

## Construction of a Vector for the Constitutive Expression of Human Papilloma Virus type 16 Genes in *Salmonella Enterica* Serovar Typhi Strain Ty21a

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### Abstract

*Salmonella enterica* Serovar Typhi strain Ty21a (*S. Typhi* Ty21a) is the only licensed live-attenuated oral vaccine against Typhoid fever in humans. It is also an oral delivery vehicle for heterologous antigen administration. This article describes the development of a constitutive expression vector, pSalEx, which is derived from pBR322 based vector, pProEx-HTb, for inducible expression of genes in *E. coli*. Repressor element, *lac I*, was removed from pProEx-HTb using PCR and restriction digestion. The pSalEx vector is smaller compared to the parent vector, pProEx-HTb, allowing larger inserts to be cloned and expressed. Expression of the major capsid protein (L1) of HPV16 under  $P_{trc}$  promoter of pSalEx was comparable to the constitutive expression of HPV16L1 from the pFS14nsd under the strong  $P_{tac}$  promoter. We demonstrate that pSalEx may be used for expression of heterologous genes in Ty21a and generally in other strains and species of bacteria belonging to the family *Enterobacteriaceae*.

**Keywords :** HPV 16 genes, pSalEx, *S. Typhi* Ty21a, Constitutive expression.

### Introduction

Effective oral vaccines offer significant advantages over the conventional parenteral

vaccines. The notable advantages of oral vaccine are the ease of administration with little or no intervention from medically skilled personnel and less stringent regulatory compliance compared to parenteral vaccines. Development of recombinant oral vaccines based on bacterial delivery vehicles that express either heterologous antigens or carry DNA for delivery into host cells are gaining prominence the world over. Bacterial vectored vaccine development has mostly been centered on *Listeria*, *Shigella*, *Lactobacilli* and *Salmonella* (1, 2, 3, 4). The live-attenuated Typhoid fever bacterial vaccine *Salmonella enterica* serovar Typhi strain Ty21a has been reasoned as a logical choice for heterologous antigen delivery (5, 6, 7). The attenuation of the bacteria generated by chemical mutagenesis has proven to be extremely safe while being fairly immunogenic. There has been no report of any major adverse affect from the millions of vaccinated populace in the nearly three-decade long use of the vaccine (8). Several target antigens of various pathogens have been expressed in the *S. Typhi* Ty21a. The recombinant *S. Typhi* has been proven efficacious in eliciting immunological responses specific to the expressed antigens (7, 9, 10, 11). Some of the promising candidate vaccines have progressed to clinical trials in humans (12, 13, 14).

Construction of a vector for the constitutive expression

Vectors originating from pUC backbone have generally been used for expression of heterologous proteins (15). Although, the pUC series of vectors were developed for cloning in the laboratory host strains of *E. coli* the vectors are compatible for use in *Salmonella*. Both *E. coli* and *Salmonella* belong to the family of *Enterobacteriaceae*, the group of gram-negative gut microbial flora that share significant genetic homology and plasmid compatibility (16, 17). The native *E. coli* plasmids with ColE1 origin of replication or vectors constructed for genetic manipulations bearing ColE1 derived replication origins are transmissible, stably maintained and well-partitioned during replication in *Salmonella* (18). However, there are no vectors available commercially that can constitutively express recombinant proteins under a strong promoter like  $P_{lac}$  or  $P_{trc}$  promoters.

This article details the construction of a vector for constitutive expression of heterologous genes in the Ty21a vaccine strain; and most likely in the other species of *Salmonella* and also other gram negative bacteria. A pBR322 based inducible expression vector (pProEx-HTb from Invitrogen) was chosen for modifications enabling constitutive expression of the cloned genes. We show that a simple reconstruction strategy of removing the *lac* I gene from the vector by employing the powerful tools of recombinant DNA technology- a hi-fidelity PCR followed by restriction enzyme cleavage and ligation enables constitutive expression of genes from the vector.

