

New enzyme belonging to the family of molybdenum-free nitrate reductases¹

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A novel molybdenum-free nitrate reductase was isolated from the obligate chemolithoautotrophic and facultative anaerobic, (halo)alkaliphilic sulphur-oxidizing bacterium *Thioalkalivibrio nitratireducens* strain ALEN 2. The enzyme was found to contain vanadium and haem *c* as cofactors. Its native molecular mass was determined as 195 kDa, and the enzyme consists of four identical subunits with apparent molecular masses of 57 kDa.

Apart from nitrate, the enzyme can utilize nitrite, chlorate, bromate, selenate and sulphite as electron acceptors. Moreover, it also has a haloperoxidase activity.

Key words: haem-containing enzyme, nitrate reductase, nitrite reductase, vanadium-containing enzyme.

INTRODUCTION

The universal role of molybdenum in nitrate reduction has been recognized for a long time. Almost all nitrate reductases that have been described at present contain molybdenum cofactor as a structural unit [1–3]. Recently, however, new nitrate-reducing enzymes have been discovered in vanadate- and iron-reducing bacteria that do not contain a molybdenum cofactor [4,5]. This finding has raised many questions. In particular, it is unclear whether these alternative nitrate reductases are widespread, which type of bacteria are able to produce them and under which conditions. It could be speculated that these unusual nitrate reductases may be utilized by bacteria living under unusual (i.e. extreme) conditions, such as conditions of extreme pH and temperature, and high concentrations of toxicants.

In the present study we have investigated a bacterium living under conditions of extremely high pH and salinity. The microbiology and biochemistry of alkaliphily have been studied thoroughly over the last 20 years using non-halophilic, alkaliphilic *Bacillus* species that can be easily obtained from non-specific environments, such as neutral soils. Only more recently has attention been drawn to (halo)alkaliphilic bacteria populating specific alkaline environments, e.g. soda lakes, where most of the important functional units are represented by specific groups of (halo)alkaliphilic bacteria [6,7]. In particular, the cultivable forms of sulphur-oxidizing bacteria in soda lakes are represented by two different groups of (halo)alkaliphilic, chemolithoautotrophic bacteria that have been described recently as two new genera (*Thioalkalimicrobium* and *Thioalkalivibrio*; members of the gamma subdivision of the Proteobacteria [8]). One of the species of the *Thioalkalivibrio* genus, *Tv. denitrificans*, is capable of anaerobic growth with reduced sulphur compounds as electron donors and nitrite or N₂O as an electron acceptor, but it was unable to reduce nitrate [9]. Recently, a new (halo)alkaliphilic representative of the *Thioalkalivibrio* genus was obtained from an Egyptian soda lake which was capable of anaerobic growth with nitrate and thiosulphate, and which was a member of a denitrifying consortium (D. Y. Sorokin, A. N. Antipov and J. G. Kuenen, unpublished work). Its role in the process was to reduce nitrate to nitrite. Here we describe a novel type of nitrate

reductase from this (halo)alkaliphilic, nitrate-reducing sulphur bacterium, *Tv. nitratireducens*, containing vanadium and haem *c* instead of a molybdenum cofactor.

EXPERIMENTAL

Culture and growth conditions

Tv. nitratireducens strain ALEN 2 was isolated from an anaerobic enrichment culture containing thiosulphate as the energy source and nitrate as the electron acceptor at pH 10, and 0.6 M total Na⁺ inoculated with a sediment sample from the hypersaline soda lake Fazda (located in Wadi Natrun, Egypt). The culture was routinely cultivated and maintained in the laboratory, as described previously [8]. The cells for isolation of nitrate reductase were obtained by growing strain ALEN 2 under limited aeration on medium containing the following (in g/l): Na₂CO₃, 20; NaHCO₃, 10; NaCl, 5; K₂HPO₄, 1; KNO₃, 2; MgCl₂ · 6H₂O, 0.1; and 2 ml/l of trace-elements solution [10]. Thiosulphate (30 mM) served as the energy source. The latter three components were added after sterilization from concentrated stocks.

