Bacterial Symbionts of the Brown Planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae)[†]

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Received 17 September 2009/Accepted 8 January 2010

The brown planthopper (*Nilaparvata lugens* Stål), the most destructive pest of rice, has been identified, including biotypes with high virulence towards previously resistant rice varieties. There have also been many reports of a yeast-like symbiont of *N. lugens*, but little is known about the bacterial microbes. In this study, we examined the bacterial microbes in *N. lugens* and identified a total of 18 operational taxonomic units (OTUs) representing four phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*) by sequencing and analyzing 16S rRNA gene libraries obtained from three populations of *N. lugens*, which were maintained on the rice varieties TN1, Mudgo, and ASD7. Several of the OTUs were similar to previously reported secondary symbionts of other insects, including an endosymbiont of the psyllid *Glycapsis brimblecombei*, an *Asaia* sp. found in the mosquito *Anopheles stephensi*, and *Wolbachia*, found in the mite *Metaseiulus occidentalis*. However, the species and numbers of the detected OTUs differed substantially among the *N. lugens* populations. Further, *in situ* hybridization analysis using digoxigenin-labeled probes indicated that OTU 1 was located in hypogastrum tissues near the ovipositor and ovary in biotype 1 insects, while OTU 2 was located in the front of the ovipositor sheath in biotype 2 insects. In addition, masses of bacterium-like organisms were observed in the tubes of salivary sheaths in rice plant tissues that the insects had fed upon. The results provide indications of the diversity of the bacterial microbes harbored by the brown planthopper and of possible associations between specific bacterial microbes and biotypes of *N. lugens*.

Close associations between insects and the microbes they harbor appear to be common. Symbionts have been found to contribute to the nutrition, development, reproduction, speciation, and defense against natural enemies of their host insects (1, 11, 18, 30, 39). The small brown planthopper (*Laodelphax striatellus*) and the white-backed planthopper (*Sogatella furcifera*) also reportedly harbor an alphaproteobacterial *Wolbachia* symbiont (29) that can be transferred horizontally between different insect species and that affects its hosts' sexual reproduction, cytoplasmic incompatibility, and immune responses (21, 38, 39).

The brown planthopper, *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is a monophagous insect herbivore of rice (13) that feeds on rice phloem and causes serious damage to rice crops. *N. lugens* reportedly harbors an intracellular, eukaryotic "yeast-like symbiont" (YLS) in the fat body, which plays a key role in recycling uric acid (3, 33). However, little is known about bacterial symbionts in *N. lugens*.

It has been well recognized that diversity exists within insect species and that "biotypes" or populations that are adapted to or that prefer a particular host can frequently develop (10, 12). The behavioral and physiological responses during insect establishment on plants are feeding, metabolism of ingested food, growth, adult survival, egg production, and oviposition (34). In *N. lugens*, the biotype is assigned to a population with

the ability to damage varieties of rice that carry resistance genes and that were previously resistant to it (5). It has been claimed that some biotypes of *N. lugens* differ in small morphological features, isozymes, and DNA polymorphisms (6, 25, 36). However, the precise nature of the virulence-conferring mechanisms in *N. lugens* biotypes (and their modes and stability of inheritance) is not clear. It is interesting to survey symbionts in different biotype populations of *N. lugens*.

Generally, the 16S rRNA gene has been used as a molecular marker enabling the detection of as-yet-uncultured microbes, and it facilitates a profound investigation of microbial diversity (2, 22, 44). We initiated a study using molecular methods to investigate the bacterial symbionts of *N. lugens*. The major objective of this study was to identify bacterial microbes in *N. lugens*. The identified bacterial microbes appeared to be associated with different populations of *N. lugens* and in some cases were located in specific tissues, according to *in situ* hybridization (ISH) analyses.

MATERIALS AND METHODS

***N. lugens* insect populations.** Insects from three populations of *N. lugens*, designated biotypes 1, 2, and 3, were maintained at the Genetics Institute, Wuhan University, on three rice varieties: TN1 (a susceptible variety) for biotype 1, Mudgo (carrying the resistance gene *Bph1*) for biotype 2, and ASD7 (carrying the resistance gene *bph2*) for biotype 3 (5). The temperature in the insectary was kept at 25°C. Samples of a field population were collected in rice fields in Wuhan, China, in 2009.

