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In vitro sulfotransferase activity of *Rhizobium meliloti* NodH protein: Lipochitooligosaccharide nodulation signals are sulfated after synthesis of the core structure

(*nod* genes/nodulation/symbiosis/host specificity/*Medicago sativa*)

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ABSTRACT **The *Rhizobium* common *nod* gene products NodABC are involved in the synthesis of the core lipochitooligosaccharide (Nod factor) structure, whereas the products of the host-specific *nod* genes are necessary for diverse structural modifications, which vary in different *Rhizobium* species. The sulfate group attached to the *Rhizobium meliloti* Nod signal is necessary for activity on the host plant alfalfa, while its absence renders the Nod factor active on the non-host plant vetch. This substituent is therefore a major determinant of host specificity. The exact biosynthetic pathway of Nod factors has not been fully elucidated. In particular, it is not known why some chemical modifications are introduced with high fidelity whereas others are inaccurate, giving rise to a family of different Nod factor structures produced by a single *Rhizobium* strain. Using protein extracts and partially purified recombinant NodH protein obtained from *Escherichia coli* expressing the *R. meliloti nodH* gene, we demonstrate here NodH-dependent *in vitro* sulfotransferase activity. Kinetic analyses with Nod factors, chitooligosaccharides, and their deacetylated derivatives revealed that Nod factors are the preferred substrate for the sulfate transfer. Moreover, the tetrameric Nod factor, NodRm-IV, was a better substrate than the trimer, NodRm-III, or the pentamer, NodRm-V. These data suggest that the core lipochitooligosaccharide structure must be synthesized prior to its host-specific modification with a sulfate group. Since in *R. meliloti* tetrameric Nod factors are the most abundant and the most active ones, high affinity of NodH for the appropriate tetrameric substrate guarantees its modification and thus contributes to the fidelity of host-specific behavior.**

Establishment of nitrogen-fixing symbiosis between rhizobia and leguminous plants relies on a molecular communication via the reciprocal exchange of signal molecules (for review, see ref. 1). The bacterial nodulation (*nod, nol*) genes, induced by host plant flavonoids, are involved in the synthesis and excretion of lipochitooligosaccharide signals (Nod factors). Purified Nod signals are potent plant morphogens triggering several of the developmental processes that lead to the formation of root nodules. Nod signals are major determinants of host specificity—i.e., each *Rhizobium* species produces characteristic Nod factors carrying a combination of structural modifications not found in other species or strains. For instance, Nod signals of *Rhizobium meliloti* carry a sulfate group at the C-6 position of the reducing *N*-acetylglucosamine (GlcNAc) residue (2, 3). This substituent is necessary for biological activity on the host plant alfalfa (*Medicago sativa*), but it renders the molecule inactive on the non-host plant vetch (*Vicia sativa*) (4).

The role of many nodulation gene products in Nod factor biosynthesis has been elucidated by *in vitro* assays or was inferred from genetic data. NodM is a glucosamine synthase providing precursor molecules for Nod factor synthesis (5–7). NodC is involved in the synthesis of the chitooligosaccharide backbone (8, 9). NodB is a chitooligosaccharide deacetylase (10) providing the acceptor for the subsequent transfer of the acyl chain by NodA (11). NodL transfers an acetyl group to the 6-O position at the nonreducing end of the oligosaccharide backbone (12). NodE and NodF are necessary for the synthesis of specific polyunsaturated fatty acids (13–15). NodPQ and NodH are required for the production of sulfated Nod factors (4). The NodP and NodQ proteins are involved in sulfate activation by producing 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (16, 17), while the NodH protein shows sequence similarity to conserved motifs of mammalian sulfotransferases (4).