Preparation of cell extract

The cells were collected by centrifugation at 10000 *g* for 15 min, washed with mineral base medium, resuspended in 25 mM sodium phosphate buffer, pH 7.0, and then disintegrated using a French press. The crude extract was centrifuged at 15000 *g* for 30 min. The cell extract was separated further into the membrane and soluble fractions by ultracentrifugation at 150000 *g* for 2 h.

Electrophoresis using polyacrylamide gels

Native electrophoresis was performed with 11 × 11 cm plates using a gradient polyacrylamide gel (5–15% polyacrylamide) in Tris/HCl buffer, pH 8.8, as described by Davis [11]. The current employed was 40 mA in the separating mode. The following standard set of proteins (supplied by Serva, Heidelberg, Germany) was used: catalase (240 kDa), aldolase (147 kDa), BSA (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase

Abbreviation used: ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid).

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(29 kDa). SDS/PAGE was performed using a gradient polyacrylamide gel (5–20%), as described by Laemmli [12]. β -Galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and α -lactalbumin (14.2 kDa) were selected as standards. The silver-staining procedure was used to visualize the protein bands [13].

Detection of enzymic activities in PAGE

To determine reductase activity in PAGE, the reaction mixture containing 0.2 M sodium phosphate buffer, pH 7.0, 20 mM KNO_3 (or, alternatively, 1 mM NaNO_2 , 2 mM Na_2SeO_4 , 2 mM KClO_3 , 2 mM KBrO_3 or 2 mM Na_2SO_3), 5 mM Methyl Viologen and 10 mM sodium dithionite was used. The gels were immersed in this mixture and incubated until transparent bands appeared against the blue background of the gel as a result of reductase activity. Thereafter, the gels were fixed first in 0.05% (w/v) triphenyltetrazolium chloride and subsequently in 5% (v/v) acetic acid.

To detect haloperoxidase activity, the gels were soaked in a solution containing 1 mM *o*-dianisidine, 100 mM KBr and 100 mM potassium phosphate, pH 7.4, and, subsequently, in 1 mM H_2O_2 . In the presence of a bromoperoxidase, *o*-dianisidine is brominated and turns brown [14].

Assay of (halo)peroxidase activity *in vitro*

Activity as iodoperoxidase was measured by following the conversion of I^- into I_3^- at 350 nm (ϵ 26400 $\text{M}^{-1} \cdot \text{cm}^{-1}$) using H_2O_2 as the electron acceptor. Bromination activity was measured spectrophotometrically at 290 nm in an assay system containing 50 μM monochlorodimedone (ϵ 20.2 $\text{mM}^{-1} \cdot \text{cm}^{-1}$), 2 mM H_2O_2 and 10 mM KBr in 100 mM phosphate buffer (pH 6.0) [14]. Peroxidase activity was measured with *o*-dianisidine or 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 25 °C at 460 nm or 405 nm respectively, and the reaction mixture contained 1 mM *o*-dianisidine or ABTS, 100 μM H_2O_2 and 5 mM sodium acetate buffer, pH 5.0.

Staining of the vanadium-containing proteins in PAGE

A simple method for detection of the vanadium-containing proteins has been developed. This is on the basis of the ability of tropolone to form an insoluble coloured complex with vanadium under strongly acidic conditions [15]. After electrophoresis, the gel was immersed in 12.5% (w/v) trichloroacetic acid containing 0.2 g/l tropolone. Blue-coloured bands appeared in the presence of vanadium-containing proteins within an incubation time of 10 min at 20 °C. The gels were either stored in 5% (v/v) acetic acid or allowed to dry. The vanadium-binding protein [16] was used as a positive control.

Absorption spectra

The air-oxidized and dithionite-reduced absorption spectra of the native, purified enzyme were recorded on a Hitachi 557 spectrophotometer, as described previously [5]. The haem was identified by alkaline pyridine haemochrome difference spectra, as described by Berry and Trumpower [17]. Horse-heart cytochrome *c* (Serva) was used as the standard.

N-terminal sequence analysis

The subunit of the reductase was blotted from the SDS/polyacrylamide gel on to a PVDF membrane (Immobilon-P⁸⁰;

Millipore, Bedford, MA, U.S.A.). N-terminal amino acid sequencing was performed by the method of Edman [18].

