Pseudomonas fluorescens proliferates in a mouse organ homogenate at low temperature

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Abstract. In this study we observed the proliferation of *Pseudomonas fluorescens* (*P. fluorescens*) in mouse organ homogenates at 4˚C. *P. fluorescens* secreted a protease possessing properties different from those of the mammalian tissue proteases. The specificity of this protease required a basic amino acid residue at the P_1 position at a pH optimum of 6.0. The specificity of the protease was similar to that of trypsin, but the pH optimum was different. The protease mildly degraded elastin-Congo red; this suggests that the protease serves as an alternative for elastase in the case of *P. fluorescens* strains that lack virulent elastase. The protease was identified as an alkaline protease of *P. fluorescens* by liquid chromatography-tandem mass spectrometry analysis. Our results show that proteome analysis of the soluble proteins is useful in identifying bacterial species, particularly the bacterial contaminants in samples containing antibiotics.

Introduction

Pseudomonas species are gram-negative bacteria indigenous to our environment, existing in, for example, drinking-water biofilms, the human skin and saliva (1-3). Indigenous microbiota have several beneficial effects on host physiological functions. Colonizing bacteria play a principal role in the postnatal maturation of the mammalian immune system (4,5). *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Pseudomonas fluorescens* (*P. fluorescens*) strains cause opportunistic infections. These organisms are generally able to acquire multidrug resistance (MDR) and cause nosocomial infections. Therefore, contamination by these species is one of the major problems in many hospitals (6). *P. fluorescens* is a member of the fluorescent pseudomonas group. The organism is generally considered to have a low level of virulence (7). Strains of *P. fluorescens* have frequently been identified as contaminants on human skin and as agents causing pseudobacteremia and procedure-related infections in hospitalized patients. In industrial environments, such as the dairy industry, the presence of *P. fluorescens* is undesirable since it is responsible for product spoilage due to the production of extracellular, heat-resistant lipases and proteases (8). *P. aeruginosa*, a species related to *P. fluorescens*, is an opportunistic pathogen that causes a variety of diseases, particularly in immunocompromised patients such as those suffering from cystic fibrosis where these bacteria preferentially colonize the bronchopulmonary tract (9). *P. aeruginosa*, a leading nosocomial pathogen, may acquire MDR. Infections caused by *P. aeruginosa* are often severe and life threatening. Moreover, they are difficult to treat because of their limited susceptibility to antimicrobial agents and the emergence of antibiotic resistance at a high frequency during therapy (10,11), resulting in severe adverse outcomes (12). *P. aeruginosa* has 2 types of extracellular Zn-metalloproteases, aeruginolysin (13) and pseudolysin (14). These proteases belong to the serralysin (15) and elastase families, respectively. Both proteases are virulence factors, contribute to tissue destruction, and assist in bacterial invasion during infection (16). The specific *in vivo* targets of aeruginolysin are not clearly known; the prospective candidates include the Clq and C3 proteins of the serum complement (17) as well as γ interferon (18). Pseudolysin is one of the strongest virulence factors among the toxins of *P. aeruginosa*. It degrades the elastin of the human lung and also other matrix proteins, including laminin and collagen types III and IV (19,20).

Materials and methods

All reagents were of analytical grade, and experiments involving animals were carried out in accordance with the guidelines established at Tohoku University, based on regulations and laws set by the Japanese government.

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Abbreviations: Boc, butyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; Glt, glutaryl; MCA, 4-methylcoumaryl-7-amide; MDR, multidrug resistance; MES, 2-morpholinoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Suc, succinyl; Z, benzyloxycarbonyl

Key words: pseudomonas fluorescens, multidrug resistance, opportunistic infection, extracellular metalloprotease, indigenous bacterium, elastase

Source. A brain was removed from a specific-pathogen-free (SPF) mouse, and the whole lateral hemisphere was immediately homogenized in 9 volumes of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl. The homogenate was centrifuged at 10,000 x g for 10 min at 4˚C. The precipitate was suspended in 9 volumes of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl. This solution was used as a source of indigenous microbes and as the culture media.

