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Characterization of PB-CS01, A Novel Photobacterium Strain Isolated from Commercial Pork

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Abstract: A Gram-negative, rod-shaped bacterial strain named PB-CS01 which is bioluminescence-positive was isolated from contaminated commercial pork that probably had been exposed to seafood. Optimal growth of strain PB-CS01 requires the presence of 3.0% (w/v) NaCl and a temperature of 20°C. Phylogenetic analysis based on *16S rRNA* gene sequences of strain PB-CS01 and other *Photobacterium* species showed that the novel isolate belongs to the genus *Photobacterium*. Sequence similarity analysis between PB-CS01 and other species indicates that the closest relatives of strain PB-CS01 are *Photobacterium phosphoreum* ATCC 11040 (99.9%), *Photobacterium kishitanii* pjapo.1.1 (99.8%) and *Photobacterium iliopiscarium* ATCC 51760 (99.5%). The most abundant fatty acids were summed feature 3 (50.77%; $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:0}$ (15%). The fatty acid profile is similar to that of the genus *Photobacterium* but this report is the first to describe $C_{16:1}\omega6c$ as one of the compositions of summed feature 3 of the genus *Photobacterium*. The G+C content of the genomic DNA of strain PB-CS01 was 44.8 mol%. Overall, strain PB-CS01 is a novel *Photobacterium* species.

Key words: Photobacterium, PB-CS01, phenotype, phylogeny, species

INTRODUCTION

The genus *Photobacterium* belonging to the Gammaproteo bacteria was first proposed by Beijerinck. There are currently 18 accepted *Photobacterium* species with validly published names such as *Photobacterium phosphoreum* (the type species), *P. kishitanii*, *P. gaetbulicola* and *P. jeanii* (Ast *et al*., 2007; Chimetto *et al*., 2010; Kim *et al*., 2010). Members of the genus *Photobacterium* were usually isolated from aquatic and marine environments (Thompson *et al*., 2005; Seo *et al*., 2005a; Park *et al*., 2006; Ast *et al*., 2007). This genus of *Photobacterium* belongs to the family Vibrionaceae in combination with other genera that also contain species isolated from these habitats.

In this study, the characterization of a novel isolate designated PB-CS01 was carried out. Interestingly, it was isolated from commercial pork that probably had been contaminated by fresh seafood in a supermarket in Changsha, Hunan province, China. Phylogenetic analysis and chemotaxonomic characterization indicates that this novel isolate belongs to the genus *Photobacterium* in combination with the morphological and biochemical characteristics.

MATERIALS AND METHODS

Bacterial strains and growth conditions: Strain PB-CS01 which was isolated from commercial pork in a supermarket was stored at 4°C and found to emit blue fluorescence. Samples were collected with sterile swabs and subsequently placed onto LB agar plates (10 g tryptone, 5 g yeast extract, 10 g NaCl, distilled water added to 1 L, pH 7.0) for 2 days. Growth of PB-CS01 was measured at temperatures from 5-37°C and its tolerance of various NaCl concentrations (0-5%) was also evaluated.

Phenotypic characterization: To characterize the phenotype of strain PB-CS01, standard phenotypic tests were performed (Smibert and Krieg, 1994; Zhang *et al*., 2008). Cellular morphology of this novel strain was determined by electron microscopy (JSM-6380 LV, Japan Electronics). Gram staining was performed as Gerhardt earlier described (Gerhardt *et al*., 1981). The morphology, size and pigmentation of colonies were observed on LB agar plates after incubation for 2 days at 20°C.

Phenotypic characterization of strain PB-CS01 was performed as described. Catalase activity was examined on freshly growing colonies using a 3% (v/v) hydrogen peroxide solution (Zhang *et al*., 2010).

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Hydrolysis of gelatin and nitrate reduction were studied as described by Bruns *et al.* (2001). To test if PB-CS01 produces acid from carbohydrates, methods described by Gordon were used in this study. Utilization of various substrates for growth was determined as described by Gordon and Mihm (1957). The API ZYM system (bioMerieux) was used to determine enzyme activity. To understand its antibiotic sensitivity, a bacterial suspension was spreaded onto LB agar plates supplemented with the following antibiotics (μg mL^{-1}): ampicillin (10), chloramphenicol (30), kanamycin (30), streptomycin (10), cephalothin (10), lincomycin (2), furazolidone (300), rifampin (5) and doxycycline (30). The plates were then placed at incubator at 20°C for 2 days.