Analysis

The metals in purified enzyme were determined using inductively coupled plasma MS on a ELAN 6100 DRC ISP MS spectrometer (PerkinElmer, Norwalk, CT, U.S.A.). Nitrate, nitrite, N_2O and protein concentrations were determined by spectrophotometric and GC methods, as described previously [9].

RESULTS AND DISCUSSION

Tv. nitratireducens ALEN 2 was capable of active nitrate reduction to nitrite in micro-oxic and anaerobic cultures, and in anaerobic cell suspensions in the presence of thiosulphate as the electron donor. However, measurements of activity of nitrate reduction *in vitro* in cell extracts of strain ALEN 2 with reduced Methyl Viologen as the artificial electron donor yielded quite unexpected results. In the presence of nitrate, rapid Methyl Viologen oxidation was observed in the soluble fraction of the ALEN 2 extract, but nitrite was not detected as the usual product of nitrate reductase activity. Replacement of nitrate with nitrite in the reaction mixture resulted in extremely high rates of Methyl Viologen oxidation, concomitant with nitrite consumption. The nitrate reductase activity estimated from the nitrate-consumption rates gave values of 0.05–0.08 $\mu\text{mol} \cdot \text{mg}^{-1}$ of protein $\cdot \text{min}^{-1}$, whereas the nitrite reductase activity was within the range of 8–10.5 $\mu\text{mol} \cdot \text{mg}^{-1}$ of protein $\cdot \text{min}^{-1}$. N_2O accumulation in the gas phase was registered after the reaction was terminated. These data suggested that bacterial cells of strain ALEN 2 possess both nitrate and nitrite reductase activities, despite the overall inability of whole cells to use nitrite as the electron acceptor *in vivo*.

After native electrophoresis of the soluble fraction in PAGE, three protein bands with molecular masses of 195, 111 and 42 kDa each demonstrated both nitrate and nitrite reductase activity (Figure 1). It is well known that nitrate reductase and

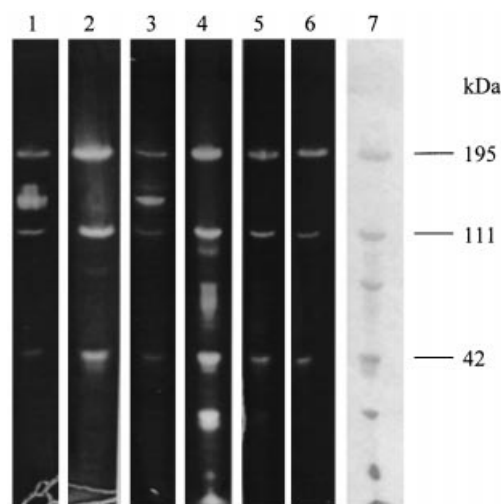


Figure 1 Reductase activity of extract from *Tv. nitratireducens* ALEN 2 in polyacrylamide gels with different substrates

Substrates investigated were as follows: NO_3^- (lane 1), NO_2^- (lane 2), ClO_3^- (lane 3), BrO_3^- (lane 4), SeO_4^{2-} (lane 5) and SO_3^{2-} (lane 6). Haloperoxidase activity is shown in lane 7. The reductase activity was determined at 70 °C at pH 7.2; haloperoxidase activity was determined at 25 °C at pH 7.0.

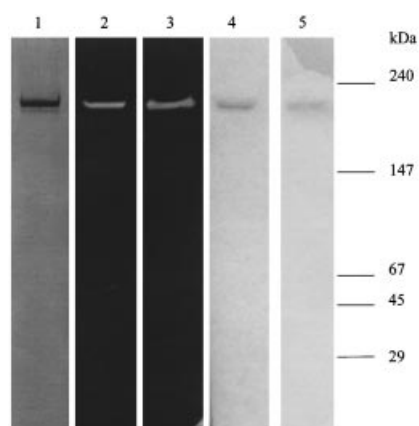


Figure 2 PAGE of purified nitrate reductase from *Tv. nitratireducens* ALEN 2

Lane 1, protein stained with silver; lane 2, identification of nitrate reductase activity; lane 3, identification of nitrite reductase activity (other activities are not shown); lane 4, determination of vanadium in polyacrylamide gels with tropolone; lane 5, red-coloured band (colour not shown) indicative of the presence of haem in the enzyme studied.

