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Full Length Research Paper

Novel approaches toward the development of an oral post-exposure DNA vaccine for latent tuberculosis using *Salmonella typhimurium* ∆*aroA* vector

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Tuberculosis remains one of the major causes of global public health problems. There is no effective vaccine for the disease until now. Many reports show that DNA vaccines are promising to induce protection against *Mycobacterium tuberculosis* (*M. tb*); however, the efficiency of DNA vaccine is limited due to inadequate delivery systems. Among others, live attenuated bacterial vectors such as *Salmonella enterica typhimurium* (*S. typhimurium*) have significant promise as efficient mucosal delivery vehicles for DNA vaccines. In this study, we constructed recombinant attenuated *S. typhimurium* DNA vaccines carrying genes encoding resuscitation promoting factor (Rpf)-like proteins of *M. tb* on eukaryotic expression plasmid agianst latent tuberculosis and evaluated the plasmid stability and growth curve assays of the recombinant *Salmonella* vaccine constructs *in vitro*. Four Rpf gene fragments (*RpfB*, *RpfC*, *RpfD*, *RpfE*) associated with latency were amplified from genomic DNA of the H37Rv strain of *M. tb,* cloned into eukaryotic expression plasmid (pVR1020) and verified by sequencing. In later studies, we will demonstrate the potential use of the *Salmonella*-mediated DNA constructs as candidate post-exposure vaccines against tuberculosis through testing their immunogenicity and effectiveness for oral delivery in eukaryotic systems.

Key words: Latent tuberculosis, resuscitation promoting factor (Rpf), DNA vaccine, recombinant *Salmonella typhimurium*.

INTRODUCTION

Tuberculosis (TB) remains one of the major causes of health problems and mortality worldwide. Approximately one–third of the world's population is infected by *Mycobacterium tuberculosis* (*M. tb*), the causative agent of TB (Barnes and Cave, 2003). The ability of the bacteria to persist in the host for decades after infection in a latent [non-replicating persistent (NRP)] state before reactivating to cause disease is central to the threat of human health (Stewart et al., 2003). Individuals with latent TB harbor a 2 to 23% lifetime risk of developing reactivation TB during their lifetime and more considerably, this risk elevates up to 10% annually due to the synergistic pathology of co-infection with human

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Abbreviations: *M. tb*, *Mycobacterium tuberculosis*; **Rpf,** resuscitation promoting factor.

immunodeficiency virus (HIV) (Frieden et al., 2003).

The efficacy of the only currently available TB vaccine, Bacillus Calmette-Guerin (BCG), still remains controversial, especially against pulmonary TB in adolescents. Thus, the development of a more effective TB vaccine is required to combat the global threat of TB (Hawkridge and Mahomed, 2011). Several alternative TB vaccine candidates are currently being tested experimentally as pre-exposure or post-exposure or booster preparations, including subunit, live attenuated, recombinant BCG, and DNA vaccines (Hawkridge and Mahomed, 2011; Orme, 2011; Walzl et al., 2011; Yuan et al., 2011).

DNA vaccines represent novel vaccine approach for TB, and are currently under rigorous investigation (Liu et al., 2008; Ly and McMurray, 2008; Lowrie, 2006; Saha et al., 2011; Yuan et al., 2011). Consequently, DNA vaccines expressing a number of antigens, individually or in combination, such as Ag85B, MPT64 and MPT83, hspX (16-kDa α-crystallin), Hsp65 (65-kDa), ESAT-6 (6-kDa) and ESAT-6-Ag85B have shown various degrees of immunogenicity and prophylactic efficacy in a murine TB model. The development of post-exposure DNA vaccine has also began long time ago and has been tested in chronic animal models (Lowrie et al., 1997). In recent studies, DNA vaccines have been used to prevent endogenous reactivation of TB in a murine model of latent TB, to prevent exogenous reinfection in drug-cured murine, or to function in a therapeutic fashion to alter the course of disease in a chronically infected murine model (Lowrie, 1999; Repique et al., 2002). Nevertheless, DNA vaccination in humans has so far yielded disappointing results (Sharma and Khuller, 2001) and the safety and efficacy of post exposure vaccination have been challenging (Moser et al., 1990).

Despite their immunogenicity, the quality, quantity and delivery route used hamper the utility of DNA vaccines to generate sufficient immune responses in humans and non-human primates (Donnelly et al., 2003; Ingolotti et al., 2010; Liu et al., 2008). Therefore, the development of efficient and cost effective delivery mechanisms to improve and increase DNA vaccine potency has been an active area of recent investigation. These approaches included improved DNA vaccine delivery methods (Cui and Mumper, 2003; Huang et al., 2010; O'Hagan et al., 2004a, 2004b).

