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COCCOMYXA ACTINABIOTIS SP. NOV. (TREBOUXIOPHYCEAE, CHLOROPHYTA), A NEW GREEN MICROALGA LIVING IN THE SPENT FUEL COOLING POOL OF A NUCLEAR REACTOR[1]

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Life can thrive in extreme environments where inhospitable conditions prevail. Organisms which resist, for example, acidity, pressure, low or high temperature, have been found in harsh environments. Most of them are bacteria and archaea. The bacterium *Deinococcus radiodurans* is considered to be a champion among all living organisms, surviving extreme ionizing radiation levels. We have discovered a new extremophile eukaryotic organism that possesses a resistance to ionizing radiations similar to that of *D. radiodurans*. This microorganism, an autotrophic freshwater green microalga, lives in a peculiar environment, namely the cooling pool of a nuclear reactor containing spent nuclear fuels, where it is continuously submitted to nutritive, metallic, and radiative stress. We investigated its morphology and its ultrastructure by light, fluorescence and electron microscopy as well as its biochemical properties. Its resistance to UV and gamma radiation was assessed. When submitted to different dose rates of the order of some tens of mGy · h^{-1} to several thousands of Gy · h^{-1}, the microalga revealed to be able to survive intense gamma-rays irradiation, up to 2,000 times the dose lethal to human. The nuclear genome region spanning the genes for small subunit ribosomal RNA-Internal Transcribed Spacer (ITS) 1-5.8S rRNA-ITS2-28S rRNA (beginning) was sequenced (4,065 bp). The phylogenetic position of the microalga was inferred from the 18S rRNA gene. All the revealed characteristics make the alga a new species of the genus *Coccomyxa* in the class *Trebouxiophyceae*, which we name *Coccomyxa actinabiotis* sp. nov.

Key index words: ***Coccomyxa*; extremophiles; green microalga; ionizing radiation; radioresistance**

***Abbreviations*: ITS, Internal Transcribed Spacer**

Conditions for life in the early days of Earth probably involved high temperatures, acidity, radiations, and pressure. These external stresses result in damaging the cell, whether it be in its metabolites, proteins, or DNA. A number of resistance mechanisms must have been found for life as we know it today to exist. However, the more complex the organism is, the harder it is to maintain cell integrity. Most of

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the extremophile organisms found up to now belong to archaea and bacteria, the predominant form of life on Earth, considered to be among the most ancient organisms living on our planet. For example, bacteria of the genus *Thiobacillus* can live in highly acidic conditions (González-Toril et al. 2003), *Bacillus infernus* survives high pressure (Sharma et al. 2002), archaea such as *Haloarcula* need high salt concentrations to live (Oren et al. 1999), other bacteria such as *Aquifex pyrophilus* high temperature (Tehei and Zaccai 2007) and *Aquaspirillum arcticum* low temperature (Gounot 1991). Some organisms resist huge doses of UV or ionizing radiations. Among the extremophiles, *Deinococcus radiodurans* (Anderson et al. 1956), remains one of the most famous bacteria – nicknamed "Conan the Bacterium" (Huyghe 1998) and listed in The Guinness Book Of World Records – as it survives extreme ionizing radiation doses, of up to 20,000 Gy, that is two thousand times the dose lethal to human (Daly et al. 2004, Daly 2009). The extremophile archaeon *Thermococcus gammatolerans* is almost as radioresistant as *D. radiodurans* (Confalonieri and Sommer 2011). Among radiation-resistant species also stand the cyanobacterium *Chroococcidiopsis* sp. (Billi et al. 2000), the archaea *Pyrococcus furiosus* (DiRuggiero et al. 1997) and *Halobacterium* sp. (Kottemann et al. 2005), and the fungus *Alternaria alternata* (Mironenko et al. 2000) that withstand ~2,500, 3,000, 5,000, and 3,000 Gy, respectively. Some micro-animals, although very few, are also known to be capable to survive huge gamma-radiation doses. The tardigrade *Milnesium tardigradum* survives radiation doses of up to 5,000 Gy but this makes it sterile (Horikawa et al. 2006), the bdelloid rotifer *Adineta vaga* up to 9,000 Gy and is still partially fecund following a 1,000 Gy irradiation (Krisko et al. 2012). The dispersion of radioresistant species in the phylogenetic tree suggests that they have acquired this property independently. The mechanisms underlying the radioresistance properties have extensively been studied in *D. radiodurans*, far less in other organisms. Various strategies have been selected during evolution including different mechanisms of protection against oxidative stress, an efficient conventional DNA repair tool box, original DNA repair mechanisms, and a condensed nucleoid (Confalonieri and Sommer 2011). The extreme resistance to ionizing radiation may have different evolutionary origins. It may have evolved as an adaptation to the non-periodic short-term gamma-radiation maxima that possibly occurred several times during evolutionary history (Melott and Thomas 2009) or to high natural ionizing radiation levels found in some marine sediments (Slade and Radman 2011). It also may be a consequence of the evolutionary adaptation to desiccation which is a more common physiological stress as dry environments are more prevalent on Earth than strongly radiative ones. Radiation resistance and desiccation resistance are actually functionally related phenomena. They are both accompanied by the production of reactive oxygen species and extensive DNA breakage. Radiation resistance of bdelloid rotifers is thought to be a consequence of their evolutionary adaptation to survive episodes of desiccation (Krisko et al. 2012). However, although all known naturally occurring radiation-resistant bacteria are also desiccation resistant, it is not the case of artificial ones. Furthermore, some desiccation-tolerant bacteria are not radiation resistant (Slade and Radman 2011).

By investigating species growing in highly radioactive environments and in particular in nuclear facilities, we discovered a unique eukaryotic microalga, which withstands radiation levels comparable to *D. radiodurans* (Rivasseau et al. 2010, 2011, 2013). This unicellular freshwater green microalga was isolated from the water pool used to cool spent fuel elements of a nuclear reactor. Species that are able to live in such environment have to be adapted to conditions which are lethal to other organisms. The microalga described in this work was actually the only autotrophic eukaryote identified in the pool of this nuclear facility, although the possibilities for microbial contamination of the pool water were numerous. Radioresistant bacteria such as *Kineococcus radiotolerans* (Phillips et al. 2002) and fungus such as *Alternaria alternata* (Mironenko et al. 2000) had previously been found in such environments, the former isolated from a storage site of radioactive wastes, the second from the Chernobyl site after the nuclear disaster. A variety of bacteria belonging for example to the genera *Bacillus*, *Burkholderia*, *Micrococcus*, *Pseudomonas*, *Ralstonia* or *Staphylococcus* have been identified in a French storage pool and in the cooling pool of a Spanish nuclear power plant (Galès et al. 2004, Chicote et al. 2005, Sarro et al. 2005). Another eukaryote, the microalga *Trachydiscus minutus*, have also been isolated from the cooling pool of a Czech nuclear power plant (Přibyl et al. 2012) but its radioresistance has not been assessed. However, eukaryotes discovered in such environments still remain few compared to bacteria and even fewer have been the subject of extensive studies.

In the present work, we characterized this freshwater green microalga. We investigated its morphology, its biochemical properties and we conducted molecular phylogenetic analyses based on the gene corresponding to 18S rRNA and to ITS1-5.8S rRNA-ITS2. As this microalga was isolated from a very peculiar place presenting extreme conditions for life, namely high ionizing radiation rates, lack of nutrients, and presence of toxic radioactive metals, we investigated its resistance to nutritive, radioactive, and UV stresses. On the basis of all these results, we concluded that this strain is a new green microalga species of the genus *Coccomyxa* in the class *Trebouxiophyceae* with exceptional radioresistance properties, and we name it *Coccomyxa actinabiotis* sp. nov.

MATERIALS AND METHODS

Experimental material, collection, and growth conditions. The new algal strain was collected from the water pool used to store spent fuel elements in a research nuclear reactor in France. It was isolated from a microalgal biofilm growing on a projector immersed 3 m below the surface water. The strain was deposited in the Culture Collection of Algae and Protozoa in Oban (CCAP; http://www.ccap.ac.uk) under accession number CCAP 216/25.

We also investigated two other strains belonging to the genus *Coccomyxa*, obtained from the Culture Collection of Algae in Göttingen (Sammlung von Algenkulturen Göttingen [SAG]; http://www.epsag.uni-goettingen.de). Strain SAG 216/2 isolated by Chodat (pre 1909) in the freshwater Lake Geneva in Switzerland is the authentic strain of *Coccomyxa chodatii* Jaag (1933). Strain SAG 216/5 isolated as a phycobiont of lichen by Jaag in Switzerland is the authentic strain of *Coccomyxa peltigerae* Warén (1920).

