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Genomic Analysis of *Sphingopyxis* sp. USTB-05 for Biodegrading Cyanobacterial Hepatotoxins

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Abstract: *Sphingopyxis* sp. USTB-05, which we previously identified and examined, is a well-known bacterial strain for biodegrading cyanobacterial hepatotoxins of both nodularins (NODs) and microcystins (MCs). Although the pathways for biodegrading the different types of [D-Asp<sup>1</sup>] NOD, MC-YR, MC-LR and MC-RR by *Sphingopyxis* sp. USTB-05 were suggested, and several biodegradation genes were successfully cloned and expressed, the comprehensive genomic analysis of *Sphingopyxis* sp. USTB-05 was not reported. Here, based on second and third generation sequencing technology, we analyzed the whole genome of *Sphingopyxis* sp. USTB-05, which is 4,679,489 bp and contains 4,312 protein coding genes. There are 88 protein-coding genes related to the NODs and MCs biodegradation, of which 16 genes (*bioA, hmgL, hypdh, speE, phy, spoC, murD, glsA, ansA, ocd, crnA, ald, gdhA, murC and murI*) are unique. These genes for the transformation of phenylacetic acid CoA (PA-CoA) to CO<sub>2</sub> were also found in *Sphingopyxis* sp. USTB-05. This study expands the understanding of the pathway for complete biodegradation of cyanobacterial hepatotoxins by *Sphingopyxis* sp. USTB-05.

Keywords: *Sphingopyxis*; cyanobacterial hepatotoxins; bacterial biodegradation; genome analysis; phenylacetic acid

Key Contribution: The whole genome sequence of *Sphingopyxis* sp. USTB-05 was obtained and provided an overview of genomic data. The complete biodegradation of several major cyanobacterial hepatotoxins to CO<sub>2</sub> by *Sphingopyxis* sp. USTB-05 was clarified.

1. Introduction

With the rapid development of the world’s agricultural and industrial sectors, a large amount of wastewater and domestic sewage containing nitrogen and phosphorus are discharged into water bodies, resulting in increasing natural water eutrophication. Record-breaking harmful algal blooms and other severe impacts are becoming increasingly frequent [1]. Cyanobacterial hepatotoxins including microcystins (MCs) and nodularins (NODs) are derived from cyanobacteria and are highly toxic, causing a risk to humans and aquatic animals. At least 279 variant structures of MCs have been reported [2]. NODs were also identified to have 12 variant structures [3]. Microcystin-LR (MC-LR), MC-RR, MC-YR, and [D-Asp<sup>1</sup>] NOD have been found and studied. The World Health Organization (WHO) prescribed that the concentration of MC-LR in our drinking water should not be higher than 1.0 µg/L [4]. The lethal dose concentration of nodularins caused by poisoning episodes in certain places is around 50 µg/kg [5,6].

Biodegradation is an efficient and environmentally friendly method to eliminate hepatotoxins. Since *Sphingomonas* ACM-3962 was first reported as a biodegradable MC-LR bacterium in 1994 [7], a variety of bacterial strains for biodegrading MCs from different ecosystems have been found. The majority of strains have been identified as *Sphingomonas* and...
Sphingopyxis of the family Sphingomonadaceae [8–11]. In surface water, Sphingomonas sp. ACM-3962 could biodegrade 1 mg/L MC-LR after a delay period of 2-8 days [12]. However, Sphingomonas sp. ACM-3962 has been demonstrated to biodegrade MC-LR, but not [D-Asp] NOD [12]. Further studies found that three enzymatic reaction processes at least involve in MCs biodegradation by Sphingomonas sp. ACM-3962 [13], as well as gene clusters involved in MCs biodegradation (mlrD, mlrA, mlrB and mlrC) [12,14,15]. Currently, more than 29 MCs biodegrading strains have been identified in Sphingomonadaceae, including 23 strains containing mlr gene clusters and 6 strains having biodegradation gene clusters other than mlr gene clusters [16–18]. Although studies have shown that MCs have many biodegradation pathways, the majority of them have yet to be thoroughly defined [19–21].

