Tn5-mutagenesis and identification of *atr* operon and *trpE* gene responsible for indole-3-acetic acid synthesis in *Azospirillum brasilense* Yu62 *

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Abstract To bring more information about synthesis of indole-3-acetic acid (IAA) from *Azospirillum brasilense*, a Tn5-insertion library of *A. brasilense* Yu62 was constructed and subjected to screening for IAA producing mutants. Two mutants with decreased IAA levels, named as A3 and A24, were isolated. The sequence analysis of loci tagged showed that the Tn5-1063a was located in the *atrA* gene encoding GntR family transcriptional regulator and *trpE* gene encoding component I of anthranilate synthase respectively. At the same time, *atrB* encoding phosphotransferase and *atrC* encoding aminotransferase were cloned downstream the *atrA* gene and *atrA*, *atrB* and *atrC* were clustered in an operon. Mutagenesis and complementation studies showed that *atrA* and *atrC* were involved in IAA synthesis. IAA levels of *trpE* mutant and wild-type strain could be improved by adding anthranilate into the medium.

Keywords: **indole-3-acetic acid (IAA), IAA mutants, *Azospirillum brasilense*, *atrABC cluster*, *trpE*, Tn5 transposon.**

Azospirillum brasilense is a nitrogen-fixing microorganism that has a potential use as an inoculant for promoting plant growth in grass and cereals[1]. In the recent decades there has been increasing evidence that besides nitrogen-fixation, synthesis and export of indole-3-acetic acid (IAA) in *Azospirillum* play an important role in the observed plant growth promotion[2,3]. In bacteria several IAA biosynthesis pathways, such as indole-3-acetamide (IAM) pathway, indole-3-pyruvic acid (IPyA) pathway, tryptophan (Trp) side chain pathway (TSO), tryptamine (TAM) pathway and indole-3-acetonitrile (IAN) pathway, have been proposed according to their intermediates, and more than one pathway may exist in certain species[4,5]. In *Azospirillum*, attempts to isolate a mutant completely deficient in IAA production have not been successful[6,7], suggesting that more than one pathway is involved in IAA synthesis. Biochemical and genetic evidence for IPyA pathway has been provided and this pathway was found to be the main route for IAA production in the presence of exogenous Trp in *A. brasilense*[4]. In general, the first step in IPyA pathway is the conversion of Trp to IPyA catalyzed by amiontransferase. Then IPyA decarboxylase (IPDC) catalyses the conversion of IPyA to indole-3-acetaldehyde (IAald) and the latter can be oxidized to IAA by non-specific aldehyde dehydrogenase. Two aromatic amino acid amionotransferases have been purified from *A. brasilense* UAP 14[8]. The *ipdC* gene, encoding a key enzyme of the IPyA pathway, has been cloned in the *A. brasilense* strain Sp245 and Sp7[9,10], and an *ipdC* mutant of strain Sp245 produced only 10% of the wild-type IAA level[11]. Though it was demonstrated that IAM pathway did exist in *A. brasilense*[12], The genes of the IAM pathway coding for the Trp monoxygenase and indoleacetamide hydrolase have not yet been isolated[12,13], and the IAM pathway could account for only less than 0.1% of IAA synthesis[14]. Using permeabilised cell suspensions of wild type or the *ipdC* mutant strain, IAA production was also detected with TAM and IAN as the substrates[5].

The molecular background and mechanism of regulation for IAA synthesis are still not well understood. The *ipdC* gene is up to now the only cloned gene involved in IAA biosynthesis in *A. brasilense*. We carried out Tn5 transposon mutagenesis and isolated two mutants showing decreased IAA production. The genes involved in IAA synthesis were identified and characterized in this study.

