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Expression of recombinant *Bordetella pertussis* filamentous hemagglutinin (FHA) antigen in Live Attenuated *Salmonella typhimurium* Vaccine Strain

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Abstract

Filamentous hemagglutinin (FHA) is considered as an essential immunogenic component for incorporation into acellular vaccines against *Bordetella pertussis*, the causative agent of whooping cough. Classically, antipertussis vaccination has employed an intramuscular route. An alternative approach to stimulate mucosal and systemic immune responses is oral immunization with recombinant live vaccine carrier strains of *Salmonella typhimurium*. An attenuated live *Salmonella* vaccine strain (*Δcya Δcrp*) expressing recombinant FHA (rFHA) was developed. Stable expression of rFHA was achieved by the use of balanced-lethal vector-host system, which employs an *asd* deletion in the host chromosome to impose an obligate requirement for diaminopimelic acid. The chromosomal *Δasd* mutation was complemented by a plasmid vector possessing the asd^+ gene. A 3 kb DNA fragment encoding immuno dominant region of FHA was subcloned in-frame downstream to the ATG translation initiation codon in the multicopy Asd^+ pYA3341 vector to create pYA3457. *Salmonella* vaccine harboring pYA3457 expressed approximately 105 kDa rFHA protein. The 100% maintenance of pYA3457 in vaccine strain was confirmed by stability examinations. Additionally, a recombinant plasmid pYA3458 was constructed to overexpress His (8X)-tagged rFHA in *Escherichia coli*. His-tagged rFHA was purified from the *E. coli* strain harboring pYA3458 using Ni^{2+}-NTA affinity purification system.

Key words – *Salmonella, Bordetella,* vaccine, immune response, pertussis

Introduction

Salmonella enterica serovar Typhimurium (*Salmonella typhimurium*) causes self-limiting gastroenteritis in humans and a typhoid-like systemic disease in mice. Orally administered *S. typhimurium* colonizes the gut-associated lymphoid tissue (Peyer's patches) and the secondary lymphatic tissues including the liver and spleen, and elicits anti-*Salmonella* immune responses during pathogenesis [6,10]. The immune responsiveness of orally administered *Salmonella* has been applied to develop live attenuated oral *Salmonella* vaccines [5]. In general, recombinant *Salmonella* vaccines are constructed by introduction of mutations in the genes required for virulence, such as the genes for adenylate cyclase (*cya*) and cyclic AMP receptor protein (*crp*) [5]. Crp is a global regulator involved in a variety of biological functions including carbohydrate utilization [1]. Chromosomal deletions (*Δ*) of *cya* and *crp* render *S. typhimurium* completely avirulent in mice [5]. These attenuated strains can then be used to deliver foreign antigen.

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The gene for aspartate β-semialdehyde dehydrogenase (*asd*) is an essential gene for bacteria *in vivo* and is the basis for the balanced-lethal host-vector system that has been successfully used to express recombinant antigens from plasmids (Asd^+ plasmids) that are retained *in vivo* in *asd* gene deleted *Salmonella* vaccine strains without the need of antibiotic selection pressure [11]. This is important, since governmental regulatory agencies do not permit expression of drug resistance genes in live bacterial vaccines. As *Δcrp* and *Δasd* mutations are frequently introduced to construct *Salmonella* live vaccine strains, a simple and efficient strategy to incorporate such mutations lacking antibiotic resistance markers into other *Salmonella* strains has been developed by our research group [7].

Bordetella pertussis is the primary etiologic agent of human pertussis or whooping cough [13]. Although whooping cough is considered classically a childhood disease [12], it can occur at any age [14]. As a highly contagious disease, whooping cough is spread by aerosols or direct contact with nasopharyngeal secretions of an infected individual. The conventional pertussis vaccines are whole cell vaccines which are prepared from suspensions of *B. pertussis* cells inactivated by physical and chemical treatments. Whole cells vaccines have been associated with several types of deleterious effects, such as erythema and neurologic side effects [4]. The demand for a new vaccine with minimal adverse reactions was the force behind the development of purified component (acellular) pertussis vaccines. Because *B. pertussis* virulence factors, pertussis toxin, filamentous hemagglutinin (FHA), pertactin and fimbriae are known as protective antigens against pertussis infection, these factors are now widely employed in the manufacture of acellular pertussis vaccines. FHA is a rod-shaped, nontoxic cell surface associated protein which agglutinates erythrocytes from various animal species [2].

