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RESEARCH ARTICLE

Analyses of Bacterial Community Dynamics Present in *Culex quinquefasciatus* Collected from Tamil Nadu, India - A Metagenomic Approach

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ABSTRACT

Culex quinquefasciatus are among the most important vectors of arboviral diseases worldwide. Recent studies indicate that diverse midgut microbiota of mosquitoes significantly affects development, digestion, metabolism, and immunity of their hosts. Here, we studied the bacterial diversity found in midgut part of *C. quinquefasciatus* to understand the host and microbe interaction. The adult *C. quinquefasciatus* mosquitos were collected from Loyola College Campus, Chennai, using ovitraps, and midgut part was extracted; moreover, the DNA templates were isolated and amplified by polymerase chain reaction. The DNA amplicons were sequenced by Illumina MiSeq gene sequencer. The total of 279,157 reads was classified into 85, the bacterial genera of *Pseudomonas, Klebsiella, Staphylococcus,* and *Aeromonas* predominantly found to be high when compared to the other bacterial genera. The present data strongly encourage further investigations to verify the potential role of the detected bacteria in mosquito for the transmission of several vectoral diseases.

Keywords: Illumina MiSeq, *Klebsiella*, midgut, *Pseudomonas*, vectoral diseases

INTRODUCTION

Mosquitoes are well known as vectors of many human and animal pathogens worldwide. *Culex quinquefasciatus* is a peridomestic mosquito seldom found far from human residence or activity, and readily feeds on avian, mammalian, or human hosts. The world has seen recent outbreaks and emergences of several tropical diseases caused by arboviruses and transmitted by mosquitoes. Most species are described are in the genera *Culex* including several blood-feeding members able to transmit pathogens to humans and animals, a great concern for public health.[1] The later causes lymphatic filariasis (LF) in humans, and presently, over 120 million peoples are infected with filarial worm. LF is a major public health problem in India and worldwide, it is estimated that 1.3 billion people from 83 countries are living at the risk of infections. However, in India, LF is endemic in 17 states and six union territories, and is responsible for one-third of the global disease burden with about 554.2 million people at risk of infection, with 31 million parasite carriers and 23 million case of symptomatic filariasis.[2] The observations over 100 years have shown that the epidemiology of dengue varies a great deal with respect to both geography and time. This is due to not only to modifications in human ecology (population increase, urbanization, and more frequent travel) but also the ecological adaptations of certain mosquito species.[3]

Mosquitoes can acquire bacteria transtadially (larvae to adult mosquitoes through bacteria in water) and through sugar feeding as adults.[4] Little is known about the midgut microflora of *Culex* mosquitoes and very few studies have been conducted to study the midgut microbiota of *Culex* mosquitoes.[5,6]

Mosquitoes serve as obligate intermediate hosts for numerous diseases that collectively represent a major cause of human mortality and morbidity worldwide. There have been attempts to generate transgenic mosquitoes refractory for the transmission of pathogens.[7] The midgut bacterial flora of the mosquito can

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be used to introduce gene products that will inhibit the development of pathogens inside the mosquitoes.[8,9] A comprehensive study has been conducted by us on the diversity of microbiota in the midgut lumen of *C. quinquefasciatus* to ascertain their potential role in disease transmission and for their exploitation in vector management. Metagenomics enables us to study microorganisms by deciphering their genetic information from DNA that is extracted directly from communities of environmental microorganisms, thus bypassing the need for culturing isolation. This discipline builds on the successes of culture-independent surveys of environmental samples.[10] To understand bacterial diversity in the midgut of mosquitoes, several laboratory-collected mosquito studies were conducted. Dependent and independent culture cultivation methods are particularly useful approaches in attempting to make a complete assessment of the bacterial mosquito species.[11]

Bacterial communities are classically assessed through culture-dependent methods based on isolation on solid medium, sometimes after enrichment by growth in liquid medium. However, it is now obvious that the microbial diversity is poorly represented by the cultured fraction, and culture has been shown to explore <1% of the whole bacterial diversity in environmental samples.[12] The complete metagenomic approach will give the total gene content of a community, thus providing data about biodiversity function and interactions.[13] For the purpose of biodiversity studies, metagenomics can focus on one common gene shared by all members of the community. The most commonly used culture-independent method relies on amplification and analysis of the 16S rRNA genes in a microbiota. Recent metagenomic studies on mosquito midgut have revealed the presence of a diverse microbiota, which can significantly affect the development, digestion, metabolism, immunity, and other physiological functions of their hosts.

