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Abundance of *Bdellovibrio bacteriovorus* in Porcine Gastrointestinal (GI) Tract and the Isolation, Characterization and Predation Ability of Porcine Stomach-derived Strain

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ABSTRACT

Bdellovibrio bacteriovorus is a kind of Gram-negative predator that feeds on other Gram-negative bacteria. Due to this, it is recognized as a potential alternative agent for antibiotics, which could alleviate the emergence of multiple drug-resistant (MDR) Gram-negative bacterial infections in animal industry. Detailed studies about *B. bacteriovorus* in porcine GI tract have rarely been reported. The research here mainly focuses on detection of the abundance of *B. bacteriovorus* in porcine GI tract, and the isolation, characterization and predation ability of stomach-derived *B. bacteriovorus*. In our study, total bacterial DNA was extracted from each segment in porcine GI tract, which was then analyzed by qPCR. Results strongly suggested that *B. bacteriovorus* did exist in porcine GI tract, and there was a significant difference between its quantity in cecum (10e4.32 copies g^{-1} contents) and duodenum (10e2.77 copies g^{-1} contents) ($P<0.05$). Meanwhile, *B. bacteriovorus* was isolated from digesta in porcine stomach, whose presence was further confirmed by transmission electron microscope (TEM) and 16S rDNA sequence analysis. To testify the predation ability of isolate, *E. coli* and *salmonella* were fed to it at 37°C. On account of this, the isolate could potentially play some role in controlling the gram-negative bacterial infections in animal industry.

INTRODUCTION

Antibiotics have been critically important in animal disease treatment and growth promotion for more than 60 years [1]. Their effects on promoting animal growth rate, improving feed efficiency and reducing animal mortality, with the only sub-therapeutic level in feeds, have been well documented [2]. Despite the clear benefits of antibiotics to animal husbandry, there are some potential problems that may affect the health of human and animals. A growing body of direct and indirect evidence in past 40 years has suggested the correlation between the use of animal husbandry antibiotics and the rise and spread of associated resistance genes in animal [3,4]. Specifically, antibiotics use accelerates the frequency of horizontal gene transfer and resistance gene fixation in genomes, which is believed to be the major cause for the emergence of multiple drug-resistant (MDR) Gram-negative bacteria [5,6]. Furthermore, the total use of antibiotics in animal husbandry reportedly accounts for as much as half of all antibiotics produced in China. Liberal and continuous use of antibiotics may contribute to a reservoir of MDR bacteria, which has emerged to be a serious threat to both human and animals health. As a result, an alternative antibiotics agent is urgently on demand in animal husbandry [7,8].

Bdellovibrio bacteriovorus is tiny (0.2-0.5 μm × 0.5-2.5 μm), uniflagellate, highly motile Gram-negative bacteria, belonging to a group of bacterial predators called *Bdellovibrio* and like organisms (BALOs), which prey naturally on other Gram-negative bacte-

ria [9,10]. As the most characterized member of this group, *B. bacteriovorus* can attach to and enter the prey periplasm, multiply by using up the host nutrients, then lyse the cell to release the progeny that will continue to seek out new prey to invade. Numerous studies indicate that *B. bacteriovorus* control a broad range of Gram-negative bacteria both *in vitro* and *in vivo*. It was reported to suppress the MDR bacterial infections as well. Moreover, recent publications have demonstrated the prohibitive effect of *B. bacteriovorus* against Gram-positive strains, such as *Staphylococcus aureus*, where *B. bacteriovorus* could effectively inhibit the formation of *S. aureus* biofilms and reduce their virulence. Nevertheless, *B. bacteriovorus* was found to have no negative effect on higher organisms, including animal and human cells. Therefore, *B. bacteriovorus* has been as the wise candidate of probiotic or living antibiotic to restrain bacterial infections in animal husbandry [11-19].

