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Brief Communication

Human Symbiont *Bacteroides thetaiotaomicron* Synthesizes 2-Keto-3-Deoxy-D-Glycero-D-Galacto-Nononic Acid (KDN)

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SUMMARY

The proper functioning of the human intestine is dependent on its bacterial symbionts, the most predominant of which belong to the Phylum *Bacteroidetes*. These bacteria are known to use variable displays of multiple capsular polysaccharides (CPs) to aid in their survival and foraging within the intestine. *Bacteroides thetaiotaomicron* is a prominent human gut symbiont and a remarkably versatile glycophile. The structure determination of the CPs, encoded by the eight CP loci, is the key to understanding the mechanism of this organism's adaptation on a molecular level. Herein, we report the bioinformatics-based discovery and chemical demonstration of a biosynthetic pathway that forms and cytidylates 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN), most likely for inclusion in the CP encoded by *B. thetaiotaomicron* CP locus 7.

INTRODUCTION

The development and function of the human intestine are largely dependent on the resident bacterial community (Backhed et al., 2005; Comstock and Kasper, 2006; Xu et al., 2003; Xu et al., 2004; Mazmanian and Kasper, 2006). These bacteria engage in a lifelong symbiotic relationship that provides the host with a defense against bacterial pathogens, digestion of dietary nutrients, synthesis of vitamins, and development of immune tolerance. The physiology of a human can be strongly affected by the composition of the intestinal microbiota, which is constantly changing due to the prevailing environment of bacterial phages, dietary intake, and toxins (e.g., antibiotics). An imbalance contributes to obesity and inflammatory bowel disease (Mazmanian et al., 2008; Turnbaugh et al., 2006, 2008; Ley et al., 2006).

The most predominate intestinal bacteria belong to the Phylum *Bacteroidetes*, which are unique in their ability to use variable displays of multiple capsular polysaccharides (CPs) to aid in their survival and foraging within the intestine (Krinos et al., 2001; Coyne and Comstock, 2008). *B. thetaiotaomicron* possesses a large repertoire of genes for optimal flexibility in response to conditions and nutrient availability in the gut. The structure and function determination of the CPs encoded by the eight CP loci is key to understanding its inhabitation of the human intestine on a molecular level (Xu et al., 2007). Herein, we report the results of the bioinformatics-based discovery and chemical demonstration of a novel biosynthetic pathway that forms and cytidylates 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) (Figure 1, **1**), most likely for inclusion in the CP encoded by *B. thetaiotaomicron* CP locus 7 (Xu et al., 2007) (Figure 2). The presence of the KDN unit, which distinguishes this CP from those encoded at other loci, enables mimicry of the human epithelial cell polysialic acid. Thus, the KDN unit is important to the specific function of this CP.

The CMP-KDN synthesis unit encodes three previously uncharacterized proteins: BT1713, BT1714, and BT1715 (ExPasy accession numbers Q8A712, Q8A711, and Q8A710). BT1714 is a distant sequence homolog (~30% identity) of the human N-acetylneuraminate (Neu5NAc) (Figure 1, **2**) synthase and the bacterial Neu5NAc, legionaminic acid (Figure 1, **3**), and pseudaminic acid (Figure 1, **4**) synthases. BT1713 and the *E. coli* 2-keto-3-deoxy-D-manno-octulosonic acid (Figure 1, **5**) (KDO) 8-phosphate phosphatase (28% sequence identity) belong to the Yrb C0 subfamily of the haloalkanoate dehalogenase superfamily (HADSF). BT1713 is more distantly related to the human Neu5NAc-9-P phosphatase (HADSF subfamily C1) of the mammalian CMP-Neu5NAc biosynthetic pathway (Figure 3). BT1715 is a member of the cytidyltransferase family, which includes (the eukaryotic and/or prokaryotic) CMP-KDO, CMP-Neu5NAc and CMP-legionaminic acid synthases. The sequence of BT1715 has diverged to the extent that it is difficult to recognize its relationship to any known sialic acid-CMP synthetases. Because BT1714 is not a homolog of KDO 8-P synthetase, and because BT1713 demands a phosphorylated sialic acid substrate (which excludes the legionaminic or pseudaminic acid pathways because both are 6-deoxysugars that cannot be phosphorylated at C(6) (Glaze et al., 2008; Liu and Tanner, 2006), our search of possible pathway products focused on a sialic acid that possesses the 9-carbon ketoacid frame of the CMP-Neu5NAc which is also present in CMP-KDN.

