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Molecular evidence of genetic modification of *Sinorhizobium meliloti*: enhanced PCB bioremediation

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Abstract The genome of the nitrogen-fixing soil bacterium *Sinorhizobium meliloti* does not possess genes for bioremediation of aromatic pollutants. It has the well-known ability to interact specifically with the leguminous alfalfa plant, *Medicago sativa*. Our previous work has shown enhanced degradation of the nitroaromatic compound 2,4-dinitrotoluene (DNT) when a plasmid containing degradative genes was introduced in it. In this study we report molecular evidence of the transfer of a polychlorinated biphenyl (PCB)-biodegradative plasmid pE43 to *S. meliloti* strain USDA 1936. Several standard analytical tests and plant growth chamber studies were conducted to test the ability of *S. meliloti* to degrade 2′,3,4-PCB congener. Alfalfa plant alone was able to degrade 30% of PCBs compared with control. No enhanced dechlorination was noted when alfalfa plant was grown with wild-type *S. meliloti*, and when alfalfa plant was grown with the *S. meliloti* electrotransformants (genetically modified) dechlorination of PCBs was more than twice that when alfalfa plant was grown with wild-type *S. meliloti*. When alfalfa plant was grown with uncharacterized mixed culture (containing nodule formers), almost equally significant PCB degradation was observed. The significance of this work is that the naturally occurring nitrogen-fixing soil bacterium *S. meliloti* (genetically modified) has the ability to enhance fertility of soil in association with the leguminous alfalfa plant while simultaneously enhancing bioremediation of PCB-contaminated soils. Enhanced bioremediation of PCB and robust alfalfa plant growth was also noted when uncharacterized mixed cultures containing alfalfa plant nodule formers were used.

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Introduction

Polychlorinated biphenyls (PCBs) are a class of potent environmental toxicants, which are particularly toxic due to their tendency to biomagnify at progressively higher trophic levels. Our biosphere contains 750,000 tons of released PCBs (http://www.bio.nagaokaut.ac.jp/~mitsui-l/BphC/background.html). The toxicological properties of a class of PCB congeners are largely influenced by the aqueous solubility and subsequent bioavailability. A variety of human health effects have been attributed to PCB exposure, including reproductive and birth defects, damage to the kidney, the nervous system and the immune system, and cancer.

Currently (as of 9 August 2005), there are 520 PCB-contaminated sites on the Environmental Protection Agency (EPA) Superfund National Priority Listing (NPL) (http://www.epa.gov/superfund/sites/query/basic.htm). Most of the sites are under remedial action orders that will cost millions to billions of US dollars and involve removal and disposal of contaminated buildings, facilities, property, soils and sediments. Aggressive engineering methods, e.g. excavation, are suitable for "hot spot" removal, but less-expensive containment and treatment technologies are required for remediation of surrounding areas or sites contaminated at a lower level. PCBs can be degraded by high temperature (1200°C) [3]. However, one of the degraded products, dioxin, is more poisonous than the original PCB.

Bioremediation may provide a safe and cost-effective alternative to current methods for PCB cleanup. Bacteria have been shown to be able to degrade PCB compounds directly and cometabolically under anaerobic and aerobic conditions under both laboratory and field conditions [4]. Bacteria have been demonstrated to effectively metabolize and mineralize PCB compounds in

both pure and mixed cultures under laboratory conditions. However, bacterial bioremediation in the field is often confounded by numerous factors such as microbial competition, fluctuating environmental conditions, or limited and sporadic metabolic resources. Plants have been shown to enhance or stabilize bacterial degradation of various organic pollutants, including PCBs and polycyclic aromatic hydrocarbons (PAHs). In a preliminary note we have reported enhanced dechlorination of PCBs using genetically modified *Sinorhizobium meliloti* [7]. In this report we provide molecular evidence of electroporation and have shown that the nitrogen-fixing soil bacterium *S. meliloti*, with added genes for PCB degradation in conjunction with the symbiont alfalfa plant, can enhance PCB degradation. In addition, comparative studies of plant growth are presented.

Materials and methods

PCB extraction and measurement

A hexane/acetone (50:50) mixture was used for PCB extraction from soils (5 ml solution/1 g soil)with constant shaking for 10–30 min. The homogenate was passed through a 0.22 μm filter, and the filtrate was collected in an amber-colored glass vial with a screw cap. For high-performance liquid chromatography (HPLC) analysis, using Hewlett-Packard HP 1050 series machine equipped with a UV detector (Palo Alto, Calif.), the mobile phase consisted of acetonitrile and water, run at 90% (v/v) acetonitrile at a flow rate of 1.0 ml/min. The filtrate (5 μl) was eluted at room temperature through a 4.6 mm×250 mm, 5 μm particle size (LC18) column (St. Louis, Mo.) and detected at 254 mm.

