**Desulfovibrio aminophilus sp. nov., a Novel Amino Acid Degrading and Sulfate Reducing Bacterium from an Anaerobic Dairy Wastewater Lagoon**

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**Summary**

A mesophilic strain of sulfate-reducing bacterium, designated ALA-3T (T = type strain), was isolated from an anaerobic lagoon of a dairy wastewater treatment plant. The curved, Gram-negative, non-sporoforming cells (0.2 × 3.0-4.0 μm) existed singly or in chains, and were motile by single polar flagella. Optimum growth occurred at 35 °C and pH 7.5 on a medium containing lactate and sulfate. Thiosulfate or sulfite but not elemental sulfur, nitrate, or fumarate could also replace sulfate as an electron acceptor. Formate, alanine, aspartate, leucine, isoleucine, valine, and methionine, H₂/CO₂ and ethanol also served as electron donors with sulfate as an electron acceptor. Pyruvate, casamino acids, peptone, serine, glycine, cysteine and threonine were fermented. Sulfite and thiosulfate were disproportionated to sulfate and sulfide. The G+C content of the DNA was 66 mol % G+C. Phylogenetic analysis revealed that *Desulfovibrio africanzts* was the nearest relative (similarity of 89%). Strain ALA-3T is physiologically and phylogenetically different from other *Desulfouibrio* species, and is designated *Desulfovibrio aminophilus* sp. nov. (DSM 12254).

**Key words:** Sulfate-reducing bacteria – Amino acid utilization – Thiosulfate – Sulfite disproportionation

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**Introduction**

The anaerobic degradation of proteins has not been studied as extensively as carbohydrate fermentation though the input of proteins into anaerobic environments is large (McInerney, 1988). Because of the presence of proteins in almost any ecosystem, the turnover of amino acids is a very important microbiological process (Girbal et al., 1997). The degradation of amino acids by anaerobic bacteria has been investigated most comprehensively in fermentative *Clostridium* species (Baker, 1981; Elsdén et. al., 1979), but other anaerobes are also known to utilize amino acids especially in the presence of external electron acceptors or in mixed cultures with hydrogen scavengers (Stams, 1994). In contrast, the use of amino acids by sulfate-reducing bacteria has been poorly studied, whereas their activities regarding amino acid degradation has been clearly established in marine sediments (Hansen and Blackburn, 1995). Only a few members of the genera *Desulfo bacterium, Desulfo tomaculum* and *Desulfovibrio* are known to utilize single amino acid (Rees et al., 1998; Stams et al., 1985; Stams and Hansen, 1986; van der Maarel et al., 1996). Furthermore, recent reports have shown that the addition of thiosulfate to the growth medium enabled the utilization of amino acids by non sulfate-reducing bacteria such as *Thermoanaerobacter brockii* (Faudon et al., 1995; Fardeau et al., 1997a) and *Dethiosulfovibrio peptidevorum* (Magot et al., 1997). Thus, these data suggest an important role of sulfate and thiosulfate in the degradation of proteinaceous compounds. In our present study, we isolated and characterized a sulfate-reducing bacterium, designated strain ALA-3T, which degraded peptides and amino acids as an energy source.


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Genes for ectoine synthesis in *Halomonas elongata* 497
FICHE DESCRIPTIVE


Titre original : *Desulfovibrio aminophilus* sp. nov., a novel amino acid-degrading and sulfate-reducing bacterium from an anaerobic dairy wastewater lagoon.


Titre en Français : *Desulfovibrio aminophilus* sp. nov., une nouvelle bactérie sulfato-réductrice dégradant les acides aminés, isolée d’une lagune anaérobie d’effluents de laiterie

Mots-clés matières : *Desulfovibrio aminophilus*, phylogénie, taxonomie, anaérobiose, sulfato-réduction, acides aminés (10 au plus)

Résumé en Français :
(150 mots maximum)

Plan de classement : Monde végétal et Animal - Fermentations
Materials and Methods

Sample source: Sludge samples were collected from an anaerobic lagoon of a dairy wastewater treatment plant (Santa Fe de Bogota, Colombia) by completely filling 100 ml bottles to the brim. The samples were transported to the laboratory and maintained at 4 °C until used. The pH and temperature from where the sample was collected was 6.8 and 24°C, respectively.

