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Revisiting Methanotrophic Communities in Sewage Treatment Plants

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The methanotrophic potential in sewage treatment sludge was investigated. We detected a diverse aerobic methanotrophic community that potentially plays a significant role in mitigating methane emission in this environment. The results suggest that community structure was determined by conditions specific to the processes in a sewage treatment plant.

Next to water vapor and carbon dioxide, methane is one of the most potent greenhouse gases that contribute to radiative forcing (1). Sewage treatment accounts for around 4% of the total methane produced (500 to 600 Tg methane) globally every year (2) and will likely increase with the growing need for clean water and sanitation to sustain the world's growing population (3). Although methane emitted from sewage treatment plants (STPs) may be captured for energy generation, methane slippage occurs and dissolved methane in the effluent can be discharged without recovery (4). This raises the question of whether methanotrophs play a role in attenuating methane emission from these environments. Indeed, biological methane oxidation is an important process, consuming around 80% of the methane entering an activated-sludge reactor (4).

Taxonomically, aerobic proteobacterial methanotrophs have been grouped into type I (*Gammaproteobacteria*) and type II (*Alphaproteobacteria*). Type I methanotrophs have been further divided into type Ia and type Ib on the basis of *pmoA* gene phylogeny. Additionally, *Methylocella* and *Methylocapsa*, belonging to *Beijerinckiaceae*, fall within the class *Alphaproteobacteria*. These methanotrophs possess a well-known physiology (5, 6). Besides, *Crenothrix polyspora* and *Clonothrix fusca* are known proteobacterial methane oxidizers found mainly in groundwater and wells (7, 8). Only recently have nitrite-driven anaerobic methanotrophs belonging to the phylum NC10 been discovered and now include a cultured representative, "*Candidatus* Methylomirabilis oxyfera" (9). "*C.* Methylomirabilis oxyfera" is unique in the way it generates molecular oxygen intercellularly and uses it subsequently for methane oxidation (9, 10). Both aerobic proteobacterial methanotrophs and "*C.* Methylomirabilis oxyfera" possess a particulate methane monooxygenase (pMMO), the key enzyme in methane oxidation. The *pmoA* gene encodes the β subunit of the particulate form of MMO and is present in the vast majority of methanotrophs, making it a suitable marker for culture-independent studies (11, 12). *Methylocella* and *Methyloferula*, however, possess only the soluble form of the MMO (13, 14). In addition to the class *Proteobacteria* and the phylum NC10, acidophilic and thermophilic verrucomicrobial methanotrophs were discovered, but they have so far been detected only in extreme environments (15, 16).

Previous interest in methanotrophs in groundwater or sewage treatment systems stems from their ability to cometabolically degrade chlorinated aliphatic hydrocarbons (e.g., trichloroethylene, 1,1,1-trichloroethane) (6, 17). However, little attention was given to their role in methane oxidation, and to our knowledge, no other study has yet described the diversity and abundance of aerobic methanotrophs in STPs. This necessitates qualitative and quantitative assessment of the methanotrophic communities in wastewater sludge. Sewage treatment systems are characterized by a high nutrient turnover rate, high shear force, and alternating oxic and anoxic periods or processes. Hence, they provide a dynamic yet homogenized model system in which to study microbial community structure and population dynamics (18, 19). Overall, we aimed to (i) determine the diversity and abundance of the bacterial methane-oxidizing communities in activated and anaerobic digester sludges and biomass from oxygen-limited autotrophic nitrification/denitrification (OLAND) rotating biological contactors and (ii) determine the potential for methane oxidation in these sludges under oxic conditions.

Wastewater sludges were sampled from compartments of different STPs that represent the different processes in domestic wastewater treatment (see Fig. S1 in the supplemental material). The characteristics of the wastewater sludge used are given in Table 1. The OLAND rotating biological contactors used are well characterized and were used to treat wastewater with high nitrogen concentrations (20). Typically, these reactors contain a community of aerobic and anaerobic ammonium oxidizers (21). Triplicate batch incubations (working volume, 8 ml) were performed with 120-ml serum bottles under approximately 25% methane by volume in air on a shaker (120 rpm) in the dark at 28°C for each sludge. DNA was extracted from the starting material and after incubation (12 days) with the QBIOgene soil extraction kit with minor modifications (MP Biomedicals) (22, 23) and stored at −20°C for further molecular analyses. For details of the methodology used and the subsequent molecular analyses, see the supplemental material.