PCB-degrading *ohb* genes and procedure for electroporation

We used one family of PCB-degrading genes, *ortho*-halobenzoate 1,2-dioxygenase (*ohb*) genes. Partial information of this *ohb* gene family is given in Table 1 which was adapted from GenBank accession number AF121970.

To prepare cells for electrotransformation, a 1:100 dilution of an overnight culture of *S. meliloti* was inoculated and grown at 37°C in a shaker until an OD_{600nm} up to 0.6 was obtained. The culture was chilled on ice for 15 min and centrifuged in a cold rotor at 4000 *g* for 15 min. The cell pellet was suspended in 500 ml ice-cold sterilized water and centrifuged again. The last step was repeated with 250 ml ice-cold water. The pellet was drained well, suspended in 1 ml ice-cold water, and glycerol was added to a final concentration of 10%.

The plasmid pE43 was isolated [4, 8] from *E. coli* (a gift from J. Tiedje) following a standard protocol [6]. The plasmid pE43 carried the *Pseudomonas aeruginosa* strain 142 *ohb* genes cloned into the broad-host-range vector pSP329, carrying a multiple cloning site and *lacZ* a-complementation fragment cloned into the *Hae*II site of the plasmid pTJS75, a derivative of R-factor RK2 [5].

Sinorhizobium meliloti recipient cells (60 μl) and plasmid pE43 (1 μl) were added into an electroporation cuvette placed in a Gene Pulser electroporator (Bio-Rad Laboratories, Philadelphia, Pa.). The mixture was pulsed twice at 2.5 kV. Following the addition of 1 ml SOC medium (Quality Biological, Gaithersburg, Md.), the mixture was incubated for 1 h at 37°C and then plated on selective plates in the presence of 10 μg/ml tetracycline.

PCR (polymerase chain reaction) and electrophoresis

In order to provide molecular evidence of electrotransformation, we designed specific primers covering part of the *ohb* B gene (Table 1) using web primer software (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer). DNA sequences from the partial *ohb* B gene from 3701 to 4000 bp were used as the PCR template. The sequence of the forward primer was 5′-AAAGGCGTGCCC-TTCCTG-3′ (3702–3719) and the same for the reverse primer was 5′-TGGTAGGGGTCTCGCGAATT-3′ (3985–3966). The GenBank accession number was AF121970 as described previously [8]. The PCR procedure was followed by 94°C for 5 min, 35 cycles of 94°C 1 min, 60°C 1 min, 72°C 2 min, and then 72°C 10 min. Electrophoresis parameters were 2.0% agarose gel (low EED), 50 V , and 2 h.

Table 1 Partial information about *ohb* genes (the primers for PCR synthesis as described in the text were designed from this information adapted from GenBank accession number AF121970)

Gene	Number of bases	Length (bp)	Product
OhbA	2783–3313	530	*Ortho*-halobenzoate 1,2-dioxygenase beta-ISP protein OhbA
OhbB	3354–4640	1286	*Ortho*-halobenzoate 1,2-dioxygenase alpha-ISP protein OhbB

Table 2 Summary of HPLC analyses of PCB dechlorination[a] after 6 weeks. Control soil was free from any PCB and was spiked with 0.33 ppm 2′,3,4-PCB congener (0.33 ppm is equivalent to 0.33 ng 2′,3,4-PCB per gram soil). All tests were repeated three times ($N=3$). One-way ANOVA showed significant differences ($P=0.0034$)

Treatment	Soil PCB remaining (ppm)	SEM
Control	0.32	0.04
Alfalfa + *S. meliloti* (pE43)	0.11	0.01
Alfalfa + *S. meliloti* (wild-type)	0.24	0.05
Alfalfa + bacterial culture[a]	0.09	0.01
Alfalfa only	0.23	0.03

[a]Mixture of several culturable microbes isolated from PCB-contaminated soil obtained from the US Army

