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Polyphasic Assignment of a Highly Proteolytic Bacterium Isolated from a Spider to *Serratia proteamaculans*

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Abstract A bacterial strain named HY-3 that produces a highly active extracellular protease was isolated from the digestive tract of a spider, *Nephila clavata*. The bacterium was a Gram-negative, oxidase-negative, catalase-positive, nonhalophilic, nitrate-reducing, facultative anaerobe. Transmission and scanning electron microscopies demonstrated that the isolate was non-spore-forming, straight, rod-shaped, and motile by peritrichous flagella. The G+C content of the DNA was 57.0 mol%. The isoprenoid quinone type was ubiquinone with 8 isoprene units (Q-8). The morphological and biochemical characteristics including the predominant fatty acid and phospholipids profiles placed the isolate HY-3 in the family *Enterobacteriaceae*. Further biochemical characterization and phylogenetic studies including determination of an almost complete 16S ribosomal DNA sequence suggested that the bacterium was closely related to the genus *Serratia*. DNA-DNA hybridization analysis revealed that this extracellular protease-producing strain belongs to *Serratia proteamaculans*, which is also known for its association with insects.

Key words: *Serratia proteamaculans*, protease, phylogeny, spider

The genus *Serratia* is characterized as a Gram-negative, oxidase-negative, catalase-positive, heterotrophic, facultatively anaerobic bacterium that belongs to the family *Enterobacteriaceae*. *Serratia* has been studied for its industrial applicability, since *Serratia* produces therapeutically potential antibiotics including the red pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) and carbapen-2-em-3-carboxylic acid [19, 40, 45, 46], and excretes many degradative enzymes including proteases, chitinases and lipases. *Serratia* occupies many diverse habitats including water, plants, animals, and hospitalized human patients. Many *Serratia* strains have also been isolated from a variety of insects, establishing mutualistic or pathogenic relationships. Strains of *Serratia marcescens* were isolated from hemolymphs of a boll weevil [4, 38] and the intestinal canals of larval silkworms [33]. *S. marcescens* strains were highly pathogenic when infected through food or injected into the haemocoel of *Drosophila melanogaster* [33]. *Serratia entomophila* and *Serratia proteamaculans* are used as effective biological pesticides since the bacteria cause the amber disease resulting in growth inhibition and death in the New Zealand grass grub [16, 17, 42]. Other phylogenetically close bacteria, *Photorhabdus luminescens* and *Xenorhabdus nematophila*, are carried with a mutualistic relationship by entomophagous nematodes that invade susceptible insects. Subsequent release of the bacteria from the nematodes into insect haemocoels causes growth inhibition and death of the insects [2, 3, 32].

In a previous research, we isolated a number of bacteria from the midgut of a golden orb-web spider, *Nephila clavata*, and identified their physiological characteristics [35]. Out of twelve isolates containing lipase or protease acivities on agar plate media, an enteric bacterium excreted an industrially potential exoprotease at high levels [23]. The protease, which belongs to the serralysin subfamily of metalloproteases, is highly active at wide pH and temperature ranges [23]. Serralysins exhibit broad specificities in the cleavage of oxidized insulin B and show caseinolytic activities in broad pH and temperature ranges [27]. The serralysin metalloproteases are used widely as anti-inflammatory agents because of their ability to hydrolyze

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inflammatory peptides such as bradykinin and histamine [44]. In this research, polyphasic approaches with many morphological and biochemical characteristics suggested that the bacterium designated HY-3 was closely related to the genus *Serratia*. Further phylogenetic analysis by 16S rDNA sequence determination and DNA-DNA hybridization demonstrated that the bacterium belongs to *Serratia proteamaculans*.

MATERIALS AND METHODS

Isolation, Culture Conditions, and Growth

Golden orb-web spiders, *Nephila clavata*, were collected in the field of Daejeon, Korea. Isolation of strain was carried out as in the previous study [35]. Nutrient broth was used to determine the optimum growth at various pH and temperature ranges. Amount of growth was monitored by measuring the optical density at 520 nm. Biochemical tests were performed at 30°C.

