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Short Communication

First Isolation of Carbon Dioxide-Dependent *Proteus mirabilis* from an Uncomplicated Cystitis Patient with Sjögren's Syndrome

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SUMMARY: An uncomplicated cystitis caused by CO_2-dependent *Proteus mirabilis* was observed in a 64-year-old Japanese female patient with Sjögren's syndrome in the Aomori Kyoritsu Hospital, Aomori, Japan. The initial *P. mirabilis* isolate came from a midstream urine specimen containing large numbers of Gram-negative, rod-shaped organisms that failed to grow on both Drigalski agar and sheep blood agar incubated in ambient air. The organism did grow when the urine was cultured overnight on blood agar under anaerobic conditions. Hence, we believed that the organism was an anaerobe. Further investigation revealed that the isolate grew on sheep blood agar along with swarming when the atmospheric CO_2 concentrations were increased to 5%. Initially, we failed to characterize or identify the *P. mirabilis* isolate or determine its antimicrobial susceptibilities using the MicroScan WalkAway-40 System because the isolate did not grow in the system. However, the isolate was subsequently identified as *P. mirabilis* based on its morphological, cultural, and biochemical properties by using the commercially available kit systems, Quick ID-GN and ID-Test EB-20. This identification of the isolate was confirmed by sequencing the 16S rRNA gene of the organism. To our knowledge, this is the first clinical isolation of capnophilic *P. mirabilis*.

It is well established that some pathogens such as *Brucella* spp., *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Helicobacter pylori* require carbon dioxide for growth. In clinical microbiology laboratories, other bacterial strains that require carbon dioxide for growth have been isolated and designated as CO_2-dependent, dwarf, G-variant, or small-colony variant.

Capnophilic *Staphylococcus aureus* strain was first documented by Hale (1) in 1951 and subsequently followed by isolation of such staphylococcal isolates by Thomas and Cowlard (2) in 1955. CO_2 dependence is not usually associated with *Enterobacteriaceae*. Further, *Klebsiella* strains requiring CO_2 for growth have been documted by Barker et al. (3), and CO_2-dependent *Escherichia coli* have been isolated by Eykyn and Phillips (4). However, little information is available on CO_2-dependency among *Proteus* spp. In this study, we have described the clinical isolation of the first absolutely capnophilic *Proteus mirabilis* from the urine of an outpatient. The organism failed to grow on sheep blood agar or on Trypticase-soy broth media (Eiken Chemical Co., Tokyo, Japan) in ambient air, even after 7 days of prolonged incubation.

A 63-year-old female patient with Sjögren's syndrome was admitted to the Aomori Kyoritsu Hospital, Japan, on April 1, 2009 for urinary problems. Until her admission, she had only been treated with traditional Chinese herbal remedies for Sjögren's syndrome. She had received no other medication including antibiotics. When she was seen in the outpatient clinic, she had a slight pain while urinating and a constant urge to urinate, which suggested a urinary tract infection; however, she had no history of fever. A mid-stream-urine specimen was submitted to the bacteriological division of the clinical laboratory in Aomori Kyoritsu Hospital for microbiological examination.

Although microscopic examination of the urine revealed abundant leucocytes with Gram-negative rod-shaped bacterial cells, no growth of organisms was observed in the urine inoculated on modified Drigalski agar (Nippon Becton Dickinson Co., Tokyo, Japan)

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and sheep blood agar (Trypticase Soy Agar II with 5% sheep blood; Nippon Becton Dickinson) after overnight incubation at 35°C under ambient air. In a subsequent study, anaerobic incubation of urine-inoculated sheep blood agar in the anaerobic chamber yielded numerous swarming colonies after overnight incubation at 35°C, implying that these colonies might be anaerobic because of the inability of the organisms to grow in an aerobic condition. However, the isolate was finally found to be strictly capnophilic. Further, no other microbial species were detected. The Neg Combo 6.11J panels in the automated microbiological instrument, MicroScan WalkAway System (Siemens Healthcare Diagnostics, Tokyo, Japan) could not correctly identify the isolate or determine its antimicrobial susceptibilities, because the isolate could not grow in the system. In contrast, both the Quick ID-GN and the ID-Test EB-20 (Nissui Seiyaku Co., Tokyo, Japan) kit panels yielded *P. mirabilis* with high probabilities. In order to ensure the identification of the isolate, the 16S rRNA gene was directly sequenced as described previously (5) using a Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif., USA) and a model 3100 DNA sequencer instrument (Applied Biosystems). The sequence was retrieved from the Ribosomal Database Project databases (6). Comparative sequence analysis showed 99% 16S rRNA sequence similarity to that of the type strain of *P. mirabilis* ATCC29906 (DDBJ/EMBL/GenBank accession no. AF008582). Further analysis of the isolate was performed with a matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) (Microflex mass spectrometer; Bruker Daltonics Japan, Yokohama, Japan) using alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics) and the Bruker BioTyper database and software, version 2.0 (Bruker Daltonics). The isolate was identified as *P. mirabilis* with an excellent score of 2.335 when compared with *P. mirabilis* DSM788. The isolate was finally identified as CO_2-dependent *P. mirabilis* based on morphological, cultural, and biochemical properties of the isolate along with the comparative sequence of the 16S rRNA genes and the MALDI-TOF MS analysis. The isolate was highly susceptible to many antibiotics including penicillins, cephems, aminoglycosides, tetracyclines, and new quinolones, when examined in the 5% CO_2-enhanced atmosphere.