Coccomyxa chodatii and *C. peltigerae* strains were cultivated in Bold Basal Modified medium (BBM) (Bischoff and Bold 1963) purchased from Sigma-Aldrich (Saint-Louis, MO, USA). *Coccomyxa actinabiotis* strain was grown in deionized Milli-Q water (MilliQ filtration system; Millipore, Molsheim, France) and in BBM. The microalgal sample just harvested from the pool emitted ionizing radiation at a dose rate of 0.15 $mGy \cdot h^{-1}$ owing to the radionuclides it had accumulated. It was hence cultivated in a radioactively controlled laboratory until its growth allowed us to work with a sample in non-controlled zone. Irradiation experiments were also performed in a radioactively controlled zone. In the controlled zone, microalgae were grown at 23°C under an illumination of 300 μmol photons $\cdot m^{-2} \cdot s^{-1}$ in Erlenmeyer flasks regularly stirred. In non-radioactive laboratories, all microalgae were cultivated at 21°C with an illumination of 70 μmol photons $\cdot m^{-2} \cdot s^{-1}$ provided by a panel of cool 36W white and fluorescent tubes (respectively Philips TL-D36W-640; Philips, Amsterdam, the Netherlands and Osram L 36W/77; Osram, München, Germany) in liquid culture medium in flasks aerated on an orbital platform shaker Innova 2300 (New Brunswick Scientific, Enfield, CT, USA) at 120 rpm. To isolate the axenic strain, labeled as CCAP 216/25, six successive plating and colony isolation on solid agar plates containing BBM nutritive medium were carried out.

Morphological and biochemical investigations. To investigate the cell morphology, microalgae directly sampled from the pool were observed in a controlled radioactive laboratory using a Euromex XE1100 optical microscope (Labomoderne, Paris, France) and an Olympus C4040Z camera (Olympus, Tokyo, Japan). Cultures of the isolated strain (CCAP 216/25), performed in non-radioactive zone, were observed using an Olympus BX51 epifluorescence microscope equipped with Nomarsky differential interference contrast optics and an U-MNU2 filter cube (360–370 nm band pass excitation filter, 400 nm dichromatic beam splitter and 420 nm barrier emission). Photographs were taken using an Olympus DP71 high resolution digital camera. Intracellular lipid droplets were observed in fluorescence microscopy owing to their yellow autofluorescence, the chloroplast owing to its red autofluorescence. The nucleus was visualized in fluorescence microscopy after DNA staining by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Starch was observed after Lugol's staining (I_2 + IK) which resulted in a brown to dark coloration of the granules in light microscopy.

Pigment extraction and HPLC analysis. Prior to HPLC analysis, pigments of *C. actinabiotis* were extracted by a methanol/chloroform mixture. Culture samples (typically 500 mg fresh weight) were centrifuged at 2,000*g* for 5 min at 4°C. An equivalent volume of MilliQ water (500 μL) was added to the pellet, which was then frozen in liquid nitrogen and ground to powder in a mortar. The powder was transferred into a glass tube (Corex, Gentaur, Paris, France) chilled with liquid nitrogen. Out of liquid nitrogen, 1 mL chloroform and 3 mL methanol were quickly added. The mixture was rapidly homogenized below 0°C using a spatula. The tube was sealed and vortexed for 10 min at room temperature. After addition of 1 mL of chloroform and 1 mL of milliQ water, the sample was vortexed and centrifuged at 7,800 *g* for 10 min at 4°C to separate the hydrophobic and hydrophilic phases. The hydrophobic chloroform phase was taken; chloroform was evaporated using a Speedvac and extracts were stored under argon atmosphere in the dark at −20°C before analysis. Just prior to HPLC injection, samples were diluted in 200 μL dimethylformamide and filtered in a 1.5 mL microtube by centrifugation.

HPLC analysis was carried out using a Varian system equipped with a UV-visible diode array detector (Varian, Palo Alto, CA, USA). Pigments were separated on a YMC 250 × 4.6 mm C30 bonded silica based reversed phase column (YMC, Kyoto, Japan) using a mobile phase consisting of a mixture of methanol (solvent A), water/methanol/ammonium acetate 20/80/0.2 v/v/v (solvent B) and methyl terbutyl ether (solvent C). All solvents were of HPLC grade. The injected sample (50 μL) was eluted by an isocratic elution of solvents A/B in proportions 95/5 v/v from 0 to 12 min followed by a gradient elution by solvents A/B/C in proportions ranging from 80/5/15 to 30/5/65 v/v/v from 12 to 30 min. Analytes were identified by their retention time and by their absorption spectra compared to reference spectra. Mass concentration of the pigments was calculated in the injected sample in $\mu g \cdot L^{-1}$ by comparison of their peak area to the surface of the peaks obtained at 450 nm for standard analytes.

Transmission and scanning electron microscopy. For transmission electron microscopy (TEM), the *C. actinabiotis* cells (CCAP 216/25) were fixed for 1 h at room temperature in 2% (v/v) glutaraldehyde (Merck, Fontenay sous bois, France) in 0.1 M sodium cacodylate buffer (pH 6.5). The samples were subsequently washed three times with 0.2 M cacodylate buffer and post-fixed with 1% osmium tetroxide in cacodylate buffer for 2 h at 4°C. After three buffer rinses, the samples were included in agar and dehydrated in ascending sequences of ethanol and infiltrated with ethanol/Epon resin mixture. Finally the algal strains were embedded in Epon. Ultrathin sections (60 nm) were prepared with a diamond knife on a UC6 Leica ultramicrotome (Leica, Solms, Germany) and collected on 200 μm mesh formvar-coated copper grids. Ultrathin sections were post-stained with 5% uranyl acetate in water and 0.4% lead citrate before being examined on a Philips CM120 transmission electron microscope operated at 80 kV.

For scanning electron microscopy (SEM), *C. actinabiotis* cells cultivated as described above were allowed to sediment on silicon nitride plates (Silson, Blisworth, UK) and cryo-dessicated at −80°C after cryofixation at −160°C into isopentane chilled with liquid nitrogen. SEM images were obtained without sample metallization using a LEO 1530 scanning electron microscope (Zeiss, Göttingen, Germany) operating at 20 kV.

DNA extraction, amplification, sequencing, and analysis. Total DNA of the strain CCAP 216/25 was isolated using the Wizard®Genomic DNA Purification Kit (Promega, Charbonnières, France), following the instructions provided by the manufacturer. The sequence of nuclear genome region spanning the genes for 18S ribosomal RNA-ITS1-5.8S rRNA-ITS2-28S rRNA (500 first bases; 4,065 bp) was amplified by PCR using *Pfu* DNA polymerase (Promega) and the pair of primers EAF3/ITS055R, conventionally used to amplify eukaryotic rRNA (Pröschold et al. 2001). The PCR products of 4 kb were sequenced in both directions at GATC Biotech (Konstanz, Germany).

Similarly, the DNA of the same ribosomal region (18S rRNA-ITS1-5.8S rRNA-ITS2-28S rRNA) of *C. chodatii* and *Coccomyxa peligerae* was amplified and sequenced as described above, yielding sequences of 3,013 and 3,103 bp, respectively. These newly determined sequences were employed for comparison.

The newly obtained sequences, excluding the primer regions, of the nuclear genome region spanning the genes for 18S ribosomal RNA-ITS1-5.8S rRNA-ITS2-28S rRNA (500 first bases) were deposited at the EMBL/GenBank databases under accession numbers FR850476 (*C. actinabiotis* strain CCAP 216/25), FN597598 (*C. chodatii* strain SAG 216/2), and FN597599 (*C. peligerae* strain SAG 216/5).

The BLASTn algorithm (Altschul et al. 1990) was used to search in the databases the sequences of ribosomal RNA genes exhibiting identity with the sequence of the microalga CCAP216/25. The 18S rRNA sequence (FR850476.1, 2,786 bases) was input into the nucleotide BLASTn version 2.232 sequence search algorithm (Zhang et al. 2000) available at the NCBI (http://blast.ncbi.nlm.nih.gov) and optimized for somewhat similar sequences and cross-species searches, using default parameters. Environmental samples were excluded from the procedure. The alignment was performed on the GenBank/EMBL/DDBJ/PDB/RefSeq databases with a total of 31,076,527 reference sequences. In addition, the reference *Chlamydomonas reinhardtii* alignment was forced by inputting specifically its sequence, and the newly obtained sequences for *C. chodatii* and *C. peltigerae* were added as they provide longer sequences than those currently available at the NCBI. This initial search returned 252 Blast hits, which alignments were exported as a FASTA sequence list.

These alignments were aligned using the Clustal Omega software (Goujon et al. 2010) and results were input into FastTree version 2.1.7 (Price et al. 2010) to infer the approximately maximum-likelihood phylogenetic tree with the generalized time-reversible (GTR) model of nucleotide evolution in the CAT approximation and a Jukes-Cantor distance metric. This procedure produces P-values computed using the Shimodaira-Hasegawa test (Shimodaira 2002) on the nearest neighbor interchanges, providing results equivalent to the PhyML support value. The branch length was finally rescaled using the Gamma20-based likelihood correction (GTR+CAT+Γ).

