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Construction of attenuated Salmonella typhimurium Strain expressing *Helicobacter pylori* conservative region of adhesin antigen and its immunogenicity

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Abstract

AIM: To construct a non-resistant and attenuated *Salmonella typhimurium* (*S. typhimurium*) strain which expresses conservative region of adhesion AB of *Helicobacter pylori* (*H pylori*) and evaluate its immunogenicity.

METHODS: The AB gene amplified by PCR was inserted into the expression vector pYA248 containing *asd* gene and through two transformations introduced into the delta *Cya*, delta *Crp*, delta *Asd* attenuated Salmonella typhimurium strain, constructing balanced lethal attenuated Salmonella typhimurium strains X4072 (pYA248-AB). Bridged ELISA method was used to measure the expression of AB antigen in sonicate and culture supernatant. According to the method described by Meacock, stability of the recombinant was evaluated. Semi-lethal capacity test was used to evaluate the safety of recombinant. The immunogenicity of recombinant was evaluated with animal experiments.

RESULTS: The attenuated *S. typhimurium* X4072 (pYA248-AB) which expresses AB was successfully constructed. Furthermore, bridged ELISA assay showed that the content of AB in recombinant X4072 (pYA248- AB) culture supernatant was higher than that was in thallus lytic liquor. And after recombinant X4072 (pYA248- AB) was cultured for 100 generations without selection pressure, the entire recombinant bacteria selected randomly could grow, and the AB antigen was defected positive by ELISA. The growth curve of the recombinant bacteria showed that the growth states of X4072 (pYA248) and X4072 (pYA248-AB) were basically consistent. The survival rate of C57BL/6 was still 100%, at 30 d after mice taking X4072 (pYA248-AB) 1.0×10^{10} cfu orally. Oral immunization of mice with X4072 (pYA248-AB) induced a specific immune response.

CONCLUSION: *In vitro* recombinant plasmid appears to be stable and experiments on animals showed that the recombinant strains were safe and immunogenic *in vitro*, which providing a new live oral vaccine candidate for protection and care of *H pylori* infection.

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INTRODUCTION

The discovery of *Helicobacter pylori* (*H pylori*) has brought about a revolution in the research of etiological factor and prevention and cure of chronic gastritis, peptic ulcer and the associated diseases[1,2]. It has been confirmed that *H pylori* is the main cause of chronic gastritis and peptic ulcer, and an important factor for the infection of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma[3-7]. In 1994, the World Health Organization has defined it as a class 1 carcinogen, and direct evidence of its carcinogenesis has recently been demonstrated in an animal model and a retrospective cohort study and nested case-control study in China[8,9]. Data of epidemiology showed that 50 percent of the population all over the world is infected with *H pylori*, and China has a high infection rate[10]. Based on it, eradication of *H pylori* is one of the main methods for preventing and treating the above-mentioned diseases. At present, the widely used method for eradication of *H pylori* in clinical practice is the antibiotic treatment, though high the eradication rate is, such problems as high expenses, yearly reduced eradication rate resulting from gradually increasing drug-resistant strains, side effects of drugs and low patient compliance have still not been solved[11-13]. Immunization against *H pylori* infection has been one of the most prospective treatments. In view of the fact that the conservative region of four confirmed adhesins is outer membrane protein and porin type component, outer membrane protein and porin are the excellent candidate components vaccination[14-16]. In addition, the attenuated Salmonella typhimurium strain expressing foreign antigen is a very hopeful new-generation of vaccine. Experiments on human body indicated that the attenuated Salmonella typhimurium strain has very good endurence and immunogenicity, which can be used to transmit foreign antigen, therefore, the problem of adjuvant as well as the problem of high cost for taking subunit protein vaccine orally are solved. At present, the research of attenuated Salmonella typhimurium strain in application to *H pylori* vaccine has been done, but non-resistant and attenuated Salmonella typhimurium strain containing the balanced lethal system has not yet been applied to the research and production of *H pylori* vaccine. We attempted to construct the non-resistance and attenuated Salmonella typhimurium strain expressing AB, and to study its biological properties to pave the way for further research of biological treatment.

MATERIALS AND METHODS

Materials

The strains and plasmids used in the experimental processes are showed in Table 1.