This midgut microbiota has also been suggested to alter the competency of mosquitoes to transmit pathogens such as arboviruses.[14-16] In this present study, the attempts were made to analyze the bacterial community dynamics in midgut part of wild-type *C. quinquefasciatus* by collecting ovitraps.

MATERIALS AND METHODS

Mosquito collections

The laboratory-bred pathogen-free strains of mosquitoes were reared in the vector control laboratory, Department of Zoology, Annamalai University. Mosquitoes were held at 28 ± 2°C, 70–85% relative humidity, with a photoperiod of 12-h light and 12-h dark. The larvae were fed on dog biscuits and yeast powder in the 3:1 ratio. At the time of adult feeding, these mosquitoes were 3–4 days old after emergences (maintained on raisins and water) and were starved for 12 h before feeding. Each time, 500 mosquitoes per cage were fed on blood using a feeding unit filled with parafilm as membrane for 4 h. *Aedes aegypti* feeding was done from 12 noon to 4:00 pm and *C. quinquefasciatus* were fed during 6:00–10:00 pm. A membrane feeder with the bottom end fitted with parafilm was placed with 2.0 ml of the blood sample (obtained from a slaughterhouse by collecting in a heparinized vial and stored at 4°C) and kept over a netted cage of mosquitoes. The blood was stirred continuously using an automated stirring device, and a constant temperature of 37°C was maintained using a water jacket circulating system. After feeding, the fully engorged females were separated and maintained on raisins.[17]

Mosquitoes gut dissection

Before the dissection for midgut extraction, the mosquitoes were surface sterilized with 70% ethanol for 2–10 min, which effectively surface sterilized the mosquitoes but did not affect the midgut bacteria (unpublished data).[5] After surface sterilization, mosquitoes were rinsed twice in sterile saline solution (0.85% NaCl). The dissected midguts were placed in 200-µl aliquots of NaCl solution prepared the day before under sterile conditions. Eppendorf tubes were reclosed immediately after midguts were put in the solution. After each dissection, forceps and needles were sterilized thoroughly in 70% ethanol to prevent contamination.

DNA extraction from mosquito gut

Obtained midguts were stored in −80°C until used for DNA extraction. The first is that DNA

must be extracted from the widest possible range of microorganisms to represent the original microbial population. The total microbial DNA was extracted by adapting small changes in the protocol described by Broderick *et al*.[18] In brief, the sample was suspended in 200 ml of digestion buffer and samples were incubated with occasional shaking in microtubes hermetically sealed for 1 h at 37°C. The tubes were centrifuged at 13,000 rpm for 30 min at −4°C; then, the supernatant was transferred to sterile centrifuge tubes. The samples were extracted with an equivalent phenolic centrifuge:chloroform:isoamyl alcohol (25:24:1) at 13,000 rpm for 15 min at −4°C. The supernatant was transferred to 1.5 µl of eppondrof tube and incubate at −80°C for 30 min. Again, it was centrifuged at 13,000 rpm for 30 min at 4°C. Reveal the supernatant of suspended in 20 ml of TE buffer (ph - >8.0) store at −20°C and use more.

DNA quality and quantity

The absorption spectrum of DNA extracts (230–280 nm and 260–230 nm) was determined using Nanodrop(R) ND-1000 spectrophotometer (Eurofins Genomics Bioinformatics Lab) according to the manufacturer's instructions. Pure DNA is known to produce 260/280 and 260/280 nm ratios 1:80.[19] DNA was visualized by electrophoresis of 5-µl aliquots through 1.2% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide, and DNA was quantified (µg DNA 0.1 g^{-1} fresh gut content) as previously described.

