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# MIZORIBINE IS EFFECTIVE AGAINST *LECLERCIA ADECARBOXYLATA* INFECTION

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**Keywords:**

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**ABSTRACT:** *Leclercia adecarboxylata* is a gram-negative bacillus. *L. adecarboxylata* has been isolated from various environments, such as food and water, and clinical specimens from patients with various diseases and ailments, such as pyelonephritis and sepsis. Although *L. adecarboxylata* infections are generally treated with β-lactam antibiotics, the emergence of multidrug-resistant strains reported in recent years has presented a major challenge in the clinical treatment of the infections. Thus, I searched for potential candidate compounds for developing new antibiotics that are effective against multidrug-resistant *L. adecarboxylata*. In a docking simulation performed to extract an inhibitor of the molecular chaperone GroEL, mizoribine was identified as a "hit."Notably, when *L. adecarboxylata* was cultured in a mizoribine-containing medium, its growth was inhibited at mizoribine concentrations of ≥0.63 mmol/L. considering these findings, I propose that mizoribine could serve as an effective new therapeutic agent for *L. adecarboxylata* infection. In the future, it will be necessary to investigate the effects of mizoribine on other bacterial species.

**INTRODUCTION:** *Leclercia adecarboxylata*, a gram-negative, facultative anaerobic bacterium, was isolated from drinking water in 1962 and initially named *Escherichia adecarboxylata*, in the order *Enterobacterales*; however, the name was changed to *L. adecarboxylata* in 1986 [1, 2, 3]. Since, then, *L. adecarboxylata* has been detected in diverse natural environments (such as natural surface waters, soils, and plant surfaces), animal sources, and foods [4]. *Leclercia adecarboxylata* infections in humans are rare, but humans can be infected under conditions of reduced immunity and overuse of non-steroidal anti-inflammatory drugs [1, 5].

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Moreover, in recent years, multidrug-resistant strains of *L. adecarboxylata* have emerged, such as strains producing extended-spectrum β-lactamase (ESBL) ormetallo-β-lactamase (MBL) and the spread of such drug-resistant strains increases the risk of refractory *L. adecarboxylata* infection [6, 7]. Therefore, it is critical to search for new antibiotics that can be used to control *L. adecarboxylata* infection completely. Here, I sought to identify novel anti-*L. adecarboxylata* therapeutic drug candidates.

## MATERIALS AND METHODS:

**Reagent:** Mizoribine was purchased from Tokyo Chemical Industry (Lot number: 55MRH-SQ; Tokyo, Japan).

**Strain, Culture Conditions:** *Leclercia adecarboxylata* NBRC102595, the strain type, was purchased from the Biological Resource Center, National Institute of Technology and Evaluation (NBRC) and cultured under aerobic conditions in

modified cyclodextrin agar or liquid medium; the cyclodextrin medium was supplemented with ammonium sulfate (4.00 g/L), D-glucose (18.2 g/L) and sodium aspartate (7.15 g/L) [8]. *Leclercia adecarboxylata* was grown for 3 days at 25°C or 37°C on a modified cyclodextrin agar medium; the bacterial cells were harvested, suspended in modified cyclodextrin broth lacking or containing mizoribine added at 0.63 or 1.25 mmol/L, inoculated at 1× $10^8$colony-forming units (CFU)/mL into 3 mL of modified cyclodextrin broth in 25$cm^2$ flasks (Thermo Fisher Scientific, Waltham, MA, USA) and cultured for 3 days at 25°C or 37°C under static conditions.

**SDS-PAGE, Protein Identification:** Proteins were extracted from cultured *L. adecarboxylata* cells through centrifugation (13,500rpm, 5 min, 4°C) by using Ez Bact Yeast Crusher (Atto, Tokyo, Japan) and then fractionated using SDS-PAGE as described by Laemmli [9]. Electrophoresis was performed at 200 V for 10 min, and gels were stained overnight with Coomassie Brilliant Blue. For protein identification, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine amino acid sequences (Genomine, Kyungbuk, Korea).

**Docking Simulation:** The docking software SeeSAR10 (BioSolvel T, Nordrhein-Westfalen, Germany) was used to extract compounds that could potentially bind to GroEL. Because the three-dimensional structure of GroEL produced by *L. adecarboxylata* was not available, Escherichia coli GroEL's structure is 96.28% identical in amino acid sequence to GroEL from *L. adecarboxylata*, was downloaded from the Protein Data Bank and used for the docking simulation. The ADP binding site of E. coli GroEL was used for the compound binding site. A database (1,000,000 compounds) owned by Namiki Shoji (Tokyo, Japan) was used as the compound database.

