

Research article

## Phylogenetic relationships and taxonomic position of *Chlorella*-like isolates from low pH environments (pH < 3.0)

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### Abstract

**Background:** Little is known about phytoplankton communities inhabiting low pH environments such as volcanic and geothermal sites or acidic waters. Only specialised organisms are able to tolerate such extreme conditions. There is, thus, low species diversity. We have characterised the previously isolated acid tolerant *Chlorella*-like microalgae *Viridiella fridericana* and *Chlorella protothecoides* var. *acidicola* by microscopical and biomolecular methods in order to assess their phylogenetic relationships.

**Results:** Both isolates belong to the trebouxiophycean lineage of chlorophytes. 18S and ITS1 sequence data clearly confirm that *Viridiella fridericana* constitutes a new genus apart from the morphologically similar and likewise acid tolerant microalga *Chlorella saccharophila*. *Chlorella protothecoides* var. *acidicola* on the other hand is not a variety of *Chlorella protothecoides* but falls within a heterogeneous cluster consisting of *Nannochloris*, "*Chlorella*" spec. Yanaqocha, and *Koliella*, and is most closely related to algae which were also isolated from extreme environments.

**Conclusions:** The distribution of acid tolerant strains in the 18S rRNA tree shows that acquisition of acid tolerance was unlikely a monophyletic event in green microalgae. We propose that different strains have independently adapted to extreme environments. Some of them have spread worldwide and were able to colonise other extreme habitats. Considering the problems of successfully isolating acid tolerant strains, acidic soils could represent an unsuspected source of biological diversity with high potential for biotechnological utilisations.

### Background

Very low pH environments can be found in volcanic and geothermal sites of Italy. They are generated by either sulphur springs emitting a continuous outflow of water at temperatures higher than 80°C, or fumaroles, which are

mostly areas of small bubblers and steam vents. The *in situ* oxidation of H<sub>2</sub>S arising from the outlets of these geothermal systems causes the production of large amounts of H<sub>2</sub>SO<sub>4</sub> and, in turn, low pH on rocks and soil surrounding the offspring. Generally, the pH values remain below

2.5 for 10–25 square meters around the outflow of H<sub>2</sub>S. Temperature, on the other hand, declines from 60°C to 25–30°C within a few meters apart from the source of volcanic activity [1].

The distribution of algal flora on these soils is strictly dependent on the above two parameters. In the close vicinity of hot springs and fumaroles, soil temperature and pH are almost constant at 40°C and 1.5, respectively, thus enabling the exclusive presence of three thermoacidophilic red microalgae, *Cyanidium caldarium*, *Galdieria sulphuraria* and *Cyanidioschyzon merolae* [2,3]. At increasing distances, pH and temperature may dramatically change, also as a consequence of the season and rainfalls. At temperatures below 30°C and pH values between 1.8 and 2.5, coccoid green algae such as *Chlorella* and related genera represent a major component of algal populations [4].

The occurrence of *Chlorella* in low pH environments seems to be restricted to acidic soils, whereas in acidic waters it has been rarely recorded [5]. Here, the most widespread species belong to different diatom genera and to several *Chlamydomonas*, *Ochromonas* and *Euglena* species [6,7]. On the basis of a combination of morphological, biochemical and eco-physiological features [8], the *Chlorella*-like strains so far isolated from the acidic microsites have been attributed to three taxa; two of these, *Viridiella fridericiana* and *C. protothecoides* var. *acidicola* seem to be endemic to acidic soils.

*V. fridericiana* is a monospecific genus erected on the basis of the absence of a pyrenoid in the chloroplast and of sporopollenin in the cell wall [9]. It does not produce secondary carotenoids, and shows a pH limit for growth below pH 1.0 [10]. On the other hand, our knowledge on *C. protothecoides* var. *acidicola* is scanty; it has been only preliminarily investigated by light microscopy (LM), whereas neither Transmission Electron Microscopy (TEM) nor Scanning Electron Microscopy (SEM) studies have been carried out. *C. protothecoides* var. *acidicola* seems to be morphologically identical to *Auxenochlorella* (= *Chlorella*) *protothecoides*; the only diacritical character for separating this variety is a pH limit for growth at 2.0 compared to 3.5–4.0 for *A. protothecoides* [11]. The third taxon is represented by "*C.*" *saccharophila*, a species which has been recently excluded together with "*C.*" *protothecoides* from the genus *Chlorella* [12], and which is well known as the lowest pH-resistant "*Chlorella*" species [13]. Although the type strain, SAG 211-9a, and other strains of "*C.*" *saccharophila* available from Culture Collections were isolated from non-acidic environments such as tree sap, they show the same morphological features and the same growth limits of pH, salinity and temperature as found in our isolates from acidic soils (unpublished results).