Although the biosynthetic function of most of the *nod* gene products has been described, relatively little is known about their substrate specificity. All *Rhizobium* species analyzed so far produce a family of structurally related lipochitooligosaccharide derivatives (1). These differ substantially in their activity toward host or non-host plants, and this depends on the length of the oligosaccharide chain, the nature of the fatty acyl substituent, or the presence or absence of the O-acetyl group (3, 14, 18). It is possible that Nod factors of low activity compete with active ones and might thus influence the efficiency of nodule induction. Therefore, it is important to know which parameters determine, for example, the length of the oligosaccharide chain and the efficiency of each type of chemical substitution. With respect to the oligosaccharide chain length, it has been shown by *in vitro* studies that NodC does not produce molecules containing more than five GlcNAc residues (8), whereas NodA transfers the acyl chain more efficiently to deacetylated chitotetraose than to deacetylated tri- or pentasaccharides (19, 20). Thus, the enzymatic properties of NodC and NodA might determine the degree of polymerization found in the chitooligosaccharide backbone of the Nod factors. However, it remains to be shown why some *Rhizobium* species preferentially produce tetrameric and others produce pentameric Nod factors.

Here, we demonstrate NodH-dependent *in vitro* sulfotransferase activity and provide evidence that sulfation takes place after the synthesis of the core Nod factor structure has been completed. Implications for the accuracy of Nod factor sulfation and host specificity are discussed.

MATERIALS AND METHODS

Preparation of Cell Extracts and Partial Purification of NodH. *Escherichia coli* strains containing the plasmid pJS402.2

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Abbreviation: PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

or pJS417.2, expressing NodH or a deletion derivative under the control of the tandem *lpp–lac* promoters, were used for the preparation of cell extracts (21). One liter of LB medium containing ampicillin (100 μg/ml) was inoculated with 10 ml of an overnight culture and bacteria were cultivated for 7 hr at 37°C with vigorous shaking. Bacteria were harvested by centrifugation at 11,000 × *g* at 4°C for 15 min, suspended in 10 ml of ice-cold 20 mM Tris·HCl (pH 7.5), and centrifuged again. After suspension to a total volume of 14 ml in 20 mM potassium phosphate (pH 7.3) containing 1 mM EDTA (buffer A), cells were sonicated for 10 min by a Vibra Cell with a 170-mm conical microprobe (Sonics & Materials, Danbury, CT). After centrifugation at 40,000 × *g* at 4°C, the supernatant was fractionated by three ammonium sulfate precipitation steps. Material precipitating between 25% and 60% saturation was pelleted by centrifugation, redissolved in 1.5 ml of buffer A, and dialyzed against the same buffer. The cell-free extract was stored in aliquots at −20°C. For further purification of NodH protein, 0.5 ml of the cell extract was fractionated on an FPLC Superdex 75 HR 10/30 column (Pharmacia) at a flow rate of 1 ml/min with buffer A as eluent. Fractions of 1 ml were collected and tested for sulfotransferase activity using NodRm-IV as substrate. The active fractions were pooled and concentrated (Centricon 10; Amicon) to 0.25 ml.

Substrates. NodRm-IV and NodRm-V were obtained by mild acid hydrolysis from the sulfated NodRm-IV(S) and -V(S) (18). NodRm-III and NodRm-II were produced by chitinase digestion from NodRm-V(S) and NodRm-IV(S), respectively (22). NodRm-IV(Ac) and NodRm-III(Ac) were prepared by *in vitro* O-acetylation using NodL protein, which was kindly provided by G. Bloemberg and H. P. Spaink (University of Leiden, The Netherlands; ref. 12). Stock solutions of Nod factors (1 mM) in dimethyl sulfoxide were prepared.

Radiolabeled chitooligosaccharides were prepared by N-acetylation of chitosan oligosaccharides with [1-^{14}C]acetic anhydride (11). Chitooligosaccharides deacetylated at the nonreducing GlcNAc residue were obtained by enzymatic deacetylation with NodB protein (10). Specific radioactivities obtained were 31,000 cpm/nmol of $(GlcNAc)_4$, 22,000 cpm/nmol of $(GlcNAc)_5$, 23,000 cpm/nmol of $GlcN(GlcNAc)_3$, and 18,000 cpm/nmol of $GlcN(GlcNAc)_4$.

Chitooligosaccharides (pure grade) were purchased from Seikagaku Kogyo (Tokyo). PAPS was obtained from Sigma and a stock solution of 5 mM in 50% ethanol was prepared.

