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Alteration in apyrase enzyme attenuated virulence of *Shigella flexneri*

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ABSTRACT

Virulence of *Shigella* is attributed to the genes presence in chromosome or in the megaplasmid. The *apy* gene which is located in the megaplasmid of *Shigella* species encodes for apyrase enzyme, a pathogenesis-associated enzyme causing mitochondrial damage and host cell death. In this study we constructed an *apy* mutant of *Shigella flexneri* by insertional activation using a kanamycin resistant gene cassette. The wild type *apy* gene of *S. flexneri* 2a was PCR amplified, cloned and mutated with insertion of kanamycin resistant gene cassette (*aph*A). The mutated construct (*apy: aph*A) was subcloned into a conjugative suicidal vector (pWM91) at the unique *Sma*1 and *Sac*1 sites. The mutation of the wild *apy* gene in the construct was confirmed by DNA sequencing. The mutated construct was introduced into wild type *S. flexneri 2a* by conjugation with *Escherichia coli*. After undergoing homologous recombination, the wild *apy* gene was deleted from the construct using the sucrose selection method. Non-functional activity of the apyrase enzyme in the constructed strain by colorimetric test indicated the successful mutation of the apyrase enzyme. This strain with mutated *apy* gene was evaluated for its protective efficacy using the guinea pig keratoconjunctivitis model. The strain was Sereny negative and it elicited a significant protection following challenge with wild *S. flexneri* strain. This apy mutant strain will form a base for the development of a vaccine target for shigellosis.

1. Introduction

Shigellosis, also known as bacillary dysentery, accounts for high levels of childhood mortality and morbidity [1,25]. The causative agent, *Shigella* spp., is a highly virulent pathogen with infectious dose of as low as 10 bacteria [11]. Shigellosis causes dysentery with frequent mucoid bloody stools, abdominal cramps and fever [26]. The choices of antibiotics to treat shigellosis are becoming limited due to increase in emergence of multidrug resistant strains [19]. Several strategies have been employed towards development of vaccines targeting for shigellosis and some are undergoing field trials, but no licensed vaccine currently exists. Some of the vaccines developed for *Shigella* include live attenuated, conjugate, broad-spectrum, and proteasome-based vaccines [3,16,18,27]. The greatest barrier to the use of shigellosis vaccines in developing areas is the poor immune responses to oral vaccines in children, who have minimal maternal antibodies and are susceptible to infection [13].

The mechanism of pathogenicity of *Shigella* spp. and its associated virulence factors has been well studied. Studies at the microscopic level in conjunction with the *in vitro* cell assays have shown the overall pathogenesis of shigellosis includes several important steps: (a) invasion of colonic epithelial cells by phagocytosis; (b) intracellular multiplication and spread of bacteria; and (c) re-infection of the adjacent cells by the bacteria. The combination of these events finally results in host cell death.

Numerous virulence genes have been identified in *Shigella flexneri*, and majority of these genes are located on the 220 kb pINV virulence plasmid [10]. The *apy* (*phoN2*) gene, which is located in this plasmid, encodes the apyrase enzyme [5]. Apyrase is a 27 kDa protein that utilizes the Sec machinery for its secretion across the inner membrane of the host cell [14,23]. suggested a possible role for apyrase in the dramatic decrease in dNTPs levels in host cells during

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intracellular multiplication. A study on structure and functions of apyrase reported that expression of *Shigella's* apyrase attributes in the mechanism of *Shigella* virulence factor and is perhaps a novel variant of bacterial acid phosphatases [2]. A study investigates the role of apyrase in the mechanism of pathogenicity of *S. flexneri* demonstrated that it is necessary for proper unipolar IcsA localization, thus required for efficient cell-to-cell spreading [21,22].

Such knowledge in mechanism and role of apyrase in pathogenicity of this bacterium has made it possible to construct a novel live attenuated vaccine. In this study, we proposed that mutation in the *apy* gene will lead to attenuation of virulence in *S. flexneri* and that this mutant strain could be used as a base in development of live attenuated oral vaccine against this organism.

2. Material and methods

2.1. Strains and culture media

Table 1 provides details about the bacterial strains and plasmids used in this study. All bacterial strains were grown in Luria–Bertani (LB) medium. Wild type *S. flexneri* SH057 were obtained from Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia and maintained in 30 μg/ml tetracycline, as this strain is resistant to tetracycline. *Escherichia coli* strains were maintained on LB agar, and plasmids containing transformants were selected on LB agar containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml). SFUSM1 mutants were maintained on LB agar supplemented with tetracycline (30 μg/ml) and kanamycin (50 μg/ml), as this *apy* mutant strain is resistant to kanamycin.