Table 1 Strain (plasmid) and genotype

Strain (plasmid)		Genotype	Source
E coli X6097		*Asd*$^-$	Dr. Roy Curtiss
Salmonella			
typhimurium	X3181	Wild strain	Institute for the control of biological product, Ministry of Health
	X3730	*GalE*$^-$, *hsd*$^-$, *asd*$^-$	Dr. Roy Curtiss
	X4072	*Cya*$^-$, *Crp*$^-$, *Asd*$^-$	Dr. Roy Curtiss
pET-22b (+)-AB		Amp^r, *AB*	Construction in the previous research[17-20], Dr. Roy Curtiss
pYA248		*Asd*$^+$	Construction in the research
pYA248-AB		*AB*	

Thirty specific-pathogen free male C57BL/6 mice, aged 4 wk, were purchased from Animal Center of our institute. Restriction enzymes, such as *EcoR* I, *Sal* I *etc*. and T4 DNA ligase, Vent DNA polymerase *etc*. were purchased from New England Biolabs corporation, Promega corporation and Sino-American Hua Mei Biotechnology Company, respectively. The goat anti-rabbit IgG-HRP and IgA-HRP were purchased from Sino-American Hua Mei Biotechnology Company. Our institute provided anti-AB antibody and AB antigen. DAP (50 mg/L) was purchased from Sigma corporation. Other reagents were analytically pure reagents produced in China.

Recombinant DNA techniques

The alkaline lysis method was chosen for rapid and large-scale preparations of plasmid DNA as described previously[21]. In accordance with the the gene sequence in conservative region, we designed specific primers and added appropriate restriction enzyme sites on its 5' termini, which was synthesized by Shanghai Boya *Co*. The sequences of primers were as follows: conservative region 1: 5'-CCG GAA TTC AAC GCG CTC AAC AAT CAG-3'; conservative region 2, 5'-CAC GTC GAC CTA GAA TGA ATA CCC ATA AG-3'. Conservative region 1 and conservative region 2 contained *Eco*R I and *Sal* I sites, respectively. The template was pET-22b (+) -AB. PCR was performed by the hot start method. The PCR condition was that after initial denaturing at 95 °C for 30 s, each cycle of amplification consisted of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and polymerization at 72 °C for 30 s, and a further polymerization for 10 min after 35 amplication cycles. PCR products were separated by electrophoresis analysis on a 8 g/L agarose gel. The PCR product was harvested from agarose gel, digested with *Eco*R I and *Sal* I, and inserted into the *Eco*R I and *Sal* I restriction fragments of the expression vector pYA248 using T4 DNA ligase. The resulting plasmids pYA248-AB were transformed into *E.coli* X6097, Salmonella typhimurium X3730 and X4072 one by one and positive clone was screened through assay of double restriction enzyme digestion. DNA sequence was performed with the DNA automatic sequencer.

Assay of AB protein expressed by recombiant strain

Recombinant strain S typhimurium X4072 (pYA248-AB) cells, after being inoculated in the culture medium of LB liquids, were cultured with shaking at 37 °C for 15-16 h, and then centrifuged, to collect thallus and supernatant. Thallus, after being washed with saline once and centrifuged, suspended with deionized water. The suspension was sonicated on ice for three min, and centrifuged, to obtain the supernatant. Bridged ELISA was used to measure the expression of AB artigen in sonicate and culture supernatant.

Assay of stability of recombinant strain

The experiment testing stability of recombinant strain was performed according to the method described by Meacock[37,38]. Ten percent of thallus, incubated with shaking at 37 °C overnight, was inoculated in LB culture medium containing DAP and was further cultured for 12 h, from which the another 10 percent was inoculated in LB culture medium containing DAP and cultured for another 12 h, which was continued in the same way for up to 50 h. By this time, recombinant X4072 (pYA248-AB) cells had been cultured for 100 generations, and then it was diluted to 10^6-fold. A total volume of 100 μL of the diluted solution was taken and paved on LB agar plate containing DAP. After being cultured overnight, 100 single thalluses were randomly selected and transferred on LB agar plate without containing DAP. Bacteria could not grow on agar plate without containing DAP liquids, means plasmid loss; through this method stability of recombinant plasmid was verified. Meanwhile, ELISA was used to measure the expression of AB antigen in the culture supernatant of these 100 thalluses.

Assay of growth curve of recombinant strain

Single clone *S.typhimurium* X4072 (pYA248) and *S.typhimurium* X4072 (pYA248-AB) were selected and inoculated in culture medium of LB liquid, respectively. After being cultured with shaking at 37 °C for 10 h, 50 μL of it was inoculated in 5 mL culture medium of LB liquid, followed by culture with shaking at 37 °C. The value of A_{60} was measured once every other hour, from which growth curve was drawn.

