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Complete genome sequence and expression profile of the commercial lytic enzyme producer

*Lysobacter enzymogenes* M497-1

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Received 14 June 2016; Editorial decision 17 November 2016; Accepted 19 November 2016

Abstract

*Lysobacter enzymogenes* M497-1 is a producer of commercialized achromopeptidase and is expected to harbour genes encoding various other antimicrobial enzymes. Here, we present the complete sequence of the genome of M497-1 and the expression profiles of the genes for various antimicrobial enzymes. Of the 117 peptidase-encoding genes found in the 6.1-Mb genome of M497-1, 15 genes (aside from the gene encoding the achromopeptidase) were expressed at a level higher than that of the average ribosomal protein genes in the 24-h culture. Thus, the strain was found more valuable than hitherto considered. In addition, M497-1 harbours 98 genes involved in the biosynthesis of various natural products, 16 of which are M497-1-specific across 4 *Lysobacter* species. A gene cluster starting at LEN_2603 through LEN_2673 among the 98 genes closely resembled the lysobactin biosynthesis gene cluster of *Lysobacter* sp. ATCC 53042. It is likely that M497-1 may produce lysobactin or related antibacterial compounds. Furthermore, comparative genomic analysis of M497-1 and four other *Lysobacter* species revealed that their core genome structure comprises 3,737 orthologous groups. Our findings are expected to advance further biotechnological application of *Lysobacter* spp. as a promising source of natural bioactive compounds.

Key words: *Lysobacter enzymogenes*, genome sequence, lytic enzyme, RNA sequencing, metabolic potential
1. Introduction

Lysobacter enzymogenes M497-1 (formerly Achromobacter lyticus) was originally isolated from a Japanese soil sample and identified as A. lyticus within the class Betaproteobacteria by Isono et al. It was registered as a producer of the bacteriolytic enzyme lysozyme endopeptidase (US patent no. 3,649,454) in 1972. The achromopeptidase (lysozyme endopeptidase) produced by this strain has broad bacteriolytic specificity and has been used for the lysis of anaerobic gram-positive rods, including various Actinomycetales and Bifidobacterium species, and gram-positive cocci, such as Micrococcus radiodurans, Staphylococcus aureus, and Peptococcus saccharolyticus. The commercialized achromopeptidase is widely employed in metagenomic analyses of the gut microbiome, which contains anaerobic cocci and rods insensitive to lysozyme, because it enables stable and highly efficient lysis (>90%) of human intestinal microorganisms. Other types of proteolytic enzymes produced by strain M497-1 include α- and β-lytic proteases.

Lysobacter species share some properties, including the ability to lyse prokaryotic and eukaryotic microbes, high genomic G + C content (65–70%), and gliding motility, with plant-associated myxobacteria, although Lysobacter species have never been observed to form fruiting bodies, which are typical for myxobacteria. Consequently, Lysobacter species have been occasionally misidentified as myxobacteria in the older literature. But this taxonomic problem was resolved in 1978 by creating a new genus, Lysobacter, within the class Gammaproteobacteria. To clarify the taxonomic position of A. lyticus M497-1, which produces lytic enzymes similar to those of Lysobacter species, we conducted a sequence homology search based on the 16S rRNA gene amplified by PCR from M497-1 DNA remaining in the commercial crude enzyme preparation, and we found 99.9% identity with the 16S rRNA of L. enzymogenes (formerly Myxobacter) strain 495T.

L. enzymogenes is a versatile bacterium producing various types of lytic enzymes specific not only for bacteria but also for eukaryotic cells. Consequently, several L. enzymogenes strains have been characterized as biological control agents against plant diseases caused by fungi such as Bipolaris sorokiniana and Uromyces appendiculatus, filamentous protists such as Pythium aphanidermatum (class Oomycota), and roundworms such as the sugar-beet cyst nematode Heterodera schachtii and the root-knot nematode Meloidogyne javanica. L. enzymogenes-derived chitinases can inhibit conidial germination of plant pathogens and degrade egg shells and hatched juveniles of nematodes, while β-1,3-glucanases from the bacterium can lyse the cell walls of pathogenic fungi. Furthermore, Lysobacter species are known to synthesize other antimicrobial agents such as tripropeptins and lycosin E, which are active against methicillin- and vancomycin-resistant S. aureus, respectively.