**Crystal Violet Assay:** *Leclercia adecarboxylata* NBRC102595 was cultured on modified cyclodextrin agar as described above in the subsection on culture preparation. In the case of cultures prepared using modified cyclodextrin broth, the obtained bacterial suspensions (1 × $10^7$ CFU/mL) were dispensed into 96-well polystyrene plates at 200 µL/well and cultured for 48 h at 25°C or 37°C under aerobic conditions. Next, the cultures were removed, the wells were all washed twice with Milli-Q water and then a crystal violet aqueous solution (0.1% w/v) was added to each well (200 µL/well) and the mixture was allowed to stand at ambient temperature (~25°C) for 30 min. After staining, the crystal violet aqueous solution was removed, the wells were again washed twice with Milli-Q water and then ethanol was added to each well (200 µL/well) and the mixture was allowed to stand at the ambient temperature for 30 min. Lastly, the optical density of the samples at 590 nm was measured on a SpectraMax190 Microplate Reader (Molecular Devices, Tokyo, Japan) to detect the amount of biofilm in the 96-well plates.

**Statistical Analysis:** Results are presented as means ± standard deviation. Data were analyzed using the statistical program Sigma Plot 14 (Systat Software, Berkshire, UK) and $P<0.05$, was considered statistically significant. Differences between groups were examined for significance using Student's and Dunnett's tests.

**RESULTS AND DISCUSSION:** To identify potential target proteins for anti-*L. adecarboxylata* drugs, the protein profiles of *L. adecarboxylata* cultured at 25°C and 37°C were compared. A protein of 60 kDa was expressed at a higher level after culturing *L. adecarboxylata* at 37°C than after culturing at 25°C **Fig. 1**. LC-MS/MS analysis of the 60 kDa band revealed that this protein was GroEL.

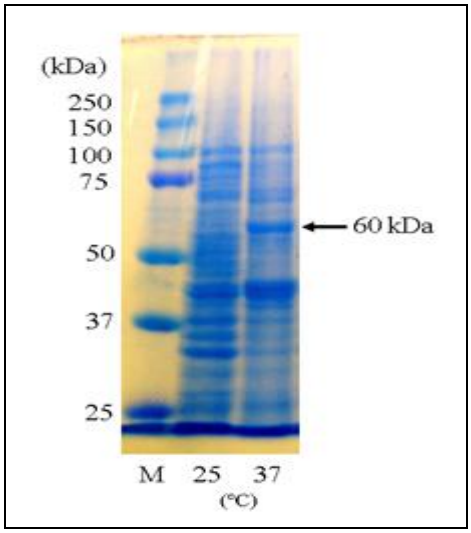


**FIG. 1: SDS-PAGE ANALYSIS OF *LECLERCIA ADECARBOXYLATA* PRROTEIN**

**Profiles Note:** *Leclercia adecarboxylata* NBRC102595 was cultured under aerobic conditions in modified cyclodextrin broth and grown for 3 days at 25°C or 37°C; subsequently, proteins extracted from the bacterial cultures were separated using SDS-PAGE and stained with Coomassie Brilliant Blue. These results imply that GroEL expression is also up-regulated when *L. adecarboxylata* is transmitted from the environment into the human body. GroEL is a molecular chaperone that functions in protein folding [10,] and thus GroEL production could be increased because the temperature rise might disrupt the three-dimensional structure of proteins and enhance the demand for chaperone-dependent folding. Furthermore, because GroEL is also involved in bacterial aggregation, [11] detection of the temperature rise could enhance GroEL production and promote the aggregation of *L. adecarboxylata*. Therefore, biofilm formation at 37°C and 25°C was compared. A significantly higher amount of biofilm was formed in *L. adecarboxylata* cultures at 37°C than in cultures at 25°C **Fig. 2.** Biofilm formation occurs when the bacteria attaches, and aggregates at the site of infection [12]. These findings indicate that GroEL significantly contributes to *L. adecarboxylata* biofilm formation.

