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Producing *Proteus mirabilis* Isolate with Inability to Grow under
pH-Indicator Dyes from the Urine of an Octogenarian Male-Patient
with Acute Pyelonephritis

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SUMMARY

A capnophilic Gram-negative rod was recovered from the urine of an octogenarian male patient with acute pyelonephritis. The isolate also demonstrated to produce CTX-M-2-type-extended-spectrum-β-lactamase. Interestingly, the isolate failed to grow on modified Drigalski (BTB) and MacConkey agars even in the CO₂-enriched atmosphere. The investigation of this phenomenon revealed that the pH-indicator dyes, bromothymol-blue and/or crystal-violet, incorporated into the agars should serve to inhibit the growth of the isolate. Although routine identification with Vitek® 2 Compact systems was unsuccessful, the isolate was finally identified as *Proteus mirabilis* by sequencing the 16S rRNA genes and by the MALDI-TOF MS analysis. The carbonic anhydrase (CA) region, responsible for the CO₂ requirement, could not be amplifiable from approximately 2,000bp upstream to 2,000bp downstream. This could possibly be ascribed to the large-scale deletion or mutation of the DNA sequences containing the CA gene region. In fact, no revertant with ability to grow under the atmosphere without addition of CO₂ emerged. However, the revertant, capable of growing both on BTB and MacConkey agars, emerged at frequencies less than 10⁻⁹. Therefore, the genes responsible for the highly sensitive reactions to pH-indicator dyes might not be linked to those of the CA genes.
Capnophilic *Escherichia coli* and *Proteus mirabilis* isolates have been reported from urines (1-3). We encountered such capnophilic *P. mirabilis* capable of producing extended-spectrum-β-lactamase (ESBL) as a causative agent of acute pyelonephritis. Additionally, the isolate could not grow on modified Drigalski (BTB) (Kyokuto Pharmaceutical Ind., Tokyo, Japan) and/or MacConkey agars (Eiken Chemical Co., Ltd., Tokyo, Japan).

The patient was an octogenarian Japanese male with urinary tract infection accompanied by body temperature of 38 to 40°C. He was admitted in June 2016 to Nakatsugawa Municipal General Hospital, Nakatsugawa, Japan, for fever up to 38.5°C. Direct microscopy of the Gram-stained urine preparation revealed abundant leucocytes with Gram-negative rod-shaped cells, thus confirming the data on admission representing high values of white-blood cell-counts ($1.05 \times 10^9$/$L$) and C-reactive protein (9.62 mg/L). After incubation in CO$_2$-incubator (Sanyo electric Co., Tokyo, Japan) at 35°C for 48hrs, non-hemolytic and swarming Gram-negative short-sized rods appeared at cell-counts of $10^7$ cfu/ml on sheep blood agars (Eiken Chemical Co.). Interestingly, the isolate failed to grow neither on BTB nor MacConkey agars after overnight-incubation in CO$_2$-enriched atmosphere.

Biochemical characterizations and antimicrobial susceptibility of the isolate, using the Vitek® 2 Compact (bioMérieux Japan Ltd.) system, were unsuccessful due to its inability to proliferate in the system. The comparative sequence analysis of the isolate showed 99.9% 16S rRNA sequence similarity to that of the *P. mirabilis* AOUC-001, complete genome. The isolate was further analyzed with a matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) (Microflex mass spectrometer; Bruker Daltonics Japan, Yokohama, Japan), yielding the data demonstrating almost the same pattern as *P. mirabilis* with an excellent score of 2.289. We concluded that the isolate be identified as *P. mirabilis* and designated the isolate as *P. mirabilis* NA2609. Antimicrobial susceptibilities were determined.
using the Dry-Plate Eiken DP-31 (Eiken Chemical Co.), incubated at 35°C for 18hrs in 5% CO₂. 

*P. mirabilis* NA2609 exhibited resistance to many β-lactams, including piperacillin, cefazoline, cefotiam, cefotaxime, ceftazidime, ceftazidime, sulbactam/ampicillin, and aztreonam, but susceptible to flomoxef and meropenem. Successive disc-diffusion screening tests demonstrated positive results for ESBL production. By means of the ESBL gene-analysis, *P. mirabilis* NA2609 was found to carry ISEcp1-bla⁻CTX-M-2 ESBL. The empiric prescription was changed from ampicillin/sulbactam on admission to meropenem. The patient’s fever and symptoms subsided after 5 days of administration.