In this report, I describe the construction of a multivalent-recombinant oral live *S. typhimurium* vaccine strain which stably expresses an immuno dominant region of *B. pertussis* FHA, and the purification of rFHA. The DNA encoding this fragment was cloned into a high-copy-number Asd^+ vector (pUC replicon based) in the avirulent *Δcya Δcrp Δasd S. typhimurium* χ8296.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. typhimurium* cultures were grown at 37℃ in Lennox broth or L-agar [8]. When required, tetracycline was added to culture media at 15 μg/mℓ. Diaminopimelic acid (DAP) (Sigma, St Louis, MO) was added (50 μg/mℓ) for the growth of Asd^- strains [11]. *Salmonella* strains were grown with aeration from a nonaerated static overnight culture. For the induction of rFHA expression, IPTG (1 mM) was added into the 0.5 OD_{600} culture and then continued the culture for 4 h.

General DNA procedures

DNA manipulations were carried out as described in the procedures of Sambrook *et al.* [16]. Transformation of *E. coli* and *Salmonella* was done by electroporation (Bio-Rad, Hercules, CA). Transformants containing Asd^+ plasmids were selected on L agar plates without DAP. Only clones containing the recombinant plasmids were able to grow under these conditions. PCR amplification was employed to obtain DNA fragments for cloning and for verification of chromosomal deletion mutations. The PCR conditions were as follows: denaturation at 95℃ for 20 sec; primer annealing at 55℃ for 20 sec; polymerization at 72℃ for 2 min; and a final extension at 72℃ for 10 min. Nucleotide sequencing reactions were performed using ABI prism fluorescent Big Dye Terminators according to the manufacturers instructions (PE Biosystems, Norwalk, Conn.).

Table 1. Bacterial strains, and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
χ 6212	F⁻ Φ80 (*lacZYA-argF*) *endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ *asdA4*	11
S. typhimurium χ 8296	SL1344 *hisG* Δ*crp-28* Δ*asdA16* Δ*cya-27 cfs*	Megan Health Inc.
Plasmids		
pYA232	Low-copy-number (pSC101 *ori*) plasmid containing *lacI*^{q} repressor gene;Tc^r	11
pYA3341	Asd$^+$, pUC*ori*	Lab collection
pYA3457	2.9 kb *B. pertussis fha* gene was cloned into pYA3341 Asd$^+$ (pUC *ori*)	This study
pYA3458	2.9 kb PCR-amplified *B. pertussis fha* gene into pYA3341 Asd$^+$ (pUC*ori*); His (8X)-tagged rFHA expression	This study
pYAP18	*B. pertussis fha* in pYA292	Lab collection

[a]Tc, tetracycline.

SDS-PAGE and Immunoblot analysis

Protein samples were boiled with loading buffer for 5 min and then separated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie brilliant blue R250 (Sigma) staining. For immunoblotting, proteins separated by SDS-PAGE were transferred eletrophoretically to nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumin in 10 mM Tris-0.9% NaCl (pH 7.4) and incubated with mouse monoclonal antibody specific for FHA (12H3C) [3] and then with a peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad). Immuno-reactive bands were detected by the addition of 4-chloro-1-naphthol (Sigma) in the presence of H_2O_2. The reaction was stopped after two minutes by washing with several large volumes of deionized water.

Results and Discussion

Construction of the rFHA-expressing plasmid

A highly immunogenic domain of FHA (970 amino acid residues at the C-terminus of the mature FHA) was selected to use as a test antigen in antigen delivery by a *Salmonella* carrier. A 2,910 bp *Xho*I-*Pst*I DNA fragment of the *fha* gene of *B. pertussis* was isolated from the plasmid pYAP18 and then cloned into *Sal*I and *Pst*I sites of pYA3341 plasmid, resulting in pYA3457 (Fig. 1). The recombinant plasmid was confirmed by restriction enzyme digestion analysis with *Eco*RI and *Pst*I. The in-frame cloning of the rFHA was confirmed by nucleotide sequencing. Transcription promoted by Ptrc promoter can be stopped by the 5ST1T2 transcriptional terminator.