Subsequent microbiological investigation of the capnophilic isolate was carried out along with two clinical strains of *P. mirabilis*, and their colony characteristics and biochemical properties were compared. These two strains were isolated in Shinshu University Hospital, designated as SH1339 and SH1440, respectively, and stored in commercially available cryogenic freezer beads (Microbank vials; Pro-Lab Diagnostic, Richmond Hill, Ontario, Canada) at −80°C in a deep freezer. Prior to investigation, a bead from each frozen cryogenic vial was removed and cultured on sheep blood agar at 37°C overnight in a CO_2-incubator (Sanyo Electric Co., Tokyo, Japan) with 5% CO_2-enhanced atmosphere. Each single *P. mirabilis* colony including the capnophilic isolate was subcultured on a sheep blood agar overnight in the CO_2-incubator. The resulting bacterial lawns were subjected to the experiments described below.

Blood agars (Nippon Becton Dickinson) seeded with the capnophilic isolate and the two reference strains, SH1339 and SH1440, were incubated overnight in a 5% CO_2 environment at 30°C, 35°C, and 42°C. As shown in Table 1, the isolate grew well only at 35°C, although both the clinical strains (SH1339 and SH1340) revealed sufficient growths at every temperature with swarming. Further experiments were carried out on these three strains by seeding them on blood agars. Overnight incubation was performed in ambient air containing varying amounts of CO_2 at 35°C. As shown in Table 2, the capnophilic isolate grew well with swarming at 35°C in an environment with more than 3.0% CO_2. No visible growth of the isolate was observed in CO_2 concentrations of less than 1.0%, while the two clinical strains revealed stable growth with swarming regardless of CO_2 concentrations. At CO_2 concentrations of between 1% and 3%, the isolate grew pin-point colonies (colonies of

Table 1. Effect of temperature under 5% CO_2-added circumstances on the formation of normal-sized swarming colonies after overnight-incubation on sheep blood agars

Incubation temperature	Capnophilic isolate (this study)	*Proteus mirabilis* SH1339	*Proteus mirabilis* SH1340
30°C	+	++	++
35°C	++	+++	+++
42°C	±	++	++

±, subtle swarming; +, weak swarming; ++, good swarming; +++, ample swarming.

Table 2. Effect of CO_2 concentration on the formation of normal-sized swarming colonies after overnight-incubation at 35°C on sheep blood agars

	Ambient air	Swarming growth at following CO_2 (%) concentrations								
		0.5	1.0	1.5	2.0	3.0	4.0	5.0	10.0	20.0
Capnophilic isolate (this study)	−	−	P.P.*	P.P.*	P.P.*	−	−	−	−	−
Proteus mirabilis SH1339	+	+	+	+	+	+	+	+	+	+
Proteus mirabilis SH1340	+	+	+	+	+	+	+	+	+	+

All of the appearing colonies without swarming were pin-point colonies.
Sheep blood agar: Trypticase soy agar II with 5% sheep blood.
−, no growth; +, growth with swarming; P.P.*, pin-point colonies without swarming.

Table 3. Effect of pH on the formation of normal-sized swarming colonies after overnight-incubation at 35°C on sheep blood agars

pH	Swarming growth at following pH under 4% and 5% CO_2 concentration														
	pH 5.0			pH 6.0			pH 7.0			pH 8.0			Nippon Becton Dickinson		
CO_2 (%)	Ambient air	4	5	Ambient air	4	5	Ambient air	4	5	Ambient air	4	5	Ambient air	4	5
Capnophilic isolate (this study)	−	−	+	−	+	+	−	+	+	−	+	+	−	+	+
Proteus mirabilis SH1339	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus mirabilis SH1340	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

−, no growth; +, growth with swarming.

about 0.15 mm in diameter) without swarming, despite the use of blood agars. Typical normal-sized swarming colonies of the capnophilic isolate were obtained after overnight incubation in the presence of CO_2 at the concentrations more than 3%, although the two reference clinical strains grew well with swarming regardless of the presence or the concentrations of CO_2.