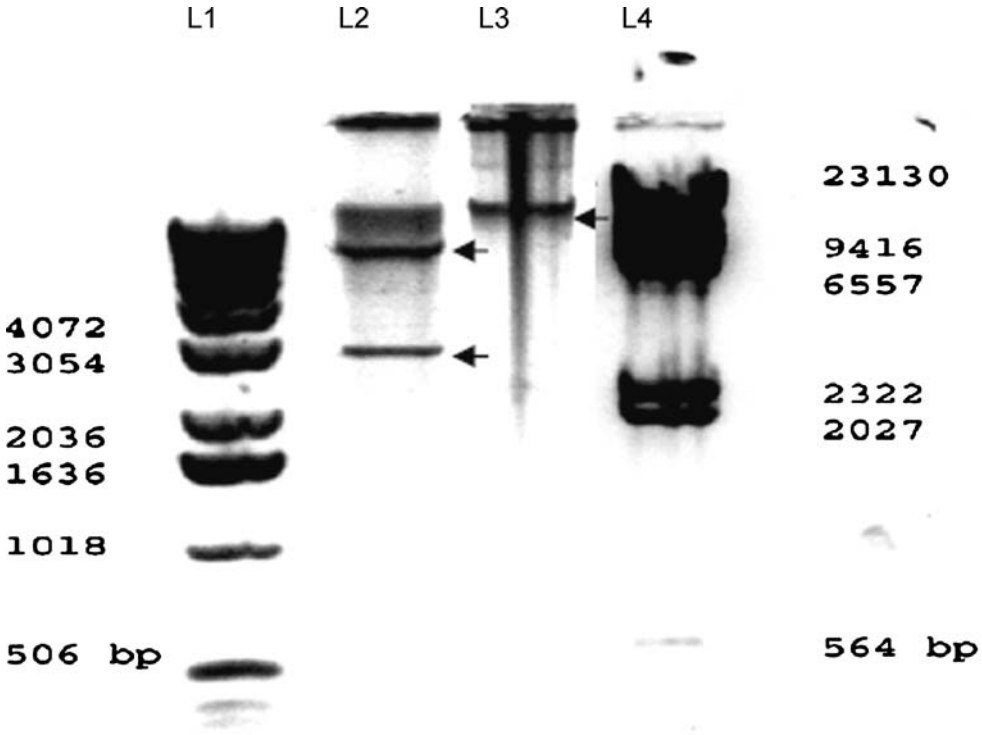

Fig. 1 Agarose gel electrophoresis of plasmids isolated from transformed (electrotransporated) bacteria. Restriction digestion and molecular weight evidence indicate the successful electrotransformation of the plasmids. *L1* DNA ladder (Promega); *L2* pE43 cut by Kpnl and Xbal with two bands, 3.5 and 9.5 kb (*arrows*); *L3* pE43 uncut, 13 kb; *L4* λ phage DNA cut by Hind III enzyme

Plant experiments and source of soil

The plant studies were done in Leonard jars containing sterile vermiculite and soil with a known PCB as described previously [1, 2]. Each Leonard jar contained 300 g clean soil obtained from US Department of Agriculture (USDA) (courtesy of D. Kuykendel) with 0.33 ppm 2′,3,4-PCB congener. Five different treatments were evaluated as shown in Table 2. Alfalfa seeds were surface-sterilized with ethanol and were seeded in sterile water and hydrogen peroxide. Plants were grown in a plant growth chamber (Envircom 2145 equipped with a programmable light and temperature cycle). Soil contaminated with PCBs (57 ppm) was obtained from the Army Waterways Experiment Station, Vicksberg, Miss. (courtesy of E. Tatem).

