein A these antibodies would have been lost during purification and therefore go undetected. Improved methods for detecting gerbil antibodies should alleviate this problem. The nature of the protective immune response in the gerbil model is clearly still a problem that needs to be resolved.

This work has demonstrated that oral immunization with *Salmonella* expressing the GalNAc lectin has potential as a protective vaccine. Future work will include creating a more stable infective strain, increasing the expression level of the lectin fragment in bacteria, defining the nature of the protective immune response, and developing a mucosal model of disease.

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