Sulfotransferase Assay. Various concentrations of Nod factors (1–25 μM) were incubated with fractionated cell extract at a final dilution of 0.5% (vol/vol) in 20 mM potassium phosphate (pH 7.3) containing 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 mM PAPS. After incubation at 30°C for 1–4 hr, the lipophilic substrates and products were extracted with 1-butanol, dried under reduced pressure, and redissolved in 1 μl of dimethyl sulfoxide. The samples were diluted to 50 μl in mobile phase [acetonitrile/water (36:64, vol/vol) containing 40 mM ammonium acetate] and fractionated by reverse-phase HPLC (22). Sulfated products were identified by cochromatography with authentic standards.

^{14}C-labeled chitooligosaccharides (25–200 μM) were incubated with partially purified NodH protein at a final dilution of 2% (vol/vol) in the same buffer as described above but containing 0.5 mM PAPS. After incubation at 30°C for variable times, samples of 1–2 μl were analyzed by TLC (silica gel 60; Merck) using 1-butanol/ethanol/water (45:30:25, vol/vol) as mobile phase. After autoradiography for 2 days with a Kodak XAR-5 film, the ratio of products and substrates was evaluated by the Bio-Image system (Millipore).

For both Nod factors and chitooligosaccharides, incubation times were chosen that allowed the determination of the initial reaction velocities (10–30% conversion). Michaelis and inhibitor constants (K_m and K_i) were calculated by linear regression from double reciprocal Lineweaver–Burk and Dixon plots, respectively.

RESULTS

NodH-Catalyzed *in Vitro* Sulfation of Nod Factors. An ammonium sulfate-fractionated enzyme preparation was obtained from *E. coli* cells expressing the *R. meliloti nodH* gene or a deletion derivative under the control of the tandem *lpp–lac* promoter. Substrates derived from *R. meliloti* Nod factors were obtained either by chemical desulfation, hydrolysis with chitinases, or *in vitro* O-acetylation as described in *Materials and Method.* Nod factors were incubated with proteins in the presence or absence of PAPS, and the butanol-extractable compounds were analyzed by reverse-phase HPLC (Fig. 1). Upon addition of a protein preparation from a *nodH* deletion derivative or in the absence of PAPS no sulfated products were formed (Fig. 1 *A* and *B*). In the presence of NodH protein and PAPS the tetrameric nonsulfated Nod factor, NodRm-IV, was converted to the sulfated derivative (Fig. 1*C*). To analyze the substrate specificity of the transfer reaction, several different Nod factors were tested. Incubation of the pentamer, NodRm-V, or the trimer, NodRm-III, led to