The main goal of this paper is to assess the phylogenetic relationships of our acid tolerant isolates with members of the genus *Chlorella sensu lato*, and to ascertain if the colonisation of acidic soils of geothermal environments is a monophyletic event. Moreover, molecular data could provide evidence to validate the establishment of *V. fridericiana* and *C. protothecoides* var. *acidicola*. To investigate these aspects, 18S rRNA sequences of authentic strains of *V. fridericiana* and *C. protothecoides* var. *acidicola* were determined. 18S sequences are routinely used to assess the phylogenetic position of coccoid green algae and an increasing body of sequences is becoming available [14–16]. The first attempt to use 18S sequences to infer phylogenetic relationships within *Chlorella* dates back to the beginning of the last decade [17], whereas a comprehensive data set of 18S rRNA sequences of *Chlorella sensu lato* has recently been presented, leading to a systematisation of the taxonomy of this genus [12].

When more than one isolate from low pH soils was available, as in the case of "*C.*" *saccharophila* and *V. fridericiana*, the Internal Transcribed Spacer 1 (ITS1) was also sequenced to reveal molecular differences within a population that have not been observed with usual morphological and biochemical methods. Data from ITS1 sequences have been a valuable guide to identify different isolates of Volvocacean species [18], and also within *Chlorella*, ITS1 comparisons became a useful tool to assign strains at the species level, although not sufficient to resolve phylogenetic relationships among species [19].

Finally, in the case of *C. protothecoides* var. *acidicola*, the need for a combined analysis of molecular and cytological parameters prompted us to investigate the morphology and ultrastructure of this strain by LM, TEM and SEM.

