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Phylogenetic characterization and morphological and physiological aspects of a novel acidotolerant and halotolerant microalga *Coccomyxa onubensis* sp. nov. (Chlorophyta, Trebouxiophyceae)

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Abstract The genus *Coccomyxa* comprises green microalgae, which can be found worldwide in remarkably versatile aquatic and terrestrial ecosystems including symbiotic associations with a number of different hosts. In this study, we describe a new species, *Coccomyxa onubensis*, based on 18S and ITS ribosomal DNA (rDNA) sequence data. *Coccomyxa onubensis* was isolated from acidic water, and its ability to adapt to a wide range of acidic and alkaline pH values and to high salinity was analyzed. The long-term adaptation capacity of the microalga to such extreme conditions was evaluated by performing continuous repeated batches at selected salt concentrations and pH values. Adapted cultures of *C. onubensis* were found to yield high biomass productivities from pH 2.5 to 9, with maximum yields at acidic pH between 2.5 and 4.5. Moreover, *C. onubensis* was also found to adapt to salinities as high as 0.5 M NaCl, reaching biomass productivities that were similar to those of control cultures. Ultrastructural analysis by transmission electron microscopy of *C. onubensis* cells adapted to high salinity showed a robust response to hyperosmotic shock. Thus, *C. onubensis* was found to be acidotolerant and halotolerant. High biomass productivity over a wide range of pH and salinities denotes *C. onubensis* as an interesting candidate for various biotechnological applications including outdoor biomass production.

Keywords *Coccomyxa* · Green algae · Phylogeny · Acidotolerance · Halotolerance · Biotechnology

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Introduction

Mass cultivation is the first step in the biotechnological use of microalgae at large scale. Suitable environmental and cultivation conditions to speed up biomass production are crucial for developing production processes that are efficient in costs and yield (Forján et al. 2015). Large scale production of microalgae is challenging due to limitations to growth under harsh outdoor conditions. Extremophile microalgae have the ability to cope with such harsh conditions. These microalgae have developed the ability to grow under high or low temperature, under high or low pH, in the presence of metal ions, under high irradiance, and also in the presence of a number of other extremophile microorganisms in the same habitats (Varshney et al. 2015). Despite the natural abilities of these microalgae that are promising for biotechnological applications, little attention has so far been paid to the potential of extremophile microalgae.

The conditions at which extremophile microalgae develop in their natural extreme habitats are mostly far from being optimal for growth. In the laboratory, extreme conditions can be simulated, which are tuned with local climate and water conditions and simultaneously lead to high productivities of extremophile microalgae while preventing growth of competitive contaminants (Varshney et al. 2015). Salinity, pH, light

irradiance, and solved metal concentrations are among such physicochemical conditions. For instance, Río Tinto, a river in Southwestern Spain, is an example of a highly acidic aquatic environment that contains solved metals at concentrations that are commonly toxic to life and hosts a wide diversity of microorganisms with several microalgal species among them (Amils and Fernández-Remolar 2014). The microalga *Coccomyxa* sp. ACCV1, isolated from the acidic waters of Río Tinto (Garbayo et al. 2012), has been reported to develop better under cultivation conditions that are different from those typical of the natural acidic aquatic habitat which, for instance, include nitrogen limitation and toxic levels of solved iron and copper (Garbayo et al. 2012; Vaquero et al. 2012; Ruíz-Domínguez et al. 2015). *Coccomyxa* sp. ACCV1 grew well at pH values as low as 2.5 (Garbayo et al. 2012), but the optimal pH for high biomass production was not determined. The plasma membrane of extremophile microalgae from low-pH environments shows very low permeability to protons, which allows the cytoplasmatic pH to be kept neutral (Gimmler et al. 1988; Gross 2000). Therefore, the proton concentration in the culture medium causes significant osmotic pressure, which makes microalgae from acidic habitats moderately halotolerant (Albertano et al. 1990; Gross 2000). As a consequence, microalgae from acidic habitats might be more applicable for outdoor biomass production, as seawater could eventually become a suitable resource for the algal production process.

Recently, the taxonomy of the genus *Coccomyxa* was revised by Darienko et al. (2015) using an integrative approach and DNA barcoding (Darienko et al. 2015). The ITS2 ribosomal DNA (rDNA) barcode allowed them to distinguish a total of seven species; three of them were newly described. Their study included far more than hundred partial or complete rDNA sequences from uncultured strains as well as several sequences from cultured strains deposited in GenBank. Among the latter was our sequence from *Coccomyxa* sp. isolated from Río Tinto. Darienko et al. (2015) recognized this strain as a yet to be described new species with a distinct barcode. Genus affiliation of our strain was already confirmed by a partial 18S rDNA sequence (GU265559; Garbayo et al. 2012). Garbayo et al. (2012) and Ruíz-Domínguez et al. (2015) tentatively designated this strain as *Coccomyxa onubensis* and *Coccomyxa* sp. (strain onubensis), respectively, without actually performing phylogenetic analyses and without a formal description. *Coccomyxa onubensis*, therefore, is a *nomen nudum*, but according to the International Code of Nomenclature for Algae, Fungi, and Plants (McNeill et al. 2012), it can be used for a later formal description, if the description refers to the same strain used before.

In this paper, we formally describe the Río Tinto isolate as a new species, *Coccomyxa onubensis* sp. nov., and analyze its ability to adapt to a wide range of pH values and to high salinity. The long-term adaptation capacity of the microalga to such extreme conditions is evaluated by performing continuous repeated batches at different salinities and pH values.