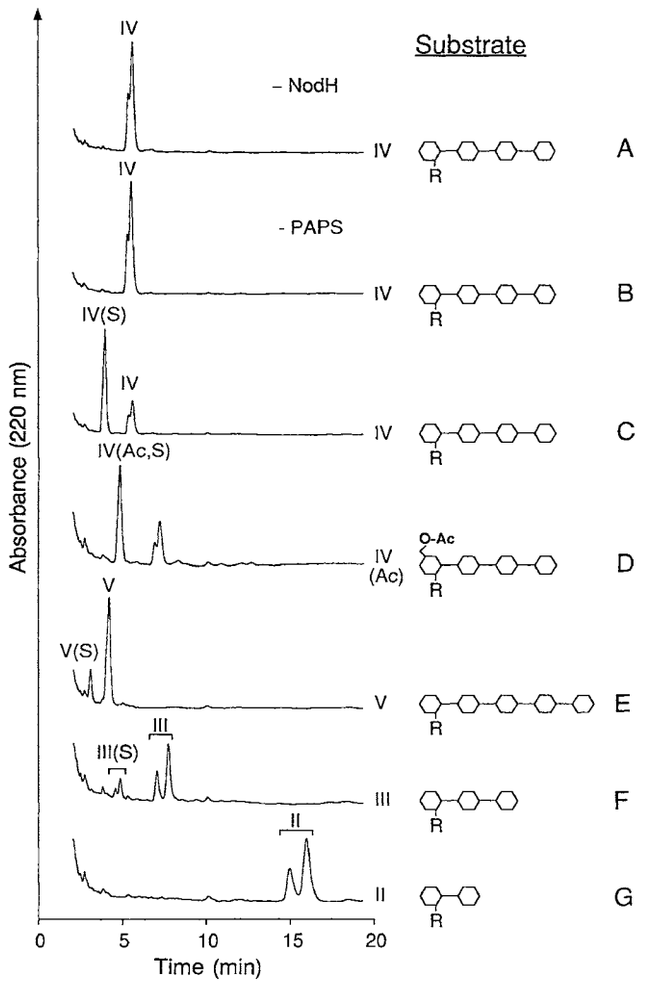

FIG. 1. Reverse-phase HPLC of lipochitooligosaccharides and sulfated products. NodRm-IV, -IV(Ac), -V, -III, or -II (2.5 μM) was incubated for 4 hr with PAPS and enzyme preparation from *E. coli* expressing the *R. meliloti nodH* gene (*C–G*). Incubation with proteins derived from *E. coli* containing the *nodH* deletion plasmid pJS417.2 (*A*) or incubation in the absence of PAPS (*B*) did not yield sulfated products. Duplicate peaks result from separation of anomers. Schematic Nod factor structures are outlined at right. R, C16:2 fatty acid.

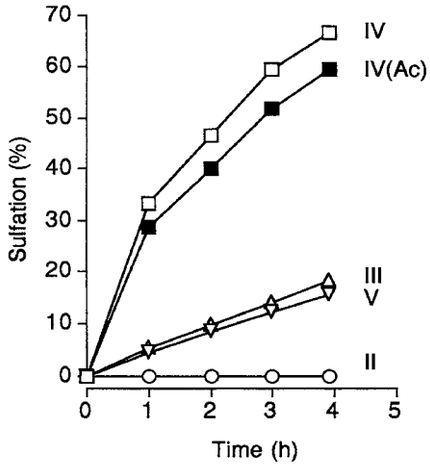

FIG. 2. Time course of the sulfate transfer reaction with 2.5 μM NodRm-IV, -IV(Ac), -V, -III, and -II.

the formation of substantially lower amounts of sulfated products than observed with NodRm-IV (Fig. 1 *E* and *F*). Thus, NodRm-IV was a better substrate for NodH than NodRm-V or NodRm-III, and the dimer, NodRm-II, was not sulfated (Fig. 1*G*). The O-acetyl group at the nonreducing end of NodRm-IV(Ac) (Fig. 1*D*) and NodRm-III(Ac) (data not shown) did not significantly influence the sulfate transfer reaction. The time course shown in Fig. 2 shows that sulfation catalyzed by NodH is dependent on the oligosaccharide chain length. The sulfate transfer reaction was saturable, yielding a K_m of 3.7 μM for NodRm-IV, compared with 14.6 μM for NodRm-V and 10.0 μM for NodRm-III (Fig. 3). These data show that Nod factors are substrates for the NodH-catalyzed sulfate transfer reaction. Moreover, the best substrates contain a tetrasaccharide backbone yielding NodRm-IV(S) and NodRm-IV(Ac, S), which are also the most abundant Nod factors produced by *R. meliloti*.

Inhibition of Nod Factor Sulfation by Chitooligosaccharides. To determine whether chitooligosaccharides are also substrates for NodH, increasing amounts of these carbohydrates or their derivatives deacetylated at the nonreducing terminus by NodB were added to the reaction mixture containing NodRm-IV (Fig. 4). While chitobiose, $(GlcNAc)_2$, did not significantly inhibit Nod factor sulfation, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$ as well as the deacetylated oligosaccharides $GlcN(GlcNAc)_3$ and $GlcN(GlcNAc)_4$ were able to inhibit the reaction. However, a concentration of about 100-fold in excess of NodRm-IV was required to achieve 50% inhibition. As determined by Dixon plots, the inhibition was

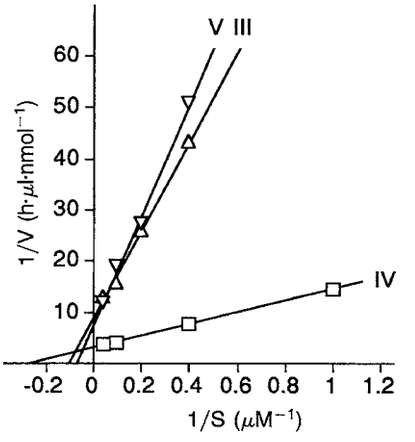

FIG. 3. Kinetics of the sulfate transfer reaction using NodRm-IV, -V, and -III as substrates. The double reciprocal plot was used for the determination of K_m values.