Total DNA extraction. For each biotype of *N. lugens*, 30 female adults were collected, frozen at –20°C for 5 min, immersed in 5% NaClO, and rinsed with sterile distilled water five times to remove surface microorganisms. Total DNA was isolated from each surface-disinfected *N. lugens* insect, using a DNeasy blood and tissue kit (Qiagen), as follows. For isolation of DNA from individual insects, a disinfected insect was crushed in liquid nitrogen using a grinding rod in a centrifuge tube, 200 µl cetyltrimethylammonium bromide (CTAB) buffer (2% [vol/vol] CTAB, 20 mM EDTA, 100 mM Tris HCl, pH 8.0, 1.4 M NaCl) was

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[‡] Published ahead of print on 22 January 2010.

TABLE 1. Bacterial microbes identified in *N. lugens* populations

OTU ^a	Representative clone	Class	Sequence database match ^b	Detected in biotype ^c :		
				1	2	3
1	TM31	<i>Gammaproteobacteria</i>	Secondary bacterial symbionts of <i>Glycapsis brimblecombei</i> ; 99; EU043378	+	—	—
2	B31	<i>Alphaproteobacteria</i>	<i>Asaia krungthepensis</i> ; 96; AB292240	—	+	—
3	TM86-2	<i>Alphaproteobacteria</i>	<i>Wolbachia</i> of <i>Metaseiulus occidentalis</i> ; 99; AY754820	—	—	+
4	TM57	<i>Gammaproteobacteria</i>	<i>Enterobacter asburiae</i> strain J2S4; 99; EF221358	+	+	—
5	TM58	<i>Gammaproteobacteria</i>	Uncultured <i>Citrobacter</i> sp. clone AV.4R-S-I13; 99; EU341179	+	—	+
6	TM86-22	<i>Gammaproteobacteria</i>	<i>Aeromonas hydrophila</i> strain ATCC 7966; 99; X60404	+	+	+
7	TM86-140	<i>Alphaproteobacteria</i>	<i>Brevundimonas diminuta</i> ; 98; DQ979376	+	—	—
8	TM86-5	<i>Firmicutes</i>	<i>Exiguobacterium acetylicum</i> strain DSM 20416; 97; DQ019167	+	—	—
9	TM78-8	<i>Gammaproteobacteria</i>	<i>Acinetobacter</i> sp. Dui-5; 99; EF031061	—	+	+
10	TM85-7	<i>Gammaproteobacteria</i>	<i>Haemophilus parainfluenzae</i> CCUG 12836; 99; AY362908	—	—	+
11	TM85-8	<i>Actinobacteria</i>	<i>Brachybacterium arcticum</i> strain Lact 5.2; 98; AF434185	—	—	+
12	TM85-110	<i>Firmicutes</i>	<i>Streptococcus salivarius</i> strain ATCC 7073; 99; AY188352	—	—	+
13	TM85-82	<i>Alphaproteobacteria</i>	<i>Sphingomonas</i> sp. oral clone AV069; 99; AF385529	—	—	+
14	TM85-87	<i>Bacteroidetes</i>	Uncultured bacterium clone IYF36; 96; DQ984575	—	—	+
15	TM85-114	<i>Actinobacteria</i>	<i>Arthrobacter</i> GOL01; 99; AY940423	—	—	+
16	TM85-136	<i>Alphaproteobacteria</i>	<i>Paracoccus</i> sp. R24652; 96; AM083998	—	—	+
17	TM85-77	<i>Gammaproteobacteria</i>	<i>Stenotrophomonas</i> sp. OS17; 98; EF491967	—	—	+
18	TM85-92	<i>Gammaproteobacteria</i>	<i>Acinetobacter</i> sp. An17; 98; AJ551155	—	—	+

^a The OTU was determined using the DOTUR program based on a 2% distance level.

^b Organism; percent identity; GenBank accession no. The sequences were determined based on the highest similarity scores for both type and nontype strains in the NCBI database.

