

IDENTIFICATION, CULTIVATION CONDITION OF *PARASEVETIBACTER* SP C101 AND ANTIMICROBIAL ACTIVITY OF THE BACTERIAL CULTURE EXTRACT

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SUMMARY

During the recent decade, uncultured bacteria have been special interest as potential candidates for discovering novel antibacterial compounds. Two strains C101 and C102 were negative Gram bacteria, only growing on low nutrient media as R2A/3, NB/3, LB/10 and R4/10 compared to the usual. On R2A/3 medium, colonies of the isolates were round, convex, lemon yellow color with the size of 1-1.5 mm after six days of incubation at 28°C. Cells were 0.2-0.3 × 0.8-1.3 µm. The strains C101 and C102 were able to grow at temperature ranging 15-37°C (optimum at 25-28°C), pH 5-8 (optimum in pH 6-7). The sequences of 16S rRNA genes from strain C101 (MT756087) and C102 (MT756088) shared 100% identity. Analysis of full-length 16S rRNA gene sequence of strain C101 via using NCBI Blast, EzTaxon Database revealed the highest similarity of 99.18-100% to uncultured clones, and 97.86% to type species as *Parasegetibacter terrae* SGM2-10^T. Genetic sequence analysis data showed that strain C101 should be considered a novel candidate species of the genus *Parasegetibacter*. Antibacterial compound was extracted from culture of strain C101 in R4/10 medium for ten days of shaking incubator at 28°C and exhibited susceptible activity to inhibit *Bacillus anthracis* KEMB 211-146 at a concentration of 2 µg/L and *Staphylococcus aureus* ATCC 6538 at 4 µg/L; intermediate inhibiting *Bacillus subtilis* KEMB 51201-001 at 8 µg/L, *Staphylococcus epidermidis* ATCC 14990 at 8 µg/L, and *S. aureus* CCARM 3155 at 16 µg/L; inhibition of *S. aureus* CCARM 3095 at 64 µg/L, *S. aureus* CCARM 3192 at 32 µg/L, and *S. epidermidis* CCARM 3710 at 64 µg/L.

Keywords: 16S rRNA gene sequence, antibacterial activity, slow-growing bacteria, uncultured bacteria

INTRODUCTION

Over the past 10 years, the number of newly discovered natural antibiotics has tended to decrease sharply, while pathogenic bacteria have evolved rapidly, allowing them to survive better in adverse conditions. As a result, many antibiotic resistant pathogens appear such as

methicillin-resistant *Staphylococcus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), vancomycin-resistant *Staphylococcus* (VRSA), and multi-drug resistant (MDR), extremely-drug resistant (XDR), extended spectrum β-lactamase (ESBL) (Davies, Davies, 2010; Tacconelli *et al.*, 2018; Centers for Disease Control and Prevention, 2019). Consequence of antibiotic

resistance bacteria have killed an estimated 8.6 million people each year in the world (Wang *et al.*, 2016). As predicted by 2050, approximately 10 million people will die within a year, if there are no timely solutions (de Kraker *et al.*, 2016).

Through molecular analysis of microbial community in soil revealed that one gram of soil may contain $10^7 \sim 10^9$ cells (Baldrian, 2017; Tecon, Or, 2017), traditional isolation methods reach only less than 1% of bacterial community so far (Pham, Kim, 2012; Hug *et al.*, 2016). More than 99% of bacterial species have not been cultured or difficulty in maintaining pure cultures in laboratories, called uncultured bacteria (Ward *et al.*, 1990; Rappé, Giovannoni, 2003; Lewis, 2013; Ling *et al.*, 2015; Hug *et al.*, 2016; Ling *et al.*, 2015). Thousands of active substances have been discovered and applied in pharmaceutical industry from the 1% of successfully cultured bacteria (Bérdy, 2005; Masschelein *et al.*, 2017). Therefore, there will be a lot of potential for discovering novel natural antibiotics from uncultured bacteria, and this is one of the effective solutions to prevent the problem of drug resistance (Ling *et al.*, 2015; Dela-Cruz, 2015; Lewis, 2017). In a previous study, we have successfully isolated two bacterial strains. In this study, the bacteria were revealed to belong to the genus *Parasegetibacter* and antibacterial activity of extract derived from the bacterial culture was investigated.