Polymerase chain reaction (PCR) amplification

The PCR reaction of bacterial 16S rRNA gene V3 and V4 region was performed containing 12.5 µl of ×2 KAPA HiFi HotStart ReadyMix, 5 µl (1 µM) of forward primer GCCTACGGGNGGCWGCAG 3' and reverse primer 5' ACTACHVGGGTATCTAATCC 3', and 2.5 µl of DNA template (5 ng/µl in 10 mM Tris pH 8.5) to a final volume of 25 µl. The PCR protocol was performed in triplicate using the following conditions: 5 min at 98°C for initial denaturing, followed by 25 cycles of 98°C for 30 s, 50°C for 30 s, and 72°C for 30 s with the final extension for 5 min at 72°C. The Illumina sequencing adapter ligated reverse primer contained a 6-bp barcode specific for sample identification.[20] After amplification, PCR products were pooled and purified using the PCR Cleanup Kit (Axygen Biosciences, Union City, CA, USA). Bacterial PCR products were pooled separately to sequence in their runs, respectively.

Cluster generation and sequencing

After obtaining the peak size from Tape Station profile, libraries were loaded onto MiSeq at an appropriate concentration (10–20 pM) for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse direction on MiSeq. The kit reagents were used in the binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after resynthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

Bioinformatics and statistical analysis

Bacterial sequences were analyzed using the UPARSE pipeline.[21] Briefly, paired-end reads were merged into single sequences, the low-quality merged sequences (maximum expected error >1, <370 bp for bacterial) were removed from downstream analysis. After removing the chimera, sequences with ≥97% similarity were clustered into operational taxonomic units (OTUs). The OTU representative sequences were assigned using the ribosomal database project classifier to identify bacterial taxonomies with a confidence threshold of 0.5.[22] The Chao1, goods coverage, Shannon, and Simpson indexes were calculated to estimate alpha-diversity of each sample using MOTHUR.[23]

RESULTS

The Nanodrop readings showed that the quantity and quality id DNA, the readings showed that the quantity of 20.2 ng/µ1 [Table 1]. QC pass DNA sample was processed for first amplicon generation followed by next-generation sequencing library preparation using Nextera XT Index Kit (illumine Inc.). The mean of the library fragment size

Table 1: Nanodrop reading for quality checking

NanoDrop readings (ng/µ1)	NanoDrop $ODA_{260/280}$	NanoDrop $ODA_{260/230}$	Remark
20.2	1.89	1.23	QC pass

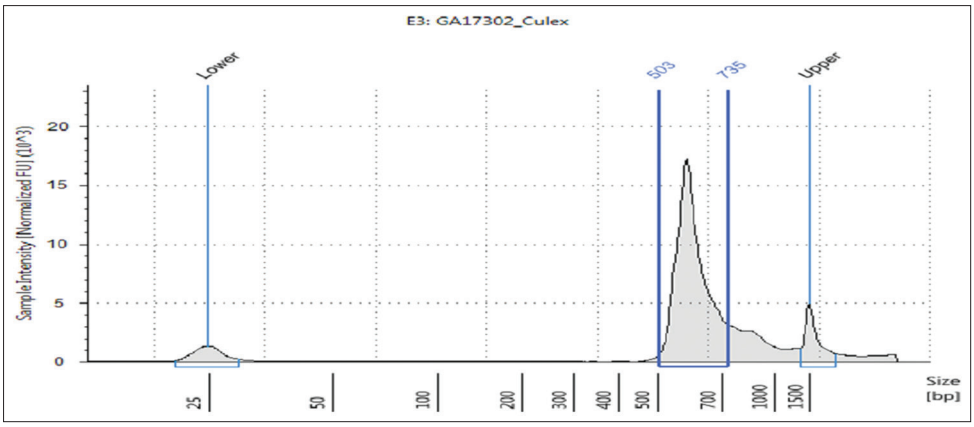

Figure 1: Library profile of a sample of *Culex quinquefasciatus* on Agilent tape station using D1000 screen tape

distribution is 610 bp. Libraries were sequence of MiSeq using 2×300 bp chemistry [Figure 1].

The total read counts and read bases were found to be 139,580 and 38,889,789 in *C. quinquefasciatus* gut part, after the chimera filter, it was found to be 279,157 and 72,286,401, respectively [Figure 2]. Metagenomic analysis of gut sample from *C. quinquefasciatus* showed the presence of different bacterial communities.