^c +, detected; —, not detected.

added, and it was lysed at 56°C for 1 h. Total DNA was then extracted with two equal volumes of chloroform-isoamyl alcohol (24:1) and precipitated with cold ethanol.

Construction and analysis of 16S rRNA gene libraries. 16S rRNA gene libraries were constructed for each of the three biotypes of *N. lugens* by amplifying their 16S rRNA genes from the purified total DNA using the universal primers 27F/1492R (44), a PTC-100 thermal cycler (MJ Research), and reaction mixtures with a total volume of 50 µl containing 10 µM each primer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.25 µM MgCl₂, 1× PCR buffer, and 3 units of *Taq* DNA polymerase (Fermentas). The PCR conditions were 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s, with a final extension step of 72°C for 10 min. The amplified products were cloned into T-vector (Promega) and transformed into *Escherichia coli*. The 27F/1492R primers were then used to identify positive clones containing the expected DNA insert. The restriction patterns of the PCR products were examined by amplified ribosomal DNA restriction analysis (ARDRA) (19) using the enzymes HhaI, HaeIII, and RsaI (Fermentas) and then sequenced. Chimeric sequences were removed with the Chimera Detection tool (<http://rdp8.cmc.msu.edu/cgiis/chimera.cgi?su=SSU>) of Ribosomal Database Project II release 10 (RDP-II) (7). The remaining sequences were compared to known sequences in the NCBI and RDP-II databases and aligned by Clustal X (41). Distance matrices were constructed using the DNADIST program of the PHYLIP package (15). Operational taxonomic units (OTUs) were determined by DOTUR (35) and reported based on a 2% distance level. Phylogenetic trees were constructed using the MrBayes inference program (see the supplemental material).

Bacterial-infection frequencies. In order to estimate bacterial-infection frequencies, 50 individual insects representing each biotype and 126 individual insects from a field population were examined. Total DNA obtained from each of the insects was isolated according to the method described above, and infection frequencies were determined by PCR using relevant primers (see Table 2) and the above-mentioned PCR amplification program, except that the annealing temperature was 52 to 60°C, as appropriate.

In situ hybridization. Female adult insects were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 48 h under vacuum. The fixed insects were dehydrated by passage through an ethanol series (30%, 50%, 70%, and 100%) and then embedded in paraffin. The specimens were cut in series of 8-µm-thick sections and mounted on glass slides coated with 2× poly-L-lysine solution (Sigma). Digoxigenin (DIG)-labeled single-stranded antisense probes and control sense probes were synthesized by *in vitro* transcription using a DIG RNA Labeling Kit (Roche) and then incubated (at 100 ng/µl) with the sections at 60°C for 12 h. After treatment with 2% bovine serum albumin (BSA) and 2% blocking agent (Roche), the hybridized probes were detected, and their

locations were stained by adding anti-Dig-AP (Roche), followed by nitroblue tetrazolium salt and bromo-4-chloro-3-indolyl-phosphate. Finally, hybridization signals were observed using an Olympus BX-51 microscope.

Transmission electron microscopy. To examine bacteria that might have been associated with salivary sheaths left by insects that had fed on rice plants by transmission electron microscopy (TEM), rice leaf sheaths infested by *N. lugens* were double fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in PBS (for 24 h under vacuum and then overnight at 4°C), followed by 1% osmium tetroxide in 0.1 M Pb overnight at 4°C. They were then dehydrated by passage through an ethanol series and embedded in Spurr resin (Fluka). Ultrathin (0.05-µm) sections were stained with uranyl acetate and examined under a Hitachi D-1000 transmission electron microscope.

Nucleotide sequence accession numbers. Eighteen unique 16S rRNA gene sequences of bacterial microbes were deposited in the GenBank database under accession numbers FJ774959 to FJ774976.