Expression of rFHA in *E. coli* and *S. typhimurium*

To observe rFHA expression, plasmid pYA3457 was introduced into *E. coli* χ 6212 and *S. typhimurium* χ 8296 (Δ*crp* Δ*asdA16*) vaccine strain. Expression of rPspA was monitored by comparing the protein profiles of strains harboring pYA3457 (rFHA) and strains harboring pYA3341 (vector alone). Both *E. coli* χ 6212 and *S. typhimurium* χ 8296 harboring pYA3457 expressed rFHA as approximately 106 kDa which is agreed to calculated size of the rFHA (Fig. 2). Westernblot analysis with the FHA specific monoclonal antibody 12H3C confirmed that the expressed

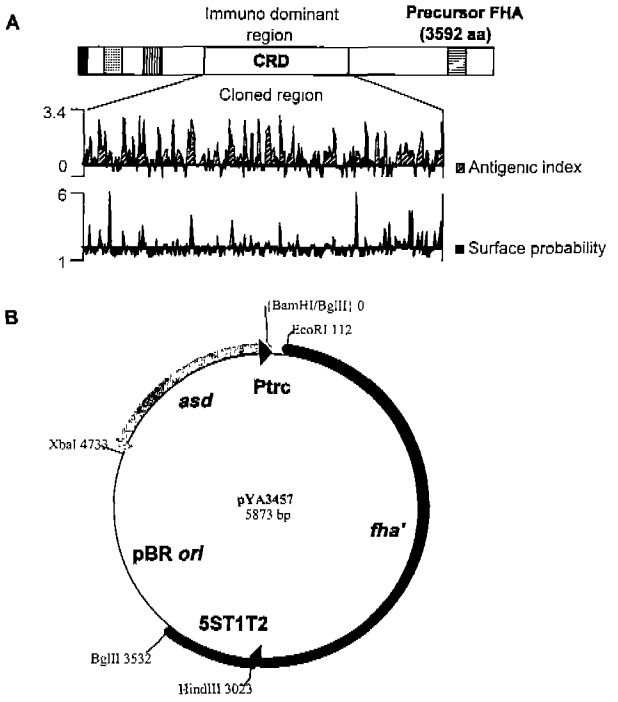

Fig. 1. Recombinant plasmid pYA3457 for rFHA expression. A. FHA region used in this study. Functional domains of native FHA from *B. pertussis* are diagramed; black box, signal sequence; dotted box, A repeat; vertical lined box, B repeat; gray box, immuno dominant region; horizontal lined box, proline-rich region. Bioinformatical analyses of the rFHA are presented. Analyses were performed with the Protean module of the Lasergene sequence analysis software. B. The map of recombinant plasmid pYA3457. The cloned fragment included the immunogenic region of FHA. CRD, carbohydrate recognition domain.

106 kDa protein is the rFHA derived from pYA3457. In the analyses of Coomassie blue-stained SDS-PAGE, the amount of rFHA was very low (Fig.). Although *fha* gene is derived from the high G+C content (70%) *Bordetella* species, there was no apparent rare codons within the *fha* gene. As expected, the rFHA expressed in χ8296 vaccine strain was not secreted into the culture supernatant (data not shown). By comparison of rFHA expression in *E. coli*, the *Δcya Δcrp-28* contained in χ8296 did not influence the expression of rFHA in the *Salmonella* vaccine strain. To examine the stability of plasmid pYA3457 in *Salmonella* χ8296 *in vitro*, the χ8296 cells containing pYA3457 were cultured with daily