nitrite reductase are different enzymes, possessing a very narrow substrate specificity towards their electron acceptors. So far, only one enzyme combining both activities in a single protein has been described [5]. The reduction of various oxoanions, such as selenate, selenite, perchlorate, etc., is accomplished by specialized reductases that are mostly molybdenum-containing, with rather strict substrate specificities [19,20]. In order to characterize the specificity of the nitrate/nitrite reductase enzyme in ALEN 2,

the following oxoanions were used: ClO_3^- , BrO_3^- , SeO_4^{2-} and SO_3^{2-} . Surprisingly, each one of the three polypeptides with nitrate/nitrite reductase activity was able to reduce all the above-mentioned oxoanions effectively (Figure 1).

In all cases, the reductase activity of the enzymes studied was rather high. On the basis of these results, two explanations can be afforded: either the nitrate/nitrite reductase protein bands represent different molecular forms of the same enzyme, or they are different isoenzymes. In order to clarify this, a comparative study of three nitrate/nitrite reductase proteins was carried out. The characterization was initiated by the isolation of a reductase protein of molecular mass 195 kDa in a homogeneous state using preparative electrophoresis (Figure 2). Its native molecular mass was confirmed to be 195 kDa, and the enzyme was identified as a homotetramer composed of four identical subunits of molecular mass 57 kDa (Figure 3, centre panel). It can exist in the form of active dimers or active monomers, and in all three forms (native tetramer, dimer and monomer) it possessed both nitrate and nitrite reductase activities (Figure 3, left- and right-hand panels). A similar structure and properties (i.e. catalytic activity of separate subunits) have been reported previously for a molybdenum-free, periplasmic nitrate reductase from vanadate-reducing denitrifier *Pseudomonas isachenkovii* [4].

The nitrate/nitrite reductase from ALEN 2 was found to be highly stable. It exhibited both activities after denaturing SDS/PAGE, including treatment with SDS (1%, w/v), but without boiling (Figure 3, right-hand panel). Maximum reductase activity for all substrates studied (nitrite, nitrate, chlorate, bromate, sulphite and selenate) under non-denaturing conditions was observed at 70 °C. In contrast, under denaturing conditions (after SDS/PAGE), the optimal nitrite reductase activity was observed at 30 °C. Such a temperature shift might be explained by an easing of the conformational transition from a catalytically active to a catalytically inactive form in the presence of detergent.