The Chao1 index was calculated to estimate the bacterial richness in sample. The Shannon and Simpson diversity indexes were used to evaluate the bacterial diversities found in sample. The Good's coverage index represents the relative measure of how well the sample represents the larger environment. The results showed that the Chao1 richness index of bacterial community was contributed as 678.43 ± 33. Both diversity indices showed that the least values represent that wide range of bacterial diversities was found in midgut part of *C. quinquefasciatus* [Table 2].

Bacterial community structure was examined by relative abundance within the sample. The phylum level distributions of bacteria are shown in Figure 3. The phylum Proteobacteria was highly found with the abundance of 92.95% which is followed by Bacteroidetes (6.88%) and Firmicutes (0.12%). Other bacterial phyla were showed very least occurrence (<0.01%) in midgut part of *C. quinquefasciatus*. The results revealed that the Proteobacteria phylum was most dominant bacterial phylum when compared with other bacterial phyla.

The class level distributions of bacteria are shown in Figure 4. The class Gammaproteobacteria was highly found in sample with the abundance of 92.95% which is followed by Flavobacteriia (6.87%). Other bacterial classes had very least occurrence (<0.01%) in midgut part of *C. quinquefasciatus*. The results revealed that the bacterial class Gammaproteobacteria was most dominant bacterial class when compared with other bacterial classes.

The family level distributions of bacteria were showed that the family Enterobacteriaceae was highly found in sample with the abundance of 54.66% which is followed by Pseudomonadaceae (22.77%), Xanthomonadaceae (10.70%), Weeksellaceae (6.87%), and Aeromonadaceae (4.37%). Other bacterial families showed very least occurrence (<0.20%) in midgut part of *C. quinquefasciatus* [Figure 5]. The results revealed that the bacterial class Enterobacteriaceae was most dominant bacterial family when compared with other bacterial families. Figure 6 showed that the presence of the pathogenic species belongings to the family Enterobacteriaceae was highly presented in the midgut of *C. quinquefasciatus*. In terms, the human pathogens were highly enriched in the midgut of selected mosquito because its feeding of blood from humans; moreover, it influences the microbial communities present in midgut.

DISCUSSION

It has been reported that the gut bacteria of mosquitoes play a significant role in the vector-parasite interaction.[24] The present

Table 2: Summary and indexing of the NGS

Sample name	Goods coverage	Richness estimator	Diversity index	
		Chao1	Shannon	Simpson
Culex quinquefacitatus	0.98±0.03	678.43±33	6.23±0.1	0.97±0.05