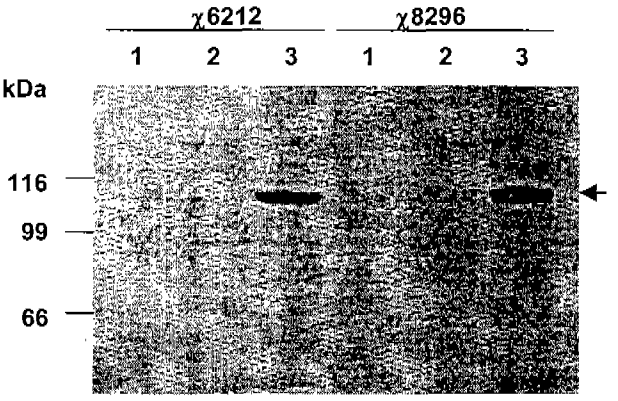

Fig. 2. Expression of rFHA in both *E. coli* and *S. typhimurium*. Protein samples were prepared from *E. coli* χ6212 and *S. typhimurium* χ8296 cells grown in LB at 37°C by the procedures described in Materials and Methods. Fractions equivalent to 30 ㎖ volume of 0.8 OD_{600} culture were analyzed by SDS-PAGE and the rFHA was detected by immunoblot with FHA specific monoclonal antibody 12H3C. Standards are indicated to the left. Lanes; 1, without any plasmid; 2, pYA3341 (vector control); 3, pYA3457. Arrow indicates the rFHA.

passage for five consecutive days in L broth containing DAP. All of examined *Salmonella* χ8296 clones (300 clones/day) kept the Asd^+ plasmid pYA3457, indicating that pYA3457 was very stable in the χ8296 vaccine strain. Cells obtained from the last day culture of the stability test expressed similar amounts of the 105 kDa rPspA compared to those from the first day (data not shown), suggesting stable expression of rFHA without rearrangements.

Regulated expression of rFHA

To examine the regulated expression of rFHA, a plasmid pYA232 carrying $LacI^q$ repressor was introduced in the *E. coli* χ6212 harboring pYA3457 for the tight repression of the P_{trc} promoter activity on pYA3457. The rFHA production was induced by adding 1 mM IPTG into the broth culture. After 4h induction, cells were harvested and subjected to SDS-PAGE and Westernblot analysis (Fig. 3). In contras to the samples grown in absence of IPTG, only sample grown in the presence of IPTG inducer produced 105 kDa rFHA protein, sug-

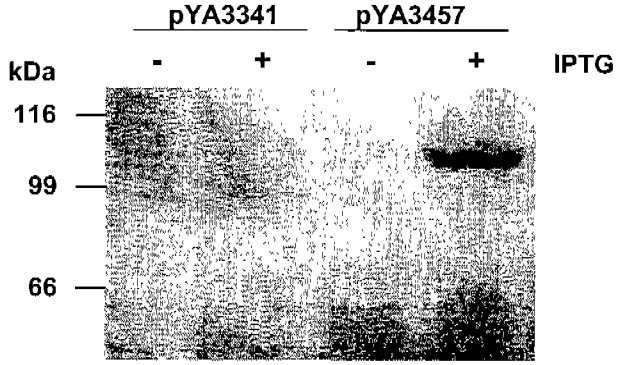

Fig. 3. Regulated expression of rFHA.
Plasmids pYA3341 (vector) and pYA3457 were introduced into *E. coli* χ6212 harboring pYA232 (*lacI*^{q}). IPTG dependent rFHA expression was examined in the culture with (+) or without (-) IPTG. For the derepression of LacI, 1 ㎖ IPTG was treated for 4h at 37°C. The rFHA was detected by immunoblot. Standards are indicated to the left.

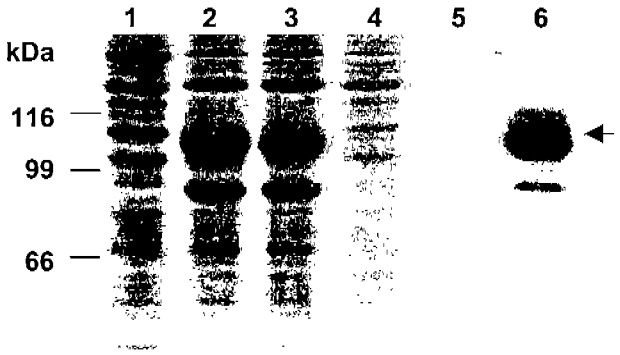

Fig. 4. Purification of rFHA by Ni^{2+}-affinity system.
E. coli χ6212 harboring pYA3458 was cultured in 500 ㎖ LB at 37°C. According to manufacturers protocol (Qiagen, Valencia, Calif.), rFHA protein was purified by an affinity purification system basis on the interaction between histidine tagged rFHA and Ni^{2+} charged support. Fractions were subjected to SDS-PAGE analysis. The gel was stained with Coomassie brilliant blue. Molecular markers are indicated to the left. Purified rFHA proteins are indicated. Lanes; 1, negative control; 2, total proteins; 3, cytoplasm; 4, column flow-through; 5, after wash column; 6, purified protein with 250 mM imidazole.

