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Full Length Research Paper

Identification and phylogenetic analysis of a bacterium isolated from the cloaca of Chinese alligator

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Wild populations of Chinese alligator (*Alligator sinensis*) are on the edge of extinction. Through a release project lunched by the State Forestry Administration (SFA) of China, some captive-bred alligators will be selected and released into the wild to supplement and renew natural populations. Before releasing, health screening protocols on individuals are very necessary. In this paper, bacterium ASC10 was isolated from the cloaca of Chinese alligators during heath screening. According to the traditional bacteriology approach, the strain ASC10 displayed the characteristics of Enterobacteriaceae, and partial similarity of *Proteus vulgaris*. In order to confirm the further phylogenetic status of this strain, 16S rRNA gene sequences was used and aligned with related bacterial strains. According to the analysis based on the phenotypes and 16S rRNA gene sequences, strain ASC10 shows a close relative relationship with Genus *Proteus* and it has a far relationship with other genera of Enterobacteriaceae. In terms of results, it is confirmed that strain ASC10 belongs to a new species of Genus *Proteus*.

Key words: Chinese alligator, 16S rRNA gene, bacterium, phylogenetic analysis.

INTRODUCTION

As the most endangered species of the global 23 crocodilian species (Thorbjamarson et al., 1992), Chinese alligator (*Alligator sinensis*) is an endemic species in China. In recent years, because of fragmentation of their habitat, the effect of human urbanization and industrialization, the distribution area of the Chinese alligator has decreased rapidly. The wild Chinese alligators are on the verge of extinction (Thorbjamarson et al., 2002; Chen et al., 2003). In 2001, Chinese alligator was listed as one of the 15 priority salvation species of the project-China's wild animal and plant protection and nature reserve construction. To enhance the conservation strength and enlarge the population size of the wild Chinese alligators, the project for the conservation and releasing into the wild of Chinese alligators was launched in 2003 by the State Forestry Administration (SFA) of China. Before releasing captive animals, it is important to know if these animals are healthy (Plowright, 1988; May, 1991; Woodford and Kock, 1991; Mills, 1999).

Bacteriological approach is widely used in bacterial identification, whereas, with the development of molecular biology techniques, homology analyses based on 16S rRNA gene sequences have became the “golden index” in the field of bacterial identification (Shen and Feng, 2004). 16S rRNA gene is the skeleton of the small subunit ribosome and the necessary location for protein biosynthesis, its coding gene existence in all bacteria. In a long period of time, 16S rRNA gene select pressure is bigger, the sequence variation is slower; 16S rRNA gene have the feature of molecular chronometers, it can span the perspective of evolution of human life; 16S rRNA gene sequences have different evolution rate region, so it can be used in systems classification study of different evolution extent organism (Ochman and Wilson, 1987). Because of the length of nucleotide sequence is moderate and the structure is complete, the 16S rRNA gene was as the common section in the classification study of bacteria. Homology analyses based on 16S rRNA gene sequences cannot only accurate identification for new isolated bacteria but also revise the inadequate bacteria classification (Dutly and Altwegg, 2001).

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In this study, In order to avoid the alligators introducing pathogenic bacteria into the wild environment and endangering the native wild populations, the pathogenic bacteria from the cloaca of alligators were screened and detected, and an uncertain strain ASC10 was isolated ultimately. Therefore, the aim of this study was to identify the strain and serve to the release project by combining some approaches of physiology and biochemistry, as well as molecular biology.

MATERIALS AND METHODS

Bacterial strain ASC10 was isolated from the cloaca of Chinese alligators for release in Anhui Research Center for Chinese Alligator Reproduction (ARCCAR) in April of 2006. Bacteria medium consists of beef extract of 5 g, peptone of 10 g, NaCl of 5 g, H_2O of 1000 ml, and Agar 2% at pH 7.2.

According to Common method of determinative bacteriology (1978), Elementary knowledge of bacteria classification (Wang, 1977), Bergey's Manual of Determinative Bacteriology (1984, 1994), Manual of common determinative bacteriology (Dong and Cai, 2001), strain ASC10 was identified and classified primarily through morphological observation, including size measure, Gram stain, flagellum stain, then tested by physiological and biochemical characteristics detection.

Inoculate bacterial strain maintained on medium plate at 28°C overnight culture. The bacterial strain was suspended in 1 mL volumes with 0.9% sterile sodium chloride solution. After the removing of cellular debris by centrifugation (4°C, 8500×g for 10 min) (Xia et al., 2005), the bacteria sedimentation was prepared by resuspension of cells in a 5% (w/v) solution of Chelex-100 (Bio-Rad, Hemel Hempstead, UK) 400 µL and boiling for 10 min, ice bath for 1 min, centrifugation (12 000×g for 10 min) at room temperature (25°C) (Fu et al., 2002). The supernatant was used as the source of template DNA for PCR, which was stored at -20°C.