Then, we examined the effect of temperature on the growth behaviors of *P. mirabilis* NA2609 after overnight-incubation of sheep blood agars, seeded with *P. mirabilis* NA2609 and the two reference *P. mirabilis* ATCC29906 and ATCC43071 strains purchased and stocked in Shinshu University. The plates were incubated overnight in 5% CO₂ at 30°C, 35°C, and 42°C, respectively. As shown in Table 1, *P. mirabilis* NA2609 actually grew at 30 and 35°C, although both *P. mirabilis* ATCC29906 and ATCC43071 revealed sufficient growths at every temperature, coincident with our previous report (3).

We investigated the effect of carbon-dioxide on the growth of *P. mirabilis* NA2609 and the reference *P. mirabilis* ATCC29906 and ATCC43071. Experiments were performed by seeding them on blood agars. Growth results were read after overnight-incubation at 35°C in the CO₂-incubator adjusted to CO₂-concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 10, and 20%, respectively. *P. mirabilis* NA2609 grew well at 35°C in an environment with more than 4.0% CO₂, as shown in Table 2, also coinciding with our previous results (3), although the two reference ATCC strains grew well regardless of CO₂-concentrations.

As *P. mirabilis* NA2609 failed to grow not only on BTB but also on MacConkey agars, growth-inhibitory effects of pH-indicator dyes, bromothymol-blue, crystal-violet, and neutral-
red were investigated on the Nutrient agars (Eiken Chemical) incorporating varying concentrations of dyes, also using the above described ATCC strains as the reference growth-control. As shown in Table 3, *P. mirabilis* NA2609 failed to grow on either the agars with more than the 0.004%-concentration of the bromothymol-blue or those containing more than the 0.0002%-concentration of crystal-violet, both the concentrations of which were those incorporated into BTB and MacConkey agar media, respectively. In contrast, when examined on MacConkey agars, neutral-red incorporated at concentration of 0.003% gave no growth-inhibitory effect on *P. mirabilis* NA2609 alike with the two ATCC strains. However, growth was suppressed at the slightly elevated concentrations. These findings clearly demonstrated that the growth failures of NA2609 on the isolation media, BTB and/or MacConkey agars were brought about by the presence of bromothymol-blue and crystal-violet dyes incorporated.

In the subsequent study, stability of *P. mirabilis* NA2609 was assessed to determine the emergence of revertants capable of growing without additional CO₂ and/or those able to grow either on BTB or MacConkey agars. No revertants were detected concerning the capnophilic property. In contrast, a single revertant with ability to grow on either BTB or MacConkey agar emerged at frequencies less than 10⁻⁹, the revertant of which was affirmed to produce ISEcp1-*blaCTX-M-2*-type ESBL. The genetic clonality between the revertant and *P. mirabilis* NA2609 was confirmed by Dienes test (4) and Pulsed-Field-Gel-Electrophoresis analyses (3) (data not shown).

Concerning the capnophilic property, we tried to detect the carbonic anhydrase (CA)-encoding *can* gene and the neighboring region in the *P. mirabilis* NA2609 isolate.

In consequence, the *can* gene was proved to present in ATCC 29906 and the 2 clinical *P. mirabilis* strains (data not shown), although un-detectable in DNAs from *P. mirabilis* NA2609. Moreover, as for the neighboring region of *can* genes from approximately 2,000bp upstream to
2,000bp downstream, the DNAs from ATCC 29906 and the 2 clinical strains were found amplifiable. In contrast, the DNAs from P. mirabilis NA2609 were also un-amplifiable (data not shown).

The proliferation of E. coli was recently reported dependent on the presence of bicarbonate (5). Indeed, CA metalloenzymes are assumed to catalyze the hydration-dehydration of carbon dioxide-bicarbonate (5-6). These enzymes are well known to support various physiological functions, including respiration and CO₂ transport.

The major metabolic pathway for CO₂ production is the Krebs cycle, and the inducible decarboxylases should supply CO₂ when the CO₂ level is lowered (7). Cellular levels of CO₂ and HCO₃⁻ are important for growth (8). CO₂/bicarbonate intracellular levels are low in general, and the spontaneous reactions fail to support the growth in ambient air. Therefore, the can gene is essential for the bacterial growth at low CO₂-concentrations (9).

