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Full Length Article

Attenuated virulence of pigment-producing mutant of *Aeromonas veronii* bv. *sobria* in HeLa cells and Nile tilapia (*Oreochromis niloticus*)

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Virulence

Abstract *Aeromonas* species are potential water/foodborne pathogens, whereas *Aeromonas veronii* bv. *sobria* is one of the most virulent species to human and fish. Most current experimental evidence has publicized that suicide plasmid dependent IS*1*-element untargeted integration into *A. veronii* bv. *sobria* ATCC 9071T strain was recently used to generate brown pigment-producing and spontaneous pelleting (BP^+SP^+) mutant. Current study was conducted to compare virulence of wild-type ATCC 9071T strain and its BP^+SP^+ mutant with respect to cytotoxicity in HeLa cells and lethality in Nile tilapia. It was found that the cytotoxicity of wild-type ATCC 9071T strain to HeLa cells has reached 75% versus 50% for the cytotoxicity of BP^+SP^+ mutant. Further, the median lethal dose (LD_{50}) of wild-type ATCC 9071T strain in Nile tilapia was 8.25 Log_{10} colony-forming units (CFU)/ml, compared to 9.16 Log_{10} CFU/ml for the LD_{50} of BP^+SP^+ mutant. Thus, current study supports the notion that non pigment-producing *Aeromonas* strains are more virulent than pigment-producing ones.

1. Introduction

For several decades, *Aeromonas* species have been recognized as water/foodborne human enteric pathogens [1]. It is well documented that several *Aeromonas* spp. were implicated in the occurrence of several diseases in aquatic animals. Aeromonads have significantly caused mass mortalities in both wild and

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farmed freshwater/marine fish species with consequent catastrophic economic losses at the aquaculture sector [2].

The production of brown water-soluble pigment is one of nine key differential characteristics of typical non motile *Aeromonas salmonicida* subsp. *salmonicida* from atypical strains [3]. Other subspecies of *A. salmonicida* and atypical *A. salmonicida* are normally weak or slow producers of pigment [4]. However, non-pigment producing strains of *A. salmonicida* subsp. *salmonicida* were first isolated from outbreaks of furunculosis in salmonids reared at several fish farms located on Southern Finland. The mortalities in the affected farms varied from 8% to 90%. In experimental challenge, non-pigment producing strains were found to be highly pathogenic to rainbow trout [5].

Few years later, four non-pigment producing isolates and two pigment producing isolates of *A. salmonicida* subsp. *salmonicida* were isolated from diseased farmed Atlantic salmon. Non-pigment producing isolate produced a significantly higher mortality in the experimentally infected fish compared to the mortality caused by pigment-producing strain [6]. On the contrary, there are several strains of motile *Aeromonas* spp. such as *A. bestiarum*, *A. media* and *A. eucrenophila,* produced brown to dark brown pigments on trypticase soy agar (TSA) similar to the melanin like pigment produced by *A. salmonicida* [7,8].

Interestingly, Gram-negative pathogens belonging to the genus *Aeromonas* are variable in harboring insertion sequence (IS) elements that play an important role in the generation of dysfunctional relatives of known genes [9–12]. Furthermore, a recent report showed that suicide plasmid dependent IS*1*-element untargeted integration into *Aeromonas veronii* bv. *sobria* ATCC 9071T strain generates brown pigment-producing and spontaneous pelleting (BP^+SP^+) mutant [9]. Therefore, current study was designed to compare virulence of wild-type *A. veronii* bv. *sobria* ATCC 9071T strain and its BP^+SP^+ mutant *in vitro* HeLa cells and *in vivo* Nile tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Bacterial strains were cultured in tryptic soy agar and/or broth medium (TSA or TSB) (Difco Laboratories, Detroit, MI) at 28 °C. When required, media were supplemented with 100 μg/ml of ampicillin (Amp.).

2.2. Transmission electron microscopy

The samples were processed using the method adopted from Martinez et al. [14]. Overnight cultures were diluted in a ratio of 1 ml broth culture/100 ml fresh TSB medium and cultivated to mid-log phase (OD_{600} nm = 0.6). Formvar-coated copper grids were incubated with cultures for 2 min. Then, grids were fixed in 4% paraformaldehyde solution and washed three times for 1 min each in 0.1 M sodium cacodylate. Whole-cell preparations were negatively stained with 2% solution of potassium phosphotungstate (PTA) pH 6.5 for 2 min. Bacteria were then viewed on a Hitachi H7100 transmission electron microscope (TEM).