Media, Culture Conditions and Isolation: The Hungate technique (HUNGATE, 1969) was used throughout these studies. The basal medium consisted of (per liter) 0.2 g of KH₂PO₄, 0.3 g of NH₄Cl, 1.0 g of NaCl, 0.4 g of MgCl₂ - 6H₂O, 0.5 g of KCl, 0.15 g of CaCl₂ - 2H₂O, 1 ml of trace element solution (IMHOFF-STUCKLE and PFENNING, 1983), 1.0 ml of 0.1% resazurin. The pH was adjusted to 7.2 with 10 M KOH. The medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Aliquots of 5-ml from the medium were dispensed into Hungate tubes under a stream of O₂-free N₂ gas, and subsequently the gas phase was replaced with N₂-CO₂ (80:20). Prior to inoculation, 0.15 ml of 2% Na₂S (5 mM) as energy source and electron acceptor were used (BAK and PFENNING, 1987). Disproportionation was verified by measuring optical density increase and the formation of sulfate and sulfide.

Analytical Techniques: Duplicate culture tubes were used throughout this study. Growth was measured by inserting tubes directly into a model UV-160A spectrophotometer (Shimadzu Corp., Kyoto, Japan) and measuring the optical density at 580 nm. Sulfide was determined photometrically (CORD-RUWISCH, 1983). Sulfate was measured according to the method of MAASEN and AAMAND (1991) and fermentation products determined as described by FARDEAU et al., 1993. Amino acid concentrations were determined by High Pressure Liquid Chromatography (MOORE et al., 1958). Growth and product formation were analyzed after 2 to 3 weeks of incubation at 37 °C. Cytochromes and desulfoviridin were determined as described by POSTGATE (1959). Cell morphology was determined using a Nikon phase-contrast microscope. For electron microscopy, cells were negatively stained with 1% sodium phosphotungstic acid (pH 7.2) as described previously (FARDEAU et al., 1997b).

Growth Characterization: Growth at various temperatures, pH and in sodium chloride was studied using basal medium containing 20 mM lactate and 20 mM thiosulfate as electron donor and acceptor respectively. For pH studies, the medium was adjusted with sterile anaerobic stock solutions of 1 M HCl, in sodium chloride was studied using basal medium containing lactate (20 mM). Fermentation tests with lactate, pyruvate, propionate, butyrate, valerate, fumarate, succinate, malate, amino acids, glutamate, valine, serine, arginine, aspartate, leucine, threonine, histidine, glycine, cysteine, isoleucine, methionine, lysine, proline, histidine, glucose, fructose, malate, succinate and fumarate were conducted using the basal medium.

Strain ALA-3T was subcultured at least once under the same experimental conditions. For disproportionation of thiosulfate and sulfite studies, basal medium containing acetate (5 mM) as a carbon source and either thiosulfate (20 mM) or sulfite (5 mM) as energy source and electron acceptor were used (BAK and PFENNING, 1987). Disproportionation was verified by measuring optical density increase and the formation of sulfate and sulfide.

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Nucleotide sequence accession number: The 16S rRNA gene sequence of strain ALA-3T has been deposited in the GenBank data base under accession number AF067964.

Results

Isolation

Cultures were regarded as positive after incubation at 37°C for 2 weeks based on growth, acetate and H₂S production. Microscopic examination of the highest dilution positive culture revealed the presence of two bacterial morphotypes, viz. rod-shaped and vibrio-shaped cells. Roll tubes inoculated with this culture also showed two distinct types of colonies. One type of colony was small, white and lense-shaped, and the other was 1 mm in diameter, round, whitish and possessed smooth edges. The latter colony type was isolated in pure culture, designated ALA-3T and characterized further.

Morphology, physiology and G+C content

Cells of strain ALA-3T were Gram negative non-sporulating vibrios, 3.0 to 4.0 μm long and 0.2 μm wide (Fig. 1). Strain ALA-3T was motile by means of a single polar flagellum (data not shown).

Strain ALA-3T was a strictly anaerobic chemooorganotrophic bacterium. The optimal growth temperature was 35°C (temperature range was between 25 and 40°C). The optimum pH for growth was around 7.5 (pH growth range between 6.7 to 8.0). Strain ALA-3T did not require NaCl for growth but tolerated up to 2% NaCl with optimum growth occurring in the presence of 0.05 to 0.75% NaCl. The cells contained c-type cytochromes and desulfoviridin. The G+C content of strain ALA-3T is 66 mol%.