Materials and methods

Microorganism and standard culture conditions

Coccomyxa onubensis ACCV1 (=SAG 2510) was isolated from acidic waters of the Tinto River (Huelva, Spain). *Coccomyxa* cells were grown in 1-L Erlenmeyer flasks in batch mode. Unless otherwise indicated, the cultures were grown at pH 2.5 in a culture medium based on K9 mineral medium (Silverman and Lundgren 1959), modified according to the following composition: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01 g $CaCl_2$, and 5-mL Hutner solution (Hutner et al. 1950) in distilled water up to a final volume of 1 L. The Hutner solution was prepared as described in Garbayo et al. (2012). The cultures were incubated at 27 °C, continuously illuminated at 150 μmol photons m^{-2} s^{-1} with white fluorescent lamps, and bubbled with air containing 5 % (*v*/*v*) CO_2. Bacterial contamination in the cultures was prevented by using 0.45-μm air filters in the air supply line.

DNA extraction, PCR amplification, cloning, and sequencing

Cells were harvested by centrifugation and mechanically ground with tiny glass beads with a diameter of about 0.2 mm and extraction buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % sodium dodecyl sulfate) (MO BIO Laboratories, USA) in 1.5-mL Eppendorf tubes in a cell mill (Qiagen, Germany) for 10 min at 30 Hz. DNA purification and precipitation were done by standard procedures. The precipitate was air-dried and resuspended in 50 μL of Milli-Q sterilized water for further use.

For amplification of the 18S rDNA, two conserved eukaryote-specific primers (forward primer, 5′ WACCTGGT TGATCCTGCCAGT 3′, 5′ PCR: Huss et al. (1999); reverse primer, 5′ ATATGCTTAAATTCAGCGGGT 3′, NLR 204/21: Van der Auwera et al. (1994)) were used to amplify the complete 18S and ITS rDNA. Thirty-five cycles were run in a Biometra T3000 thermal cycler (Labrepco, USA) using Phusion High-Fidelity DNA Polymerase (2 U μL^{-1}) with 5 s of denaturation at 98 °C (30 s for the first cycle), 20-s annealing at 55 °C, and 120-s extension at 72 °C followed by another 300 s after the last cycle. The amplified products were analyzed on a 1.8 % agarose gel stained with

ethidium bromide. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) and cloned with a Zero Blunt Topo Cloning Vector Kit (Invitrogen, USA) following the instructions by the manufacturers. The insert nucleotide sequences were determined with the universal primers M13 forward and reverse as well as some intern sequencing primers listed in Huss et al. (1999) by GATC Biotech (Constance, Germany). The sequence was submitted to GenBank with the accession number HE617183.

Phylogenetic analyses

The 18S + ITS rDNA sequence (without intron) of *C. onubensis* ACCV1 was manually aligned on a MicroVAX computer using the sequence editor program by Olsen et al. (1992) with representative sequences of all currently described *Coccomyxa* species (Darienko et al. 2015). For the phylogenetic analyses, ITS1 was excluded, as this region could not be unambiguously aligned with all reference sequences. For the alignment of the ITS2 region, the secondary structure was used according to Darienko et al. (2015). Phylogenetic trees were inferred from all positions available for 18S, 5.8S, and ITS2 rDNA (2249 nt) by the neighbor-joining (NJ), the maximum parsimony (MP), and the maximum likelihood (ML) method using the PAUP program version 4.0b 10 (Swofford 2002). One thousand bootstrap replicates were carried out for each method. ML was used to infer the tree topology shown in Fig. 2 by selecting empirical base frequencies, setting the transition/transversion (ti/tv) ratio to 2, and assuming a gamma distribution of 0.5. For the NJ bootstrap analysis, the HKY85 correction was used to convert pair-wise sequence similarities into evolutionary distances, starting trees were obtained via NJ, and the tree bisection–reconnection (TBR) branch-swapping algorithm was selected. In the MP analysis, starting trees were obtained via random stepwise addition of taxa repeated ten times, gaps were treated as a "fifth base," and TBR was selected.

Repeated-batch cultures at different pH values

Experiments at different pH values were performed in 1-L flasks in repeated-batch mode. The algal cultures were grown in repeated cycles (a total of 5) for 72 h per cycle. After each cycle, the cultures were diluted with fresh culture medium to 0.23 g L^{-1} biomass concentration. The pH of the cultures was measured several times a day and corrected by addition of either HCl or NaOH if necessary. The cultures were incubated in a culture room under the standard culture conditions described above. To calculate the average biomass productivity (g L^{-1} day^{-1}) of each 72-h cycle in repeated-batch mode, the increase in biomass per liter at the end of a cycle was divided by the cycle duration (3 days). Three replicates were done for each experiment.

Repeated-batch cultures for algal adaptation at increasing salinity

Experiments at different salinities were performed in 1-L flasks in repeated-batch mode. The culture media were prepared at pH 2.5 with different salinities, 100, 300, 400, and 500 mM NaCl. The growth experiments were performed in the following: (a) non-adapted batch cultures, to which NaCl was added at the selected concentration just at the start of the experiment, and (b) adapted cultures, in which NaCl was gradually increased up to the selected concentration. For that, the NaCl concentration of the repeated-batch culture was held constant until constant productivity was reached. Then, the salinity was increased and the process repeated again. A culture was considered adapted once the biomass productivity was constant and the maximum quantum yield (F_v/F_m) was at least 0.6. The algal cultures were incubated in a culture room under the standard culture conditions described above. Average biomass productivity was calculated as described before. Three replicates were done for each experiment.

Light and electron microscopy

Photomicrographs of *C. onubensis* were taken from both control culture cells and NaCl-adapted culture cells using an Olympus BX-61 microscope with a CCD Colour-View-II camera (Soft Imaging System, Germany) and the CellSens analysis imaging system (Olympus, Japan). For transmission electron microscopical observations, we followed the method described by Nishikawa et al. (2006). The algal cells were collected by centrifugation (1,957×*g*, 1 min) from each culture (repeated-batch cultures treated with 400 mM NaCl and untreated cultures). The algal cells were fixed with 1 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4 °C. The cells were then washed three times for 5 min using the same buffer. The samples were postfixed with 1 % osmium tetroxide in 0.2 M cacodylate buffer at 4 °C for 1 h. Samples were washed with the same buffer, dehydrated in a graded ethanol series, and embedded in Epon 812 (Electron Microscopy Science, USA). Ultrathin sections of 80–90 nm obtained by an ultramicrotome (Leica, Germany) and placed on copper grids were stained with aqueous 1 % (*w*/*v*) uranyl acetate and lead citrate. Transmission electron micrographs were observed with

a JEM 1011 (Jeol Ltd., Japan) electron microscope using an accelerating voltage of 80 kV. Several photographs of entire cells and of local detailed structures were taken at random, analyzed, and compared to investigate the effect of NaCl on different subcellular structures.

