

Short communication:

The diversity of ureolytic bacteria isolated from limestone in East Java, Indonesia based on amino acid sequences encoded by *ureC*

ENNY ZULAIKA^{1,*}, M. A. PRIO UTOMO¹, N. HIDAYATUL ALAMI¹, N. DWIANITA KUSWY TASARI¹,
MAYA SHOVI TRI¹, RIDHO BAYUAJI², ENDRY N. PRASETYO¹

¹Department of Biology, Faculty of Sciences, Institut Teknologi Sepuluh Nopember. Jl. Arif Rahman Hakim, Sukolilo, Surabaya, East Java, Indonesia. Tel.: +62-31-5947319, Fax.: +62-31-5947319, *email: ennyzulaika123@gmail.com

²Department of Civil Engineering Infrastructure, Faculty of Vocations, Institut Teknologi Sepuluh Nopember. Keputih, Sukolilo, Surabaya 60111, East Java, Indonesia.

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Abstract. Zulaika E, Utomo MAP, Alami NH, Kuswytasari ND, Shovitri M, Bayuaji R, Prasetyo EN. 2019. Short communication: The diversity of ureolytic bacteria isolated from limestone in East Java, Indonesia based on amino acid sequences encoded by *ureC*. *Biodiversitas* 20: 2316-2320. Ureolytic bacteria isolated from limestone are capable to produce urease enzyme which can break down urea into carbonate (CO₃²⁻), has been utilized for various building material bioremediation and restoration. In this present study, we figured out the diversity and genetic relationship of α sub-unit *ureC* gene among six ureolytic bacteria (JA1, JB2, JB3, JA4, AK4, and SU1) which were isolated from limestone area in East Java province. PCR was conducted to detect the gene which encoded active site of urease, *ureC*. Followed by sequences translation using BLAST-X (Basic Local Alignment Search Tool) based on the name and function of formed proteins and then aligned to the conserved domain database. Furthermore, the functions and characters of formed proteins were described. Based on PCR results, all isolates showed 340 bp DNA band which indicate the presence of *ureC* gene. The results of BLAST-X, JB2 isolates showed 100% similarity with the α sub-unit *ureC* gene from *Lysinibacillus sphaericus* B1-CDA (WP_054549252.1). Whereas, JA1 isolates showed 88% similarity (lowest) with the α sub-unit *ureC* gene from *Bacillus cihuensis* FJAT-14515 (WP_028391929.1). The present study reveals that *ureC* phylogeny can be used in order to investigate ureolytic bacteria species which isolated from calcareous area in East Java province.

Keywords: Bacteria, diversity, *ureC* gene, ureolytic

INTRODUCTION

Calcium carbonate (CaCO₃) is one of the most abundant materials in nature and main material of cement (Ozen et al. 2013; Jeong et al. 2017). It is massively used for cement-based materials like concrete and mortar because of its remarkable durability (Chunxiang et al. 2009). CaCO₃ formation through a biomineralization process generating heterogeneous accumulation which consists of organic and inorganic compounds that reflect the environment where it finds (Skinner and Ehrlich 2014). Bacteria is one of the microorganisms that can induce the biomineralization process of CaCO₃ in nature and is widely found in calcareous habitats (Jimenez-Lopez et al. 2008). CaCO₃ precipitation which performed by heterotroph bacteria occurs through nitrogen and sulfur cycle. Induction of CaCO₃ biomineralization related to sulfur cycle by means of sulfate-reducing bacteria occurs in anoxic conditions (Castanier et al. 1999). While, CaCO₃ precipitation related to the nitrogen cycle might occur through the oxidative amino acid deamination process and urea hydrolysis in aerobic conditions. On the other hand, nitrate reduction occurs in anaerobic or microaerophilic conditions (Castanier et al. 1999). However, the biomineralization process of CaCO₃ mainly appears

through urea hydrolysis process by urease producing bacteria (Hammes et al. 2003; De Jong et al. 2010).