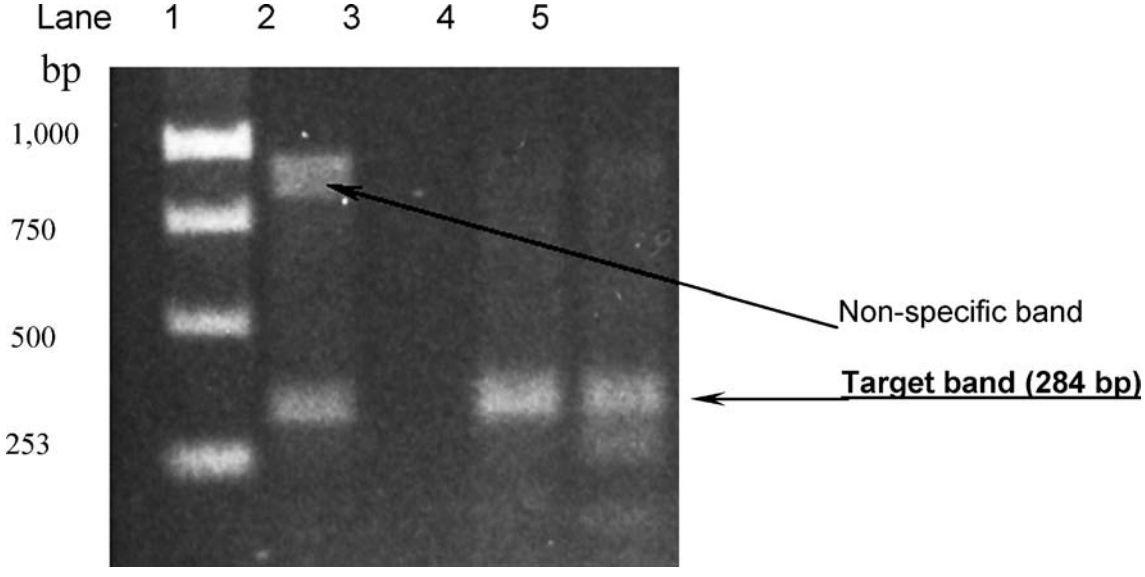

Fig. 2 PCR analysis of the transformants from the alfalfa rhizosphere indicates the presence of stable transformants containing PCB-degrading genes. *Lane 1* DNA ladder (Promega); *lane 2 E. coli* (pE43); *lane 3 S. meliloti* wild-type; *lane 4 S. meliloti* electrotransformant; *lane 5 C. testosteroni* strain VP44 (pE43)