In the presence of thiosulfate or sulfate as electron acceptor, strain ALA-3T grew on lactate and did not require yeast extract (though 0.2% yeast extract enhanced growth). Strain ALA-3T grew with ethanol and H₂/CO₂ only with added acetate or yeast extract. Furthermore, the following amino acids are only used in the presence of 0.2% yeast extract: alanine, leucine, isoleucine, valine, aspartate, and methionine with sulfate as an electron acceptor (Table 1). Glutamate was converted mainly to pyroglutamate and the small amounts of acetate and propionate and sulfide produced could be a result of partial oxidative deamination.

In the absence of sulfate, strain ALA-3T used pyruvate, peptone and casamino acids. Serine, glycine, threonine and cysteine were used in the presence of yeast extract (0.2%). Serine, glycine and cysteine were fermented to acetate. Threonine was fermented to acetate and traces of propionate. Only pyroglutamate was detected from glutamate utilization in the absence of sulfate.

Sulfate, thiosulfate and sulfite were utilized as electron acceptors but not elemental sulfur, nitrate and fumarate. Thiosulfate and sulfite were disproportionated to sulfate and sulfide.

16S rRNA sequence analysis

An almost complete 16S rRNA gene sequence comprising of 1530 nucleotides, corresponding to positions 21 to 1539 respectively [E. coli numbering, according to Winker and Woese, (1991)], was determined. Sequence alignment and subsequent comparisons with sequences of representative members of domain Bacteria consistently placed strain ALA-3T as a member of the family Desulfovibrionaceae. Maximum likelihood analysis using fastDNAml also gave similar results. Further analysis indicated that the closest relative of strain ALA-3T was D. africana (similarity of 89%). The clustering of strain ALA-3T with the sole lineage represented by D. africana is interesting but cannot be predicted with confidence as bootstrap analysis (100 data sets) was poor (48%) indicating the possibility that other close relatives are yet to be identified.

Fig 1. Phase-contrast photomicrograph of cells of strain ALA-3T from the exponential growth phase. Bar, 10 μm.
Table 1. End-products of metabolism of strain ALA-3T in the presence of sulfate.

<table>
<thead>
<tr>
<th>Culture conditionsa</th>
<th>Substrate utilized (mM)</th>
<th>Products formed (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetate</td>
</tr>
<tr>
<td>Lactate</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Formate</td>
<td>15</td>
<td>0.0</td>
</tr>
<tr>
<td>H₂ + CO₂ + Acetate</td>
<td>nd</td>
<td>0</td>
</tr>
<tr>
<td>Alanineh</td>
<td>7.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Leucineh</td>
<td>6.5</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucineh</td>
<td>5.3</td>
<td>0</td>
</tr>
<tr>
<td>Valineh</td>
<td>3.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Aspartateh</td>
<td>4.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Methionineh,2</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>Glutamateh</td>
<td>7.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Serineh,3</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Cysteineh,3</td>
<td>5.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Glycinenh,3</td>
<td>9.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Threoninenh,3</td>
<td>4.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

- Results were recorded after 2–3 weeks incubation at 37 °C; 10 mM sulfate was added unless otherwise indicated. All values are corrected for the small amounts of products formed in the control tubes. The following substrates were tested and were not utilized by strain ALA-3T: arginine, lysine, proline, histidine, gelatin, casein, glucose, fructose, galactose, ribose, maltose, mannitol, acetate, propionate, butyrate, valerate, fumarate, malate, succinate, n-propanol, butanol, glycerol or benzoate.
- Tested in the presence of 0.2% Y.E.
- Abbreviations: f, formate; p, propionate; pGlu, pyroglutamate; iv, isovalerate; 2-mB, 2-methylbutyrate; iB, isobutyrate.
- Tested in the presence of 20 mM sulfate.
- An unidentified end-product peak was detected by HPLC.
- These substrates are also fermented; lactate, fructose, malate, succinate and fumarate were tested but were not fermented.

Discussion

Strain ALA-3T used a wider range of energy substrates than that reported for other Desulfovibrio species. It had, in particular, an important property of oxidizing or fermenting proteinaceous compounds such as peptides and amino acids (Table 1). Our results indicated that the growth of strain ALA-3T on amino acids depended on the concentration of yeast extract. For example, growth was obtained with 0.2% but not with 0.02% yeast extract on alanine, glutamate and valine. Strain ALA-3T reduced sulfate and thiosulfate and disproportionated thiosulfate and sulfite, suggesting that it may play a decisive role in the regulation of the electron flow in the sulfur cycle and also in protein turnover.

