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In Vitro and in Vivo Stability of Plasmids in Attenuated Salmonella enterica serovar typhimurium Used as a Carrier of DNA Vaccine is Associated with its Replication Origin

Takeshi HAGA[1], Shino KUMABE[1], Ai IKEJIRI[1], Yuya SHIMIZU[1], Huang LI[1], Yoshitaka GOTO[1], Hidenori MATSUI[2], Hironori MIYATA[3], and Tomoyuki MIURA[4]

[1] *Department of Veterinary Microbiology, University of Miyazaki, 1–1 Gakuen Kibanadai Nishi, Miyazaki 889-2192,* [2] *Kitasato Institute for Life Sciences, Kitasato University, 5–9–1 Shirokane,Minato-ku, Tokyo108-8641,* [3] *Animal Research Center, University of Occupational and Environmental Health, 1–1 Iseigaoka, Yahatanishi-ku, Kitakyushu, 807-8555, and* [4] *Laboratory of Primate Model, Experimental Research Center for Infectious Disease, Institute for Virus Research, Kyoto University,53 Shougoin-kawaharamachi, Sakyo-ku, Kyoto 606-8507, Japan*

Abstract: *The ability of live attenuated* Salmonella enterica *serovar typhimurium (*S. *typhimurium) as a carrier of DNA vaccine was evaluated using model plasmid encoding beta-galactosidase (β-Gal) and BALB/c mice. We constructed pBRCMVβ. β-Gal expression apparatus having a replication origin from low copy pBR322. Comparison of the plasmid stability showed that pBRCMVβ remained stable in* Salmonella *even after oral administration, while pUC-based pCMVβ tended to be lost quickly. However, titers for β-Gal specific IgG in sera did not significantly increase in mice orally administered* S. *typhimurium harboring pBRCMVβ. These data suggest that the stability of plasmid in* S. *typhimurium is associated with its replication origin. Further studies are required to scientifically establish this methodology.*

Key words: *DNA vaccine, plasmid stability,* Salmonella *typhimurium*

The use of live attenuated intracellular bacteria as vehicles to deliver plasmid DNA vaccine is a novel and attractive approach of inducing effective immunity [2]. After the release of the plasmid DNA from the bacteria, the plasmid-encoded antigens can be expressed directly by the host cell. These bacteria infect antigen presenting cells (APCs) such as macrophages and dendritic cells, so that the plasmid DNA vaccine is delivered effectively into APCs. These bacteria also act as a natural adjuvant and activate immune responses.

S. typhimurium is a typical intracellular bacteria, and was first used to show that bacterial DNA vaccine de-

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Address corresponding: T. HAGA, Department of Veterinary Microbiology, University of Miyazaki, 1–1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan

livery induced immune responses against vector-encoded antigens and protection in an animal model of infectious disease [1, 2]. However, the mechanism underlying plasmid delivery by *S.* typhimurium is unclear, and a recent report showed a controversial result. Garmory *et al.* reported that *S.* typhimurium harboring model plasmid failed to induce an antigen-specific immune response [3]. Therefore, we examined and evaluated the ability of live attenuated *S.* typhimurium to deliver plasmid DNA in a mouse model. In this paper, we report that the stability of plasmid in *S.* typhimurium is associated with its replication origin.

Female BALB/c AnNCrlCrlj *(H-2^d)* mice were purchased from Charles River, Japan and kept on a 14 h light 10 h dark schedule in an air conditioned animal facility at Frontier Science Research Center, University of Miyazaki. Mice were always allowed to adapt for more than 1-week after arrival, and animals at six to ten weeks of age were used for the experiments. This experiment was approved by the Animal Care and Use Committee, University of Miyazaki.