Assay of immunology of recombinant strain

Three groups of 5 mice including controls were used as follows: (1) PBS control group was non-immunized mice that received PBS; (2)Salmonella control group was immunized with attenuated *S. typhimurium* X4072 (pYA248) strain; (3) The vaccine group was immunized with *S. typhimurium* X4072 (pYA248-AB) strain expressing AB. Prior to immunizations ,the mice were left overnight without solid food and 4 h without water. A total volume of 100 μL of 30 g/L sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Immediately after stomach neutralization, mice from PBS control group received 100 μL PBS, and mice from the Salmonella control group and Salmonella vaccine group, received 1.0×10^9 colony forming units (c.f.u) of *S. typhimurium* strain X4072 (pYA248) and *S. typhimurium* strain X4072 (pYA248-AB), respectively, in a total volume of 100 μL. Water and food were returned to the mice after immunization. At 4 wk after immunization, mice were sacrificed by terminal cardiac puncture under metoxyfluorance anesthesia and the small intestines were taken from mice to prepare intestine fluid. Indirect ELISA was used to evaluate serum samples and intestine fluid from mice for AB-specific IgG or IgA. Purified *H pylori* AB was used as the coating antigen in ELISA immunoassays.

RESULTS

Construction of recombinant plasmid containing asd gene and encoding AB gene

The size of plasmid pYA248 was 3.0 kb. Promotor was Ptrc and multiclonal points included *Eco*R I *Hin* d III and *Sal* I *etc*. and pYA248 was digested with *Eco*R I and *Sal* I, and large E-S

sequences were recovered as vector. The same enzymes were used to completely digest AB PCR product and mixed with vector for connection after being recovered, and then transformed into *E. coli* X6097 by the means of $CaCL_2$. Transformer could grew on LB agar plate without containing DAP and further extracted plasmid for verification. The results of double restriction enzyme digestion of recombinant plasmid pYA248-AB are showed in Figure 1.

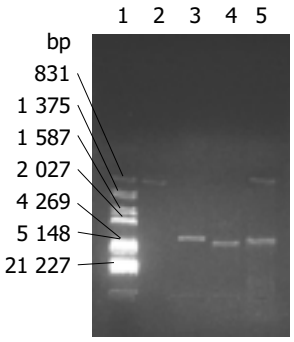

Figure 1 Double restriction enzyme digestion map of recombinant plasmid PYA248-AB. Lane1: DNA marker; Lane2: PCR product; lane3: pYA248 / *Eco*RI; lane4: pYA248-AB / *Eco*RI; lane5: pYA248-AB / *Eco*RI+*Sal*I.

Construction of recombinant strain

Recombinant plasmid extracted from *E.coli* X6097 (pYA248-AB) was transformed into *S.typhimurium* X3730 and X4072 by the transformation of electricity one by one. Owing to the difference between two kinds of *S.typhimurium*, the best transformational conditions were worked out. By adopting the first condition: cuvette gap 0.2 cm, voltage 2.5 kV, field strength 12.5 E, capacitor 25 μF, resistor 200 Ω, time constant 4.5-5.0 ms, recombinant plasmid was transformed into *S.typhimurium* X3730 and then separated from it. By adopting the second condition: cuvette gap 0.2 cm, voltage 2.4 kV, field strength 12.0 E, capacitor 25 μF, resistor 400 Ω, time constant 9-13 ms, recombinant plasmid was transformed into *S. typhimurium* X4072, from which recombinant strain *S.typhimurium* X4072 (pYA248-AB) was obtained.

Assay of AB protein expressed in recombinant strain

The results of AB expressed in X4072 (pYA248-AB) culture and sonicate supernatant assayed by bridged ELISA are shown in Table 2. The fact that the content of AB in recombinant germ X4072 (pYA248-AB) culture supernatant was higher than that in sonicate supernatant indicated that AB existed mainly in culture supernatant was expressed in the form of secretion.

Table 2 Expression of AB in X4072 (PYA248-AB) assayed by bridged ELISA

Sample	A_{492nm}	P / N
Negtive control of X4072 (pYA248) culture supernatant	0.04	
Negtive control of X4072 (pYA248) sonicate supernatant	0.04	
Positive control of BL21 (pET-AB) periplasm	1.08	27.0
X4072 (pYA248-AB) culture supernatant	0.96	24.0
X4072 (pYA248-AB) sonicate supernatant	0.78	19.5

Assay of stability of AB antigen expressed in recombinant strain

After recombinant germ pYA248-AB was cultured 100 generations, 100 bacteria were randomly selected and then transferred onto LB agar plate containing DAP. All of them could grow and the AB antigen was detected positive by ELISA. For the recombinant the mean of the value of A_{492nm} plus or minus standard deviation was 0.979±0.052, and the value of A_{492nm} of negative control X4072 (pYA248) was 0.040, which indicated that recombinant germ, after seeing cultured for 100 generations, could stably exist in attenuated *S.typhimurium* strain.