Over 40 species within the genus Lysobacter have been registered in the NCBI taxonomy database, and recently, the complete genomes of 4 species (L. antibioticus, L. capsici, L. enzymogenes, and L. gummosei) have been sequenced. The genome sequence data are very useful to elucidate the intragenus as well as intraspecies genomic diversity. Here, we compared the genomes of M497-1 and 4 other Lysobacter species to determine the core genomic structure of the genus Lysobacter, and explored the genetic, metabolic, and physiological potential of M497-1, which could be exploited in biotechnological applications.

2. Materials and methods

2.1. Strain and cultivation

L. enzymogenes strain M497-1 was kindly supplied by Wako Pure Chemical Industries (Osaka, Japan), Ltd. This strain was deposited to the American Type Culture Collection as Lysobacter sp. (ATCC 21456). M497-1 was aerobically grown at 30°C in 500-ml flasks containing 100 ml of medium for 24 or 48 h. For DNA analysis, the bacteria were grown in CY medium containing 0.5% casitone and 0.1% yeast extract, and for RNA expression analysis, they were cultured in medium containing 1.5% glycerol, 0.3% NaCl, 0.5%, 1-glutamate monohydrate (pH 7.4), and 1.5% cotton seed meal (Traders Protein, Southern Cotton Oil Company, Memphis, TN, USA).

2.2. DNA and RNA purification

Genomic DNA was extracted and purified using the Nucleospin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer’s protocol. RNA was extracted and purified from 24-h and 48-h M497-1 cultures using the RNeasy Protect Bacteria Mini kit and QIAzol Lysis Reagent (Qiagen, Tokyo, Japan) according to the manufacturer’s protocol. DNase I-treated RNA was re-purified using the RNeasy Mini Cleanup Kit (Qiagen) and rRNA was removed using the Ribo-Zero rRNA Removal Kit (Gram-Negative Bacteria) (Epicentre, Madison, WI, USA).

2.3. Sequencing, assembly, and data analyses

For whole-genome shotgun sequencing, we combined the Roche GS FLX (Roche Diagnostics K.K., Tokyo, Japan) and Sanger sequencing technologies, which produced ~53- and 23-fold genome coverage, respectively. First, GS FLX reads with an average length of 231 bp were assembled using the Newbler programme version 2.3, and 385 contigs with an N50 length of 33,738 bp were generated. Next, we performed end-sequencing of 77,952 and 1,920 clones from 2- to 35–40 kb (fosmid) insert-size libraries, respectively, using an ABI 3730xl sequencer (Thermo Fisher Scientific K.K., Yokohama, Japan), and hybrid assembly was carried out by the KB Basecaller and Phrap/Consed programmes. The remaining gaps between contigs were filled in using PCR and Sanger sequencing of amplified products. In addition, low-quality genomic regions were sequenced to increase the genome coverage.

RNA-seq libraries were prepared from 24- to 48-h cultures according to the standard Illumina protocol and cDNA libraries were checked for quality and quantity using the DNA-100 kit (Agilent Technologies Japan, Ltd., Tokyo, Japan) and a 2100 Bioanalyzer. Each library was sequenced with the Illumina Sequencing Kit v2 on one lane of a MiSeq desktop sequencer (Illumina K.K., Tokyo, Japan) to obtain 150-bp average paired-end reads. The value of Reads Per Kilobase per Million mapped reads (RPKM) was calculated according to the standard method. The RPKM ratio was calculated by dividing the RPKM of each gene by the mean RPKM of all ribosomal proteins, and was used to determine relative gene expression levels. Promoter-like sequences were detected with GENETYX-MAC version 18 (Tokyo, Japan) and PePPER (http://pepper.mogenrug.nl/index.php/prokaryote-promoters). Peptidase genes were identified by BLAST searches and from the MEROPS database of proteolytic enzymes; MEROPS identifiers were assigned on the basis of a phylogenetic tree: an identifier was assigned to all sequences derived from the same node as a holotype.