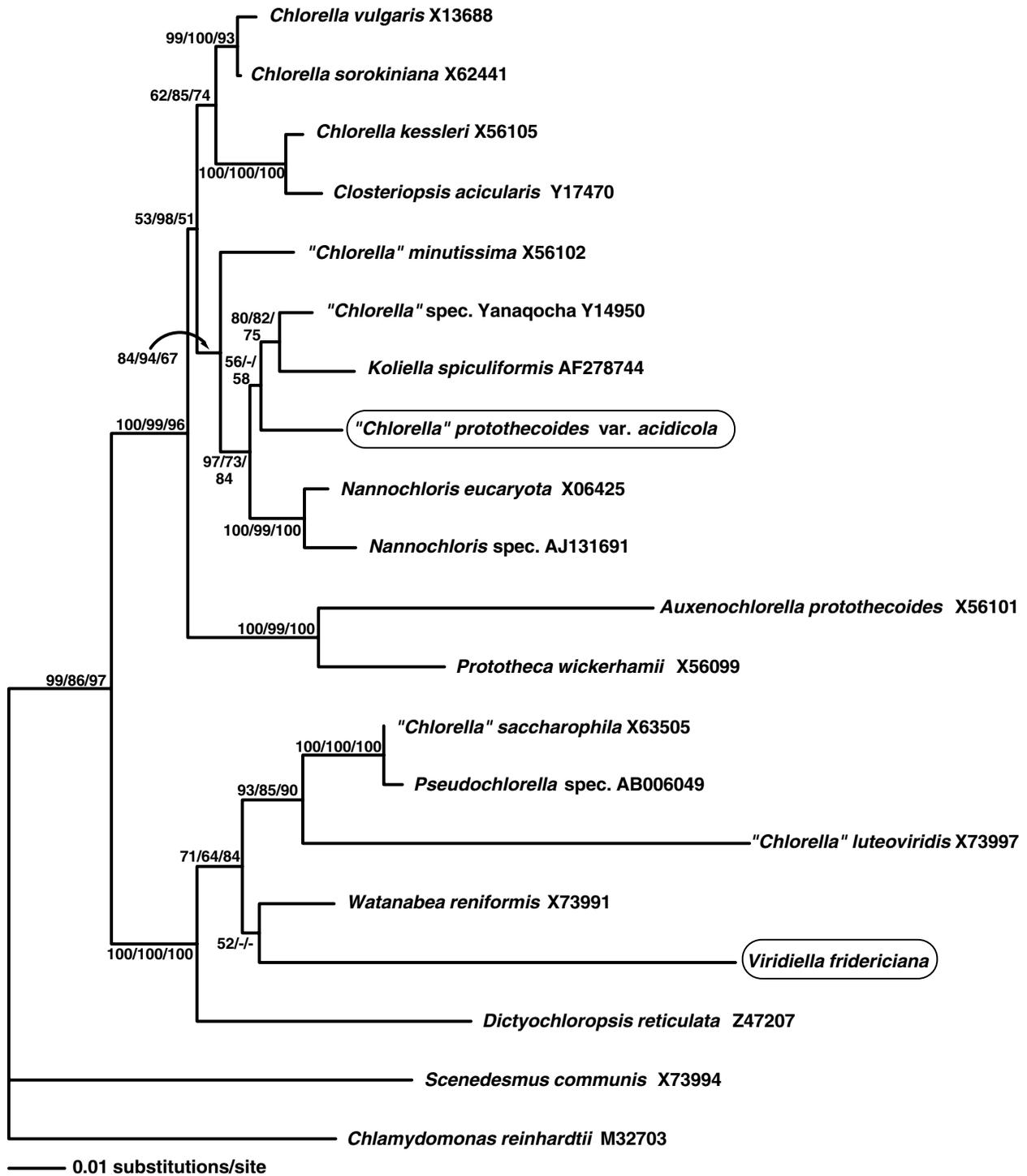
## Results

### *Viridiella fridericiana* and "*Chlorella*" *saccharophila*

#### *Molecular and phylogenetic studies*

ITS1 lengths were 289 and 261 bp for "*C.*" *saccharophila* SAG 211-9a and *V. fridericiana* 237, respectively, and the GC content ranged from 61 mol% for the former species to 59 mol% for the latter. We found no sequence divergence of ITS1 among the strains of "*C.*" *saccharophila* isolated from acidic soils and the authentic strain of the species, as well as among those belonging to *V. fridericiana*. On the other hand, the identity of ITS1 sequences (Clustal W alignment) between "*C.*" *saccharophila* and *V. fridericiana* was only 35,7%. This indicates that the two taxa are clearly separated; a similar distance is found between the ITS sequences of "*C.*" *saccharophila* and "*C.*" *protothecoides* var. *acidicola*.

The 18S rRNA tree in Fig. 1 confirms that *V. fridericiana* and "*C.*" *saccharophila* are not closely related. Although



**Figure 1**  
**Maximum likelihood tree inferred from 18S rRNA gene sequences of various trebouxiophycean algae.** The chlorophytes *Scenedesmus communis* and *Chlamydomonas reinhardtii* were used as outgroups. Bootstrap support of respectively maximum parsimony, neighbor joining, and maximum likelihood analyses is shown along nodes if > 50%. Branch lengths are proportional to the number of substitutions/site (note scale bar). Sequences determined in this work are encircled. Reference sequences were taken from GenBank and are identified by their accession number.

**Table 1: Biochemical and physiological characters of "C." minutissima, A. protothecoides and "C." protothecoides var. acidicola**

Species	Sec car.	NO <sub>3</sub> <sup>-</sup>	Thiam.	B12	pH	%NaCl	°C
"C." minutissima <sup>1</sup>	-	+	-	-	5.5	1	32
A. protothecoides <sup>1</sup>	-	-	+	-	3.5–4.0	3–4	28–34
"C." protothecoides var. acidicola	-	-	+	-	2.0	3–5	34

<sup>1</sup>Data from [12]. Sec car. = Secondary carotenoids, NO<sub>3</sub><sup>-</sup> = nitrate reduction, Thiam. = thiamine requirement, B12 = vitamin B12 requirement, pH = limit of growth at pH, %NaCl = limit of growth at %NaCl, °C = upper temperature limit of growth.

both algae belong to the same subgroup within the trebouxiophytes, a close relationship on the genus level is excluded. The long branch of *Viridiella*, and also of "C." *luteoviridis*, hints to a higher mutation rate of their ribosomal RNA compared to most other algae included in Fig. 1. Limited taxon sampling as an alternative explanation can be largely ruled out as we have included all available trebouxiophycean and several chlorophycean 18S rRNA gene sequences in a first search for related algae resulting in the same long branch leading to *V. fridericana*. For sake of clarity, only those sequences which appeared significant for demonstrating the phylogenetic position of our isolates within the trebouxiophytes, were included in Fig. 1. Moreover, the 18S rRNA gene of *Viridiella*, in addition to base substitutions in variable regions, contains several mutations at sites that are otherwise highly conserved in most green algae, which is a typical character of fast evolving genes. It is well known that long branches may generate artificial tree topologies [20]. However, considering the available data, it is obvious that *V. fridericana* represents a distinct lineage justifying the establishment of a new genus *Viridiella* by [9] also on a molecular basis. *Watanabea reniformis* (formerly designated as *Chlorella saccharophila* strain SAG 211-9b, but excluded from the genus *Chlorella*[21]) was found to be most closely related in the maximum likelihood analysis, but this relationship is not significantly supported by either bootstrap analysis.