RESULTS

Analysis of bacterial microbes in *N. lugens*. 16S rRNA gene sequences, ca. 1.5 kb long, were amplified from the three biotype libraries and, in total, 28 restriction patterns were identified. The corresponding clones were sequenced, and 28 sequences were obtained, with lengths ranging from 1,466 bp to 1,506 bp. Of these sequences, two were found to be chimeric and were discarded. The remaining 26 were grouped into 18 OTUs by DOTUR (Table 1) and assigned to four phyla: *Proteobacteria* (13 OTUs; 72%), *Firmicutes* (2 OTUs; 11%), *Actinobacteria* (2 OTUs; 11%), and *Bacteroidetes* (1 OTU; 6%). Thus, the bacterial microbes in *N. lugens* appeared to be dominated by *Proteobacteria* (more specifically, gamma- and alphaproteobacteria, which accounted for eight and five of the proteobacterial OTUs, respectively). These sequences were analyzed with known sequences in the NCBI and RDP-II databases. The results indicated that some of the OTUs were similar to secondary symbionts reported in other insects, i.e., *Arsenophonus*, *Asaia*, and *Wolbachia*, and some of OTUs were related to gut-associated microbes, i.e., *Aeromonas* and *Citrobacter* (Table

TABLE 2. Infection frequencies of bacterial microbes detected in *N. lugens* populations

Primer name	Targeted sequence	Sequence (5'–3')	Infection frequency detected in biotype or population ^a				Reference
			1	2	3	Field	
27F/1492R	Eubacteria 16S rRNA partial sequence	AGAGTTTGATCCTGGCTCAG ACGGTTACCTTGTAGGACTT	60/67	47/51	43/48	121/126	44
Ars23s-1/Ars23s-2	<i>Arsenophonus</i> 23S rRNA partial sequence	CGTTTGATGAATTCATAGTCAAA GGTCTCCAGTTAGTGTTACCCAAC	34/60	0/51	0/48	30/126	40
16Swup/16Swlo	<i>Alphaproteobacteria</i> 16S rRNA partial sequence	GCCTAACACATGCAAGTCGAA AGCTTCGAGTGAAACCAATTCCC	0/67	20/51	2/48	68/126	17
99F/994R	<i>Wolbachia</i> 16S rRNA partial sequence	TTGTAGCCTGCTATGGTATAACT GAATAGGTATGATTTTCATGT	0/67	0/51	2/48	46/126	29
81F/691R	<i>Wolbachia</i> <i>wsp</i> gene partial coding sequence	TGGTCCAATAAGTGATGAAGAAAC AAAAATTAAACGCTACTCCA	0/67	0/51	2/48	46/126	46

^a The denominators are the total numbers of samples, and the numerators are the numbers of samples in which the bacterium was detected.

1). A phylogenetic tree of the 8 gammaproteobacterial sequences was constructed (see Fig. S1 in the supplemental material). The *Wolbachia*-like sequences (OTU 3) were grouped into supergroup B (see Fig. S2 in the supplemental material).

Bacterial diversity associated with different populations of *N. lugens*. Analysis of the detected bacterial complements in three *N. lugens* populations that were maintained on different rice varieties showed that the OTUs were not homogeneously distributed among them. Of the 18 OTUs, only one (OTU 6) was detected in all three biotypes, one (OTU 4) was detected in biotypes 1 and 2, one (OTU 9) in biotypes 2 and 3, and another (OTU 5) in biotypes 1 and 3. *Proteobacteria* almost completely dominated in biotypes 1 and 2, in which five out of six (83%) and all four (100%) OTUs were proteobacterial, respectively. Greater diversity in bacterial microbes was found in biotype 3, in which, of 13 OTUs, 9 belonged to *Proteobacteria* (69%), 2 to *Actinobacteria* (15%), and 1 each (8%) to *Firmicutes* and *Bacteroidetes*. The results show that the bacterial symbionts hosted by the different biotypes of *N. lugens* differed substantially.

The proportions of biotypes 1, 2, and 3 and the field population, in which bacteria were detected by PCR with relevant primers for OTU 1 (*Arsenophonus*), the alphaproteobacterial microbes, and OTU 3 (*Wolbachia*), indicated that the infection frequencies of OTU 1 were 56.1% and 23.8% in the biotype 1 and field populations, respectively; Alphaproteobacteria were present at 42.6% and 54% in the biotype 2 and field populations, respectively; and OTU 3 was present at frequencies of 4.7% and 36.5% in the biotype 3 and field populations, respectively (Table 2). The results show that each bacterial microbe did not occur in all insects and that there were specific mi-

crobe-biotype associations in the laboratory populations, but each microbe was detected in the field population.