To capture aerobic methanotroph diversity, a *pmoA*-based diagnostic microarray analysis was performed as described before (22, 23), with the A189f/T7_A682r primer combination. The amplicons were derived from three DNA extractions of the starting material and from three batch incubations of each sludge sample after 12 days. Analysis of the standardized microarray data was

Received 6 November 2012 Accepted 12 February 2013
Published ahead of print 15 February 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03426-12.

doi:10.1128/AEM.03426-12

TABLE 1 STP wastewater sludge characteristics and total methane consumed over 12 days of incubation[a]

Sample description	City, country	pH	DO[b] (mg O_2 liter^{-1})	EC_c[c] (mS cm^{-1})	VSS[d] (mg ml^{-1})	Nutrient contents (mM)[f]				CH_4 uptake measurement[g]		
						PO_4^{3-}	SO_4^{2-}	NO_3^-	NH_4^+	Lag (h)	Total CH_4 consumed (mmol g VSS^{-1})	Temp range (°C)
Anoxic activated sludge	Ghent, Belgium	7.37	0	0.88	0.64	BDL[e]	0.66	0.58	1.13 ± 0.09	<94	160.32 ± 17.79	9–16[h]
Returned activated sludge	Ghent, Belgium	7.35	0–5.0	0.99	2.90	BDL	1.06	0.62	0.18 ± 0.03	<94	34.66 ± 0.94	9–16[h]
Anaerobic digester sludge	Ghent, Belgium	7.70	0	5.27	21.56	BDL	0.30	0.77	53.41 ± 1.12	<94	22.09 ± 1.02	34
OLAND (lab scale)	Ghent, Belgium	7.57	1.0	8.92	2.04	1.34	37.29	17.94	1.98 ± 0.02	≤117	85.16 ± 2.69	28
OLAND (industrial scale)	Sneek, The Netherlands	5.78	0.6	4.73	1.74	5.63	3.41	58.38	15.68 ± 0.29	≤168	35.19 ± 5.71	27

[a] The means ± standard deviations shown are for three samples each. Samples were normalized per gram of VSS (21).
[b] DO, dissolved oxygen.
[c] EC_e, electrical conductivity.
[d] VSS are particulate organic solids, excluding soluble solids and inorganic solids.
[e] BDL, below detection limit.
[f] Endpoint nutrient concentrations (after 12 days of incubation) are shown in Fig. S4 in the supplemental material.
[g] Temporal changes in methane uptake are shown in Fig. S3 in the supplemental material.
[h] Sampled in March 2012; subject to seasonal temperature fluctuations.

done in R ver. 2.10.0 (24) using heatmap.2 as implemented in gplots ver. 2.7.4. The *pmoA* gene is present in virtually all methanotrophs, allowing a wider coverage of the methanotroph inventory than *mmoX* (a gene encoding the soluble form of MMO), which is confined to only some methanotrophs. *Methylocella*-like *mmoX* sequences have been retrieved from diverse environments (25), but their ecological relevance at circumneutral pH remains uncertain (26). Hence, we focused on the *pmoA* gene diversity of the potentially active community after 12 days of *in vitro* incubation. Although we detected a broadly similar methanotrophic composition comprising both types I and II, a cluster analysis of standardized microarray data revealed grouping of the activated and anaerobic digester sludges and OLAND sludges, respectively (Fig. 1). The methanotrophic communities in the returned activated and anaerobic digester sludges were largely similar but differed in their relative distribution in these sludges (Fig. 1). Similar clusters were observed in the starting material (see Fig. S2 in the supplemental material). Although the microarray covers *Crenothrix*-like and verrucomicrobial methanotrophs, the corresponding probes did not show a hybridization signal (Fig. 1; see Fig. S2). Despite the different working scales and pHs, it appears that in the OLAND reactor biomass, a specific methanotrophic community was enriched after incubation, largely made up of *Methylocaldum*-affiliated type Ib (probes Mcl404, Mcl408, and P_MclE302) and a unique type II community (probes peat264, Msi232, and P_Msi423). Interestingly, this community seems to be depauperate in *pmoA2* (probe P_NMsiT.271). *pmoA2* encodes an isozyme of pMMO that en-