## Materials and Methods

**Plasmids and Bacterial hosts:** The live-attenuated *S. Typhi* Ty21a vaccine strain and the constitutive expression vector for expression pFS14nsd was obtained from Dr. Denise Nardelli, CHUV- Lausanne, Switzerland. The pProEx-HTb and *E. coli* strain Top10 were procured from Invitrogen® Corporation (USA).

**Primers and PCR:** Primers, *Mlu* I Forward primer, binding to the nucleotide sequence region

4750 to 4769 of pProEx-HTb (5' ATCTATACGCGTAATTAATGTGAGTTA GCGCG 3') and *Mlu* I Reverse primer, binding to the nucleotide sequence region, 3627 to 3646 of pProEx-HTb (5' AGTTCTACGCGTTGAATT GACTCTC TTCCG GG 3') were obtained from Bioserve™. Both primers were incorporated with *Mlu* I restriction site. The Polymerase chain reaction was performed with the HotStar Hi-fidelity polymerase kit™ (Qiagen). The conditions set for PCR were as follows- a) Initial denaturation at 96°C for 5 min. b) Thirty five cycles of denaturation at 96°C for 30 sec; annealing at 60 °C for 30sec and extension at 72 °C for 4min. c) Final extension of primers at 72 °C for 10min

**Re-circularization of vector:** The PCR amplified product was digested with the restriction enzyme, *Mlu* I (New England Biolabs, USA) according to the manufacturer's instruction. The digested product was then ligated with the  $T_4$  DNA ligase (Roche Applied Sciences) following the procedure outlined by the manufacturer. Competent, *E. coli* Top 10 cells were transformed with the ligated product and then plated on Luria-Bertani Agar containing ampicillin (50µg/ml). Plasmid from the broth culture of a single colony of transformed Top 10 cells was extracted using Hispeed Plasmid Maxi Kit (Qiagen).

**HPV genes:** The codon-optimized HPV16 L1 gene in the pFS14nsd vector was obtained from Dr. Nardelli, CHUV, Lausanne. The codon-optimized HPV 16 E6 and E7 genes for expression in *Salmonella* Typhi were obtained as synthetic constructs cloned in a plasmid from GeneArt™, Germany.

**Cloning:** Plasmids containing HPV16 L1, E6 and E7 genes were restriction digested with *Nco* I and *Hind* III enzymes from New England Biolabs® Inc. for 3 hrs at 37°C in the compatible buffer (Buffer-2) according to the manufacturer's instructions. The pSalEx vector was similarly digested with the corresponding *Nco* I and *Hind* III enzymes. Vector and gene ligation reactions

were carried out with the Rapid Ligation Kit from Roche according to the manufacturer's instructions. Competent *E. coli* Top 10 cells were transformed with the ligated products and plasmids were purified either using the Qiaprep Plasmid Miniprep or the Hispeed Plasmid Maxi kit from Qiagen.

**Expression:** Competent *S. Typhi* Ty21a cells were electroporated with pSalEx clones of HPV16 L1S, E6 or E7. Cell lysates of the recombinant Ty21a thus obtained were screened for expression by western blotting procedure with the PVDF membranes according to the standard procedures involving electro-blotting (19). For qualitative comparison of expression, western blotting of the whole cell lysates of Ty21a recombinant containing the HPV16 L1S gene in the pFSnsd vector and the recombinant culture containing the gene in pSalEx was performed. Expression of HPV 16 L1S gene was probed with the commercial monoclonal antibody specific to protein, CAMVIR-1™ (Novus Biologicals). HPV 16 E6 and E7 genes were probed with monoclonal antibodies specific to the respective proteins procured from Santa Cruz Biotechnology, Inc.