The resulting phylogeny tree was imported into the Archaeopteryx/ATV software (Zmasek and Eddy 2001). As the tree contained 252 sequences, too numerous to be all shown, neighboring tree branches having a low support value were merged in order to highlight similar species and reveal the taxonomy tree. The rationale was to favor valid ICN species, and merge branches with similar substitutions per site. Finally, the tree contains 61 species with proper name, sequence reference and culture collection number. The branches have been ordered as to follow as much as possible the clade arrangement proposed by Pröschold et al. 2011 and Darienko et al. 2015. The resulting cladogram is shown in Figure 5. The full cladogram is supplied as Appendix S1 in the Supporting Information in Nexus and Newick format.

Assessment of resistance to UV and to γ-radiation. To assess resistance to UV radiation, a microalgal sample of 1 cm thickness was grown in BBM and illuminated for 10 min with a Zeiss UV lamp delivering a 800 $\mu W \cdot cm^{-2}$ flux at a distance of 2.54 cm. Experiment was carried out in controlled zone. Growth after acute irradiation was measured by cell density using a Malassez counting plate and compared to that of a control culture.

To assess algae resistance to gamma ionizing radiation, microalgae were exposed at different dose rates for various times. Microalgae were exposed to dose rates of 0.8, 20, and 60 $mGy \cdot h^{-1}$ for 30 d, in controlled zone at a light intensity of 400 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The growth during irradiation, measured by the cell density, was compared to that of a control culture. Very high dose rates of 1,000 and 4,000 $Gy \cdot h^{-1}$ were also applied to the microalgae for short periods of 20 and 5 h, respectively. For these experiments, cells were concentrated to $1–2 \times 10^9$ cells $\cdot mL^{-1}$ in water and inserted inside a spent nuclear fuel element providing the intense γ-radiation flux. The same experiment was carried out by maintaining the algae sample inside the fuel element delivering the 4,000 $Gy \cdot h^{-1}$ flux for various controlled times, yielding different doses ranging from 300 to 10,000 Gy. Microalgae were allowed to recover in non-controlled zone in BBM diluted twice after these acute irradiations. We assessed the cell growth during recovery by density measurement and compared the results to that of the control sample, as described in Rivasseau et al. 2013.

RESULTS AND DISCUSSION

Coccomyxa actinabiotis Rivasseau, Farhi et Couté **sp. nov.** Figures 1–6

Description: Micro-organismus unicellularis, viridis, photosyntheticus, dulcaquatilis, ovalis et non mobilis. Longitudo: (5)-5,9-7,7-(9-10) μm; latitudo: (2,5)-3,2-4,1-(4,6) μm. Cellula cum uno nucleo et uno chromatophoro, et cum amylo in chromatophoro. Multiplicatio vegetativa divisione cellulae et emissione autosporarum. Analyticum genetivorum naturalium characterum studium de 18SrRNA-ITS1-5.8SrRNA-ITS2-28SrRNA (initium) illam micro-algam *Coccomyxae* (Trebouxiophyceae) generis esse monstrat. Proxima genetivis naturalibus characteribus genera: *Coccomyxa polymorpha* Darienko & Pröschold basi CAUP H5101 (HG972979), *Coccomyxa rayssiae* Chodat & Jaag basi UTEX273 (HQ317304), *C. chodatii* Jaag basi SAG 216-2 et *C. peltigerae* Warèn basi SAG 216-5. Dissimilis est omnium generis familiarum quod differunt ITS 1 et ITS 2 et quod adsunt duo compotia circiter 500 nucleolorum fragmina in sequentia ARNr 18s equivalente. Egregia adversus atomos transvertentes radios resistentia est: medius algarum populus aliquanto numero 10,000 Grays superest. Vitae locus: frigidarium machina fractorum atomorum vetula nocibiliaque reliqua salvans, cum aquae pH = 5.2 usque ad 5.5, cum inter 1 et 1.5 $\mu S \cdot cm^{-1}$ ductione, cum medietate calorum = 25°C et cum inter 0.1 $mGy \cdot h^{-1}$ et 4,000 $Gy \cdot h^{-1}$ atomos transvertentibus radiis. Ejus genetivis naturalibus characteribus egregiaque adversus atomos transvertentes radios resistentia novum genus est, *C. actinabiotis* n. sp. Nomine.

Description: Unicellular freshwater eukaryotic green microalga measuring 6.8 ± 0.9 μm × 3.8 ± 0.6 μm, containing a parietal chloroplast with starch. The main pigments determined by HPLC are chl *a* and *b*, lutein, violaxanthin and β-carotene. It multiplies by autospore production inside the autosporangium with (2–4) spores. Cells gather in colonies in a mucilaginous jelly in the pool but are isolated in culture.

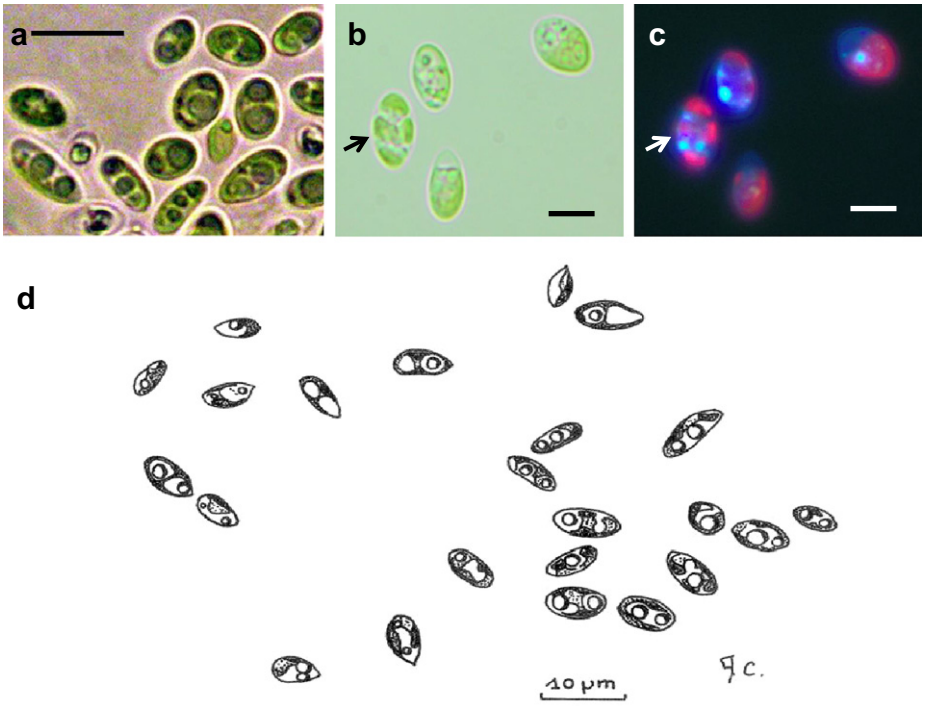

FIG. 1. Overview of populations of *Coccomyxa actinabiotis* cells (a) directly sampled from the storage pool of spent nuclear fuels, observed by photonic microscopy, scale bar: 10 µm. (b–d) after cultivation in BBM nutritive medium, observed by (b) photonic and (c) fluorescence microscopy after DAPI staining. The autofluorescence of chl is visible in red. Arrow points to a dividing cell containing four spores. One chloroplast is visible in each cell and spore, scale bars: (b and c) 5 µm.

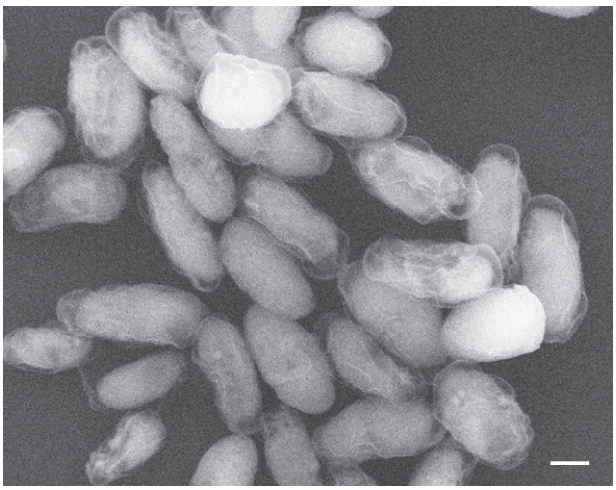

FIG. 2. Overview of a population of *Coccomyxa actinabiotis* cells grown in diluted BBM nutritive medium observed by scanning electron microscopy, scale bar: 2 µm.

Habitat: Freshwater storage pool containing spent nuclear fuel elements. The water is slightly acidic (pH, 5.3 ± 0.2), demineralized (conductivity, 1.2 ± 0.2 $\mu S \cdot cm^{-1}$), oligotrophic (10 $mg \cdot L^{-1}$ nitrate; <0.2 $mg \cdot L^{-1}$ phosphate) and contains radionuclides originating from the dissolution and activation of spent nuclear fuel elements materials. The pool water is in contact with air, continuously illuminated (200 µmol photons $\cdot m^{-2} \cdot s^{-1}$), and maintained at 25 ± 3°C. The spent nuclear fuel elements generate radiologic dose rates varying between 70 $\mu Gy \cdot h^{-1}$ close to the pool walls and surface and several hundred $Gy \cdot h^{-1}$ close to the elements.