Fig. 2. Dendrogram based on 16S rRNA sequence data indicating the position of Desulfovibrionaminophilus strain ALA-3T within the members of Desulfovibrio and related genera. All the sequences used in the analysis, with the exception of those of D. oxyclinae, D. profundus, D. fructosovorans and D. alcoholovorans (GenBank accession numbers U33316, U90726, AF050101 and AF053751 respectively), were obtained from the Ribosomal Database Project, version 5.0 (MAIDAK et al., 1996). Bootstrap values greater than 90% (generated from 100 data sets) are shown at the branching points. The triangle indicates the cluster for the genus Desulfomicobilium. Escherichia coli was used as the outgroup member. Scale bar indicates 10 nucleotide substitutions per 100 nucleotides.
Table 2. Differentiating characteristics of strain ALA-3<sup>T</sup> and other amino acid utilizing sulfate-reducing bacteria.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strain ALA-3&lt;sup&gt;T&lt;/sup&gt;</th>
<th>Desulfovibrio 2002&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Desulfovibrio 2002&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Desulfo-</th>
<th>Desulfo-</th>
<th>Desulfo-</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>acrylicus&lt;sup&gt;c&lt;/sup&gt;</td>
<td>vacuolatum&lt;sup&gt;d&lt;/sup&gt;</td>
<td>bacterium niacin&lt;sup&gt;e&lt;/sup&gt;</td>
<td>tomatium ruminis&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Habitat</strong></td>
<td>anaerobic waste water lagoon</td>
<td>marine sediments</td>
<td>marine sediments</td>
<td>anoxic marine sediments</td>
<td>marine sediments</td>
<td>marine sediments</td>
</tr>
<tr>
<td><strong>Optimal temperature</strong></td>
<td>37 °C</td>
<td>30–37 °C</td>
<td>25–30 °C</td>
<td>29 °C</td>
<td>37 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td><strong>G+C content (mol%)</strong></td>
<td>66.0</td>
<td>47.2</td>
<td>42.9</td>
<td>45.1</td>
<td>45</td>
<td>45.8</td>
</tr>
<tr>
<td><strong>Amino acids used with SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</strong></td>
<td>ala, val, leu, lie, asp, met</td>
<td>ala, ser, gly (+) val, leu, lie, cys, thn, asp</td>
<td>ala, ser, gly (+) val, leu, lie, cys, thn, asp</td>
<td>gly, ser, ala, cys</td>
<td>ala, glu, gin, gly, lie, leu, pro, val, ser</td>
<td>glu ala</td>
</tr>
<tr>
<td><strong>Other substrates used with SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>formate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>lactate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ethanol</td>
<td>+ (with acetate or YE)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>acetate</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>pyruvate</td>
<td>+</td>
<td></td>
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<td></td>
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<tr>
<td>fatty acids C-atoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acids used without SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
<td>ser, gly, cys, thr</td>
<td>ser</td>
<td>ser</td>
<td>n.r.</td>
<td>none</td>
<td>n.r.</td>
</tr>
<tr>
<td>Casamino acids used without SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
<td>+</td>
<td>n.r.</td>
<td>n.r.</td>
<td>+</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Peptide used without SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
<td>+</td>
<td>n.r.</td>
<td>n.r.</td>
<td>+</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
</tbody>
</table>

n.r. not reported.

<sup>a,b</sup> After Stams et al. (1985).

<sup>c</sup> After Van der Maarel et al. (1996).

<sup>d</sup> After Widde (1988); Rees et al. (1998).

<sup>e</sup> After Immhoff-Stücke and Pfennig (1983).

<sup>f</sup> After Stams and Hansen (1986).

Symbols: +, utilized; (+)<sup>a</sup>, autotrophic growth; (+), poorly utilized; -, not utilized.

n.d. not determined.
Strain ALA-3T fermented peptone and casamino acids with production of acetate and hydrogen. Sulfate or thiosulfate was not required for casamino acids and peptone utilization, but its presence improved growth during which sulfide was produced. Acetate, isovalerate, propionate and traces of succinate were produced from the oxidation of 1% peptone or 1% casamino acids in the presence of sulfate suggesting that more than one amino acid contained in both compounds were being utilized leading to the production of mixed fatty acids.