MATERIALS AND METHODS

Materials

Media used, including R2A (3.12 g/L, M1687, Mimedia, India), nutrient broth NB (8 g/L 105443, Merck), Luria-Bertani (LB) (tryptone: 10 g/L; NaCl: 10 g/L; yeast extract: 5 g/L), R4 (glucose: 10g/L; yeast extract: 1g/L; casamino acid: 0.1g/L; proline: 3g/L; MgCl₂.6H₂O: 10 g/L; K₂SO₄: 0.2 g/L; CaCl₂.2H₂O: 4 g/L; TES: 5.6 g/L), and Mueller–Hinton (275730, BD DIFCO). In this study, except for the Mueller–Hinton medium, the above media are made nutrient-poor as R2A/3 including (per L) 0.167 g casein acid

hydrolysate, 0.167 g yeast extract, 0.167 g proteose peptone, 0.167 g dextrose, 0.167 g starch, 0.1 g K₂HPO₄, 0.008 g MgSO₄, 0.1 sodium pyruvate; NB/3 containing per L) 1,67 g peptone from meat, 1 g meat extract; LB/10 including (per L) 1 g tryptone, 1 g NaCl, 0.5 g yeast extract; and R4/10 containing (per L) 1 g glucose, 0.1 g yeast extract, 0.01 g casamino acid, 0.3 g proline, 1 g MgCl₂.6H₂O, 0.02 g K₂SO₄, 0.4 g CaCl₂.2H₂O, 0.56 g TES.

Two strains C101 and C102, were isolated from soil according to method of Nguyen *et al.* (2018) and stored in 20% glycerol at -86°C until used.

Microbial testing: *S. aureus* ATCC 6538 (penicillin resistance), *S. epidermidis* ATCC 14990, *S. aureus* CCARM 3095 (cephalothin, erythromycin, norfloxacin, and oxacillin resistance), *S. aureus* CCARM 3192 (cephalothin, erythromycin, norfloxacin, and oxacillin resistance), *S. aureus* CCARM 3155 (cephalothin, and erythromycin resistance), *S. epidermidis* CCARM 3710 (cephalothin, erythromycin, norfloxacin, oxacillin, tetracycline, and chloramphenicol resistance), *B. subtilis* KEMB 51201-001, and *B. anthracis* KEMB 211-146. These bacteria are provided by The American Type Culture Collection (ATCC), Culture Collection of Antimicrobial Resistant Microorganisms (CCARM) and Korea National Environmental Microorganisms Bank (KEMB).

Methods

Phenotypic characteristics of strains C101 and C102

Gram-staining, catalase-, oxidase reaction were carried out according to previously study by Krieg, Padgett (2011). Cell morphology was estimated by scanning electron microscope (SEM). Different types of media (R2A/3, NB/3, LB/10, and R4/10) were used to test the growth of the isolates. The growth of these strains was also investigated at different temperatures (15, 20, 25, 28, 30, 37, 40, and 45°C), various pH values (pH 4.0–9.0; at intervals of 1 pH unit by HCl 1N and

NaOH 1N) using R2A/3. The tests were checked after six days of incubation at OD₆₀₀.

Optimal media for producing antimicrobial compound of strains C101 and C102

Pure colonies of strains C101 and C102 were cultivated on R2A/3 at 28°C, 160 rpm, for 4 days. Then, 7% (v/v) of the suspension was inoculated on R2A/3, NB/3, LB/10 and R4/10 (pH adjusted to 6.8 for each medium), cultivated at 28°C, 160 rpm, ten days. Removal of cell biomass was conducted by centrifugation at 13.000 rpm, 60 µL of cultured solution was added on paper disc (6 mm, WHA2017006). The paper disc was placed on Mueller–Hinton agar plate containing *S. epidermidis* CCARM 3710 at concentration of 10⁵ CFU/mL. Checking inhibition zone after 24h, 37°C.