Urease is a metalloenzyme that requires nickel ion to hydrolyze urea into ammonia and carbamate, then it will be spontaneously hydrolyzed to form carbonic acid and the other ammonia molecules (Andrews et al. 1984). Urease from *Sporosarcina pasteurii* which has been successfully purified and crystallized is heteropolymer protein ($\alpha\beta\gamma$)₃ that consists of three polypeptide chains with a molecular weight: α = 61.4 kDa, β = 14.0 kDa, and γ = 11.1 kDa (Benini et al. 1999). The active site contains two nickel ions banded with three to four amino acids (Benini et al. 1999). Urease is encoded by three structural genes: *ureA* genes encode γ subunit, *ureB* encode β subunits, and *ureC* encode α subunits (Joners and Mobley 1989). Furthermore, there are several additional proteins encoded by accessory genes namely *ureD*, *ureE*, *ureF*, *ureG*, and *ureH* which contribute in nickel ions transport (Ni²⁺) towards the center of active site of urease (Mobley et al. 1995). *ureC* gene is the largest gene encoding urease functional subunit and contains many conserved areas. It possesses many priming sites and it can be used as gene marker to study bacterial diversity (Gresham et al. 2007).

Six bacterial isolates JA1, JB2, JB3, JA4, AK4, and SU1 are successfully isolated from calcareous area in East

Java province, Indonesia (Utomo 2018). Those bacterial isolate showing precipitation zone around their colony when cultivated on CaCO₃ precipitation selective media as described by Shiping et al. (2015). The objective of present study is to detect their ureolytic ability in Christensen Agar and determine the diversity and genetic relationship of *ureC* gene from CaCO₃ forming isolates which isolated from calcareous area in East Java, Indonesia.

MATERIALS AND METHODS

Materials

Urease qualitative test was performed on isolates JA1, JB2, JB3, JA4, AK4, and SU1 which originated from Jaddih, Bangkalan and Suci, Gresik lime hills area in the Province of East Java, Indonesia; while AK4 isolated from new stalagmites of Akbar cave, Tuban, East Java, Indonesia (Utomo 2018).

Procedure

Qualitative urease test

The morphological characteristics of CaCO₃ precipitating bacterial colony from East Java limestone area following Harley and Prescott (2002) protocols are described in Table 1. Urease activity detection was performed by employing Christensen agar which contains urea 20.0; NaCl 5.0; peptone 1.0; glucose 1.0; KH₂PO₄ 2.0; phenol red 0.012; and agar 15.0 (g/L) according to the protocol of Atlas (2010). All ingredients were sterilized by autoclaving, except 20% urea solution which sterilized through membrane filtration. Bacterial isolates were incubated at 37° C and observed regularly for the production of color. Bacteria that can hydrolyze urea produce cherish pink color after 24 hours incubation.

Genomic DNA extraction and PCR

DNA template for amplification process from CaCO₃ forming isolates was obtained by extracting total genomic DNA in accordance to manufacturers protocol (Qiagen®, Germany) with some minor modifications. Amplification of *ureC* gene was performed in 50 µL reaction mixture containing 25 µL of OneTaq® Quick-Load 2X Master Mix with Standard Buffer (New England BioLabs®, US), 1 µL of forwarding ureC-F primer (5'-TGGGCCTTAAAATHC AYGARGAYTGGG-3'), 1 µL of ureC-R reverse primer (5'-GGTGGTGGCACACCATNANCATRTC-3 ') (Reeds 2001), 2 µL of template DNA, and nuclease-free water until the total volume of reaction mixture reaches 50 µL. PCR was then performed on the thermocycler under condition: initial denaturation at 94°C (30 seconds), followed by 35 cycles consisting of denaturation (94 °C; 30 seconds), annealing (50°C; 50 seconds), extension (68°C; 1.5 minutes), and final extension (68°C; 10 minutes). Electrophoresis was carried out in ThermoFischer® 1.5% agarose at voltage 100V/cm for 30 minutes (Caccamo et al. 2001). The 340 bp *ureC* gene DNA band that appeared was then purified using the AccuPrep® PCR Purification Kit (Bioneer®, South Korea).