Time course of the peptidase activity in culture medium. The mouse brain homogenate was incubated at 4 and 37˚C for specific time intervals. The homogenate was then centrifuged at 15,000 x g at 4˚C for 10 min. The supernatant was used as a crude enzyme solution and assayed for protease activity.

Enzyme assay. The hydrolysis of the fluorogenic peptide substrates was spectrofluorimetrically measured at 37˚C. The reaction mixtures contained 945 μl of 50 mM MES-NaOH buffer (pH 6.0), 5 μl of 10 mM substrate solution, and 50 μl of sample enzyme in a total volume of 1 ml. The increase in fluorescence intensity produced by substrate cleavage during the incubation was measured at an emission wavelength of 440 nm with excitation at 360 nm using a fluorescence spectrophotometer. One katal of the enzyme activity was defined as the amount of enzyme that liberates 1 mol of 4-methylcounmaryl-7-amide (MCA) from a fluorogenic peptide substrate at 37˚C and a pH of 6.0.

Purification procedure. The mouse brain homogenate incubated at 4˚C for 24 h was used as the starting material for the purification of the enzyme. All subsequent procedures were carried out at 4˚C. The homogenate was centrifuged at 15,000 x g for 10 min. The supernatant was then applied to a HiLoad 16/60 Sephadex 200 column (1.6 cm x 60 cm; GE Healthcare, Uppsala, Sweden) equilibrated with 25 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. The enzyme was eluted using equilibration buffer. The fractions exhibiting Boc-Val-Leu-Lys-MCA hydrolyzing activity were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 7.0 (buffer A). The solution was applied to a HiTrap Q column (5 ml; GE Healthcare), and the enzyme was eluted using a linear gradient of NaCl at a concentration of 0-250 mM in buffer A. The active fractions were pooled and dialyzed against buffer A and then applied to a Resource Q column (1 ml; GE Healthcare). Elution was performed using a linear gradient of 20-200 mM NaCl. The purified enzyme migrated as a single band when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7% gel) performed by Laemmli's method (21), as observed by Coomassie brilliant blue staining. Protein concentrations were measured by Bradford's method (22) using bovine serum albumin (BSA) as the standard protein.

Effects of inhibitors on activity. To investigate the inhibition of the protease, the enzyme was treated with various inhibitors at 37˚C for 30 min at pH 6.0 and the residual enzyme activity was measured. Control runs (100% activity) were conducted by replacing the inhibitor solutions with distilled water. The following components were used: pepstatin A (3 μg/ml), leupeptin (3 μg/ml), phenylmethylsulfonyl fluoride (PMSF, 5 mM), antipain (3 μg/ml), E-64 (0.1 mM), and ethylenediaminetetraacetic acid (EDTA, 1 mM).

Determination of protein sequence. SDS-PAGE was carried out by Laemmli's method. The final acrylamide concentration of the separating gel was 12% (w/v) and that of the stacking gel was 4% (w/v). All samples were mixed with the same volume of 2X Laemmli sample buffer (0.1 M Tris-HCl buffer (pH 6.6), 200 μM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue, and 20% glycerol), and denaturated at 98˚C for 10 min. After loading the samples, electrophoresis was carried out at a constant voltage of 80 V for 3 h. After the electrophoresis, the gel was fixed for 1 h in 50% v/v methanol and 10% v/v acetic acid followed by staining with SYPRO Ruby stain (Bio-Rad Laboratories, Hercules, CA) overnight. The background stain was removed by incubation in 10% v/v acetic acid and 7% v/v methanol for 1 h. The gels were stored in water at 4˚C. The stained band observed at approximately 45 kDa on the SDS-PAGE gel was excised and chopped into small pieces. The gels were dried on a centrifugation evaporator without heating. Five microliters of 0.2% (v/v) Tween-20 containing 20 mM sodium bicarbonate buffer (pH 8.0) was added to swell the gel. A 5-μl volume of trypsin solution (1 ng/μl) was added to the above solution, and the mixture was incubated at 37˚C for 30 min. The gel was filled with 200 μl of ammonium bicarbonate buffer (pH 8.0) and incubated at 37˚C for 18 h. The digestion was stopped by the addition of 5 μl of formic acid. The sample was injected into a nano-LC system that was directly coupled to an electrospray ionization mass spectrometry (ESI-MS, HCT Ultra, Bruker Daltonik GmbH, Bremen, Germany). The MS and MS/MS data were acquired and processed automatically using the DataAnalysis software. Database searching was carried out using Mascot 2.0 (Matrix Science Ltd, UK, www.matrixscience.com) against the nonredundant protein sequence database of the National Center for Biotechnology Information (NCBI). All the peptide sequences were checked manually.