As first described by Darji et al. (1997), one novel approach is the use of live attenuated *Salmonella enterica typhimurium* (*S. typhimurium*) *ΔaroA* SL7207 bacterial strains for the delivery of DNA through the mucosa. *Salmonella* strains have been used to deliver DNA vaccines of variety of pathogen and cancer origins (Schoen et al., 2004; Dietrich et al., 2003; Xu and Ulmer, 2003). They are easy to handle in the laboratory and several well-characterized attenuated strains are available (Dunstan et al., 1998). The *aroA* mutant strains of *S. typhimurium* were among the first attenuated strains (Stocker, 1988). Mutant *Salmonella* strains with a blocked aromatic pathway (Hoiseth and Stocker, 1981) lack the *aroA* gene encoding an essential aromatic acid vital for survival and growth. Orally delivered *S. typhimurium ΔaroA* harboring eukaryotic expression plasmid were shown to cross the gut epithelium via M cells and efficiently convey foreign DNA under eukaryotic promoter control to macrophages and dendritic cells by releasing their plasmid DNA when they die due to their *aroA* attenuation (Clements, 1987). In so doing, the host cells are transfected with the plasmids, antigen expression takes place and all of the specific arms of the immune system are potentially stimulated (Darji et al., 1997; Hess et al., 2000).

Recently, five resuscitation promoting factor (*rpf*) gene homologues, Rv0867c (*rpfA*), Rv1009 (*rpfB*), Rv1884c (*rpfC*), Rv2389c (*rpfD*), and Rv2450c (*rpfE*), have been described in *M. tb* (Mukamolova et al., 1998, 2002a), and it was described that these genes are characteristic of latency and important for resuscitation to the survival of the bacteria (Gupta et al., 2010; Kana et al., 2008; Mukamolova et al., 2002b). Although, the approach of post-exposure vaccine still remains an area of active and cautious research, there is hope that vaccines that include such latency-associated antigens may generate immune responses that provide protection against reactivated TB. Such vaccines are suggested to boost the immune response of individuals with latent TB infection and could reduce progression of latent infection to active disease. The fact that Rpf proteins of *M. tb* are associated with NRP- TB and are immunogenic secreted products (Fan et al., 2010, 2008; Yeremeev et al., 2003), they are becoming a center of interest in vaccine development against latent TB. We were, therefore, interested to construct a pre-clinical recombinant post-exposure DNA vaccine candidate in live attenuated *S. typhimurium aroA* vector for effective delivery against latent TB. In this study, we constructed recombinant live *S. typhimurium aroA* DNA vaccine strains carrying the *M.tb* H37Rv *rpfB*, *rpfC*, *rpfD* and *rpfE* genes for potential oral delivery of the DNA vaccine candidates to eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains and plasmids

M. tb H37Rv strains were obtained from Armauer Hansen Research Institute (AHRI) TB laboratory originally from ATCC number 27294. Chemically, competent DH5α *Escherichia coli* strains were purchased from Invitrogen, Germany. *S. typhimurium aroA* mutant and the eukaryotic expression vector (pVR1020) were kind gift of the laboratory of Prof. Trindad Chakraborty, Institute of Medical Microbiology, Justus Leibeg University Giessen, Germany.

Construction of recombinant plasmids

Four out of the five *rpf* genes of *M. tb* H37Rv strain: *rpfB*, *rpfC*, *rpfD*

Table 1. Primers used for PCR amplification of *rpf* genes of *M. tb* H37RV strain.

Primer target and direction	Sequence (5' →3')	Expected PCR product
Rv1009c-F	TGACATGGATCCTGCAAAACGGTGACGTTGACCGT	*rpfB* (1020 bp)
Rv1009c-R	CATGATGGATCCTCAGCGCGCACCCGCTCGTCGAG	
Rv1884c-F	TGACATGGATCCTCAGCGCGGAATACTTGCCTGAAT	*rpfC* (531 bp)
Rv1884c-R	CATGATGGATCCGTGCATCCTTTGCCGGCCGACCA	
Rv2389c-F	TGACATGGATCCTCATCAATCGTCCCTGCTCCCCGAAC	*rpfD* (455 bp)
Rv2389c-R	CATGATGGATCCATGACACCGGGTTTGCTTACTAC	
Rv2450c-F	TGACATGGATCCTCAGCCGCGGCCGCAGACC	*rpfE*(432 bp)
Rv2450c-R	CATGATGGATCCGACGACGCGGGCTTGGACCCAA	

and *rpfE* were PCR amplified from genomic DNA of *M. tb* H37Rv strain with Taq DNA polymerase (Invitrogen, Germany) by using primers (Table 1) designed from the *M. tb* genome sequence database. All genes were amplified without signal sequences with both 5' and 3'primers containing *BamHI* sites. PCR reactions were performed using a gradient PCR system with a preheating stage of 15 min at 95°C followed by 30 cycles of 1 min denaturation at 94°C; 30 s annealing at different temperatures for each gene (68°C for *rpB* and *rpfE*; 60°C for *rpfC*; and 56°C for *rpfD*) and 1.5 min extension time at 72°C with a final extension at 72°C for 10 min. The PCR product of each was visualized on 1.2% agarose stained with ethidium bromide.