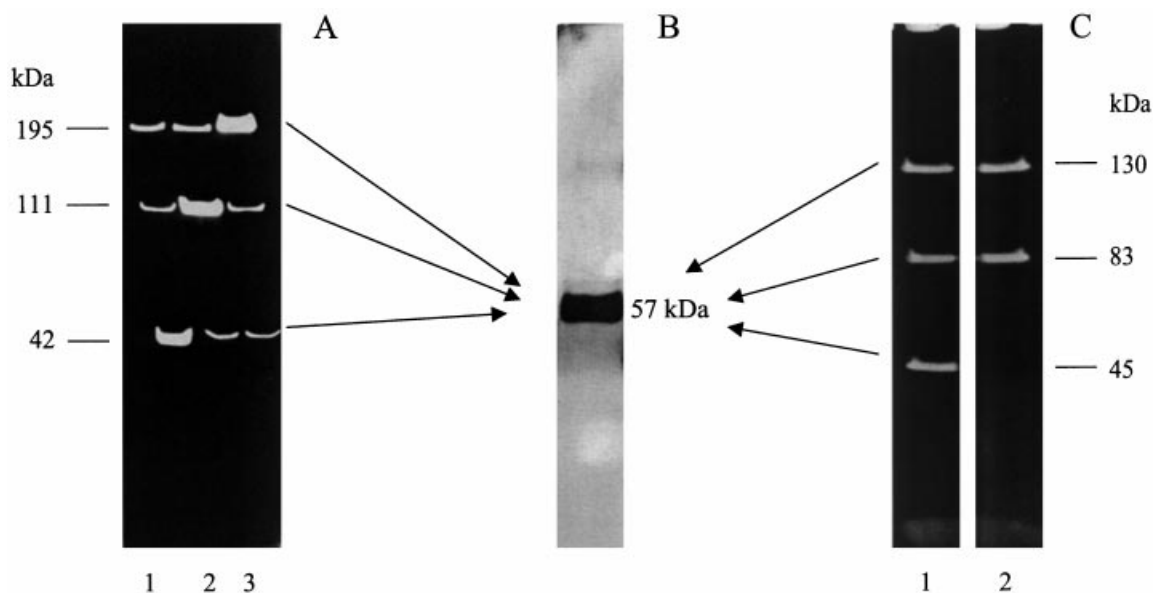


Figure 3 Molecular properties of the enzyme studied

Left panel: existence of the reductase in different molecular forms (native electrophoresis, stained for nitrate reductase activity). In this experiment, the purified enzyme was used. Each form (dimer or monomer) was isolated and again applied to polyacrylamide gels. Lane 1, isolated monomer form; lane 2, isolated dimer form; lane 3, tetramer form. Centre panel: SDS/PAGE of the purified reductase after boiling, stained with silver. Right panel: reductase activity after SDS/PAGE without boiling. Lane 1 shows nitrate reductase activity; lane 2 shows nitrite reductase activity.

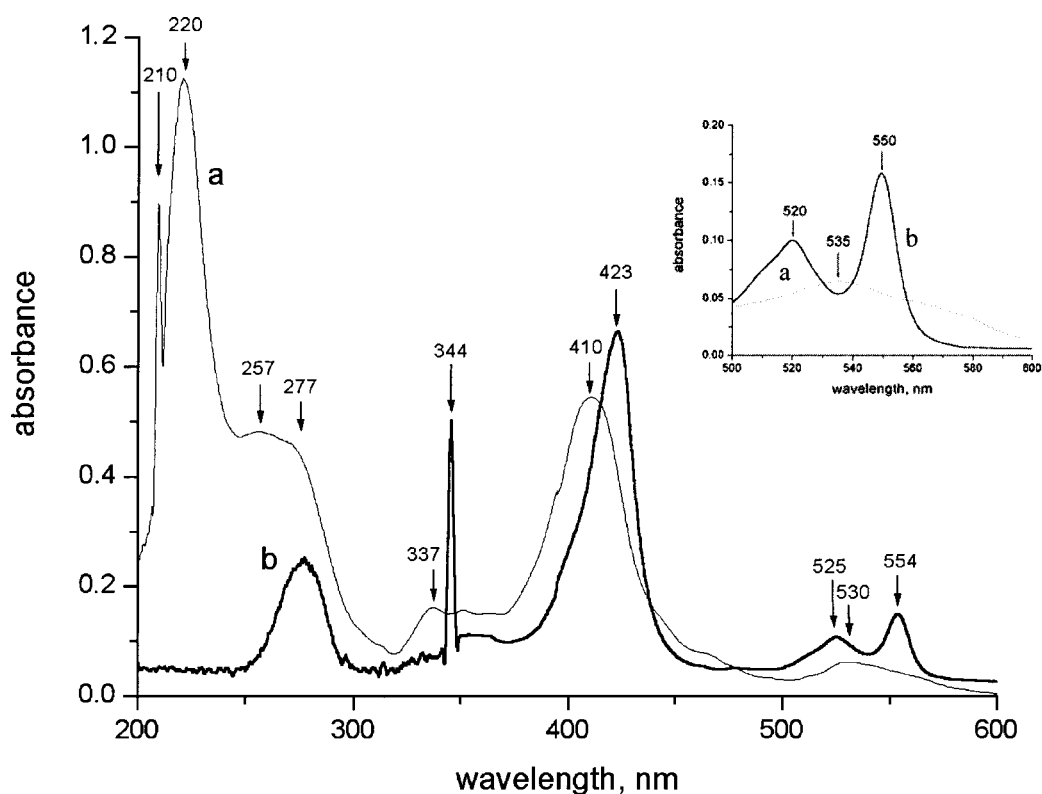


Figure 4 Difference spectra of air-oxidized (trace a) and dithionite-reduced (trace b) forms of purified nitrate reductase

The inset shows the final identification of the type of haem in pyridine.