Dry weight measurements

To measure dry weight, 10-mL samples of each culture were used. The samples were filtered through Whatman filters of 47-mm diameter and 0.7 μm pore size. The filters containing the wet algal biomass were dried at 100 °C for 24 h (Vaquero et al. 2013).

Maximum quantum yield

Maximum quantum yield (F_v/F_m) of photosystem II (PSII) was measured to evaluate the photosynthetic performance of the algal cells, which is related to the physiological status (Maxwell and Johnson 2000). F_v/F_m was determined using the pulse amplitude modulation (PAM) technique. The chlorophyll fluorescence was measured using an AquaPen AP-100 (Photon Systems Instruments, Czech Republic) device. The measurements were performed with 2-mL samples of algal culture. The samples were dark-adapted for 15 min (F_o) prior to the application of a saturating pulse of actinic light (F_m). The maximum quantum yield was calculated according to Cuaresma et al. (2011), where $F_v/F_m = (F_m - F_o) / F_m$ (Cosgrove and Borowitzka 2011).

Statistics

Statistical analysis of the data in Figs. 4 and 5 was carried out. For this purpose, one-way analysis of variance (ANOVA) was used. Assumptions of normality were checked with the Shapiro–Wilk test. Significance was determined at $p < 0.05$. The data in Figs. 4 and 5 represent the mean values and standard deviations (SD) of three independent experiments.

Results

Morphology

The morphology of *C. onubensis* was investigated by light and transmission electron microscopy for cells cultivated under standard conditions and under different salinities as described in "Materials and methods" section. Light microscopy of exponentially growing cells cultivated under standard conditions revealed the typical shape characteristic for the genus *Coccomyxa*: most mature vegetative cells were of elongated ovoid shape with a large chloroplast that occupied more than half of the cell volume (Fig. 1a, b). The average size of mature vegetative cells cultivated under standard conditions was 6.9 ± 1.1 μm × 3.0 ± 0.3 μm. A few vacuoles were observed in the cytoplasm of each cell grown under standard conditions (Fig. 1a, b). Cultivation under high salinity (400 mM NaCl) resulted in the following morphological changes: (a) slight increase in cell size, (b) increase in the number of vacuoles, (c) more spherical appearance of cell shape (Fig. 1c, d), and (d) protrusion at one cell end (Fig. 1c, d). The size of cells cultivated under high salinity increased significantly up to 9.6 ± 1.5 μm × 4.7 ± 1.1 μm.

To study the phenotypic plasticity of *C. onubensis*, the effect of high salinity on the ultrastructure was determined by transmission electron microscopy and compared to control culture cells (Fig. 2). Figure 2a, b is derived from longitudinal and cross sections through cells from control batch cultures and shows a unicellular microalga with a typical large chloroplast that surrounds the nucleus and occupies approximately half of the total cell volume. Thylakoids and several starch bodies are obvious. The microalga was ellipsoidal in shape (Fig. 2c) and showed a distinct but apparently thin cell wall (Fig. 2b; see also Garbayo et al. 2012). Cells from cultures grown under salt stress (Fig. 2d–f) contained lipid droplets, and occasionally electron-dense deposits in the cytoplasm were observed (Fig. 2d, e). A protrusion at one cell end was observed in some of the cells under high salinity (Fig. 2f). Typical reproduction with two to four autospores formed by oblique division within the mother cell was observed (Fig. 2d).

Phylogenetic analyses

The complete 18S and ITS rDNA sequence of *C. onubensis* was determined (HE617183). The 18S rDNA contained 1801 nt excluding a 437-nt-long group I intron located at position 1512 (*E. coli* numbering). The ITS1, 5.8S, and ITS2 regions were 643 nt long. The sequence was aligned with representatives of all *Coccomyxa* species recently defined by Darienko et al. (2015), and a ML phylogenetic tree was inferred (Fig. 3). This tree shows that our isolate from Río Tinto together with an isolate from a tree bark (deposited in GenBank as HE586515) constitutes a new species *C. onubensis* with a distinct ITS2 barcode defined as BC-10 by Darienko et al. (2015). Although species

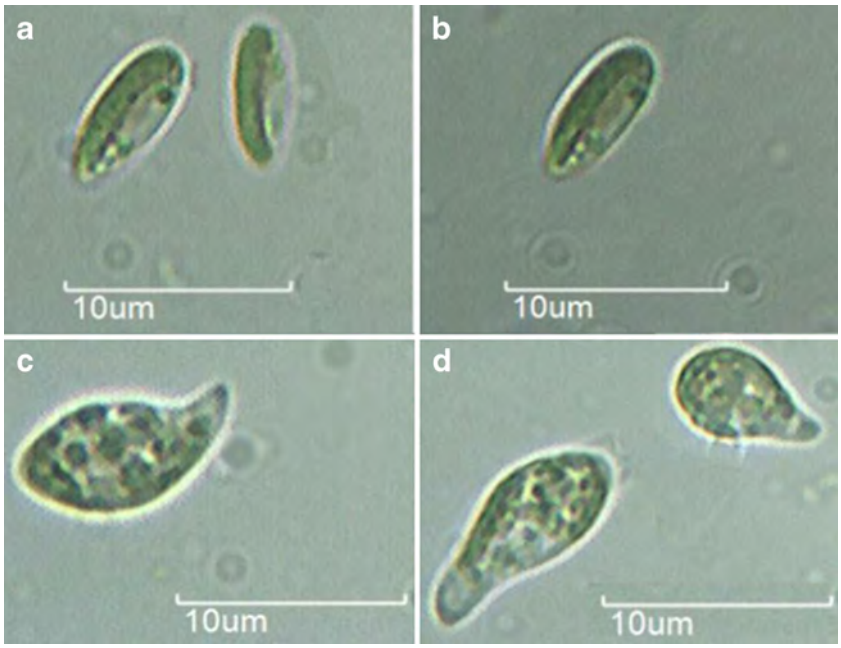

Fig. 1 Morphology of *Coccomyxa onubensis* sp. nov. Large chloroplast and elongated ovoid shape of control culture cells (**a**, **b**). Higher cell size, protrusion at one cell end, and spherical-like cell shape (**c**, **d**) of cells grown under high salinity