Each of the four *rpf* gene (*rpfB* to *E*) with incorporated *BamHI* sites was digested with *BamHI* (ROCHE, Germany) and then cloned into the *BamHI* predigested and dephosphorylated eukaryotic expression vector pVR1020 (VICAL, Germany) using T_4 DNA Ligase (Invitrogen, Germany) following standard cloning procedures (Sambrook and Russell, 2000). The recombinants were designated as pVR1020::*rpfB*, pVR1020::*rpfC*, pVR1020::*rpfD* and pVR1020::*rpfE*. The recombinant pVR1020 plasmids were used to transform chemically competent *E. coli* DH5α strain by heat shock at 42°C. Individual clones were selected and characterized by PCR and *BamHI* restriction digestion from Luria Bertani (LB) agar plates incubated overnight at 37°C and containing Kanamycin (50 µg/ml, Invitrogen, Germany). The recombinant plasmid pVR1020 constructs were further confirmed by sequencing at the Institute of Medical Microbiology, Justus Liebig University, Giessen.

Construction of recombinant *S. typhimurium* ΔaroA strains

Each recombinant plasmid (pVR1020::*rpfB*, pVR1020::*rpfC*, pVR1020::*rpfD* and pVR1020::*rpfE*) isolated from *E. coli* cells and the empty pVR1020 plasmids (negative control) were used to transform *S. typhimurium ΔaroA* SL7207 strains by electroporation (Gene-Pulser, Bio-Rad, Germany) with the conditions: voltage, 1.8 Kv; capacitor, 25 µF; resistor, 200 Ω and time constant, 4.2 to 4.4 min. Recombinant plasmids were once again analyzed by PCR and *BamHI* restriction digestion and true recombinant *S. typhimurium ΔaroA* vaccine constructs were stored in LB broth with 25% glycerol aliquots at -80°C until use.

Plasmid stability and growth curve assays

Plasmid stability and growth curve assays were basically conducted according to Bai et al. (2004). Briefly, each of the recombinant *S. typhimurium ΔaroA* vaccine constructs was grown in Kanamycin (50 µg/ml) containing LB broth at 37°C without shaking for 16 h to an OD_{600} value of approximately 0.7. Further, 100 µl of 10^{-5} and 10^{-6} dilutions of each recombinant *Salmonella* was plated on LB agar plates with and without kanamycin, plasmid stability was confirmed by the colony forming unit (CFU) generated after 15 h of growth (OD600 = 0.6) at 37°C over five consecutive days on subcultures. Besides, growth curve of each recombinant *S. typhimurium ΔaroA* was assayed by determining OD_{600} values obtained every hour from 12 to 16 h of culture, which were used to draw the growth curves of the recombinant *S. typhimurium ΔaroA* constructs. The wild *Salmonella ΔaroA* SL7207 strain was grown without antibiotics for use as negative control for both experiments.

Statistical/data analysis

Graphpad prism (Version 4.0) (Stata Corporation, Texas, USA) was used to compare the difference in growth characteristics of recombinant *Salmonella* and the wild mutant strain from their OD values. All values with P value < 0.05 were considered statistically significant. Nucleotide sequence and alignment analyses of the *rpf* genes were done using the DNASTAR SeqMan I and VectorNTI softwares (Lasergene, USA).

RESULTS

Construction of recombinant *E. coli* and *S. typhimurium* ΔaroA

The genes encoding the four Rpfs: *rpfB*, *rpfC*, *rpfD* and *rpfE* containing the *BamHI* sites were PCR amplified from *M. tb* H37Rv genomic DNA with HotStar Taq DNA polymerase and bands corresponding to the expected sizes of the genes were observed on agarose gel (Figure 1). The purified four *rpf* gene fragments cloned into the 5.047 kb eukaryotic expression vector, pVR1020 (Figure 2), were used to transform chemically competent *E. coli DH5α* and *S. typhimurium aroA* mutant SL7207 strains. Both PCR (data not shown) and *BamHI* digestion (Figure 3A and B) confirmed the successful constructions of the recombinant plasmids: pVR1020::*rpfB*, pVR1020::*rpfC*, pVR1020::*rpfD*, and

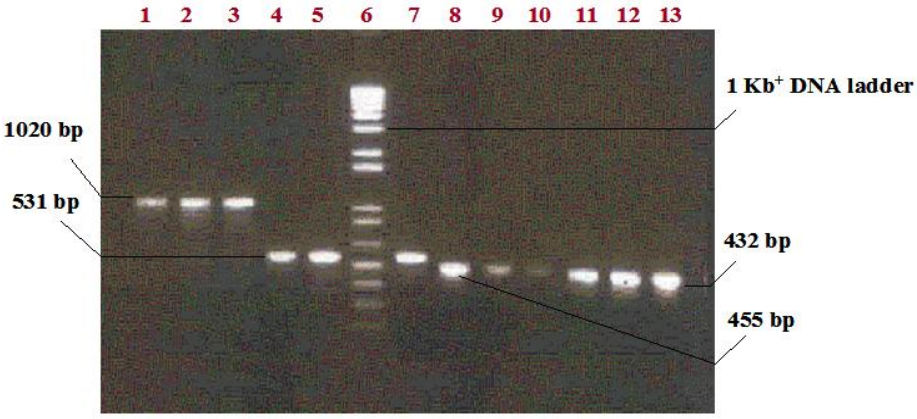

Figure 1. Agarose gel electrophoresis analysis of PCR products. Lanes 1 to 3, *rpfB* (1020 bp); lanes 4, 5 and 7, *rpfC* (531pb); lane 6, 1kb plus DNA ladder; lanes 8 to 10, *rpfD* (455 bp); lanes 11 to 13: *rpfE* (432 bp).