Phylogenetic analyses

Genomic DNA of strains C101 and C102 was isolated by using the method of Sambrook, Russell (2001). Components and conditions of PCR reaction to amplify 16S rRNA of these strains were performed according to description of Klindworth *et al.* (2013), using forward primer 27F-AGAGTTTGATCMTGGCTCAG and reverse primer 1492R TACGGYTACCTTGTTACGACTT (Lane, 1991). PCR product was sequenced via Applied Biosystems 3730 xl DNA analyzer using Big Dye terminator cycle sequencing kit v.3.1 (Applied Biosystems). Full 16S rRNA gene sequences of the isolates were searched for the most nearly exact matches through NCBI Blast (<https://blast.ncbi.nlm.nih.gov/Blast>) and EzTaxon (<https://www.ezbiocloud.net/>). Tree topology of the isolates and related taxa was established using MEGA 7 (Kumar *et al.*, 2016).

Fermentation and extraction of antimicrobial compound

The isolate showing strong antimicrobial activity was cultivated on R2A/3, 28°C, 160 rpm for four days. Seven percent (v/v) of the culture was inoculated on R4/10 for ten days of shaking incubation. Fermented culture (2L) was centrifuged to remove cell biomass. Culture

supernatant was reduced to 1 L by using rotary evaporator at 40°C under vacuum. Supernatant was extracted with diethyl ether thrice (1:1, v/v), then evaporating solvent to collect crude compound. The residue was dissolved in distilled water before filtering through a 0.22 µm membrane filter, and lyophilized.

Minimum inhibitory concentration (MIC)

The crude compound was dissolved in dimethyl sulfoxide. MIC values were determined by the method of CLSI (2012). Cephalothin and norfloxacin were used as positive controls. Bacterial cells for each test were prepared at 5×10⁵ CFU/mL.

RESULTS AND DISCUSSION

Phenotypic characteristics of strains C101 and C102

Two strains C101 and C102 were isolated from soil collecting in Tan Cuong, Thai Nguyen, Vietnam using intensive soil extract medium (ISEM) (Nguyen *et al.*, 2018). Two isolates were not able to grow on complete media according to the manufacturer or previously published. Instead of two isolates only grew on poor nutrient (R2A/3, NB/3, LB/10 and R4/10). In many media, high nutrient content could inhibit growth of previously uncultured soil bacteria, while using less nutrient-poor media than normal were considered to be one of effective methods for isolating and culturing previously uncultured bacteria (Pham, Kim, 2012; Pulschen *et al.*, 2017).

Strains C101 and C102 were Gram negative, catalase, and oxidase positive. Colonies of the isolates were round, convex, lemon yellow color, size of colonies ranging from 1-1.5 mm after six days of incubation at 28°C on R2A/3 medium. Cells were 0.2-0.3 × 0.8-1.3 µm (Figure 1). Both isolates were able to grow on R2A/3, NB/3, LB/10 and R4/10 (optimum in R2A/3 at 15-37°C (optimum at 25-28°C), pH 5-8 (optimum in pH 6-7). As shown in Figure 2, the isolates grew slowly on R2A/3 medium, lag phase took at 3rd day and reached to the stationary phase at 5-7th days of incubation.

Suitable media for producing antimicrobial compound of strains C101 and C102

Fermented culture of strains C101 and C102 in R4/10 medium showed the inhibition of *Staphylococcus epidermidis* CCARM 3710 growth. Inhibition zones were 4.5 and 3.8 mm, respectively (Figure 3). While, the inhibition was not detected in fermented culture of two isolates in R2A/3, NB/3 or LB/10. As reported by Masschelein *et al.* (2017) most negative Gram bacteria have genome sizes ranging from 400 kb to more than 6 Mb, G+C ratio from 25

to 80%, thus negative Gram bacteria may easier to adapt to living conditions than Gram positive bacteria. Therefore, by different methods, including using a medium it is possible to activate silent gene clusters encoding for synthesis of secondary metabolites as an effective method to find novel antimicrobials. Typical examples are odorhabdin produced from *Xenorhabdus nematophila* K102 (Pantel *et al.*, 2018) or darobactin from *Photorhabdus khanii* HGB1456 (Imai *et al.*, 2019).