The *Escherichia coli* (*E. coli*) strain, XL1-Blue, was used as a host during the cloning experiments. Three attenuated strains of *S.* typhimurium were used as live vaccine vector in this study. *S.* typhimurium 2337-65 derivative auxotrophic *aroA* strain SL7207 {*hisG46* DEL407[*aroA*:: Tn*10*(Tc^r)]}, which is widely used for similar experiments [1], was kindly provided by B.A.D. Stocker, Stanford University. *S.* typhimurium *phoP*-deficient strainχ3306 *phoP* {*phoP*:: *aphT* ΔTer} [5] and *rpoE*-deficient strainχ3306 *rpoE* {*rpoE*:: *aphT*} [10] were derivatives of strain SR-11. All bacteria were routinely grown in Luria-Bertani (LB) broth [1% Bacto tryptone (Difco Laboratories, MI, USA) 0.5% Bacto yeast extract (Difco Laboratories) 1% sodium chloride (Nacalai Tesque, Kyoto, Japan)] or on an LB agar plate at 37°C.

The eukaryotic expression vector pCMVβ (Clontech, CA, USA) was purchased and used as a model for DNA vaccine. This pUC-based plasmid produces β-Gal under the control of CMV IE promoter and has been used in many published studies [1, 7, 9]. pBRCMVβ was constructed by inserting the *Eco*RI-*Sal*I fragment of pCMVβ containing eukaryotic CMV promoter, β-Gal coding region and polyA signal, into pBR322 backbone including its replication origin. As negative controls, pCMVΔ and pBRCMVΔ were also constructed by removing the β-Gal coding region at *Eco*R I sites, respectively. Each plasmid was introduced into bacteria by electroporation (BTX, CA, USA).

To examine transfer of the plasmid from attenuated *S.* typhimurium to mouse cells, *in vitro* gene transfer assay was carried out using mouse peritoneal exudates macrophage [1]. To induce inflammatory exudates-derived macrophages, 1 ml of 3% (w/v) Brewer's thioglycolate medium (Sigma, MO, USA) was injected into the peritoneal cavities of BALB/c mice 7 days prior to the harvest. Cells suspended in RPMI medium [RPMI 1640 with 2 mM L-glutamine and sodium bicarbonate (Sigma) containing 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL Life Technologies, Auckland, New Zealand)] at the concentration of 2×10^5/ml were allowed to adhere to Lab-Tec glass chamber (Nalgenunc, Denmark) for 2 h at 37°C. *S.* typhimurium harboring pCMVβ which were cultured in LB broth with ampicillin (100 μg/ml) with shaking for 18 h at 37°C were added to the cells at a multiplicity of infection (MOI) of 1, 5, 10 and 20 for 15 min. The medium was then replaced by RPMI medium supplemented with gentamycin (50 μg /ml) to kill extracellular bacteria, and cells were incubated for 3 to 4 h at 37°C. To block intracellular bacterial multiplication, tetracycline was added to the cell culture at a final concentration of 20 μg per ml of the medium. Incubation was continued for another 24 h. After 2–3 washes with PBS, cultured cells were fixed with acetone/methanol [1:1 (vol/vol)] for 15 min at 4°C. After 2 3 washes with PBS, X-gal substrate (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 100 mg/ml X-gal in PBS) was added. After overnight incubation at 37°C, β-Gal expressing cells were observed by light microscopy.

To observe *in vivo* transfer of plasmid and immune induction, *S.* typhimurium harboring plasmid was orally administered [5]. Mice were deprived of drinking water and food for 5–6 h prior to the administration, and fed 30 min after the infection. Fifteen minutes before the infection, mice received 50 μl of 10% $NaHCO_3$ by Pipetman (Gilson) to neutralize gastric acid. Animals were then fed with 20 μl of PBS with 0.01% gelatin (BSG) containing approximately 5×10^8 CFU of recombinant *S.* typhimurium by Pipetman. Exponential-growth phase *S.* typhimurium harboring the plasmid was inoculated 3 times at 15-day intervals.

To determine bacterial counts and plasmid stability, samples were plated on LB agar plate with or without ampicillin. The ampicillin resistant gene present on plasmid pCMVβ and pBRCMVβ was used as an indicator of plasmid presence. The ampicillin resistant colony forming unit (CFU) and the total number of CFU were compared. Plasmid stability in *Salmonella* was first assessed by serial passage *in vitro*. After the plasmid transformation, a single colony on LB-agar plate supplemented with 100 μg/ml of ampicillin was cultured in antibiotics-free LB broth at 37°C overnight. Passage was done using 1,000-fold dilution of the overnight culture with fresh LB broth without ampicillin. To assess *in vivo* stability, the bacteria from mouse organs were recovered by homogenization of the sample in ice-cold BSG.