Type locality: Freshwater pool used to cool the spent nuclear fuel elements of a nuclear research reactor, Grenoble, France. Latitude 45°12′ N, longitude 5°41′ E, altitude 214 m a.s.l.

Holotype: Figures 1, a and d; 3, a and b.

Reference strain: The living strain has been deposited in the CCAP (http://www.ccap.ac.uk) under accession number CCAP 216/25.

Etymology: The specific epithet reflects the ability of the strain to live in an ionizing radiation environment.

The microalga was harvested from a biofilm growing on an underwater projector used to illuminate the cooling pool of a nuclear research reactor. The water filling the pool was slightly acidic (pH = 5.2–5.5), in contact with air, and maintained with a very low conductivity of 1–1.5 $\mu S \cdot cm^{-1}$ by mean of filters and an ion-exchange resin purification chain. The water contained 1.32 $mg \cdot L^{-1}$ chlorides and 10.3 $mg \cdot L^{-1}$ nitrates. Phosphate, nitrite, sulfate, and fluoride concentration was below the 0.2 $mg \cdot L^{-1}$ detection limit. The water also contained dissolved radionuclides among which

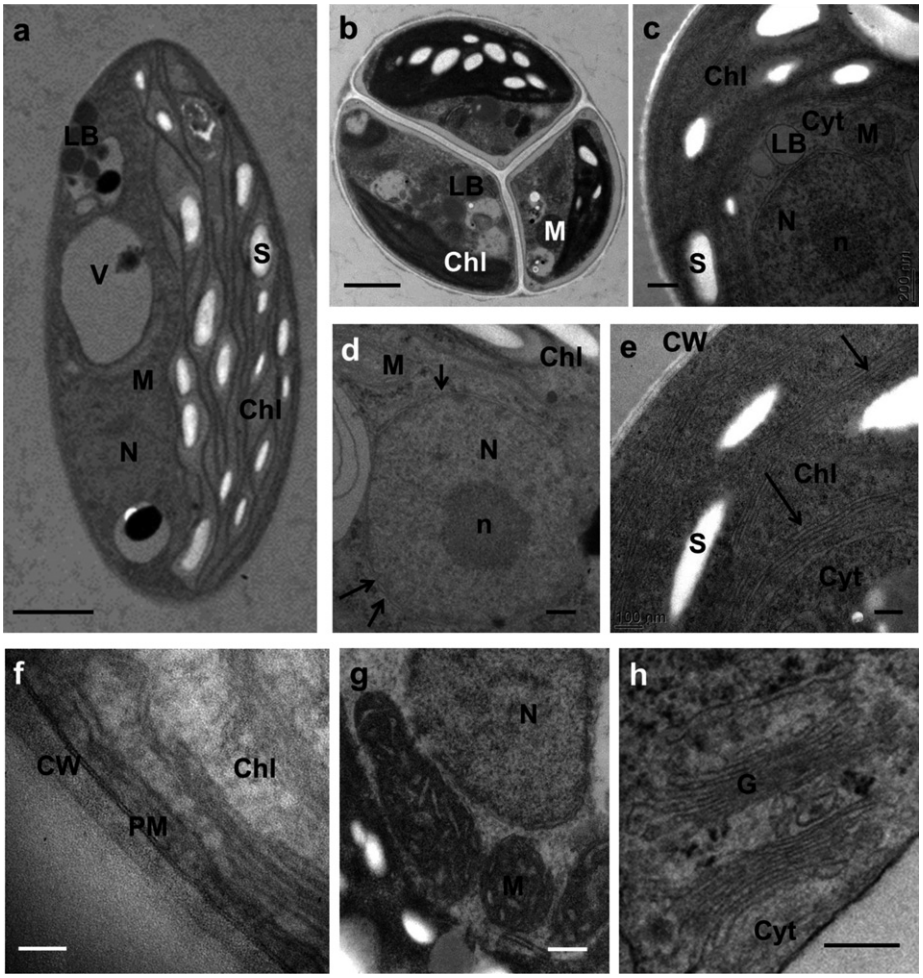

FIG. 3. Ultrastructure of *Coccomyxa actinabiotis* cells observed by transmission electron microscopy. Chl, chloroplast; Cyt, cytosol; CW, cell wall; G, Golgi apparatus; LB, lipid bodies; M, mitochondria; N, nucleus; n, nucleolus; PM, plasma membrane; S, starch granules; V, vacuoles. (a) TEM image of a whole cell showing the nucleus, the parietal chloroplast with starch granules, mitochondria, vacuoles and lipid bodies. (b) TEM image of cell division showing three spores in a cyst. Cell wall is splitting around each spore. A parietal chloroplast, mitochondria and lipid bodies are observed in each spore. (c) Detail of a single cell showing the nucleus with condensed chromatin and the nucleolus, surrounded by its double membrane, the chloroplast with starch granules, a mitochondrion, and lipid bodies in the cytosol. (d) Detail of a nucleus. Nucleolus and condensed chromatin are visible. Arrows point at nuclear pores crossing the nuclear envelope. (e) Detail of a chloroplast fragment with thylakoid lamellae (arrows). Starch granules are inserted between the thylakoids. (f) Detail of the plasma membrane and of the cell wall. The lipid bilayer of the plasma membrane is visible. (g) Detail of three mitochondria with intramitochondrial cristae. (h) Detail of the Golgi apparatus in the cytosol. Scale bars: (a and b) 1 μm, (c–e) 200 nm, (f) 50 nm, (g and h) 200 nm.

radioactive metals originating from the dissolution and activation of spent nuclear fuel element materials. The water temperature varied between 23 and 30°C with a mean value around 25°C. The pool was continuously illuminated with a 200 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} flux at the water surface. The radiologic activity in the pool originated from the spent nuclear fuel elements stored inside. The dose rate varied from 70 μGv $\cdot$ h^{-1} close to the pool walls and surface, up to several hundred grays per hour in the vicinity of the fuels elements. The pool constituted a very peculiar biotope to live in, combining several stresses, namely the presence of ionizing radiation, a lack of minerals leading to nutritive stress and the

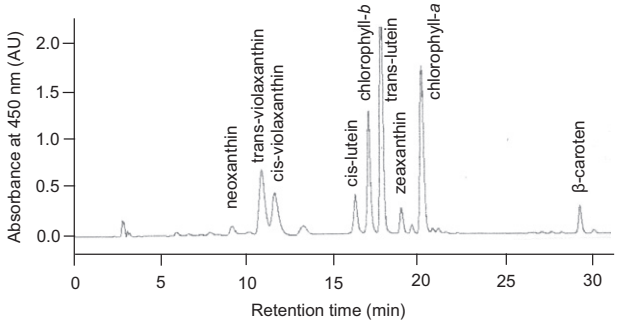

FIG. 4. Pigment analysis of *Coccomyxa actinabiotis* by HPLC followed by UV-visible detection at 450 nm after methanol/chloroform extraction.