Sahuquillo-Arce, J. M. et al. (10) recently documented that the two capnophilic E. coli strains could not be detected by PCR the can gene. Likewise, can gene of P. mirabilis NA2609 was also undetectable by PCR, although can gene of ATCC 29906 and the 2 clinical strains were found amplifiable. Notwithstanding the repeated trials, we could not detect the revertants with ability to grow in ambient air. This fact suggested that the capnophilic property of P. mirabilis NA2609 might be due to the fairly large-scale deletion or mutation of the DNA sequences containing the CA gene region.

In the meanwhile, concerning the unique property of exhibiting high sensitivity against the dyes, five drug transporters comprising of ABC-, RND-, MF-, SMR- and MATE-types in E.coli strains have been genetically investigated (11). Among them, the RND-type-transporter was known involved in the release of compounds including dyes. Presumably, the P. mirabilis also holds a drug transporter, alike with the E. coli, and the drug transporter of P. mirabilis NA2609
might have been accompanied by some abnormality. We therefore speculate that something abnormal might have occurred in the drug transporter of the *P. mirabilis* NA2609 isolate, although the additional mechanisms other than the RND-type transporter might also be existed.

It is noteworthy that only one revertant capable of growing on BTB and MacConkey agars emerged at frequencies less than $10^{-9}$. This findings implied that in part the mutated region of the corresponding gene should be rather small as compared with that of the *can* gene.

Isolations of capnophilic *Enterobacteriaceae* from urines have rarely been documented. The low isolation frequency might have been ascribed to the laboratory status that the incubations of routine urine cultures have not always been performed in CO$_2$-incubator. Moreover, we should recognize the possible inability to isolate the causative pathogens due to the growth inhibitory effect of the dyes, crystal violet or bromothymol blue, incorporated into isolation media plates. As the failure to grow on those media is indeed an unusual property, such strains are apt to be missed or overlooked. Although we fortunately isolated the strain NA2609 because the blood agar without any kind of dye incorporated was used in this case of urine material, in cases of fecal samples of suspected pathogens, such as diarrheagenic *E. coli*, we could never have isolated such strains because such blood agars are not included in the usual set of isolation media. The implication of this type of pathogens is an important finding, indicating that clinical microbiologists should pay appropriate attention to the isolation of such bacteria. Our case report is a noteworthy and useful piece of information in the field of clinical microbiology.

**Ethical statement.** The ethical committee waived the need for written consent regarding the research of bacterial isolates; the personal data related to the clinical information were anonymized.
Conflict of interest. None to declare.

REFERENCES:


Table 1.

Effects of temperatures under 5% CO₂-added circumstances on the proliferation after overnight-incubation on sheep blood agars

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th><em>Proteus mirabilis</em> NA2609 (this study)</th>
<th><em>Proteus mirabilis</em> ATCC29906 ¹)</th>
<th><em>Proteus mirabilis</em> ATCC43071 ¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>35°C</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>42°C</td>
<td>±</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

±: subtle growth, +: weak growth, ++: good growth, +++: ample growth

¹): Reference *Proteus mirabilis* ATCC strains for growth control
Table 2

Effect of CO$_2$ concentration on the proliferation of the capnophilic isolate and the reference ATCC strains after overnight-incubation at 35°C on sheep blood agars

<table>
<thead>
<tr>
<th></th>
<th>Ambient air</th>
<th>Growth at following CO$_2$ (%) concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC29906 1)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC43071 1)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1): See footnote of Table 1. 2): pin-point colonies
Table 3  Growth inhibitory behavior in the presence of pH-indicator dyes, bromothymol-blue, crystal violet, and neutral red, at concentrations incorporated in isolation agar media against the isolate and the reference ATCC strains

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>pH-indicator-dyes</th>
<th>Bromothymol-blue</th>
<th>Crystal-violet</th>
<th>Neutral-red</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.004% 1)</td>
<td>0.0001% 2)</td>
<td>0.0002%</td>
<td>0.003% 2)</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA2609 (this study)</td>
<td>+++</td>
<td>No-growth</td>
<td>±</td>
<td>No-growth</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 29906 3)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 43071 3)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

1): concentration (%) incorporated into modified Drigalski-agar  
2): concentration (%) incorporated into MacConkey agar 
3): See footnote of Table 1.