The forward primer of PCR amplification of 16S rRNA gene sequence is 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' (according to *E. coli* 16S rRNA 8 - 27 bp location). The reverse primer is 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (according to *E. coli* 16S rRNA 1492 – 1510 bp location) (Martin and Collen, 1998). A 25 µL reaction mixture of PCR was run with 10 – 100 ng of template DNA, 2.5 µL 10×reaction buffer, 1.0 µL 25 mmol/L $MgCl_2$, 0.5 µL 10 mmol/L dNTPs, 1 µL 10 µmol/L each primer, 1 U Taq DNA polymerase, and sterile double distilled water to make up a final volume of 25 µL. A DNA thermal cycler (PTC-200, MJ Research Inc, USA) used for thermal amplification was programmed for the following: an initial denaturizing for 5 min at 94°C followed by 31 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extending for 1.5 min at 72°C, finally reactions were held at 72°C for 10 min. The PCR products were separated by electrophoresis in 1% agarose gel. The gels were stained with ethidium bromide and photographed on a UV transilluminator (UVP Inc, USA). PCR products were purified by PCR Cleanup Kit (Axygene, Biotech Ltd, Hangzhou, China) and sequenced with ABI 3730 DNA Analyzer (Shanghai Sangon Biotechnology Co., Shanghai, China).

By the software of DNAStar (version 5.01) and Chromas (version 2.22), the sequences were analyzed. Finally, the 16S rRNA gene sequence of strain ASC10 was obtained. The determined nucleotide sequence was submitted to the GenBank with the accession number EU040283.

The sequence data were aligned with Clustalx 1.8 (Thompson et al., 1997). All variable characters, parsimony-informative characters, and the numbers of TIs and TVs were estimated from pairwise comparisons of sequences with Mega 3.1 (Kumar et al., 2004) based on Kimura Parameter (Kimura, 1980). Phylogenetic analyses were carried out using maximum-likelihood (ML) method (Felsenstein, 1981) and Maximum Parsimony (MP) method (Swofford, 1998). The MP and ML analyses with the nucleotide data set were conducted with PAUP*4.0beta (Swofford, 2003). Bootstrap analysis of 1 000 replicates was performed to estimate robustness of the tree. The Bayesian analysis chooses the GTR+I+G model by Akaike information criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). The accession numbers of 16S rRNA of all strains cited in this paper were showed in Table 1.

RESULTS

Strain ASC10 was cultured on bacteria medium for 24 h at room temperature, the colony was big with the size of about 0.4~0.8 µm × 1.0~2.2 µm; moreover, which was erose, convex, gray, dry, and toothed. The bacterium was a Gram-negative bacillus and periplasmic flagellum without spore. The physiological and biochemical characteristics of the strain were given in Table 2.

To obtain the taxology group, the PCR product of 16S rRNA gene was sequenced. The size of 16S rRNA gene sequence is 1400 bp. By using the phylogenetic software of Mega 3.1, sequences from 23 bacteria were analyzed. Of 1318 sites in the 23-taxon data matrix, 212 (16.1%) were variable and 141 (10.7%) were parsimony informative. Transition/Transversion is 42/28. The average composing of G+C percentage is 54.3%, from 53.3 to 55.4% (ASC10 is 53.4%).

From three kind of phylogenetic trees, all the bacterial species were divided into two clades which clustered a sister group. Strain ASC10 was grouped together with *Proteus vulgaris*, which formed a sister group with Swine mature bacterium RT-13A, Swine mature bacterium RT-4A, Swine mature bacterium RT-6A and Swine mature bacterium RT-10A. That is to say, strain ASC10 has a close relationship with Genus *Proteus* and swine mature bacterium.

According to these results, thereby it can be inferred and confirmed that ASC10 belongs to Enterobacteriaceae.

DISCUSSION

By the analysis of bacteriology, it shows that strain ASC10 has the typical characteristics of physiology and biochemistry of Enterobacteriaceae, therefore strain ASC10 can be classified as Enterobacteriaceae primarily. Moreover, the results displayed that the physiological and biochemical characteristics of strain ASC10 were similar to the Genus *Proteus*, besides phenylanine ammonia-lyase-positive and oxidase-negative (Bergey et al., 1994). According to the phylogenesis trees (Figures 1, 2 and 3) based on the 16S rRNA gene sequences, strain ASC10 has a close relationship with the *P. vulgaris*, but far from other genera of Enterobacteriaceae. Hence, it can be inferred and confirmed that ASC10 belonged to Genus *Proteus*, which will enrich the classification basis of Genus *Proteus*.