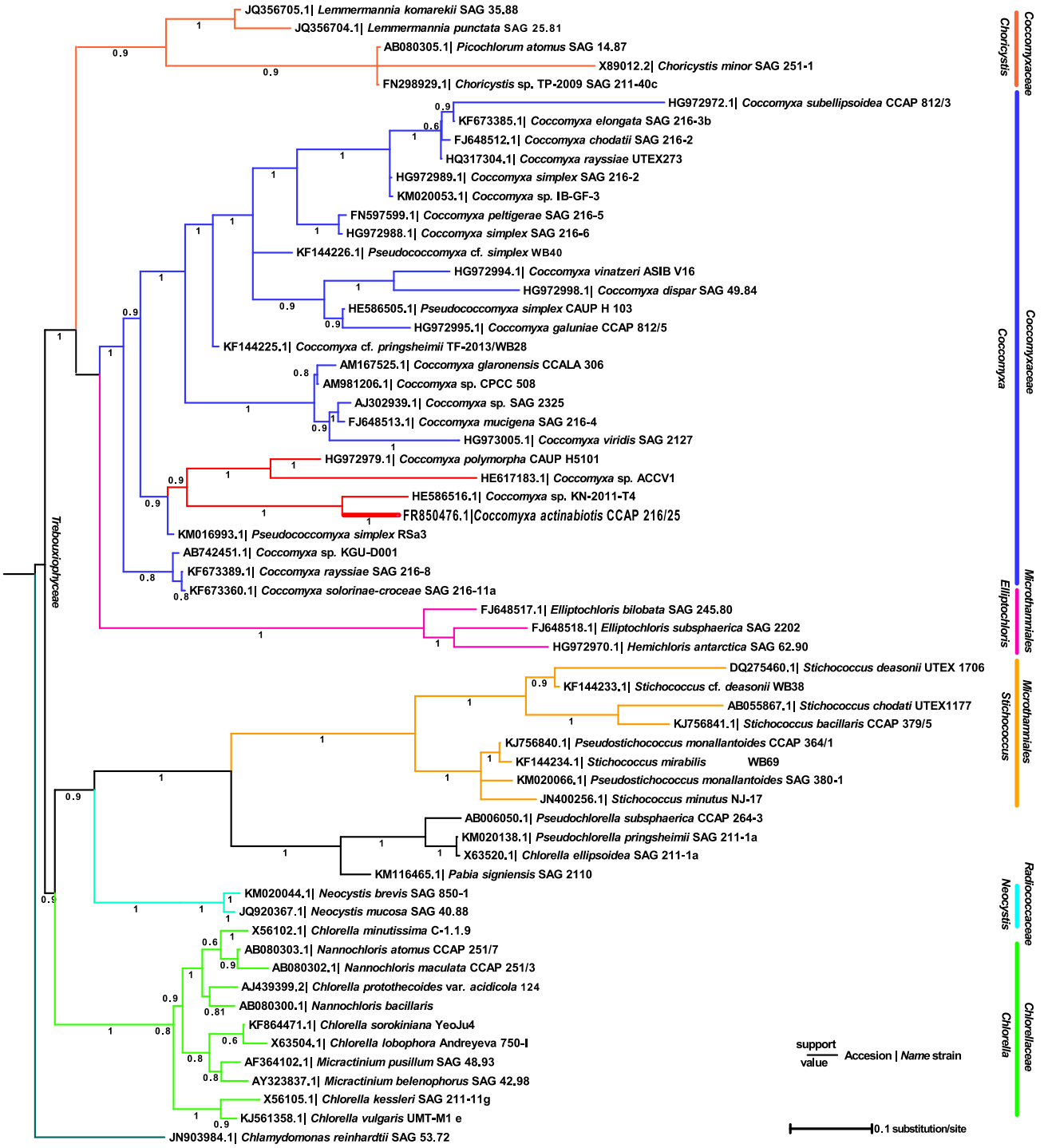

FIG. 5. Phylogenetic tree of *Coccomyxa actinabiotis* obtained after pair-wise DNA sequence alignment of the total 18S rRNA gene sequence FR850476.1 (BLASTn) by using a maximum likelihood phylogenetic approach. The scale bar corresponds to a 0.1% substitution ratio. Colors on the online version correspond to clades following as much as possible the order proposed by Pröschold et al. 2011 and Darienko et al. 2015,. The support values correspond to the Shimodaira-Hasegawa test (Shimodaira 2002) bootstrap *P*-values, as used in PhyML 3. The values have been rounded to simplify the layout of the tree.

presence of radioactive metals inducing oxidative and ionizing stress. In this hostile environment, the presence of life would result probably both from an adaptation and an acclimatization to the very specific physico-chemical conditions.

Microalgae directly collected from this biofilm were observed using light microscopy (Fig. 1a) and presented the same morphological characteristics as the isolated strain (described below). Some differences distinguished cells freshly harvested from the

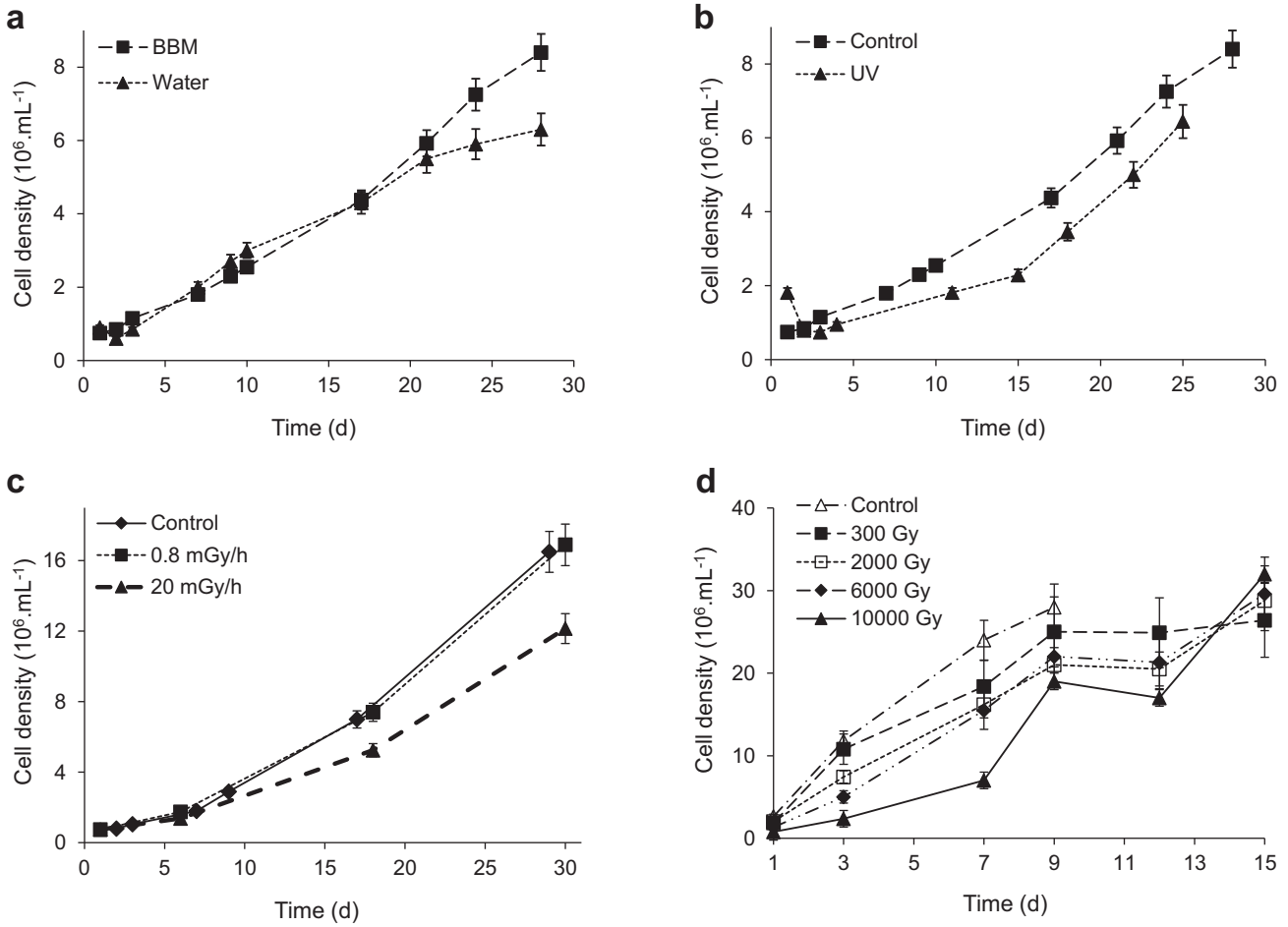

FIG. 6. Growth of *Coccomyxa actinabiotis* cells under control and stress conditions. (a) Compared growth of *C. actinabiotis* cells in BBM nutritive medium and in deionized water. (b) Growth of *C. actinabiotis* cells after an acute UV irradiation at time zero, compared to control. Following irradiation, cells were allowed to recover in BBM medium. (c) Growth of *C. actinabiotis* cells in BBM medium during a continuous gamma irradiation at different dose rates. (d) Growth of *C. actinabiotis* cells during recovery after an acute gamma irradiation at a dose rate of 4,000 Gy · h^{-1} at time zero, compared to growth of control (not irradiated) cells. Different radiation doses in the range 300–10,000 Gy were applied at time zero. Following irradiation, cells were allowed to recover in BBM medium diluted twice with water. At time 12 d, BBM growth medium was added to the algal suspensions. The gray (Gy) is the measure unit of the absorbed dose of ionizing radiation. The absorbed dose represents the mean energy imparted to matter per mass unit. It is expressed in joules per kilogram, or grays.

storage pool from those grown in a mineral growth medium classically used for algae, namely BBM nutritive medium. The former had large reserve granules and were mostly agglomerated together, held by a mucilaginous jelly. The latter were more pigmented and appeared bright green, generally well separated. Their normal growing rate in the pool was slow, typically with a doubling of the population per week that could reach 2–3 d when cultured in BBM. Microalgae were stressed in their original biotope by the physico-chemical conditions of the pool whose two main components were radioactivity and oligotrophy.