***16S rRNA* gene sequencing and phylogenetic analysis:** The *16S rRNA* gene of strain PB-CS01 was amplified by PCR with universalprimers 27F (5'-AGAGTTTG ACCTGGCTCAG-3') and 1492R (5'-GGTTACC TTGTTACGACTT-3') and the resulting PCR product was sequenced directly using the method described by Lu *et al.* (2001). Nucleotide sequences were determined automatically using Applied Biosystems DNA sequencers (Model 377). The resultant *16S rRNA* gene sequence was submitted to the blast program and compared with those available from the GenBank database to determine an approximate phylogenetic affiliation for strain PB-CS01. To determine the classification of strain PB-CS01, multiple sequence alignments with CLUSTAL_X (Thompson *et al.*, 1997) were first performed and then the software package Phylip 3.63 was used for phylogenetic analysis (Felsenstein, 1993). The neighbor-joining method (Saitou and Nei, 1987) which is based on a distance matrix that was corrected by Kimura (1980)'s two-parameter model was used to construct the phylogenetic tree. In order to evaluate the robustness of the tree's topology, Bootstrap analysis with 1000 resamplings was used (Felsenstein, 1985).

Chemotaxonomic characterization and DNA G+C content: Cellular fatty acid methyl esters were prepared and analyzed using gas chromatography according to the instructions of the Microbial Identification System (MIDI). Fatty acid analysis was performed using the Sherlock System (Microbial ID). The DNA G+C content was determined according to the thermal denaturation method described by Marmur and Doty (1962) using DNA form *Escherichia coli* K-12 as a control.

Nucleotide sequence accession numbers: The *16S rRNA* gene sequence of Photobacterium strain PB-CS01 was deposited in GenBank under accession number JN665072.

RESULTS AND DISCUSSION

Phenotypic characteristics: PB-CS01 is a Gram-negative, rod-shaped strain. After growth for 2 days on LB agar plates, colonies are cream-colored and opaque with a smooth surface, circular form and convex elevation. These colonies observed on the plate have entire margins and are 2.0-3.0 μm in diameter as shown in Fig. 1. In the dark room, colonies of PB-CS01 exhibited blue luminescence as shown in Fig. 2, though the molecular mechanism of this bioluminescence is unclear. Strain PB-CS01 can grow between 5 and 22°C and between 1.5 and 4% NaCl (w/v). Optimal growth occurs at 20°C and in the presence of 3% (w/v) NaCl. Antibiotic assays revealed that this strain is susceptible to chloramphenicol, kanamycin, streptomycin, cephalothin, lincomycin, furazolidone, rifampin and doxycycline but this strain is resistant to ampicillin.

As indicated in Table 1 are the morphological physiological and biochemical characteristics of strain PB-CS01 including characteristics for differentiating this strain from other *Photobacterium* species. The test for enzyme activity showed that this bacterium can produce catalase, arginine dihydrolase and lysine decarboxylase although, it did not exhibit the ability to produce H_2S. The reduction of nitrate to nitrite and the hydrolysis of gelatin occur in this bacterium. Acid production from D-sorbital is positive in PB-CS01 which is the clearest differentiating characteristic between strain PB-CS01 and its closest phylogenetic neighbors, *P. phosphoreum* and *P. iliopiscarium*. Strain PB-CS01 is able to utilize D-fructose, D-mannose and maltose as carbon sources but it rarely attacks D-cellobiose, D-galactose, sucrose and L-arabinose.

Phylogenetic and chemotaxonic characteristics: In the resulting phylogenetic tree based on *16S rRNA* gene

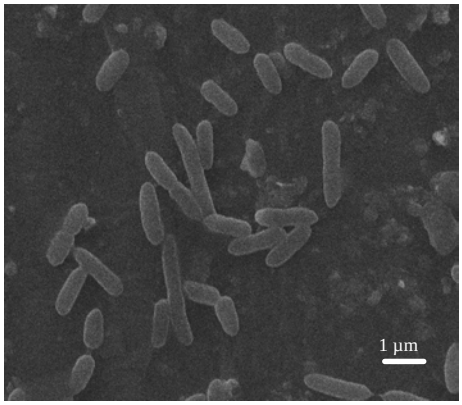

Fig. 1: Electronic micrograph of strain PB-CS01 grown in LB medium

Table 1: Differential phenotypic characteristics of strain PB-CS01 and related *Photobacterium* species