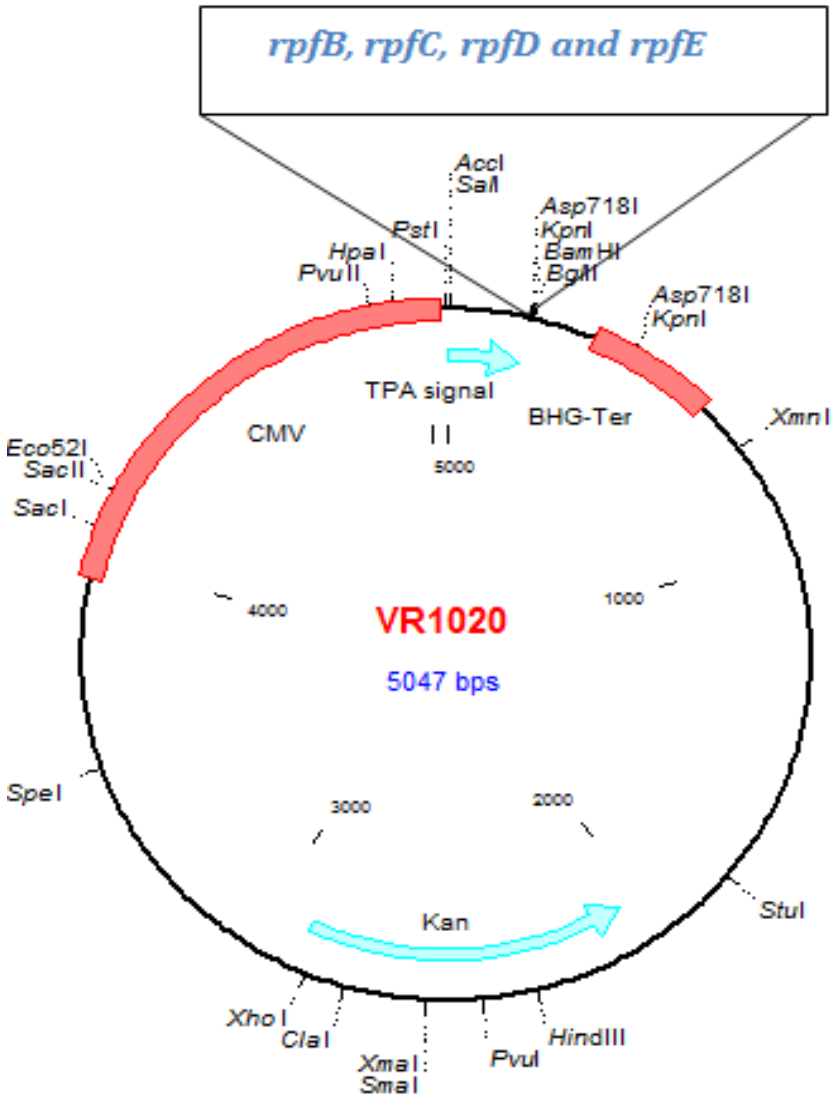

Figure 2. Schematic diagram of pVR1020 plasmid.