RESULTS AND DISCUSSION

In order to identify the pathway product, the three genes were cloned via a PCR-based strategy and expressed in *E. coli*, and the protein products BT1714, BT1713, and BT1715 were purified using standard column chromatography procedures and

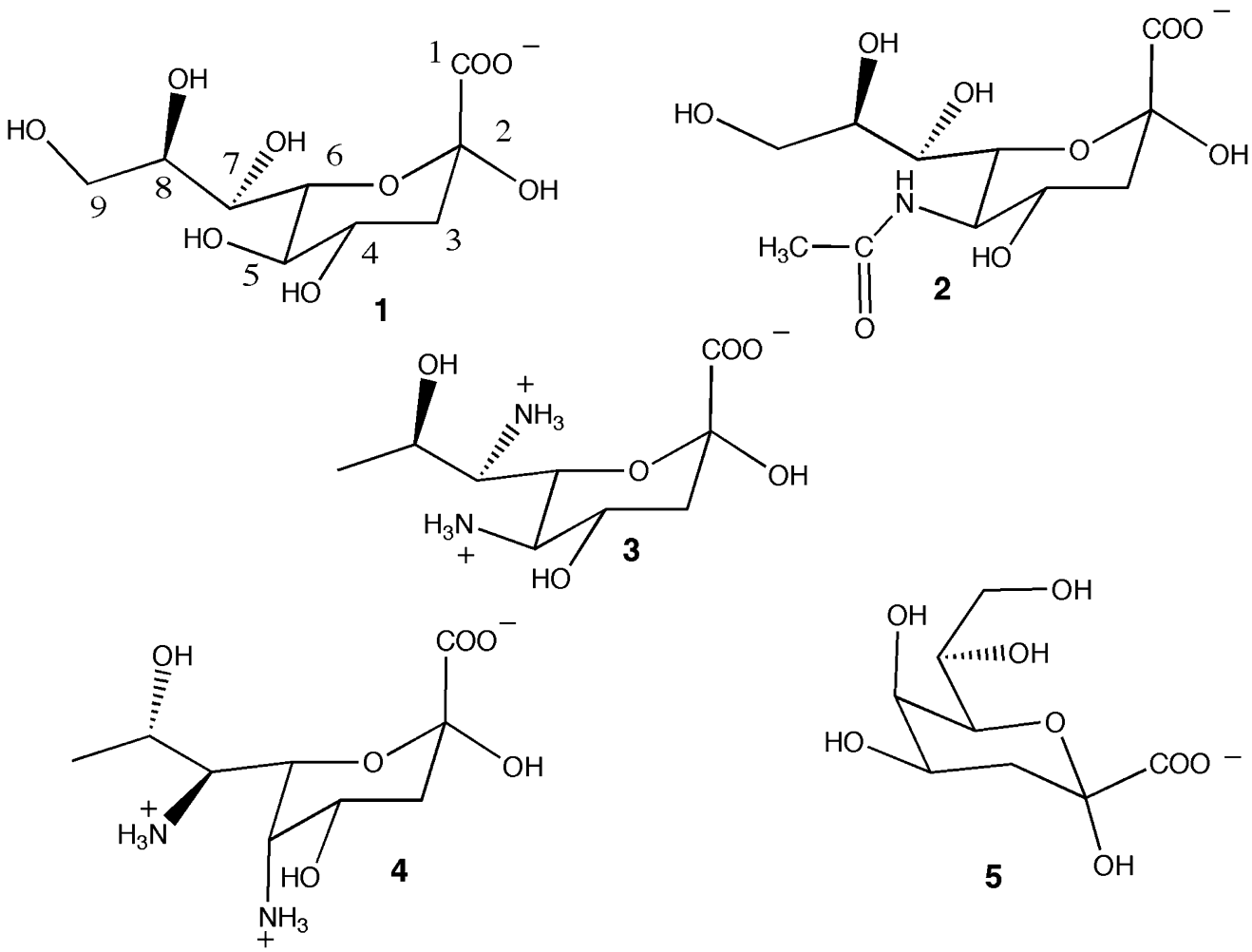

Figure 1. The Structures of Known Sialic Acids: KDN (1), Neu5NAc (2), Legionaminic Acid (3), Pseudaminic Acid (4), and KDO (5)

subjected to substrate specificity analyses (details provided in Supplemental Data available online).

BT1714 Biochemical Function

The BT1714-catalyzed reaction between PEP and mannose 6-phosphate, which is anticipated to produce KDN 9-P, was monitored at 340 nm using a BT1713/Neu5NAc aldolase/NADH-based coupled assay. Reaction solutions contained 15 μM BT1714, 2 mM $MgCl_2$ and varying concentrations of one substrate, and a fixed concentration of the second substrate in 50 mM K^+HEPES (pH 7.5). The steady-state kinetic constants determined at 25°C and pH 7.5 from the initial velocity data