B. bacteriovorus is ubiquitous in the terrestrial and aquatic environment such as soil, rhizosphere and water, etc. To date, the majority of the studies relating to *B. bacteriovorus* are based on the isolates from the natural environment like soil and water, while the animal GI tract-derived strains have rarely been involved. Compared with traditional isolates (from water and soil), isolates from animal GI tract should be more suitable for applying to animal husbandry, due to their survivability in the acidic and thermal environment of animal GI tract. This has made GI tract-derived isolates a potent candidate in restraining the pathogen infections, especially the Gram-negative bacteria, in animal or even human gut. Our study aims to detect the presence of *B. bacteriovorus* in porcine GI tract, we focus on the isolation, identification and predation ability of porcine stomach-derived strain. The work presented here highlights the potential use of porcine stomach-derived *B. bacteriovorus* as a promising alternative agent for controlling Gram-negative bacterial infections in the animal industry [20-25].

MATERIALS AND METHODS

Ethical Statements

All surgical and animal care procedures throughout this study followed protocols approved by the China regulations and Institutional Animal Care.

Samples collection: Digesta were randomly collected in GI tract (stomach, duodenum, ileum, cecum, and colon) of four Erhualian pigs at the same farm. Part of samples was used for total community DNA extraction for detecting the abundance of *B. bacteriovorus* in each intestinal segment by qPCR; the rest of it was conducted to isolate *B. bacteriovorus* by the double-layer technique. Samples kept cold storage during transportation to the laboratory.

Digesta DNA Extraction

Total community DNA was extracted by QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's instructions. After extraction, DNA concentration was quantified by a Nano-Drop spectrophotometer (Thermo Fisher Scientific, Delaware, USA) at 260 nm, along with the 260/280 ratio. DNA samples were stored at-20°C.

Quantitative-PCR

A quantitative PCR (qPCR) was implemented with the standard curve to assay the abundance of *B. bacteriovorus* in our samples. Bdellovibrio-specific primers and hydrolysis probe were used to amplify a *B. bacteriovorus* specific region of 16S rDNA sequence. Briefly, primers used were Bd347F (3'-GGAGGCAGCAGTAGGGAATA-5') and Bd549R (5'-GCTAGGATCCCTCGTCTTACC-3'), while the probe was Bd396P (5'FAM-TTCATCACTCACGCGGCGTC-TAMRA3'). The qPCR reaction mix was made up with 10 μL of Premix Ex TaqTM (Probe qPCR) (Takara Bio. Inc., Dalian, China), 1.8 μL (900 nM) for both primers, 0.1 μL (50 nM) for the probe, and 4 μL for the template. The final reaction volume was adjusted to 20 μL with ddH_2O. Thermal cycling conditions were: a 95°C initial denaturation step (2 min), followed by 50 repeats of a 95°C denaturation step (15 s) and a 60°C annealing and extension step (1 min). Data were collected during the annealing phase. Triplicates were performed for each sample. To construct the standard curve, a positive plasmid was quantified by spectroscopy at 260 nm and 10-fold serially diluted in the range of 109-103 copies. The abundance value was expressed as 'number of Lg (copies g^{-1}) of sample [26-29].

Bacterial Strains and Isolation Procedure

The rest of digesta from porcine GI tract was used for *B. bacteriovorus* isolation. *Pseudomonas stutzeri* were used as prey 0.5 g of samples were aseptically added into 5 mL sterile saline. Vortex the dilutions to homogenize thoroughly and incubate them with *Pseudomonas stutzeri* using the double-layer technique (Stolp and Starr 1963). Specifically, 500 μL of each dilution was mixed with 300 μL of a suspension of *Pseudomonas stutzeri* in a 0.4% molten diluted nutrient agar, and the mixture was propagated onto a 1% diluted nutrient bottom agar plate, then plates were left undisturbed to solidify and incubated at 37°C for 48-96 h for the formation of plaques. Plaques were visible during 48 to 60 h and they were considered as potential *B. bacteriovorus* after further expansion for several days. For purification, an individual plaque was purified by successive cultivation, until lytic plaques on an agar plate were uniform in size.

Electron Microscopy

To examine the morphology of attack-phase *B. bacteriovorus*, one drop of fresh lysate was placed on a microscope copper grid for 2 min at room temperature. The grid was then lifted gently with a pair of forceps, and the excess liquid was removed by

blotting. Samples were then counter-stained with a 1% (wt vol^{-1}) phosphotungstic acid solution (pH=6.4) for 30 min and examined with a HITACHI H-7650 transmission electron microscope.