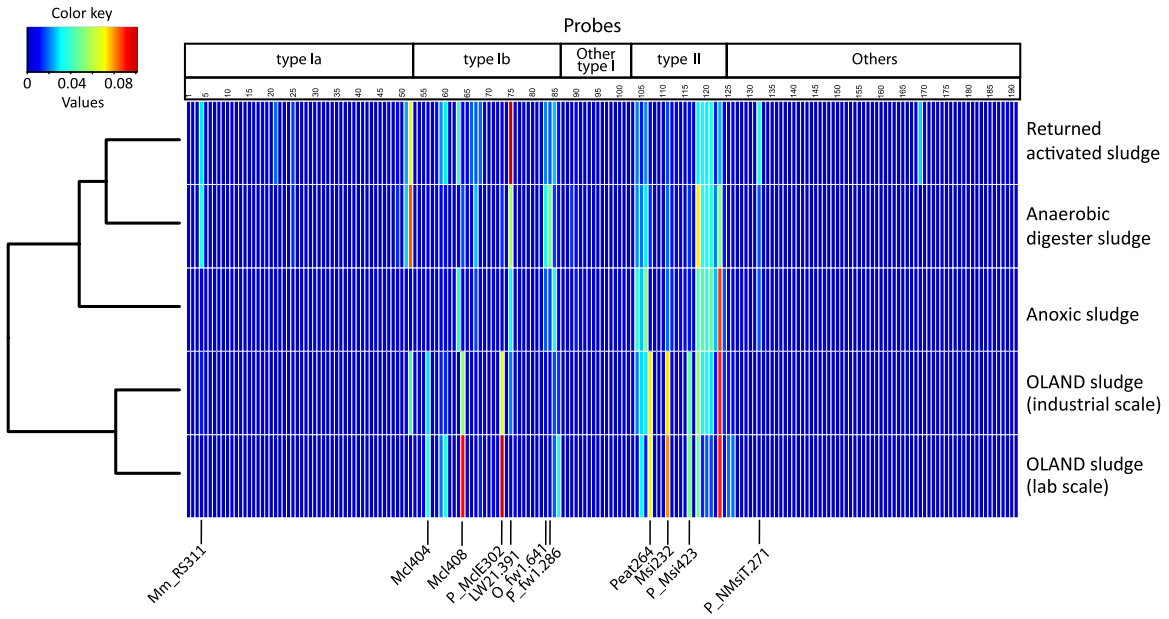

FIG 1 Cluster analysis of standardized microarray data showing the diversity of the aerobic methanotrophic community in wastewater sludge after incubation. Probe names and their intended specificities are shown (see Table S1 in the supplemental material). The results shown are averages of triplicate analyses of each sludge sample. Red and blue indicate the highest and no hybridization signal, respectively. These probes represent type I and II methanotrophs. Probes targeting *amoA* (a gene encoding ammonia monooxygenase), *pmoA2*, verrucomicrobial methanotrophs, and environmental sequences clustered between *amoA* and *pmoA* are grouped as "Others."

ables methane oxidation and growth at low methane concentrations (27) and is so far confined to type II methanotrophs (28). The dominant type Ib methanotrophs in the OLAND reactor biomass were *Methylocaldum*-affiliated microorganisms, whereas all other samples were dominated by environmental *pmoA* lineages without closely related cultivated representatives (freshwater lineages 1 and 2) (29), suggesting that the different processes favored distinct members of a subgroup.