Morphology

Colonial and cellular morphological characters were investigated by using standard procedures [9, 22, 39]. To examine flagella by transmission electron microscopy, cells were negatively stained with 1% phosphotungstic acid and photographs were obtained with a Philips CM20 transmission electron microscope operated at 80 kV. For scanning electron microscopy, cells were fixed for 2 h in a buffer containing 2% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.4), dehydrated by using increasing concentrations of ethanol, dried in a critical point apparatus, and sputtered with gold. Scanning microscopic photographs were taken with a Philips SEM515.

Biochemical Tests

Oxidase and catalase activities, indole, DNase, H_2S production, nitrate reduction, and denitrification were determined according to Gerhardt *et al.* [15]. Decarboxylation of amino acids (Sigma Chemical Co., St. Louis, MO, U.S.A.) and growth in the presence of KCN were tested by the method of Edwards and Ewing [9]. Citrate utilization was investigated by Simmons citrate agar, and Christensen urea agar was used to assay for urease [9]. Acid production from carbohydrates was determined by the procedure of Leifson [25]. Additional biochemical tests were performed by using API 20E (bioMerieux, Lyon, France) and Biolog GN (Biolog Inc., Hayward, CA, U.S.A.) test kits according to the manufacturer's specifications.

DNA Base Composition

The isolate and *E. coli* K12 used as a standard were grown to the late-logarithmic phase in Luria-Bertani broth at 28°C. DNA was purified by the method of Mamur [29], and diluted to a concentration of 50 μg/ml in 0.1× SSC buffer (15 mM of NaCl and 1.5 mM of trisodium citrate, pH 7.0). The G+C content of the DNA was determined from the midpoint value (*Tm*) of the thermal denaturation profile using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech Ltd., Cambridge, U.K.) equipped with a programmable Peltier temperature control unit. The mean G+C content was calculated by the equation described by Mamur and Doty [30], as modified by De Lay [6].

Analyses of Cellular Fatty Acids, Isoprenoid Quinones, and Phospholipids

The culture for fatty acid analysis was grown on trypticase soy agar at 28°C for 24 h. Fatty acid methyl esters were prepared from fresh wet biomass according to Guckert *et al.* [18] and separated by using a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and a 5% silica capillary column fused with phenyl methyl silicone (0.2 mm by 25 m). The fatty acid methyl esters were identified and quantified by the standard MIS library generation software (Microbial ID, Inc., Newark, U.S.A.). The resultant profiles were compared with those of a commercial bacterial library (MIDI Aerobe Library, version 3.8).

Isoprenoid quinones were purified by using the procedure of Collins and Jones [5]. The crude isoprenoid quinones were purified by thin-layer chromatography using Kieselgel 60 F254 plates (Merck, Darmstadt, Germany). Purified menaquinone and ubiquinone were identified by a mass spectrometer (JEOL SX102, Tokyo, Japan) with ionization energy of 70 eV.

Polar lipids were extracted from 100 mg of freeze-dried cells by the method of Tindall [41]. The polar lipids were separated by two-dimensional silica gel thin-layer chromatography (Merck 5554). The total lipids and specific functional groups were detected by using molybdophosphoric acid (for total lipids), Zinzadze reagent (for phosphate), ninhydrin (for free amino groups), periodate-Schiff reagent (for α-glycols), and alpha-naphthol-sulfuric acid (for glycolipids).

Sequence Alignment and Phylogenetic Analysis of the 16S rRNA Gene Sequences

Chromosomal DNA was extracted as described by Beji *et al.* [1]. The almost complete 16S rRNA gene was determined using universal primers [24]. The 16S rDNA sequence was aligned with those in the Ribosomal Database Project [28] and GenBank. Phylogenetic analyses were performed using the Fitch-Margoliash [13], maximum parsimony [12], and neighbor-joining [37] methods. Evolutionary distance matrices were generated according to Jukes and Cantor [20]. The topology of the resultant neighbor-joining tree was evaluated by bootstrap analyses [10] based on 1,000 resamplings. The PHYLIP package [11] was used for all phylogenetic analyses.