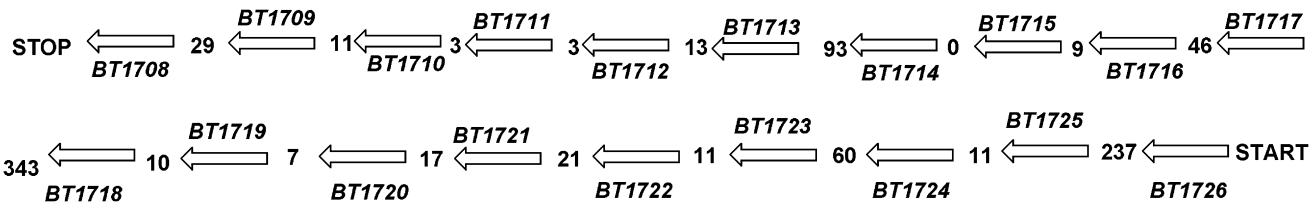

Figure 2. A Map of the *B. thetaiotaomicron* Capsular Polysaccharide Biosynthesis Locus 7

The arrows represent the structural genes and the numbers in between the arrows are the number of intervening nucleotides. The gene function annotation derived from blast searches of nonredundant gene data bases is as follows: BT1726 integrase, BT1725 transcriptional regulator, BT1724 transcriptional regulator, BT1723 O-antigen export protein, BT1722 O-antigen chain length determinant protein, BT1721 nucleotidyl transferase, BT1720 phosphoenolpyruvate phosphomutase, BT1719 phosphonopyruvate decarboxylase, BT1718 2-aminoethylphosphonate transaminase, BT1717 capsular polysaccharide repeat unit transporter ("flippase"), BT1716 CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase, BT1715 CMP-sialic acid synthetase, BT1714 sialic acid synthase, BT1713 HADSF phosphatase, BT1712 gylcosyltransferase, BT1711 unknown, BT1710 capsular polysacharride polymerase, BT1709 gylcosyltransferase, and BT1708 gylcosyltransferase.