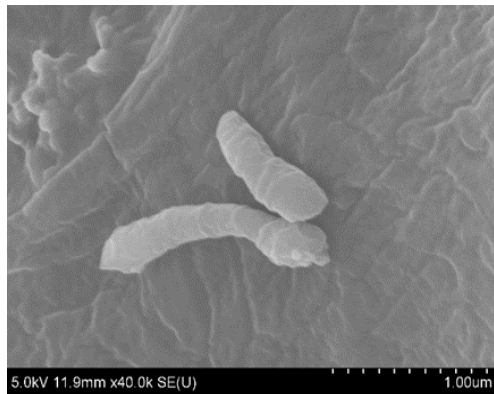


Figure 1. Cell morphology of strain C101 in R2A/3 broth at 28°C after six days of incubation.

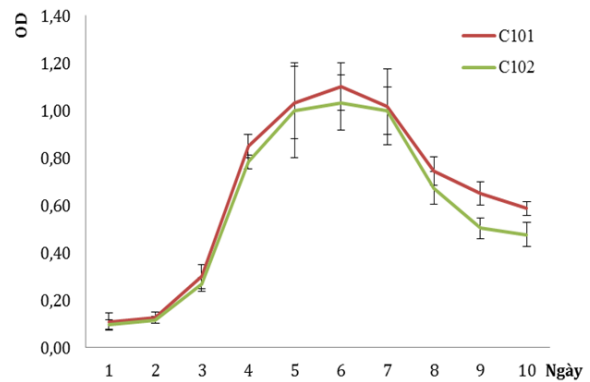


Figure 2. Growth characteristic of strains C101 and C102 in R2A/3 broth.

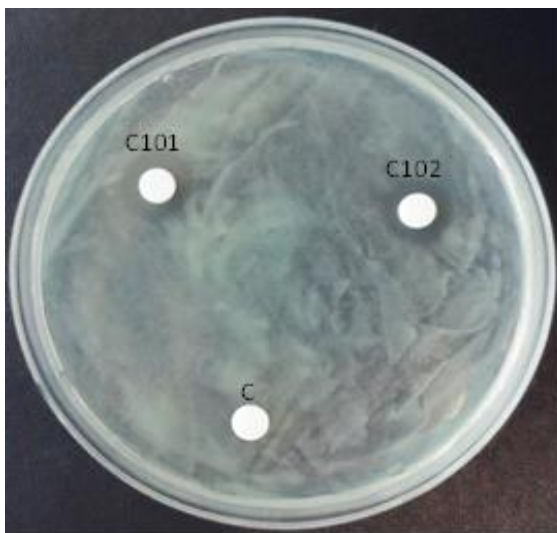


Figure 3. Inhibition zone of fermented culture of strains C101 and C102 to inhibit *Staphylococcus epidermidis* CCARM 3710, C: control (R4/10 medium only).

Phylogenetic analyses

Full-length 16S rRNA gene sequences of strains C101 (Genbank accession number: MT756087) and C102 (Genbank accession number: MT756088) were 1465, 1469 bp, respectively. There was 100% similarity between 16S rRNA sequence of strain C101 and C102 via analysis of Nr BlastN. The obtained results from Nr BlastN showed that the 16S rRNA gene sequence of C101 was the most identical (100% to the corresponding gene from uncultured bacterium clone V8-55 (GQ487995), 99.25% to uncultured *Bacteroidetes* clone TH451 (AJ888562), 99.18% to uncultured bacterium clone GZ8 (JX133428), and 99.18% to uncultured bacterium clone WIFD26 (HQ450144). Based on these results, strain C101 belongs to a previously uncultured bacterium.

