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ORIGINAL PAPER

Cohnella pontilimi sp. nov., isolated from tidal-flat mud

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Abstract

A Gram-positive, aerobic, endospore-forming, rod-shaped bacterial strain, CAU 1483 ^{T}, was isolated from tidal-flat mud in the Republic of Korea. It grew optimally at 30 °C, in a pH 7.0 medium with 2% (w/v) NaCl. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain CAU 1483 ^{T} formed a separate clade within *Paenibacillaceae* together with members of the genus *Cohnella*. Strain CAU 1483 ^{T} exhibited the highest 16S rRNA gene sequence similarity (97.1%) to *C. candidum* 18JY8-7 ^{T}. Whole genome of strain CAU 1483 ^{T} was 4.29 Mb in size with a 53.7 mol% G + C content, and included 4046 coding sequences and included 4046 coding sequences, some of which associated with stress response. The average nucleotide identity and digital DNA–DNA hybridization similarity between strain CAU 1483 ^{T} and related members of the genus *Cohnella* were 71.8–74.9% and 22.6–33.9%, respectively. The major respiratory quinone present in this strain was menaquinone-7. Strain CAU 1483 ^{T} contained anteiso-$C_{15:0}$ and iso-$C_{16:0}$ as the major fatty acids, while its polar lipids consisted of phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, lysyl-phosphatidylglycerol, phosphatidylcholine, three unidentified aminophospholipids, two unidentified lipids and an unidentified phospholipid. Peptidoglycan type was A1γ *meso*-Dpm. On the basis of taxonomic characterization, strain CAU 1483 ^{T} constitutes a novel species, for which the name *Cohnella pontilimi* sp. nov. is proposed. The type strain of this novel species is CAU 1483 ^{T} (= KCTC 43047 ^{T} = NBRC 113953 ^{T}).

Keywords *Cohnella pontilimi* · Paenibacillaceae · Tidal-flat mud · 16S rRNA gene sequence · Genome

Introduction

The genus *Cohnella*, belonging to the family Paenibacillaceae together with the class Bacilli of the phylum Firmicutes, was proposed by Kämpfer et al. (2006) with the description of *C. thermotolerans* as the type species. At the time of writing, the genus *Cohnella* comprises 31 validly published members (https://www.bacterio.net/genus/cohnella), which have been isolated from a wide range of environmental habitats. Members of the genus *Cohnella* are gram-positive, aerobic, thermotolerant and rod-shaped, and can be motile or non-motile, and spore forming or endospore forming. During the investigation of novel bacteria, a bacterial isolate, designated CAU 1483 ^{T}, was retrieved from tidal-flat mud in Modo Island, Republic of Korea, and phylogenetically affiliated to members of the genus *Cohnella*. In the present study, the taxonomic position of strain CAU 1483 ^{T} was determined through a polyphasic approach, which included phylogenetic analysis based on the 16S rRNA gene sequences, as well as phenotypic and chemotaxonomic characterization.

Materials and methods

Bacterial strain isolation and culture conditions

A mud sample was collected from Modo Island (37°32′12.28″ N, 126°24′51.47″ E) in the Republic of Korea,

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serially diluted, spread on BD™ Difco™ R2A agar (MA; BD Difco, Sparks, MD, USA) according to Kim and Kim (2016), and incubated at 30 °C for 7 days. Isolates were randomly selected, grown at 30 °C on R2A and then subsequently purified on R2A medium. The obtained pure colonies were preserved in 25% (v/v) glycerol stocks at − 80 °C. Strain CAU 1483 ^{T} has been deposited in the Korean Collection for Type Cultures (KCTC; Jeongeup, Korea) and the National Institute of Technology and Evaluation Biological Resource Center (NBRC; Tokyo, Japan) as KCTC 43047 ^{T} and NBRC 113953 ^{T}, respectively. For comparative analysis, *C. candidum* KCTC 33969 ^{T}, *C. thermotolerans* DSM 17683 ^{T} and *C. lubricantis* LMG 29763 ^{T} were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), the KCTC and the Belgian Co-ordinated Collections of Micro-organisms (BCCM; Ghent, Belgium), respectively.