2.2. Sereny test

This animal study was conducted in accordance with the requirement of the rules and regulation by Animal Ethics Committee Universiti Sains Malaysia (AECUSM) approval protocol PPSG/07(A)/044. *S. flexneri* strains (SH052, SH057, SH060, and SH062) were tested via inoculation into guinea pig eyes as follows. The above bacterial strains were grown overnight in LB broth and were brought to 10^8 CFU in normal saline (0.9%) by measuring optical density (OD) at 600 nm. Each eye of a Hartley guinea pigs ($n = 6$) was inoculated in the conjunctival sac with 10^8 CFU of one of the wild strains. Guinea pigs were examined daily for 5 days, and their inflammatory responses were graded according to Hartman et al. [9]. Development of the disease was rated as follows: 0: no disease or mild; 1: mild conjunctivitis or late development and/or rapid clearing of symptoms; 2: keratoconjunctivitis without purulence; and 3: fully developed keratoconjunctivitis with purulence. The strain that showed the highest degree of conjunctivitis was used for the mutation of *apy* gene.

2.3. Construction of apy mutants

Fig. 1 illustrates the procedure used to construct the *apy* mutant. The sequence for the *apy* gene was obtained from GenBank (accession number U04539). The primers used in this study are described in Table 2. The 738 bp coding region for the *apy* gene was PCR amplified using the primers ApyF (5′-ATGAAAACCAAA AACTTTCTTC-3′) and ApyR (5′-TGGGGTCAGTTCATTGGTA-3′) from wild type *S. flexneri* 2a SH057. The *apy* gene fragment was cloned into the pTZ57R vector using the pTZ T/A cloning kit (Fermentas, USA). This procedure resulted in the recombinant plasmid, pTZ-*apy*.

To mutate the *apy* gene, a 1.1 kb *aphA* gene cassette that encodes for kanamycin resistance was PCR amplified from pCR2.1-TOPO (Invitrogen, USA) using KS1 (5′-TCG AGC TCT AGA AGC TTC AGG GCGCAA GGG CTG CT-3′) and KR1 (5′-TCG AGC TCTAGA AGC TTC AGA AGA ACT CGT CAA GAA G-3′) primers, which were based on published sequence (http://www.invitrogen.com/content/sfs/vectors/pcr2.1toposeq.txt). The *aph*A PCR product was blunt-ended using T4 DNA polymerase and then blunt-end ligated at the *Hpa*I site (located at position 564 with reference to the GenBank sequence) of the *apy* gene in pTZ-*apy* to create the recombinant plasmid pTZ-*apy*-*aph*A. The recombinant plasmid was confirmed by insertion of *aph*A gene (~2 kb) within the *Hpa*I site of the wild *apy* gene. The *apy*-*aph*A region from pTZ-*apy*-*aph*A was excised using *Sma*I and *Sac*I. It was then subcloned into the plasmid pWM91 at the same restriction site. The resultant recombinant plasmid construct, pWM91-*apy*-*aph*A, was transferred into the *E. coli* cells (BW20767-λ pir) host for further replication. This constructed plasmid was conjugatively transferred to wild type *S. flexneri* 2a SH057 by the direct mating method as follows. A single colony of the donor strain, *E. coli* BW20767, containing pWM91-*apy*-*aph*A was grown in LB agar containing kanamycin and ampicillin. The recipient, wild type *S. flexneri* 2a SH057, was grown on LB agar containing tetracycline. For the mating experiment, the donor strain was spread in LB agar in a small area (1.5 cm × 1.5 cm). The recipient strain then was spread over the donor strain. Plates were incubated at 37 °C for approximately 5 h, and the mixture culture was scooped from the plate and transferred into 1 ml pre-warmed LB broth. The culture was vortexed briefly before undergoing 10 fold dilutions. For each dilution, 100 μl was spread on LB agar plates containing kanamycin (50 μg/ml), ampicillin (100 μg/ml), and tetracycline. Merodiploids (the clones which has both wild *apy* and mutated *apy* gene) were selected from the antibiotic plates and their identity was confirmed by PCR.

2.4. Deletion of the wild apy gene

Confirmed merodiploids were subjected to sucrose selection in order to drive the second recombination, which resulted in the

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Source or reference
E. coli Top10	*F-merA Δ* (*mrv hsdRMS mcrBC*) *φ80lacZΔM*15 *Δlac X*74 *deOR recA1 araD*139 *Δ*(*ara-leu*)7697 *galU galK rpsL*(*strR*) *endA1 nup*G	Invitrogen
E. coli BW 20767	*RP*4 *2tet: mu-1kan::Tn7integrant leu* 63::*IS10 rec A*1 *cre* (510 *hsdR*17 *end A1 Zbf*-5 *uid*) (*ΔMluI*): *pir thi*	Gift from Dr. William Metcalf, University of Illinois [15]
SH057	Wild type, *Shigella flexneri 2a*	This study
SFUSM1	Mutated *Shigella flexneri 2a, apy::aph*A	This study
Plasmids		
pTZ57R	InsT/A clone™ PCR Product Cloning Kit	Fermentas
pCR2.1 TOPO	*LacZα* m13R T7 promoter m13F-20 M13F-40 MCS f1 origin KanR, AmpR *ColE1* origin	Invitrogen
pTZ-*apy-aphA*	pTZ-*apy-aphA* with *aphA* at polished *Nde*I site of the *apy* gene, Amp^R, Kan^R	This study
pWM91- *apy-aph*A	pWM91- *apy-aph*A with *apy-aph*A fragment cloned at *Sac*I and *Sma*I site, Amp^R, Kan^R	This study