2.4. Orthologous analysis and estimation of gene clusters for biosynthesis of natural product

Orthologous groups (OGs) for M497-1 and 4 other Lysobacter species were constructed using the rapid classification programme DomClust on the Microbial Genome Database (MBGD) server. A core genome is defined as a set of genes (OGs) syntenically conserved in at least half of the compared strains. In our study, a set of
genes in the syntenic regions shared by at least three strains was defined as the core genome for the five analyzed Lysobacter strains. A set of syntenic regions and the consensus order of OGs in these regions designated ‘core genome structure’ were created using the CoreAligner programme based on conserved linkages between orthologous genes in each chromosome. Among the OGs in the core genome, those present in all the genomes were called ‘universal core genome’. Non-ribosomal protein synthase (NRPS)- and polyketide synthase (PKS)-encoding genes were manually annotated using web-based prediction programmes. Gene clusters related to biosynthesis of bioactive natural product were estimated based on gene direction and continuity in the M497-1 genome and compared with previously identified gene clusters.

2.5. Evaluation of the metabolic and physiological potential

The metabolic and physiological potential of L. enzymogenes M497-1 was investigated using Metabolic and Physiological Potential Evaluator (MAPLE) ver. 2.1. Genes were mapped to functional modules defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) (pathways: 235, complexes: 284, functional sets: 149, signatures: 40) and the module completion ratio (MCR) was calculated according to a Boolean algebra-like equation described previously.

The MCR patterns of M497-1 were compared with those of other Lysobacter species. L. antibioticus M497-1 and C3, the former has 638 CDSs fewer than the latter. M497-1 and C3 shared 4,043 OGs, while they had 488 and 1,022 unique genes, respectively. Among 488 M497-1 genes unique to the 5 Lysobacter spp. (L. enzymogenes M497-1 and C3, L. antibioticus, L. capsici, L. gummosus), 8 encoded peptidases and 16 encoded NRPSs and PKSs, while the function of 338 unique genes (70%) is not known. Among the functionally unknown genes, 11 showed an RPKM ratio higher than 2 in 24-h culture; LEN_1053 had the highest RPKM ratio (186) among all genes identified in the M497-1 genome. The original RPKM values and RPKM ratios for all genes are listed in Supplementary Table S1.

3. Results and discussion

3.1. General features of the L. enzymogenes M497-1 genome

The genome of L. enzymogenes M497-1 consists of a single circular chromosome (6,096,022 bp) with a mean G + C content of 69.4% (Fig. 1A). We identified 4,891 protein-coding sequences (CDSs), 55 transfer RNA genes, and 6 rRNA genes comprising 2 rRNA operons (Table S1). Although there was no significant difference in genome size between L. enzymogenes strains M497-1 and C3, the former has 638 CDSs fewer than the latter. M497-1 and C3 shared 4,043 OGs, while they had 488 and 1,022 unique genes, respectively. Among 488 M497-1 genes unique to the 5 Lysobacter spp. (L. enzymogenes M497-1 and C3, L. antibioticus, L. capsici, L. gummosus), 8 encoded peptidases and 16 encoded NRPSs and PKSs, while the function of 338 unique genes (70%) is not known.

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3.2. Bacteriolytic enzymes

One hundred seventeen genes encoding peptidases, including the commercialized lysyl endopeptidase (LEN_1308), were identified in the M497-1 genome. These peptidase genes were classified into 56 families according to the MEROPS peptidase database. The largest family, S08A, comprised 21 peptidases, and the S01A family contained 70 genes encoding the TonB-dependent family of proteins, of which 52 and 41 were included in the core and universal core gene clusters, respectively (Supplementary Table S2). Considering that Lysobacter spp. are inhabitants of soil and water, which are characterized by low bioavailability of iron, the high number of TonB-dependent proteins should be helpful for iron acquisition. In fact, 7 genes included in the core OGs (LEN_0009, LEN_0351, LEN_3594, LEN_3807, LEN_4109, LEN_4624, and LEN_4786) in the M497-1 genome showed an expression level equal to or higher than that of ribosomal proteins in 24-h culture; among them, the highest RPKM ratio (more than 10) was observed for LEN_4109.