### "Chlorella" protothecoides var. acidicola

#### Ecophysiological and biochemical characters

Table 1 summarises biochemical and ecophysiological characters of "C." *protothecoides* var. *acidicola* compared with those of "C." *minutissima* and *Auxenochlorella protothecoides*, two of the most closely related algae for which these data are available. "C." *protothecoides* var. *acidicola* shares numerous features with *A. protothecoides*, such as the inability to grow on nitrate, the upper limit of temperature for growth, and the tolerance to high concentrations of NaCl. As far as the need for thiamine is concerned, "C." *protothecoides* var. *acidicola* does not strictly require thiamine for growth, even though growth is promoted by the presence of this vitamin.

#### Light microscopy

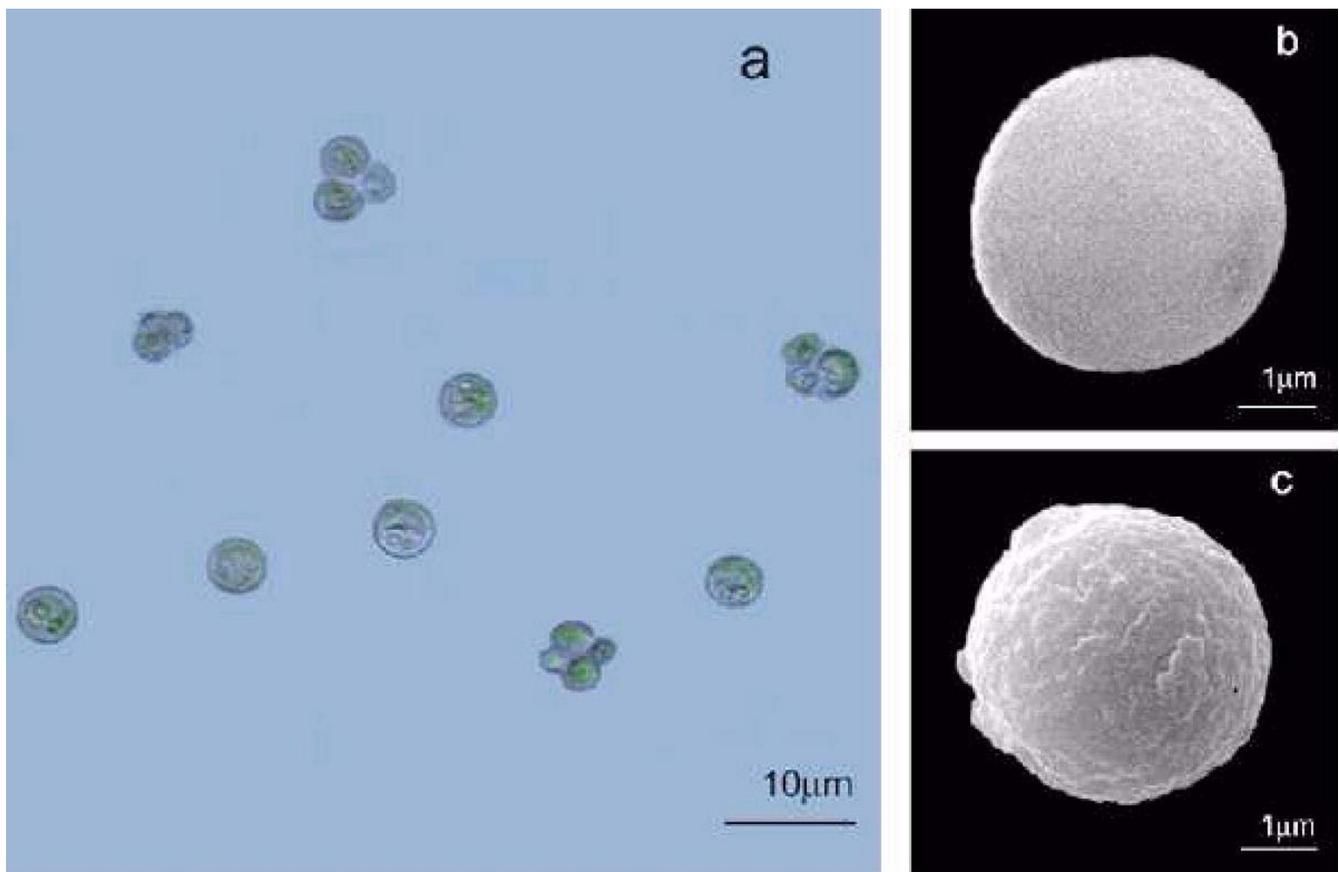
In exponentially grown cultures, "C." *protothecoides* var. *acidicola* consists of single cells, spherical to oval, 4.5–6.7 µm in diameter, with a thin cell wall. No mucilage surrounds the cells which are filled with a single parietal chloroplast, bright green, cup shaped, and without pyrenoid. Colourless oily droplets may occur in the cytoplasm. The propagation mode is by autospore. Sporangia are 8–10 µm in diameter and contain 4 (rarely 8) autospores, spherical to oval, 3–4 µm in diameter, with a single cup shaped chloroplast, which are released through rupture of the mother cell wall (Fig. 2a). Residues of sporangial cell walls are visible and keep the autospores together for a certain time (not shown).