Table 1 Bacterial strains used in this study.

Name	Relevant characteristics	Reference
ATCC 9071T	*A. veronii* bv. *sobria* type strain, isolated from frog red leg disease	[13]
BP^+SP^+ mutant	Brown pigment-producing and spontaneous pelleting mutant strain derived from ATCC 9071T strain	[9]

2.3. Cytotoxicity assay in HeLa cells

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM: Sigma–Aldrich, St Louis, MO, USA) plus 10% fetal bovine serum (FBS: Sigma–Aldrich) without antibiotic at 37 °C in an atmosphere of 5% CO_2 and humid air in Corning 96 well plates (Sigma–Aldrich). Confluent cultures were infected for 3 h at a multiplicity of infection (MOI) of 10 with an overnight culture of wild type ATCC 9071T strain or BP^+SP^+ mutant [15]. Non-infected cells were used as a control. Cell death or cytotoxicity was classically evaluated by the quantification of plasma membrane damage using the Promega CytoTox 96 kit (Promega, Corporation Madison, WI), which enzymatically assess lactate dehydrogenase (LDH) released from damaged cells into the supernatant according to the manufacturer's instructions.

Displayed data were obtained from three independent experiments. The results were shown as the mean ± SD. The statistical significance of the results was assessed by Student's *t*-test, calculated using Excel software.

2.4. Experimental challenge in Nile tilapia

Experimental challenge of live fish was regulated by the ethical guidelines provisioned by the ethical committee of the World Fish Center, Abbassa, Egypt. A total of 220 apparently healthy Nile tilapia (*O. niloticus*) cultured fish of both sexes (average weight, 100 ± 4 g) were divided into 22 equal groups (10 fish each). They were reared in glass aquaria ($50 \times 60 \times 120$ cm^3), fed on a balanced commercial diet at a ratio of 3% of body weight per day. The water was partially renewed daily and monitored regularly; the temperature was maintained at 27 ± 3 °C. Fish groups were maintained in the aquaria for couple weeks for adaptation.

Suspensions of *A. veronii* bv. *sobria* ATCC 9071T wild type and BP^+SP^+ mutant strains were prepared by cultivation in TSA medium for 24 h at 28 °C, then collected, washed, and suspended in sterile normal saline (0.85% NaCl) and matched against McFarland standard tubes. Fish groups were then experimentally infected by an intra-peritoneal (I.P.) injection with 1 ml of the bacterial suspensions at different concentrations of 10^5, 10^6, 10^7, 10^8 and 10^9 colony-forming units (CFU)/ml. Each concentration was injected into replicate fish groups. The last two groups were used as control (injected with sterile normal saline). Fish groups were monitored for two weeks and percentages of lethality were reported to calculate the median lethal dose (LD_{50}) according to Reed and Muench [16]. At the end of the experiment, all fish groups were scarified and hygienically disposed.

3. Results and discussion

Aeromonas veronii bv. *sobria* ATCC 9071T strain belongs to DNA hybridization group HG8 and serogroup O:11, is a non-pigment producing and non-spontaneous pelleting, has a surface array protein (S-layer). Such strain is considered by most investigators to be more closely similar to clinical *A. sobria* strains [17–20].

Aeromonas strains are variable in their susceptibility for suicide plasmid dependent IS*1*-element untargeted integration. The susceptible species such as *A. veronii* bv. *sobria* ATCC 9071T strain has been changed to BP^+SP^+ mutant, which mimics pigment-producing/spontaneous pelleting strains. These strains are naturally occurring among heterogeneous group of foodborne aeromonads [9].

Noteworthy, non-brown pigment-producing strains of *A. salmonicida* subsp. *salmonicida* are significantly more virulent than brown pigment-producing one [6]. Controversially, non-spontaneous pelleting *Aeromonas* spp. strains are more virulent to mice than spontaneous pelleting one [19,21]. Incredibly, such two virulence markers (brown pigment-production and spontaneous pelleting) are displayed in BP^+SP^+ mutant.