16S rRNA gene sequence analysis

The total genomic DNA of strain CAU 1483 ^{T} was extracted with a genomic DNA extraction kit (iNtRON, Seongnam, Korea). The 16S rRNA gene was amplified by PCR with universal primers (Lane 1991). The PCR products were then purified and directly sequenced by an automatic DNA sequencer (3730 DNA Analyzer; Applied Biosystems, Foster City, CA, USA). Multiple alignments between the sequence of CAU 1483 ^{T} and those of members of the genus *Cohnella*, retrieved from the EzBioCloud database (http://www.ezbiocloud.net/eztaxon), were generated through the Clustal X 2.1 software (Larkin et al. 2007) and the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi.). Evolutionary distance matrices were calculated through the neighbour-joining method described by Jukes and Cantor (1969). Phylogenetic trees were constructed with MEGA 7 based on the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) algorithms from the PHYLIP v.3.66 package (Felsenstein 1989). The topology was evaluated through a bootstrap resampling method based on 1000 replicates (Felsenstein 1985).

DNA G + C content and whole-genome sequencing analysis

The whole-genome sequence and the mol% G + C content of CAU 1483 ^{T} were examined using an Illumina HiSeq 2500 System (Illumina, San Diego, USA). De novo assembly of the sequencing data was performed with SPAdes v.3.13.0 (http://cab.spbu.ru/software/spades). The average nucleotide identity (ANI) between the genomic sequences of CAU 1483 ^{T} and those of closely related strains was calculated using the OrthoANI algorithm of the EzBioCloud web service (www.ezbiocloud.net/sw/oat) (Lee et al. 2016), whereas digital DNA–DNA hybridization (dDDH) values were calculated by the Genome-to-Genome Distance Calculator v.2.1 (http://ggdc.dsmz.de/ggdc.php) (Meier-Kolthoff et al. 2013). The genome of strain CAU 1483 ^{T} was annotated using the RAST Server (Brettin et al. 2015) and subsequently analysed in detail using the SEED database (Overbeek et al. 2014). Predicted bacterial species biosynthetic gene clusters were identified with antiSMASH v.5.1.0 (Blin et al. 2019). Proteome comparison between strain CAU 1483 ^{T} and its most closely related strain, *C. candidum* 18JY8-7 ^{T}, was carried out through the PATRIC website (www.patricbrc.org) (Wattam et al. 2017).

Phenotypic and biochemical analyses

For colony morphology characterization, the novel strain was cultured on R2A plates at 30 °C for 2 days. Motility was tested in cells grown in semisolid (0.3% agar) MA media (Bowman 2000). A Gram staining kit (bioMérieux, Craponne, France) was used to determine if bacteria were Gram positive or Gram negative. Cell morphology was observed with a DM1000 light microscope (Leica, Wetzlar, Germany) and a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan). The growth of strain CAU 1483 ^{T} under aerobic and anaerobic conditions was tested at various temperatures (4, 10, 20, 30, 37 and 45 °C). The pH of the medium was adjusted after sterilization to pH 4.5–11.0 (at 0.5 unit intervals) using sodium acetate/acetic acid, 1 M HCl for acid conditions and 1 M NaOH for alkaline conditions. Tolerance to NaCl concentrations of 0–15% (w/v, at 1% intervals) was investigated by observing growth in the R2A broth. Oxidase activity was determined with 0.1% tetramethyl-p-phenylenediamine while catalase activity was monitored by bubble production from cells placed in 3% (v/v) H_2O_2 (Cappuccino and Sherman 2005). Hydrolyses of urea, gelatin and starch were examined with standard methods (Smibert et al. 1994). Additional physiological, biochemical and enzymatic analyses were carried out using the commercial test kits API® 20 NE, API® 50 CH and API® ZYM (bioMérieux), according to the manufacturer's instructions.