### Results and Discussion

Live-attenuated salmonella as an oral delivery vehicle of heterologous antigens from pathogens to confer immunological resistance to diseases is an attractive proposition. The approach requires that the heterologous antigen be expressed in adequate amounts to bring about an effective immune response to the antigen. An *in vivo* inducible system where the bacteria allows heterologous gene expression only after infection of the host cells is the ideal situation for evoking a robust immune response specific for the expressed antigen. However, construction of an inducible system is complex. It involves an intricate approach that is needed to strike the right balance between a strong, tightly regulated promoter system and the fitness of the bacteria. The hall-mark of attenuated vaccines is the poor survival ability inside the host cell thereby causing

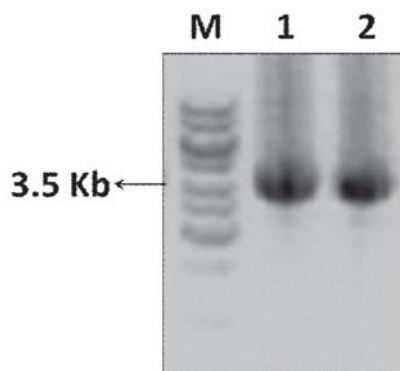
temporal limitation for optimal expression of the foreign antigen. A simpler alternative is to constitutively express the antigen under a strong promoter.

We envisaged that a constitutive vector suitable for expression in *Salmonella* can be constructed from an *E. coli* expression vector. The basis for such an idea originates from the use of plasmids based on ColE1 origin of replication in *Salmonella* for protein expression or as vectors for carrying heterologous DNA (15). The suitability of ColE1 origin for maintenance and replication of plasmids in *Salmonella* precludes the need for identification, isolation and selection of *Salmonella* specific plasmids. The published report on a pBR322 based pFS14nsd vector system with P<sub>tac</sub> as the promoter for expression of a modified HPV16 L1 gene lends credence to the suitability of modified *E. coli* promoter systems for expression of proteins in *Salmonella* (10). Most *E. coli* inducible expression vector systems have been ingeniously adopted from the *in vivo* regulatory mechanism of the *lac* operon (20). Inhibition of expression before induction is mediated by the *lac I* or the modified *lac I<sup>q</sup>* gene in these vectors, through its translated product the protein Lac I (or Lac I<sup>q</sup>) (20). Therefore, the removal of *lac I* gene or abolishing its expression in an expression vector should logically render constitutive expression of the gene in *Salmonella*.

We chose the inducible expression vector, pProEx-HTb (currently discontinued by Invitrogen) for the modification into a constitutive vector. This vector is derived from the pBR322 vector. The hi-fidelity PCR with forward and reverse primers that flanked the 5' upstream and 3' down-stream of the *lac I* gene in the pProEx-HTb vector respectively amplified the ~ 3.8Kb length vector sequence minus the *lac I* as seen in Fig 1. The Mlu I site incorporated in both the primers enabled efficient ligation and re-circularization of the amplified product. The deletion of the *lac I* gene in the re-circularized pSalEx was verified by sequencing analysis (data not shown). The pSalEx vector retains all the

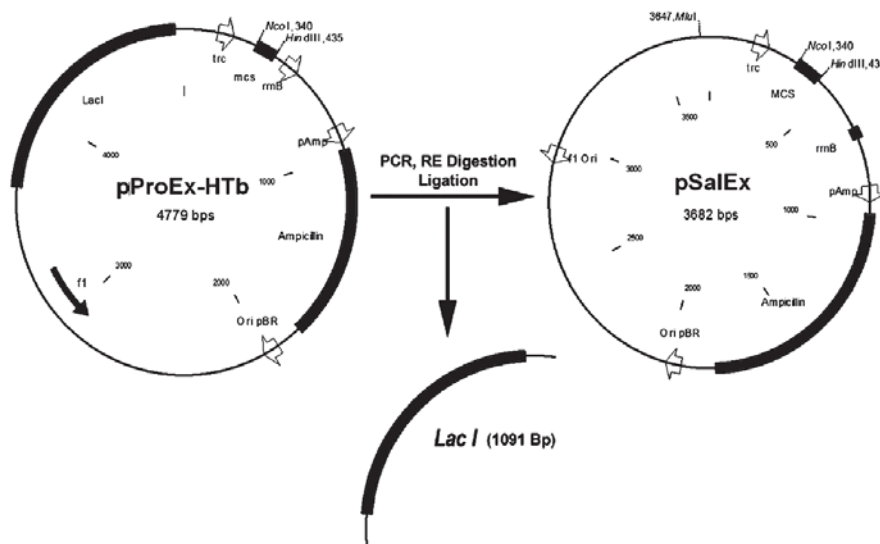
features of the pProEx including the multiple cloning site (MCS) and P<sub>trc</sub> promoter upstream of the MCS. A conceptualized picture of the resulting vector from re-circularization of the PCR amplified product minus the *lac I* gene is illustrated in Fig 2.