delimitation of morphologically simple and asexually reproducing green microalgae like *Coccomyxa* or *Chlorella* is now completely defined on the basis of compensating base changes (CBCs) in conserved regions of ITS2, expressed as ITS2 DNA barcodes (Darienko et al. 2015 and references therein), we found four more strains in the database, for which no ITS sequence is available, but which most likely also belong to *C. onubensis*. Table 1 shows that the 18S rDNA sequences of these strains display less nucleotide differences to *C. onubensis* than to all other next closely related *Coccomyxa* species.

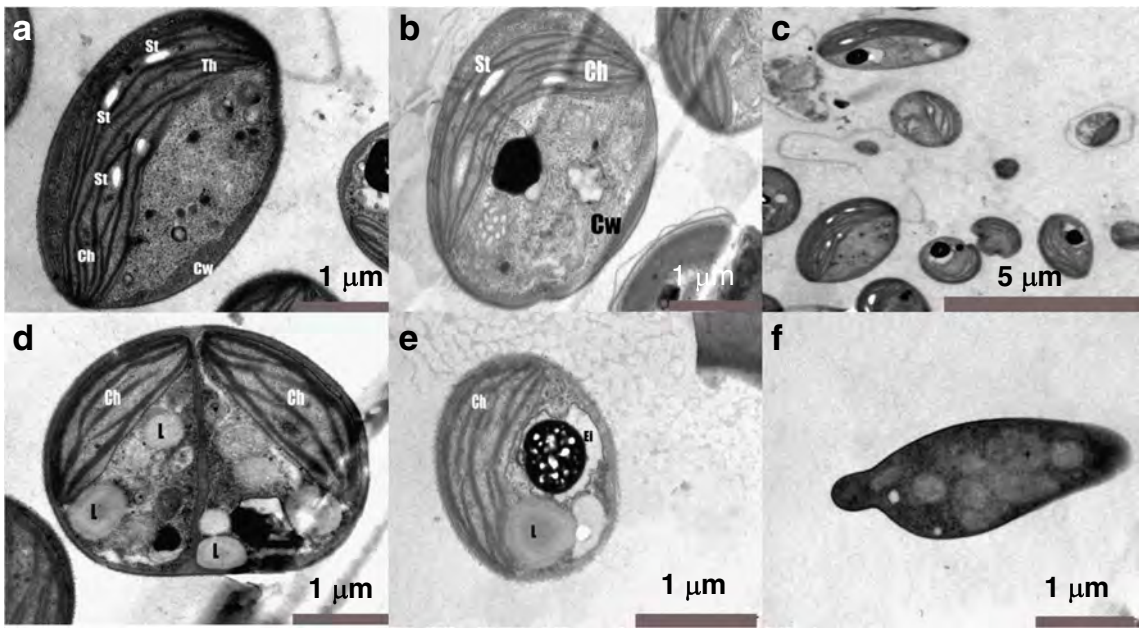

Fig. 2 Transmission electron microscopy of *Coccomyxa onubensis* sp. nov. **a**–**c** Control cells from cultures without added NaCl. **d**–**f** Cells adapted to salt stress (400 mM NaCl) with lipid droplets (**d**, **e**) and electron-dense inclusion (**e**). *Scale bars*: **a**, **b**, **d**–**f** 1 μm; **c** 5 μm. *Ch* chloroplast, *Th* thylakoids, *L* lipid droplets, *St* starch grains, *EI* electron-dense inclusion, *CW* cell wall