In 2010, a bacterial strain of Sphingopyxis sp. USTB-05 (GenBank accession number: EF607053) was successfully isolated from Lake Dianchi in Yunnan Province of China [22,23], and it was capable of biodegrading both MCs and [D-Asp] NOD [22–27]. Initial concentrations of 19.5 mg/L MC-YR, 79.5 mg/L MC-RR, 43.6 mg/L MC-LR and 25.2 mg/L [D-Asp] NOD were completely biodegraded by Sphingopyxis sp. USTB-05 within 4 d [23–25,27]. Further studies indicated that MC-LR, MC-YR, MC-RR and [D-Asp] NOD could be biodegraded at more rapid rates by crude enzymes of Sphingopyxis sp. USTB-05 with initial MC-YR, MC-LR, MC-RR and [D-Asp] NOD concentration of 14.8, 19.5, 28.4, 25.2 mg/L being completely removed within 12 h [22,23,25,27].

Furthermore, the functions of enzymes encoded by the USTB-05-A, USTB-05-B, and USTB-05-C genes were validated in E. coli via heterologous expression [28–34]. The first enzyme encoded by USTB-05-A catalyzes the first step in the biodegrading of MC-YR, MC-LR, MC-RR and [D-Asp] NOD, hydrolyzes the cyclic hepatotoxins into linear hepatotoxins as the first product [28–32]. The second enzyme encoded by USTB-05-B can transform linear MC-YR, MC-LR and MC-RR to a tetrapeptide by breaking the Ala-Tyr, Ala-Arg bonds [27,28,33]. The third enzyme encoded by USTB-05-C can cleave Adda-Glu peptide bonds and convert the tetrapeptide of Adda-Glu-Mdha-Ala to Adda [33].

Although the bacterial strains, enzymes, and genes for biodegrading both MCs and NODs in Sphingomonadaceae have been well studied, research on the genome of bacterial strains for biodegrading hepatotoxins is rarely reported, and a large number of genes encoding enzymes for the biodegradation pathway of hepatotoxins are rarely clarified. To fully comprehend the biodegradation process, it is essential to identify the corresponding genes and enzymes for biodegrading hepatotoxins through genomic data mining. We analyzed the whole genome of Sphingopyxis sp. USTB-05, which 88 genes related to the biodegradation of both NODs and MCs, 16 of which are unique (bioA, hmgL, hypdh, speE, nspC, phy, spuC, murD, glsA, ansA, ocd, crnA, ald, gdhA, murC, and murI). These genes for the transformation of phenylacetic acid CoA (PA-CoA) to CO₂ were also found in Sphingopyxis sp. USTB-05.

2. Results

2.1. General Genome Features of Strain USTB-05

The genome of Sphingopyxis sp. USTB-05 (4.679 Mb), with an overall GC content of 64%, accounts for 62.39% of the total encoding sequences (Figure 1 and Table 1). Without a CRISPR site, the genome sequences of strain USTB-05 comprises 4312 predicted protein-encoding sequences. Forty-eight tRNAs and one tmRNA were identified (Table 1). The 16S rRNA of strain USTB-05 is one copy gene within a single genome.
Figure 1. Circular representation of the single chromosome of Sphingopyxis sp. USTB-05. From inner to outer ring: circles 1 illustrates position in megabases (black); circles two and three denote GC Content and GC Skew, respectively; circles four and five indicate forward and reverse strand CDS (purple), tRNA (light purple), rRNA (light blue), respectively; circle six is COG analysis of reverse strand CDSs; circle seven is COG analysis of forward strand CDSs. Abbreviations: L, replication, recombination, and repair; E, amino acid transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; K, transcription; M, cell wall, membrane, envelope biogenesis; S, function unknown; H, coenzyme transport and metabolism; F, nucleotide transport and metabolism; P, inorganic ion transport and metabolism; O, posttranslational modification, protein turnover, chaperones; C, energy production and transformation; T, signal transduction mechanisms; J, translation, ribosomal structure, and biogenesis; I, lipid transport and metabolism; U, intracellular trafficking, secretion, and vesicular transport; D, cell cycle control, cell division, chromosome partitioning; V, defense mechanisms; N, cell motility; G, carbohydrate transport and metabolism; B, chromatin structure and dynamics.