In order to test the suitability of the vector we chose the clinically relevant human papilloma virus genotype 16 (HPV 16) antigens, L1, E6 and E7 for expression in the vector. HPV infection causes cervical cancer in women and the genotype 16 of the virus is the most predominant type associated in cancer cases world-wide (21, 22). Recombinant Ty21a expressing the HPV16 L1 protein has been shown to elicit immune response in mice (10). Although its efficacy in humans would only be decided in planned clinical trials it holds promise as an oral vaccine and a cost-effective alternative to the virus like particle (VLP) based prophylactic vaccines (23, 24). We have attempted to correlate, although qualitatively, the expression of L1 from pSalEx to that seen in the candidate vaccine.



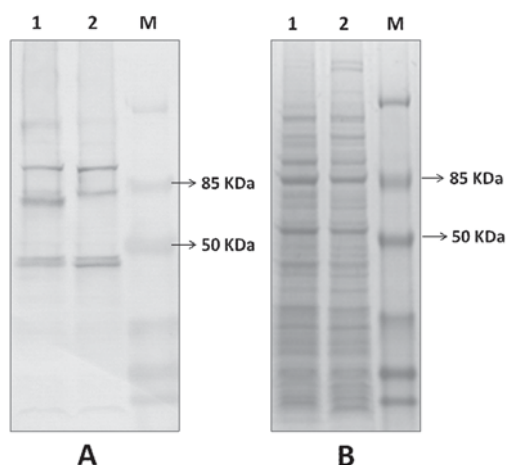
**Fig. 1.** Hi-fidelity PCR of pProEx-HTb with primers flanking the *lac I* gene. Lane M: Molecular weight DNA marker; Lane 1 and 2: Amplified pProEx-HTb vector region of ~3.8Kb devoid of *lac I* gene.

The HPV 16 LIS gene in the pFS14nsd vector is cloned in the *Nco I* and *Hind III* sites and the sub-cloning of the gene in pSalEx was also carried out in the same sites. We sought to determine the ability of pSalEx to render expression of HPV16 LIS by the standard

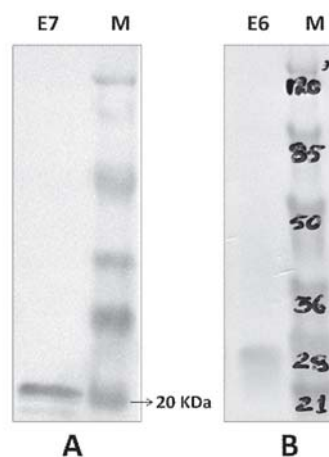


**Fig. 2.** Schematic illustration of the modification of pProEx-HTb to the constitutive expression vector pSalEx. *lac I* is removed by a PCR reaction with primers flanking the gene. *Mlu I* enzymatic digestion produces cohesive ends that enable efficient joining of the linear DNA. The T4 DNA ligase treatment covalently links the ends creating the re-circularized vector

Western blotting procedure and also make a qualitative comparison of the expression to that seen from the  $P_{tac}$  promoter of the pFS14nsd vector (Fig. 3). However, quantitative analysis would help determine the effect of concentration on immune response to an antigen *in vivo*. The western blot profile of the HPV16L1 proteins (Fig. 3) is typical for the whole-cell bacterial lysates of L1 expressing recombinant Ty21a. Though the blot was probed with the monoclonal antibody, CAMVIR-1, specific for a linear epitope in the L1, the profile obtained is consistently of more than one band apart from the expected ~57.0 KDa (HPV16 L1 gene is 1515 bp in length). Fig. 3 shows a similar profile for pSalEx-HPV16L1 and pFS14nsd-HPV16L1 recombinant Ty21a indicating a similar levels of expression from the pSalEx vector to that of pFS14nsd.