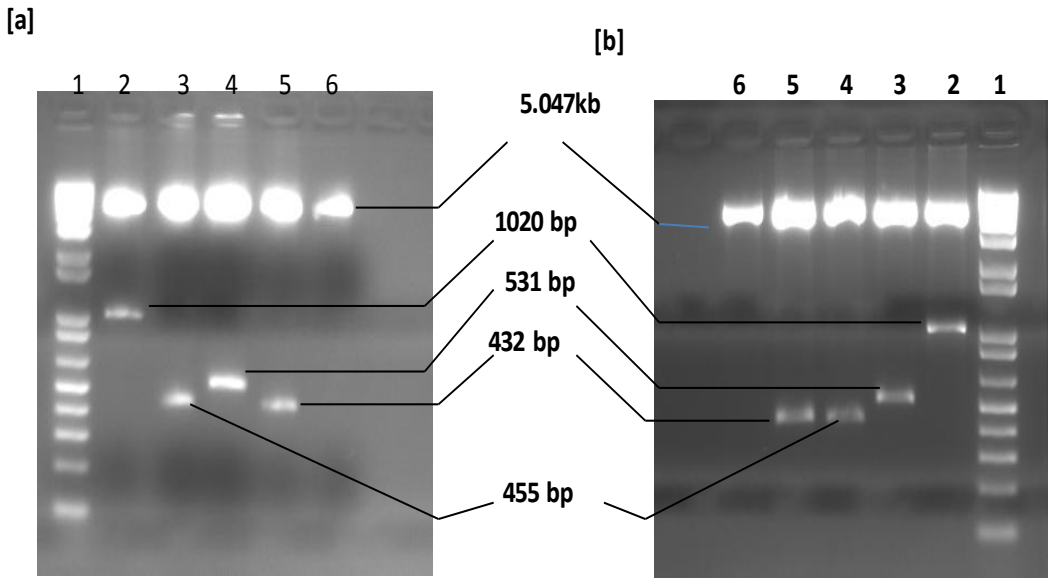

Figure 3. Analysis of recombinant *E. coli* (A) and *Salmonella* (B) with *BamHI* digestion. (A) Lane 1, 1kb^{+} DNA ladder; lane 2, pVR1020::*rpfB*; lane 3, pVR1020::*rpfD*; lane 4, R1020::*rpfC*; lane 5, pVR1020::*rpfE*; lane 6, pVR1020. (B) Lane 1, 1 kb ladder; lane 2, pVR1020::*rpfB*; lane 3, pVR1020::*rpfC*; lane 4, pVR1020::*rpfD*; lane 5, pVR1020::*rpfE*; lane 6, pVR1020.

pVR1020::*rpfE* with the correct orientation and sizes of the cloned *rpf* genes in both *E. coli DH5α* and *S. typhimurium aroA* strains. The recombinant plasmid pVR1020 constructs containing the coding regions of the four *rpf* genes (*rpfB, rpfC, rpfD and rpfE*) were confirmed by sequencing and the recombinant plasmids showed 100% homology indicating that there were no changes in the nucleotide sequences of the cloned *rpf* genes as shown for *rpfC* and *rpfE* as representative gene sequences (Figure 4A and B).

Plasmid stability and growth curve assays

Plasmid stability assay was performed to assure the stable expression of antigens encoded by the *rpf* genes cloned into the eukaryotic expression plasmid, pVR1020. The results of the growth for the recombinant *Salmonella* SL7207 *ΔaroA* constructs carrying pVR1020::*rpfB* to *E*, and the empty pVR1020 in LB medium containing kanamycin and CFU of each on LB agar plates with and without kanamycin have depicted that the recombinant plasmids could still stably exist in the attenuated *S. typhimurium aroA* strain (data not shown).

Those *Salmonella* grown on LB agar plates with kanamycin were considered to also retain the plasmid, while those grown on Kanamycin free plates were not. The values of OD_{600} of the *Salmonella* constructs and the mutant wild type strain grown in LB medium (1:1000 dilution without shaking at 37°C) were recorded every 1 h interval from 12 to 16 h of culture time. The patterns of the growth curve drawn from OD values against culturing time are depicted in Figure 5. The OD values of the recombinant *Salmonella* did not show significant differences (P value > 0.05) compared to that of the mutant wild type SL7207 *aroA* strain and that carrying empty pVR1020 vector.

DISCUSSION

Post-exposure prophylactic DNA vaccines have raised a substantial promise for combating latent TB. The use of live *aroA*-deficient strains of a *S. typhimurium* carrier system constitutes a novel approach to achieve efficient introduction of DNA constructs mucosally in order to specifically target antigen presenting cells for the immunogenicity of the resulting DNA vaccine delivered orally by *S. typhimurium* (Gahan et al., 2009). Thus it is attractive to contemplate the potential use of mutant *Salmonella* vectors for the delivery of latency associated immunodominant Rpf of *M. tb* under the control of eukaryotic expression plasmids to elicit cell mediated responses against latent TB.

With an objective of developing an efficient pre-clinical post-exposure TB vaccine delivery system, we constructed DNA vaccine encoding Rpf of *M. tb* in live *aroA* mutant *S. typhimurium* vectors. The recombinant plasmids pVR1020::*rpfB*, pVR1020::*rpfC* pVR1020::*rpfD*, pVR1020::*rpfE* and pVR1020 in *S. typhimurium aroA* mutant strain constructed in this study were found to be stable *in vitro* (data not shown). When cultured under