Table 1. Genome characteristics of Sphingopyxis sp. USTB-05.

| Category   | Sphingopyxis sp. USTB-05 |
|------------|--------------------------|
| bases      | 4,679,489                |
| tmRNA      | 1                        |
| tRNA       | 48                       |
| CDS        | 4312                     |
| GC(%)      | 64%                      |
| plasmid    | 0                        |

2.2. Gene Ontology Annotation

Gene ontology (GO) is a standardized gene functional classification system that tends to a dynamic-updated controlled vocabulary. In the GO database, gene functions are categorized as biological processes, cellular components, and molecular functions. The GO analysis indicates that a total of 4,731 GO terms are associated with all unigenes (Figure 2, Supplementary Table S1). According to the secondary classification of the GO terms, all
unigenes are sorted into 49 functional groups. Biological process is the main category of GO annotations (2,012, 42.53%) unigenes, followed by cellular component (1,787, 37.77%) and molecular function (932, 19.70%). Most of the biological process categories are represented by the cellular process (29.08%) and metabolic process (27.98%), suggesting that the bacterium has strong metabolic activity. There are also some subcategories including response to stimulus (8.20%), cellular component organization or biogenesis (6.51%), biological regulation (6.46%), regulation of biological process (5.22%), growth (4.77%) and localization (3.28%). Cell (32.51%), cell part (32.51%), membrane (12.53%) and protein containing complex (7.67%) are the cell gene clustering of three main components. The catalytic activity (51.50%) and binding (34.23%) represent most of the molecular function category, forecasting that the bacterium has a high degree of molecular catalysis (Figure 2).

Figure 2. Distributions of second level GO of *Sphingopyxis* sp. USTB-05 genome sequence. The y-axis indicates the GO ontology; the x-axis represents the number of unigenes in a category.

2.3. Cluster of Orthologous Groups Classification

The Cluster of Orthologous Groups (COG) is a database used to classify gene products based on their homology. Unigenes *Sphingopyxis* sp. USTB-05 are annotated in the COG database is 62.39%. A total of 4040 classified unigenes are divided into 25 functional categories. The four main groups of amino acid transport and metabolism (352, 8.71%), transcription (309, 7.65%), lipid transport and metabolism (273, 6.76%), and function unknown (825, 20.42%) are the most prevalent. The biodegradation of Adda is completed by carbohydrate transport and metabolism (4.85%), this is also a key category to consider. In addition, the biodegradation of cyanobacterial hepatotoxins is dependent on various biological enzymes during cellular processes and signaling (20.15%). Thus, posttransla-
tional modification, protein turnover, chaperones (4.06%) are also considered an important functional group (Figure 3, Supplementary Table S2).

Figure 3. COG functional classification of Sphingopyxis sp. USTB-05. The columns represent the number of unigenes in each subcategory.

2.4. Genes and Gene Clusters Associated with Hepatotoxins Biodegradation

The whole genome of Sphingopyxis sp. USTB-05 contains many genes related to nutrient absorption, including ssuA, modA, potD, etc. They are all classified as the ABC transporters pathway. Sphingopyxis sp. USTB-05 primarily has five metabolic processes (alanine, aspartate, and glutamate metabolism; arginine and proline metabolism; D-glutamine and D-glutamate metabolism; degradation of aromatic compounds; and valine, leucine, and isoleucine biosynthesis), all of which are related to the biodegradation of hepatotoxin (Table 2).

Table 2. Genes related to cyanobacterial hepatotoxin biodegradation in the genome of Sphingopyxis sp. USTB-05.

| Pathway                                      | Genes                                                                 |
|----------------------------------------------|----------------------------------------------------------------------|
| ABC transporters                             | ssuA, ssuC, ssuB, modA, modB, modC, potD *, potC, potB, porG, malK, msnX, snoK, thuK, mlaD, mlaE, mlaF, pslS, pslC, pslA, pslB, lolC, lolE, lolD, ccmC, ccmB, ccmA, lptF, lptB, lptG, fisX, fisE, ftsE, lapB |
| Alanine, aspartate and glutamate metabolism | ald, pyrB, purA, argG, purB, argH, asnB, ansA, racD, nadB, gabD, gdhB, gdhA, gdhB, glmS, glmA, purF, cad |
| Arginine and proline metabolism              | astB, astA, cma, proB, putA, proC, phy, pip, ocd, hypdh, amiE, spuC, nspC, speE, mruI, mruD, mruC |
| D-glutamate and D-glutamate metabolism       | mleE, bkdA1, pddH, bkdB, ivd, paaF, pccB, hmgL, scoA, atoB, mmsB, bioA, bccA, sbm, mce, aspC, glsA, mssA, proA |

* The bold fonts indicate that these genes are only present in Sphingopyxis sp. USTB-05 genome, but not in Spingomonas morindae sp. NBD5.