#### Electron microscopy

SEM micrographs reveal a cell wall which is smooth or covered by an irregular network of subtle ribs (Figs. 2b,2c). Smooth and "rough" cell walls were present in samples of the same culture, and apparently, they can be observed in cells of similar size. TEM micrographs show that sporangia of "C." *protothecoides* var. *acidicola* may contain autospores of different size (Fig. 3a). The autospores have a central nucleus and a single parietal chloroplast lacking a pyrenoid, with some interthylacoidal starch grains. A mitochondrion is also visible near the concave side of the chloroplast. Moreover, TEM indicates that the cell wall is composed of an inner granulo-fibrillar polysaccharide layer and an outer trilaminar (TL) layer (Fig. 3b). As reported for *Auxenochlorella protothecoides*[22], the external surface of the TL-layer after fixation with glutaraldehyde-OsO<sub>4</sub> is slightly undulated and the polysaccharide layer is not clearly visible. After fixation with glutaraldehyde-OsO<sub>4</sub>-tannic acid, a film of electron dense material external to the TL-layer is recognisable, probably due to a precipitation of tannic acid; moreover, the inner polysaccharide layer becomes more evident.

#### Phylogenetic position

In spite of the various features shared between "C." *protothecoides* var. *acidicola* and *A. protothecoides* (see above), molecular evidence suggests that both are not closely related. The phylogenetic tree in Fig. 1 places "C." *protothecoides* var. *acidicola* inside a cluster that contains two

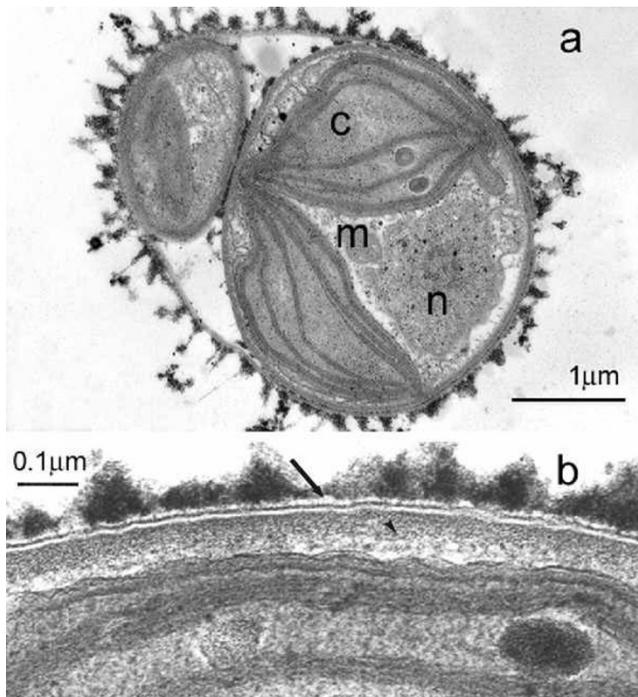


**Figure 2**  
**LM and SEM micrographs of "C." protothecoides var. acidicola strain I24.** (a) LM micrograph under culture conditions. (b) – (c) SEM micrographs showing the different morphology of the outer cell wall: smooth cell wall in (b); cell wall covered by a network of ribs in (c).

*Nannochloris* strains as well as "*Chlorella*" spec. Yanaqocha and *Koliella spiculiformis*. Our analyses show a weak tendency, although not significantly supported by bootstrap values, to affiliate "*C.*" *protothecoides var. acidicola* with the latter two algae which are also known to colonize extreme environments. "*C.*" spec. Yanaqocha is a UV-B tolerant alga isolated from the Andean lake Yanaqocha (3980 m above sea level; [23]), and *Koliella* species, although polyphyletic [24], have been isolated e.g. from the Himalayan Yala Glacier (5100–5700 m above sea level; [25]) and also from acidic waters [http://www.npsumava.cz/gabreta/hejzlar.html]. Interestingly, a microalga isolated from the German Lake Lugteich (Lusatia, Saxony) and designated as "*Chlorella*" spec. Pi98/29 turned out to be very closely related to "*C.*" *protothecoides var. acidicola* with just one difference in their 18S rRNA genes (Hepperle, pers. commun.). Lake Lugteich is a highly eutrophic and extremely acidic mining lake (pH 2.6) which was characterised with respect to its phytoplankton communities [7].