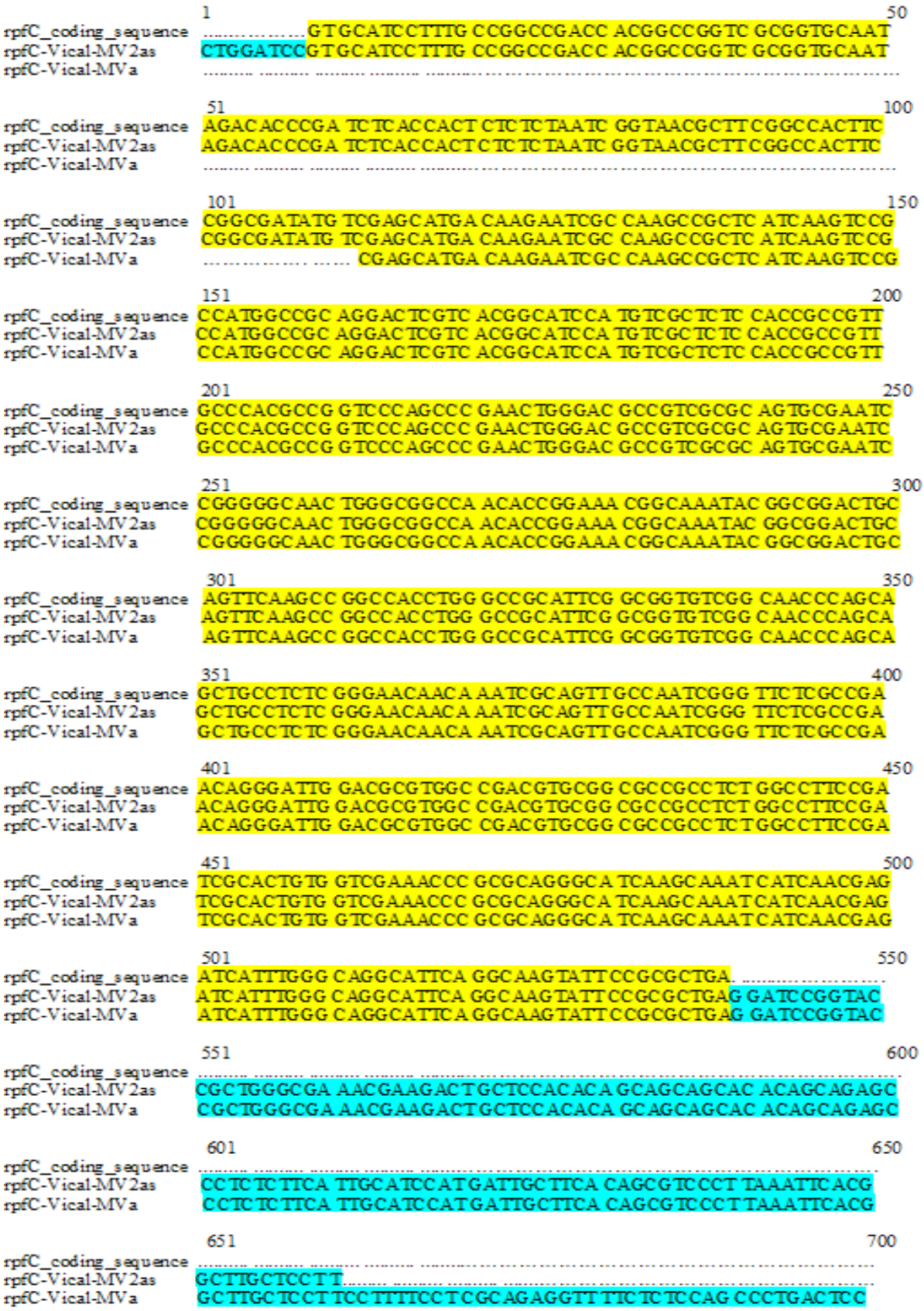

Figure 4A. Nucleotide sequence alignment of *rpfC* using the NCBI's database and BLAST program.