Table 1. The morphology of CaCO₃ precipitating bacteria

Isolates	Colony morphology		
	Form	Elevation	Margin
JA 1	Irregular	Flat	Undulate
JB 2	Irregular	Flat	Erose
JB 3	Circular	Convex	Entire
JA 4	Circular	Convex	Entire
AK 4	Irregular	Flat	Erose
SU 1	Irregular	Raised	Erose

Sequencing and ureC gene sequence analysis

The pure *ureC* gene fragment was sequenced through ABI 3730XL DNA analyzer (Bioneer®, South Korea). Amplicons sequence were then translated into amino acid sequence by using BLASTX. The sequence of deduced amino acid is recognized based on the name and function of the formed protein which founded by alignment process on CCD (Conserved Domain Database) at NCBI. The formed proteins were then described by its functions and characters.

Construction of ureC phylogenetics tree

Deduced amino acid sequence were used for constructing *ureC* phylogenetics tree. The neighboring relative of each sequence was obtained from GenBank using BLASTP tool (www.blast.ncbi.nlm.nih.gov/Blast). The relative sequence was then aligned using ClustalW program. The phylogenetic tree was constructed using *Neighbor-Joining* method by MEGA7 program. Boostrap with 1000 replication was applied to evaluate the formed phylogenetics tree branches.

RESULTS AND DISCUSSION

Urease screening

The six CaCO₃ forming bacteria isolates were able to hydrolyze urea. Figure 1 shows the color of Christensen urea agar media turned into cherish pink after 24 hours of incubation at 37°C.

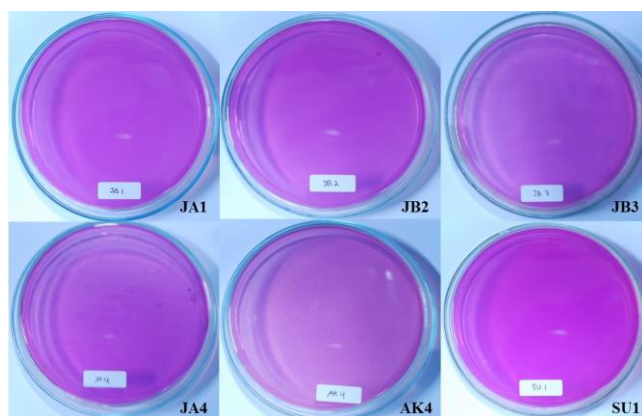


Figure 1. All tested bacterial isolates (JA1, JB2, JB3, JA4, AK4, and SU1) are capable to turn the medium color into cherish pink

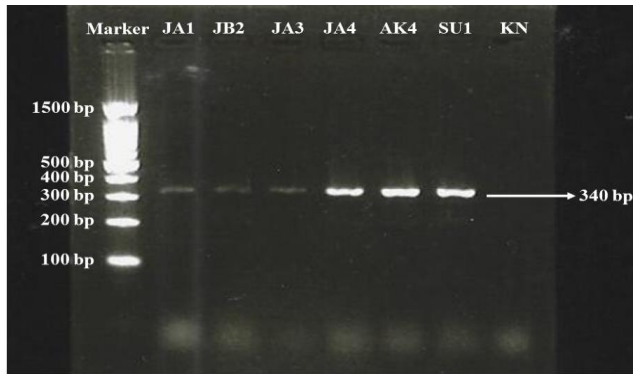


Figure 2. Electrophoresis result of *ureC* in 1.5% agarose gel. All of the tested isolates showed 340 bp band. Promega® BenchTop 100 bp was used as marker. * KN: Negative control

Genomic DNA extraction and PCR result

Electrophoresis results of *ureC* detection showed that all isolates possess the *ureC* in their genomic DNA. Figure 2 shows the molecular weight of the *ureC* gene is 340 bp (Reeds et al. 2001).