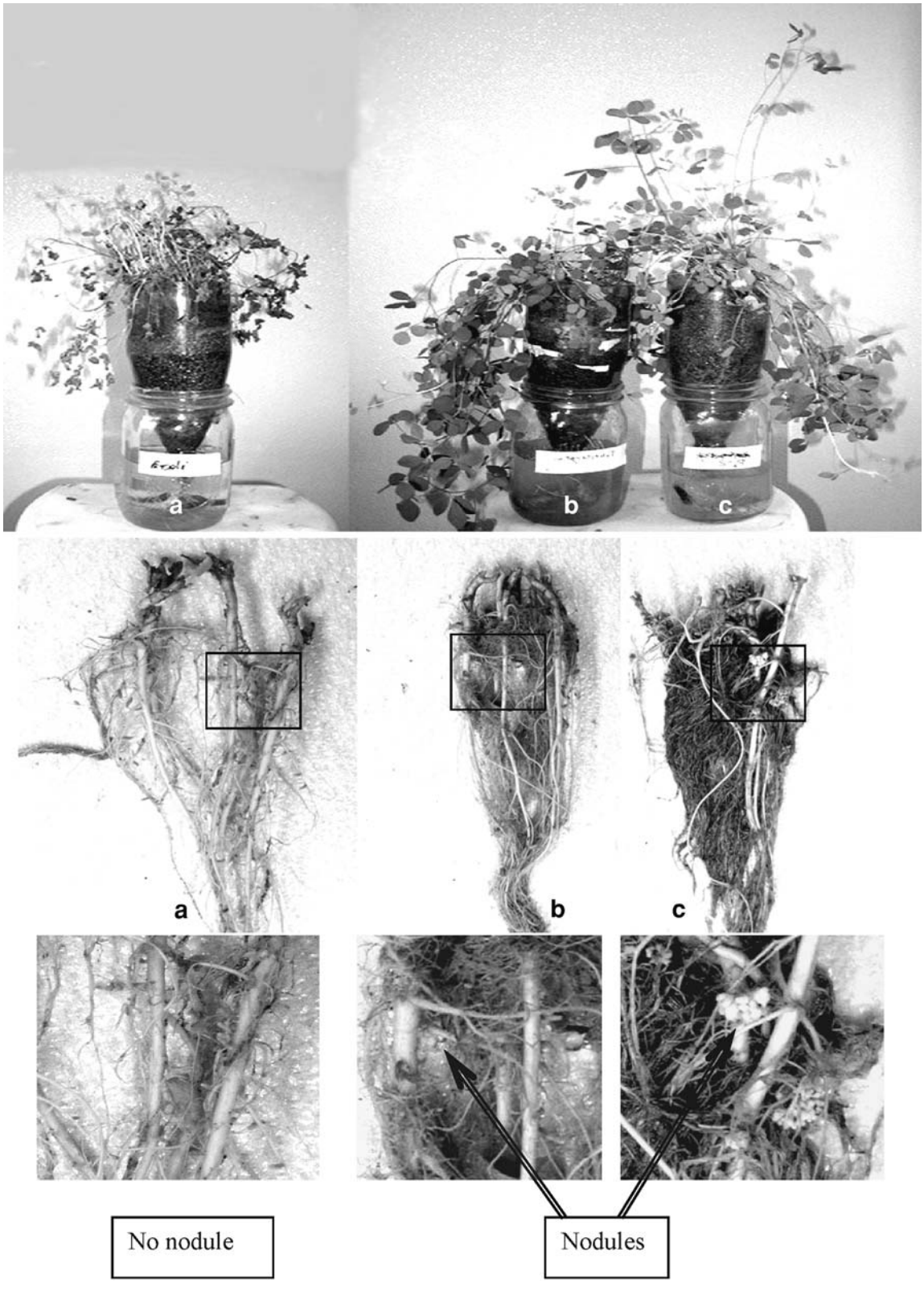

Fig. 3 The top photographs show plant growth: *Jar A* transformed *E. coli* (pE43) containing PCB-degrading *ohb* genes with alfalfa plants; *jar B* transformed *S. meliloti* (pE43), a nitrogen-fixing bacterium containing PCB-degrading genes with alfalfa plants; *jar C* uncharacterized microbial culture isolated from PCB-contaminated soil. Bottom photographs show growth of alfalfa roots in the presence of different microbial inocula. *Root A* transformed *E. coli* (pE43) containing PCB-degrading genes; *root B* transformed *S. meliloti* (pE43) containing PCB-degrading genes; *root C* uncharacterized microbial culture isolated from PCB-contaminated soil

Table 3 Summary of typical data from one complete set of plant growth studies. Studies were repeated two to three times and deviations were not more than 5%

	E. coli (pE43)	*S. meliloti* (pE43)	Soil (mixed culture)	Alfalfa plant (only)
Length (cm)				
Root	32	47	43	38
Shoot	34	43	53	33
Weight (g)				
Root	12.6	20.2	29.3	17.9
Shoot	17.9	24.6	26.3	20.2
Total	30.4	44.8	55.6	38.1

Results and discussion

Molecular evidence of electrotransformation

The presence of plasmid pE43 after electrotransformation was tested by restriction enzyme double digestion (Fig. 1). As expected, approximately 13 kb plasmid (uncut) electrotransformant showed two bands of 3.5 and 9.5 kb when cut with *Kpn*I and *Xba*I enzymes. Figure 1 provides molecular evidence of successful electrotransformation of the entire plasmid pE43.

Figure 2 shows the molecular evidence for the existence of the specific nucleotide sequence of part of the *ohb* B gene in the plasmid. The target PCR product was 284 bp as shown in Fig. 2. The presence of three PCR target bands of the same size (284 bp) proves that the *ohb* genes were present in the *S. meliloti* electrotransformant (pE43), *E. coli* (pE43) and *Comamonas testosterone* strain VP44 (pE43).

Measurement of PCB degradation in different alfalfa rhizosphere soil samples

Alfalfa plants were grown in sterile soil spiked with 0.33 ppm 2′,3,4-PCB and inoculated with *S. meliloti* or mixed microbial cultures obtained from PCB-contaminated soil. PCB extracted from four treated soil samples and from control soils was compared by HPLC after 6 weeks of growth. The results are summarized in Table 2.

The HPLC chromatogram showed that uncharacterized microbial mixtures were also very effective PCB degraders.

Alfalfa plant growth studies

Alfalfa plants were grown for 8 weeks in Leonard jar setups in humidity-controlled growth chambers at 30/25°C for 8/16-h day/night cycles. Each jar contained sterile soil: vermiculite (50:50) spiked with 0.33 ppm 2′,3,4 PCB congener and inoculated with respective bacterial cultures (Fig. 3). Table 3 summarizes the lengths and weights of the alfalfa plants. The best growth of plant roots and shoots and the best weights were obtained when mixed soil bacteria were used (Table 3). Genetically modified *S. meliloti* (the alfalfa symbiont) was second best in terms of root growth (Fig. 3). Interestingly, however, both *S. meliloti* (pE43) and the mixed culture, when grown with symbiont alfalfa plant, showed robust plant growth as well as PCB bioremediation. The products of the mixed bacterial culture provided a better environment for alfalfa plant growth but the presence of the PCB degradative gene in the culture also powerfully enhanced bioremediation. Figure 3 shows some typical nodules in roots when grown with known *S. meliloti* (pE43) and also with the uncharacterized mixed bacterial culture. This suggests that the uncharacterized mixed culture also contained nodule-forming bacterial symbionts.

Conclusion

Alfalfa plants with the nitrogen-fixing symbiont *S. meliloti* with an added gene for PCB degradation significantly accelerated bioremediation. The alfalfa rhizosphere consists of mixed cultures including nitrogen-fixing soil bacterium containing PCB-degrading gene, provide cometabolic products for robust growth of leguminous plants along with significantly enhanced bioremediation. These studies show that naturally occurring soil bacteria can be used in large-scale field applications for bioremediation of several environmentally toxic aromatic pollutants. Field trials and toxicity tests are required before application of *S. meliloti* (pE43) nitrogen-fixing bacteria can be used for large-scale PCB remediation in contaminated soils.

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