Chemotaxonomic characterization

For chemotaxonomic characterization, strain CAU 1483 ^{T} and its closely related reference strains *C. candidum* 18JY8-7 ^{T}, *C. thermotolerans* CCUG 47242 ^{T} and *C. lubricantis* KSS-154-50 ^{T} were harvested after growth in R2A at 30 ℃ for 2 days. Fatty acid methyl esters were extracted according to the standard protocol of the Sherlock Microbial Identification System v.6.2B. Cellular fatty acids were separated through a gas chromatography system (6890 N GC System

with 7683 Autosampler; Agilent Technologies, Carpinteria, CA, USA) as described previously (Sasser 1990). The cell wall peptidoglycan of strain CAU 1483 ^{T} was analysed by the Identification Service of the DSMZ (Braunschweig, Germany) as described by Schumann (2011). The polar lipids of strain CAU 1483 ^{T} were identified by two-dimensional thin-layer chromatography (Minnikin et al. 1980). The plate was sprayed with an ethanolic solution of 10% molybdatophosphoric acid, ninhydrin, α-naphthol/sulfuric acid, molybdenum blue and Dragendorff's reagent (Sigma-Aldrich, St. Louis, MI, USA) for separation of total lipids, amino lipids, glycolipids, phospholipids and phosphatidylcholine, respectively. Respiratory quinones were extracted as described by Collins and Jones (1981) and analyzed by reversed-phase high-performance lipid chromatography (Kroppenstedt 1982).

Nucleotide sequence and whole-genome shotgun project accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1483 ^{T} is MH892071. The whole-genome sequence has been deposited in GenBank/EMBL/DDBJ under the accession number SUPK00000000.

Results and discussion

Phylogenetic characterization

The nearly complete sequence of the 16S rRNA gene of strain CAU 1483 ^{T} (1520 bp) was obtained and compared with the corresponding sequences of the most closely related strains, retrieved from the GenBank database. According to 16S rRNA gene sequence similarity, strain CAU 1483 ^{T} was most closely related to *C. candidum* 18JY8-7 ^{T} (97.1%), *C. thermotolerans* CCUG 47242 ^{T} (95.1%) and *C. lubricantis* KSS-154-50 ^{T} (95.0%). The neighbour-joining tree indicated that that CAU 1483 ^{T} formed a distinct lineage within the family *Paenibacillaceae* and clustered with the members of the genus *Cohnella* (Fig. 1). The trees obtained by applying the maximum-likelihood and maximum-parsimony algorithms showed the same topology.

Morphological, physiological and biochemical analyses

Detailed comparisons of physiological and biochemical characteristics between strain CAU 1483 ^{T} and the reference species are shown in Table 1. Cells of strain CAU 1483 ^{T} were gram-negative, non-motile, spore-forming and rod shaped, with a size of 1.5–2.6 × 0.5–0.8 μm (Supplementary