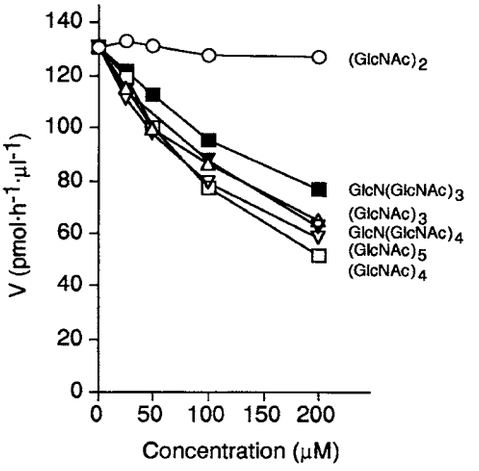

FIG. 4. Inhibition of NodRm-IV sulfation at 2.5 μM by chitooligosaccharides, $(GlcNAc)_n$, and derivatives deacetylated at the nonreducing terminus, $GlcN(GlcNAc)_n$.

competitive and apparent K_i values varied between 77 μM for $(GlcNAc)_4$ and 170 μM for $GlcN(GlcNAc)_3$. A 500-fold excess of $(GlcNAc)_4$ resulted in 90% inhibition (data not shown).

These data suggest that chitooligosaccharides may bind to NodH and may serve as substrates for the sulfate transfer reaction. However, the chitooligosaccharides have a much lower affinity for NodH than the N-acylated chitooligosaccharides.

Sulfation of Chitooligosaccharides. To demonstrate that chitooligosaccharides may indeed serve as substrates for NodH, we used ^{14}C-labeled oligosaccharides to facilitate the detection and quantification of substrates and products after TLC. Preliminary experiments had shown that the amount of protein needed for sulfation of chitooligosaccharides was higher than for the Nod factors. Therefore, NodH was partially purified by gel filtration on FPLC Superdex 75 HR. Chromatographic data of the active fraction indicated a molecular mass of about 60 kDa for the native NodH protein (data not shown). The molecular mass predicted from the primary structure (26 kDa) or determined by SDS/PAGE (28.6 kDa; ref. 21) suggests that NodH acts as a dimer.

$(GlcNAc)_4$ and $(GlcNAc)_5$ as well as $GlcN(GlcNAc)_3$ and $GlcN(GlcNAc)_4$ were converted to sulfated products in the presence of NodH and PAPS (Fig. 5). Kinetic analyses with chitooligosaccharides as substrates yielded K_m values of 140 μM for $(GlcNAc)_4$, 190 μM for $(GlcNAc)_5$, 170 μM for $GlcN(GlcNAc)_3$, and 160 μM for $GlcN(GlcNAc)_4$ (data not shown). These data indicate that the affinity of NodH for chitooligosaccharides is >1 order of magnitude lower than for Nod factors. The same is true for chitooligosaccharides deacetylated at the nonreducing terminus. This suggests that in the pathway of Nod factor biosynthesis, NodH preferably sulfates the basic lipochitooligosaccharide molecule instead of the biosynthetic intermediates.

DISCUSSION

We have demonstrated by *in vitro* studies that the host-specific *nod* gene product NodH exhibits sulfotransferase activity. The enzyme can catalyze the transfer of a sulfate group from PAPS preferentially to lipochitooligosaccharides rather than to chitooligosaccharides or the deacetylated intermediates of Nod factor biosynthesis. Although the latter two can serve as substrates for the sulfotransferase, their affinity for NodH, as estimated by Michaelis–Menten kinetics, is >40-fold lower than that of NodRm-IV. *In vivo* labeling studies with D-[^{14}C]glucosamine have recently shown that, at least in *Rhizobium leguminosarum* bv. *viciae*, chitooligosaccharide deriv-