gesting that the transcription of *fha* is under the control of P_{trc} promoter.

Purification of the rFHA

For overexpression of histidine (8X)-tagged rFHA, a fragment of the *fha* gene of *B. pertussis* was PCR-amplified from pYAP18 template DNA using a pair of primers ([N-terminal] 5ATGGGAATTCGCCATCACCATCACCATCACC ATCATTCCACCATCGAG3, and [C-terminal] 5AGCGATGGATCCAACTCGTT CGTCAAGGCG3). The 0.4 kb *Eco*RI-*Xho*I PCR-amplified fragment was replaced with the 0.4 kb *Eco*RI-*Xho*I fragment of pYA3457, resulting in pYA3458 (Table 1). The N-terminal primer contains an *Eco*RI site and eight consecutive histidine codons (alternate use of CAT and CAC) for histidine (8X) tagging at the N-terminus. In-frame cloning was confirmed by nucleotide sequencing. *E. coli* χ6212 harboring pYA3458 expressed a high amount of histidine (8X)-tagged rFHA. According to manufacturers protocol (Qiagen, Valencia, Calif.), rFHA protein was purified by an affinity purification process with Ni^{2+}-nitrilotriacetic acid-agarose support (Fig. 4). No or little contaminated protein species were observed in the purified protein fraction. Approximately 3 ㎎ of rFHA was purified from 500 ㎖ culture. Immunoblot with a 12H3C FHA monoclonal antibody confirmed the purified proteins as rFHA.

The *S. typhimurium* vaccine χ8296 expressing rFHA antigen can be used in the study examining immune responses after oral immunization. There are several advantages in the use of *Salmonella* vaccine-mediated foreign antigen delivery, such as fear free in vaccination, cost effective in vaccine preparation, omitting antigen purification steps and adjuvant activity by *Salmonella* carrier itself. Additionally, the ability of live recombinant *Salmonella* vaccines to colonize the gut-associated lymphoid tissue (Peyer's patches) and the deep tissues following oral administration is beneficial in that it elicits all arms of the immune response, including mucosal, humoral and cellular immunities [9]. Because *B. pertussis* colonizes the ciliated epithelium of the human upper respiratory tract [15,17], strong secretory IgA-mediated mucosal immunity is preferred for the pertussis vaccine to prevent colonization in respiratory mucosal. Accordingly,

the *Salmonella*-rFHA vaccine constructed in this study has a potential to be developed as pertussis vaccine. Using the *Salmonella*-rFHA vaccine constructed in this study, immune response to the delivered rFHA will be examined after oral immunization in mice. The purified rFHA antigen proteins will be used to coat microtiter plates in the ELISA analysis.