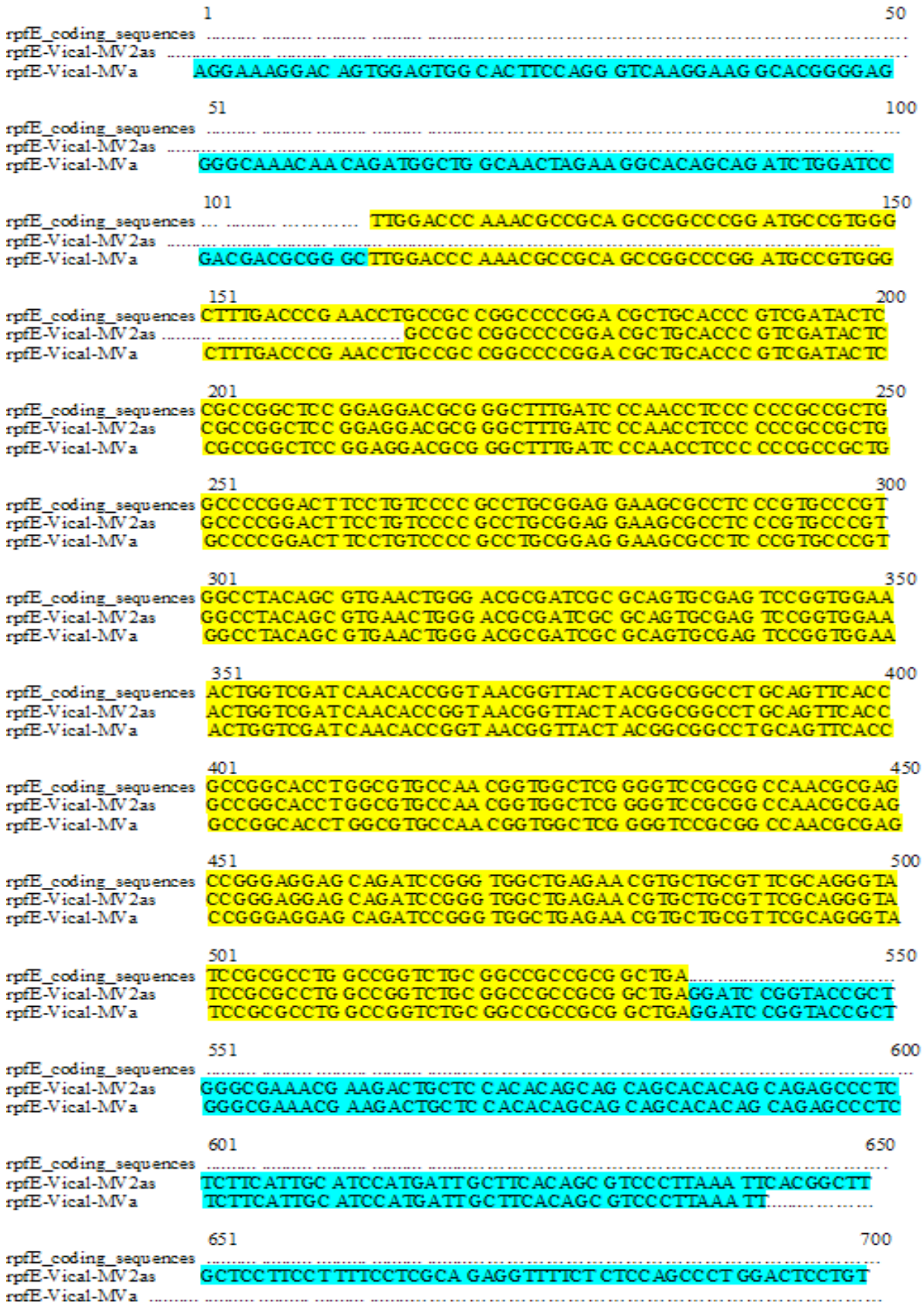

Figure 4B. Nucleotide sequence alignment of *rpfE* using the NCBI's database and BLAST program.

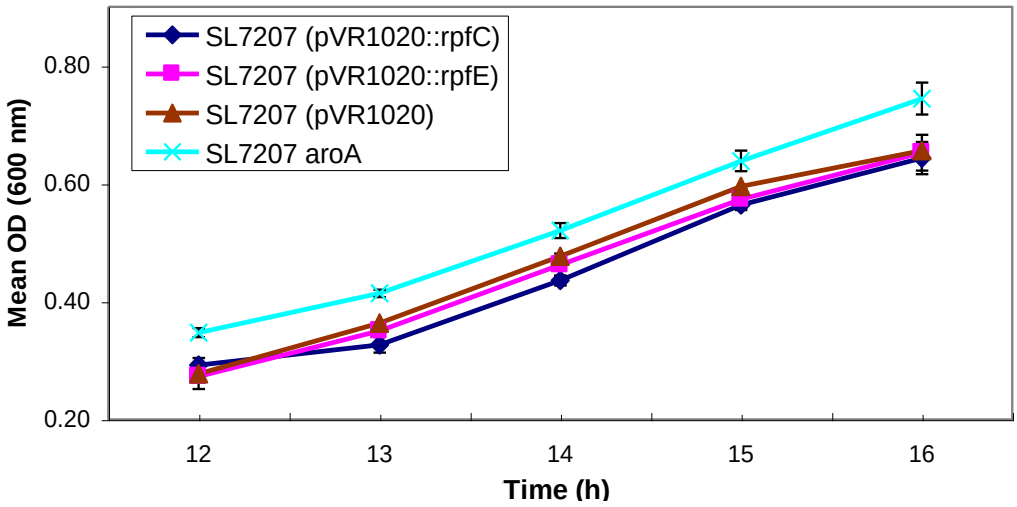

Figure 5. Growth curve assay of recombinant *Salmonella ΔaroA* constructs the wild mutant strain. Mean ± standard deviation optical density at 600 nm (37°C) from five independent experiments.

kanamycin selection pressure, the plasmid persisted within the *Salmonella* for several generations as confirmed by CFU counts on LB agar plates. Maintenance of stable expression of the *rpf* gene in the host cells is one of the qualities desired in vaccine delivery system, for it gives a sufficient stimulation time to the immune system after delivery. Similar studies have shown substantial *in vitro* plasmid stability in bacterial vectors under ampicillin selection pressure (Xu et al., 2005; Gahan et al., 2007). The construction of a live recombinant attenuated *S. typhimurium* DNA vaccine strain expressing HpaA protein of *H. pylori* (Xu et al., 2005), similarly obtained a stable recombinant plasmid *in vitro* by growing the recombinant bacteria for several generations. Gahan et al. (2007) have also examined the impact of plasmid stability on oral DNA delivery by the attenuated *Salmonella enterica serovar typhimurium* vaccine strain BRD509 carrying the C fragment of tetanus toxin under control of the cytomegalovirus (CMV) promoter that plasmid copy number was found to impact on plasmid stability and the induction of antigen-specific humoral responses.