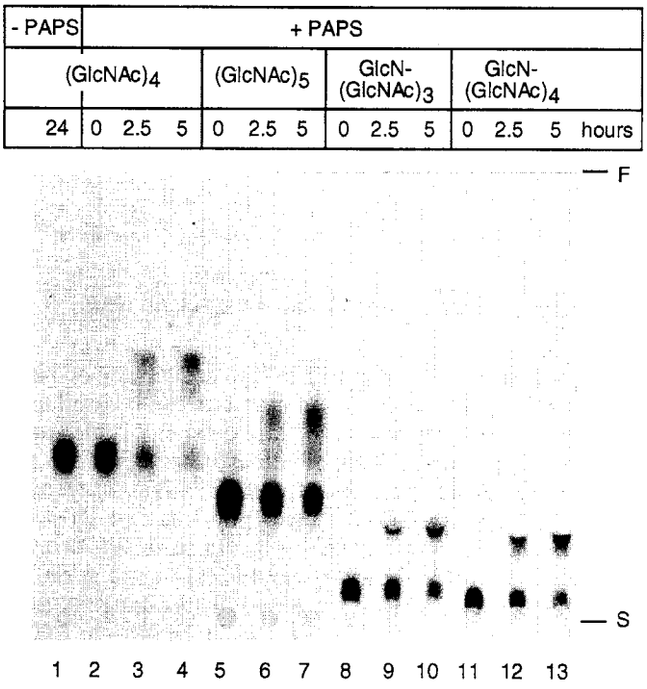

FIG. 5. TLC of ^{14}C-labeled $(GlcNAc)_4$, $(GlcNAc)_5$, $GlcN(GlcNAc)_3$, and $GlcN(GlcNAc)_4$ and their reaction products after incubation with partially purified NodH in the presence of 0.5 mM PAPS. Incubation times are indicated. Lane 1, incubation of $(GlcNAc)_4$ for 1 day in the absence of PAPS. S and F indicate start and solvent front, respectively.

atives accumulate within the bacterial cell in much lower amounts than the acylated oligosaccharides (9). Therefore, it is very likely that also *in vivo*, sulfation takes place only after the lipochitooligosaccharide core structure has been synthesized. Moreover, tetrameric Nod factors, NodRm-IV and NodRm-IV(Ac), were sulfated more efficiently than the trimeric or pentameric forms. The specificity of NodH may guarantee the efficient conversion of nonsulfated tetramers into the sulfated products, which are the most abundant and most active ones in *R. meliloti*.

R. meliloti strains carrying mutations in the *nodH* gene are unable to produce sulfated Nod factors (4) and do not nodulate *Medicago*, but instead gain the ability to induce nodules on *Vicia* (21). Strains carrying mutations in the *nodP* or *nodQ* gene are impaired in their ability to sulfate Nod factors. They produce a mixture of both sulfated and nonsulfated molecules (4) and are thus able to form nodules on both *Medicago* and *Vicia* (17). Therefore, the fidelity of Nod factor sulfation is an important parameter determining host specificity.

Interestingly, the *nodPQ* genes are redundant—i.e., a second pair of genes is present on the second megaplasmid of *R. meliloti*. The presence of two functional loci in addition to the housekeeping sulfate activation locus (*saa*; ref. 17) seems to indicate an evolutionary advantage for efficient Nod factor sulfation that guarantees the interaction of *R. meliloti* with its specific host plant.

In contrast to *nodPQ*, the *nodH* gene is present as a single copy. However, the substrate specificity of NodH provides a means to ensure the accuracy of Nod factor sulfation. Unlike acylation, where an array of different structures becomes irreversibly fixed by the inaccuracy of the NodA-catalyzed acyl chain transfer, the highly efficient sulfate transfer allows a quantitative conversion of nonsulfated precursors. It appears that other steps in the biosynthesis of *R. meliloti* Nod factors—e.g., the synthesis of the chitooligosaccharide backbone by NodC or the NodL-catalyzed O-acetylation—are less accurate. However, this leads to the synthesis of Nod factors that still show significant activity on alfalfa—e.g., the non-O-acetylated pentamer NodRm-V(S) (3).

In summary, we have demonstrated the *in vitro* sulfation of lipochitooligosaccharides by *R. meliloti* NodH protein and have defined some of the properties of the sulfotransferase. The *in vitro* sulfate transfer reaction may be used in a future total enzymatic synthesis of Nod signals and their analogues.

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