Table 1. The data of 16S rRNA gene sequences used in the study.

Name of species	Accession number of Gen Bank
Proteus vulgaris	DQ826507
Proteus mirabilis	EF091150
Swine manure bacterium RT-13A	AY167953
Swine manure bacterium RT-10A	AY167951
Swine manure bacterium RT-6A	AY167947
Swine manure bacterium RT-3C	AY167939
Xenorhabdus innexi	AJ810292
Morganella morganii	DQ358146
Klebsiella pneumoniae	X87276
Arsenophonus nasoniae	M90801
Citrobacter freundii	AF025365
Edwardsiella tarda	EF091710
Salmonella typhimurium	NC003197
Escherichia coli	AF053965
Shigella boydii	X96965
Yersinia pestis	AL590842
Serratia marcescens	EF208030
erwinia carotovora	NC004547
Enterobacter aerogenes	AY825036
Hafnia alvei	DQ412565
Pectobacterium chrysanthemi	AF373173
vibrio cholerae	NC009457
ASC10	EU040283

Table 2. The physiological and biochemical characteristics of ASC10 strain.

Test item	Result	Test item	Result
Catalase	+	D(+)-Mannose	-
Oxidase	+	Lactose monohydrate	±
Acid-fast stain	-	Fructose	±
Methyl red	-	L(+)-Rhamnosemonohydrate	-
Gelatin liquidized	-	Sorbitol	-
H_2S	-	Esculin	+
Lactic acid	-	Sugar	+
Nitrogen-Fixing	-	Maltose	+
Hydrolysis cellulose	-	D-Galactose	+
Phenylanine ammonia-lyase	-	Erythritol	-
Glucose	+	Dulcitol	-
D-Xylsoe	±	Inulin	-
L-arabinose	-	Inositol	-
D-Mannitol	-		

+ = Positive; – = negative; ± = feeble ferment.

Enterobacteriaceae include Genera *Escherichia*, *Edwardsiella*, *Citrobacter*, *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Hafnia*, *Serratia*, *Proteus*, *Yersinia* and *Erwinia* in Bergey's Manual of Determinative Bacteriology (8th Edition) (Buchanan and Gibbons, 1984). In this paper, Genera *Morganella*, *Xenorhabdus*, *Arsenophonus* and *Pectobacterium* are not referred in 8th Edition. *Morganella* was classified as Genus *Proteus* in 8th Edition, but the *Morganella* was separated from it in 9th Edition, and Genus *Proteus* was classified again. The classification in

