A hemin auxotrophic Enterobacter cloacae clinical isolate with increased resistance to carbapenems and aminoglycosides

Tatsuya Tada, Kohei Uechi, Isamu Nakasone, Zenji Miyazato, Takashi Shinzato, Kayo Shimada, Mitsuhiro Tsuchiya, Teruo Kirikae, and Jiro Fujita

Abstract

Small-colony variants (SCVs) were obtained from an Enterobacter cloacae clinical isolate in Okinawa, Japan. One variant showed auxotrophy for hemin with a deletion of 20 365 nucleotides, dosC-ydiK-mmuP-mmuM-tauA-taub-tauC-tauD-hemB-yaiT-yaiV-ampH-yddQ-sbmA-yaiW-yaiY-yaiZ, including hemB, and was more resistant to aminoglycosides and carbapenems, but more susceptible to aztreonam, than the parent strain.

Small-colony variants (SCVs) of bacteria grow more slowly on routine media and yield smaller colonies than normally growing parent strains [1, 2]. SCVs were first described in 1910 as an aberrant form of Salmonella enterica serovar Typhi [1]. Since then, SCVs have been isolated from Gram-positive bacteria, including Lactobacillus acidophilus, Staphylococcus aureus, Staphylococcus epidermidis and Staphylococcus capitis; and from Gram-negative bacteria, including Brucella melitensis, Burkholderia cepacia, Enterobacter aerogenes, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella serovars, Serratia marcescens, Shigella spp., Stenotrophomonas maltophilia and Vibrio cholera [1, 3–5].

SCVs are often found after prolonged administration of aminoglycosides, antifolate agents and several other classes of antibiotics [2]. Many of these SCVs were found to acquire auxotrophy for growth factors, such as hemin, menadione and thymidine [1].

Enterobacter cloacae NCGM-RYU195 was isolated from a urine sample obtained from an inpatient in a hospital in Okinawa, Japan. The isolate was obtained by active surveillance for carbapenem-resistant Gram-negative bacteria at the hospital. A MacConkey agar supplemented 2 µg ml⁻¹ meropenem was used as the screening medium. Its antimicrobial susceptibility was determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [6]. The minimum inhibitory concentrations (MICs) of antibiotics were determined using Mueller–Hinton (MH) broth with and without 0.5 µg ml⁻¹ hemin and culturing at 37 °C for 24 and 48 h. The growth of NCGM-RYU195 and its SCV, NCGM-RYU195scv, was assessed by culture in Luria–Bertani (LB) broth with and without 0.5 µg ml⁻¹ hemin for 8 h at 37 °C. The whole genomes of these isolates were deposited in GenBank under accession number DRA005419. To determine the size of the plasmid harbouring blaIMP-4, DNA plugs of NCGM-RYU195 and NCGM-RYU195scv, digested with S1 nuclease, were prepared and separated by pulsed-field gel electrophoresis, and Southern hybridization was performed using the probes of blaIMP-4.

The growth of NCGM-RYU195 was similar in the absence and presence of hemin, with doubling times of 20.1 and 21.8 min, respectively. In contrast, the growth of NCGM-RYU195scv was dependent on hemin, with doubling times of 19.7 and 66.9 min, respectively, in the presence and absence of hemin (Fig. 1), indicating that this SCV is a hemin auxotrophic isolate.

The results or the determination of MICs after 24 and 48 h incubation in the presence or absence of hemin are shown in Table 1. Comparisons of the MICs for the SCV and
parent in the presence of hemin for 24 h found that the SCV was more resistant to aminoglycosides (amikacin, arbekacin, gentamicin, kanamycin and tobramycin) and carbapenems (doripenem, imipenem and panipenem) than the parent strain (Table 1). Overall, these results indicate that NCGM-RYU195scv is relatively resistant to these aminoglycosides and carbapenems, in both the presence and absence of hemin.

The strains NCGM-RYU195 and NCGM-RYU195scv were found to possess identical drug-resistance genes; three β-lactamase-encoding genes, including \( \text{bla}_{\text{ACT-24}} \), \( \text{bla}_{\text{IMP-4}} \) and \( \text{bla}_{\text{OXA-2}} \), and three aminoglycoside modification enzyme-encoding genes, including \( \text{aac(6')-Ib} \), \( \text{aadA1} \) and \( \text{aadB} \).