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1 Materials and methods

1.1 Strains, plasmids and culture conditions

The bacterial strains and key plasmids used in this work are listed in Table 1. A map that indicats the position of the kanamycin cassettes (*km* cassettes) used for mutants constructions is presented in Fig. 1. *A. brasilense* Yu62 is able to produce more than 50 μg/mL IAA into the medium supplemented with exogenous Trp[15]. The rich culture medium used was LB medium for *Escherichia coli* and LD medium for *A. brasilense*[16]. For the IAA production minimal medium (MAZ)[17] was used, supplemented with 37 mmol/L malate as carbon source, 100 μg/mL Trp as IAA precursor and 10 mmol/L NH_4Cl as nitrogen source. Antibiotics were used as following final concentrations: ampicillin (Ap) 25 μg/mL and nalidic acid (Nx) 5 μg/mL for *A. brasilense* Yu62 wild-type; kanamycin (Km) 30 μg/mL for *A. brasilense* mutants and complementary strains, and 50 μg/mL for *E. coli*; tetracycline (Tc) 12.5 μg/mL for complementary strains and for *E. coli*.

Table 1. Strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics	Source/reference
A. brasilense		
Yu62	Wild type, Ap^r, Nx^r	Yang et al.[15]
A3	IAA mutant, Ap^r, Nx^r, Km^r	This study
A24	IAA mutant, Ap^r, Nx^r, Km^r	This study
B78	Yu62 derivative, *atrB-km* mutant	This study
C39	Yu62 derivative, *atrC-km* mutant	This study
E. coli		
S17-1	*pro thi hsd recA* RP4-2Tc::Mu-Km::Tn7 Sm^r Tp^r Tra^+	Simon et al.[18]
DH5α	Host for recombinant plasmids *lacZ* α complement	Hanahan et al.[19]
Vectors		
pRL1063a	Carrying Tn5-1063, Km^r	Wolk et al.[20]
pUC4K	Sources of *km* cassettes	Pharmacia
pPHU281	Suicide vehicle, *lacZ* mob, Tc^r	Hübner et al.[21]
pLAFR3	Broad host range vector for complementation, Tc^r	Staskawicz et al.[22]
Other Plasmids		
pPHU281-*atrB*	pPHU281 derivative containing the *atrB* region	This study
pPHU281-*atrC*	pPHU281 derivative containing the *atrC* region	This study
pPHU281-BK	pPHU281 derivative containing the *atrB* gene inactivated by *km* cassette	This study
pPHU281-CK	pPHU281 derivative containing the *atrC* gene inactivated by *km* cassette	This study
pLAFR3-*atrA*	pLAFR3 derivative containing the *atrA* region, for complementation	This study
pLAFR3-*atrC*	pLAFR3 derivative containing the *atrC* region, for complementation	This study

1.2 Tn5 mutagenesis and selection of IAA mutants

Plasmid pRL1063a carrying Km^r-transposon Tn5 was introduced into recipient *A. brasilense* Yu62 by biparental mating with *E. coli* S17-1 as a donor as described by Abdel-Salam and Klingmüller[7]. Transconjugants were selected on plates containing Ap, Nx and Km. Colonies were streaked twice on the selective medium to obtain single colonies. IAA-secretion mutants were selected by measuring their IAA production in MAZ medium with colorimetric and HPLC assays.

1.3 Colorimetric assay for indolic compounds

Indolic compounds were estimated using the colorimetric assay as described[23]. The PC reagent (12 g/L $FeCl_3$ in 7.9 mol/L H_2SO_4) was used and indolic compounds were determined spectrophotometrically at 540 nm as described[5].

1.4 Verification of IAA levels by HPLC

Since colorimetric assay is a preliminary and rapid method and only gives a preliminary quantification of indolic compounds including IAA, IAM, IPyA and IAald, IAA levels were verified by HPLC. The samples for HPLC were prepared as described[5]. HPLC analysis was performed on a 4.6 mm × 15 cm, 5 μm Agilent C-18 reverse column on a Waters Liquid Chromatograph. The mobile phase was methanol: 1% acetic acid in water 40:60 (vol/vol), at 1 mL/min flow rate. Eluates were detected by absorbance at 280 nm. IAA was determined based on a standard

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curve.