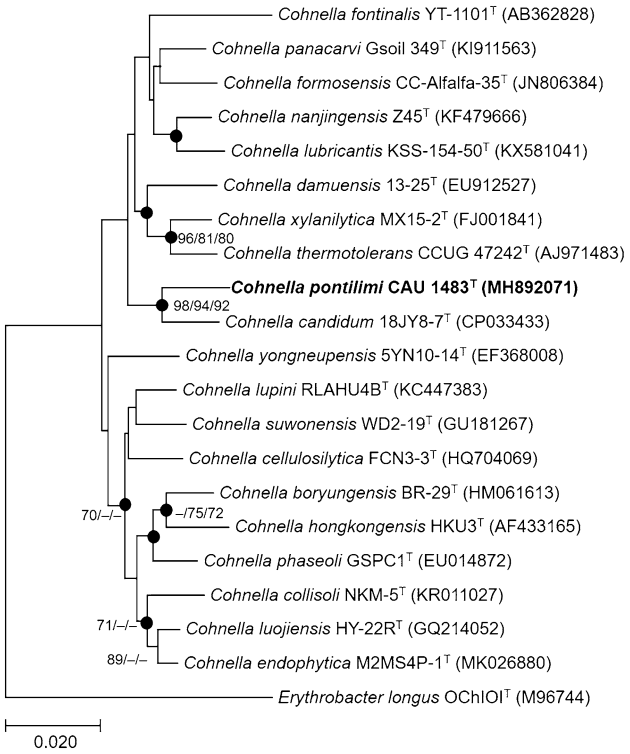

Fig. 1 Phylogenetic tree constructed by neighbour-joining based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 1483 ^{T} and its closely related species of the genus *Cohnella*. Bootstrap values refer to neighbour-joining, maximum-likelihood and maximum-parsimony analyses (NJ/ML/MP) of 1000 resampled datasets; only values > 70% are given. The sequence determined in this study is shown in bold. Bar, 0.02 nucleotide substitutions per position. *Erythrobacter longus* OChIOI^{T} is used as an outgroup organism

Fig. S1). Colonies were white, round, smooth, raised and with entire margins after incubation for 2 days at 30 °C in R2A. Strain CAU 1483 ^{T} could grow at 25–30 °C (optimum, 30 °C), at pH 5.0–9.5 (optimum, pH 6.5–7.5) and at a 0–4% (w/v) NaCl concentration (optimum, 2%). The details of morphological, physiological and biochemical results are shown in Table 1. Strain CAU 1483 ^{T} had many same features with the three reference strains. For example, all strains were positive for C4 esterase, C8 esterase lipase, naphthol-AS-BI-phosphohydrolase and acid phosphatase activities, and could hydrolyze esculin, starch, D-mannitol and inositol. However, strain CAU 1483 ^{T} differed from *C. candidum* 18JY8-7 ^{T}, *C. thermotolerans* CCUG 47242 ^{T} and *C. lubricantis* KSS-154-50 ^{T} by its inability to display α-galactosidase and β-glucosidase activities. In addition, strain CAU 1483 ^{T} could also be distinguished from the reference strains since it scored positive for the hydrolysis of methyl-α-D-mannopyranoside. The list of all negative traits from commercial kits between CAU 1483 ^{T} and reference strains are shown in Supplementary Table S2.

Table 1 Differences in properties between strain CAU 1483 ^{T} and three closely related species of the genus *Cohnella*

Characteristic	1	2	3	4
Temperature range (˚C)	25–30	18–42	20–55	20–45
Optimum	30	30	30	28
pH range	5.0–9.5	5.5–8.0	6.0–8.0	5.5–6.5
NaCl tolerance (%)	0–4	6.5	0–3	1–3
Catalase	+	+	ND	−
Acid production				
D-fructose	+	+	−	+
D-manitol	+	+	−	+
D-maltose	+	−	−	+
D-Glucose	−	−	−	+
D-arabinose	−	−	−	+
D-Mannose	+	−	−	+
D-Raffinose	−	−	−	+
Gentiobiose	−	−	−	+
methyl-α D-mannopyranoside	+	−	−	−
D-Trehalose	−	+	−	+
Enzyme activity				
Leucine arylamidase	−	−	+	−
β-galactosidase	−	+	+	+
α-galactosidase	−	+	+	+
β-glucosidase	−	+	+	+
DNA G + C content (mol%)	53.70	57.00[a]	59.00[b]	ND[c]

Strains: 1, CAU 1483 ^{T}; 2, *C. candidum* KCTC 33969 ^{T}; 3, *C. thermotolerans* DSM 17683 ^{T}; 4, *C. lubricantis* LMG 29763 ^{T}. Data derived from this study unless otherwise indicated. All strains were Gram-positive, and scored positive for oxidase, citrate hydrolysis, C4 esterase, C8 esterase-lipase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, as well as for the hydrolysis of trisodium citrate, D-mannitol, inositol and esculin. In contrast, these strains were negative for hydrolysis of gelatin and urea, as well as for C14 lipase activity. +, positive; w, weakly positive; -, negative; ND, no data

[a]Maeng et al. (2019); [b]Kämpfer et al. (2006); [c]Kämpfer et al. (2017)