β-Gal specific antibody titers were determined by ELISA [6]. Ninety-six-well Nunc-immuno MaxiSorp assay plates (Nunc, Denmark) were coated with 50 μl of β-Gal (5 μg/ml) (Roche Diagnostics) in coating buffer (0.1 M Na_2HPO_4, pH 9.0) per well. After overnight incubation at 4°C, β-Gal was removed, and plates were blocked with 10% FCS in PBS for 1 h at 37°C. One hundred microliters of serial two-fold dilution of the serum in PBS with 1% bovine serum albumin (BSA) were added per well, and incubated for 2 h at 37°C. After four washes with PBS-0.05% Tween 20 (PBST), biotinylated γ-chain-specific goat anti-mouse IgG (Sigma) was added as a secondary antibody. Plates were further incubated for 2 h at 37°C. After four washes with PBST, 100 μl of peroxidase-conjugated streptavidin (Amersham, UK) dilated at 1:1,000 in PBS containing 0.1% BSA was added, and the plates were incubated for 1 h at room temperature (r.t.). After four washes with PBST, reactions were developed with ABTS [2, 2'-azimobis-(3-ethylbenzthinzoline-6-sulfonic acid)] in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2 (Kirkegaard & Perry Laboratories, MD, USA) for 30 min. at r.t. ABTS Peroxidase Stop Solution (Kirkegaard & Perry Laboratories) was added to each well (100 μl /well). Within 30 min, the optical density (OD) of each well was determined using a microplate reader (Bio-Rad, CA, USA) at 405 nm wavelength. The titer was determined by the highest dilution of the serum showing an OD value of 0.1 above that of the normal serum according to a previous report [6]. β-Gal monoclonal antibody (Clone GAL-13, mouse IgG1, Sigma) was used as the standard.

β-Gal activity was occasionally detected from peritoneal exudates-derived macrophage like cells infected with SL7207 and χ3306 *phoP* harboring pCMVβ 24 h after infection (Fig. 1 A and B). In this study, however, no expression of β-Gal was detected by using χ3306 *rpoE*.

During the experiments, pCMVβ was found to be lost in *S.* typhimurium, but not in *E. coli*. In an attempt to stabilize the plasmid, pBRCMVβ with low copy pBR322 [8] backbone was constructed. β-Gal was detected when pBRCMVβ was transfected into Cos7 cells [4] by lipofectin (data not shown) indicating its ability to express β-Gal in mammalian cells. Although pUC-based pCMVβ in *Salmonella* tends to be lost with time, pBRCMVβ with pBR322 backbone was stable both *in vitro* (Fig. 2A) and *in vivo* (Fig. 2B).

To investigate if the attenuated *S.* typhimurium strains transformed with the low copy plasmid pBRCMVβ could induce antigen-specific humoral response, the production of anti β-Gal specific IgG was examined using the ELISA technique [6]. The specific antibody in the serum of some mice administered SL7207 harboring pBRCMVβ showed positive. However, the antibody titer increased even in some negative control animals inoculated with SL7207 harboring pBRCMVΔ. These results suggest that the antibody detected in this system may have been derived from the background. As posi-