1.5 Construction of the *atrB* and *atrC* mutants

Primer P1: 5′-CGTTGAATTCCCCATCCGCTTCCTCAG-3′ (*Eco*RI site underlined) and primer P2: 5′-AATTCTGCAGGTAGGCGTCCAGATAGCG-3′ (*Pst*I site underlined) were designed for amplifying *atrB* gene. Primer P3: 5′-CGCTGAATTCCATGACCATGATCAACGCCT-3′ (*Eco*RI site underlined) and primer P4: 5′-CGTTCTGCAGAAGCGCCGTCACTTACAGC-3′ (*Pst*I site underlined) were designed for amplifying *atrC* gene. The PCR products were cloned into the suicide vector pPHU281 to yield plasmids pPHU281-*atrB* and pPHU281-*atrC*. *km* cassettes from pUC4K were inserted into the *atrB* and *atrC* genes of plasmids pPHU281-*atrB* and pPHU281-*atrC* to yield plasmids pPHU281-BK and pPHU281-CK respectively. The resulting plasmids were transferred into *E. coli* S17-1 to generate the *atrB* and *atrC* mutants by biparental mating with *E. coli* S17-1 and *A. brasilense* Yu62. The disrupted *atrB* and *atrC* were integrated into the genome of *A. brasilense* Yu62 by double cross-over recombination, respectively. The two double crossing-over mutants, named B78 and C39 (Fig. 1), were selected on medium containing Km and verified by PCR amplification and DNA-DNA hybridization (data not shown).

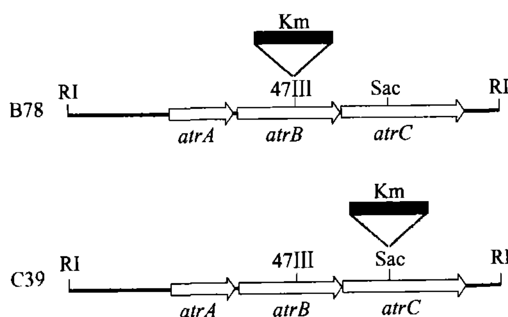

Fig. 1. Physical map of the *atr* cluster and the B78 and C39 mutants. RI, *Eco*RI; 47III, *Eco*47III; Sac, *Sac*II.

1.6 Recombinant DNA techniques and sequence analysis

Isolation of chromosomal DNA and plasmids, restriction enzyme digestions, ligations, transformation, PCR amplification and Southern blotting were performed as described[24]. Plasmids were transferred by conjugation from *E. coli* S17-1 to *A. brasilense* strains[7]. Sequencing was done by Bioasia Co., Shanghai, China. BLAST searches and sequence analysis were performed on the NCBI server (http://www.ncbi.nlm.nih.gov/), http://smart.embl-heidelberg.de/ and http://cubic.bioc.columbia.edu/predictprotein/. The probe for Southern blotting was the PCR product with the plasmid pRL1063a as template and P1: 5′-TTTGTTCGGCTTGGTATCGC-3′ and P2: 5′-ATTGCTTAGGTCCATTCTCA-3′ as primers.

1.7 Complementation study

To complement the *atrA* and *atrC* mutants respectively, A 1379 bp fragment of *artA* was amplified with the primers: 5′-AATTGGATCCAGCGGGTGATGATAGAG-3′ (*Bam*HI site underlined) and 5′-CGTTAAGCTTACTTCAGCACATAGCCC-3′ (*Hin*dIII site underlined); the *atrC* gene was recovered from the plasmid pPHU281-*atrC* by excision with *Eco*RI and *Pst*I. The PCR fragment of *atrA*, digested with *Bam*HI and *Hin*dIII, and the fragment of *atrC* were cloned into the corresponding sites of the broad host range vector pLAFR3, to yield plasmids pLAFR-*atrA* and pLAFR-*atrC* respectively. These constructs were then introduced into the mutants A3 and C39 to do complementation.

2 Results

2.1 Isolation of IAA mutants

Mutagenesis was carried out by conjugative transfer of Tn5 from *E. coli* S17-1 to *A. brasilense* Yu62 as described in materials and methods. More than 5000 Km^r transconjugants were obtained and screened for mutants with abnormal IAA production. Two mutants with decreased IAA production were selected by measuring the IAA production with colorimetric and HPLC assays. Southern blotting analysis with Tn5 fragment as a probe showed that only one hybridizing band was in each mutant strain and no hybridizing band was in the wild-type strain, suggesting that the IAA mutants are single transposon insertion mutants (Fig. 2).