Elastin-Congo red assay. The elastolytic activity of the enzyme was tested by a modification of the procedures of Rust *et al* (23). Ten milligrams of elastin-Congo red (Sigma Chemical Co., St. Louis, MO) was suspended in 0.9 ml of 50 mM sodium acetate buffer (pH 6.0). The digestion reaction was initiated by the addition of the enzymes (final concentration of 1 μM) and then incubated at 37˚C for 18 h. The reactions were carried out in the absence or presence of 1 mM EDTA. Finally, the reaction mixtures were centrifuged at 10,000 x g for 15 min, and the absorption of the supernatants at 490 nm was recorded.

Results and Discussion

Proliferation of a microorganism in a mouse brain homogenate. A mouse brain homogenate was prepared and the time courses of the peptidase activity were recorded over a period of 24 h at 4 and 37˚C, respectively. When the homogenate was incubated at 4˚C, its hydrolytic activity against Boc-Val-Leu-Lys-MCA, Boc-Arg-Val-Arg-Arg-MCA, and Boc-Gly-Arg-Arg-MCA at pH 6.0 increased

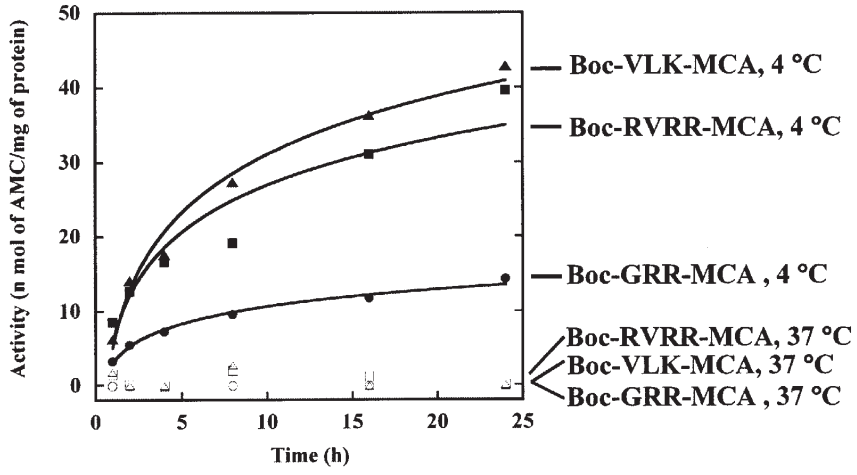

Figure 1. Curve demonstrating the increase in protease activity in a mouse brain homogenate at 4˚C (closed symbols) and 37˚C (open symbols). The mouse brain homogenate was incubated at 4 and 37˚C respectively in order to increase the amount of indigenous bacteria and its secreted proteases. The homogenate supernatant was assayed for protease activity at 37˚C for 1 h at pH 6.0. The synthetic substrates Boc-Val-Leu-Lys-MCA (circle), Boc-Arg-Val-Arg-Arg-MCA (square) and Boc-Gly-Arg-Arg-MCA (triangle) were used as substrates. The activity was defined as the increase in fluorescence intensity per total protein. The collected data from Boc-Val-Leu-Lys-MCA, Boc-Arg-Val-Arg-Arg-MCA, and Boc-Gly-Arg-Arg-MCA at 4˚C were approximated to logarithmic curves.