References

1. Botsford, J. L. and J. G. Harman. 1992. Cyclic AMP in prokaryotes. *Micro. biol. Rev.* **56**, 100-122.
2. Brennan, M.J. and R.D. Shahin. 1996. Pertussis antigens that abrogate bacterial adherence and elicit immunity. *Am. J. Respir. Crit. Care Med.* **154**, 145-149
3. Brown, D.R. and C.D. Parker. 1987. Cloning of the filamentous hemagglutinin of *Bordetella pertussis* and its expression in *Escherichia coli*. *Infect Immun.* **55**, 154-161.
4. Cherry, J.D. 1996. Historical review of pertussis and the classical vaccine. J. *Infect. Dis.* **174**, 259-263.
5. Curtiss, R. III, T. Doggett, A. Nayak and J. Srinivasan. 1996. Strategies for the use of live recombinant avirulent bacterial vaccines for mucosal immunization, pp. 499-511. *In* Kiyono, H. and M. F. Kagnoff, (eds.), *Essentials of mucosal immunology*, Academic Press, San Diego, CA.
6. Gálan, J. E. and P. J. Sansonetti. 1996. Molecular and cellular bases of *Salmonella* and *Shigella* interaction with host cells, pp. 2757-2773. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbaeger (eds.), *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed. ASM press, Washington, D. C.
7. Kang, H. Y., C. M. Dozois, S. A. Tinge, T. H. Lee and R. Curtiss III. 2001. Transduction-mediated transfer of unmarked deletions and point mutations through the use of counterselectable suicide vectors. Submitted to *J. Bacteriol.*
8. Lennox, E.S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190-206.
9. Medina, E. and C. A. Guzman. 2001. Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. *Vaccine* **19**, 1573-1580.
10. Mittrucker, H. W. and S. H. Kaufmann. 2000. Immune response to infection with *Salmonella typhimurium* in mice. *J. Leukoc. Biol.* **67**, 457-463.
11. Nakayama, K., S. M. Kelly and R. Curtiss III. 1988. Construction of an Asd^+ expression-cloning vector: Stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *Bio/ Technology* 6, 693-697.
12. Olson, L. C. 1975. Pertussis. *Medicine (Baltimore)* **54**, 427-469.
13. Preston, N. W. 1988. Pertussis today, pp. 1-18. *In* A. C. Wardlaw and R. Parton (eds.), *Pathogenesis and immunity in pertussis*, Hohn Wiley and Sons, New York, N.Y.
14. Robertson, P.W., H. Goldberg, B.H. Jarvie, D.D Smith and L.R. Whybin. 1987. *Bordetella pertussis* infection: a cause of persistent cough in adults. *Med. J. Aust.* **147**, 522-525.
15. Salyers, A.A. and D.D. Whitt. 1994. Whooping cough (*Bordetella pertussis*), pp. 157-168. *In* Bacterial pathogenesis-a molecular approach, American Society for Microbiology, Washington, D.C.
16. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. Weiss, A.A., and Hewlett, E.L. 1986. Virulence factors of *Bordetella pertussis*. *Annu. Rev. Microbiol.* **40**, 661-686.

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초록 : 약독화 *Salmonella typhimurium* 생백신 균주에서 *Bordetella pertussis*의 filamentous hemagglutinin (FHA) 항원의 발현

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백일해 원인균 *Bordetella pertussis* 에 대한 비세포성 백신과 관련하여 filamentous hemagglutinin (FHA)는 중요한 면역구성원 역할을 한다. 전통적으로 백일해 예방은 근육주사를 통하여 수행되어왔으나, *Salmonella typhimurium* 이 중재하는 경구투여 재조합 생백신은 점막을 비롯한 면역 반응계를 유도할 수 있는 또 하나의 방법이 될 수 있기에, 약독화 *Salmonella* 백신주 (*Δcya Δcrp*) 에서 재조합 FHA (rFHA)이 안정적으로 발현하는 장치를 개발하였다. 즉 염색체상의 *asd* 결손주에서 diaminopimelic acid 의 공급을 유도할 수 있는 balanced-lethal vector-host 장치를 통하여 안정된 재조합 FHA가 발현되었으며, 이는 플라스미드에 위치하는 asd^+ 유전자에 의하여 *Δasd* 돌연변이체는 정상적인 생장을 보여준 것이다. multicopy Asd+ pYA3341의 단백질 합성 개시 암호 ATG 의 downstream에 3kb의 FHA 특이항원 부위를 클로닝하여 pYA3457을 제작하였으며, 이 플라스미드를 소유한 *Salmonella* 백신주는 약 105 kDa 의 rFHA 를 생산하였다. 재조합 플라스미스상에서 생성된 rFHA는 100%의 효과를 나타내는 안정성을 보여주었으며, 부가적으로 재조합 플라스미드 pYA3458는 *Escherichia coli* 에서 발현의 극대를 위하여 His (8X)-tagged rFHA 상태로 제작되었으며, pYA3458 를 소유한 *E. coli* 에서His-tagged rFHA는 Ni^{2+}-NTA affinity 분리장치를 이용하여 순수 분리되었다.