2.2 Characterization of IAA mutants

The two Tn5-insertion mutants A3 and A24 showed normal growth behavior and decreased IAA production compared with those of the wild-type strain (Fig. 3). The result that no mutants were totally devoid of IAA production was in agreement with the previous report of Hartmann and Zimmer[6].

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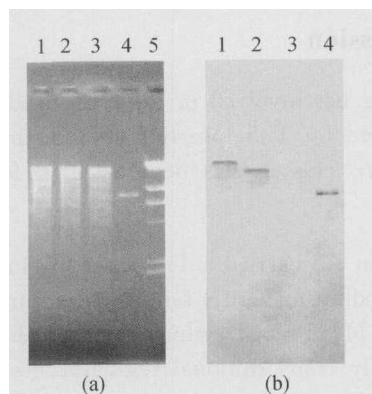

Fig. 2. Southern blotting analysis of *Eco*RI digested DNA fragments of the wild-type and mutant *A. brasilense*. (a) Agarose gel electrophoresis; (b) southern blot hybridization result. 1, A3; 2, A24; 3, wild-type; 4, pRL1063a; 5, λ DNA/*Hin*dIII.

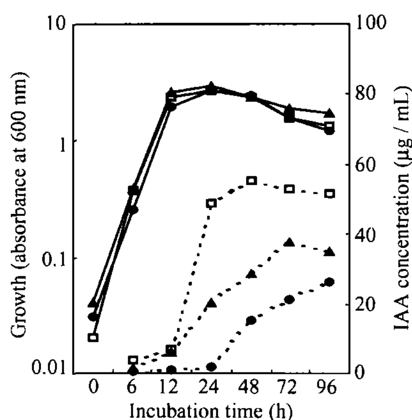

Fig. 3. Growth (—) and indole-3-acetic acid (IAA) production (---) curves of *A. brasilense* wild-type (□) and mutants A3 (●), and A24 (▲).

2.3 Cloning and sequence analysis of the genes involved in IAA synthesis

To characterize the genes of *A. brasilense* Yu62 involved in IAA synthesis, the *Eco*RI fragments of the chromosomes from the two mutants carrying a single transposon were cloned. Because the plasmid pRL1063a is a modified Tn5 transposon containing an origin of replication that functions in *E. coli*, DNA contiguous with the transposon can be recovered by excision, recircularization, and transferred to *E. coli*[20]. The transferred plasmids in *E. coli* can be sequenced directly with the primers (5′-TATCAATGAGCTCGGTACCC-3′ and 5′-GATGAAGAGCAGAAGTTATC-3′) that were designed based on the sequence of pRL1063a. The DNA sequences flanking the transposon were determined by splicing the sequencing results on the forward repeated positions caused by Tn5 insertion.

A 4370 bp DNA sequence flanking the Tn5 in mutant A3 was determined. DNA analysis showed that it includes 3 ORFs (GenBank accession number AY850387), which were designated as *atrA* (from 1087 bp to 1770 bp), *atrB* (from 1814 bp to 2911 bp) and *atrC* (from 2918 bp to 4234 bp). The 3 ORFs clustered together in an operon with the *atrA* gene interrupted by Tn5 insertion. The deduced amino acid sequences of *atrA*, *atrB* and *atrC* showed the highest identities with GntR family transcriptional regulator, phosphotransferase and aminotransferase, respectively.

The disrupted gene by Tn5-insertion in mutant A24 was *trpE* (GenBank accession number AY850391), whose predicted product has significant similarity with component I of anthranilate synthases (TrpEs) from many bacteria, such as 61% identity with *Rhodospirillum rubrum* TrpE and 58% identity with *Rhodobacter sphaeroides* TrpE.

2.4 Characterization of the *atr* mutants

To confirm that one or more genes of the *atr* operon are responsible for the IAA⁻ phenotype, two mutant strains were generated by inserting *km* cassettes into the *atrB* and *atrC* to yield the strains B78 and C39. The results indicated that the *atrB* mutant strain B78 kept the same IAA level compared with that of the wild type. But the *atrC* gene mutant C39 produced even lower IAA level than the *atrA* mutant did (Fig. 4).