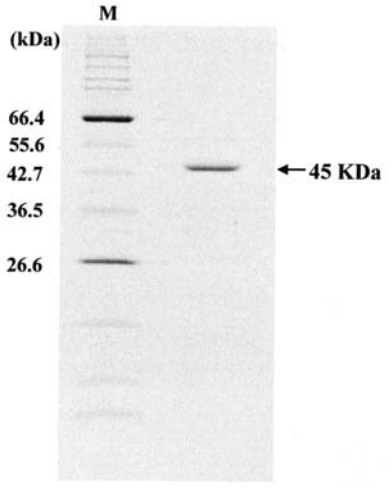

Figure 2. SDS-PAGE analysis of the protease from a mouse brain homogenate. Approximately 2 μg of the purified protein was separated under reducing-gel conditions (7% gel) and stained with Coomassie brilliant blue. M represents the molecular mass marker. The homogeneous band was detected at 45 kDa.

logarithmically over the 24-h time course (Fig. 1), while at 37˚C, the peptidase activity did not increase. These results indicate that a certain psychrophilic microorganism preferentially proliferates in the mouse homogenate at low temperature and secrets a protease. It is considered that this microorganism exists as an indigenous bacterium; however, it proliferated only under temperature conditions equivalent to that of the mouse body. Diverse microbiota exist in the mammal intestine, stomach and skin (24-27). As the average body temperature of a mouse is approximately 37˚C, there is little information regarding the latent bacteria growing in the organs of this mammal at low temperature.

Purification of the protease and its enzymatic properties. In order to purify the protease that degrades Boc-Val-Leu-Lys-MCA at pH 6.0, successive chromatographies were performed. A single band migrating during SDS-PAGE was obtained, as shown in Fig. 2. A 377-fold purification over the starting material was attained with approximately 29% recovery (Table I). The specific activity of the enzyme toward Boc-Val-Leu-Lys-MCA was 4.36×10^{-3} kat kg^{-1} protein. Its molecular mass was estimated to be 45 kDa by SDS-PAGE. To investigate the inhibition of the purified protease, the enzyme was treated with various inhibitors at 37˚C for 30 min at pH 6.0 and the residual activity was measured. The enzyme activity was completely inhibited by antipain (3 μg/ml) and EDTA (1 mM). Moreover, the activity appeared to be unaffected by pepstatin A (3 μg/ml), leupeptin (3 μg/ml), PMSF (5 mM), and E-64 (0.1 mM). The residual activity of the protease treated with these inhibitors was more than 90%. Antipain is an aldehyde-type of inhibitor with an effective arginal residue, and it inhibits serine and cysteine proteases. The unidentified protease was considered to be a metalloprotease with an affinity towards antipain since EDTA completely inhibited its activity. To investigate the substrate specificity, 22 types of fluorogenic peptide substrates were used (Table II). The protease hydrolyzed the substrates containing the basic amino acid residues arginine or lysine at the P_1 position and the amino acids with basic or large side chains at the P_2 position. However, it did not hydrolyze substrates possessing non-basic amino acids at the P_1 position and small size amino acids at the P_2 position; this indicates that the enzyme has trypsin-like substrate specificity at a pH optimum of 6.0. Highly specific activity was observed against substrates with dibasic amino acids at the P_1 and P_2 positions, such as Boc-Gly-Arg-Arg-MCA and Boc-Arg-Val-Arg-Arg-MCA. The 45-kDa metalloprotease with trypsin-like substrate specificity was not observed in the mouse brain homogenate at an acidic pH.

Identification of the microorganism. To identify the microorganism that proliferates in a mouse brain homogenate, the purified enzyme was degraded for a definite period by trypsin and analyzed by liquid chromatography-tandem mass

Table I. Purification of a protease that cleaves the carboxyterminal end comprised of a pair of basic amino acids at pH 6.0.

	Total Protein (kg)	Total activity (mol s^{-1})	Specific activity (mol s^{-1} kg^{-1})	Yield (%)	Purification (fold)
Crude[a]	6.40×10^{-5}	7.39×10^{-10}	1.16×10^{-5}	100	1
Sephadex 200	1.30×10^{-5}	5.84×10^{-10}	4.49×10^{-5}	79	4
HiTrap Q	3.04×10^{-7}	2.68×10^{-10}	8.83×10^{-5}	36	76
Resource Q	4.96×10^{-8}	2.16×10^{-10}	4.36×10^{-3}	29	377

[a]Mouse brain homogenate incubated for 3 days at 4˚C in order to increase indigenous bacteria and its secreted proteases.