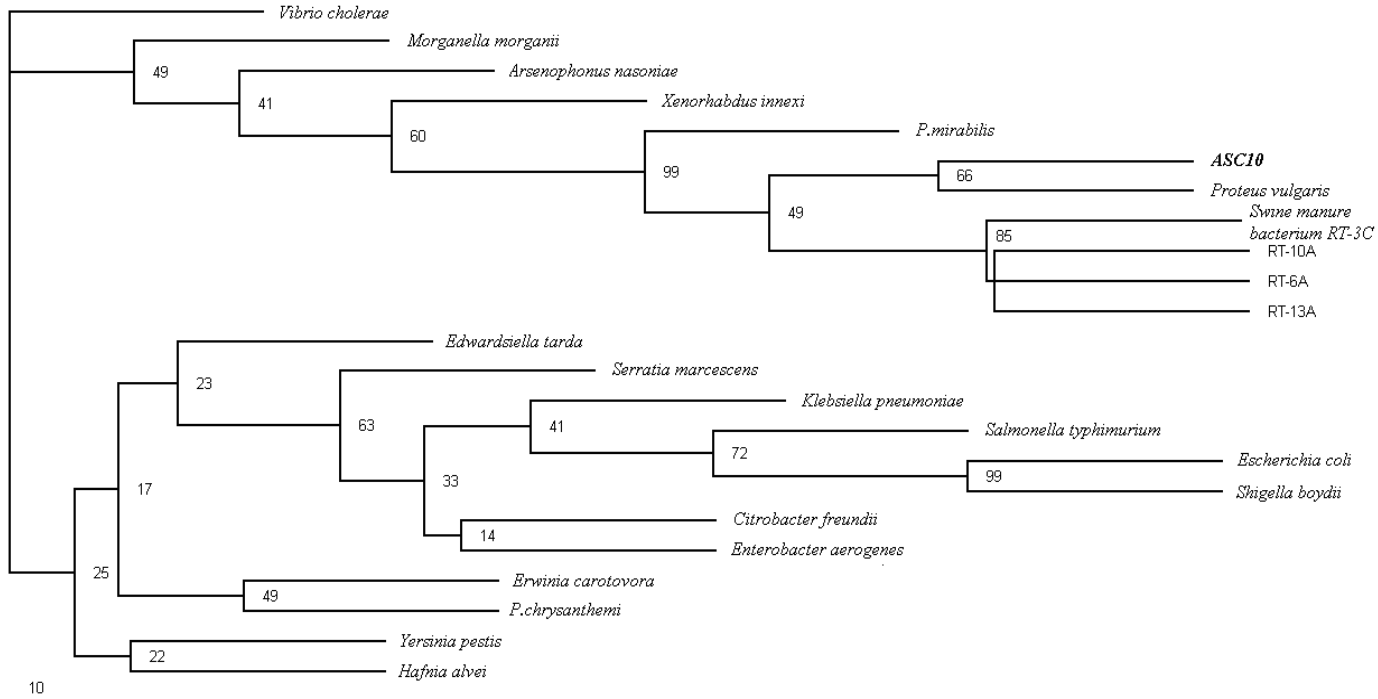

Figure 1. The Maximum-parsimony tree constructed based on nucleotide sequences of the 16S rRNA gene with confident values of bootstrap 1 000.

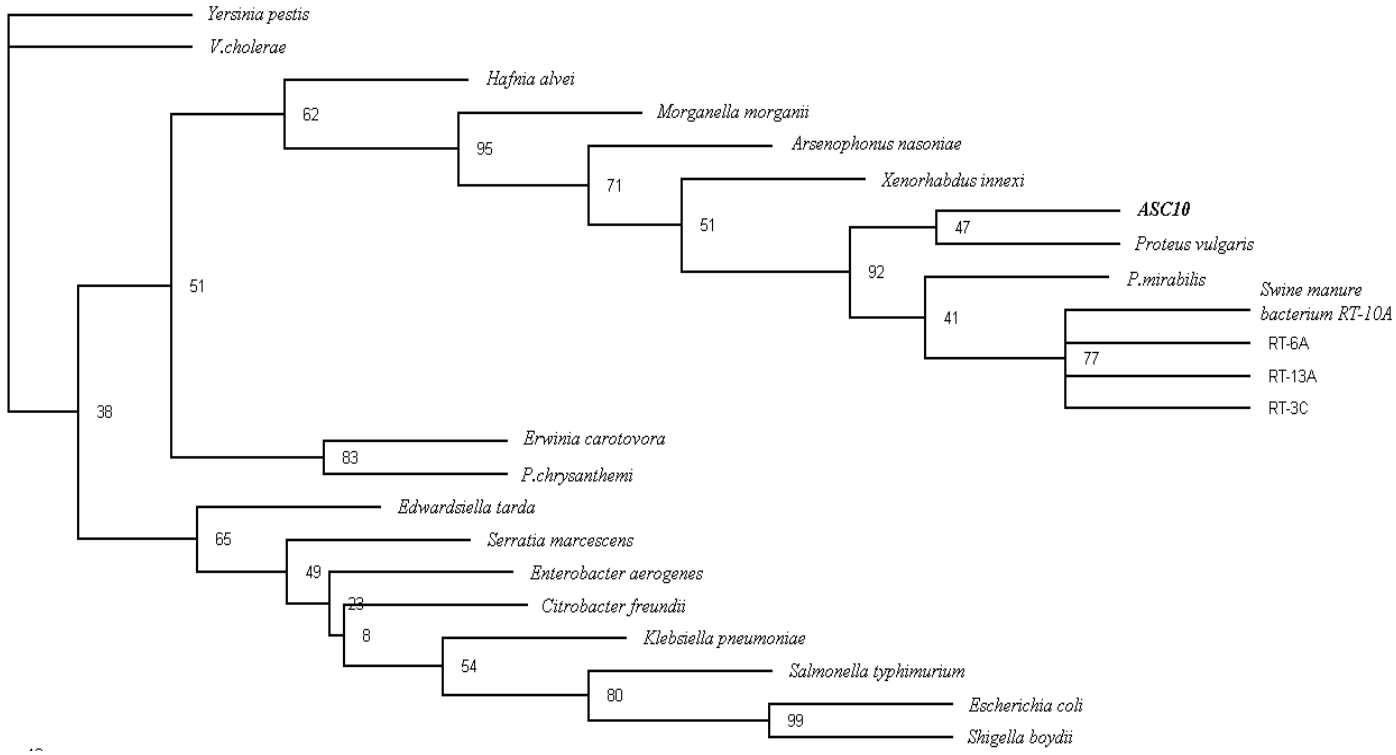

Figure 2. The Maximum-likelihood tree constructed using nucleotide sequences of the 16S rRNA gene with confident values of bootstrap 1 000 indicated above each branch.

