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# SEQUENCING RELATIONSHIPS BETWEEN OF NINE METALLO-B-LACTAMASE (MBL)-PRODUCING *CHRYSEOBACTERIUM INDOLOGENES* STRAINS IN KOREA

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## ABSTRACT

The genus *Chryseobacterium* (previously *Flavobacterium*) can be mostly found in soil and water. Some species of this genus are found in some foodstuffs. We applied PCR to the detection of the metallo-beta-lactamase gene, *bla*IND, in clinically isolated 31 *Chryseobacterium indologenes* strains. This study used our previous data (16S rDNA of metallo-β-lactamase of *C. indologenes*) for further analysis of studies. Aligned nucleotides of 16S rDNA of metallo-β-lactamase were varied within *C. indologenes* varying from 746 bp in *bla*IND 10 to 877bp in *bla*IND 11. The probability of changing from C to T was 21.7 percent, higher than other substitution probabilities. Number of segregating sites of *C. indologenes* was 349 and nucleotide diversity ($\pi$) was 0.175. Under the neutral mutation hypothesis, the probability that Tajima test statistic (D) is positive (1.065) is large than 0.5. In the hierarchy of strains, base arrangement showed polymorphism. Significant differences were expressed in synonymous/replacement ratio of them at the 0.05% level. There was heterogeneity in the sequencing type of IND enzymes. The heterogeneity of these sequences for IND enzymes may cause differences in function. The molecular identification of *C. indologenes* together with sequence heterogeneity of other metallo-enzymes may help to design novel metallo-β-lactamase inhibitors of clinical relevance.

**KEYWORDS:** *Chryseobacterium indologenes*, Metallo-β-lactamase, Nucleotide diversity.

## INTRODUCTION

*Chryseobacterium* spp. are Gram-negative, aerobic, non-fermentative, oxidase-positive and catalase-positive non-motile bacilli that produce yellow to orange pigment. *Chryseobacterium indologenes* (previously classified as *Flavobacterium indologenes*) is also a Gram-negative rod organism and a group of nonmotile.[1] *C. indologenes* could be recovered from water systems and humid surfaces such as moisture hospital environments. *C. indologenes* has been considered opportunistic infection to the susceptible patients such as the infant, the elder, the immuno-suppressed patient and the long-term inpatient as an uncommon pathogen.[2] In approximately half of *C. indologenes* infection cases according to a clinical case report, the organism is present on an indwelling device, and severe illness such as immunocompromised problems is the result in half of cases.[3]

Metallo-enzymes are enzyme proteins containing metal ion cofactors, which have been found in several microbial species associated with infections in humans.[4,5,6] The enzymes are transfer of class B β-lactamase-encoding genes to clinically obnoxious species.[4,5] MBLs have extremely diverse structures and are carried by various organisms including human pathogens. The β-lactamases could hydrolyze carbapenem preferentially and showed broad spectrum of activity. They were used together with their resistance to the potent mechanism-based inactivators of active-site serine β-lactamases.[6]

*C. indologenes* typically exhibits resistance to multiple antibiotics.[7,8] The species is naturally resistant to aminoglycosides and possesses chromosomal metallo-beta-lactamases.[9] Antibiotics with variable activity include the fluoroquinolones, trimethoprim-sulfamethoxazole, and rifampin. In the last decade, it has been phenotypically and genotypically differentiated from other members of this group.[10,11]

In this work, we report the genetic variation of a *C. indologenes* strain from Korea that produces a new IND-type variant by Yum.[12] The molecular identification of *C. indologenes* together with those of other metallo-enzymes may help to design novel metallo-β-lactamase inhibitors of clinical relevance.

## MATERIALS AND METHODS

This study used our previous data (16S rDNA of metallo-

β-lactamase of *C. indologenes*) for further analysis of studies.[12] It was conducted on 31 separate *C. indologens* from the nation's tertiary university hospitals and used a base sequence analysis of 16S rDNAs.