Macroscopically, the colony of BP^+SP^+ mutant strain mimics atypical *A. salmonicida* in producing a diffusible brown melanin pigment on TSA medium (Fig. 1a), as the BP^+SP^+ mutant strain lost their ability to hydrolyze L-tyrosine amino acid with much increase of brown pigmentation of the medium [9]. In broth culture, BP^+SP^+ mutant strain secrets significant higher quantity of PilF homologous protein than the wild-type strain. Increased secretion of PilF homologous protein by BP^+SP^+ mutant strain is suggested to increase the surface piliation which mediates spontaneous pelleting and rough colony morphology [9].

Ultra-microscopically, the TEM micrograph of wild-type ATCC 9071T strain showed long polar lophotrichous flagella and short pilus-like cell surface appendages (Fig. 1b). Whereas, TEM micrograph of BP^+SP^+ mutant show distorted flagella and long pilus-like cell surface appendages (Fig. 1c). These results support the notion that BP^+SP^+ mutant manifested SP^+ phenomenon in broth culture due to increased assembly of pilus-like structures and impaired assembly of the flagella.

In typical *A. salmonicida* A449 strain, an IS-element dependent plasmid rearrangement causes the loss of the Type Three Secretion System (TTSS) [22]. Also, the endogenous IS-element (IS*AS1*) interrupts the *AbcA* gene encoding an ATP-binding cassette transport protein required for biogenesis of smooth lipopolysaccharide [23]. However, in atypical *A. salmonicida* strains, *tapA* gene, which encodes the type IV pilus subunit protein, is interrupted by ISAsa4 element [24].

The current study has proved that the suicide plasmid dependent IS*1*-element untargeted integrations resulted in the attenuation of the virulence of ATCC 9071T strain *in vitro*, as the cytotoxicity of wild-type ATCC 9071T strain to HeLa cells is minimized from 75% to 50% after mutating to BP^+SP^+ mutant (Fig. 2). Interestingly, *A. veronii* bv. *sobria* exerts a cytotoxic effect on HeLa cells through AexU TTSS-effector protein which disrupts the actin cytoskeleton by down-regulation of Rac1 small GTPase protein [15].

IS*1*-element containing pYAK1 suicide plasmid was used successfully in *A. hydrophila* ATCC 19570 strain to generate *pepO* gene deletion mutant without IS*1*-element untargeted

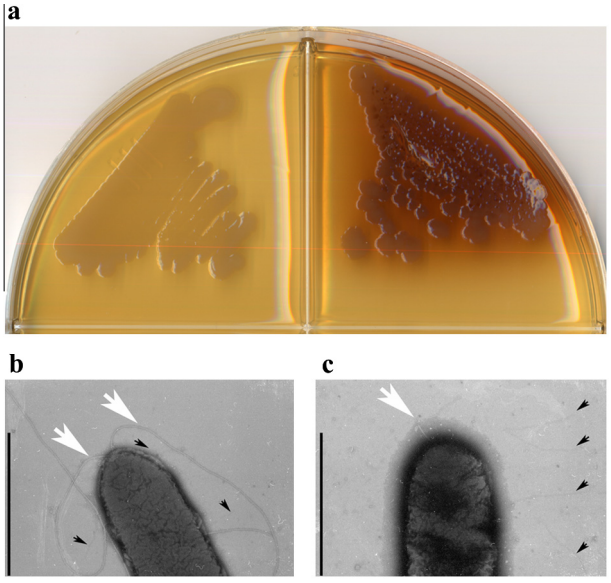

Figure 1 Macroscopic and ultramicroscopic appearance of wild-type *A. veronii* bv. *sobria* ATCC 9071T strain and its BP^+SP^+ mutant. (a). Photograph of TSA divided plate; the left side shows original whitish smooth non-pigment-producing colonies of wild-type ATCC 9071T strain, and right side shows rough brown pigment-producing colonies of BP^+SP^+ mutant. (b). TEM micrograph of wild-type ATCC 9071T strain showing the two polar lophotrichous flagella (white arrow) and short pilus-like cell surface appendages (black arrows). (c). TEM micrograph of BP^+SP^+ mutant strain showing short polar flagella (white arrow) and long pilus-like cell surface appendages (black arrows). PTA stain, ×20,000. Scale bars = 2 μm.