In the KEGG pathway database, we performed comparative analysis of these functional genes from Sphingopyxis sp. USTB-05 and Spingomonas morindae sp. NBD5 (Figure S2). These genes potD, potC, potB, porG, malK, msnX, snoK, thuK, and lapB in the metabolic pathways of ABC transporters are peculiar to Sphingopyxis sp. USTB-05. cma, phy, ocd, hypdh, spuC, nspC, and speE are specific to Sphingopyxis sp. USTB-05 in the metabolic pathways of arginine and proline. ald, ansA, and gdhA are unique to Sphingopyxis sp. USTB-05 in the metabolic pathways of alanine, aspartate and glutamate. mruI, mruC, and mruD are characteristic to Sphingopyxis sp. USTB-05 in the metabolic pathways of D-glutamine and D-glutamate. glsA, bioA, and hmgL in the biodegradation pathway of
valine, leucine and isoleucine may be involved in the biodegradation of cyanobacterial hepatotoxins. Interestingly, *Sphingopyxis* sp. USTB-05 were predicted to contain a complete set of genes involved in phenylacetate biodegradation, in addition to the gene encoding AMP-forming phenylacetyl-CoA ligase (PA-CoA ligase; EC: 6.2.1.30) (Figure 4). *paaR, paaE, paaC, paaB, paaA, paaG, and paaN* of phenylacetate biodegradation are important in *Sphingopyxis* sp. USTB-05.

**Figure 4.** The metabolism pathway for biodegrading cyanobacterial hepatotoxins by *Sphingopyxis* sp. USTB-05. In this purposed metabolism pathway, MC-LR, MC-YR, MC-RR and [D-Asp\(^1\)]NOD are enzymatically hydrolyzed by glutamate protease USTB-05-A to produce linearized MC-LR, MC-YR, MC-RR and [D-Asp\(^1\)]NOD, which are further biodegraded by USTB-05-B or USTB-05-C to tetrapeptide or Adda. Tetrapeptide is disassimilated to Adda by tetrapeptidase. After, Adda is biodegraded into PA, which is further biodegraded by the unannotated enzymes to PA-CoA. Then PA-CoA is biodegraded to acetyl-CoA by the corresponding Phenylacetic-CoAase; 2-(1,2-Epoxy-1,2-dihydrophenyl) acetyl-CoAase; 2-Oxepin-2(3H)-ylideneacetyl-CoAase; 3-Oxo-5,6-dehydrosuberyl-CoA semialdehydease and 3-Oxo-5,6-didehydrosuberoyl-CoAase. Ultimately, acetyl-CoA is completely converted to CO\(_2\) via the TCA cycle. The dashed line indexes the sites of bond-breakage. The red boxes represent that these genes are found in *Sphingopyxis* sp. USTB-05 genome. The purple or white boxes represent that these genes are not noted in *Sphingopyxis* sp. USTB-05 genome.

3. Discussion

Biodegradation is an efficient way to remove cyanobacterial hepatotoxins from water bodies. 16S rRNA-oriented phylogeny is used to evaluate the taxonomic placement of bacteria [10]. In 2010, 16S rDNA sequence analysis showed that the *Sphingopyxis* sp. USTB-05 was most similar to the reference strain *Sphingopyxis* sp. C-1 [22]. However, evolutionary relations among the *Sphingopyxis* genus of MC-biodegrading bacteria were re-evaluated.
Sphingopyxis sp. USTB-05 is the most similar the reference strain Sphingopyxis chilensis S37, having a similarity of 99.29. Recent studies demonstrate that genome-wide phylogeny can improve the phylogenetic accuracy and preferably delimit the species borderlines [35]. Sphingopyxis sp. USTB-05 whole-genome sequencing enriches the whole genome data of cyanobacterial hepatotoxins biodegrading bacteria. For instance, the taxonomic study of Sphingosinicella sp. B9, Sphingopyxis sp. C-1 and Novosphingobium sp. MD-1 suggests that they are phylogenetically different from previously described species [36–39].