Sequencing and *ureC* sequence analysis

All sequences which evaluated with BLASTX are sequences encoding α sub-unit of urease protein. The amount of predicted deduced amino acid through BLASTX ranges from 90-107 amino acids. Meanwhile, the similarity analysis of amino acid sequence compared with amino acid sequences which deposited in NCBI database showed similarity value range from 88-100%. The urease subunit α sequence from JB2 isolate possesses 100% similarity with the *ureC* α subunit gene from *Lysinibacillus sphaericus* B1-CDA (WP_054549252.1), whereas the amino acid sequence urease α subunit in JA1 isolates possess similarity of 88% (lowest) with α subunit *ureC* gene from *Bacillus*

cihuensis FJAT-14515 (WP_028391929.1). The complete results are shown in Table 2.

ureC gene phylogenetic tree

Phylogenetic tree of the *ureC* in Figure 3 shows the bootstrap value varies between 36% -100%. Phylogenetic tree branch considered to be consistent if it has bootstrap value above 70% (Hedges 1992). There is JB3 and JA4 sequence which possess bootstrap value below 70% with their relative sequence. All of our tested isolates belong to Firmicutes phylum and divided into two different families are Bacillales (genus *Bacillus*, *Virgibacillus*, and *Lysinibacillus*) and Planococcaceae (genus *Sporosarcina*). In the *ureC* phylogenetic showed JA1 isolates had the closest relation with *B. cihuensis*, JB2 isolates with *L. sphaericus*, JB3 isolates with *V. necropolis*, JA4 isolates with *Sporosarcina* sp., AK4 isolates with *Bacillus* sp., and SU1 isolates with *Bacillus lentus*.

Discussion

The formation of CaCO_3 can be induced through ureolytic bacterial activity, this material can be used as ingredient in paper, polymers, and anticorrosive paints industry (Rohaya et al. 2010; Hosada et al. 2003; Olad and Rashidzadeh 2008). Urea is a nitrogen source that can easily be hydrolyzed to form ammonia and is then assimilated into bacterial cell proteins formation (Wagner et al. 2010). Bacterial isolates namely JA1, JB2, JB3, JA4, AK4, and SU1 are ureolytic bacteria group, since they can turn the color of Christensen's medium into cherish pink. Hydrolyzed urea leads to ammonium ion accumulation, so that the medium alkalinity got increased. Phenol red is pH indicator that contained in the medium, when the medium alkalinity was increased, it will turn from yellow into cherish pink (Prescott 2002). Bacteria that capable to hydrolyze urea are easily found in various types of environment (Fujita et al. 2000).

Table 2. BLASTX result of *ureC* from NCBI

Isolate	Protein name	The most similar sequence ureC	Identity	Similarity
JA1	Urease subunit alpha	<i>Bacillus cihuensis</i> FJAT-14515 (WP_028391929.1)	84/95	88%
JB2	Urease subunit alpha	<i>Lysinibacillus sphaericus</i> B1-CDA (WP_054549252.1)	93/93	100%
JB3	Urease subunit alpha	<i>Virgibacillus necropolis</i> LMG 19488 (WP_089533948.1)	84/92	91%
JA4	Urease subunit alpha	<i>Sporosarcina</i> sp. HY08 (WP_067407747.1)	89/90	99%
AK4	Urease subunit alpha	<i>Bacillus</i> sp. FJAT-29937 (WP_066290964.1)	101/107	94%
SU1	Urease subunit alpha	<i>Bacillus lentus</i> NBRC16444 (WP_066141981.1)	85/93	92%