DNA-DNA Hybridization

The DNA-DNA hybridization was performed on a nylon membrane (Hybond-N^+, Amersham) using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals, Germany) according to the manufacturer's protocol. The membranes were then pre-hybridized in a hybridization solution at 52°C for 30 min. The actual pre-hybridization was carried out in a hybridization solution containing labeled DNA (25 ng/ml) at 52°C for 16 h. After hybridization, the membranes were washed twice in a primary washing solution (2×SSC and 0.1% SDS), and then subsequently washed twice in a secondary solution (0.5×SSC and 0.1% SDS) at 68°C. Detection reagents were added to the membranes for 5 min at room temperature, and then the membranes were exposed to autoradiography film (Hyperfilm-ECL, Amersham) for 10 min, and the signal intensities were determined by the TINA 2.0 program. The signal produced by self-hybridization was taken as 100%, and percentage homology values were calculated for the duplicate samples.

Nucleotide Sequence Accession Number

The complete 16S rRNA gene sequence of strain HY-3 has been deposited in the GenBank database under accession number U93263.

RESULTS

Isolation and Morphological Characteristics of the Isolate

In a previous study, Moon *et al.* [35] collected a total of 10^3–10^5 colonies and identified 22 bacteria from the digestive tract of a spider, *Nephila clavata*, collected in the field. We isolated a bacterium that formed a clear zone with a diameter of approximately 5 mm after 2-day incubation in skim milk plate. This observation indicated that the bacterium excretes an extracellular protease at high levels. To further identify the strain designated as HY-3, the morphological characteristics were observed by microscopy. The organism was Gram-negative and motile, and formed a smooth, round, convex, pale pink colony on nutrient agar plates (data not shown). The strain grew relatively well between 5 to 40°C at pH 6.0 to 10.0, and the optimum growth temperature and pH were 25°C and pH 8.0, respectively. Scanning electron micrographs demonstrated that the strain HY-3 was a short rod, 0.5 to 0.8 by 0.9 to 2.0 μm in size (Fig. 1A). Transmission electron microscopy after negative staining with phosphotungstic acid showed that the isolate had peritrichous flagella (Fig. 1B).

Chemotaxonomic Characteristics

The morphological and chemotaxonomic characteristics clearly placed the isolate in the family *Enterobacteriaceae*