Comparative genomic analysis

The genome of strain CAU 1483 ^{T} consisted of 24 contigs with an N50 of 430 044 bp and a k-mer coverage of 370×. The draft genome of CAU 1483 ^{T} was 4,289,335 bp long and contained 4046 total coding sequences, including genes coding for 20 rRNAs (six 5S rRNAs, five 16S rRNAs and nine 23S rRNAs) and 54 tRNAs. According to whole-genome sequencing, the DNA G + C content of CAU 1483 ^{T} was 53.7 mol%. The whole-genome sequence of strain CAU 1483 ^{T} has been deposited in GenBank under the accession number SUPK00000000. The genome of strain CAU 1483 ^{T} displayed 1397 predicted gene functions. In particular, SEED subsystem analysis highlighted a total of 37 genes associated with stress response, specifically involved in osmotic stress, oxidative stress, detoxification and periplasmic stress (Supplementary Fig. S2). The antiSMASH analysis indicated that the genome of strain CAU 1483 ^{T} includes five biosynthetic gene clusters encoding a type III polyketide synthase and enzymes for bacteriocin, terpene, phosphonate and linear azol(in)e-containing peptide synthesis. The values of ANI between CAU 1483 ^{T} and its most closely related strains, *C. candidum* 18JY8-7 ^{T} and *C. thermotolerans* CCUG 47242 ^{T}, were 74.9 and 72.4%, respectively. Both values were significantly lower than the threshold of 95% above which two strains can be assigned to the same species (Kim et al. 2014). The ANI values relative to the comparison of CAU 1483 ^{T} with other members of the genus *Cohnella* are shown in Supplementary Fig. S3, Supplementary data. Furthermore, according to digital DNA–DNA hybridization (dDDH), the similarity between strain CAU 1483 ^{T} and other members of the genus *Cohnella* was 18.0–33.9% (Supplementary Table S1), which is significantly lower than the threshold of 70% above which two strains can be attributed to the same species (Goris et al. 2007). Finally, the genomic map of CAU 1483 ^{T} based on protein sequences revealed that strain CAU 1483 ^{T} displays 10 − 80% identity with the *C. candidum* 18JY8-7 ^{T} strain (Supplementary Fig. S4). Altogether, these results suggested that strain CAU 1483 ^{T} represents a distinct species of the genus *Cohnella*.

Chemotaxonomic analysis

The most abundant quinone in strain CAU 1483 ^{T} was menaquinone-7, which is in line with the genus *Cohnella*. Moreover, the predominant fatty acids of strain CAU 1483 ^{T} were anteiso-$C_{15:0}$ (55.6%), iso-$C_{16:0}$ (18.9%) and iso-$C_{14:0}$ (7.8%). The overall fatty acid profile of strain CAU 1483 ^{T} was significantly similar to those of the closely related strains *C. candidum* KCTC 33969 ^{T}, *C. thermotolerans* DSM 17683 ^{T} and *C. lubricantis* LMG 29763 ^{T}. Detailed cellular fatty acid profiles of strain CAU 1483 ^{T} and its closely related strains are shown in Table 2. In contrast, the polar lipid profile of strain CAU 1483 ^{T} consisted of phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, three unidentified aminophospholipids, lysyl-phosphatidylglycerol, phosphatidylcholine, two unidentified lipids and an unidentified phospholipid. The polar lipid profile of strain CAU 1483 ^{T} was significantly similar to those of other members of the genus *Cohnella*, but CAU 1483 ^{T} differed from the three reference strains owing to the absence of two unidentified phospholipids (Supplementary Fig. S5). The cell wall peptidoglycan of strain CAU 1483 ^{T} contained diaminopimelic acid (Dpm), alanine (Ala) and glutamic acid (Glu) in a molar ratio of 1.4: 1.0:1.0. The particle hydrolysis detected peptides M-Ala, Ala-Glu, Ala-Dpm, Ala-Glu-Dp, Dpm-Ala-Dpm, Glu-Dpm-Ala-Dpm and Ala-Glu-Dpm-Ala. These data suggested that the composition of major