PCR Amplification

To avoid potential amplification from bacterial prey DNA, the *Bdellovibrio*-specific primer (primer 842R; 5'-CGWCACT-GAAGGGGTCAA-3') and the bacteria-specific primer (primer 63F; 5'-CAGGCCTAACACATGCAAGTC-3') were performed in PCR to amplify the 16S rDNA partial sequence of the stomach-derived isolate (Jurkevitch and Ramati) [22]. The primers were synthesized by Invitrogen Bio. Inc. (Shanghai, China). PCR (25 µL mixtures) were performed by using a Programmable Cyclic Reactor (Biometra, Goettingen, Germany). Each reaction mixture contained 12.5 µL 2X reaction buffer, 1.25 U of Premix Taq polymerase (Takara Bio. Inc., Dalian, China), 0.5 µL each primer, 1 µL template about fresh lysate, and 10.5 µL ddH_2O. The PCR cycle consisted of a 95°C initial denaturation step (3 min), 34 cycles of a 94°C denaturation step (1 min), a 53°C annealing step (50 s) and a 72°C extension step (30 s) and a final extension step for 10 min at 72°C. PCR products were electrophoresed on a 2% agarose gel to check for successful amplification. *B. bacteriovorus* 109J were used as the positive control, provided by Field Bio. Inc. (Nanjing, China).

Phylogenetic Analysis of 16S rDNA Sequence

The PCR products of stomach-derived isolate were directly sequenced by Invitrogen Bio. Inc. (Shanghai, China). The 16S rDNA partial sequence obtained was then deposited in Genbank database. BLAST-N search was used to identify the species as most related to isolate S1. Additionally, a phylogenetic tree was constructed using the Neighbor-joining method with Maximum Composite Likelihood model and a bootstrap analysis of 1000 replicates by Mega6 [23].

Predation Ability of Isolate S1 against *E.coli* and Salmonella

The predation ability of isolate S1 on *E.coli* and *Salmonella* was assessed by its ability to form clear lytic halos on a lawn of preys with the double-layer technique. Those two preys were initially isolated from the feces of piglets in Jiangsu province in this laboratory, and prey cells were grown for 12 h in LB (Luria-Bertani) broth (Haibo Inc., Qingdao, China) to reach the concentration of 10^6 CFU mL^{-1}. 0.3 mL of fresh prey cells were added to 0.5 mL of the fresh attack-phase *B. bacteriovorus* and 5 mL of 0.4% molten diluted nutrient agar as mentioned above. They were mixed and poured over the pre-warmed bottom agar. Plates were also incubated for 48-96 h at 37°C. Prey bacteria were regarded as being susceptible if any clearly appeared plaque on the double layer agar plates, and unsusceptible if otherwise. Each prey bacteria group had six replicates.

Statistical Analyses

Analysis of variance (ANOVA) and t-test were carried out in this study, statistical analyses were performed using GraphPad Prism (version 6.01 for Windows) and a P value less than or equal to 0.05 was considered significant.

RESULTS AND DISSCUSION

Abundance of ***B. bacteriovorus*** in Porcine GI Tract

B. bacteriovorus abundance was assessed by qPCR to investigate its presence in porcine GI tract. The primers and probe were used for the qPCR as the previous study reported (Iebba et al.). As shown in **Figure 1**, *B. bacteriovorus* were clearly present in all five intestinal segments, i.e., 10e3.15 copies g-1 contents in stomach, 10e2.77 copies g^{-1} contents in duodenum, 10e3.94 copies g^{-1} contents in ileum, 10e4.32 copies g^{-1} contents in cecum, and 10e3.82 copies g^{-1} contents in colon. It's noteworthy that the highest level was in cecum and lowest in duodenum (P<0.05).

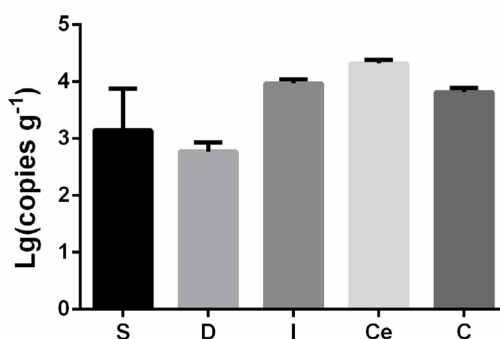

Figure 1: Abundance of *B. bacteriovorus* in porcine GI tract. S denotes stomach, D denotes duodenum, I denotes ileum, Ce denotes cecum, C denotes colon.