The capnophilic isolate and the two reference strains, SH1339 and SH1440, were inoculated on heart-infusion agar (Eiken Chemical) incorporating 5% defibrinated sheep blood adjusted to the pH of 5.0, 6.0, 7.0, and 8.0. The inoculated agars were incubated overnight in a 5% CO_2-enhanced envionment. As shown in Table 3, the capnophilic isolate failed to grow at pH 5.0 in 4% CO_2, however, the isolate demonstrated good growth with swarming under 5% CO_2. In contrast, the two reference strains grew with swarming, regardless of the pH.

We examined the isolates on minimal media in a 5% CO_2-enriched atmosphere to determine whether the capnophilic isolate was, in addition to being CO_2 auxotrophic, an additional auxotrophic mutant requiring particular growth factors such as vitamin K, purine, pyrimidine, L-arginine, succinate, biotin, vitamin B_{12}, oxaloacetate, citrate, iso-citrate, α-ketoglutarate, fumarate, malonate, sodium bicarbonate (Na_2CO_3), or urea, depending upon the site of the mutation. The minimal media used were those described by Vogel and Bonner (7) in 1956 and by Kawakami et al. (8) in 1972. As a result, the minimal media, when incubated in the 5% CO_2-enhanced atmosphere, supported the growth of the isolate as well as the two reference strains, SH1339 and SH1440. That is, no additional auxotrophic property was observed in addition to CO_2 requirement.

In subsequent studies performed without the addition of CO_2 gas, we investigated whether or not the isolate could grow on the minimal media with or without supplementation of the compounds above described. We prepared nine types of test media (A, B, C, D, E, F, G, H, and I), by adding the above-mentioned substrates to the minimal media. Medium A contained vitamin K, purine, pyrimidine, L-arginine, and succinate; medium B contained vitamin K, purine, pyrimidine, L-arginine, succinate, and biotin; medium C contained pyruvate; medium D contained biotin and vitamin B_{12}; medium E contained oxaloacetate, citrate, D/L-iso-citrate, α-ketoglutarate, fumarate, and malate; medium F contained oxaloacetate, citrate, D/L-iso-citrate, α-ketoglutarate, fumarate, malate, and vitamin B_{12}; medium G contained Na_2CO_3; medium H contained all of the 15 substances; and medium I contained urea. The capnophilic isolate failed to grow on any of the media in ambient air without the addition of CO_2. In addition, the isolate failed to grow in a 3% (w/v) solution of Na_2CO_3 in ambient air.

An interesting observation, called the CO_2 effect, was reported in 1968 (9) that some auxotrophic mutants of *E. coli* grew on a minimal medium without growth factors when the gas phase is supplemented with CO_2. In such strains, when the gas phase was not supplemented with CO_2, most of the mutants responded to other specific growth factors depending upon the locus of their mutation (9). However, our capnophilic *P. mirabilis* isolate revealed no response to the CO_2-auxotroph, definitely restricting to the property of CO_2-rquirement. Moreover, CO_2-supplementation could not be replaced by the addition of any of the 16 substances we tested. This suggested that the isolate was an obligate CO_2-mutant because it could not grow in minimal or complete sheep blood agar media without supplementation of CO_2. All of the experiments were carried out by using fresh colonies grown after recovery from frozen cryogenic vials. However, the capnophilic property was stably maintained across subsequent generations.

Moreover, even in the presence of 5% CO_2, as shown in Table 1, the growth of the capnophilic isolate was weaker than that of the two reference strains. This showed that CO_2 itself did not completely compensate for the defect in the CO_2 mutant. This might imply the possibility that CO_2 supplementation may result from the stimulation of enzymatic reactions in our capnophilic isolate of *P. mirabilis*. Capnophilic *E. coli* (4) and *Klebsiella ozaenae* (3) strains have been reported, and no other species in the family *Enterobacteriaceae* was documented to date. To our knowledge, we reported the first case of uncomplicated cystitis due to capnophilic *P. mirabilis* in a patient with Sjögren's syndrome. Since CO_2 dependence is an unusual property, the isolate may possess distinct metabolic pathways when compared with reference *P. mirabilis* strains. Therefore, further characterization of the isolate may potentially reveal a novel *Proteus* sp.

Although the capnophilic *P. mirabilis* isolate was unable to grow in broth media unless supplemented with CO_2, this isolate actually caused cystitis. Therefore, it must possess some mechanism to compensate for CO_2

dependence during its growth in the case of urinary tract infection.

These findings indicate the possibility that the CO_2 dependence may occur among species other than *P. mirabilis* in the family *Enterobacteriaceae*. In clinical bacteriology laboratories, the first step in processing urine material is direct microscopic examination of a Gram-stained preparation. In cases of occurrences of discrepancies between Gram's stain and culture findings, additional CO_2-enhanced or anaerobic incubations should be carried out. We should reaffirm the fundamental importance of direct Gram's staining of the urine specimens.

Conflict of interest None to declare.

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