Microalgae were then cultured on solid agar plates containing BBM medium. We isolated the axenic strain CCAP 216/25 after successive plating of colonies, grew it in liquid BBM in non-radioactive conditions, and identified it at the morphological, biochemical, and phylogenetic levels. Light and fluorescence microscopy as well as scanning and transmission microscopy were used to investigate the morphology and the ultrastructure of the alga (Figs. 1b–3). It was a unicellular freshwater eukaryotic green microalga with a regular ellipsoidal shape (Figs. 1b and 2). Its average length was 6.8 ± 0.9 μm and the average width 3.5 ± 0.6 μm. However, according to the stage of growth, it was possible to observe a largest average length up to 9–10 μm, or a smaller one of the order of 5 μm especially just after division. The cell contained one cup-shaped parietal chloroplast occupying most of the cellular volume and highlighted by the autofluorescence of chl in fluorescence microscopy and by transmission electron microscopy (Figs. 1, b and c; 3a). Each cell and each spore contained a single chloroplast. Starch granules were inserted between

the thylakoid lamellae (Fig. 3e). Starch was brought to light by optical microscopy observation of cells after Lugol's staining, which revealed the presence of elongated granules of a brown to black color localized in the chloroplast. The cells were uninuclear and the nucleus was generally centered between the chloroplast ends. The single nucleolus and condensed chromatin were often visible (Fig. 3, c, d and g). A thin plasma membrane measuring 5 ± 0.5 nm surrounded the protoplast (Fig. 3f). Cells multiplied by autospores production inside the autosporangium with two to four spores (Figs. 1, b and c; 3b). Cells contained lipid bodies and vacuoles (Fig. 3, a–c) whose volume increased in aging cells. Lipid droplets were present both in plastid and in the cytosol. On some TEM images, the fusion of lipid droplets in the chloroplast into bigger ones was observed. These lipid droplets yielded a yellow-green autofluorescence when observed in fluorescence microscopy. This phenomenon has recently been described for several unicellular microalgae of the classes Xanthophyceae and Eustigmatophyceae (Řezanka et al. 2010, Přibyl et al. 2012) but it had not been reported for strains of the class Trebouxiophyceae yet. We also observed lipid autofluorescence in another species of the genus *Coccomyxa*, namely *C. chodatii*. This autofluorescence may be a characteristic of this genus as it had not been observed in other strains of Trebouxiophyceae tested (Přibyl et al. 2012).

The main pigments identified by HPLC were chl-*a*, chl-*b* and lutein followed by violaxanthin, β-carotene, zeaxanthin and neoxanthin (Fig. 4 and Table 1). It is noteworthy that the analytical protocol employed allows to separate the cis/trans isomers of lutein and violaxanthin. These profiles are also observed for higher plants and green microalgae (Roy et al. 2011). The same pigments, with the exception of zeaxanthin, were identified in other *Coccomyxa* species, for example, *Coccomyxa elongata* Jaag, *Coccomyxa simplex* (Pringsheim) Mainx (Whittle and Casselton 1969) and a *Coccomyxa* species parasitic of blue mussel (Zuykov et al. 2014). In the latter, however, the proportions of the three main pigments were different from those obtained in *C. actinabiotis*, with a concentration ratio chl-*a*/chl-*b*/lutein of ~5/10/2 evaluated from the results of Zuykov et al. 2014, whereas it was ~10/4/2 in *C. actinabiotis* (Table 1). Chl-*a* and chl-*b* represented, respectively, 55% and 23% of the total detected pigments. Among carotenoids, lutein and violaxanthin were predominant, representing, respectively, 52% and 35% of the total detected carotenoids.

TABLE 1. Pigment analysis of *Coccomyxa actinabiotis* using HPLC followed by UV-visible detection. Operating conditions described in the Materials and Methods section.

Pigment	Retention time (min)	Concentration related to total pigments (%)	Concentration related to total carotenoids (%)
Neoxanthin	9.45	0.7	2.9
Trans-violaxanthin	11.14	4.6	20.4
Cis-violaxanthin	11.91	3.4	15.1
Cis-lutein	16.57	2.0	8.9
Chl-*b*	17.34	22.9	
Trans-lutein	18.08	9.8	43.3
Zeaxanthin	19.22	1.0	4.3
Chl-*a*	20.35	54.5	
β-carotene	29.56	1.1	5.0

The isolated microalga is therefore a taxon of unicellular eukaryotic freshwater green algae, classified in the phylum of Chlorophyta as it contained chl *a* and *b* and carotene as photosynthetic pigments and starch as reserves. Based on morphological and biochemical observations, the microalga was identified as part of the Protista within plant affinity kingdom, phylum Chlorophyta, genus *Coccomyxa*, with a morphology not fully similar with respect to already described *Coccomyxa* species. However, the plasticity of *Coccomyxa* strains is usually high, particularly depending on growth conditions (Darienko et al. 2015). The cells of the species described in this work became more spherical in a medium containing diluted see water at a 15 $g \cdot L^{-1}$ salinity, as what has been observed for other *Coccomyxa* species (Darienko et al. 2015).

In order to identify the strain CCAP 216/25 at the phylogenetic level, we analyzed the genes of the RNA of the small subunit of the ribosome (18S rRNA) and of the ITS 1 and 2 region, which are conventionally used to assess the evolutionary relationship between species. The nuclear genome region spanning the genes for 18S ribosomal RNA-ITS1-5.8S rRNA-ITS2-28S rRNA (beginning) was thus amplified and sequenced. The PCR products obtained from two independent cultures were of 4 kb (Fig. S1 in the Supporting Information), that is, 1 kb longer than what is classically described for microalgae. The sequence (Fig. S1) was compared with the nucleotide collection (GenBank/EMBL/DDBJ/PDB/RefSeq databases) available at the NCBI using the BLASTn algorithm. This search revealed that the species most similar to the isolated strain belonged to the genus *Coccomyxa*. However, the level of similarity was only ~64% for most hits, with the exception of the sequence of a *Coccomyxa* sp. (KN-2011-T4) which matched 97% of the CCAP 216/25 sequence and will be discussed below. Indeed, the 18S rRNA sequence of the CCAP 216/25 strain contained two insertions of 545 and 436 nucleotides at position 1175 and 2327, respectively, and shown in italics in Figure S1in the Supporting Information, which were not found in other *Coccomyxa* species. The comparison of these two insertions with the nucleotide collection partially matched a number of sequences in fungi and a few eukaryotes, but only on, at most, half of the insertion sequence. If included in the rRNA, one insert would be located between the two stems 33 and 34 in the rRNA

TABLE 2. Comparison of the sequences corresponding to the genes of the RNA of the small ribosomal subunit (18S rRNA) and of the whole ITS1-5.8S-ITS2 region from the microalga CCAP 216/25 (1–2,385 nucleotides) and from other *Coccomyxa* and green microalga species listed in databases carried out by multiple alignment of the sequences using the software Clustal Omega (1.2.1) and expressed as percentage of identity.

Strain	Accession no.	Length (bp)	SSU rRNA	ITS1-rRNA-ITS2
Coccomyxa polymorpha CAUP H5101	HG972979	2,446	99.60	84.22
Coccomyxa sp. KN-2011-T4	HE586516	3,440	98.91	98.38
Coccomyxa ACCV1	HE617183	2,925	98.85	82.42
Coccomyxa simplex	HG972980	2,444	98.33	76.41
Coccomyxa rayssiae isolate UTEX273	HQ317304	1,793	98.33	nd
Coccomyxa chodatii SAG 216/2	FN597598	3,013	98.04	76.43
Coccomyxa peltigerae SAG 216/5	FN597599	3,103	97.70	75.93
Coccomyxa glaronensis CCALA 306	AM167525	1,797	97.63	nd
Coccomyxa subellipsoida SAG 216/7	HG972976	3,208	97.47	75.33
Chlamydomonas reinardtii CCAP 11/32CW15	FR865576	2,500	92.22	63.21

The two specific inserts in the SSU RNA sequence of the CCAP 216/25 strain and of *Coccomyxa* sp. KN-2011-T4 were removed. The accession numbers of the sequences used for the comparison together with their nucleotide number are indicated. The sequences determined in this work were used for *Coccomyxa chodatii* strain SAG 216/2 and *Coccomyxa peligerae* strain SAG 216/5. For the comparison of the ITS region, some data are missing (nd = not determined) because the published sequences are too short to cover the whole region.

secondary structure, the other on the stem 50 when taking the model of the SSU rRNA secondary structure of *Micractinium pusillum* strain CCAP 248/5 (accession number FM205836). The extension of the 3′ end of the 18S rRNA gene sequence into a putative group I intron is common in the class *Trebouxiophyceae* (Friedl et al. 2000, Neustupa et al. 2011). Introns mostly come from close association with lichens. Some trebouxiophytes which live in a lichen have introns which come from an ascomycete (T. Pröschold, personal communication). However, the algal strain described in this work is an aquatic free-living species. Among the genus *Coccomyxa*, a few species possess introns. One of them, *Coccomyxa pringsheimii* strain SAG 216/7 (currently regarded as *Coccomyxa subellipsoida*; Darienko et al. 2015; accession number HG972976) is photobiont of lichen *Botrydina vulgaris* (Karsten et al. 2005) and displays two insertions, one of 377 bp upstream of the first intron of *C. actinabiotis*, the other in a similar position as the second intron of *C. actinabiotis*. Another one, *Coccomyxa* sp. KN-2011-T4 (HE586516) is a terrestrial epiphytic species and possesses two insertions at the same positions as *C. actinabiotis*.