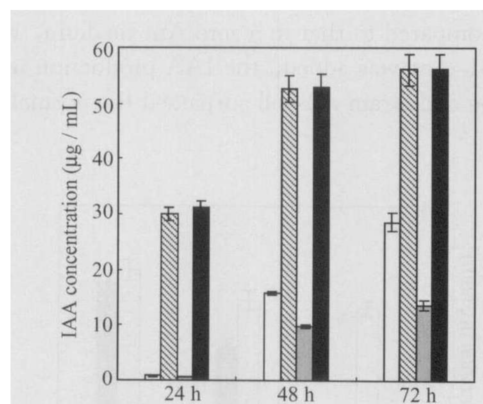

Fig. 4. Comparison of IAA production of A3 mutant (white bars), B78 mutants (hatched bars), C39 mutant (gray bars) and wild type (black bars).

2.5 Complementation for *atrA* and *atrC* mutants

To further confirm the *atrA* and *atrC* are involved in IAA production, complementation studies were performed. As shown in Fig. 5, IAA produc-

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tions of both the *atrA* and *atrC* mutants were nearly completely restored by complementation with pLAFR-*atrA* carrying *atrA* gene and pLAFR-*atrC* carrying *atrC* gene, respectively. The data suggested that the *atrA* and *atrC* genes were indeed involved in IAA synthesis.

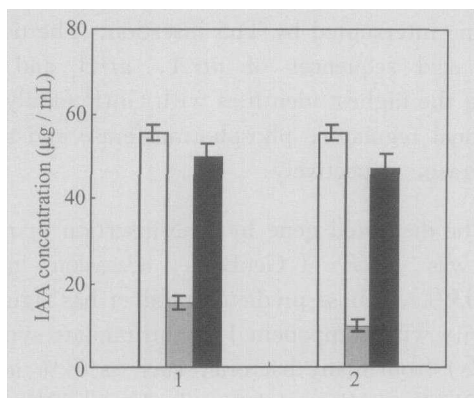

Fig. 5. IAA production in wild-type (white bars), mutants (gray bars) and complementary strains (black bars) after 48 h of culture. 1, wild-type; A3 mutant; A3 complemented by pLAFR-*atrA*. 2, wild-type; C39 mutant; C39 complemented by pLAFR-*atrC*.

2.6 Effect of anthranilate on IAA production

Because the interrupted *trpE* gene of the mutant strain A24 encodes the anthranilate (Ant) synthase component I, the effect of Ant on IAA production of wild-type and the mutant strain A24 was tested by supplementing different quantities of Ant into the MAZ medium (Fig. 6). When 10 μg/mL Ant was added in the medium, IAA production recovered largely compared to that in a zero Ant medium, when 50 μg/mL Ant was added, the IAA production in the wild-type and strain A24 all surpassed the normal level.

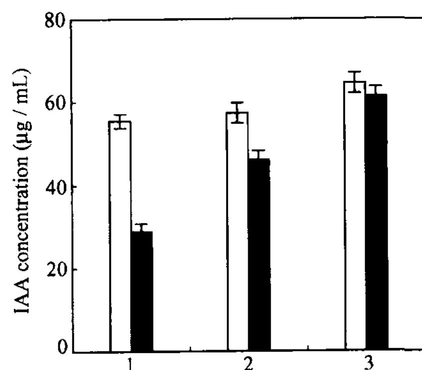

Fig. 6. IAA production of wild-type (white bars) and strain A24 (black bars) after 48 h of culture. 1, No Ant; 2, 10 μg/mL Ant added; 3, 50 μg/mL Ant added.

3 Discussion

Two genes involved in the IAA production have been isolated by Tn5 insertion method in this work and the *atr* cluster was demonstrated for the first time.