The growth curve of all recombinant *S. typhimurium* constructs compared to the controls (the wild mutant strain of *Salmonela* without and with empty plasmid) showed no statistically significant difference in growth ($P > 0.05$). This suggests that the recombinant *Salmonella* carrying pVR1020::*rpfB* and pVR1020::*rpfC*, and pVR1020 pVR1020::*rpfD* and pVR1020::*rpfE* are metabolically as competent as the *Salmonella* carrying the empty plasmid pVR1020, indicating that the presence of *rpf* gene did not impose considerable physiological burden on the bacteria. The fact that these recombinant *Salmonella* behave physiologically like the wild mutant means that they are able to traverse the mucosal blanket following their normal infection route and finally get to antigen presenting cells (APCs) to release the DNA plasmids of a vaccine potential. The eukaryotic expression plasmid, pVR1020, used in this study harbors the human tissue plasminogennic encoding activator (tPA). Plasmids encoding a secreted form of the protein by fusing the gene to the signal sequence of tPA are generally more immunogenic than plasmids encoding a mature form of the protein (Baldwin et al., 1999). More recently, Parida et al. (2005) evaluated the feasibility and efficacy of attenuated *aroA* mutant of *S. typhimurium* delivery strategy with DNA encoding Ag85A of *M. tb* using different eukaryotic expression plasmids and showed that pVR1020 containing tPA signal gives a much-accentuated immune response in mice as compared to the conventional expression plasmid, pCMV-β. Therefore, though this could be part of future investigation, it could be speculated that DNA vaccines that elicit protective responses against Rpf(s) could be maintained by using pVR1020 with tPA signal sequence. Previous vaccine development using Rpf was based on the protype Rpf protein from *Micrococcus luetus* (Mukamolova et al., 2002b) and a subunit vaccine of the Rpf-like protein family from *M. tb* (Fan et al., 2008, 2010; Yeremeev et al., 2003). The data presented in this study provides the first report of a framework exploration of mutant *S. typhimurium aroA* SL7207 strains as bacterial vaccine carriers for post-exposure DNA vaccine encoding Rpf of *M. tb.* Previously, the cloning of the *rpf* genes has been limited to only a portion of the genes (Mukamolova et al., 2002a, 2002b). This is the first attempt to clone virtually the complete coding sequences of the *rpf* genes excluding the N-terminal signal sequence into a eukaryotic expression vector with the objective of developing a pre-clinical post-exposure DNA vaccine

escape system against NRP TB using *S. typhimurium* vaccine vehicle for oral immunization. Therefore, the successful cloning of the whole coding sequences of the *rpf*-genes would relatively provide more detailed understanding of the immunogenicity of the Rpf proteins compared to the truncated forms of these proteins (Downing et al., 2004).

Earlier studies have shown that *S. typhimurium aroA* strains have been utilized for DNA vaccine delivery by using different expression vectors and antigens (Darji et al., 1997; Motameni et al., 2004; Bai et al., 2004; Xu et al., 2005; Huang et al., 2010). The current study has valuable future applications for exploring the potential of attenuated *S. typhimurium* as a delivery vehicle to efficiently deliver *rpf* DNA vaccines to professional antigen presenting cells through oral delivery. In our next studies, we will demonstrate the gene transfer potential of *S. typhimurium aroA* SL7207 strain to deliver *M.tb* DNA vaccines to eukaryotic system.

Conclusion

An improved vaccine against TB should preferably be cost-effective and simple to administer for use in low income countries, where the vaccine is most needed. DNA vaccine is relatively easy to prepare; avoids complicated protein purification processes and the use of chemical adjuvant. Recent studies on novel TB vaccines reveal that DNA vaccination is one of the most commonly useful approaches and several investigators are pursuing the development of prophylactic DNA vaccines for TB to protect against virulent challenge in animal models. However, the selection of appropriate protective antigens and vaccine delivery system remain as one of the major challenges in the efforts made to control the disease, particularly reactivated TB in latently infected population.

In this study, we developed a new Salmonella-based TB DNA vaccine delivery system with the cloning of full coding sequences of four out of the five latency–associated *rpf* genes of *M. tb* into the eukaryotic plasmid, pVR1020. These recombinants are believed to accentuate vaccine potential compared to the previously cloned short or truncated forms of these genes. Recombinant *Salmonella* carrying these transgenic plasmids encoding *rpfB-E* would serve as novel approach to mucosal delivery of DNA vaccines against latent TB if proof of principle can be established in animal studies. Our further studies will explore the *in vitro* and *in vivo* immunogenicity and protective efficacy of orally administered live recombinant *S. typhimurium* carrying pVR1020::*rpfB-E* in a murine TB model.

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