Growth curve of recombinant strain

Single clone X4072 (pYA248) and X4072 (pYA248-AB) were selected and then inoculated in the culture medium of LB liquid. After being cultured at 37 °C overnight, 50 μL of it was inoculated in 5 mL culture medium of LB liquid and then cultured with shaking at 37 °C. The value of A_{600} was measured and recorded once every other hour, from which growth curve (Figure 2) was drawn. It can be discovered from Figure 2 that the growth states of all bacteria were basically consistent, that is, metabolism of the bacteria were basically unaffected by transforming recombinant plasmid into attenuated *S. typhimurium* strain.

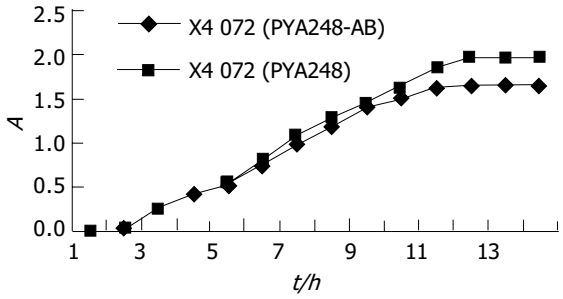

Figure 2 A growing curve of X4072 (PYA248) and X4072 (PYA248-AB).

Safety of recombinant strain

The results of experiments testing safety of C57BL/6 mice taking recombinant strain orally are shown in Table 3. After taking virulent strain X3181×10^7 c.f.u, all the mice died within 5 d, but after taking recombinant strain X4072 (pYA248-AB) 1.0×10^{10} c.f.u, the survival rate was still 100 percent. It can be noticed from the table that the number of live bacteria of C57BL/6 mice taking recombinant strain orally was 10^3 fold more than that of the lethal dose of virulent strain X3181.

Table 3 Survival of mice after oral inoculation with virulent and recombinant *S.typhimurium* strains

Strain	Relevant phenotype	Inoculating dose (c.f.u)	Observation intervals (d)	Survial (live / total)
X3181	wild type	1.0×10^7	5	0 / 5
		2.6×10^5	30	2 / 5
X4072 (PYA248-AB)	$Cya^-Crp^-AB^+$	1.0×10^{10}	30	5 / 5

Immunology of recombinant strain

The positive results defected by ELISA had colors, but the negative results had no colors, or weak colors. The five mice (group 3) serums and intestinal fluid immuned with vaccine *S. typhimurium* X4072 (pYA248-AB) showed positive results. In contrast, the mice serums and intestinal fluid of PBS control group and Salmonella control group showed negative results. It indicated *S.typhimurium* X4072 (pYA248-AB) could enable the organism to generate specific mucous membrane and humoral immunity.

DISCUSSION

S. typhimurium strain, through adhesion, invasion and inhabitation in intestine-associated lymph tissue, can be phagocyted by macrophage and M cells in Peyer node and through mesentery

lymph node reaches liver and spleen, which further stimulate other organs and tissue to develop mucous membrane, cell and body fluid immunization responses effectively. In recent few years, the research and production of gastrointestinal tract vaccine with attenuated *S. typhimurium* strain as the release system have become a new trend in the research of a new-type recombinant live vaccine taken orally. In it, attenuated *S.typhimurium* strain as a kind of vaccine carrier carrying foreign antigen is, up to now, the most widely used strain. This is mainly due to the fact that the model of mice infected by *S.typhimurium* strain is basically consistent with the organism of man infected by typhoid, and the virulence factor of *S. typhimurium* strain has been deeply researched[25,26]. The genes of virulence which have already been used in attenuated mutation of *S. typhimurium* strain include: *aro* gene controlling synthesis of aromatic compound[22]; gal E gene affecting synthesis of lipopolysaccharide[23]; *cya, crp* gene adjusting metabolic level of cyclic adenosine monophosphate(cAMP)[24], *pur* gene controlling sythesis of purine organism[25]; non-specific acid phosphoric acid enzyme *phoP* gene manipulating transcripition of gene and omp gene adjusting porin expression *etc*[26,27]. Many laboratories have successfully constructed attenuated and immunity *S.typhimurium* strain by mutating relative genes, which have been proved to be safe through experiments on animals, such as mice, cattle, and pig and even on human volunteers[28-33]. But when these attenuated *S.typhimurium* strains are used to express foreign protective antigen, genes encoding protective antigen are usually cloned on a vector containing drug resistance gene. In order to make sure of the stability of plasmid, antibiotic must be used as selection pressure. According to the rules stipulated in American Food and Drug Administration, drug resistance plasmid can not exist in live vaccine and the stability of recombinant plasmid can not be maintained in human beings and animals by antibiotic[28].