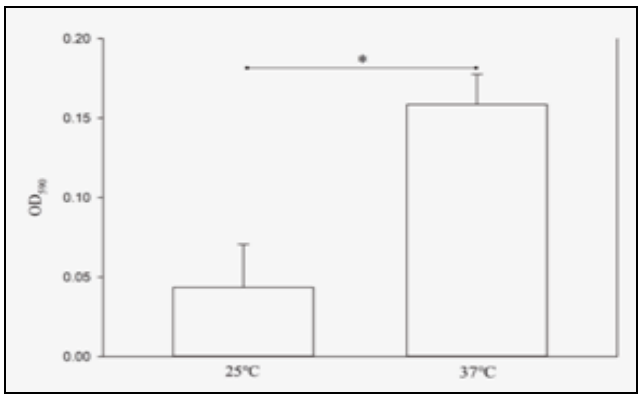


**FIG. 2: TEMPERATURE-DEPENDENT DIFFERECES**

**Amounts of *leclercia adecarboxylata* Biofilm Formation Note:** *Leclercia adecarboxylata* NBRC102595 was cultured in modified cyclodextrin broth in 96-well plates and after incubation at 25°C or 37°C for 48 h, biofilm formation was quantified based on the optical density measured at 590 nm. Data are presented as means ± standard deviation (n = 5); **P*< 0.05 (Student's *t* test): significant difference in biofilm formation at the two temperatures.

Additionally, this study revealed that mizoribine, which was identified as a GroEL-targeting drug candidate through *in-silico* screening, shows anti-*L. adecarboxylata* activity. To search for new anti-*L. adecarboxylata* drugs, GroEL was used as the target protein in docking simulations for identifying binding molecules.

In simulations performed using the three-dimensional structure of *E. coli* GroEL, which shares >96% sequence identity with *L. adecarboxylata* GroEL, mizoribine was extracted as a potential GroEL-binding compound **Fig. 3**. Notably, the growth of *L. adecarboxylata* was significantly suppressed when mizoribine was added to cultures at concentrations of ≥0.63 mmol/L **Fig. 4**.

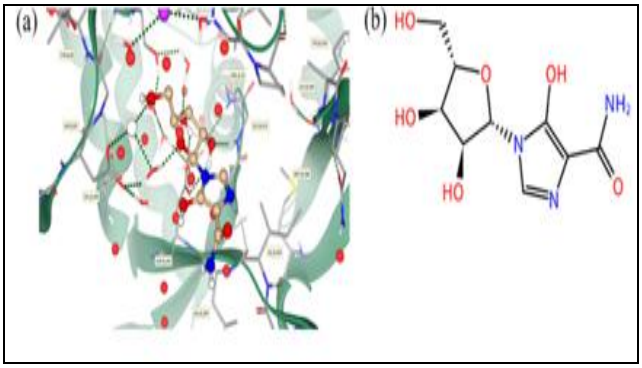


**FIG 3: *IN-SILICO* SCREENING OF ANTI-GROEL AGENT.** Note: Docking of mizoribine to *E. coli* GroEL in the predicted binding site by using docking software SeeSAR 10 (a). Structure of mizoribine (b).

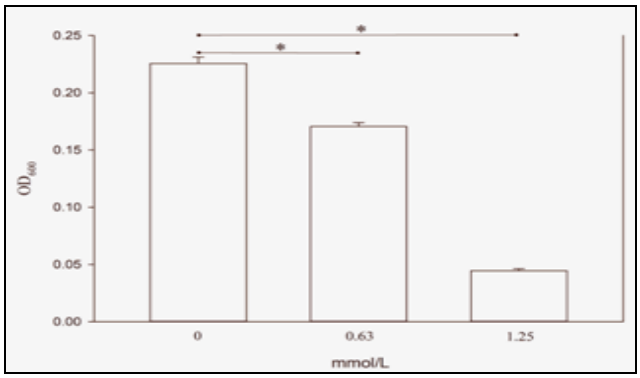


**FIG 4: ANTI-LECLERCIA ADECARBOXYLATA EFFECT OF MIZORIBINE**. Note: *Leclercia adecarboxylata* NBRC102595 was cultured in 96-well plates for 24 h at 37°C in modified cyclodextrin broth lacking or containing mizoribine at 0.63 and 1.25 mmol/L; subsequently, bacterial proliferation in the cultures was quantified based on the optical density measured at 600 nm. Data are presented as means ± standard deviation (n = 3); *P< 0.05 (Dunnett's test): significant difference in bacterial growth between mizoribine-supplemented and non-mizoribine-supplemented media.

Because mizoribine does not harbor a β-lactam ring, the drug is likely effective against ESBL, MBL, and carbapenemase-producing bacterial strains. Future studies should investigate the effects of mizoribine on other bacterial species and carefully evaluate the drug's effective dosage.

**CONCLUSION:** In conclusion, GroELis considered to play a critical role in the survival of *L. adecarboxylata* under high-temperature conditions similar to those in the human body, as well as in *L. adecarboxylata* accumulation and colonization at infection sites. Notably, this study revealed that mizoribine, identified as a potential GroEL-targeting drug, exhibits anti-*L. adecarboxylata* activity.

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