Table 2 Cellular fatty acid compositions (%) of strain CAU 1483 ^{T} and its closely related species of the genus *Cohnella*

Fatty acid	1	2	3	4
Saturated				
$C_{14:0}$	2.32	2.88	2.94	3.22
$C_{16:0}$	8.09	6.99	4.76	12.40
Branched				
Iso-$C_{14:0}$	7.77	4.99	3.11	3.17
Iso-$C_{15:0}$	2.48	3.47	1.45	1.80
Iso-$C_{16:0}$	18.90	20.96	29.74	22.34
Anteiso-$C_{15:0}$	55.56	54.20	38.94	49.44
Anteiso-$C_{17:0}$	3.34	4.19	4.60	4.74
Unsaturated				
$C_{17:1}$ *ω6c*	–	–	2.00	–
Summed features*				
8	–	TR	7.48	1.46

Strains: 1, CAU 1483 ^{T}; 2, *C. candidum* KCTC 33969 ^{T}; 3, *C. thermotolerans* DSM 17683 ^{T}; 4, *C. lubricantis* LMG 29763 ^{T}. Data derived from this study and strains were cultured under the same conditions. Fatty acids amounting to > 1% of total fatty acids in all strains are shown. – , not detected; TR, tract (< 0.5%)

*Summed features represent groups of two or three fatty acids that could not be separated by gas–liquid chromatography via the MIDI system. Summed feature 8 contains C18:1 ω7c/C18:1 ω6c

of peptidoglycan type of the strain CAU 1483 ^{T} was A1γ *meso*-Dpm.

Taxonomic conclusion

Phylogenetic analyses, conducted through the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms, based on the 16S rRNA sequences showed that strain CAU 1483 ^{T} and *C. candidum* 18JY8-7 ^{T} form a separate branch within the genus *Cohnella*. Moreover, ANI and dDDH analyses, together with morphological, physiological, biochemical and chemotaxonomic characterization, suggested that strain CAU 1483 ^{T} represents a novel species of the genus *Cohnella*, for which the name *Cohnella pontilimi* sp. nov. is proposed.

Description of *Cohnella pontilimi* sp. nov.

Cohnella pontilimi sp. nov. (pon.ti.li'mi L. masc. n. pontus, sea; L. masc. n. *limus*, mud; N.L. gen. n. *pontilimi*, of mud of the sea, where the type strain was isolated).

Cells are Gram-stain positive, aerobic, rod shaped, approximately 1.2–2.3 × 0.4–0.7 µm in size, non-motile and spore forming. Colonies appearing on R2A after 2 days at 30 °C are white, smooth and shiny. Optimum growth occurs at 30 °C on R2A at pH 6.5–7.5 with 2% (w/v) NaCl. On API® 20 NE strips, this strain is positive for β-galactosidase activity and citrate hydrolysis, but negative for arginine dihydrolase and urease activities, as well as for nitrate reduction, H_2S production, indole production and the Voges–Proskauer reaction. On API® 50 CH strips, cells are positive for D-mannitol, methyl-α-D-mannopyranoside, esculin, D-maltose, D-lactose, starch and potassium 5-ketogluconate hydrolysis. In the API® ZYM system, strain CAU 1483 ^{T} scores positive for C4 esterase, C8 esterase lipase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities. The major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{14:0}$. Moreover, the most abundant polar lipids of strain CAU 1483 ^{T} consist of phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, three unidentified aminophospholipids, lysyl-phosphatidylglycerol, phosphatidylcholine, two unidentified lipids and an unidentified phospholipid. The peptidoglycan type is A1γ *meso*-Dpm. The DNA G + C content of this strain is 53.7 mol%. The type strain, CAU 1483 ^{T} (= KCTC 49037 ^{T} = NBRC 113953 ^{T}), was isolated from a soil sample in Modo Island, Republic of South Korea.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02266-x.

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Declarations

Conflicts of interest The authors declare no competing interests.

Ethics approval Not applicable.

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