For gene detection that encodes the IND MBL from the chromosome, PCR amplification was performed using the initiator described in Yum.[12] Briefly, the final PCR cocktail of 100 μℓ contained 1 μℓ DNA, 20 pmol of each primer, and preMix with polymerase (Bioneer, Korea). The amplifying reactions were run for 35 cycles of denaturing for 30s at 94℃, primer annealing for 30s at 50℃, extension for 60s at 72℃, and elongation for 7 min at 72℃. Amplified products were resolved through a 1.5% agarose gels. PCR-amplified template was isolated and purified with the QIAquick Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA. The purified target band was cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystems Inc., Foster City, CA). Sequences were aligned and edited using the Sequencer software program (Gene Codes Corporation, Ann Arbor, MI, USA).

Substitution patterns and rates of *C. indologenes* were estimated with the Tamura-Nei Model.[13] Disparity Index analysis was conducted with nucleotide sequences.[14] Codon positions included were 1st+2nd+3rd+Noncoding for Tajima's Neutrality Test.[15] The best substitution pattern was considered on Models with the lowest BIC scores (Bayesian Information Criterion). AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) were considered for each model. The degree of non-uniformity of evolutionary rates among sites estimated by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). For the estimates of gamma shape parameter and/or the estimated fraction of invariant sites were followed. Assumed or estimated values of transition/transversion bias (*R*) are also used for each model.

Codon-based tests of neutrality for analysis between 16S rDNA of metallo-β-lactamase of *C. indologenes* sequences were conducted using the Nei-Gojobori method. A maximum parsimony tree (MP) for the phylogenetic analysis was inferred using heuristic search, branch-swapping options and tree bisection-reconnection using MEGA X.[16] Bootstrap analysis for confidence values on individual branches were determined with 100 repeated sampling of the data.

**RESULTS**

Sequences of 16S-rDNA region for thirty-one specimens of *C. indologenes* in Korea were successful in all of the strains. Aligned nucleotides of 16S rDNA were varied within *C. indologenes* varying from 746bp in *bla*IND 10 to 877p in *bla*IND 11 (Table 2). The base composition did not show a significant difference among total taxa. The mean nucleotide frequencies for thirty-one of *C. indologenes* are A = 35.4%, C = 17.8%, G = 19.5%, and T = 27.4%. Total alignment length of *C. indologenes* is 886 positions, of which 286 are parsimony-informative, 349 variables, 63 singletons, and 553 constant characters.

Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (Table 3). Each entry is the probability of substitution from one base (row) to another base (column). The probability of changing from C to T was 21.7 percent, higher than other substitution probabilities.

Number of segregating sites of *C. indologenes* was 349 and nucleotide diversity (π) was 0.175. Under the neutral mutation hypothesis, the probability that D is positive (1.065) is large than 0.5 (Table 4).

Even if statistical significance test and in the analysis of contingency tables were analyzed by Fisher's exact test. Although it is employed when sample sizes are small in practice, it is valid for all sample sizes. There is a significant difference in synonymous/replacement ratio between polymorphisms and fixed differences (Table 5).

Disparity Index per site is shown for all sequence pairs (Table 6). Twenty-one values of the them are greater than zero. Values greater than 0 indicate the larger differences in base composition biases than expected based on evolutionary divergence between sequences and by chance alone. The positive value of D was not observed in the case of deletion/insertion.

A pairwise distance (PD) based on the proportion of shared sequences was used to evaluate relatedness among nine strains (Table 6). The estimate of PD ranged from 0.003 between *bla*IND 11 and *bla*IND 12 to 0.796 between *bla*IND 7 and *bla*IND 13.