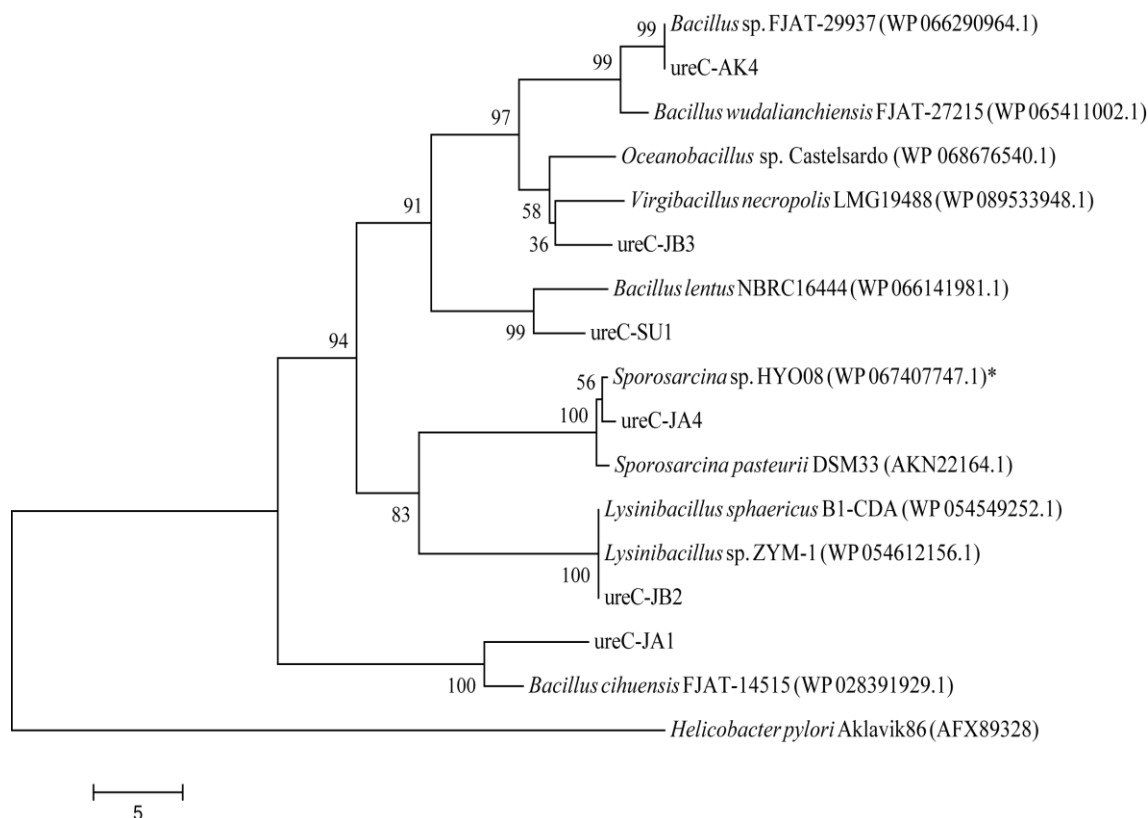


Figure 3. Phylogenetics tree from amino sequence which encoded by *ureC* gene from tested isolates and their relatives. Phylogenetic tree construction was performed by 1000 replication of bootstrap

All isolates showed positive results in the detection of *ureC* gene with 340kb of DNA after electrophoresis on 1.5% agarose gel. The 340 kb DNA band showed the number of nucleotides which flanked by the *ureC*-F and *ureC*-R primers. This primer was made using CODEHOP program through aligning of *ureC* sequences from various bacteria (Reeds et al. 2001). The *ureC* gene was chosen as urease coding gene marker, since *ureC* gene encodes the α subunit which is the active site of urease and it has function in binding of nickel, substrate, and catalytic metals (Mobley et al. 1995). The BLASTX program suggest the possible amino acids which can be formed from the sequence of *ureC* gene from those isolates; determine the name of the protein which formed from the amino acid alignment test in Conserve Domain Database; and determine the similarity of tested amino acid sequences with amino acids that deposited in protein databases (NCBI). Analysis of the *ureC* was carried out by translating into amino acid sequences because of the similarity of *ureC* at the nucleotide level is lower than at protein level, due to the degeneration of its genetic code (Reeds 2001). BLASTP results showed that the amino acid sequence formed has similar sequences with ureolytic bacteria that have been known to induce the formation of CaCO_3 such as *Bacillus*, *Lysinibacillus*, *Virgibacillus*, and *Sporosarcina* (Sarada et al. 2009; Krishnapriya et al. 2015; Silva-Castro et al. 2015; Dhami et al. 2013).

Even the results of *ureC* and 16S rRNA phylogeny analysis showed genus congruency, but there was still difference in result on JB3 isolates which showing the closest similarity with genera *Lysinibacillus* by *ureC* analysis (Utomo 2018). The different results from gene barcode (16S rRNA) and *ureC* analysis might be caused by horizontal gene transfer (HGT) phenomenon among ureolytic bacteria, instead of genetic transmission into the next generation bacteria cell (Keeling et al. 2008). Since this gene undergoes horizontal gene transfer, it might not reflect the actual composition of bacteria community. However, Su et al. (2013) state that this gene may be used better for understanding ureolytic bacteria strain wherein this ability is not possessed by the 16S rRNA gene.

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