## Discussion

The complete identity of ITS1 sequences of "*Chlorella*" *saccharophila* isolates from acidic soils with that of the authentic strain confirms the widespread distribution of this species and its ability to occupy extreme environments. Although it has been suggested that "*C.*" *saccharophila* could be a component of littoral microflora [26], this species is mainly a "luftalga", occurring on bark of trees and bare rocks, and, less frequently, on soils of temperate regions [27]. However, this alga might be distributed worldwide: in 1982, a "*C.*" *saccharophila* strain (labelled CCAP 211/57), isolated from Mt. Erebus soils, Antarctica, was deposited by P. Broady in the Culture Collection of Algae and Protozoa. There are no available molecular and cytological results confirming this attribution. Molecular studies have confirmed that "*C.*" *saccharophila* does not belong to the group of "true" *Chlorella*, and that it is distant from "*C.*" *ellipsoidea*[12] once considered a variety of "*C.*" *saccharophila*[28,29]. Moreover, the erection of *Watanabea reniformis* has recently been proposed [21], which in-



**Figure 3**  
**TEM micrographs of "C." proptothecoides var. acidicola strain 124.** (a) TEM micrograph of autospores fixed with glutaraldehyde, paraformaldehyde, and tannic acid. Note the different size of the two autospores. c = chloroplast, n = nucleus, m = mitochondrion. (b) Magnification of the cell wall structure. The arrow points at the trilaminar outer layer, the arrowhead at the granulo-fibrillar inner layer.

cludes strains of "*C.* *saccharophila*" with a low sequence identity with the authentic strain CCAP 211/9a, and a reduced tolerance to low pH values and high NaCl concentrations.

The taxonomy of Chlorellaceae *sensu* Komarek & Fott [27] is largely based on chloroplast morphology, and at the generic level on the presence or absence of a pyrenoid. Recently, studies on 18S rRNA sequences of coccal green algae belonging to Selenastraceae have demonstrated the inconsistency of several "small" genera separated on the basis of a single-character difference such as presence or absence of a pyrenoid [16]. In the case of *Viridiella*, which is morphologically separated from "*C.* *saccharophila*" only by the absence of a pyrenoid, the establishment of the genus seems to be well supported by molecular analyses (Fig. 1).

On the other hand, the comparative approach attempted to clarify the taxonomic position and the phylogenetic relationships of "*C.* *proptothecoides* var. *acidicola*" did not lead to unequivocal conclusions. From a morphological point

of view, this organism showed a number of features similar to *Auxenochlorella proptothecoides* ranging from shape and dimension of cells, lack of pyrenoid and absence of growth on nitrate. TEM observations, along with the confirmation of the absence of a pyrenoid, indicated the occurrence in the cell wall of a trilaminar layer, another characteristic shared with *A. proptothecoides*. In contrast, the occurrence of autospores of different dimensions in the same sporangium is a feature observed in other "*Chlorella*" species such as "*C.* *luteoviridis*" [30] and, most important, the genus *Auxenochlorella* is defined primarily on the strict need of thiamine for growth [22], which was not observed in our strain. Analyses of 18S rRNA gene sequences rule out a close relationship of "*C.* *proptothecoides* var. *acidicola*" with *A. proptothecoides* and indicate that our isolate is related, although weakly, to other algae apparently not related from a morphological point of view (Fig. 1). "*C.* *minutissima*" and *Nannochloris* are characterized by their small size of about 2 μm [12], whereas *Koliella* is represented either by two-celled filaments or by spindle shaped unicells, very similar to *Raphidonema* [24]. The availability of increasing information on phylogenetic relationships among coccal green algae is evidencing the difficulties arising when a traditional system of classification is compared with molecular data. In unicellular organisms lacking a sexual life cycle, species and genera boundaries often remain uncertain, even after comprehensive data sets of morphological, biochemical, ecophysiological and molecular features have been collected, probably because limitations and the way how to use this multimethod approach are not clear. At the present time, with no consensual species concept in organisms in which sexual phenomena are not existent, more and more conflicting results are expected to be found.

It is evident from the phylogenetic tree in Fig. 1 that acid tolerant strains such as *V. fridericiana*, "*C.* *saccharophila*", and "*C.* *proptothecoides* var. *acidicola*" are distributed among strains that are less tolerant or even sensitive to conditions of high acidity or salinity such as "*C.* *minutissima*" (Table 1). This indicates that acquisition of acid tolerance *per se* in this group of algae is unlikely to be a monophyletic event. Rather, different strains have apparently adapted to the specific environmental conditions they were exposed to. Once the resistance to such conditions was genetically fixed, they might have been able to spread and colonise other extreme environments eventually giving rise to monophyletic "miniclusters" of extremotolerant taxa as represented by "*C.* *proptothecoides* var. *acidicola*", "*C.* spec. Yanaqocha", and *K. spiculiformis*. An example for such a spreading is demonstrated by the occurrence of "*C.* *proptothecoides* var. *acidicola*" in volcanic sites in Italy and of the genetically closely related "*Chlorella*" spec. Pi98/29 isolated from an acidic mining lake in Germany (see Results). It has to be kept in mind, however, that the loss of a charac-

teristic such as acid tolerance is at least as likely to occur as its acquisition thus obscuring monophyletic events in gene trees.