Mutant A3 carried a Tn5 insertion in *atrA* gene which encoding for GntR family transcriptional regulator. The HTH motif, which is a typical structure of GntR family transcriptional regulator, is situated at the N-terminus of the deduced product of *atrA*. The GntR family regulators were named after *Bacillus subtilis* GntR, a repressor of the gluconate operon[25]. Six subfamilies have been described for the GntR family: FadR, HutC, PlmA, MocR, YtrA and AraR, which regulate various biological processes and important bacterial metabolic pathways. BLAST search for the AtrA sequence at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) shows that AtrA belongs to the FadR subfamily. In this subfamily, the proteins consist of an all-helical C-terminal domain and most of this subfamily proteins are involved in the regulation of oxidized substrates related to amino acids metabolism or at the crossroads of various metabolic pathways such as aspartate (AnsR), pyruvate (PdhR), glycolate (GlcC), galactonate (DgoR), lactate (LldR), malonate (MatR), or gluconate (GntR)[25]. Mutation in *atrA* resulted in IAA^- phenotype, suggesting that *atrA* is a necessary regulator in IAA synthesis.

Our results have shown that *atrB* and *atrC*, whose predicted products are phosphotransferase and aminotransferase respectively, are located just downstream of *atrA*, and the three genes are clustered in an operon. The *atrB* and *atrC* mutants have been constructed by insertion of *km* cassettes (Fig. 1). The IAA production of *atrB* mutant was normal, but that of *atrC* mutant was decreased significantly. This suggests that the aminotransferase encoded by *atrC* should be responsible for the transamination from Trp to IPyA and its activity is regulated by the GntR family regulator AtrA. Although aminotransferases have been purified from *A. brasilense*[8], the gene encoding aminotransferase is first demonstrated here. Because mutation of *atrB* did not affect IAA production, the gene *atrB* should not be related to IAA synthesis and its function needs to be determined in the future.

The disruption of *trpE* leads to the decreased

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IAA production. This result is in agreement with the report that Tn5-insertion in *trpGDC* cluster led to the decreased IAA production[26], and the IAA production can be recovered by adding anthranilate in the medium (Fig. 6). Tryptophan is synthesized from chorismate. In total, five enzymatic steps are necessary to convert chorismate to tryptophan. The first enzyme, anthranilate synthase, converts chorismate into anthranilate. The genes involved in the enzymatic reaction are *trpE* and *trpG*, which encode component I and II, respectively, of the anthranilate synthase. So the level of endogenous tryptophan decreases because of the disruption of *trpE*. On the other hand, anthranilate is also a precursor for IAA biosynthesis. Disruption of *trpE* leads to the decreased levels of the two precursors (both tryptophan and anthranilate) for IAA production.

The *trpE* gene of *A. brasilense* Yu62 contains an open reading frame encoding a 532-residue polypeptide with a calculated molecular weight of 58 kD and no leader peptide is found upstream of *trpE*. However, De Troch et al. reported that *trpE* and *trpG* were fused together in *A. brasilense* Sp7 and the fused gene *trpE(G)* encodes a 732-residue polypeptide with a calculated molecular weight of 78 kD. Upstream the fused *trpE(G)* gene is a short leader peptide coding sequence (*trpL*), which contains three consecutive tryptophan codons[27]. A shortage of tryptophan increases the ratio of uncharged to charged $tRNA^{Trp}$. If uncharged $tRNA^{Trp}$ enters the ribosome translating *trpL* RNA at the repeated tryphophan codons, the ribosome is stalled and allows the formation of an antiterminator. In excess of tryphophan, charged $tRNA^{Trp}$ is abundant and reaches the stop codon of the leader peptide. The presence of the ribosome on the stop codon of the leader peptide allows the formation of the terminator, which in combination with a stretch of uridylate residues serves as a rho-independent transcription[27]. But in *A. brasilense* Yu62, the *trpE*, which has been found in our work, is not fused together with *trpG*, and no *trpL* was found upstream the *trpE* sequence. Comparison of amino acid sequences showed that both TrpEs of *A. brasilense* have 44% identity at their last half-parts. It suggests that there may be two copies of *trpE* genes exist in *A. brasilense*. One is regulated by attenuator and another is constitutively expressed. It was reported that two copies of *trpG* genes were found in *A. brasilense*. One is fused to the *trpE* gene; another is linked to *trpDC*[26]. This shows that neither *trpE* nor *trpG* gene in *A. brasilense* is redundant.

In summary, the capacity for the IAA biosynthesis of *A. brasilense* is subject to several levels of regulation, which include the concentration of precursors and the activity of enzymes which take part in the IAA biosynthesis pathway.

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