The comparison of the sequences corresponding to the SSU rRNA of the isolated strain (after removal of the two specific inserts) and of other *Coccomyxa* species listed in databases was carried out by multiple alignment of the sequences using the Clustal Omega software. The comparison matrix is shown in Table S1 in the Supporting Information. The most similar species are *C. polymorpha* Darienko et Pröschold (2015) strain CAUP H5101 (formerly *Choricystis chodatii*; Darienko et al. 2015; HG972979), *Coccomyxa* sp. KN-2011-T4 (HE586516) and ACCV1 (HE617183), *C. simplex* (HG972980), *C. rayssiae* strain UTEX273 (HQ317304), *C. chodatii* strain SAG 216/2 (FN597598), and *C. peltigerae* strain SAG 216/5 (FN597599), with sequence identity exceeding 97.7% (Table 2). These strong identity scores obtained for the comparison of the CCAP 216/25 strain to the genus *Coccomyxa* were similar to those obtained after comparing the sequences of *Coccomyxae* between each other (Table S1) and were away from the score obtained for the sequence comparison with a unicellular microalga belonging to another genus (*C. reinhardtii* strain CCAP 11/32CW15; FR865576; Table S1). This clearly indicates that this microalga is a member of the genus *Coccomyxa*.

Furthermore, the sequence of the ITS region (ITS1-5.8S rRNA-ITS2) of the strain CCAP 216/25 was compared to that of other *Coccomyxae* (Table 2). This comparison yielded a sequence identity of 75%–84% of the sequence of the isolated strain compared to other referenced *Coccomyxae* strains, except for the *Coccomyxa* sp. KN-2011-T4 whose identity reached 98%. These values were of the same order as the scores obtained for the comparison of the ITS region between other referenced *Coccomyxae* (75%–95%; Table S2 in the Supporting Information) and very far from that obtained for comparison with other genera (of the order of 30%–60%). It should be noted that the *Coccomyxa* sp. KN-2011-T4 discovered by K. Nemjova in 2011 since the first report of *C. actinabiotis* as CCAP 216/25 in 2010 (Rivasseau et al. 2010) is very similar to *C. actinabiotis* CCAP 216/25 with respect to its rRNA gene sequence. This taxon (KN-2011-T4), analyzed at the molecular level, differed by about seven bases in the ITS2 sequence and ~50 bases in the SSU rRNA sequence, and is probably a distinct species. Moreover, its biotope is very different. But in the absence of morphological characteristics, a definitive conclusion cannot be drawn. Altogether, these results indicate that the isolated microalga belongs to the genus *Coccomyxa*. However, *C. actinabiotis* CCAP 216/25 exhibits two unique insertions of 545 and 436 bp

in the DNA sequence of its SSU rRNA and the DNA sequences of the 18S rRNA and the ITS are distinct from those of other *Coccomyxae* species listed in databases. The phylogenetic cladogram shown in Fig. 5 was inferred from the alignments of the total 18S rRNA sequence, and identified the new sequence as that of an original species belonging to the genus *Coccomyxa* (Schmidle 1901) and more particularly to the *C. polymorpha* lineage (Darienko et al. 2015), in the class *Trebouxiophyceae* (Friedl 1995). The genomic and morphological characteristics of the microalga and its remarkable properties of radioresistance described next allows to make it a new species of *Coccomyxa*, which we name *C. actinabiotis* from ancient Greek meaning "lives in rays."

The genus *Coccomyxa* comprises to date ~50 species which are distributed worldwide and display very different habitats and lifestyles (Darienko et al. 2015, Guiry and Guiry 2015, SAG Database http://sagdb.uni-goettingen.de/, CCAP database http://www.ccap.ac.uk/). They include planktonic free-living freshwater species (Chodat 1913, Verma et al. 2009) as well as terrestrial epiphytic species (Lamenti et al. 2000). They also include symbiotic species with lichens (Lohtander et al. 2003), as for instance *Coccomyxa subellipsoida* which is a phycobiont of lichen *B. vulgaris* (Zoller and Lutzoni 2003), symbiotic species with trees such as the *Coccomyxa* sp. shown to be associated with the tree *Ginkgo biloba* (Trémouillaux-Guiller et al. 2002, Trémouillaux-Guiller and Huss 2007) or with protozoans (Hoshina and Imamura 2008). Some *Coccomyxa* are parasitic species as, for example, *Coccomyxa parasitica* that infects the soft tissues of blue mussels (Mortensen et al. 2005, Rodríguez et al. 2008). Most species are free-living in freshwater or phycobionts of lichens. While *C. actinabiotis* is a freshwater free-living microalga, the phylogenetically closest species have habitats covering the diversity of the genus *Coccomyxa*. *C. polymorpha* strain CAUP H5101, formerly named *Choricystis chodatii*, is a terrestrial species, as well as *Coccomyxa* sp. KN-2011-T4, which possesses introns at the same position as *C. actinabiotis*, and was found as a terrestrial species hosted by bamboos at Singapore. *C. rayssiae*, *C. simplex*, and *C. chodatii* are free-living freshwater species isolated respectively from Romania (Chodat & Jaag 1933), Switzerland, and Lake Geneva in Switzerland (Chodat 1913). But *C. peltigerae* as well as *C. glaronensis* are phycobionts of lichen isolated respectively from Switzerland and from the Czech Republic (Jaag 1933, Thüs et al. 2011).

As the microalga described here lives in an oligotrophic environment where it is continuously submitted to ionizing radiations, we assessed the impact of these stresses on the algal growth. As mentioned earlier, the pool water contained nitrates at a concentration at least 25 times lower than that of a culture medium and potentially other essential nutrients such as phosphate and sulfate at concentration below the $0.2\ mg \cdot L^{-1}$ detection limit. Radioactive trace metallic ions such as cobalt, manganese and zinc were also present at respective average concentration of 2×10^{-13}, 1×10^{-14}, and $7 \times 10^{-15}\ mol \cdot L^{-1}$ for ^{60}Co, ^{54}Mn, and ^{65}Zn. The radioactive isotopes were accompanied by non-radioactive isotopes of these metallic elements whose concentration was below the detection limit of inductively coupled plasma-mass spectrometry. Owing to the large volume of the pool ($360\ m^3$) and to the continuous filtering resulting in the agitation of the water, essential nutrients might thus be present in sufficient amount to sustain a slow microalgal growth despite their minute concentration. The ability of other *Coccomyxa* species to grow in nutrient deficient media has already been reported. *Pseudococcomyxa adhaerens* could thus grow in distilled water with a 5–15 $\mu S \cdot cm^{-1}$ conductivity and which was regularly changed (Taylor 1965). *C. actinabiotis* was able to live in a 10 times more diluted medium. We further tested *C. actinabiotis* ability to grow in ultrapure deionized water whose conductivity was $0.05\ \mu S \cdot cm^{-1}$ in parallel to nutritive BBM medium. The growth curve shown in Figure 6a indicated that they grew from their internal reserves for more than 1 month in ultrapure water, this time laps corresponding to 3–4 divisions. The growth then slowed down, whereas it went on in nutritive medium.

The resistance of the microalga to UV and gamma radiations was evaluated. UVs are non-ionizing but high energy radiations. They are increasingly used as an alternative to the chlorination in water treatment plants, due to their efficient sterilization power against viruses, bacteria, parasites, algae spores and algae in suspension. For example, a flux of $22{,}000\ \mu W \cdot s^{-1} \cdot cm^{-2}$ is required to destroy the unicellular microalgae *Chlorella* (Collentro 1986). UV radiation is absorbed by the DNA nucleotides, whose absorption spectrum ranges from 210 to 310 nm with a maximum at around 260 nm. The most germicidal are UVc (200–280 nm). The energy of UVc brings about chemical modification in DNA and changes in its structure. It induces especially the formation of pyrimidine dimers distorting DNA, affecting replication and causing cell death or the appearance of mutants (Sinha and Häder 2002). Exposure to UV radiation also generates indirect damage to DNA and to other cellular molecules via an oxidative stress which will be discussed below (Caldwell et al. 1998). The acute UV irradiation of *C. actinabiotis* causes a decrease in the population in the day following the irradiation due to immediate cell death (Fig. 6b). The surviving *C. actinabiotis* cells continue to grow with a growth curve parallel to that of the control algae (Fig. 6b), suggesting that this microalga possesses very efficient DNA protection and repair mechanisms.