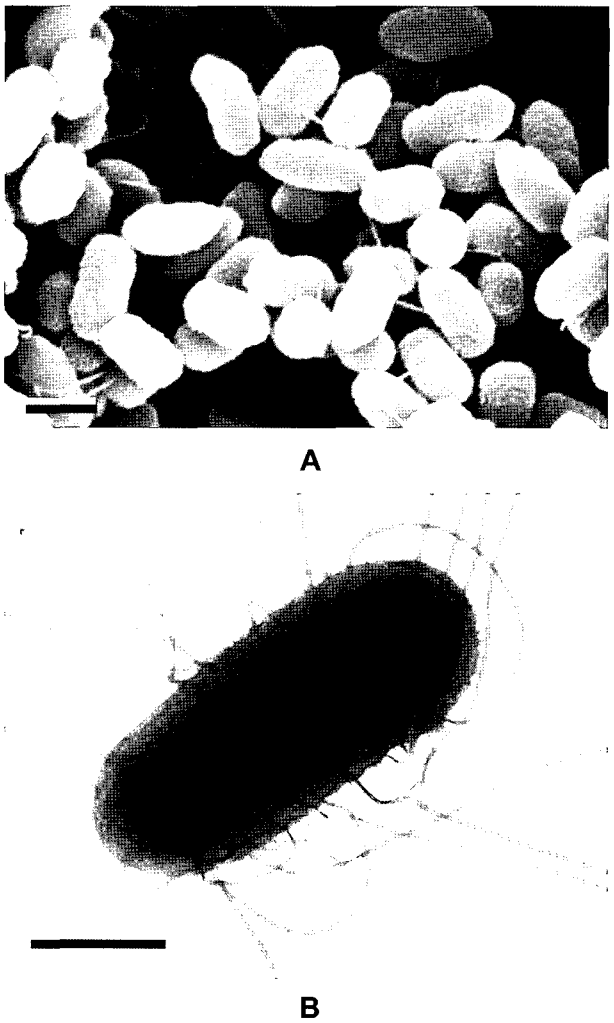

Fig. 1. Electron micrographs of the strain HY-3.
A. Scanning electron microscope image. Cells were grown overnight in NA medium at 28°C. **B.** Negatively stained cells of strain HY-3, showing peritrichous flagella. Bar=0.5 μm.

as summarized in Table 1. The strain generated a big halo with a diameter of approximately 5 mm after 2 days of incubation on 1% skim milk plates, indicative of excreting an extracellular protease. The strain had catalase and β-galactosidase activities, and reduced nitrate with gas generation. The strain produced acidic compounds after incubating in media containing many saccharides as sole carbon sources, such as D-glucose, lactose, sucrose, arabinose, raffinose, maltose, trehalose, mannose, and several alcoholic sugars such as D-mannitol, D-sorbitol, and myo-inositol. The strain produced ornithine decarboxylase, but did not produce arginine dihydrolase, lysine decarboxylase, or urease. The major isoprenoid quinone was Q-8. The isolate showed a unique fatty acid profile composed of 16:0 (32%), 16:1 w7c/15:0 iso 2OH (21%), and 18:1 w7c (11%), as shown in Table 1. The organism contained a major amount of phosphatidylethanolamine, phosphatidylglycerol, and

Table 1. Phenotypic and chemotaxonomic characteristics of strain HY-3

Characteristic	Strain HY-3
Cell size	0.5–0.8×0.9–2.0 μm
Motility	+
Yellow pigment	–
Catalase	+
Oxidase	–
Hydrolysis of	
Gelatin	+
Starch	–
Esculin	+
Urea	–
ONPG test	+
Nitrate reduction	+
Arginine dihydrolase	–
Lysine decarboxylase	+
Ornithine decarboxylase	+
Phenylalanine deaminase	–
Indole production	–
H_2S production (TSI)	–
Methyl red test	–
Voges-Proskauer reaction	+
Growth in KCN	+
DNase at 25°C	+
Utilization of	
Citrate	+
Malonate	–
Acetate	–
Gas from D-glucose	+
Fermentation of	
D-Glucose, Lactose, Sucrose	+
D-Mannitol, myo-Inositol	+
L-Arabinose, Raffinose	+
L-Rhamnose, Cellobiose	–
Maltose, Trehalose	+
Erythrytol, Melibiose, D-Arabitol	–
Glycerol, Amygdalin	+
Quinone system	Q-8
Cellular fatty acid	PE, PG, DPG
Polar lipids	$C_{16:0}$, $C_{16:1}$/iso, $C_{15:0}$, $C_{18:0}$
G+C content (mol%)	57.0 (Tm)

[a]+, positive; –, negative.