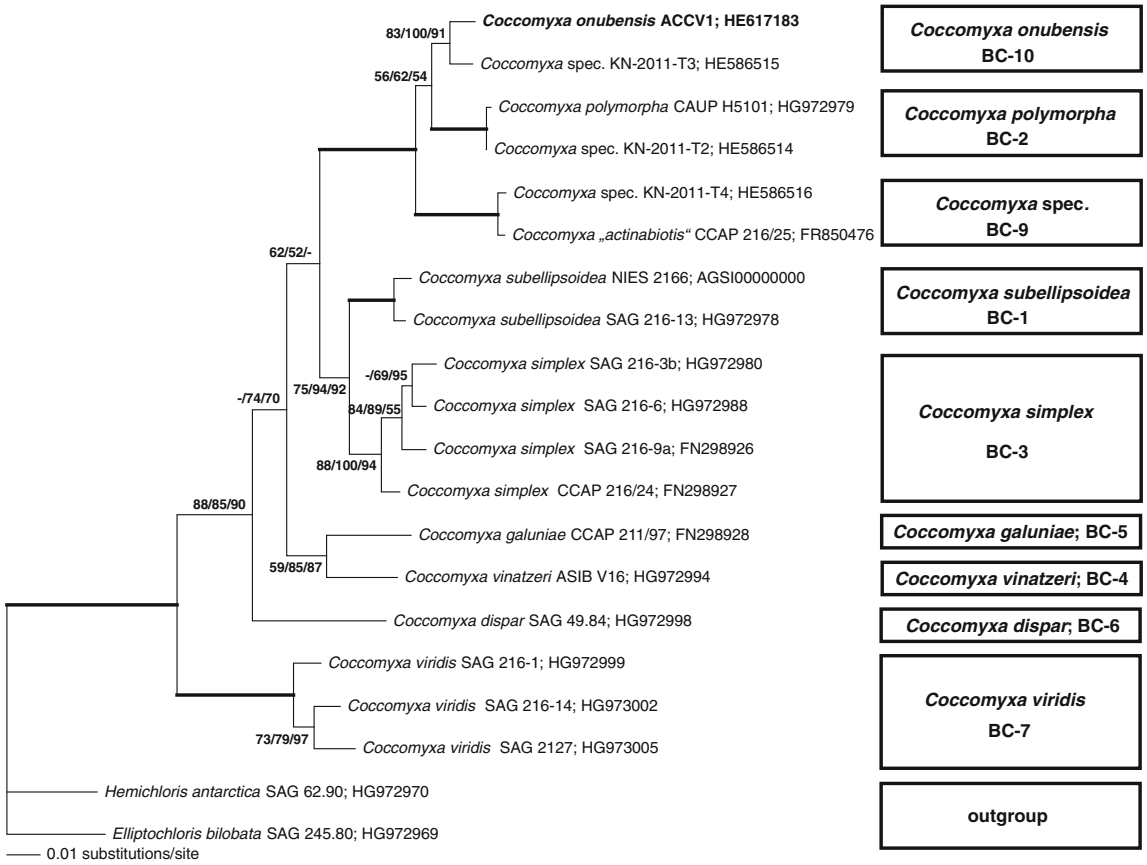

Fig. 3 Maximum likelihood tree based on 18S, 5.8S, and ITS2 rRNA gene sequences of the genus *Coccomyxa*. A sister group of *Coccomyxa* (*Hemichloris antarctica* and *Elliptochloris bilobata*) was chosen as an outgroup. Bootstrap values of each 1000 replications of ML/NJ/MP are indicated at the branches if greater than 50 %. *Thick lines* represent nodes with 100 % bootstrap support for all three methods. Branch lengths reflect the evolutionary distance indicated by the scale. The different *Coccomyxa* species are defined by distinct ITS2 barcodes (BC) as published by Darienko et al. (2015)

Table 1 18S rDNA nucleotide differences between *Coccomyxa onubensis* ACCV1, closely related strains, and representatives of next closely related described species

Strain; GenBank No./ Sequence length (nt)	*C. onubensis* ACCV1 (1797)	*C.* spec. AH4 (1526)	*C.* spec. RL75K2 (1797)	*C.* spec. AC1 (1516)	*Pseudococcomyxa simplex* Rsa3 (1707)	*C. polymorpha* CAUP H5101 (1777)	*C. subellipsoidea* NIES 2166 (1777)	*C. simplex* SAG 216-9a (1777)
C. onubensis ACCV1; HE617183	-	0	1	3	3	6	29	29
C. spec. AH4; KC155324		-	1	3	3	6	22	23
C. spec. RL75K2; HE617184			-	4	4	5	29	31
C. spec. AC1; KC155323				-	2	9	19	20
Pseudococcomyxa simplex Rsa3; KM016993					-	9	27	29
C. polymorpha CAUP H5101; HG972979						-	28	28
C. subellipsoidea NIES 2166; AGSI00000000							-	7
C. simplex SAG 216-9a; FN298926								-

Strains enclosed in the box most likely belong to *C. onubensis*

Formal taxonomic description

Coccomyxa onubensis Garbayo et al. (2012) ex Fuentes, Huss, Montero, Torronteras, Cuaresma, Garbayo et Vílchez (Fig. 1).

Synonyms: *Coccomyxa onubensis* Garbayo et al. (2012), J. Phycol. 48: 607–614 (*nom. inval.*); Vaquero et al. (2012), Process Biochem. 47: 694–700 (*nom. inval.*); Vaquero et al. (2013), J. Appl. Microbiol. 116: 839–850 (*nom. inval.*); Vaquero et al. (2014), Algal Res. 6: 70–77 (*nom. inval.*).

Diagnosis: Mature vegetative cells solitary or occasionally assembled in star-like structures, elongated ovoid, asymmetric, cell size 6.9 ± 1.1 μm × 3.0 ± 0.3 μm. Chloroplast lateral, trough-shaped, covering more than half of the cell volume. Lipid droplets present in the cytosol. Starch grains present in the chloroplast. Cell wall apparently thin. Reproduction by two to four autospores. Protoplast division is oblique. Morphologically similar to *Coccomyxa galuniae*. Exact identification possible only using ITS2 as a phylogenetic marker.

Habitat: acidic waters.

Type locality: Tinto River, Huelva, Andalucía, Southwestern Spain.

Holotype: Authentic strain SAG 2510 (=ACCV1) kept on standard freshwater medium 3NBBM+V Ag at The Sammlung von Algenkulturen (SAG), University of Göttingen, Germany.

Iconotype: (designated here in support of the holotype): Fig. 1.

Etymology: The species name was chosen in relation to Onuba, the Latin name for the Spanish City Huelva.

Adaptation of *C. onubensis* to acidic, neutral, and alkaline pH

Coccomyxa onubensis ACCV1 was isolated from an acidic environment with a pH between 2 and 3 (Garbayo et al. 2012) and was able to adapt to a wide range of pH from acidic to alkaline values and to high salinity (approximately 85 % of that of seawater, 599 mM). Cultures were prepared to study the pH dependence of biomass productivity as described in "Materials and methods" section. In the adaptation process to each selected pH value, only those cultures shifted from pH 2.5 to pH 0.5 and 1.5 did not grow. Cultures shifted from pH 2.5 to higher pH values showed immediate adaptation in terms of cell density and dry weight, until constant biomass productivities were reached in the repeated-batch cultures at each selected pH value. The biomass productivity at different pH values, calculated as described in "Materials and methods" section, is given in Fig. 4a as a percentage of the value at pH 4. There is no statistically significant difference (ANOVA) in the pH range from 2.5 to 4.5. The results show that *C. onubensis* is productive at acidic, neutral, and alkaline pH, if the cultures were previously adapted. The algal biomass productivity was higher at acidic pH above 2.5, with a maximum of 0.22 g L^{-1} day^{-1} at pH 4. The difference between the maximal and the minimal biomass productivity of the cultures adapted to acidic pH is only about 10 %. The biomass productivity of cultures adapted to neutral and alkaline pH accounted for approximately 80 % of the maximum value at pH 4. The slight differences in biomass productivity of the cultures within the pH range from 2.5 to 9 were consistent with the slight differences in the maximum quantum yield of these cultures as shown in Fig. 4b. All cultures from pH 2.5 to 9 showed F_v/F_m values above 0.6, which indicates a highly active photosynthesis.