To our best knowledge, this is the first study to confirm the existence of *B. bacteriovorus* in each segment of porcine GI tract. *B. bacteriovorus* was firstly discovered by serendipity in 1962 by Stolp and Petzold when they were trying to isolate bacteriophages

for plant pathogenic bacteria from soil [20]. After their initial discovery, *B. bacteriovorus* was isolated from various habitats and found to be widely distributed in the terrestrial and aquatic environments [21]. Numerous studies were conducted to focus on its toxicity, prey range, diversity and genomics [14,17]. However, there is little information about its distribution or characterization in porcine GI tract. Here, our results indicated that *B. bacteriovorus* indeed existed in each segment of porcine GI tract. It was worth noting that the animal stomach was not adequate for the growth of bacteria due to the low pH [26,27]. The total number of bacteria in the mammalian stomach was typically maintained at 10^3 -10^4 bacterial cells g^{-1} contents [28]. However, the result of qPCR suggested that the number of *B. bacteriovorus* in porcine stomach was 103.15 copies g^{-1} contents **(Figure 1)**, which may account for a high proportion of total bacteria in porcine stomach. Owing to this, we speculated that *B. bacteriovorus* might have a good adaptation for the acid environment of the stomach and play a role in maintaining a balance in stomach ecosystem. Moreover, the qPCR result did not show a gigantic difference in numerical values of *B. bacteriovorus* between anterior GI tract and posterior GI tract. The probable reason was that *B. bacteriovorus* was a facultative aerobic bacteria and could not exist in the posterior GI tract for a long time. This result was also consistent with Iebba's study about the abundance of *B. bacteriovorus* in the healthy human gut [13,29].

Isolation of *B. bacteriovorus* from Porcine Stomach

The confirmation of the presence of *B. bacteriovorus* in porcine GI tract was followed by isolation from each of the five segments. The plaques became visible after 48 h of incubation at 37°C, with size expanding over time and reaching 4.0-6.0 mm at 96 h **(Table 1).** Unlike the phage plaques, those plaques developed more slowly and expanded over time. Among them, isolates from porcine stomach formed the largest plaques in the shortest time, demonstrating the greatest plaques-forming tendency **(Table 1).** Based on this, the stomach-derived isolate, denoted as isolate S1, was selected for the follow-up characterization **(Figure 2A).**

Table 1: Characteristics of plaques formed by isolates from five segments in porcine GI tract

Digesta	Plaques-forming time(h)	Diameter of plaques at 96 h (mm)	Plaque morphology
Stomach	48	5.5-6.0	rounded transparent
Duodenum	48	4.0-4.5	rounded transparent
Ileum	60	4.0-4.5	rounded transparent
Cecum	60	3.5-4.0	rounded transparent
Colon	60	3.5-4.0	rounded transparent

Isolate S1 is the first reported *B. bacteriovorus* isolated from porcine GI tract. Because of the special environment in porcine stomach, it may have two potential advantages over traditional strains of *B. bacteriovorus* (derived from soil and water) when used in animal husbandry: (i) the incubation temperature for isolate S1 is 37 °C, which is closer to the temperature of animal GI tract, as opposed to the 28°C for the traditional strains [20-22], (ii) the superb adaptation of isolate S1 to lower pH affords wider tolerance to habitation environments, whereas for traditional strains (optimum pH ranging from 7.0-7.6), an acid environment could cause the shedding of flagella and dramatically reduce the predation ability [30]. Therefore, isolate S1 tends to outperform the traditional strains in maintaining the vitality and play some role in animal GI tract **(Figure 2B).**