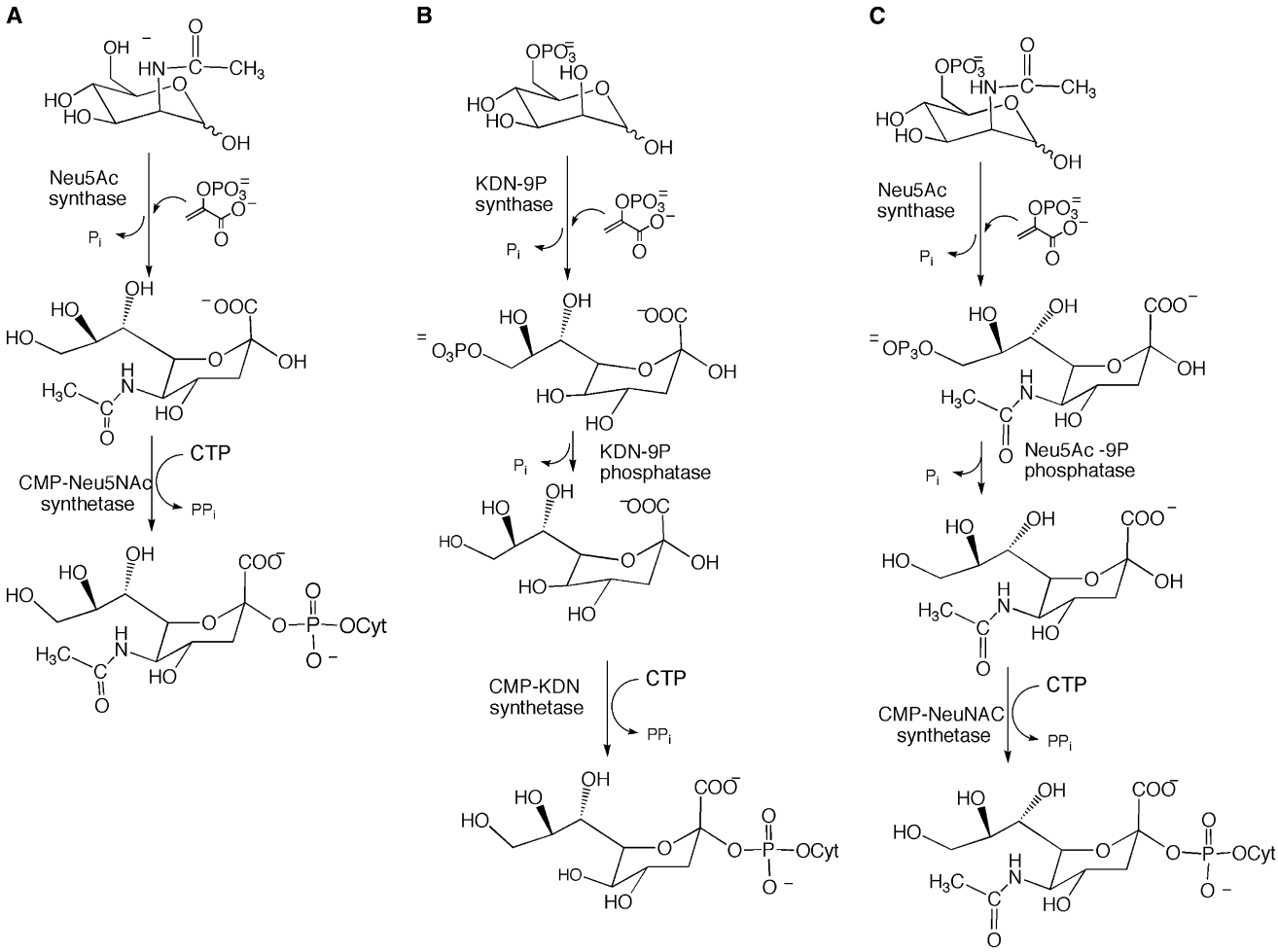

Figure 3. Synthesis and Activation of Neu5Ac in Bacteria, KDN in *B. thetaiotaomicron*, and Neu5Ac in Vertebrates

(A–C) The *E. coli* CMP-Neu5NAc biosynthetic pathway (A), the *B. thetaiotaomicron* CMP-KDN biosynthetic pathway (B), and the human CMP-Neu5NAc biosynthetic pathway (C).