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Optimum temperature (°C)	20	18	20	25	25-28	14	25	20-30	35	28	25-30	26
Catalase	+	+	+	-	+	+	+	ND	+	+	W	-
H_2S production	-	(-)	-	+	-	ND	ND	ND	-	-	-	(-)
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	ND	-	+	+	+	+	-	+	+
Lysine decarboxylase	+	(-)	+	-	-	ND	ND	-	-	-	-	(-)
Hydrolysis of gelatin	-	-	-	-	-	ND	+	-	+	+	-	-
Utilization of												
D-Fructose	+	+	+	ND	+	+	+	+	ND	+	+	+
D-Galactose	-	-	+	ND	-	+	W	+	ND	-	+	+
D-Cellobiose	-	-	-	ND	-	-	+	+	ND	-	+	-
D-Mannose	+	+	+	ND	-	+	+	+	W	-	+	+
Sucrose	-	-	-	+	+	+	+	+	ND	+	+	+
Maltose	+	+	ND	+	+	+	+	+	ND	+	+	+
L-Arabinose	-	-	-	-	-	+	-	-	ND	-	-	-
Acid production from												
D-Mannitol	-	-	-	ND	-	+	ND	+	+	+	-	-
D-Sorbitol	+	-	-	ND	-	ND	ND	-	-	-	-	-
L-Rhamnose	-	-	-	ND	-	ND	ND	V	W	-	-	-
Sucrose	-	-	-	+	+	+	ND	+	-	+	+	-
L-Arabinose	-	-	-	-	-	ND	ND	-	-	+	-	-
DNA G+C content (mol%)	44.8	39-42	38-40	40	47	43.8	45	47.6-47.9	44	49.8	48.3	40-44

Species: 1: strain PB-CS01; 2: *P. phosphoreum* (Reichelt and Baumann, 1973; Nogi *et al.*, 1998); 3: *P. iliopiscarium* (Onarheim *et al.*, 1994); 4: *P. indicum* (Johnson and Weisrock, 1969; Xie and Yokota, 2004); 5: *P. lipolyticum* (Yoon *et al.*, 2005); 6: *P. frigidiphilum* (Seo *et al.*, 2005a); 7: *P. aplysiae* (Seo *et al.*, 2005b); 8: *P. rosenbergii* (Thompson *et al.*, 2005); 9: *P. ganghwense* (Park *et al.*, 2006); 10: *P. halotolerans* (Rivas *et al.*, 2006); 11: *P. lutimaris*; 12: *P. leiognathi* (Nogi *et al.*, 1998). +: positive; -: negative; V: Variable; W: Weak; ND: No Data available; Data in parentheses are for type strains

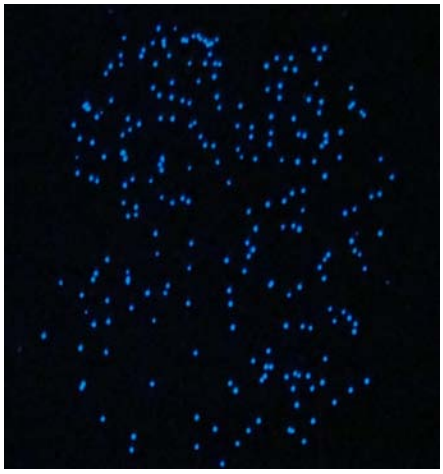

Fig. 2: Bioluminescence of colonies of strain PB-CS01 grown on an LB agar plate which was observed at dark room

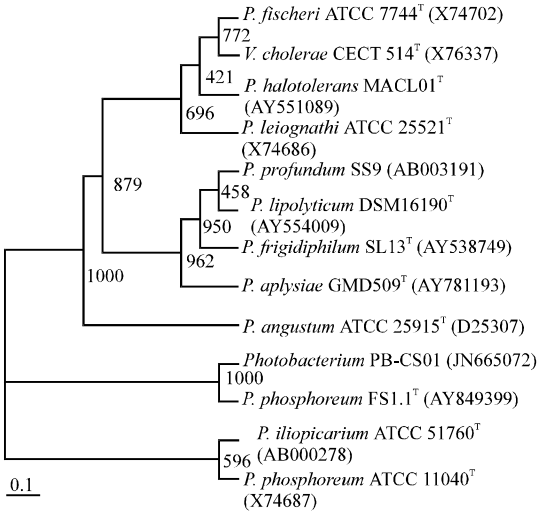

Fig. 3: Phylogenetic tree of strain PB-CS01 and other related *Photobacterium* species based on *16S rRNA* gene sequences. Bootstrap analysis with 1000 resamplings was selected for evaluating the robustness of the tree's topology and the relative values are given at the nodes