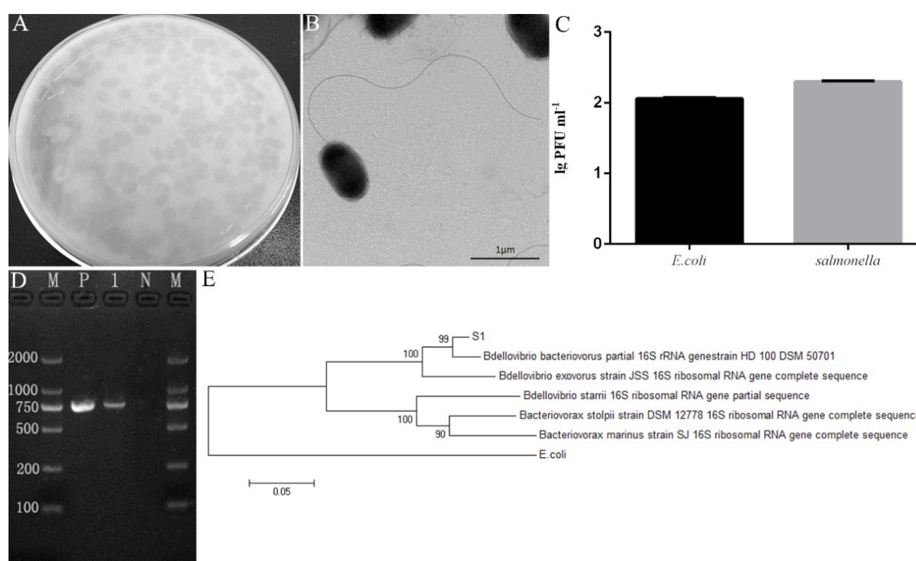

Figure 2: A. Plaques formed by stomach-derived isolate; B. Transmission electron micrograph of isolate S1 in attack phase; C. Plaques amount by isolate S1 on *E. coli* and *Salmonella* plates; D. Amplification of 16S rDNA partial sequence of isolate S1; E. Phylogenetic tree based on 16S rDNA partial sequence of isolated S1.

Identification of Isolate S1

Electron microscopic observations and molecular methods (16S rDNA sequence analysis) were used to identify isolate S1 as *B. bacteriovorus*. Fig. 2B showed that the phenotype of isolate S1 was clearly oval-shaped with a single polar sheathed flagellum at one end. As for body size, isolate S1 was about 0.7-0.8 μm in length and 0.4-0.5 μm in breadth, with a flagellum length of 4.2-4.3 μm. According to the electron microscopic observations, the morphological features of isolate S1 were consistent with the typical morphological characteristics of *B. bacteriovorus*, i.e., a small, vibrioid bacteria with a long, thick-sheathed polar flagellum. This phase was called an attack phase, where the cells are highly motile by the long polar flagellum in order to seek the prey to attack [31]. Morphologically speaking, the isolate S1 could be identified as *B. bacteriovorus*.

Electron microscopic observations alone were not enough for the identification of isolate S1, 16S rDNA sequence analysis was required as the most determinative step to confirm the bacterial strain [9]. The 16S rDNA partial sequence of isolate S1 was successfully amplified by *Bdellovibrio* specific primers (Figure 2D). The BLAST similarity search indicated that the amplified sequence displayed 97% similarity with the 16S rDNA partial sequence of *B. bacteriovorus* HD 100 in the Genbank database. Together with the phylogenetic tree, isolate S1 had a close relationship with *B. bacteriovorus* HD 100 (Figure 2E). The 16S rDNA partial sequence (approx. 0.8 kb) of isolate S1 has been submitted to the Genbank database with accession no. KX082773.

Predation Ability of Isolate S1 against *E. coli* and *Salmonella*

E. coli and *Salmonella*, which could elicit severe gastroenteritis and diarrhea of animals [32] were used to assess the predation ability of isolate S1. **Figure 2C** showed that the number of plaques was 114.5 ± 2.0 PFU mL^{-1} and 200.5 ± 1.5 PFU mL^{-1} for E.coli and *Salmonella* plates at 37°C, respectively. This indicated that the isolate S1 was capable to prey both *E. coli* and *Salmonella*. It is worth mentioning that there was a significant difference in the number of plaques between Salmonella group and *E. coli* group ($P<0.05$), which could imply that isolate S1 was more likely to prey *Salmonella* over *E.coli*. The fact that isolate S1 preys both Gram-negative bacteria suggested that it could be potentially an alternative antibiotic agent to control Gram-negative bacterial infections, especially *Salmonella*, in the animal industry **(Figures 2D and 2E)** [33,34].

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