The OLAND reactor may provide a niche for the aerobic methanotrophs, given oxic or micro-oxic areas in the structured biofilms (21). The OLAND reactor biomass, however, is not known to produce methane, with methanogenesis probably being suppressed by the continuous presence of nitrate. However, methane is supplied via the influent. The biomass in the OLAND reactors experiences continuously high ammonium concentrations, up to 1.0 to 1.1 g N liter^{-1} in the influent. Type I methanotrophs, particularly *Methylomicrobium*- and *Methylocaldum*-like methanotrophs, shown to be selectively stimulated by ammonium amendment (30), may have been favored under this condition. Moreover, intermediates and products of nitrification may have discriminated against methanotrophs, e.g., susceptible to hydroxylamine or nitrite (31, 32). Hence, a large ammonium load may be a strong selecting force for the methanotrophic community composition in these reactors. On the contrary, type II methanotrophs are thought to be present mainly as resting cells in other environments (26, 33), which is also indicated by the qPCR analysis in this study (Fig. 2).

PCR amplification targeting the *pmoA* gene belonging to NC10 was performed by using a nested approach (12). Consistent with a previous study (34), cloning and sequence analysis revealed the presence of methanotrophs affiliated with "*C.* Methylomirabilis oxyfera" in activated and anaerobic digester sludges. These sequences clustered within known "*C.* Methylomirabilis oxyfera"-like *pmoA* from other environments (Fig. 3). The biomass in the OLAND reactors did not harbor these sequences. The presence of "*C.* Methylomirabilis oxyfera"-like sequences indicates the potential for anaerobic methane oxidation at the expense of nitrite in the activated and anaerobic digester sludges.

Microarray analysis is able to resolve the methanotrophic composition down to the species level (22) but does not provide the numerical abundances of the methanotrophs detected. Hence, group-specific qPCR assays targeting type Ia (MBAC assay), type Ib (MCOC assay), and type II (TYPEII assay) methanotrophs were performed, with minor modifications as described before (23, 35), to determine the abundance and growth of the active subpopulation in the *in vitro* incubations (Fig. 2). Before incubation, the number of *pmoA* gene copies was low, often below or at the detection limit ($<10^4$ to 10^5 copies of target molecule g of volatile suspended solids [VSS]$^{-1}$). However, the *pmoA* gene copy number of sequences affiliated with type I methanotrophs markedly increased by 2 to 5 orders of magnitude during aerobic incubation with methane (Fig. 2). The sludge from the OLAND lab scale reactor was the only exception, showing a relatively stable community of type Ib and type II methanotrophs; type Ia was not detected by microarray and qPCR. Type II *pmoA* was dominant in the starting material, as shown by qPCR and microarray analyses. However, regardless of the different type II community, the respective *pmoA* copy numbers remained relatively constant or decreased during incubation. With the qPCR assays covering a high percentage of environmental sequences (23), the increase of type

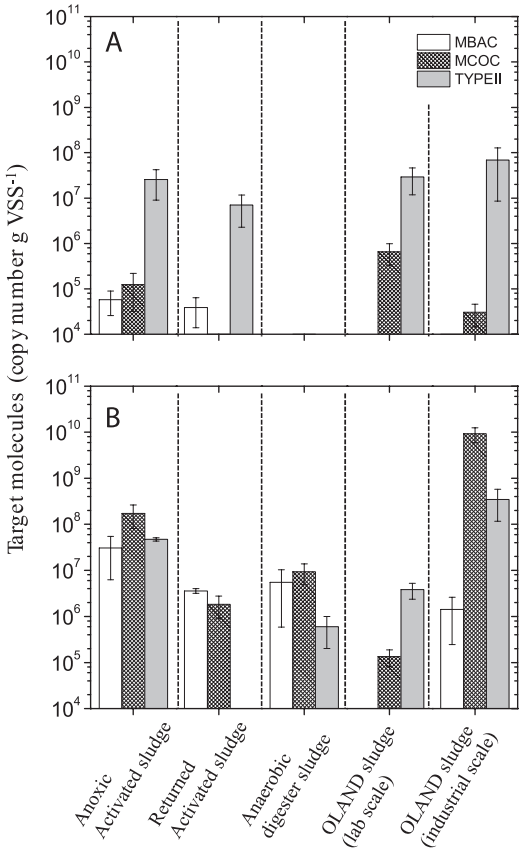

FIG 2 qPCR analysis of MBAC, MCOC, and TYPEII assays in the starting materials (A) and after 12 days of incubation (B). qPCR was performed in duplicate with each DNA extract (mean ± standard deviation, total $n = 6$).