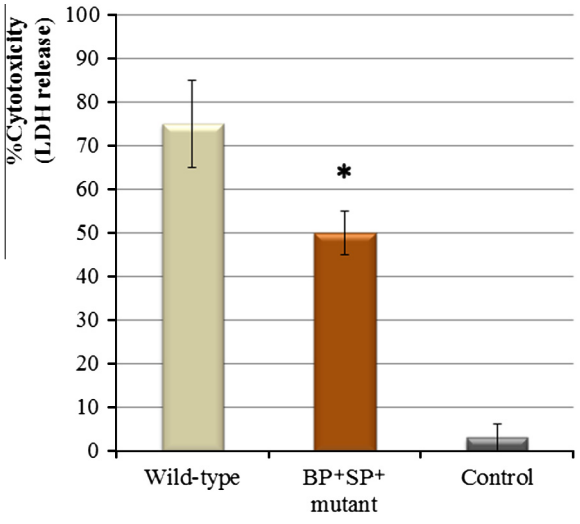

Figure 2 Measurement of lactate dehydrogenase (LDH) released from HeLa cells infected with wild-type *A. veronii* bv. *sobria* wild-type ATCC 9071T strain or its BP^+SP^+ mutant. Cytotoxicity (LDH release) was calculated as a percentage of that obtained from fully lysed cells. Significant reduction in cytotoxicity of BP^+SP^+ mutant infected cells ($P < 0.001$) was obtained. Data represent the means ± SD from three individual experiments.

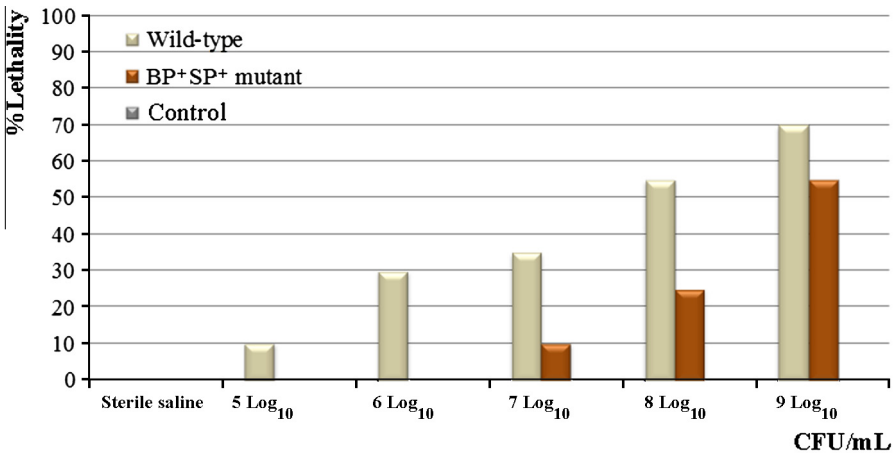

Figure 3 Lethality of wild-type ATCC 9071T strain or its BP^+SP^+ mutant in Nile tilapia. Groups (10 fish each) of apparently healthy Nile tilapia (*O. niloticus*) were infected by an intra-peritoneal injection with 1 ml of the bacterial suspensions of wild-type ATCC 9071T strain or its BP^+SP^+ mutant at different concentrations of 5 Log_{10}, 6 Log_{10}, 7 Log_{10}, 8 Log_{10} and 9 Log_{10} CFU/ml. Control groups were injected with 1 ml sterile normal saline. Infected fish groups were monitored for two weeks and lethality (%) was reported. Data represent the means from two individual experiments.

integration [9,25]. Although *pepO* gene deficient mutant strain lost its ulcerogenic property, this mutant strain caused a higher mortality in goldfish (*Carassius auratus*) than the wild-type *A. hydrophila* [25].

In vivo, wild-type ATCC 9071T strain was found to be highly virulent to Nile tilapia as the LD_{50} is 8.25 Log_{10} CFU/ml (Fig. 3). This result is closely similar to the pathogenicity of wild-type ATCC 9071T strain to mice as the LD_{50} is 7.8 Log_{10} CFU/ml [18]. But BP^+SP^+ mutant strain lost lethality in Nile tilapia at infection dose up to 6 Log_{10} CFU/ml and LD_{50} required 9.16 Log_{10} CFU/ml (Fig. 3).

To sum up, this study supports the belief that non-brown pigment producing and non-spontaneous pelleting strains are more virulent than brown pigment-producing and spontaneous pelleting aeromonads.

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