Title

Detection of in-frame mutation by IS30-family insertion sequence in the phospholipid phosphatidylglycerol synthase gene (*pgsA2*) of high-level daptomycin-resistant *Corynebacterium striatum*

Authors

Kazuyoshi Gotoh[1], I Putu Bayu Mayura[1], Yusaku Enomoto[1], Koji Iio[2], Osamu Matsushita[1], Fumio Otsuka[3], Hideharu Hagiya[3]*

[1]Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

[2]Microbiology Division, Clinical Laboratory, Okayama University Hospital, Okayama 700-8558, Japan

[3]Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

*Corresponding author:

Hideharu Hagiya, MD, PhD

19 Department of General Medicine, Okayama University Graduate School of Medicine, Dentistry and
20 Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan.
21 Tel: +81-86-235-7342 Fax: +81-86-235-7345
22 E-mail: hagiya@okayama-u.ac.jp
23
24 **Declarations**
25 **Funding**: None to report.
26 **Conflicts of interest**: None to report.
27 **Availability of data and material**: All data relevant to the study are available on reasonable request
28 to the corresponding author.
29 **Authors' contributions**: Conceptualisation: Hideharu Hagiya; Methodology: Kazuyoshi Gotoh;
30 Formal analysis and investigation: Kazuyoshi Gotoh, I Putu Bayu Mayura, Yusaku Enomoto, and Koji
31 Iio; Writing - original draft preparation: Kazuyoshi Gotoh; Writing - review and editing: Hideharu
32 Hagiya; Supervision: Osamu Matsushita and Fumio Otsuka.
33
34 **Ethics approval**: The manuscript has not been published previously, in any language, in whole or in
35 part, and is not currently under consideration elsewhere. The institutional review board of our hospital
36 approved the study (No. 2001-002). We have read and understood your journal's policies, and we

believe that neither the manuscript nor the study violates any of these.

Keywords: Antimicrobial Resistance, Daptomycin resistance, *Corynebacterium striatum*, Insertion sequence, *pgsA2* gene

Abstract

The emergence of high-level daptomycin (DAP)-resistant (HLDR) *Corynebacterium striatum* has been reported as a result of loss-of-function point mutations or premature stop codon mutations in a responsible gene, *pgsA2.* We herein describe the novel detection of an HLDR *C. striatum* clinical isolate, in which IS30-insertion was corroborated to cause destruction of *pgsA2* gene. We isolated an HLDR *C. striatum* from a critically ill patient with underlying mycosis fungoides who had been treated with DAP for ten days. With a sequence investigation, IS30-insertion was discovered to split *pgsA2* in the HLDR *C. striatum* strain, which may cause disrupted phospholipid phosphatidylglycerols (PG) production. Future studies should survey the prevalence of IS-mediated gene inactivation among HLDR *C. striatum* clinical isolates.

54 **Text**

55 Daptomycin (DAP) is a last-resort antibiotic for the treatment of gram-positive bacterial infections [1].
56 This lipopeptide-class drug works by binding to the phospholipid phosphatidylglycerols (PG) in the
57 lipid bilayer of bacterial cell membranes [2]. One possible daptomycin-resistant mechanism is an
58 impairment of the PG synthesis gene, PG synthase (*pgsA2*), which is a non-essential gene in some
59 gram-positive bacteria [3]. Previously, two research groups reported that clinical isolates of high-level
60 DAP resistant (HLDR) *Corynebacterium striatum* showed DAP-resistance through the loss-of-
61 function point mutations or premature stop codon mutations in *pgsA2* [4,5]. Because *C. striatum* is a
62 gram-positive skin commensal that causes various infections, including bloodstream infections, the
63 emergence of HLDR *C. striatum* isolates in clinical specimens is considered a risk for patients [6,7].
64 Herein, we describe the discovery of an HLDR *C. striatum* clinical isolate harbouring the insertion
65 sequence (IS) 30 family element in *pgsA2*.

66 The patient was a 57-year-old woman who had been diagnosed with mycosis fungoides.
67 Otherwise, her clinical history was unremarkable. A combination treatment of topical corticosteroid
68 and phototherapy was ineffective, and oral bexarotene was additionally introduced. However, the
69 systemic rash continued to deteriorate accompanying high fever, and the patient was referred to our
70 hospital. On admission, her vital signs were stable, but a high fever at ≥38 °C continued. The patient
71 suffered from generalised erosive lesions, erythroderma, and scaling, and was unable to move by