NGS: Next-generation sequencing

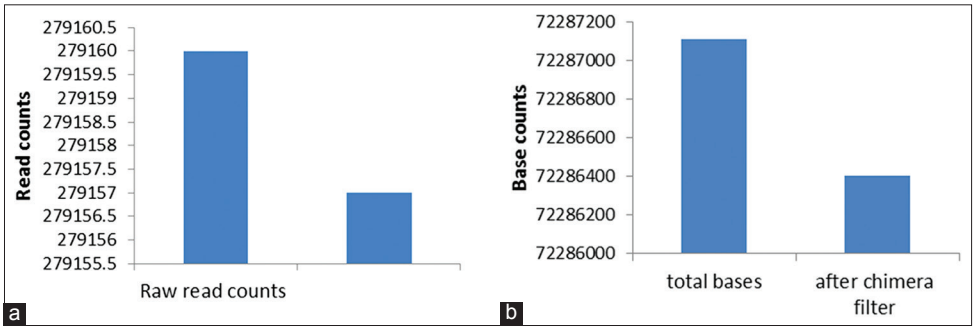

Figure 2: Next-generation sequencing reads counts (a) and base (b) summary

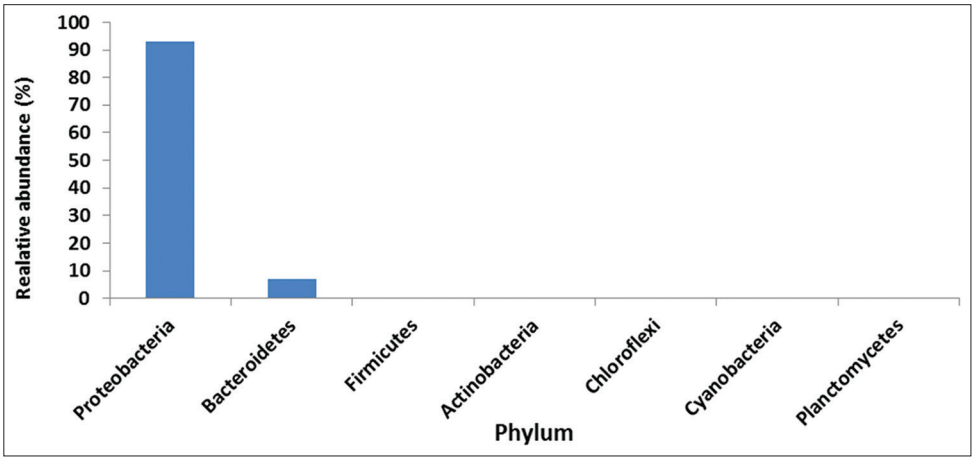

Figure 3: Relative abundance of bacterial Phylum found in midgut part of *Culex quinquefasciatus*

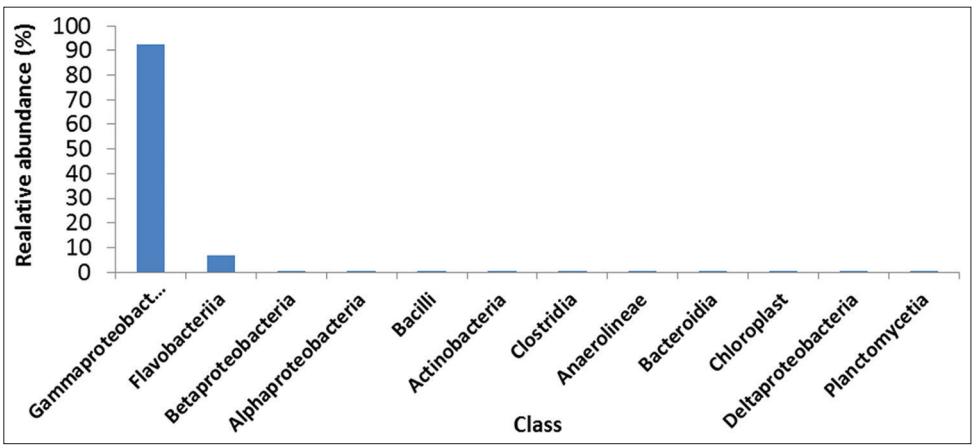

Figure 4: Relative abundance of bacterial classes found in midgut part of *Culex quinquefasciatus*

study was undertaken to study the cultivable and unculturable bacterial diversity found in selected to mosquito species *C. quinquefasciatus* gut sample using Illumina MiSeq studies. Similarly, Angelakis *et al*., 2016, used that the Illumina MiSeq platform for analyses the bacterial diversity present in different mosquitos gut part. Similarly, Satnami *et al*., 2017,[25] were used Illumina MiSeq sequencing platform for analyses the bacterial gut diversity in heavy metal remediation earthworms. In the present study, we contributed that the presence of different bacterial pathogens *Stenotrophomonas geniculate, Enterobacter cloacae, Erwinia soli, Salmonella enterica, Enterococcus asini, Bacillus*