The number of algal species inhabiting low pH environments is generally considered to be very low [31]. Our results suggest that a comprehensive list of coccoid green algae occurring in low pH habitats is far from complete. Collecting algae from acidic soils is not simple, due to the variations of pH, light intensity, and ion concentrations which can remarkably change within few centimetres, allowing the establishment of different algal populations. Moreover, the identification of coccoid green microalgae through sampling and fixation can be misleading, because morphological characters are often not sufficient for a correct taxonomic determination. On the other hand, isolation and establishment of pure cultures is frequently unsuccessful. However, acidic soils could represent an unsuspected source of biological diversity, and the isolation and characterisation of acid and salt tolerant strains could open a promising avenue of research on their potential biotechnological utilisations.

## Conclusions

The present study demonstrates that the taxonomic treatment proposed for *Viridiella fridericiana* seems to be well supported by molecular data. On the other hand, our results indicate that "*C.*" *protothecoides* var. *acidicola* can not be incorporated into the genus *Chlorella* sensu stricto and can not be regarded as a variety of *Auxenochlorella protothecoides* as previously assumed from morphological comparisons. We suggest that this taxon should be treated as a distinct species to be included in a new genus. However, this treatment also needs reconsideration of the taxonomic status of other closely related microalgae, namely "*C.*" spec. *Yanaqocha* and *Koliella spiculiformis*. Moreover, our data show that acquisition of acid tolerance was unlikely a monophyletic event in green microalgae. Several strains of different genetical equipment have apparently independently adapted to acidic environments making them interesting candidates for various biotechnological applications. Acidic soils of volcanic and geothermal sites from which our strains have been isolated may provide an unsuspected source of biological diversity for such extremely acid tolerant organisms.

## Methods

### Strains and culture conditions

The algal strains used in this study were: "*Chlorella*" *saccharophila* SAG 211-9a, 042 (Bagni di Repole, Calabria at pH 2.0), 045 (Mondragone, Campania, pH 2.5), 156 (Montefiore Conca, Emilia Romagna, pH 2.0); *Viridiella fridericiana* 035 (Bagni San Filippo, Tuscany, pH 2.5), 039 (Suio Terme, Lazio, pH 2.5), 236 (Frattocchie, Lazio, pH 2.5), 237 (Mefite di Ansanto, Campania, pH 2.0); and *Chlorella*

*protothecoides* var. *acidicola* 124 (Pisciarelli, Campania, pH 2.0). The strains are from the algal collection of the University of Naples [32] except strain 211-9a which is from the Sammlung für Algenkulturen in Göttingen, Germany. All algae were grown in Erlenmeyer flasks (1000 ml) containing 500 ml of modified Bold Basal Medium with 0.25 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as a nitrogen source and a pH adjusted to 3.0 by adding H<sub>2</sub>SO<sub>4</sub>. The cultures were bubbled with air and maintained in a temperature-controlled room at 25°C on a plexiglass shaking apparatus [28] under a photon irradiance of 150 μmol photons m<sup>-2</sup> s<sup>-1</sup> with continuous light provided by cool-light fluorescent lamps (Philips TLD30w/55). Cell density of the algal cultures was assessed at 550 nm with a colorimeter Bausch & Lomb Spectronic 20.

### Ecophysiological and biochemical tests on *C. protothecoides* var. *acidicola*

The synthesis of secondary carotenoids under nitrogen deficiency was carried out according to Kessler et al. [32]. All tests were carried out in 100 ml Erlenmeyer flasks containing 50 ml of modified Allen medium [33] at pH 3.0, except in the different pH tests, at the same culture conditions as specified above. For the salt tolerance tests, the algae were grown at different concentrations of NaCl (2,4,6, and 8%). For the tests dealing with pH limits for growth, H<sub>2</sub>SO<sub>4</sub> was added at different concentrations in modified Allen medium to obtain final pH values of 1.5, 2.0, 2.5, 3.0, 5.0, 6.0 and 7.0. During the experiments, the pH value was daily monitored in each flask. For growth tests on nitrate as sole nitrogen source, algal cultures, previously centrifuged (5000 rpm × 10 min) and washed twice with nitrogen-free medium, were inoculated in Allen medium containing 3 mM NaNO<sub>3</sub>. In all experiments a control with *C. protothecoides* var. *acidicola* containing only modified BBM medium at pH 3.0 was also tested. After sterilisation by autoclaving, the flasks were inoculated with several drops of enrichment cultures in exponential growth phase to have a cell density of 0.003 units (corresponding to 100.000 cells/ml). Growth of each flask was followed daily by measuring the cell density as described above. Ecophysiological tests were carried out in triplicate for each strain and were repeated three times. Specific growth rates were calculated for each individual flask by linear regression of logarithmic cell density data obtained during the experiments. The results were evaluated on the basis of the average of three tests and the relative standard error was never higher than 5%.