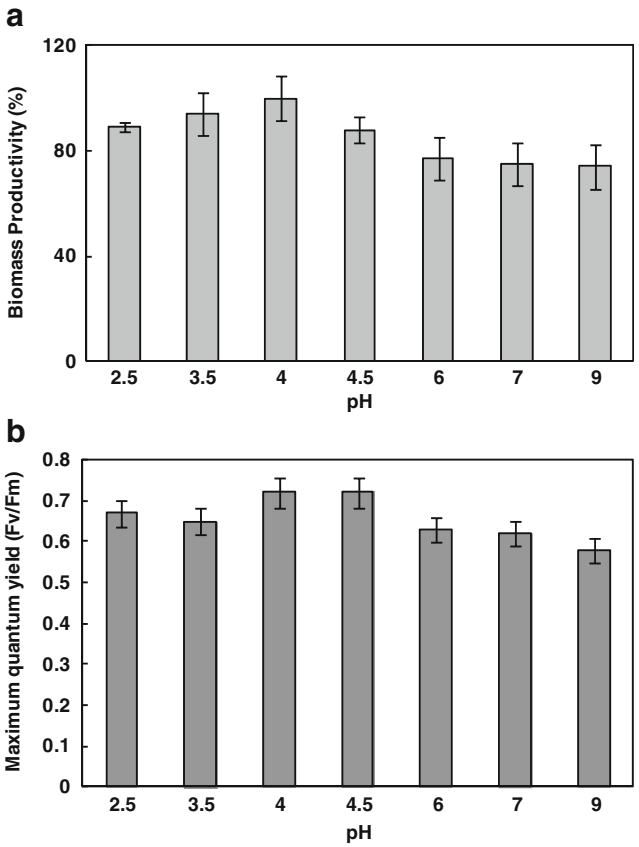

Fig. 4 Effect of pH on biomass productivity (**a**) and maximal photosynthetic efficiency (F_v/F_m) (**b**) of *Coccomyxa onubensis*. Aliquots of equal biomass from a mother culture were adapted to different pH values (from 2.5 to 9) through repeated-batch cultivation. A repeated-batch mother culture was used as control at pH 2.5. Biomass productivity of each culture is shown as percentage of the maximal productivity found at pH 4 (100 % biomass productivity = 0.22 g L^{-1} day^{-1}). Details are described in "Materials and methods" section. *Error bars* are standard deviations of three independent experiments

Adaptation of *C. onubensis* to salinity

Standard cultures of *C. onubensis* were adapted to increasing salinity following the procedures described in "Materials and methods" section. At each salinity, the biomass productivity became constant mostly after three or four 72-h growth cycles of the corresponding repeated-batch culture. Figure 5a shows the biomass productivity obtained during the steady state once the repeated-batch cultures adapted to salinities of 100 to 500 mM (so-called "adapted" cultures, A.). Moreover, productivities from standard batch cultures with the respective amount of NaCl just added before the growth experiments (so-called "non-adapted" cultures, N.A.) are also shown. The latter data therefore reflect the saline stress experienced by non-adapted cells. The direct addition of NaCl to non-adapted batch cultures resulted in a decreased biomass productivity, which became as low as 27 % of that of standard cultures. However, the gradual adaptation to increasing salinities of adapted cultures resulted in biomass productivities similar to those reached by standard control cultures. *Coccomyxa onubensis* even adapted to 500 mM NaCl, which is close to seawater salinity (599 mM), and still reached a biomass productivity of 0.17 g L^{-1} day^{-1}, approximately 80 % of that of standard cultures.

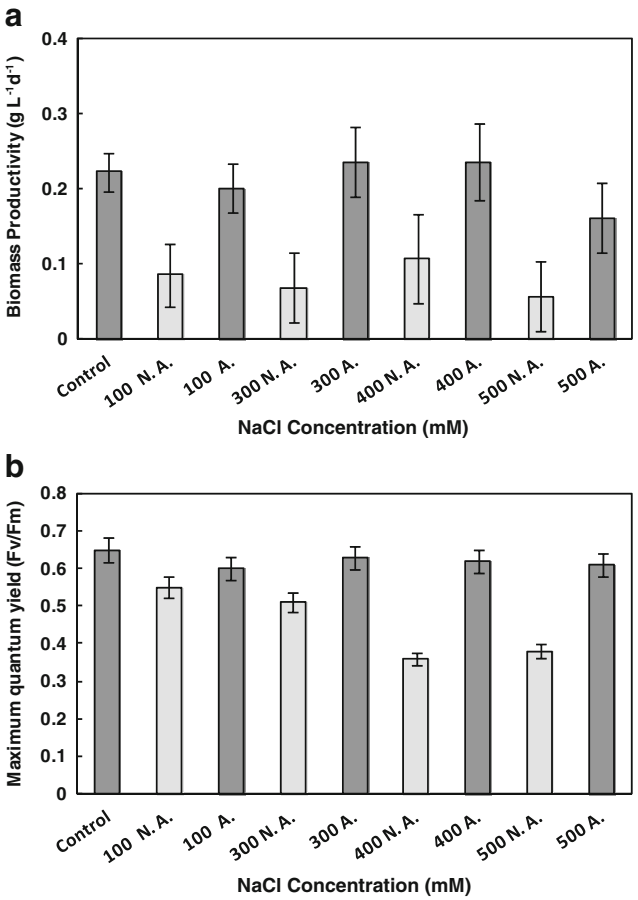

Fig. 5 Effect of salinity on biomass productivity (**a**) and photosynthetic efficiency (F_v/F_m) (**b**) of *Coccomyxa onubensis*. Aliquots of equal biomass prepared from a mother culture were adjusted to different NaCl concentrations (from 100 to 500 mM) and incubated either as batch cultures (non-adapted cultures (N.A.)) or adapted to increasing salinities as repeated-batch cultures (adapted cultures (A.)). A repeated-batch mother culture was used as control. Details are described in "Materials and methods" section. *Error bars* are standard deviations of three independent experiments