Localization of bacteria in *N. lugens*. To identify the locations of the bacteria in *N. lugens*, *in situ* hybridization was applied to tissue sections of adult females, using DIG-labeled probes. Two probes were designed based on the 16S rRNA gene sequences of OTU 1 and OTU 2, amplified from the total DNA of biotypes 1 and 2 using the primers TM31F/TM31R and B31F/B31R (Table 3), and then hybridized with tissue sections of adult female insects of biotypes 1 and 2, respectively (Fig. 1).

As shown in Fig. 1, hybridization signals from both probes were observed in the abdominal areas of insects near reproductive tissue. However, signals from probe 1 were found in hypogastric tissue near the ovipositor and ovary in biotype 1, while signals from probe 2 were found in front of the ovipositor sheath in biotype 2. The sense strands of the two probes (used as negative controls) did not detect any signals. A probe based on OTU 3 was designed to hybridize with biotype 3, but no signal was detected, presumably because of the low infection frequency of *Wolbachia* in *N. lugens* populations. The Cy5-labeled oligonucleotide probe eub338, which recognizes eubacteria, was used, but the autofluorescence signals of *N. lugens* were strong and disturbed the probe hybridization signals (data not shown).

TEM observations of rice leaf sheaths. In previous experiments, bacterium-like 16S rRNA gene sequences, which showed high similarity to the 16S rRNA gene sequences of some OTUs detected in this study, were found in rice tissues infested by *N. lugens* (data not shown). *N. lugens* sucks rice phloem sap from the rice leaf sheath, so salivary sheaths left in

TABLE 3. Probes used for *in situ* hybridization

Probe	Target sequence	Primer	Primer sequence (5'–3')	Probe length (bp)
1	OTU 1 16S rRNA	tm31-f tm31-r	TCCACGCTGTAAACGATG CTGGCAACAAAGGATAAGG	330
2	OTU 2 16S rRNA	B31-f B31-r	GGGGAGCAAACAGGATT AGTGCCCAACCAACAT	379

FIG. 1. ISH of DIG-labeled probes with tissue sections of adult female *N. lugens*, showing signals from probe 1 in a biotype 1 section (A and B) and probe 2 in a biotype 2 section (C and D). Panels B and D show $\times 200$ magnifications of the boxed areas in panels A and C ($\times 40$), respectively. The arrows indicate signals from DIG-labeled probes.

leaf sheaths of rice plants that *N. lugens* had fed upon were examined by transmission electron microscopy, and masses of round bacterium-like organisms (BLOs) were found in them (Fig. 2A and B). BLOs were also observed in rice tissues adjacent to the salivary sheaths (Fig. 2C and D). However, BLOs were not found in the leaf sheaths of rice that had not been infested by *N. lugens*. Thus, these results suggest that some BLOs might be transmitted into rice tissue while *N. lugens* feeds.

DISCUSSION

Bacteria in *N. lugens*. Previous studies of microbe-*N. lugens* interactions have focused on the yeast-like symbiont, which has been shown to be essential for the survival and development of the insect (3, 33). However, in this study, we examined 16S rRNA gene sequences to characterize bacterial associates of *N. lugens*. The bacterial sequences detected were grouped into 18 OTUs, more than two-thirds of which appeared to be members of the phylum *Proteobacteria*, while the others belonged to the phyla *Firmicutes* (2 OTUs), *Actinobacteria* (2 OTUs), and *Bacteroidetes* (1 OTU). Searches of public databases indicated these OTUs were similar to secondary symbionts or related to gut-associated microbes reported in other insects, and some OTUs had not been reported in insects before (i.e., *Haemophilus*). Another study indicated that *Cardinium* bacteria and *Wolbachia* were present in the white-backed planthopper but not in the small brown planthopper and *N. lugens* (28). *Cardinium* bacteria were not detected in our populations, and *Wolbachia* was detected at a low infection frequency (4.7%) in biotype 3 but at a higher frequency (36.5%) in the field population. The results provide useful information for further exploration of bacteria associated with *N. lugens*.