Simultaneously, using Eztaxon server database (<https://www.ezbiocloud.net/>) containing complete sequences from successfully cultured strains, 16S rRNA gene sequence of strain C101 exhibited similarities with validly species published 97.86% to *Parasegetibacter terrae* SGM2-10^T (KJ634465), 95.51% to *Parasegetibacter luojiensis* RHYL-37^T (EU877263), 95.1% to *Flavitalea populi* HY-50R^T (HM130561), 95.04% *Terrimonas soli* FL-8^T (MF595514), 94.42% to *Niastella caeni* HX-16-21^T (MK812841), etc. Comparison of the 16S rRNA similarity at the cut-off limit of 98.7%, strain C101 should be considered a novel member of the genus *Parasegetibacter* (Browne *et al.*, 2016). Genus *Parasegetibacter*, type species as *Parasegetibacter luojiensis* RHYL-37^T, was first described by Zhang *et al.* (2009) (<https://lpsn.dsmz.de/genus/parasegetibacter>). A new member *Parasegetibacter terrae* SGM2-10^T is isolated and successfully cultured in the laboratory (Kim *et al.*, 2015). *Parasegetibacter* species are slow-growing, negative Gram, family *Chitinophagaceae*, phylum *Bacteroidetes*.

Phylogenetic analysis of strains C101 and C102 was the closest match with uncultured clones, arranged with previously cultured *Parasegetibacter* species, but a distinct branch (Figure 4). Based on the identical sequences and analysis of phylogenetic, strain C101 can be considered as a candidate for a new species of the genus *Parasegetibacter*, named *Parasegetibacter* sp. C101.

Minimum inhibitory concentration testing

Crude product extracted from a culture of strain C101 has pale yellow. Results of antibacterial activity are shown in Table 1. The crude compound was able to against bacterial pathogens testing including multidrug-resistant bacteria with MIC values from 2 to 64 µg/mL. Comparing to the breakpoint of CLSI (2012), the extract was susceptible to kill *Bacillus anthracis* KEMB 211-146 and *Staphylococcus aureus* ATCC 6538; intermediate activity for *Bacillus subtilis* KEMB 51201-001, *Staphylococcus epidermidis* ATCC 14990; to be need from 16-32 µg/L of the extract to inhibit the remaining bacteria (Table 1).

Table 1. MIC values of crude compound extracted from strain C101.

Chủng	MIC (µg/ml)		
	C101	Cephalothin	Norfloxacin
<i>Bacillus subtilis</i> KEMB 51201-001	8	32	0.5
<i>Bacillus anthracis</i> KEMB 211-146	2	0.125	0.25
<i>Staphylococcus aureus</i> ATCC 6538	4	32	1
<i>Staphylococcus aureus</i> CCARM 3095	64	128	256
<i>Staphylococcus aureus</i> CCARM 3192	32	128	32
<i>Staphylococcus aureus</i> CCARM 3155	16	64	2
<i>Staphylococcus epidermidis</i> ATCC 14990	8	0.125	0.5
<i>Staphylococcus epidermidis</i> CCARM 3710	64	128	128

As known, more than 70% of commercial natural antibiotics are derived from actinomyces (Bérdy, 2005). In recent years, number of natural new antibiotics discovered has decreased sharply, therefore slowly-growing species belong to negative Gram bacteria are potential candidates for discovery of new antibiotics. Negative Gram bacteria known as group may easily reveal phenotypic characteristics via using cultural factors such as media, temperature... than positive Gram bacteria (Masschelein *et al.*,