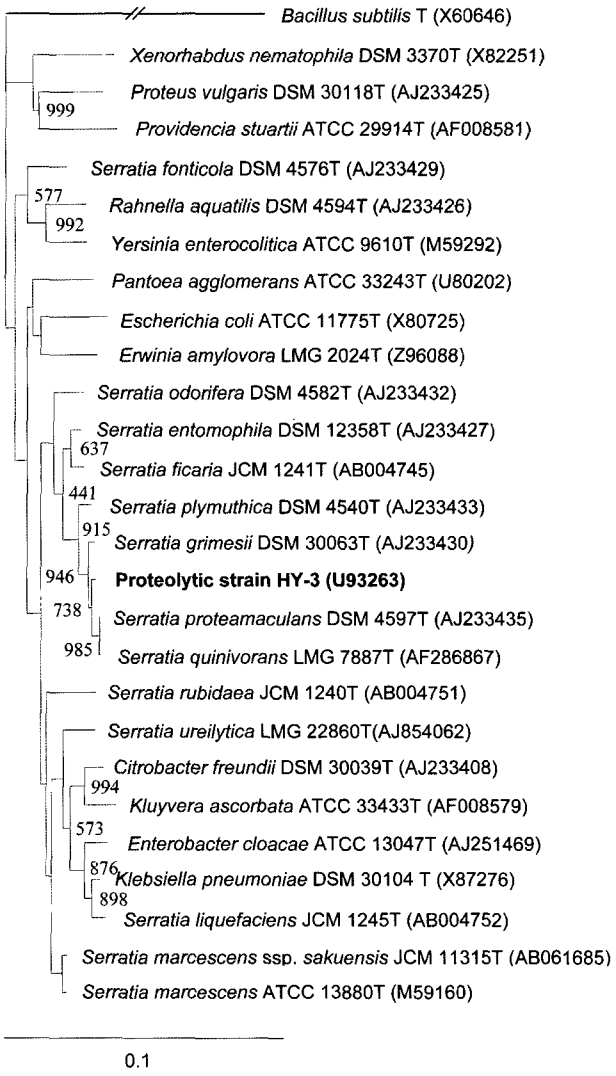

Fig. 2. Phylogenetic tree of the strain HY-3 and closely related enteric bacteria on the basis of 16S rDNA sequences.
The tree was generated by neighbor-joining algorithm of PHYLIP. The bar corresponds to 0.1 substitutions per nucleotide postion.

diphosphatidylglycerol (data not shown). The G+C content of the DNA was 57.0 mol% by *Tm*.

To examine the chemotaxonomic characters up to the species level, commercial identification systems were applied. The API 20E kit and Biolog system revealed that the strain HY-3 was closely related to *Serratia liquefaciens* with 76.1% likelihood and 0.891 similarity, respectively. However, the cellular fatty acid analysis by the standard MIS library generation software suggested that the closest neighbors of the strain were *S. liquefaciens* and *Rahnella aquatilis*, with low levels of similarities of 0.674 and 0.539, respectively. Other chemotaxonomic markers did not differentiate the strain from other genera in the family *Enterobacteriaceae*.

Phylogenetic Analysis and DNA-DNA Hybridization

A total of 1,501 nucleotides of the 16S ribosomal RNA gene were amplified by PCR and the sequence was determined and aligned with those of reference strains of the family *Enterobacteriaceae*. Based on the nucleotide sequence similarity and phylogenetic analyses using the PHYLIP package [11], the closely related species were *Serratia quinivorans* and *Serratia grimesii* (Fig. 2). The isolate exhibited 99.5 and 99.4% sequence similarity with

Table 2. 16S rRNA sequence similarity values between strain HY-3 and closely related species.

Names	Similarity to strain HY-3 (%)	No. of nucleotide differences/total no. of nucleotides compared
Serratia quinivorans DSM 4597T	99.53	7/1489
Serratia grimesii DSM 30063T	99.39	9/1485
Serratia proteamaculans DSM 4543T	99.13	13/1489
Serratia plymuthica DSM 4540T	98.99	15/1486
Serratia ficaria JCM 1241T	97.99	29/1446
Serratia entomophila DSM 12358T	97.79	33/1490
Serratia fonticola DSM 4576T	97.57	36/1483
Serratia odorifera DSM 4582T	97.04	44/1486
Serratia marcescens DSM 30121T	96.37	54/1486

S. quinivorans and *S. grimesii*, respectively, as shown in Table 2. The DNA-DNA homology level between the strain and other closely related *Serratia* species (*S. grimesii*, *S. plymuthica*, and *S. quinivorans*) were 3 to 37% (Table 3). However, the strain exhibited 97% DNA-DNA homology with *S. proteamaculans* (Table 3).