sequences as shown in Fig. 3, strain PB-CS01 fell within the clade comprising *Photobacterium* species and formed a cluster with *P. phosphoreum* ATCC 11040^T (99.9%), *P. iliopiscarium* ATCC 51760^T (99.5%), *P. kishitanii* pjapo.1.1^T (99.8%) and *P. angustum* ATCC 25915^T (97.6%) (Fig. 3). Strain PB-CS01 shared 94.3-97.8% sequence similarity of *16S rRNA* gene with other *Photobacterium* species included in the phylogenetic analyses. To further confirm the classification of strain PB-CS01 as a *Photobacterium* species, chemotaxonomic analysis was performed in the present study. The obtained results showed that strain PB-CS01 had large amounts of straight-chain and unsaturated fatty acids the major components were summed feature 3 ($C_{16:1}\omega7c$ and/or

Table 2: Cellular fatty acid compositions of strain PB-CS01 and related *Photobacterium* species

Fatty acid	1	2	3	4	5
$C_{12:0}$	5.3	6	2.4	-	3.4
$C_{12:0}$ 3-OH	4.7	9	2.6	4.0	2.1
$C_{14:0}$	7.5	11	2.4	3.8	3.6
$C_{14:1}$	-	1	-	-	-
iso-$C_{15:0}$	-	-	5.0	-	-
$C_{15:0}$	-	-	2.1	-	-
$C_{16:0}$	15.0	25	13.1	25.9	21.2
$C_{16:1}$	-	40	-	-	-
iso-$C_{17:0}$	-	-	1.8	-	-
iso-$C_{17:1}$ ω9c	-	-	1.8	-	-
$C_{16:1}$ω7c alcohol	-	-	1.2	-	-
$C_{18:0}$	1.2	-	-	-	-
$C_{18:1}$	-	3	-	-	-
$C_{18:1\omega7c}$	4.6	-	13.0	5.9	29.6
$C_{18:1\omega9c}$	1.6	-	-	-	-
Summed feature 2*	4.7	3	3.0	-	3.7
Summed feature 3*	50.8	-	43.3	51.3	27.8

Strain: 1: PB-CS01; 2: *P. phosphoreum* ATCC 11040^T (data from Nogi *et al*., 1998; Xie and Yokota, 2004; Seo *et al*., 2005a); 3: *P. lutimaris* DF-42^T (Jung *et al*., 2007); 4: *P. lipolyticum* M37^T (Yoon *et al*., 2005); 5: *P. halotolerans* LMG 22194^T (Park *et al*., 2006. Values are percentage of total fatty acids; -: Not detected or not described; *Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI System. Summed feature 2 contained $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I, an unidentified fatty acid with an equivalent chain-length of 10.928 and/or $C_{12:0}$ALDE. Summed feature 3 contained $C_{16:1}$ω7c and/or iso-$C_{15:0}$ 2-OH but the summed feature 3 of strain PB-CS01 was $C_{16:1}$ω7c and/or $C_{16:1}$ω6c

$C_{16:1}$ω6c; 50.77%), $C_{12:0}$ (5.32%), summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I; 4.68%), $C_{12:0}$ 3-OH (4.73%), $C_{14:0}$ (7.51%), $C_{16:0}$ (15%) and $C_{18:1}$ω7c (4.58%). This fatty acid profile is similar to those of other *Photobacterium* species although, there are differences in the proportions of certain fatty acids as shown in Table 2. In addition, the compositions of summed feature 3 ($C_{16:1}$ω7c and/or $C_{16:1}$ω6c) of strain PB-CS01 are different from that of other *Photobacterium* species ($C_{16:1}$ω7c and/or iso-$C_{15:0}$ 2-OH).

The DNA G+C content of strain PB-CS01 was 44.8 mol% which is within the accepted range for the genus *Photobacterium* (38-48 mol%) but this value is slightly higher than that of its closely related strains (*P. phosphoreum* was 39-42 mol% and *P. iliopiscarium* was 38-40 mol%).

CONCLUSION

In this study, results indicate that strain PB-CS01 is a Gram-negative, rod-shaped bacterium. This strain represents a novel *Photobacterium* species as revealed by its phenotypic and phylogenetic characteristics.

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