2017). Novel teixobactin is a typical example, teixobactin is extracted from previously uncultured bacteria that named as *Eleftheria terrae*, is negative Gram bacterium. Teixobactin could inhibit a broad-spectrum of pathogenic positive Gram bacteria and pathogenic negative Gram bacteria, including MRSA, VRE at the MIC value less than 0.5 µg/mL (Ling *et al.*, 2015). Elansolid A belongs to polyketides, isolated from *Chitinophaga sancti*; lysobactin was isolated from *Cytophaga* sp. PBJ-

5356/*Lysobacter* sp. ATCC53042; lactivicin were ability to inhibit MRSA (Steinmetz *et al.*, 2011; Xie *et al.*, 2012).
 from *Empedobacter lactamgenus* YK-258 that

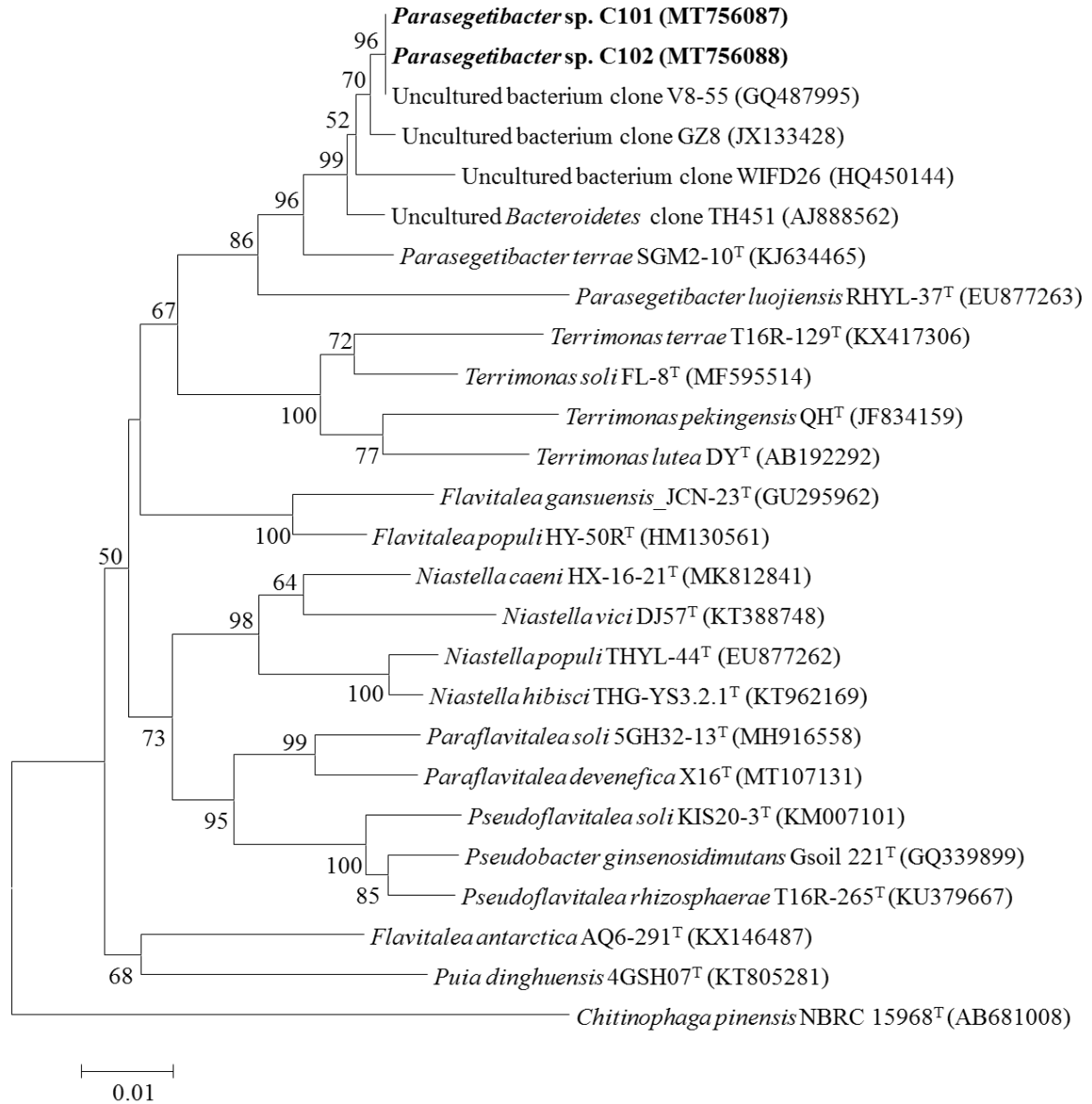


Figure 4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strains C101 and C102 with closely related taxa. Bootstrap values based on 1000 replications; only values $\geq 50\%$ are give. *Chitinophaga pinensis* NBRC 15968^T (AB681008) was used as out group. Bar, 0.01 substitutions per site.

CONCLUSION

Two strains C101 and C102 were negative Gram bacteria, could not grow on complete media, but on poor nutrient media such as

R2A/3, NB/3, LB/10 and R4/10 (optimum R2A/3) at 15-37°C, pH 5-8. The 16S rRNA gene sequences of C101 (MT756087) and C102 (MT756088) were 97.86% to type species cultivated as *Parasegetibacter terrae* SGM2-

10^T. Combination of 16S rRNA gene sequence analysis and phylogenetic taxonomy, strain C101 should be considered a novel member of genus *Parasegetibacter*.

Crude compound extracted from the culture of strain C101 was susceptible to inhibit *Bacillus anthracis* KEMB 211-146 and *Staphylococcus aureus* ATCC 6538 at a concentration of 2-4 µg/L; 8-16 µg/L for *Bacillus subtilis* KEMB 51201-001, *Staphylococcus epidermidis* ATCC 14990, and *Staphylococcus aureus* CCARM 3155; 32-64 µg/L for *Staphylococcus aureus* CCARM 3095, *Staphylococcus aureus* CCARM 3192 and *Staphylococcus epidermidis* CCARM 3710.

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REFERENCES

- Baldrian P (2017) Forest microbiome: diversity, complexity and dynamics. *FEMS Microbiol Rev* 41: 109–130.
- Bérdy J (2005) Bioactive microbial metabolites. *J Antibiot* (Tokyo) 58 1–26.
- Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD. (2016) Culturing of “unculturable” human microbiota reveals novel taxa and extensive sporulation. *Nature* 533: 543–546.