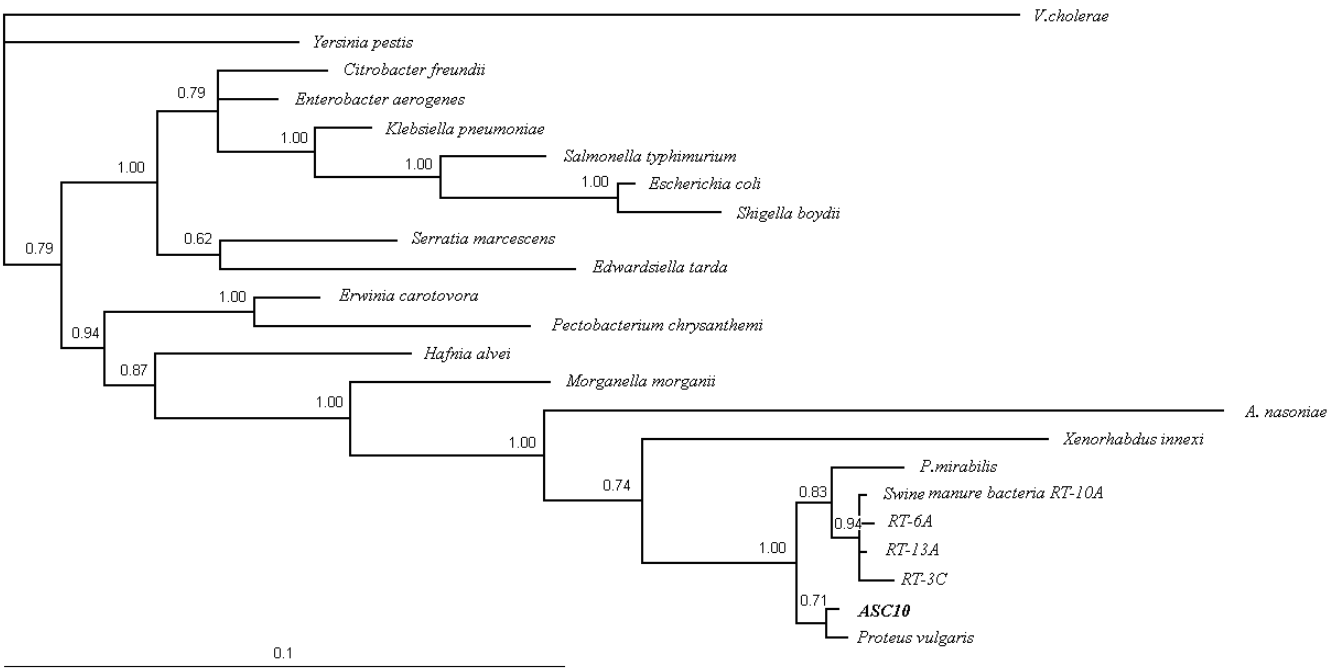

Figure 3. The Bayesian tree constructed using nucleotide sequences of the 16S rRNA gene with confident values of bootstrap above each branch.

Bergey's Manual of Determinative Bacteriology (1984, 1994) was based on the morphologic, physiological and biochemical characteristics, as well as serological and immunity proofs. Recently the 16S rRNA gene sequence with the size of 1.5 Kb was considered and widely used in bacterial taxology. It contains high conservation region which have variable nucleic acid region in different species (Kox et al., 1995). Furthermore, the most importance is that 16S rRNA gene can be sequenced easily. By combining the molecular phylogeny with traditional approaches, such as morphological, physiological and biochemical characteristics, bacteria identification can be carried out more accurately (Li et al., 2006; Mo et al., 2003).

The results of physiology and biochemistry characterristics identification of strain ASC10 have some disaccord with *Bergey's Manual of Determinative Bacteriology* (9^{th} Edition) (Bergey et al., 1994). For example phenylanine ammonia-lyase test, oxidase, methyl red, gelatin liquidized and H_2S test. According to the results of phylogenesis analysis, ASC10 has 90% similarity with genus *Proteus*. So the identification of bacteria should connect the phenotypic information of traditional classification with the identification of some molecular marker. This is useful for the new strain's finding. It also offered credible method for health screening on Chinese alligator individuals.

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