are: k_{cat} = 1.0 ± 0.1 min^{-1}, $^{(PEP)}K_m$ = 0.11 ± 0.01 mM (at 3 mM mannose 6-phosphate), $^{(mannose\ 6\text{-}phosphate)}K_m$ = 1.4 ± 0.1 mM (at 1.5 mM PEP). The maximum value of k_{cat} for the BT1714 (100 μM) catalyzed reaction between PEP (1.5 mM) and NAc-mannose-2-amine 6-phosphate (4 mM), whose anticipated product is Neu5NAc-9-P, was determined to be ~0.0035 min^{-1}. Mannose (product KDN), NAc-mannose-2-amine (product Neu5NAc), mannose-2-amine (product neuramic acid) and arabinose 5-phosphate (product KDO 8-P), arabinose (product KDO) were also tested as substrates. Reaction solutions of each of these sugars (4 mM), BT1714 (100 μM), 1.5 mM PEP, 2 mM $MgCl_2$ and 50 mM K^+HEPES (pH 7.5) were monitored using the spectrophotometric assay as well as by using high resolution mass spectral analysis of the product mixture. Because in each case no product was detected, the upper limit of k_{cat} is set at <0.0025 min^{-1}. This finding shows that BT1714 is specific for mannose 6-phosphate and, as a result, it is dedicated to KDN-9P synthesis. The catalytic efficiency of BT1714 is low but consistent with that reported for the human Neu5NAc-9-P synthase (for reaction with NAc-mannose-2-amine 6-phosphate, k_{cat} = 1.3 min^{-1} and K_m = 1.0 mM; for reaction with mannose 6-phosphate, k_{cat} = 0.6 min^{-1} and K_m = 2.6 mM). BT1714 differs from the human Neu5NAc-9P synthase because it recognizes only mannose-6-phosphate as substrate, whereas the human Neu5NAc-9P synthase shows a small preference for NAc-mannose-2-amine 6-phosphate over mannose 6-phosphate (Hao et al., 2005).

BTI1713 Biochemical Function

In order to define the substrate preference for the phosphatase BT1713, the steady-state kinetic constants for catalyzed hydrolysis of the BT1714 product KDN-9-P and the structurally related phosphorylated sialic acids Neu5NAc-9-P and KDO-8-P were determined. The three reactants were synthesized from PEP and mannose-6-phosphate, NAc-mannose-2-amine-6-phosphate or arabinose 5-phosphate using the catalysts BT1714, human Neu5NAc-9-P and *E. coli* KDO-8P synthase, respectively (the detailed procedures and spectral data for the purified compounds are provided in Supplemental Data). The BT1713 catalyzed dephosphorylation reactions were monitored at 340 nm by using a Neu5NAc aldolase/NADH-based coupled assay. Assay solutions contained 2 mM $MgCl_2$ and 50 mM K^+HEPES

(pH 7.0, 25°C). The k_{cat} and K_m values measured for KDN 9-P (72 ± 6 min^{-1} and 110 ± 10 μM), Neu5NAc-9-P (44 ± 1 min^{-1} and 120 ± 30 μM), and KDO 8-P (3.8 ± 0.2 min^{-1} and 310 ± 30 μM) show that the BT1713 substrate preference is KDN-9-p > Neu5NAc-9-p > > KDO-8-P. The kinetic constants of the human Neu5NAc-9-P phosphatase with Neu5NAc-9-P are k_{cat} = 56 s^{-1} and K_m = 90 μM (Maliekal et al., 2006) and the kinetic constant of the *E. coli* KDO-8-P phosphatase with KDO-8P are k_{cat} = 175 s^{-1} and K_m = 75 μM (Wu and Woodard, 2003). It is noteworthy that the specificity constant measured for BT1714, with its native substrate (k_{cat}/K_m = 1 × 10^4 $M^{-1}s^{-1}$), is comparable to those of HADSF phosphatases, which function in secondary degradation pathways (Tremblay et al., 2006). The greater specificity constants of the human Neu5NAc-9-P phosphatase (k_{cat}/K_m = 6 × 10^5 $M^{-1}s^{-1}$) (Maliekal et al., 2006) and *E. coli* KDO-8-P phosphatase (k_{cat}/K_m = 2 × 10^6 $M^{-1}s^{-1}$) (Wu and Woodard, 2003) suggest that they possess a more highly evolved catalytic function.