- Centers for Disease Control and Prevention, Office of Infectious Disease (2013) Antibiotic resistance threats in the United States. U.S department of health and human services. <https://www.cdc.gov/drugresistance/biggest-threats.html>.
- Clinical and Laboratory Standards Institute (CLSI) (2012) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—Ninth edition, CLSI document M07-A9 (ISBN 1-56238-783-9), Pennsylvania.
- Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74(3): 417–433.
- de Kraker ME, Stewardson AJ, Harbarth S (2016) Will 10 million people die a year due to antimicrobial resistance by 2050?. *PLoS Med* 13:e1002184.
- Dela-Cruz CS (2015) Looking at the 99% in nature to stop drug-resistant bacteria. *Sci Transl Med* 274: 274ec25.
- Hug L, Baker BJ, Anantharaman K, Brown CT, Probst AJ, Castelle CJ, Butterfield CN, Hersndorf AW, Amano Y, Ise K, Suzuki Y, Dudek N, Relman DA, Finstad KM, Amundson R, Thomas BC, Banfield JF (2016) A new view of the tree of life. *Nat Microbiol* 1: 16048. <https://doi.org/10.1038/nmicrobiol.2016.48>.
- Imai Y, Meyer KJ, Iinishi A, Favre-Godal Q, Green R, Manuse S, Caboni M, Mori M, Niles S, Ghiglieri M, Honrao C, Ma X, Guo JJ, Makriyannis A, Linares-Otoya L, Böhringer N, Wuisan ZG, Kaur H, Wu R, Mateus A, Typas A, Savitski MM, Espinoza JL, O'Rourke A, Nelson KE, Hiller S, Noinaj N, Schäberle TF, D'Onofrio A, Lewis K (2019) A new antibiotic selectively kills Gram-negative pathogens. *Nature* 576: 459–464.
- Kindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and nextgeneration sequencing-based diversity studies. *Nucleic Acids Res* 41(1): 1–11.
- Krieg NR, Padgett PJ (2011) Phenotypic and physiological characterization methods. In *methods in microbiology* (pp. 16-17). Academic Press, Oxford, UK.
- Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33(7): 1870–1874.
- Lane DJ (1991) 16S/23S rRNA sequencing. In *nucleic acid techniques in bacterial systematics* (pp. 115-175). John Wiley and Sons, New York, NY.
- Lewis K (2013) Platforms for antibiotic discovery. *Nat Rev Drug Discov* 12: 371-87.
- Lewis K (2017) New approaches to antimicrobial discovery. *Biochem Pharmacol* 134: 87-98.
- Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schäberle TF, Hughes DE, Epstein S, Jones M, Lazarides L, Steadman VA, Cohen DR, Felix CR, Fetterman KA, Millett WP, Nitti AG, Zullo AM, Chen C, Lewis K (2015) A new antibiotic kills pathogens without detectable resistance. *Nature* 517: 455–459.