The evolutionary history based on sequences of *C. indologenes* was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-3109.62) is shown. 16S rDNA of metallo-β-lactamase of *C. indologenes* used with success to reconstruct phylogenetic relationships. Initial tree(s) for the heuristic search. Neighbor-Join and BioNJ algorithms was used initial tree(s) for the heuristic search. Maximum Composite Likelihood (MCL) was also used to estimate a matrix of pairwise distances and then selecting the topology with superior log likelihood value. All 16S rDNA of metallo-β-lactamase of *C. indologenes* generated in Korea exhibited well solved topology with high bootstrap support irrespective of the methods (parsimony) and the setting used (Fig. 1). Nine strains formed two or three distinct clades with moderate bootstrap support (for convenience, only bootstrap values under parsimony criterion are presented here).

**Table 1: Strains numbers and *bla*IND of *C. indologenes* from GenBank.**

| Strains No. (specimen) | *bla*IND | No. of GenBank |
|---|---|---|
| DEUM04-066(sputum), DEUM05-054(blood), DEUM06-1048(blood), DEUM08-1666(sputum), DEUM08-1667(sputum), DEUM09-0544(sputum), DEUM09-0587(bronch), DEUM09-0659(sputum), DEUM09-0687(sputum), DEUM09-0753(sputum), | 2c | GU186042 |
| YMC94/01/3047(sputum), YMC94/12/3839(sputum) | 7 | GU186043 |
| YMC94/04/3376(sputum) | 8 | GU186044 |
| YMC96/09/3073(sputum), YMC96/09/3300(sputum) | 9 | GU186045.1 |
| DEUM05-0117(blood) | 10 | GU206353 |
| DEUM08-0001(blood) | 11 | HM2455379 |
| DEUM09-1534(sputum) | 12 | HM2455380 |
| DEUM08-0899(cerebrospinal fluid) | 13 | HM2455381 |
| EUM09-0922(sputum) | 14 | KM367709 |

**Table 2: Base frequencies and total length across nine strains of C. indologenes in Korea using 16S-rDNA.**

| Strains No./ No. of GenBank | Base (%) | | | | Total (bp) |
|---|---|---|---|---|---|
| | T | C | A | G | |
| *bla*IND/ GU186042 | 26.3 | 18.1 | 35.9 | 19.7 | 878 |
| *bla*IND/ GU186043 | 27.0 | 18.9 | 34.0 | 20.1 | 851 |
| *bla*IND/ GU186044 | 29.4 | 17.2 | 35.0 | 18.4 | 849 |
| *bla*IND/ GU186045.1 | 28.8 | 15.8 | 36.8 | 18.6 | 805 |
| *bla*IND/ GU206353 | 29.0 | 16.6 | 35.1 | 19.3 | 746 |
| *bla*IND/ HM2455379 | 26.3 | 18.2 | 35.9 | 19.5 | 877 |
| *bla*IND/ HM2455380 | 26.6 | 18.1 | 35.6 | 19.7 | 823 |
| *bla*IND/ HM2455381 | 26.2 | 18.1 | 35.9 | 19.8 | 878 |
| *bla*IND/ KM367709 | 26.8 | 19.0 | 34.0 | 20.1 | 820 |
| Mean | 27.4 | 17.8 | 35.4 | 19.5 | 836 |

**Table 3: Maximum likelihood estimate of substitution matrix.**

| Base | A | T/U | C | G |
|---|---|---|---|---|
| A | - | 6.29 | 4.10 | **6.48** |
| T/U | 8.13 | - | **14.10** | 4.48 |
| C | 8.13 | **21.65** | - | 4.48 |
| G | **11.77** | 6.29 | 4.10 | - |

**Table 4: Results from Tajima's neutrality test for 16S-rDNA sequences of C. indologenes.**

| M | S | ps | $\Theta$ | $\prod$ | D |
|---|---|---|---|---|---|
| 9 | 349 | 0.393 | 0.145 | 0.175 | 1.065 |

M = number of sites, S = Number of segregating sites, ps = S/M, $\Theta$ = ps/a1, and $\pi$ = nucleotide diversity. D is the Tajima test statistic.