In order that foreign protective antigen can be stably expressed in attenuated *S.typhimurium* strain without antibiotic, a new type of plasmid vector system has been now developed, that is, the vector-host balanced lethal system. Host strain in the vector-host balanced lethal system is a type of chromosome mutant, and the mutated gene is housing keeping gene. Because the product of its encoding catalysed the genetic metabolic reaction of bacteria, the defects of the gene will certainly result in nutritious deficiency of bacteria, therefore, it can not grow in normal culture medium. Under this condition, plasmid must be necessary for the existence of bacteria. Once the transformed plasmid is lost, bacteria will not synthesize the essential substance so as not to grow in normal culture medium. Therefore, all bacteria that can grow in normal culture medium certainly contain recombinant plasmid, so as to construct the balanced lethal system. In this system, foreign protective antigen can be stably expressed without antibiotic in host stain. And the balanced lethal system constructed by *asd* gene has been widely used, for example, Redman *et al*.[34] cloned the surface protein antigen gene of *streptococcus* to plasmid with *asd*, and then transformed it to △*cya*△*crp*△*asd* defective *S. typhimurium* strains which were taken by mice orally. The results showed that recombinant strain could induce mice to produce continuous reaction of antibody response[34]. Scholars experimented △*cya*△(*crp cdt*)△*asd* expressing hepatitis B virus core pre S protein on adult women volunteers, showing that the strain could stimulate organism to develop system and mucous immunity response, and produce a unique secreted antibody (sIgA) in urinary and reproductive tract and intestinal tract[35].

By adopting the system as described above, we successfully carried out the construction of attenuated *S. typhimurium* strain expressing AB. Because the cell wall of *S. typhimurium* strain is thick and so hard to be transformed, *E.coli* X6097 with knocked out *asd* gene was used as host strain for screening recombinant clone. The screened recombinant plasmid was transformed electrically to host *S. typhimurium* X3730. X3730 with knocked out gene *asd* is the gene *galE* mutated strain of *S. typhimurium* strain LT-2 and loses the effect of restriction but possess the effect of modification, because of which plasmid transformed into *S. typhimurium* strain can obtain the methylated model of *S. typhimurium* strain and thus stably exists in *S.typhimurium* strain. Recombinant plasmid with methylated model extracted from X3730 was transformed into final *S.typhimurium* strain X4072 without restriction and so the construction work was finished.

Experiment on animals has proved that recombinant strain is safe. It was found by ELISA that AB protein was expressed mainly in the form of secretion, but the expression level was comparatively low. For recombinant strain with vaccine construction as the aim, however, a more important issue than high level expression is the stability of expressing foreign gene, because only when engineering bacteria can exist in body for a relatively long period and stably release antigen, it can effectively stimulate the immune system response of organism and expression with too high level may destroy the biological and chemical balance of strain as a complete organism which makes it hard for *S .typhimurium* strain to maintain the stable expression of foreign gene. The recombinant strain we constructed possessed a good stability, and under the condition without selection pressure,we recombinant plasmid could exist stably, and the foreign gene could also be expressed stably whose growth curve showed no obvious changes for expression of foreign gene. The main reason for it is probably that the expression level of foreign gene is suitable to *S. typhimurium* strain. In addition, according to Sutton etal's[36] principle of immunization protection and cure of *H pylori* "less is best", that is, low dose of antigen has better immunization effect, and maybe this *S. typhimurium* strain live vaccine expressing *H pylori* antigen with a low but stable level can create better immunization effect. In this study, the serums and intestine fluid from the mice immunized with *S.typhimurium* X4072 (pYA248-AB) expressed specific antibody aimed to AB, but the serums and intestine fluid from the mice in the control group failed to express. These results suggest that *S. typhimurium* X4072 (pYA248-AB) might be a good candidate as a vaccine. However, whether it can be used as a vaccine or not need be researched further. To achieve his goal we are carrying out the associated experiments on animals now.

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