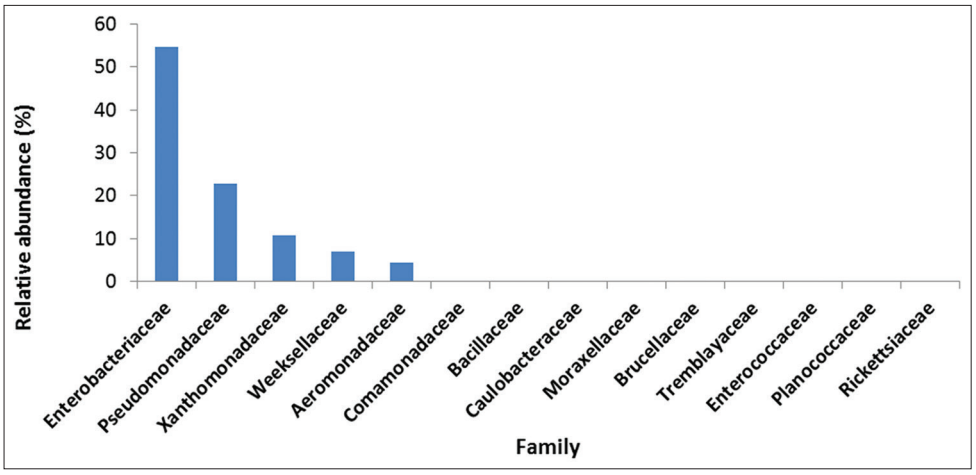

Figure 5: Relative abundance of bacterial Family found in midgut part of *Culex quinquefasciatus*

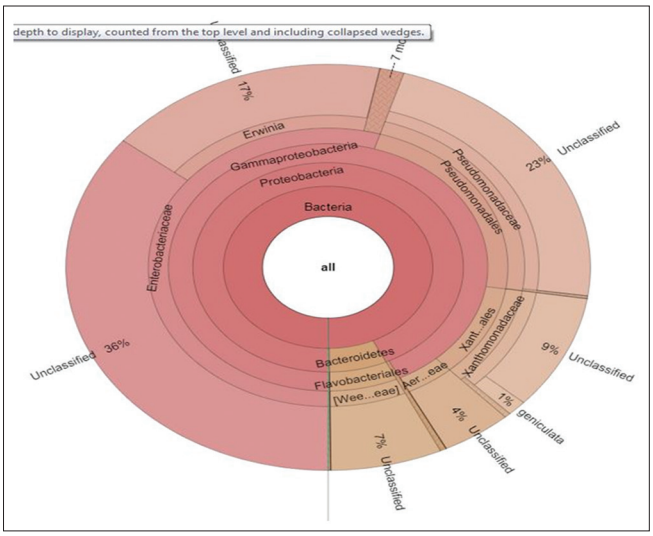

Figure 6: Krona chart explores bacterial diversity found in midgut part of *Culex quinquefasciatus*

cereus, Enterobacter turicensis, and *Enterobacter ludwigii.* Similar results were also reported by several researchers.[26-29]

Rani *et al.,* 2009,[11] reported that the vastness of pathogenic bacterial population was high in the larvae and gut part of adult *Anopheles stephensi*. The results also similarly correlated with the results obtained from the present work. The results from the work carried out by Yadav *et al.,* 2016,[30] revealed that the midgut part of different mosquito species collected from biodiversity hotspot, Bhalukpong, Arunachal Pradesh, India, has harbored to several human pathogenic bacteria. Similarly, the metagenomic results obtained from this present study showed that gut part of *C. quinquefasciatus* contains several human pathogenic bacteria.

The prevalence of bacterial pathogen belongings to the *Enterobacteriaceae* which was harbored in gut environment of *C. quinquefasciatus* found to be high and it shows that there is more prevalence potential will be occur when *C. quinquefasciatus* bites the humans. Importantly, the abundance of Enterobacteriaceae family in the mosquito midgut correlates significantly with the Plasmodium infection status. This striking relationship highlights the role of natural gut environment in parasite transmission.[26]

The analysis of conventional cultivable bacterial population in midgut of *Aedes albopictus* was studied by Yadav *et al*., 2016, reported that, based on colony morphological characteristics, we selected 113 cultivable bacterial isolates for 16S rRNA gene sequence-based molecular identification. Of the 113 isolates, we could identify 35 bacterial species belonging to 18 distinct genera under four major phyla, namely, Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Similarly, results also obtained in this present study, which the abundance of Proteobacteria phylum was to be high when compared to other phyla.

CONCLUSION

The characterization of bacterial communities present in midgut of mosquito *C. quinquefasciatus* will help to overcome the health problems caused by this mosquito to humans. Furthermore, the understanding of mosquito and pathogen interaction will give more sustainability to minimize the survivability of pathogens inside the mosquito midgut environment. Further studies are needed to investigate by physiological characteristics of the bacteria and their possible interactions with mosquito by biology and vector competence.

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