The adaptation to high salinity of *C. onubensis* requires the cells to retain the activity of the photosynthetic apparatus. In Fig. 5b, the impact of the increased high salinity on non-adapted cultures is evident by the immediate decrease of F_v/F_m, which represents the maximal PSII efficiency. Each F_v/F_m value shown in Fig. 5b for non-adapted cultures was recorded 72 h after addition of the corresponding amount of NaCl. Therefore, F_v/F_m values represent the maximum PSII efficiency of the non-adapted batch cultures after the impact of the new osmotic conditions. In non-adapted cultures with 100- or 300-mM NaCl concentrations, the F_v/F_m decreased after 72 h by about 15 and 20 %, respectively, but in cultures with 400 or 500 mM NaCl, the PSII efficiency decreased by approximately 50 % within the same time. In contrast, as also shown in Fig. 5b, the adaptation to increasing NaCl concentrations of repeated-batch cultures allowed the full recovery in terms of F_v/F_m values.

Discussion

Morphology The general characteristics of *C. onubensis* cells observed under the light microscope including a large chloroplast, visible presence of vacuoles, elongated ovoid shape, cell size, and oblique cell division (Figs. 1 and 2) are in good agreement with the observations reported by Darienko et al. (2015) for most *Coccomyxa*-like species. The typical reproduction with two to four cells formed by oblique division (Fig. 2d) was also described by Darienko et al. (2015) for other *Coccomyxa* species, who also reported some atypical autosporangia with 16–32 daughter cells for *Coccomyxa dispar* (SAG 49.84) and *Coccomyxa viridis* (SAG 216–4) photoautotrophically grown in nutrient-rich BBM medium. Such atypical autosporangia were not found in photoautotrophically grown *C. onubensis* cultures.

Cultivation of *C. onubensis* under high salinity produced significant changes in the cell morphology both inside the cell and in the cell shape. The slight increase in cell size and number of vacuoles observed for *C. onubensis* adapted to high salinity is consistent with observations for other *Coccomyxa* species (Darienko et al. 2015). *Coccomyxa onubensis* was able to grow under high salinity (500 mM), almost seawater salt concentration (Fig. 5). Therefore, the morphological changes observed under such conditions prove the high

adaptation capacity of *C. onubensis* to those harsh cultivation conditions.

The electron microscopy studies showed that *C. onubensis* cells adapted to salt stress differed in their general morphology from control culture cells, becoming slightly more spherical in appearance and with a protrusion at one cell end (Fig. 1), in accordance with observations of Darienko et al. (2015) and Muscatine et al. (1994) for other *Coccomyxa* species. A viscous material was present outside some salt-adapted cells (Fig. 2c). This material might be of polysaccharide nature and might play a relevant role in regulating the ionic balance around the cells (Bérubé et al. 1999; Ferroni et al. 2007). The increase of lipid droplets due to salt stress has been previously described in the halotolerant green alga *Dunaliella tertiolecta* (Takagi et al. 2006; Goyal 2007), in *Chromochloris* (formerly *Chlorella*) *zofingiensis*, and in *Haematococcus pluvialis*, in which salt stress induced the production of secondary carotenoids (Orosa et al. 2001; Pelah et al. 2004).

The increase of lipid droplets found in *C. onubensis* adapted to high salinity (400 mM NaCl) is also consistent with recently published data of Darienko et al. (2015) for other *Coccomyxa* species. They studied the morphological and physiological plasticity of *Coccomyxa* for more than 40 strains and reviewed the biodiversity and biogeographical distribution of *Coccomyxa* species. According to the algal response to different salinities in terms of cell size and shape, they suggested to classify strains into three types: sensitive, intermediately sensitive, and robust. Compared to salinity resistance of other *Coccomyxa* strains, *C. onubensis* might be considered as a salinity-robust species. Interestingly, all *Coccomyxa* strains found by Rodríguez et al. (2008) and Vázquez et al. (2010) as parasites of marine mussels belong to *C. viridis*, the most salinity-robust species in the study of Darienko et al. (2015).

Phylogeny The species concept for the genus *Coccomyxa* was recently evaluated by Darienko et al. (2015) using integrative taxonomy and DNA barcoding. They recognized the 18S + ITS sequence of our strain ACCV1 as a yet undescribed new species with a distinct barcode (BC-10) for ITS2. Our phylogenetic tree in Fig. 3 confirms the distinct position of ACCV1 together with *C.* spec. KN-2011-T3 (HE586515), most closely related to *Coccomyxa polymorpha* (BC-2). We therefore describe here strain ACCV1 and other strains with the same barcode as *C. onubensis*. Moreover, one of us (V.A.R.H.) has determined an 18S rDNA sequence from an uncultured environmental sample (RL75K2, deposited as HE617184 in GenBank), which most likely also belongs to the new species. Although no ITS sequence is available for this strain, its 18S sequence differs by just one nucleotide from the sequence of ACCV1 (Table 1). RL75K2 was contained in a water sample taken from "Restloch" 75, an acidic mining lake in Lusatia (Germany) with a pH of 2.4 (51° 31′ 0, 7.50″ N, 13° 42′ 57.34″ E). According to Table 1 and as described in Darienko et al. (2015), three more strains may be assigned to *C. onubensis* based on their 18S rDNA sequences: strains AH4 (KC155324; no difference to ACCV1 within 1526 determined nucleotides) and AC1 (KC155323; three differences to ACCV1 within 1516 nucleotides) were found in Guadiana pit lake (Spain) with a pH of 2.9 (Falagán et al. 2014), and "*Pseudococcomyxa simplex*" Rsa3 (KM016993) was detected in an acidic copper mine draining stream (Falagan et al., unpublished). Thus, all strains except *C.* spec. KN-2011-T3 (Fig. 3), which was isolated from a tree bark in Java, were found in extremely acidic environments. This implies that *C. onubensis* preferentially grows under acidic conditions, but due to its high adaptability, it may also be found in various other habitats.