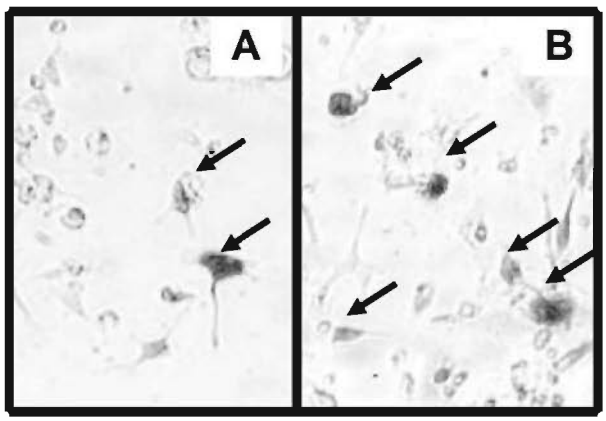

Fig. 1. Expression of β-Gal in mouse macrophage-like cells after infection with *S.* Typhimurium containing pCMVβ. Twenty-four hours after the infection of peritoneal exudates macrophage cells *in vitro* with A) χ3306 *phoP* and B) SL7207 strain. Arrows indicate positive cells.

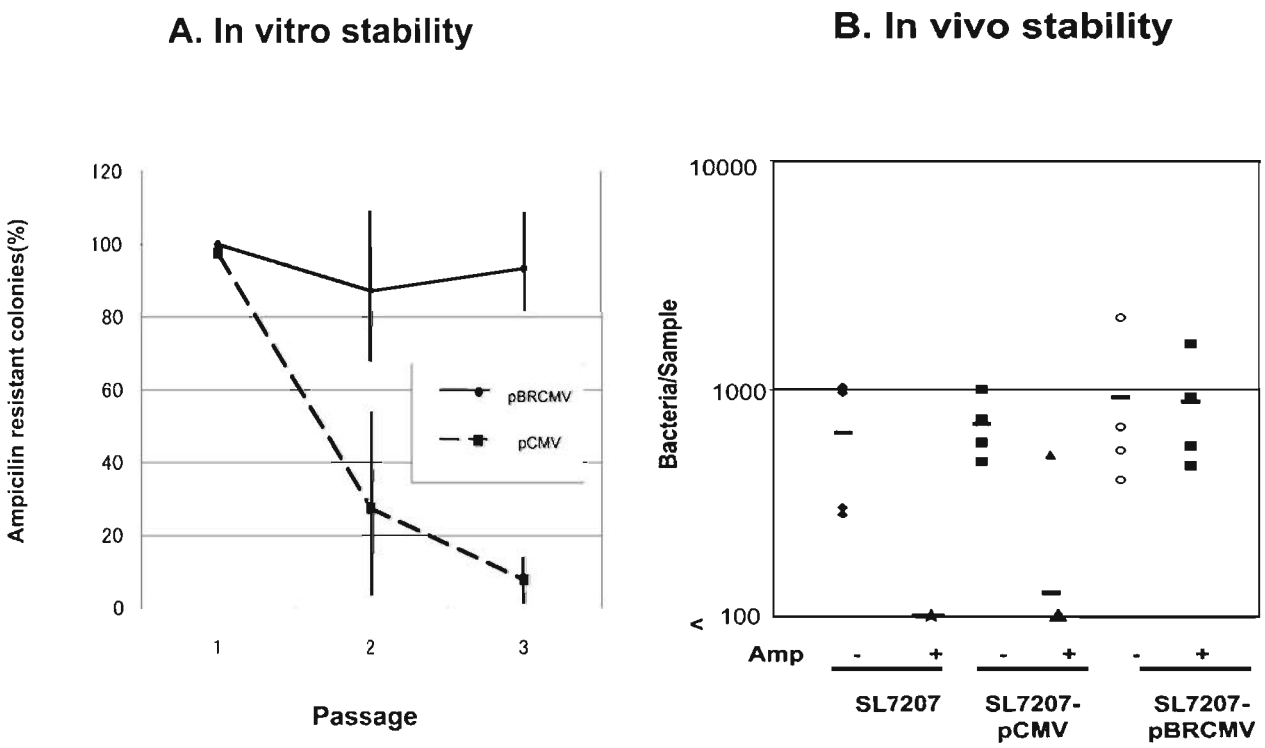

Fig. 2. Stability of pCMVβ and pBRCMVβ in *S.* typhimurium. A) After *in vitro* passage of *S.* typhimurium containing plasmid as indicated. Results of ampicilin resistant colony count divided by colony number on ampicilin negative plate are expressed as%. B) After oral administration, bacteria recovered from Peyer's patch were counted either on ampicilin positive or negative LB plate. The average of four mice samples is shown as bar (-). *S.* typhimurium containing plasmid as indicated was inoculated twice (on days 0 and 15), and the samples were collected on day 16 after the initial infection.

tive control, mice were injected with β-Gal (Roche Diagnostics; 50 μg in 10 μl of PBS) intramuscularly. The titers of β-Gal-specific antibody in the sera of these positive control animals were much higher than those from other samples (Fig. 3).