Ingram-positive bacilli, among which are various industrial enzyme producers, protease production usually starts from the early stationary phase of cultivation (20–24 h), and the productivity is maintained even at the late stationary phase (48 h). In contrast to bacilli, L. enzymogenes M497-1 easily lyses after reaching the stationary phase; cell lysis was actually observed after 24 h of cultivation (data not shown), likely due to autolysis by autoproduced peptidases.

LEN_1308, located in a peptidase island comprising nine contiguous peptidase genes, showed RPKM ratios of 14 and 1.6 in the 24- and 48-h cultures, respectively, and the expression of the neighbouring LEN_1309 and LEN_1310 genes also exceeded the average level of ribosomal proteins (Fig. 2C); moreover, potential -dependent
promoter sequences were identified upstream of these genes (Supplementary Fig. S3). Among the other genes in the peptidase island (LEN_1301–1306), another r54-dependent promoter-like region was detected for LEN_1301, suggesting polycistronic expression of the LEN_1301–1306 region; however, expression was very low compared with the average level of ribosomal proteins (Fig. 2C). On the other hand, expression of 4 peptidase genes (LEN_1590, LEN_1765, LEN_1799, and LEN_3019) was detected, while no putative promoter-like sequences were identified (Supplementary Fig. S3). Our manual search for other types of promoter-like regions was not successful either, probably because promoters depending on different σ factors are likely to have significant sequence variability.

3.3. Lytic enzymes having activity against eukaryotic cells

_L. enzymogenes_ is known to protect plant from diseases caused by various fungi, filamentous protists, and nematodes. Chitinase is a hydrolytic enzyme cleaving glycosidic bonds in chitin, a component of fungal cell walls and of exoskeleton of lower animals, including nematodes. _L. enzymogenes_ M497-1 has 3 chitinase-encoding genes belonging to glycoside hydrolase families 18 and 19; all of these genes were included in the universal core OGs (Fig. 3A). Among them, LEN_2961 demonstrated significant expression in 24-h culture, as evidenced by its RPKM ratio of 3.9, while the other two genes (LEN_3788 and LEN_4456) were expressed at levels below the average of ribosomal proteins; upstream of each gene, putative r54-dependent promoter-like sequences were detected (Fig. 3B).

The M497-1 genome also contains 4 endoglucanase/β-1,3-glucanase-encoding genes belonging to glycoside hydrolase families 6, 8, 16 and 64; 2 of these (LEN_0845 and LEN_3546) were included in the universal core OGs (Fig. 3A). _L. enzymogenes_ C3 lacks an orthologous gene corresponding to LEN_1176 (family 6), while _L. antibioticus_ 76 and _L. gummosus_ 3.2.11 do not have orthologs of LEN_4406 (family 16) (Fig. 3B). Except for LEN_3546, endoglucanase/β-1,3-glucanase-encoding genes were significantly expressed (relative RPKM ratio of 1.5 and higher in 24-h culture); the highest levels were observed for LEN_0845 (family 6), which demonstrated RPKM ratios of almost 10 and 2 in 24-h and 48-h cultures, respectively. However, no r54-dependent promoter-like sequences could be detected for these genes.