Certain amino acid metabolic processes may be involved in the biodegradation of [D-Asp$^1$]NOD, MC-LR, MC-YR, and MC-RR. Due to cyanobacterial hepatotoxins [D-Asp$^1$]NOD, MC-LR, MC-YR, and MC-RR are a class of monocyclic pentapeptide and heptapeptide compounds. According to the cyanobacterial hepatotoxins general chemical molecular structures, contain amino acids such as the following: D-isoleucine, D-alanine, D-glutamic acid, D-erythro-$\beta$-methylaspartic acid, variable L-amino acid, N-dehydrogenation alanine, Adda. The variable L-amino acids are arginine and leucine for the congener MC-LR. Adda is a remarkable C20 $\beta$-amino acid: (2S, 3S, 8S, 9S) 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4(E), 6(E)-dienoic acid [40]. MC-LR and MC-LA biodegrading bacteria have been identified in a variety of genera, including Brevibacterium sp., Rhodococcus sp. and Arthrobacter sp. [41–43], but reports of highly biodegradable hepatotoxins MCs in the Sphingomonadaceae are increasing [14]. Sphingopyxis sp. USTB-05 gene clusters for [D-Asp$^1$]NOD, MC-LR, MC-YR and MC-RR biodegradation were identified, including USTB-05-A, USTB-05-B, and USTB-05-C. Molecular studies find that Sphingopyxis sp. USTB-05 contains USTB-05-A, USTB-05-B and USTB-05-C genes, which are highly homologous to mlr A, mlr B, and mlr C, respectively [28]. The first enzyme encoded by USTB-05-A stimulates the first and most critical step in the biodegrading of [D-Asp$^1$]NOD, MC-LR, MC-YR, and MC-RR, including the stimulation of cyclic hepatotoxins to linear hepatotoxins as the first product [27,29–32]. The second enzyme encoded by USTB-05-B converts linear [D-Asp$^1$]NOD, MC-LR, MC-YR, and MC-RR to tetrapeptide by cutting off the Ala-Arg, Ala-Tyr bonds [27,28,31,33]. The third enzyme encoded by USTB-05-C cleaves the Adda-Glu peptide bond, transforming it to Adda-Glu-Mdha-Ala into Adda [33] (Figure 4).

Hashimoto et al. [44] noted that genes referred to the biodegradation of MC-LR comprised a lot more than these four genes. However, there are few genomic data on strains that biodegrade cyanobacterial hepatotoxins, limiting further research on the biodegradation mechanism. Via the KEGG database metabolic pathway annotation, the following genes may be referred to the biodegradation processes of [D-Asp$^1$]NOD, MC-LR, MC-YR, and MC-RR by Sphingopyxis sp. USTB-05. These genes gdhA, ansA, and ald in the metabolic pathways of glutamate, alanine and aspartate are referred to the biodegradation of D-glutamate, D-alanine and D-erythro-$\beta$-methylaspartate. The involving biodegradation genes of L-arginine are speE, crnA, hypdh, nspC, ocd, spuC, and phy in the metabolic pathway of proline and arginine. murC, murD, and murl take part in the biodegradation of D-glutamate. glsA, bioA and hmgL participate in the biodegradation of D-isoleucine (Table 2).