- Masschelein J, Jenner M, Challis GL (2017) Antibiotics from Gram-negative bacteria: a comprehensive overview and selected biosynthetic highlights. *Nat Prod Rep* 34(7): 712-783.
- Nguyen TM, Seo C, Ji M, Paik MJ, Myung SW, Kim J (2018) Effective Soil Extraction Method for Cultivating Previously Uncultured Soil Bacteria. *Appl Environ Microbiol* 84 (24): e01145-18.
- Pantel L, Florin T, Dobosz-Bartoszek M, Racine E, Sarciaux M, Serri M, Houard J, Campagne JM, de Figueiredo RM, Midrier C, Gaudriault S, Givaudan A, Lanois A, Forst S, Aumelas A, Cotteaux-Lautard C, Bolla JM, Lundberg CV, Huseby DL, Hughes D, Villain-Guillot P, Mankin AS, Polikanov YS, Gualtieri M (2018) Odilorhabdins, antibacterial agents that cause miscoding by binding at a new ribosomal site. *Mol Cell* 70(1): 83-94.
- Pulschen AA, Bendia AG, Fricker AD, Pellizari VH, Galante D and Rodrigues F (2017) Isolation of uncultured bacteria from antarctica using long incubation periods and low nutritional media. *Front Microbiol* 8: 1346.
- Pham VHT, Kim J (2012) Cultivation of unculturable soil bacteria. *Trends Biotechnol* 30 (9): 475-484.
- Rappé MS, Giovannoni SJ (2003) The uncultured microbial majority. *Annu Rev Microbiol* 57: 369-394.
- Sambrook J, Russell DW (2001) Molecular cloning. In *a laboratory manual* (pp. 1-170). Cold Spring Harbor Laboratory Press, New York.
- Steinmetz H, Gerth K, Jansen R, Schläger N, Dehn R, Reinecke S, Kirschning A, Müller R (2011) Elansolid A, a unique macrolide antibiotic from *Chitinophaga sancti* isolated as two stable atropisomers. *Angew Chem Int Ed Engl* 50(2): 532-536.
- Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N; WHO Pathogens Priority List Working Group (2018) Discovery, 25/26 research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* 18(3): 318-327.
- Tecon R, Or D (2017) Biophysical processes supporting the diversity of microbial life in soil. *FEMS Microbiol Rev* 41: 599-623.
- Wang H, Naghavi M, Allen C, Barber RM, Bhutta ZA, Carter A, *et al* (2016) Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Lond Engl* 388 (10053): 1459-1544.
- Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 345: 63-65.
- Xie Y, Wright S, Shen Y, Du L (2012) Bioactive natural products from *Lysobacter*. *Nat Prod Rep* 29(11): 1277-1287.