72 herself because of excruciating pain. Bacterial culture of the damaged skin detected methicillin-
73 susceptible *Staphylococcus aureus* and DAP-susceptible *C. striatum* strain. Subsequently, intravenous
74 cefazolin was initiated. Bacterial identification was performed using MALDI Biotyper (Bruker
75 Daltonics, Billerica, MA, United States) and antimicrobial susceptibility testing according to CLSI
76 document M45 (Dry Plate Eiken, Eiken Chemical Co., Ltd, Tokyo, Japan). Four weeks after
77 hospitalisation, methicillin-resistant *S. aureus* (MRSA) was isolated from a follow-up skin culture and
78 the patient was administered with 6 mg/kg per day of DAP under the diagnosis of MRSA skin and soft
79 tissue infection. Two days later, however, the patient was transferred to an intensive care unit to treat
80 the severe infection and the dose of DAP was increased to 8 mg/kg per day. Ten days after initiating
81 DAP treatment, *C. striatum* was again isolated from her skin lesion, which appeared to be resistant to
82 DAP, with a minimum inhibitory concentration (MIC) level of >16 μg/mL (Dry Plate Eiken). We
83 named this isolate as OUH2489 and confirmed its MIC using the Etest method on Mueller-Hinton
84 agar plates supplemented with 5% sheep blood (bioMérieux, Marcy-l'Étoile, France) at 37 °C in a 10%
85 CO_2 atmosphere. Etest results revealed that the strain OUH2489 exhibited an MIC value of DAP >256
86 μg/mL, suggesting an HLDR phenotype. We transferred this isolate to further investigate the
87 underlying molecular mechanisms.
88 We first suspected that the resistance was caused by a loss-of-function mutation in *pgsA2*,
89 according to the previous literature [4,5]. We performed PCR using the originally prepared primer pair

which anneals to the *pgsA2* flanking region of the reference strain: forward, 5′-CTGAGCCGAGAATCTTGCCTTT-3′ and reverse, 5′-AACGCGCCCCTAACCAATGG-3′. The cycling conditions were as follows: isothermal step at 95° C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. The amplicons were analysed using 1.5% agarose gel electrophoresis and visualised with SYBR Gold Nucleic Gel Stain (Thermo Fisher Scientific Inc., Massachusetts, USA). Unexpected DNA band (2.7 kbp) that was larger than the expected size of 918 bp were observed in HLDR isolates compared to the DAP-susceptible strain OUH1796 that possesses wild-type *pgsA2* (**Fig. 1A**). We used the DAP-susceptible *C. striatum* strain originating from another case as control. We sequenced the PCR products using the same primers and the BigDye™ terminators v.1.1 Cycle Sequencing Kit (Applied Biosystems) for reaction and 3730 DNA analyser (Applied Biosystems) for analysis. The sequence data revealed an insertion of 1,713 bp sequence at nucleotide position 185 from the start codon (GTG) in *pgsA2,* which caused an in-frame inactivation and divided the gene into an unexpected open reading frame (ORF) and the gene remnant (Δ*pgsA2*) (**Fig. 1B**). An ORF of 1,623 bp was found within the inserted region. A BLASTn sequence similarity search of this ORF using the nucleotide database showed 97.12% nucleotide similarity with IS30-family transposase of *C. diphtheriae* subsp. *lausannense* CMCNS703 (GenBank accession no. CP038789.1). Moreover, the new insertion sequence, named IS*Cs*2489, was bordered by two terminal inverted repeats (IR) and flanked on both

sides by short direct repeat sequences of 2 bp which is a common feature in IS30 [8]. Goldner *et al.* reported that *pgsA2* gene mutation is enough for PG-depletion in the bacterium [4]. Hence, we suspected that *C. striarum* OUH2489 emerged as a consequence of reduced PG synthase production that was induced by IS-induced *pgsA2* alteration.

In this brief report, we described the novel finding of IS-induced *pgsA2* destruction in a clinical strain of HLDR *C. striatum*. In accordance with previous literature [4,5], we investigated for sequence abnormality of *pgsA2* and found that intragenic IS30-insertion, instead of loss-of-function or premature stop codon mutations is the underlying mechanism. IS potentially carries various antimicrobial-resistant genes, propagating drug resistance through a horizontal gene transfer vector [9]. Based on our molecular analysis, the IS cassette was found to split the *pgsA2* in the HLDR *C. striatum* strain, resulting in disrupted production of PG, a target site of DAP. Our data endorsed one such function of the IS as a transporter of antimicrobial resistance. In the absence of further exploration to elucidate another underlying molecular mechanism, we cannot conclude that the IS30-insertion in *pgsA2* alone was accountable for the emergence of HLDR *C. striatum* strain. However, insufficient expression of *pgsA2* reduces PG synthesis [4], and it would be reasonable to conclude that the IS30-insertion in *pgsA2* was associated with the emergence of HLDR *C. striatum*.