Coccomyxa actinabiotis were further exposed to chronic and acute gamma irradiation. Gamma rays

are ionizing radiations. They collide with cell molecules such as water or DNA, creating short-lived radicals which cause further chemical changes by bonding with and/or stripping particles from nearby molecules. When collisions damage DNA, RNA or proteins without efficient repair, cell division is hampered. That is why gamma irradiation is used to preserve food and prevent the spread of invasive pests. A maximum 10 kGy dose is authorized in France for food preservation, which effectively destroys many microorganisms as the vast majority of organisms on Earth are killed by doses less than 500 Gy (Slade and Radman 2011). The *C. actinabiotis* cultures were exposed to low dose rates for long periods and to high dose rates for short periods. In the pool, the algae lived and grew for several years in a weakly radioactive environment, where they were submitted to a gamma-radiation dose rate lower than or equal to 0.15 $mGy \cdot h^{-1}$. Actually, owing to the radioactive metals they accumulated (Rivasseau et al. 2013), cells forming the colony in the pool were continuously exposed to this irradiation rate. *Coccomyxa actinabiotis* cells (CCAP 216/25) were submitted to gamma irradiation dose rates of 0.8, 20 and 60 $mGy \cdot h^{-1}$ for 30 d, yielding integrated doses of 0.57, 14, and 43 Gy. A 0.8 $mGy \cdot h^{-1}$ dose rate did not induce any visible effect as the growth of algae exposed to 0.8 $mGy \cdot h^{-1}$ was identical to that of the control (Fig. 6c). Integrated doses below 100 mSv (low doses, corresponding to 100 mGy for a gamma radiation) actually do not significantly increase the appearance of cancers in animals. The boundary of low dose rates below which no biological effects are observed can be set to 1 $mSv \cdot h^{-1}$ (corresponding to 1 $mGy \cdot h^{-1}$ for a gamma radiation). Statistically observable effects appear above 100 mSv and deterministic effects above 1 Gy (high doses). *Coccomyx actinabiotis* algae suffer little from a 20 or 60 $mGy \cdot h^{-1}$ flux applied for 1 month. They remained deep green and grew during that period. Nevertheless, the growth slightly slowed down. When exposed to the 20 $mGy \cdot h^{-1}$ flux, the final algal density after 1 month was 30% lower than that of control non-irradiated algae (Fig. 6c).

To test the microalgae resistance to a very high ionizing radiation flux, *C. actinabiotis* were introduced inside a spent fuel element providing an intense gamma-radiation flux of 1,000 or 4,000 $Gy \cdot h^{-1}$ for various durations ranging from some minutes to several hours. Following the acute irradiation, cells were allowed to recover in non-radiative conditions. Ionizing radiation doses of up to 20,000 Gy were integrated. They survived a gamma flux of 1,000 $Gy \cdot h^{-1}$ applied for 20 h; if they were then transferred into non-radioactive conditions, they recovered their growth capacity in less than 2 weeks. When submitted to a gamma flux of 4,000 $Gy \cdot h^{-1}$ for an integrated dose of 300 Gy, their growth during recovery was similar to that of the non-irradiated control (Fig. 6d). After 2 weeks, all the irradiated samples presented the same cell density, whatever the initial irradiation dose in the range 300–10,000 Gy (Fig. 6d). Earlier work revealed that *C. actinabiotis* tolerates gamma radiation with a 50% lethal dose of 10,000 Gy (Rivasseau et al. 2013), while the 50% lethal dose of ionizing radiation for algae are generally between 30 and 1,200 Gy (IAEA 1976). *Coccomyxa chodatii* was shown to withstands lower radiation levels than *C. actinabiotis*, indicating that *C. actinabiotis* most probably utilizes specific resistance and repair mechanisms (Rivasseau et al. 2013).

Most organisms are killed by such gamma-rays doses. But some are extremely resistant to radioactivity. The famous bacterium *D. radiodurans* was isolated from cans of meat subjected to sterilizing doses of gamma radiation in the 10 kGy range (Anderson et al. 1956). *Coccomyxa actinabiotis* exhibits an extreme radiation resistance similar to that of *D. radiodurans*, although its eukaryotic cell is much more complex. How can a living organism resist such radiation doses that generate several thousands of double strand breaks in its genome and even damage internal structure of glass and plastic? Ionizing radiations actually induce roughly a fixed rate of ~0.002–0.005 double strand breaks per 10^6 nucleotides per gray in many radioresistant or radiosensitive species (Olive 1998, Daly et al. 2004, Gladyshev and Meselson 2008). For more than 70 years, *D. radiodurans* has been subject to numerous studies to discover the reasons of its extreme radioresistance. Resistance and repair mechanisms including a highly condensed nucleoid preventing the dispersal of DNA fragments, multiple DNA copies, the implementation of very efficient classical DNA repair mechanisms and of specific DNA repair mechanism and a cellular response to radiation such as gene regulation have been brought to the fore (Battista et al. 1999, Cox and Battista 2005, Lovett 2006, Zahradka et al. 2006, Confalonieri and Sommer 2011, Slade and Radman 2011) but it appeared that the question by itself was not fully solved.

These studies relied on the hypothesis that DNA macromolecules were the most critical targets of radiations. DNA has actually to be reconstructed after irradiation to ensure cell survival. But over the past 10 years, a new paradigm has emerged wherein enzymes involved in DNA repair and replication are equally or even more critical targets (Daly 2009, 2012, Confalonieri and Sommer 2011, Slade and Radman 2011). The accurate and efficient functioning of these proteins is crucial to maintain genome integrity and cellular division. The prominent effect of radiation in a cell is an oxidative stress through the generation of reactive oxygen species such as hydroxyl radical, superoxide radical and hydrogen peroxide originating mainly, directly or indirectly, from water radiolysis (Daly

2009, 2012, Slade and Radman 2011). Cell molecules and particularly proteins hence have to be protected against oxidative stress. Cells have developed enzymatic or non-enzymatic ways of scavenging free radicals with antioxidant metabolites such as carotenoids and complexes of divalent manganese (Confalonieri and Sommer 2011). It has been experimentally evidenced in bacteria and yeast that high concentrations of divalent magnesium efficiently protected proteins against the oxidative stress brought about by radiation, rendering the organisms more resistant. *Deinococcus radiodurans* benefits from an unusually high level of manganese ensuring especially the protection of proteins involved in DNA repair and replication and which explains to a large extend its radioresistance (Daly et al. 2004, Daly 2009, 2012, Slade and Radman 2011).

Coccomyxa actinabiotis most probably makes use of very efficient protection and repair mechanisms to maintain the integrity of its cellular functioning and of its genomic material. The mechanisms implemented are currently unknown but we can argue that the microalga benefits from an efficient protection against oxidative stress particularly as a high ratio of manganese/iron, shown to be involved in such mechanisms (Daly 2012), has been determined in this microalgal species. Manganese actually scavenges or limits oxidative stress by forming antioxidant complexes with small metabolites as mentioned above and by substituting iron in the active center of enzymes, thus limiting the generation of reactive oxygen species depending of iron under oxidative conditions through Fenton reaction (Cox and Battista 2005, Latour 2015). Genome sequencing which is currently under way will probably provide some insight into the mechanisms implemented to ensure the exceptional radioresistance of this eukaryote.

Extreme environments constitute a unique opportunity for new knowledge in the occurrence and development of life on earth and other planets as well as for novel biotechnologies. *Coccomyxa actinabiotis* constitutes an outstanding model for fundamental and applicative research. Its radioresistance and its radionuclide accumulation properties make it an organism of choice for new decontamination processes and bioremediation technologies of radioactively contaminated environments. Its radioresistance and photosynthetic capacities could also be relevant for space exploration and for the seeding of life on planets such as Mars where *C. actinabiotis* could help in creating an aerobic environment and organic matter for soil. Moreover, further fundamental research on the origin of the outstanding properties of *C. actinabiotis* will undoubtly bring new insights into the mechanisms involved in protection against ionizing radiation and damage repair in eukaryotic cells, which could be useful in medicine, radiotherapy, and protection against nuclear radiation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Percent identity matrix of the comparison of the sequences corresponding to the SSU RNA gene of the microalga CCAP 216/25 (1–2,385 nucleotides), of other *Coccomyxa* species listed in databases, and of *Chlamydomonas reinhardtii*, carried out by multiple alignment of the sequences using the software Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Accession numbers are identical to that used in Table 2. The two specific inserts in the SSU RNA sequence of the CCAP 216/25 strain and of *Coccomyxa* sp. KN-2011-T4 were removed for the comparison.

Table S2. Percent identity matrix of the comparison of the sequences corresponding to the ITS1-5.8S rRNA-ITS2 gene of the microalga CCAP 216/25, of other *Coccomyxa* species listed in databases, and of *Chlamydomonas reinhardtii*, carried out by multiple alignment of the sequences using the software Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Accession numbers are identical to that used in Table 2.

Figure S1. Sequence of the *Coccomyxa actinabiotis* DNA region spanning the ribosomal RNA. Sequences corresponding to 18S rRNA, 5.8S rRNA, and 28S rRNA (beginning) are presented in black. Two introns, in pink italics characters, are inserted in the 18S rRNA region. Internal transcribed spacers 1 and 2 (ITS1 and ITS2), in green, separate the genes corresponding to the different rRNA subunits.

Appendix S1. Full cladogram in Nexus and in Newick format.