DISCUSSION

In this paper, we describe a bacterium designated HY-3 (KCTC 2390) isolated from the digestive tract of a spider, *Nephila clavata*, based on polyphasic evidence. The bacterium has been an attractive host, since it produces a highly active extracellular protease of industrial potential. Chemotaxonomic features of the strain such as the fatty acid profiles, carbon utilization, G+C content, and phospholipid profile demonstrated that the isolate is a member of the family *Enterobacteriaceae*. The morphological characteristics using light, scanning electron, and transmission electron microscopic techniques supported the biochemical observations. The phenotypic characteristics of the isolate were also in accordance with the general description of members of *Enterobacteriaceae* Rhan 1937[AL] that have many phenotypic properties in common: they are Gram-negative and rod shaped; do not form spores; are motile by peritrichous flagella; grow well on peptone, meat extract, and MacConkey's media; ferment D-glucose; are catalase positive and oxidase negative; and reduce nitrate to nitrite [9]. *Enterobacteriaceae* are mostly distributed in water, soil, and intestines of humans and other animals, and even found in plants including fruits, vegetables, grains, flowering plants, and trees [22].

Table 3. Level of DNA homology between HY3 and other *Serratia* spp. using HY-3 chromosomal DNA as a probe.

Strain	Similarity (%)
E. coli KCTC 2441	1
Serratia ficaria JCM 1241T	6
Serratia fonticola DSM 4576T	23
Serratia grimesii DSM 30063T	23
Serratia plymuthica DSM 4540T	3
Serratia quinivorans DSM 4597T	37
Serratia proteamaculans DSM 4543T	97
HY-3	100

Based on the chemotaxonomic characteristics by using the API 20E kit, Biolog system, and standard MIS library generation software with the cellular fatty acid analysis to identify the strain up to the species level, the strain was most closely related to *Serratia liquefaciens* with relatively high possibilities. *S. liquefaciens* strains were previously reported to be extensively associated with insects [22]. Phylogenetic analyses of the 16S ribosomal RNA gene revealed that the strain is most similar to *Serratia quinivorans* or *Serratia grimesii*. It is not likely that the strain could be *S. grimesii*, *S. plymuthica*, or *S. quinivorans*, since the strain showed 3 to 37% homology to them at the DNA-DNA hybridization experiment. It is clear that the isolate belongs to *S. proteamaculans*, based on the DNA-DNA hybridization level of 97% as well as molecular systematic and phenotypic data. The phylogenetic definition of a species is accepted when the related strains have more than 70% DNA-DNA hybridization levels [43].

We speculate that the bacterium may have a mutualistic or synergistic relationship with the spider, since the infected host did not show any observable symptom. Thus, the occurrence of the bacterium may be related to the unusual food intake mode of some spiders. Spiders prey upon insects and other small arthropods, and vomit digestive fluid, mainly consisting of proteolytic enzymes from the intestinal tract, onto the victim. Only a little is known about the source of proteolytic enzymes in spiders. As this case suggests, the proteolytic enzymes can be originated from the natural microbial population in the digestive tract of spiders. However, we do not exclude the possibility that *S. proteamaculans* KCTC 2390 can be pathogenic to spiders and insects since most enteric bacteria producing serralysin metalloproteases cause diseases in their hosts. *S. marcescens* ATCC 25419 isolated from boll weevil causes diseases in a variety of insects [7]. *S. marcescens* strains producing exoproteases are involved in pathogenesis in guinea pigs, mice [26], and insects [14]. *S. marcescens* kums3958 was

isolated from a patient with a severe corneal ulcer [31]. A phytopathogenic bacterium, *Erwinia chrysanthemi*, causes soft rot and wilt diseases in plants [8]. Several mechanisms are known of how metalloproteases are involved in virulence between infecting pathogens and their hosts [34]. In *Listeria monocytogenes*, the zinc metalloprotease is required for maturation and activation of phospholipase C [36]. Zinc metalloproteases also have a direct toxin activity as in the cases of enterotoxin in *Bacteroides fragilis* [21].

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