Adaptation of *C. onubensis* to acidic and alkaline pH Highly acidic aquatic environments host photosynthetic eukaryotic life. Acidophilic algae are adapted to pH values as low as 0.05 and unable to grow at neutral pH, while acidotolerant algae are also able to grow at neutral or even higher pH (Gross 2000). As *C. onubensis* grows over a wide pH range from 2.5 to 9, it has to be considered as an acidotolerant microalga.

Considering that the intracellular pH of microalgae from acidic environments has been found to be nearly neutral (Beardall and Entwisle 1984; Gimmler et al. 1988), life at such conditions depends on the cell capacity to reduce the proton influx and to increase the proton pump efficiency that keeps the proton efflux highly active. Several acidophilic and acidotolerant microalgae have been shown to keep a very low proton permeability coefficient across the plasma membrane, which avoids acidification of the cytosol (Gross 2000). To keep an active, intense proton efflux is an ATP-dependent process, which consumes metabolic energy of approximately at least 7 % of the total ATP generated by photosynthesis (Messerli et al. 2005), therefore reducing the energy available for growth. This is consistent with the low growth rate values found for acidophilic and acidotolerant microalgae if compared to the so-called "common" microalgae (Gross 2000; Vaquero et al. 2013).

The growth of acidophilic and acidotolerant microalgae depends on the availability of dissolved inorganic carbon, CO_2 and HCO_3^-. The bioavailability of dissolved inorganic carbon differs enormously depending on the pH of the culture medium. By far, most of the dissolved inorganic carbon at acidic pH is available in the form of CO_2. The low concentration of inorganic carbon present in acidic waters would limit the photosynthesis of algae. However, to cope with the limited carbon bioavailability, the microalgae of acidic habitats often have high-affinity mechanisms for CO_2 uptake or high-affinity Rubisco enzymes (Balkos and Colman 2007; Spijkerman 2008). In this respect, based on the genetic analysis of *Coccomyxa subellipsoidea* strain, Blanc et al. (2012) suggested the existence of a functional CO_2-concentrating

mechanism. Verma et al. (2009) proposed the presence of an external carbonic anhydrase (CA) in *Coccomyxa*, which operates at pH above 7, with a carbon transport facilitating role rather than a concentrating function. These findings might also explain partly the moderate productivity values obtained for *C. onubensis* cultured at acidic pH (Fig. 4a) and even at neutral pH and suggest that culturing *C. onubensis* in photobioreactors under intensive control of CO_2 supply might be promising for achieving high biomass productivities.

Adaptation of *C. onubensis* to salinity Salinity is a stress factor for most microalgae. The capacity of microalgae to cope with increased salinity levels in the culture medium depends on the species. Depending on their adaptability to salinity, microalgae are considered as halophilic or halotolerant (Richmond 2004). *Coccomyxa onubensis* was found to grow in culture media that contain up to 0.5 M NaCl and can therefore be considered as halotolerant. As the concentration of salt in the seawater is, on average, 35 g L^{-1}, *C. onubensis* might even be able to grow on seawater, thus saving water resources and costs for biomass production.

The capacity of *C. onubensis* to grow at approximately seawater salinity, together with its natural ability to grow at very low pH such as 2.5, allows culture conditions, which are highly unfavorable for competing microalgae. Consequently, both halotolerance and optimal growth at acidic pH of this alga are promising properties to prevent contamination also in outdoor mass production.

F_v/F_m values around 0.6–0.7 are expected for healthy microalgal cells (Young and Beardall 2003; Cuaresma et al. 2011). Thus, according to Fig. 5, *Coccomyxa* cultures adapted to high salinity can be considered photochemically active. The electron transport in photosynthesis is affected by the oxidative action of ROS and by the high concentrations of Na^+, which alter the D1 proteins and the water complex oxidation at PSII (Masojídek et al. 2000; Sudhir and Murthy 2004; Sudhir et al. 2005). High ionic strength induces the intracellular accumulation of osmoregulant molecules in microalgae that protect proteins and membranes against chemical damage produced by inorganic ions, including the oxidative damage from ROS (Fuggi et al. 1988; Varshney et al. 2015). There is published information available on the biochemical mechanisms by which halotolerant microalgae cope with high osmotic pressure (Gimmler 2001). Liu and Shen (2006) suggested that salt shock induces state II transition in dark-adapted *Dunaliella salina* cells and that ATP content depression is likely involved in the regulation. Strizh et al. (2004) found the induction of the synthesis of several proteins with molecular weights close to 100 kD in *Tetraselmis viridis*, suggesting that a novel Na^+-ATPase isoform might be induced by the alga at high NaCl concentrations. These findings might support the fact that *C. onubensis* also cope with the hyperosmotic shock produced by the addition to the culture medium of a high amount of NaCl (Fig. 5b). This might also help explaining the halotolerant behavior of the alga in addition to the accumulation of osmoregulant molecules, whose nature and applications have still to be investigated.

The phenotypic changes of *C. onubensis* cells adapted to high salinity (Fig. 2) reinforce the idea of a strong adaptation response, in good agreement with the adaptation concept stated by Darienko et al. (2015). Such a strong adaptation response enables the microalga to reach biomass productivities similar to those of control cultures, which might be of biotechnological interest.

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