Multilocus sequence typing (MLST) of these isolates was ST78 (E. cloacae MLST database, https://pubmlst.org/ecloacae/), which has been reported in France [7], Israel [7], Japan [8], Latvia [7], Spain [9] and Taiwan [10]. The \( \text{bla}_{\text{IMP-4}} \) gene was present in a class 1 integron, which had a genetic environment pf \( \text{tnpA-tnpR-intI1-bla}_{\text{IMP-4}} \)-\( \text{aac(6')-Ib-aadA1-tnpA-bla}_{\text{OXA-2}} \)-\( \text{qacEdelta1-sulI-orfX} \) (acetyltransferase)-IS6100-tnp. This genomic region encompassing nucleotides (nt) 134 to 5138 (accession no. LC198842) and containing \( \text{tnpA-tnpR-intI1-bla}_{\text{IMP-4}} \) had a nucleotide sequence that was 99.9 % identical to the region from nt 98 392 to nt 103 396 of the

Table 1. Minimum inhibitory concentrations (MICs) of the parent strain NCGM-RYU195 and the small-colony variant NCGM-RYU195scv grown in Mueller–Hinton broth and in the presence or absence of hemin

<table>
<thead>
<tr>
<th>Antibiotic (s)</th>
<th>Hemin (−)</th>
<th>Hemin (+)</th>
<th>Hemin (−)</th>
<th>Hemin (+)</th>
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<tr>
<td>Penicillin G</td>
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<td>−†</td>
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<td>−</td>
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</table>

*The ratio of ampicillin to sulbactam was 2:1.
†This MIC was not determined after 24 h because of the slow growth of the small-colony variant without hemin.
plasmid pIMP-PH114 in *Klebsiella pneumoniae* CRE114, isolated in the Philippines [11]. The region downstream of *blaIMP-4*, from nt 7969 to nt 12 282 (accession no. LC198842), including *blaOXA2*-GacEdelta1-sull-orfX (acetyltransferase)-IS6100, showed 99.8% nucleotide sequence identity with the region from nt 19 717 to nt 24 030 of the plasmid pCAV1335-92 in *K. oxytoca* strain CAV1335, isolated in 2010 in the USA (accession no. CP011614). There was no plasmid harbouring *blaIMP-4*, which suggests that the *blaIMP-4* may be located on the chromosomes (data not shown).

Compared with the parent strain, NCGM-RYU195scv had a deletion of a genomic region containing 20 365 nt, including the *hemB* gene. The deleted region contained *dscC-ydiK-mmuP-mmuM-tauA-tauB-tauC-tauD-hemB-yaiT-yaiV-ampH-yddQ-sbmA-yaiW-yaiY-yaiZ*. Among these genes, *dscC* encodes a protein associated with biofilm formation [12]; *mmuM* encodes a protein associated with methylation of homocysteine [13]; *tauABC* encodes proteins associated with taurine uptake and sulfate release from taurine [14]; *yddQ* [15], *sbmA* [16] and *yaiZ* [17] encode proteins associated with the inner membrane; and *yaiW* encodes a protein associated with the outer membrane [18].

Aminoglycoside resistance in NCGM-RYU195scv with a deletion in *hemB* may have been caused by abnormalities in *hem* genes, which are key components of the electron transfer apparatus. Aminoglycosides are positively charged antibiotics that are transported across cell membranes into the cytoplasm of bacterial cells in response to a high electrochemical gradient [19, 20]. Hemin-dependent SCVs are resistant to aminoglycosides because of their low membrane potential, which prevents the uptake of aminoglycosides and other cationic substrates [21, 22]. Carbapenem resistance in NCGM-RYU195scv with a deletion in *hemB* may have been associated with the deletion of *ampH*, which is associated with *ampC* regulation [23].

In conclusion, this study describes a hemin auxotrophic *Enterobacter cloacae* clinical isolate that was more resistant to carbapenems and aminoglycosides than its parent strain. The genome of the SCV also included a deletion of *hemB*. To the best of our knowledge, this is the first report describing a strain of *Enterobacteriaceae* with a *hemB* deletion associated with resistance to β-lactams. Heme deficiency resulting from *hemB* deletion leads to a broad range of metabolic disturbances and a lack of intracellular energy, such as ATP molecules. Reductions in intracellular levels of ATP can lead to slow growth, which increases resistance to cell wall-active antibiotics [24].

**Ethical statement**
This study was approved by the Ethical Committee, University of the Ryukyus (approval number: 890), and the Biosafety Committee, National Center for Global Health and Medicine (approval number: 28-M-053).

**References**


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