The native reductase enzyme (shown in Figure 2, lane 5) was bright red in colour. The difference spectrum of the reduced enzyme had absorption maxima at 423, 525 and 554 nm (520 and 550 nm in pyridine), which indicated the presence of a haem *c* group (Figure 4). The only known nitrate/nitrite reductase enzyme complex, isolated from the dissimilatory iron-reducing bacterium *Geobacter metallireducens* [5], also contains haem *c* in one of its subunits, but it differs from the enzyme characterized in the present study both structurally (by containing four different subunits) and functionally (by forming ammonia as a product of nitrate reduction).

Metal analysis of the 195 kDa nitrate/nitrite reductase protein of ALEN 2 revealed the complete absence of molybdenum. Instead, vanadium and iron were present at a molar ratio of 1:3. Additional evidence for vanadium presence in the enzyme was obtained by a specific staining procedure in PAGE (Figure 2, lane 4).

At present, three types of vanadium-containing enzyme species are recognized: an alternative nitrogenase [21], the vanadium-dependent haloperoxidases [22] and a vanadium-containing alternative nitrate reductase from *P. issachenkovii* [4]. None of them, however, contain haem groups. The vanadium metal in these enzymes is co-ordinated by amino acid residues. Vanadium can be co-ordinated in porphyrin structures, although its complexes have different spectral parameters as compared with iron-containing porphyrins. It might be speculated that vanadium and the haem *c* group are responsible for reduction of nitrate and nitrite respectively. As a rule, the functional groups of the molybdenum-containing nitrate reductases, such as molybdenum cofactor and haem *b*, are located in different subunits [2,3]. In the

case of the nitrate/nitrite reductase complex of ALEN 2, the two active sites are probably located on the same subunit, which provides a further indication of the unusual nature of this enzyme.

The presence of both vanadium and haem groups in the enzyme allowed the suggestion that it might have a peroxidase activity, as was the case for vanadium-dependent, haem-containing haloperoxidases [22]. Indeed, the nitrate/nitrite reductase complex from ALEN 2 was able to catalyse the reaction of halogenation, i.e. the bromination of *o*-dianisidine in the presence of H_2O_2 (Figure 1, lane 7). Enzymic reactions with Cl^- and Br^- were studied by the monochlorodimedone assay; that with I^- , by the formation of tri-iodide. The enzyme oxidized I^- and catalysed the bromination and chlorination of monochlorodimedone. The specific activities of purified enzyme were 1057, 171 and 246 units \cdot mg $^{-1}$ of protein for I^- oxidation, bromination and chlorination respectively. The enzyme is capable of oxidizing directly *o*-dianisidine or ABTS, which are typical substrates of peroxidases. The specific activities of purified enzyme were approx. 291 and 411 units \cdot mg $^{-1}$ of protein for *o*-dianisidine and ABTS respectively.

For determination of the N-terminal amino acid sequence, the homogeneous native nitrate/nitrite reductase from ALEN 2 was used (molecular mass 195 kDa). The 57 kDa subunit was obtained after SDS/PAGE of homogeneous native enzyme. The double detection of amino acids, so-called 'plugs of restriction', was not observed during analysis. This confirmed the absence of a co-migrating protein. A sequence of Glu-Pro-Gly-Glu-Asn-Leu-Tyr-Pro-Val-Asp-Ala-Met-Gln-Cys-Phe-Asp-Tyr was determined. The N-terminal amino acid sequence was compared

with the sequences of conventional molybdenum-containing nitrate reductases, nitrite reductases and haloperoxidases, available in the protein databases. The sequence in question did not show any similarity to the N-terminal sequences of known enzymes of these types. An analysis of the sequence using the SwissProt bank revealed a high degree of similarity to different peptide fragments belonging to different proteins. However, all these fragments were localized inside the amino acid sequences of the various known proteins, indicating that the isolated protein is different.

Overall, the data obtained allow us to conclude that the new enzyme complex from the (halo)alkaliphilic sulphur-oxidizing bacterium *Tv. nitratireducens* possesses an unusual combination of oxoanions polyreductase and haloperoxidase activity in a single protein. Further investigation of the genes coding for this protein is necessary to understand fully the origin and functioning of this novel enzyme.

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