**Table 5: Fisher's exact test for 16S-rDNA sequences of *C. indologenes.***

| *bla*IND | 2C | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|
| 2C | | | | | | | | |
| 7 | 0.390 | | | | | | | |
| 8 | 0.365 | 1.000 | | | | | | |
| 9 | 1.000 | 0.351 | 0.053 | | | | | |
| 10 | 0.319 | 0.355 | 0.489 | 0.025 | | | | |
| 11 | 0.373 | 0.373 | 0.365 | 1.000 | 0.373 | | | |
| 12 | 1.000 | 0.403 | 0.527 | 1.000 | 0.422 | 1.000 | | |
| 13 | 0.370 | 0.394 | 0.365 | 1.000 | 0.319 | 0.472 | 0.472 | |
| 14 | 0.481 | 0.534 | 1.000 | 0.282 | 0.340 | 0.464 | 0.389 | 0.463 |

**Table 6: Disparity index for 16S-rDNA sequences of *C. indologenes*.**

| ***bla*IND** | **2C** | **7** | **8** | **9** | **10** | **11** | **12** | **13** |
|---|---|---|---|---|---|---|---|---|
| 2c | | | | | | | | |
| 7 | 0.000 | | | | | | | |
| 8 | 0.306 | 0.496 | | | | | | |
| 9 | 0.362 | 0.628 | 0.022 | | | | | |
| 10 | 0.453 | 0.117 | 0.000 | 0.000 | | | | |
| 11 | 0.000 | 0.000 | 0.265 | 0.318 | 0.409 | | | |
| 12 | 0.000 | 0.000 | 0.153 | 0.327 | 0.421 | 0.000 | | |
| 13 | 0.000 | 0.000 | 0.352 | 0.405 | 0.503 | 0.0012 | 0.0012 | |
| 14 | 0.000 | 0.000 | 0.462 | 0.522 | 0.102 | 0.000 | 0.000 | 0.000 |

**Table 7: Pairwise distance among six groups of *C. indologenes*.**

| ***bla*IND** | **2C** | **7** | **8** | **9** | **10** | **11** | **12** | **13** |
|---|---|---|---|---|---|---|---|---|
| 2c | - | | | | | | | |
| 7 | 0.358 | | | | | | | |
| 8 | 0.351 | 0.257 | | | | | | |
| 9 | 0.329 | 0.281 | 0.224 | | | | | |
| 10 | 0.294 | 0.208 | 0.004 | 0.217 | | | | |
| 11 | 0.005 | 0.357 | 0.349 | 0.333 | 0.291 | | | |
| 12 | 0.006 | 0.333 | 0.329 | 0.325 | 0.293 | 0.003 | | |
| 13 | 0.005 | 0.360 | 0.352 | 0.333 | 0.295 | 0.009 | 0.009 | |
| 14 | 0.355 | 0.010 | 0.264 | 0.289 | 0.204 | 0.354 | 0.338 | 0.357 |

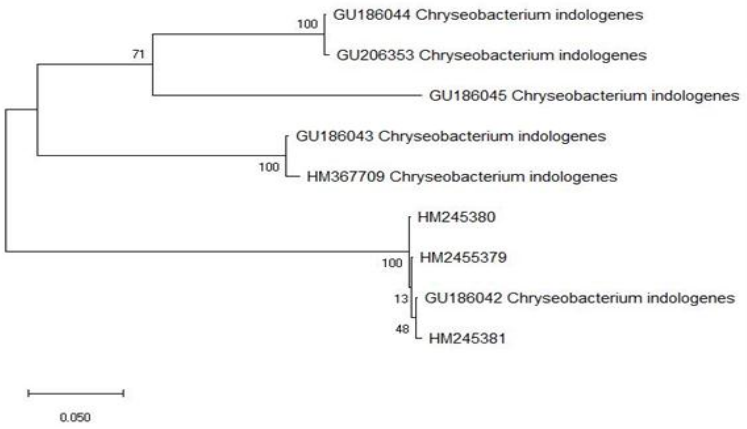


**Figure 1: The maximum parsimonious tree for nine strains of *C. indologenes* based on 16S rDNA of metallo-β-lactamase using MEGA X. The values of bootstrap were shown in side of vertical lines.**

## DISCUSSION

The process of infection and multiplication of *C. indologenes* have not been fully understood yet because this bacterium has not been frequently recovered from clinical specimens and did not occurred epidemic.[17] In 1993, Bonten et al.[18] first isolated a strain of *C. indologenes* from a tracheal aspirate in a patient with ventilator-associated pneumonia.