Alanine, valine, leucine and isoleucine were degraded respectively to acetate, isobutyrate, isovalerate and 2-methyl butyrate only in the presence of sulfate as an electron acceptor. This can be explained by the fact that oxidative deamination of these single amino acids can occur if the electrons produced in this step are effectively scavenged (STAMS, 1994; ÖRLIGGSON et al., 1995).

Although HANSEN and BLACKBURN (1995) provided evidence that sulfate reducing bacteria are involved in amino acid turnover in marine sediments, most laboratory studies have been limited to occasional characterization tests with amino acids as growth substrates (REEs et al., 1998; VAN DER MAAREL et al., 1996). STAMS et al., (1985) showed that members of the genera Desulfovibrio (strains 20020 and 20028) were able to oxidize various single amino acids such as alanine and glycine, if sulfate was present. Desulfovibrio acrylicus (VAN DER MAAREL et al., 1996) has also been reported to use amino acids such as serine, glycine, alanine and cysteine, as electron donors in the presence of various electron acceptors. Desulfothomaculum ruminis was also reported to use L-alanine as an energy source (STAMS and HANSEN, 1986). Several Desulfbacterium spp. are known to use glutamate (IMHOFF-STUCKLE and PFENNING 1983; BRYSCH et al., 1987; SCHNELL et al., 1989). In the addition, the complete oxidizer, Desulfobacterium vacuolatum (REEs et al., 1998) readily used glutamate, proline, isoleucine, alanine, glutamine, glycine, valine and serine only in the presence of sulfate. However strain ALA-3T is phenotypically, genotypically (Table 2) and phylogenetically different from all these microorganisms. Furthermore, strain ALA-3T converts glutamate to pyroglutamate and this constitutes the first report on a sulfate-reducing bacterium with this characteristic. Pyroglutamate has so far only been reported as an end-product of glutamine metabolism by a few ammonia producing bacteria from the rumen (CHEN and RUSSELL, 1988, 1989).

The utilization of peptides and amino acids in the presence of thiosulfate has been shown for non sulfate-reducing bacteria such as Thermoanaerobacter brockii (FARDEAU et al., 1997a) and Dethiosulfovibrio peptidovorans (MAGOT et al., 1997). However, strain ALA-3T, cannot be ascribed to any of these species due to its different physiological and phylogenetic characteristics (Fig 2). Therefore strain ALA-3T is described as a new species of the genus Desulfovibrio, Desulfovibrio aminophilus sp. nov.

Description of Desulfovibrio aminophilus sp. nov.

Desulfovibrio aminophilus a.mino. phi.lus. Gr. n. am-i-nus; Gr. adj. philus loving. M.L. adj. aminophilum amino acid loving. Vibrios (0.2 by 3.0-4.0 µm) motile by one polar flagellum, occur singly or in chains. Gram-negative, non-sporeforming. Strictly anaerobic. Colonies are round with entire edges, smooth, and white. Mesophilic. Optimum growth temperature is 35 °C (temperature range for growth is between 25 to 40 °C), optimum pH 7.5 (pH range for growth is from 6.7 to 8.0) and does not require NaCl for growth. Yeast extract is not required for growth on lactate, and pyruvate in the presence of sulfate, but stimulates growth. Not autotrophic. It requires yeast extract and sulfate for growth on alanine, valine, leucine, isoleucine, aspartate and methionine. In the presence of acetate as a carbon source, growth is obtained on ethanol or H2+C02 in the presence of sulfate. Sulfate, sulfite, and thiosulfate serve as electron acceptor, but not elemental sulfur, fumarate and nitrate. Thiolsulfate and sulfite are disproportionated to sulfate and sulfide. Does not ferment fumarate, malate, succinate, glucose, fructose, glyceral, but is able to ferment pyruvate, peptone, casamino acids, serine, glycine, threonine and cysteine. Desulfovirdin present. The G+C content of the DNA is 66 mol%. The type strain is ALA-3T (DSM12254). Isolated from anaerobic sludge of a dairy wastewater treatment plant of Santa Fe de Bogota, Colombia. Adverse effects on animals and humans are not known. Because of the ability of Desulfovibrio aminophilus to degrade amino acids and peptides, the possibility of harmful effects cannot be excluded. Cautionous handling and autoclaving of cultures before disposal is recommended.

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