**Fig. 3.** Western blot of bacterial cell-lysates of HPV16 L1 recombinant *S. Typhi* Ty21a. A- Blot probed with HPV16 L1 specific monoclonal antibody, CAMVIR-1. B- Corresponding SDS-PAGE stained with coomassie brilliant blue. Lane 1: Bacterial cell-lysate from *Salmonella* Ty21a transformed with pFS14nsd cloned with the HPV 16 L1 gene; Lane 2: Bacterial cell-lysate from *Salmonella* Ty21a transformed with pSalEx cloned with the HPV 16 L1 gene; Lane M: Pre-stained Protein molecular weight marker.



**Fig. 4.** Western blot of bacterial cell-lysates of HPV16 E6 and E7 recombinant *S. Typhi* Ty21a A- Blot probed with HPV16 E7 specific monoclonal antibody. B- Blot probed with HPV16 E6 specific monoclonal antibody. Lane M: Pre-stained Protein molecular weight marker; Lane E7: Bacterial cell-lysate from *Salmonella* Ty21a transformed with pSalEx cloned with the HPV 16 E7 gene; Lane E6: Bacterial cell-lysate from *Salmonella* Ty21a transformed with pSalEx cloned with the HPV 16 E6 gene

We cloned the clinically relevant, HPV 16 oncogenes E6 and E7 (25, 26) to further establish the strength of pSalEx as an expression vector. The western blot pictures (Fig. 4) confirm the expression. We need to mention here that pSalEx uses the synthetic *E. coli* promoter  $P_{trc}$  for expression.  $P_{trc}$  is identical in its nucleotide sequence to that of  $P_{tac}$  except that in  $P_{trc}$  the spacer sequences between the -35 and -10 element contains 15 bases compared to the 14 in  $P_{tac}$ . The relevance of the extended spacer on expression can only be unraveled by the quantitative determination of the expressed proteins in both pSalEx and pFSnsd.

The deletion of *lac I* does render the constitutive expression of antigens in pSalEx as confirmed by the results. But the vector requires further modification before being employed in the

development of vaccines for clinical use. The most important modification that needs to be carried out is to replace the selection marker in the vector. The ampicillin resistance gene, *bla<sup>R</sup>* is the selection marker used in pSalEx. The kanamycin resistance gene, *kan<sup>R</sup>* is the only antibiotic resistance gene approved for human use by the Food and Drug Administration (27). Therefore, *bla<sup>R</sup>* needs to be replaced with either *kan<sup>R</sup>* or a more acceptable selection marker such as genes that supplement nutritional auxotrophy. One of the characterized mutations in Ty21a is a point mutation in the isoleucine and valine biosynthetic gene, *ilvD* rendering it non-functional (8). A functional *ilvD* gene is therefore, a potential selection marker.

### Conclusion

The results reiterate that vectors containing ColE1 derived origins of replication are adept for use in *Salmonella*. It also reiterates that the *E. coli* based synthetic promoters are suitable for expression in *Salmonella*. The use of *S. Typhi* Ty21a and other attenuated strains of *Salmonella* as bacterial vehicles for oral delivery of heterologous antigens are increasingly gaining ground. Researchers across the world are keen to develop suitable expression systems to optimize antigen delivery. This study strengthens the case for exploiting the large repertoire of *E. coli* expression vectors for expression of heterologous genes in *Salmonella*. The robust tools of recombinant DNA technology namely high-fidelity PCR and generation of synthetic DNA has given limitless options for vector modifications towards that end. Further modification to replace the ampicillin resistance gene *bla<sup>R</sup>* with a clinically acceptable, selection marker would render the use of pSalEx vector in the development of recombinant Ty21a based oral vaccines.

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