*Chryseobacterium* is not a common pathogen in humans. A few cases have reported in hospital infection. Six cases have been reported in Europe, two in Australia, two in India and seven in the USA.[19] Lee and Lee[20] reported 6 cases of *Chryseobacterium indologenes* isolated from clinical specimens in Korea. In addition, peritoneal dialysis (PD)-associated peritonitis caused by *C. indologenes* is rare.[21] There were not reported *C. indologenes* being a single causative pathogen of peritoneal dialysis-associated peritonitis in Korea until 2018. Choi et al.[21] reported a case of peritoneal dialysis due to *C. indologenes* after a 16-day course of intraperitoneal antibiotic therapy.

Zeba et al.[22] reported the identification and characterization of a new IND-type variant from a *C. indologenes* isolated from Burkina Faso that is resistant to β-lactams and aminoglycosides. Many new mutations

of IND-type metallo-β-lactamases are occurring. For example, the level of sequence identity of IND-6 was 72% the same as them of IND-4 and 90% the same as IND-5. The N-terminal of the purified enzyme (IND-6) is heterogenous to other IND-type. Thus, the new variant will vary in structure and function. Actually, IND-6 observed a broad substrate profile and had overall higher turnover rates than IND-5. In addition, IND-6 showed higher activities than IND-2 and IND-5 against ceftazidime and cefepime. Although Chryseobacterium species shared high similarity in whole genomic level, they showed significant differences between IND types. These findings imply that the multiple novel resistance determinants were evolved.[23]

Genetic evolution can be seen as occurring in species with close flexible relationships. McDonald and Kreitman[24] sequenced the alcohol dehydrogenase gene of three related Drosophila species. The 43 synonymous genes were polymorphisms, 2 genes were replacement polymorphisms, 17 synonymous genes fixed differences, and 7 fixed genes were replacement differences. In this study, varying sites were not classified as synonymous or amino acid replacements. Thus, the nucleotide variation could change an amino acid and they were also classified as polymorphic (varying within a species) or fixed differences between strains of *C. indologenes*.

Finally, the sequence heterogeneity shown by IND-type enzymes may be natural variants. The variation of 16S rDNA within *C. indologenes* will be accompanied by changes in biochemical properties and makes these enzymes interesting models to investigate the role of remote mutations in metallo-β-lactamase structure and function.[22]