Table II. Substrate specificity of the *P. fluorescens* alkaline protease from mouse brain homogenate.

Substrate P_3 P_2 P_1	m katal (mol/sec kg)	%
Boc-Gly-Arg-Arg-MCA	33.2	100
Boc-Arg-Val-Arg-Arg-MCA	32.4	98
Boc-Val-Leu-Lys-MCA	5.8	18
Boc-Leu-Lys-Arg-MCA	4.6	14
Boc-Glu-Lys-Lys-MCA	2.9	9
Boc-Ala-Gly-Pro-Arg-MCA	1.0	3
Z-Phe-Arg-MCA	0.3	1
Boc-Leu-Gly-Arg-MCA	N.D.	0
Boc-Gln-Gly-Arg-MCA	N.D.	0
Boc-Leu-Thr-Arg-MCA	N.D.	0
Arg-MCA	N.D.	0
Lys-MCA	N.D.	0
Ac-Asp-Glu-Val-Asp-MCA	N.D.	0
Suc-Leu-Leu-Val-Tyr-MCA	N.D.	0
Z-Leu-Arg-Gly-Gly-MCA	N.D.	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	N.D.	0
Suc-Ile-Ile-Trp-MCA	N.D.	0
Z-Val-Lys-Met-MCA	N.D.	0
Glt-Ala-Ala-Phe-MCA	N.D.	0
Suc-Ala-Pro-Ala-MCA	N.D.	0
Suc-Ala-Ala-Ala-MCA	N.D.	0
Phe-MCA	N.D.	0

N.D., not detected.

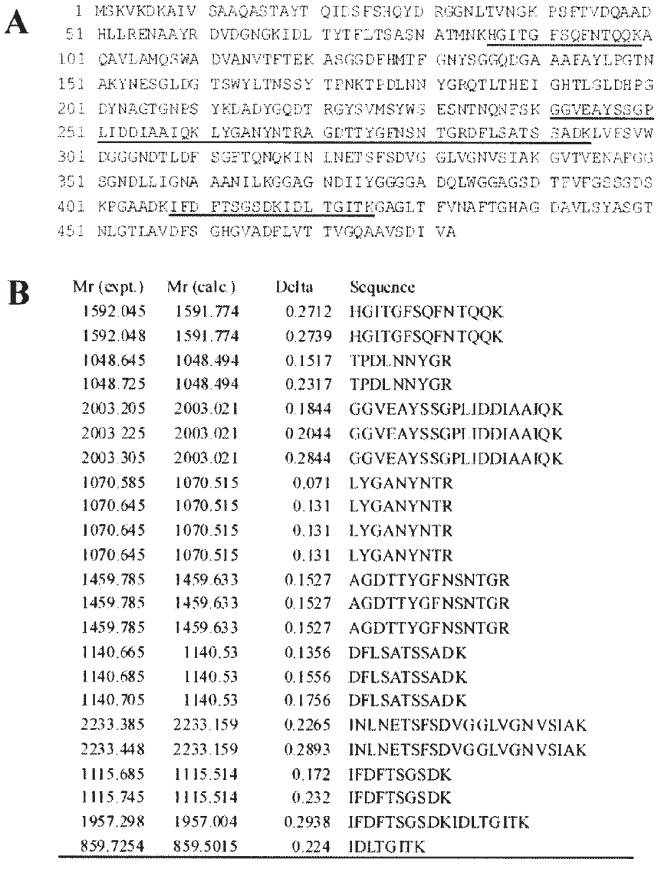

Figure 3. Identification of the protease purified from a mouse brain homogenate by LC-MS/MS and Mascot search. (A) Amino acid sequence of the *P. fluorescens* alkaline protease (Entrez protein accession ID: BAA36461). Ten sequences were detected by LC-MS/MS analysis of the trypsin-digested protein, and they matched the sequence of the *P. fluorescens* alkaline protease on the nonredundant protein sequence database of NCBI using the Mascot algorithm. The underlined regions represent the matched sequence that covers 24% of *P. fluorescens* alkaline protease. (B) Molecular masses of tryptic peptides and their agreement with the identified sequences.