Bacterial diversity and biotypes of *N. lugens*. Rice varieties resistant to *N. lugens* have been released since the 1970s (31,

32). However, their resistance has broken down due to the emergence of new biotypes of *N. lugens* (26). In addition to the apparent genetic differences between insect biotypes, several indications of associations between secondary symbionts and biotype populations in insects, particularly whitefly and aphid, have been reported (4, 37). Previous studies have detected biological differences among *N. lugens* biotype populations (6, 25, 36). The three biotypes of *N. lugens* were maintained on specific rice varieties for a long time. In this study, differences in the bacterial complements of the three biotype populations were found in the numbers of genera of OTUs detected (6, 4, and 13 in biotypes 1, 2, and 3, respectively). Furthermore, OTUs unique to each biotype population were detected: OTU 1 was detected only in biotype 1, OTU 2 (*Asaia*) only in biotype 2, and OTU 3 (*Wolbachia*) and several other OTUs only in biotype 3. However, these OTUs were all detected in the field population (Table 2), confirming that the field population may be composed of multiple biotypes (9). Bacterial microbes associated with the field populations of *N. lugens* need to be analyzed in the future.

Possible roles of bacterial microbes in *N. lugens*. The bacterial microbes may affect the host insects' reproduction. OTU 1 shares 99% nucleotide identity with the 16S rRNA gene sequences of *Arsenophonus* of the psyllid *Glycapsis brimble-combei* and the whitefly *Aleuroplatus gelatinosus* (20, 40), and OTU 3 also shares 99% nucleotide identity with the 16S rRNA gene sequence of *Wolbachia* of the mite *Metaseiulus occidentalis*, which are known to "manipulate" the reproduction and induce male offspring killing in the host arthropods (39, 40). The bacteria may also facilitate adaptation of the insects to resistant varieties of their host plants, which may partly explain why OTU 2 was detected only in biotype 2. OTU 2 shared 96% nucleotide identity with *Asaia krungthepensis*, an acetic acid bacterium of the alphaproteobacterial genus *Gluconacetobacter*. *Asaia* spp. have been isolated from tropical flowers and

FIG. 2. Transmission electron micrographs of BLOs observed in salivary sheaths (A and B) and adjacent leaf sheath tissues (C and D) of rice variety TN1 on which *N. lugens* had fed. Panels B and D show magnifications of the boxed areas of panels A and C, respectively. Bar = 1 μ m. SS, salivary sheath of the brown planthopper; LS, leaf sheath of rice.

fruit-flavored bottled water (24, 27, 45). Furthermore, they have reported associations with the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae) (26) and the mosquito *Anopheles stephensi* (Diptera: Culicidae) (14). The latter study indicated that *Asaia* was present in the female gut and the male reproductive tract. In this study, OTU 2 was located in front of the ovipositor sheath in biotype 2 females. As a putative *Gluconacetobacter*, OTU 2 may provide metabolic functions that contribute to the adaptation of biotype 2 insects to the rice variety Mudgo, which carries the resistance gene *Bph1*. However, more evidence is required to evaluate this hypothesis.

A third possible role is concerned with plant pathogens. The plant pathogens that cause sugar beet disease and strawberry marginal chlorosis disease are transmitted by the planthoppers *Pentastiridius beieri* and *Cixius wagneri* (8, 16). In this study,

OTU 1 had high similarity (98%) to the 16S rRNA gene sequences of both pathogens. Masses of BLOs were observed in salivary sheaths (Fig. 2) (42). Many studies have found that plant defensive responses induced by homopteran insects substantially overlap with responses to microbial pathogens (23, 43). However, the mechanisms whereby these microorganisms may be linked to rice responses to *N. lugens* feeding are unclear and warrant further research.

ACKNOWLEDGMENTS

We thank Qiang Fu (Chinese National Rice Research Institute) for providing the original insects and Jie Zhao, Peiying Hao, and Yan-chang Wang for advice on tissue sectioning and transmission electron microscopy.

This research was supported by the National Natural Science Foundation of China (grant no. 30570140) and a Key Grant Project of the Chinese Ministry of Education (307018).

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