## REFERENCES


1. Imataki O, Uemura M. *Chryseobacterium indologenes*, a possible emergent organism resistant to carbapenem antimicrobials after stem cell transplantation. Clin Case Rep, 2017; 5: 22–5.
2. Ozcan N, Dal T, Tekin A, Kelekci S, Can S, Ezin O, et al. Is *Chryseobacterium indologenes* a shunt-lover bacterium? A case report and review of the literature. Infez Med, 2013; 21: 312–16.
3. Olbrich P, Rivero-Garvía M, Falcón-Neyra MD, Lepe JA, Cisneros JM, Marquez-Rivas J, et al. *Chryseobacterium indologenes* central nervous system infection in infancy: an emergent pathogen? Infection, 2014; 42: 179–83.
4. Rossolini GM, Walsh TR, Amicosante G. The aeromonas metallo-b-lactamases: genetics, enzymology, and contribution to drug resistance. Microb Drug Resistance, 1996; 2: 245–52.
5. Senda K, Arakawa Y, Ichiyama S, Nakashima K, Ito H, Ohsuka S, et al. PCR detection of metallo-beta-lactamase gene (*bla*IMP) in gram-negative rods resistant to broad-spectrum beta-lactams. J Clin Microbiol, 1996; 34: 2909–13.
6. Rasmussen BA, Bush K. Carbapenem-hydrolyzing beta-lactamases. Antimicrob Agents Chemother, 1997; 41: 223–2.
7. Cascio A, Stassi G, Costa GB, Crisafulli G, Rulli I, Ruggeri C, Iara C. *Chryseobacterium indologenes* bacteraemia in a diabetic child. J Med Microbiol, 2005; 54: 677–80.
8. Chou DW, Wu SL, Lee CT, Tai FT, Yu WL. Clinical characteristics, antimicrobial susceptibilities, and outcomes of patients with *Chryseobacterium indologenes* bacteremia in an intensive care unit. Jpn J Infect Dis, 2011; 64: 520–4.
9. McKew G. Severe sepsis due to *Chryseobacterium indologenes* in an immunocompetent adventure traveler. J Clin Microbiol, 2014; 52: 4100–4101.
10. Murray PR, Pfaller MA, Tenover FC, Yolken RH. Manual of Clinical Microbiology. 6th ed., Washington DC., ASM Press: 1995, pp. 528-30.
11. Winn WC, Koneman EW, Allen SD, Janda WM, Schreckenberger PC. Koneman's Colour Atlas and Textbook of Diagnostic Microbiology. 6th ed., Philadelphia, Lippincott Williams and Wilkins: 2006, pp. 345-52.
12. 12.Yum JH. Genetic diversity of metallo-β-lactamase genes of *Chryseobacterium indologenes* isolates from Korea. Biomedical Science Letters, 2019; 25: 275-81.
13. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 1993; 10: 512-26.
14. Kumar S, Gadagkar SR. Disparity Index: A simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. Genetics, 2001; 158: 1321-7.
15. Tajima F. Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics, 1989; 123: 585-95.
16. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 2018; 35: 1547-9.
17. Palewar MS, Mudshingkar SS, Dohe V, Bharadwaj R. Infection by multidrug-resistant *Chryseobacterium indologenes* in cases of obstructive uropathy: Case series with short review. Case Report, 2017; 10: 376-80.
18. Bonten MJ, van Tiel FH, van der Geest S, Smeets HG, Stobberingh EE, Gaillard CA. Topical antimicrobial prophylaxis of nosocomial pneumonia in mechanically ventilated patients. Microbiological observations. Infection, 1993; 21: 137-9.
19. Bhuyar G, Jain S, Shah H, Mehta VK. Urinary tract infection by *Chryseobacterium indologenes*. Indian J Med Microbiol, 2012; 30: 370-2.
20. Lee SK, Lee NY. *Chryseobacterium indologenes* isolated from clinical specimens. Korean J Clin Microbiol, 1999; 2: 101-4.
21. Choi MW, Oh SS, Choi MR, Lee JH, Yang HJ, Sun IO, Lee KY. Peritoneal dialysis-associated peritonitis caused by *Chryseobacterium indologenes*. Korean J

Med, 2018; 93: 65-7.
22. Zeba B, Luca FD, Dubus A, Delmarcelle M, Simporé J, Nacoulma OG, et al. IND-6, a Highly divergent IND-type metallo—lactamase from *Chryseobacterium indologenes* strain 597 isolated in Burkina Faso. Antimicrobial Agents And Chemotherapy, 2009; 53: 4320–6.
23. Wang T, Jiang X, Feng C, Li A, Dong H, Wu S, et al. Whole genome sequencing uncovers a novel IND-16 metallo-β-lactamase from an extensively drug-resistant *Chryseobacterium indologenes* strain J31. Gut Pathog, 2016; 8: 47.
24. McDonald JH, Kreitman M. Adaptive protein evolution at the Adh locus in *Drosophila*. Nature, 1991; 351: 652-4.