spectrometry (LC-MS/MS). Ten sequences were detected by LC-MS/MS analysis of the trypsin-digested protein, and they matched with the sequence of the *P. fluorescens* alkaline protease (Entrez protein accession ID: BAA36461) on the nonredundant protein sequence database of NCBI using the Mascot algorithm (Fig. 3). The sequence coverage was 24%. The score of the Mascot search was 727 indicating high confidence. The *P. fluorescens* alkaline protease gene *($AprA_{PF33}$)* is clustered with the genes coding for the lipase $lipA_{PF33}$, the ABC-exporter $AprDEF_{PF33}$, and 2 homologues of the *Serratia* serine protease, namely, PspA and PsPB (28). The enzyme hydrolyzes azocasein optimally at pH 8.0 and therefore it is named alkaline protease (29,30). The unidentified microorganism that proliferated in the mouse brain homogenate at 4˚C was indicated to be *P. fluorescens*. It was assumed that *P. fluorescens* existed as an indigenous bacterium and proliferated when the host tissue was destroyed. These bacteria can survive in poor nutrient

conditions, and they exist in our living environment. They can grow over a wide range of temperatures (0-32˚C) (31). In spite of careful procedures and aseptic manipulations, it is possible that *P. fluorescens* contaminated the mouse brain homogenate during experimental procedures. The psychrophile may have existed as an indigenous bacterium in the skin of the SPF mouse. We consider that the proliferation of *P. fluorescens* is suppressed under the high body temperatures in a mouse and that it proliferated in the mouse brain homogenate at 4˚C.

The role of the Pseudomonas fluorescens alkaline protease. The substrate specificity of the *P. fluorescens* alkaline protease appeared to be governed by the presence of a basic residue at the P_1 position and a pair of basic amino acids at the P_1 and P_2 positions at pH 6.0 (Table II). This is in accordance with reported data for the serralysin family proteases from *P. fluorescens* 114 (32). *P. fluorescens* is considered to have a low level of virulence. On the other hand, *P. aeruginosa* is an opportunistic pathogen. The *P. aeruginosa* elastase (pseudolysin) is an extracellular metalloprotease and is one of the strongest virulence factors among the toxins of this bacterium. Elastase from *P. fluorescens* was not detected in the present study. Therefore, the elastase activity of the alkaline protease of *P. fluorescens* was examined. The enzyme appeared to hydrolyze elastin-Congo red (ΔAbs_{490} = 0.078). This activity was inhibited in the presence of EDTA, which inhibits the *P. fluorescens* alkaline protease. It was surprising that the protease hydrolyzed elastin because peptide substrates of elastase, such as Suc-Ala-Pro-Ala-MCA and Suc-Ala-Ala-Ala-MCA, were not cleaved (Table II). Elastin is an important connective tissue protein that provides elasticity and tensile strength to organs, such as the skin, lung, blood vessels and ligaments (33). It is among the most hydrophobic proteins known. Although there is some species variation, approximately 75% of the entire sequence of elastin is made up of only 4 hydrophobic amino acids (Gly, Val, Ala, and Pro). The lysine residues of elastin are oxidatively deaminated by lysyl oxidase and are involved in covalent crosslinking (34,35). The *P. fluorescens* alkaline protease does not appear to degrade elastin because it cleaves peptide substrates at the C-terminal end of basic amino acids. Trypsin, however, has a strict substrate specificity; it hydrolyzes only at the C-terminal end of the basic amino acids. It has been suggested that trypsin mildly hydrolyzes elastin (36). This is an interesting observation because *P. fluorescens* does not have an extracellular elastase; therefore, it is thought to have a low level of virulence. It is suggested that in case of *P. fluorescens* strains that do not secrete virulent elastase, the protease serves as an alternative for this enzyme.

The proteome analysis of the soluble protein in the sample. We successfully identified *P. fluorescens* contamination in a mouse organ homogenate by targeting its specific protease activity. *P. fluorescens* exists as an indigenous bacterium in our environment and can acquire MDR. It is possible that the bacterium contaminates samples and affects the compositions of proteins. Our findings provide novel insights into the bacterial biota that potentially exist in samples and demonstrate that proteome analysis of the soluble proteins is useful in the identification of bacterial species.

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