During hospitalisation, both the DAP-susceptible and DAP-resistant (OUH2489) strains were detected from bacterial cultures of the patient's damaged skin. We assume that the DAP-resistant

126 strain OUH2489 emerged or was selected at the damaged skin surface as a consequence of DAP
127 treatment, rather than by an accidental event, considering the preceding administration of DAP and
128 the alteration in *pgsA2*.

129 This report is a cue for the worldwide surveillance of IS30-insertion strain in HLDR *C.*
130 *striatum*. We suggest surveillance of two possible mechanisms, point mutations and IS-mediated gene
131 deletion, for the control of DAP resistance in this bacterium.

132

133

134 **Declarations**

135 **Funding**: None to report.

136 **Conflicts of interest**: None to report.

137 **Availability of data and material**: Accession number was obtained as *C. striatum* OUH2489 *pgsA2*-
138 flanking region sequence (MZ605120). All data relevant to the study are available on reasonable
139 request to the corresponding author.

140 **Authors' contributions**: Conceptualisation: Hideharu Hagiya; Methodology: Kazuyoshi Gotoh;
141 Formal analysis and investigation: Kazuyoshi Gotoh, I Putu Bayu Mayura, Yusaku Enomoto, and Koji
142 Iio; Writing - original draft preparation: Kazuyoshi Gotoh; Writing - review and editing: Hideharu
143 Hagiya; Supervision: Osamu Matsushita and Fumio Otsuka.

Ethics approval: The manuscript has not been published previously, in any language, in whole or in part, and is not currently under consideration elsewhere. The institutional review board of our hospital approved the study (No. 2001-002). We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these.

References

1. Mascio CT, Alder JD, Silverman JA. Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells (2007). Antimicrob Agents Chemother 51:4255-4260. doi: 10.1128/AAC.00824-07.

2. Steenbergen JN, Alder J, Thorne GM, Tally FP (2005). Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J Antimicrob Chemother 55:283-288. doi: 10.1093/jac/dkh546.

3. Hines KM, Waalkes A, Penewit K, Holmes EA, Salipante SJ, Werth BJ, Xu L (2017). Characterization of the Mechanisms of Daptomycin Resistance among Gram-Positive Bacterial Pathogens by Multidimensional Lipidomics. mSphere 2:e00492-17. doi: 10.1128/mSphere.

4. Goldner NK, Bulow C, Cho K, Wallace M, Hsu FF, Patti GJ, Burnham CA, Schlesinger P, Dantas G (2018). Mechanism of High-Level Daptomycin Resistance in *Corynebacterium striatum*. mSphere 3:e00371-18. doi: 10.1128/mSphereDirect.00371-18.

5. Hagiya H, Kimura K, Okuno H, Hamaguchi S, Morii D, Yoshida H, Mitsui T, Nishi I, Tomono K (2019). Bacteremia due to high-level daptomycin-resistant *Corynebacterium striatum*: A case report with genetic investigation. J Infect Chemother 25:906-908. doi: 10.1016/j.jiac.2019.04.009.

6. Silva-Santana G, Silva CMF, Olivella JGB, Silva IF, Fernandes LMO, Sued-Karam BR, Santos CS, Souza C, Mattos-Guaraldi AL (2021). Worldwide survey of Corynebacterium striatum

increasingly associated with human invasive infections, nosocomial outbreak, and antimicrobial multidrug-resistance, 1976-2020. Arch Microbiol 203:1863-1880. doi: 10.1007/s00203-021-02246-1.

7. Wong KY, Chan YC, Wong CY (2010). Corynebacterium striatum as an emerging pathogen. J Hosp Infect 76:371-372. doi: 10.1016/j.jhin.2010.05.018.

8. Mahillon J, Chandler M. Insertion sequences (1998). Microbiol Mol Biol Rev 62:725-774. doi: 10.1128/MMBR.62.3.725-774.1998.

9. Vandecraen J, Chandler M, Aertsen A, Van Houdt R (2017). The impact of insertion sequences on bacterial genome plasticity and adaptability. Crit Rev Microbiol 43:709-730. doi: 10.1080/1040841X.2017.1303661.

Figure legend

Fig. 1. (A) Agarose gel electrophoresis of the *pgsA2* PCR products. M, DNA size marker; second lane, strain OUH1796 as a DAP-susceptible control (isolated from another patient); and third lane, strain OUH2489 indicating the HLDR isolate. (B) Schematic representation of the *C. stratum* OUH2489 *pgsA2* gene in-frame deletion by IS*Cs*2489, as compared to the reference strain KC-Na-01 (GCA_002156805.1). IR-L and IR-R are indicating inverted repeats on the left and right terminal. DR, direct repeats.