Adda is the detoxification end-product produced by the final enzymatic reaction. Sphingopyxis sp. USTB-05 was forecasted to possess a full set of genes taken part in phenylacetic biodegradation, in addition to the gene encoding AMP-forming phenylacetyl-CoA ligase (PA-CoA ligase) (Figure 4). Recently, genes and transposable elements associated with phenylacetate biodegradation have been identified near the mlr gene cluster [36,45]. A previous report [36,37] demonstrated the identification of a set of genes involved in the phenylacetate biodegradation in Sphingopyxis sp. C-1. However, the gene encoding phenylacetyl-CoA ligase was absent. Sphingopyxis sp. YF1, which leans on the mlr biodegradable metabolic pathway, can also biodegrade Adda by the phenylacetic acid metabolism pathway [46].
4. Conclusions

The whole genome of *Sphingopyxis* sp. USTB-05 consists of a circular chromosome of 4,679,489 bp with 4312 protein-coding genes including 88 genes related to the biodegradation of both NODs and MCs, of which 16 genes (*bioA, hmgL, hypdh, speE, nspC, phy, spuC, murD, gisA, ansA, ocd, crnA, ald, gdhA, murC, and murI*) are unique. These genes for the transformation of phenylacetic acid CoA (PA-CoA) to CO$_2$ were also found in *Sphingopyxis* sp. USTB-05. This study expands the understanding of the pathway for complete biodegradation of cyanobacterial hepatotoxins by *Sphingopyxis* sp. USTB-05.

5. Materials and Methods

5.1. Bacterial Strains

*Sphingopyxis* sp. USTB-05 was isolated and identified from the sediment of Dianchi Lake in Kunming, Yunnan, China [22].

5.2. DNA Extraction and Sequencing

*Sphingopyxis* sp. USTB-05 was initially incubated on the original solid isolation media at 30 $^\circ$C for 48 h. A single colony was selected and cultured in the culture medium of previous report [22]. The genomic DNA was extracted using the Rapid Bacterial Genomic DNA Isolation Kit (CoWin Biosciences, Taizhou, Jiangsu, China) according to the manufacturer’s instructions. NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) analysis and gel electrophoresis were used to determine the purity and concentration of the DNA samples. A small fragment second-generation genomic library with a size of 350 bp was constructed using the NEBNext Ultra™ II DNA kit. The genome was sequenced by using Illumina X10 platform (Madison, WI, USA) [47]. The third-generation genomic library was structured by the standard protocol of Oxford Nanopore Technologies (ONT, Oxford, UK).

5.3. Genome Assembly and Quality Control

Prior to genome assembly, the qualities of the next-generation sequencing reads were optimized by fastp software v0.23.2 before assembly. The sequences with a quality value of Q < 25 and containing linker fragments were deleted. The first fastq formatted data for nanopore sequencing was gathered using FAST5 files included in the MinKNOW software v4.0.4 package. For genome assembly, a total of 11 Mb Nanopore long reads with an N50 length of 8 kb were produced (Figure S1). Spades software (combined with its development process) was used for hybrid assembly, while Pilon software v1.5 was used to correct the assembly results.

5.4. Genome Annotation

The online NMPDR-rust server was used to forecast the gene and coding sequence (CDS). All unigenes were functionally annotated using the Pfam and Swiss-Prot databases. Circos calling a visualization tool was effective in evidencing variation in the genome’s structure. The annotation of eggNOG of protein-coding genes was completed by blast software v2.9.0 [48]. The GO annotation of protein-coding genes were annotated using the Pfam and SwissProt databases. Kobas 3.0 software was used to document KO pathway annotations of protein-coding genes. In addition, the CRISPRFinder software v4.2.19 was used to forecast the clustered regularly interspaced short palindromic repeats (CRISPR) structure of the *Sphingopyxis* sp. USTB-05 genome [49]. The coding sequences of the genome were arranged for using MUMmer version 4.0+ and analyzed in combination with the results of the genome annotation [50].

5.5. Nucleotide Sequence Accession Number

The sequence data were submitted to NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/ (accessed on 6 March 2022)) with accession numbers CP084712, CP084930-CP084933. The sequence data will be released on 31 October 2023.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins14050333/s1. Table S1: Gene classification results: Gene Ontology (GO) classifications of genes. It includes the hierarchy of GO and gene number in this GO term; Table S2: Gene classification results: Clusters of Orthologous Group (COG) classifications of genes. It shows the COG class, abbreviation, gene numbers and IDs in the COG term; Figure S1: The length of the three generations of nanopore data is distributed in turn; Figure S2: Comparison of genes related to hepatotoxin biodegradation in the genomes of Sphingomonas marinae sp. NBDS and Sphingopyxis sp. USTB-05.

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