3.4. Natural products

Although natural products from gram-positive actinomycetes have been extensively studied,53 those from gram-negative bacteria have received little attention. However, a number of _Lysobacter_ spp. are currently recognized as promising antibiotic producers.54 In the _L.
enzymogenes M497-1 genome, we found 98 genes related to the bio-
synthesis of natural products such as lantibiotics, non-ribosomal
peptide and polyketide compounds, and siderophores (Supplementary Table S5); to the best of our knowledge, this is the
highest number of such genes identified in Lysobacter
strains. However, none of these genes, except for LEN_4535, was included
in the universal core OGs, although some of them had orthologous
relationships to those of other Lysobacter strains. Among the 98
genes, 31 encoded NRPSs, type I PKSs, and NRPS/PKS hybrids com-
prising various catalytic domains, and 12 of them, LEN_2075-2077,
LEN_2515–2517 and LEN_2548–2551 (NRPS/PKS), LEN_2803
(type I PKS), and LEN_4535 (NRPS) were well conserved at similar
positions in the genomes of L. enzymogenes stains M497-1 and C3
(Fig. 4 and Supplementary Table S6). On the other hand, M497-1
was found to have many unique genes, especially for type I PKS
and NRPS/PKS hybrids, which have not yet been identified in other fully
sequenced Lysobacter spp. (Supplementary Table S6). Furthermore,
9 out of 20 genes responsible for NRPS were also unique in M497-1.
These unique genes might have been acquired through horizontal
gene transfer because they showed 40–60% sequence identity with
those of several species within phylogenetically distant genera
Rhodococcus, Polyangium, Bradyrhizobium, except for Xanthomonas,
within the same family. Unfortunately, significant expression was observed only for the NRPS cluster comprising LEN_
4535 and LEN_4536, which showed RPKM ratios of 4–6 in 24-h
culture and 2.5 in 48-h culture although the product was not
identified in the culture medium. Although genes encoding types II
and III PKSs (which are involved in the biosynthesis of aromatic
compounds) and genes involved in the biosynthesis of terpene com-
pounds have been frequently found in Actinomycetales genomes,
such genes were not identified in the M497-1 genome. Interestingly,
the region LEN_2603–LEN_2673 (ca. 145 kb) was extremely similar
to that containing a gene cluster for lysobactin biosynthesis of
Lysobacter sp. ATCC 53042, which is a patent strain (accession no.
JF412274). The lysobactin biosynthesis cluster comprising LG3211_
2475, 2476 is also present in the L. gummosus 3.2.11 genome,37 and
these genes showed significant identities (>70%) to LEN_2615 and
LEN_2613. Through the analysis of a series of genes involved in
lysobactin biosynthesis in Lysobacter sp. ATCC 53042, it was sug-
gested that the region LEN_2608–LEN_2615 is responsible for the
biosynthesis of lysobactin or related compounds.55 Although 2
NRPSs (LybA and LybB) of Lysobacter sp. ATCC 53042 are
involved in the formation of the peptide backbone of lysobactin, 3
NRPSs (LEN_2613–2615) identified in the L. enzymogenes M497-1
genome were found to correspond to LybA and LybB; LEN_2615
corresponds to LybA, while LybB is divided into two NRPSs, LEN_
2614 and LEN_2613. Thus, L. enzymogenes M497-1 is presumably
able to produce lysobactin or related antibacterials. Notably, gene
clusters encoding other antibiotics identified in Lysobacter species,
such as heat-stable antifungal factor (HSAF)/dihydromaltophilin,
WAP-8294A2 (anti-MRSA), and phenazine,37 were not detected in
M497-1.
3.5. Metabolic and physiological potential

To compare the metabolic and physiological potential of M497-1 and other Lysobacter strains, the completion ratios of the four types of KEGG functional modules (pathway, structural complex, functional set and signature modules) were calculated with MAPLE (Supplementary Table S7).48 Most MCR patterns were similar among the 5 Lysobacter strains (Supplementary Fig. S4); however, several modules demonstrated species-specific differences (Fig. 5). Among them, there were seven pathway modules related to amino acid metabolism, nitrogen metabolism, carbohydrate metabolism, and terpenoid backbone biosynthesis. In the ornithine biosynthesis module comprising 5 reaction steps, L. enzymogenes M497-1 and L. gummosus 3.2.11 lacked amino-acid N-acetyltransferase (argA) assigned to the first reaction step. The module for dissimilatory nitrate reduction comprising two reaction steps was complete only in L. antibioticus 76, while the enzymes for each reaction step, nitrate reductase and nitrite reductase, were absent in the other 4 species. Except for L. enzymogenes strains, the other three Lysobacter spp. completed the module for D-galactonate degradation, which is one of the rare modules, i.e. completed by less than 10% of prokaryotes.49

The differences in MCR patterns among Lysobacter strains were also observed in structural-complex and functional-set modules. As a conspicuous example, only L. antibioticus 76 completed the modules for RTX (repeats in toxin) transport belonging to the type I secretion system, putrescine transport, phospholipid transport, aminoglycoside-resistant protease HtpX, and the osmotic stress response (EnvZ-OmpR) two-component regulatory system (Fig. 5). In addition, the modules for type VI secretion and simple sugar transport were completed only by L. enzymogenes C3 and L. capsici 55, respectively. Moreover, strain-specific differences between M497-1 and C3 were detected in 3 rare modules: the a-hemolysin/cyclolysin transport system and two-component regulatory systems associated with central carbon metabolism (BarA-UvrY) and chemosensing (WspE-WspRF). Thus, we could detect exact differences in the metabolic and physiological potential among five closely related Lysobacter species.