Ia/Ib methanotrophs suggests that they are the predominantly active population under the assay conditions used (Fig. 2). Generally, type I methanotrophs were found to be active in other high-methane environments (30, 33, 36–39). The proliferation of methanotrophs after methane addition indicates their potential to mitigate methane emission in wastewater sludges.

While both aerobic and nitrite-driven anaerobic methanotrophs have been detected in wastewater sludge (34, 40), a recent study indicates that aerobic methane oxidation is more important *in situ* (4). High nitrite-driven anaerobic methane-oxidizing activity has so far been documented only in enrichment cultures of "*C.* Methylomirabilis oxyfera"-like microorganisms (41–43). Hence, we focused on aerobic methane oxidation in the natural community. Methane uptake was detected in all sludges (Table 1; see Fig. S3 in the supplemental material). Total methane consumption was highest in the anoxic activated sludge, while the other types of sludge exhibited relatively lower methane uptake (Table 1). The anaerobic digester sludge and activated sludge and OLAND reactors receiving methane-rich influent are compartments with large methane loads. The potential for aerobic methane oxidation thus signifies the role of methanotrophs to mitigate methane emission in these compartments if/when electron acceptors are not limiting. Interestingly, the anaerobic digester sludge and OLAND reactor biomass exhibited methane uptake despite the potential inhibitory effects induced by (by)products of ammonia oxidation (31, 32, 44). With a relatively stable methanotrophic population