BT1715 Biochemical Function

The BT1715 (1.5 μM) catalyzed reaction between cytidine 5′-triphosphate (CTP) and KDN (prepared by using N-acylneuraminic acid adolase, as detailed in Supplemental Data) was monitored at 360 nm using an inorganic pyrophosphatase/2-amino-6-mercapto-7-methyl-purine riboside/purine nucleodide phosphorylase-based coupled assay. Assay solutions contained 10 mM $MgCl_2$ and 100 mM NaCl in 50 mM Tris (pH 7.5, 25°C). The k_{cat} = 1.02 ± 0.03 min^{-1}, $^{(CTP)}K_m$ = 0.51 ± 0.03 mM (at 6 mM KDN), $^{(KDN)}K_m$ = 1.04 mM ± 0.01 (at 0.5 mM CTP). The maximum value of the k_{cat} for BT1715 (40 μM) catalyzed reaction between CTP (0.5 mM) and 10 mM (commercial) Neu5NAc or KDO is below the detection limit of ~0.001 min^{-1}.

Conclusions

The substrate specificities of BT1714, BT1713 and BT1715 identify the physiological product of the *B. thetaiotaomicron* BP locus 7-encoded pathway as CMP-KDN. Like the more common 9-carbon sialic acid Neu5NAc, KDN (Inoue and Kitajima, 2006) is used in the synthesis of a polysialic acid glycan unit of human glycoproteins required for glycan-mediated cellular functions (Varki, 2007). The Neu5NAc and KDN in humans are products of a common pathway, as evidenced by the promiscuity of the pathway enzymes and the presence of a single copy of a sialic acid synthase gene in the human genome. Neu5NAc is synthesized by bacterial pathogens as units within cell surface CPs and/or lipopolysaccharides (Severi et al., 2007). The bacterial sialic acid synthase is homologous to the human Neu5NAc-P synthase but uses the bacterial pool of NAc-mannose to produce NeuAc (Figure 3A) rather than the phosphorylated product. The *B. thetaiotaomicron* KDN pathway (Figure 3B) most closely resembles the human Neu5NAc pathway (Figure 3C) in that the use of a phosphorylated sugar precursor demands the participation of a phosphatase (Angata and Varki, 2002). The human phosphatase (which uses a cap domain in substrate binding) and the *B. thetaiotaomicron* phosphatase (which does not possess a cap domain) evolved within separate subfamilies of the HADSF. The *B. thetaiotaomicron* pathway also is distinguished from the human pathway in that it is only capable of CMP-KDN production. Given that the *B. thetaiotaomicron* genome does not encode a second synthase homolog, we surmise that *B. thetaiotaomicron* displays KDN rather than Neu5NAc at its cell surface.

Using the BT1714 sequence as query in Blast searches of deposited *Bacteroides* genome sequences, we identified a CP locus which contains the neighboring genes encoding BT1714, BT1713, and BT1715 analogs in each of the intestinal bacterial species *B. stercoris* and *B. intestinalis*. This suggests that cell surface KDN display may be used by these symbionts as well but does not imply that all strains of *B. thetaiotaomicron* would necessarily contain this particular locus (Backhed et al., 2005).

SIGNIFICANCE

Human intestinal inflammation and inflammatory bowl disease stem in part from inappropriate immune responses to gut microbiota. The interaction of the symbiont with its host is mediated by the cell surface polysaccharides. In order to understand these interactions on a molecular level, the structures of the polysaccharides must be determined. In this paper, we have identified the pathway for biosynthesis of KDN as a distinguishing unit of one of the seven capsular polysaccharides used for variable display in *B. thetaiotaomicron*. The KDN unit might be used to protect the cell from bacteriophage invasion and/or to mimic the human epithelial cell surface. Bacterial CMP-KDN biosynthesis has not been previously described. Consequently, this work shows that such a pathway exits in a bacterium and that it is unrelated to the bacterial CMP-Neu5NAc pathway (Angata and Varki, 2002). Although the *B. thetaiotaomicron* CMP-KDN biosynthesis genes might have originated from the host CMP-Neu5NAc pathway genes via horizontal transfer, we have shown herein that the encoded enzymes have evolved to become specialized in CMP-KDN synthesis by demonstrating their specificity for the production and utilization of KDN over Neu5NAc.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/9/893/DC1/.

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