3.6. Regulatory factors

The production of bioactive products in Lysobacter strains is regulated by intracellular signaling mediated by cyclic adenosine monophosphate-receptor-like protein (Clp) and the diffusible signal factor (DSF)-dependent system.56-58 The Clp-encoding gene was identified in all Lysobacter genomes, including M497-1 (LEN_0938), and the deduced gene products were 99–100% identical to each other. LEN_0938 was highly expressed as evidenced by its RPKM ratio of 6.6, and the expression of various Clp-regulated genes, including those encoding lytic enzymes such as chitinase, endoglucanase/β-1,3-glucanase, and peptidase, was also markedly induced (Supplementary Table S2). These data are consistent with previous reports that Clp upregulated the expression of chitinase, endoglucanase/β-1,3-glucanase, and peptidase, as well as antifungal factor in L. enzymogenes C3 and OH11.57,58
Genes encoding components of the DSF-dependent system (rpfG, rpfC, rpfF, and rpfB), which is widely conserved among Lysobacter species (with the exception of L. capsici), were also identified in the M497-1 genome (LEN_2756–2759). A previous study did not detect genes corresponding to rpfC and rpfF in L. capsici 55; however, two genes (LC55x_5419 and LC55x_5418) were assigned to the rpfF and rpfC OGs, respectively, by MBGD analysis. These genes showed 30–40% sequence similarity to phylogenetically distant species within different classes such as Betaproteobacteria and Alphaproteobacteria, suggesting that they might have been acquired by L. capsici 55 through horizontal gene transfer.

L. enzymogenes OH11 produces a DSF-like protein, LeDSF3, which positively regulates HSAF biosynthesis through NRPS and the RpfC/RpfG-Clp pathway. However, in the M497-1 genome, the genes responsible for the DSF-dependent system (LEN_2756–2759) showed very low expression when compared with Clp (RPKM ratio 0.2–0.6 versus 6.6, respectively), and genes related to HSAF biosynthesis regulated by the DSF-dependent system were not identified.

With regard to antibiotic production pathways, we found 16 genes related to type I PKS and NRPS to be unique to M497-1 (Supplementary Table S6). Additionally, all of them were silent, although three other orthologous genes (LEN_4535, LEN_2614, and LEN_2615) showed an expression level greater than half the average expression level of ribosomal proteins in a 24-h culture (>0.58).

Further studies focusing on the regulatory mechanisms underlying antibiotic production in L. enzymogenes M497-1 will be required with regard to not only unique genes but also other orthologous genes to identify novel antibiotics synthesized by this strain.

In conclusion, L. enzymogenes M497-1 genome sequencing and expression profiling performed in this study revealed 16 unique genes for biosynthesis of natural product and 15 highly expressed genes encoding peptidases except for commercialized achromopeptidase. It also represented the possibility to produce lysobactin or related antimicrobial compounds. Thus, our findings are expected to promote further biotechnological application of Lysobacter spp. as a promising source of natural, bioactive compounds.

Acknowledgements
We thank Mr T. Hara of Wako Pure Chemical Industries, Ltd. for his valuable contribution to this work.

Data availability
The nucleotide sequences determined in this study were deposited in DDBJ/EBI/NCBI database. The accession number for L. enzymogenes M497-1 whole genome sequence is AP014940 and that for RNA sequences (RNA-seq) of 24- and 48-h cultures is DRA003922.
Conflict of interest

None declared.

Accession numbers

AP014940

Supplementary data

Supplementary data are available at www.dnaresearch.oxfordjournals.org.

Funding

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to H.T. (No. 20310124 and 26550053) and by a grant from the Cross-ministerial Strategic Innovation Promotion Programme to HT and WA. This work was also supported in part by grants from the collaborative research programme of National Institute for Basic Biology (NIBB) to H.T. and I.U. (No. 13-360, 14-359, and 15-355). Computational resources were provided partly by the Data Integration and Analysis Facility of NIBB.

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