The efficacy of gene transfer observed in this study was much lower than reported previously using SL7207. Paglia *et al.* reported that expression of GFP in eukaryotic expression plasmid pEGFP-C2 was detected from approximately 19% of the splenocytes on day 28 after the first vaccination [7]. We also observed that pCMVβ was unstable in *Salmonella* by examining the ratio of ampicilin resistant CFU to total bacterial CFU. To stabilize the efficacy of DNA vaccination using live attenuated *Salmonella* vector, pBRCMVβ with pBR322 backbone was constructed. Although pUC-based pCMVβ in *Salmonella* tends to be lost with time, pBRCMVβ with pBR322 backbone was stable in the bacteria both *in vitro* and *in vivo*. However, ELISA for β-Gal-specific antibody showed that there was no sig-

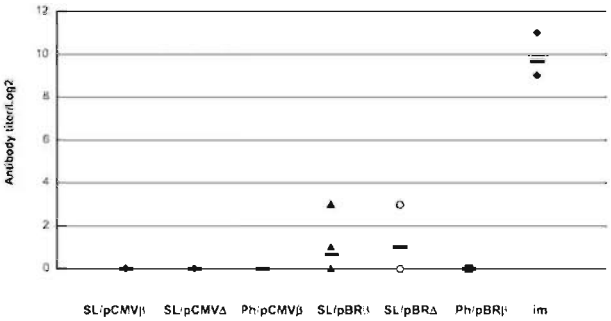

Fig. 3. Anti-β-Gal specific IgG response. ELISA was performed to detect anti-β-Gal-specific IgG in serum of the mice. *S.* typhimurium (SL: SL7207 strain, Ph: *phoP* deficient χ3306 strain) containing plasmid as indicated (pCMVβ, pCMVΔ, pBRβ: pBRCMVβ, pBRΔ: pBRCMVΔ) was inoculated twice (on day 0 and 15), and the samples were collected on day 28 after the initial infection. The average titer of four mice in each group is shown as bar (-). im: intramuscular inoculation of 50 μg of β-Gal (Roche; in 10 μl of PBS) as positive control. Antibody titer is presented as the highest dilution (Log2) of the serum showing OD value 0.1 over the normal serum.

nificant increase in the titer. The amount of DNA may not have been sufficient to achieve the expression of β-Gal [9] from pBRCMVβ, because it is thought to be a low copy plasmid [8]. As a note, there were some positive sera from pBRCMVβ group as well from the pBRCMVΔ, negative control group. This may have been due to the increase in total IgG level in those animals (data not shown).

In this study, we examined IgG response in the serum. Although there is no evidence for systemic IgG induction, we cannot exclude the possibility that a weak IgA response is elicited in the intestine, because this vaccine is administered orally.

Recently, Garmony *et al.* reported that the high copy plasmid pCMVβ is unstable in SL7207 and that the low copy plasmid pLGβ GAL, which was constructed by cloning of pCMVβ into low copy number plasmid pLG339 is stable in SL7207. However, SL7207 harboring pCMVβ or pLGβ GAL failed to induce β-Gal-specific IgG *in vivo* [3]. Our results are compatible with those observations.

In conclusion, our results suggest that plasmid is delivered into antigen presenting cells by live attenuated *Salmonella*, and that the stability of plasmid in *Salmonella* is associated with its replication origin; pUC-based pCMVβ tends to be lost, and pBR322 based pBRCMVβ is stable both *in vitro* and *in vivo*. To achieve vaccine design using the *Salmonella* delivery system, the development of stable and high copy plasmid in *Salmonella* may be required.

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