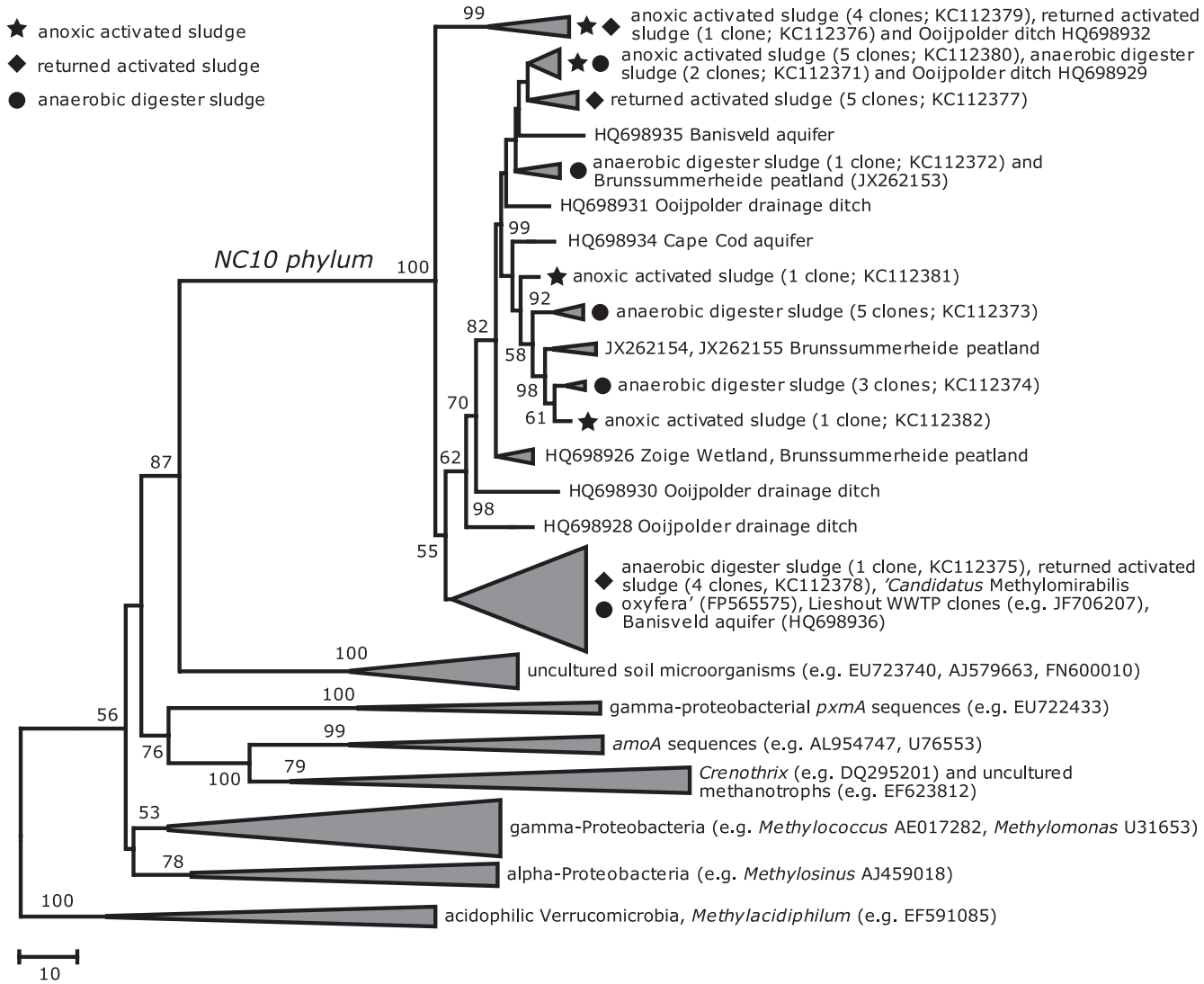

FIG 3 Phylogenetic tree depicting NC10 bacterial diversity in different compartments of STPs based on *pmoA* gene sequences. Clustering of nucleotide sequences (351 positions) was inferred by using the neighbor-joining algorithm integrated in MEGA5 (45). Bootstrap support values (1,000 replicates) of greater than 50% are indicated at the nodes. The analysis involved 158 *pmoA*, *amoA*, and *pxmA* gene sequences, but for clarity, they were grouped as shown. The scale bar shows the number of base differences between sequences.

size in the OLAND lab scale reactor (Fig. 2), the higher methane uptake suggests an increase in cell-specific activity. There was, however, a longer lag phase (Table 1) before the onset of activity in the OLAND sludge. Moreover, the decrease in nutrient concentrations after incubation (see Fig. S4 in the supplemental material) coincides with the growth of the methanotrophic population. The relatively fast response in activity (<4 days) and growth in the activated and anaerobic digester sludges indicates the presence of an active aerobic methanotrophic population that acts to attenuate methane emission when conditions turn permissive in STPs.

We investigated STPs to determine the methanotrophic potential in wastewater sludge and provide a first insight into methanotroph ecology in this environment. We demonstrated that the aerobic methanotrophic communities in STPs are diverse and that they potentially attenuate methane emission from this environment. We confirmed the occurrence of "*C.* Methylomirabilis oxyfera"-like methanotrophs in wastewater sludge. Further, results suggest that the community composition was driven by conditions specific to the processes in STPs.

Nucleotide sequence accession numbers. Representative *pmoA* gene sequences were deposited in GenBank under accession numbers KC112371 through KC112382.

ACKNOWLEDGMENTS

We thank Tim Lacoere for excellent technical assistance and Karen De Roy and Pieter van den Abbeele for proofreading the manuscript. We extend our gratitude to Levente Bodrossy (CSIRO, Tasmania, Australia) for introducing us to microarray analysis and to Claudia Lüke (Max Planck Institute, Marburg, Germany) for assistance with microarray analysis. We also thank Willy Verstraete, Brendo Meulman (Desah, Sneek, Netherlands), and Jo De Vrieze for assistance with sampling.

A.H. and N.B. are supported by research grants from the Geconcerteerde Onderzoeksactie of Ghent University (BOF09/GOA/005). S.E.V. is financially supported by the Research Foundation Flanders

(Fonds Wetenschappelijk Onderzoek). K.F.E. is supported by a grant from the Darwin Center for Biogeosciences (project 142.16.3071).

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