### Morphological observations

Algal samples of each *Chlorella* or *Viridiella* species were observed with a Leitz Aristoplan microscope equipped with Nomarski interference optics, and the number of endospores and the size of 100 cells of each strain were measured with a micrometer eyepiece. The observations

were made at different stages of the life cycle, either on cells in full exponential phase, or on cells in late stationary phase of growth.

### Scanning electron microscopy (SEM)

*C. protothecoides* var. *acidicola* cultures in late exponential growth phase were harvested by centrifugation (5000 rpm × 10 min) and fixed with 0.5% glutaraldehyde and 1% formaldehyde, dehydrated in ethanol series, washed with water and spread over supporting glass squares, dried to the critical point and shadow-cast with gold. The preparation was scanned in a Cambridge 250 Mark 3.

### Transmission electron microscopy (TEM)

The cells were fixed either with 3% glutaraldehyde + 2% formaldehyde or in a mixture of 3% glutaraldehyde + 2% formaldehyde + 0.75% tannic acid. Fixatives were dissolved in 0.05 M PIPES buffer (pH 7.4) for 2 h at 4°C. Algae were then harvested by centrifugation (5000 rpm × 5 min), rinsed with distilled water for 30 min and suspended in 2% agar. Agar blocks were washed in 0.05 M Na-cacodylate buffer (pH 7.0) and resuspended in 1% osmium tetroxide (aqueous solution) overnight at 4°C. Small blocks of agar-suspended algae were dehydrated with an ethanol series and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife, sequentially stained each 10 min with 2% uranyl acetate and 1.33% Pb citrate, and observed with a Philips CM12 electron microscope at 50 kV.

### Biomolecular studies

Cells of each *Chlorella* or *Viridiella* strain were harvested by centrifugation (6000 rpm × 5 min) and total DNA was extracted from liquid cultures following the procedure described in Doyle & Doyle [34]. 18S rRNA genes and ITS1 regions were amplified by PCR. For 18S, PCR conditions and sequencing primers were the same as previously described [12]; the ITS1 region was amplified using primers annealing with the 3' region of the 18S rRNA (5'-GGA-GAAGTCGTAACAAGGTTTCCG-3') and with the 5' region of the 5.8S rRNA (5'-ATCCTGCAATTCACACCAAG-TATCG-3'), respectively. PCR products were directly sequenced in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Cetus, Foster City, CA, U.S.A.). The 18S rRNA gene sequences of *Chlorella protothecoides* var. *acidicola* strain 124 and *Viridiella fridericiana* strain 237 were deposited in the EMBL database under the accession numbers AJ439399 and AJ439401, and the ITS1 sequences of "*Chlorella*" *saccharophila* SAG 211-9a and *Viridiella fridericiana* 237 under the accession numbers AJ439400 and AJ439402, respectively.

### Phylogenetic analyses

For the analysis of ITS1 regions, sequences were reduced to only ITS1 by comparing them with the 3' terminus of

18S rRNA and of 5.8S rRNA of *Chlorella* species available from the literature [19]. ITS1 sequences were aligned using Clustal W ver. 1.6 [35]. 18S rRNA sequences were manually aligned on a MicroVAX computer with the sequence editor program distributed by G. Olsen [36]. External primer sequences and highly variable regions that could not be aligned unambiguously were excluded from the analyses resulting in a total of 1726 positions. Phylogenetic trees were inferred from the aligned sequence data by the neighbor-joining (NJ), the maximum parsimony (MP), and the maximum likelihood (ML) method. For all methods, heuristic bootstrap analyses [37] with 1000 (NJ, MP) or 100 replicates (ML) were conducted with the PAUP program package 4.0b8a [38] on a Power Macintosh G3 computer. For the NJ analysis, the HKY85 correction was used to convert pairwise sequence similarities into evolutionary distances, starting trees were obtained via neighbor-joining, and the TBR branch-swapping algorithm was selected. In the MP analysis, starting trees were obtained via random stepwise addition of taxa repeated 10 times, gaps were treated as "fifth base", and TBR was selected. In the ML bootstrap analysis, empirical base frequencies were used, the transition/transversion (ti/tv) ratio was set to 2, a gamma distribution of 0.5 was assumed for variable sites, and addition of taxa was by neighbor joining. In contrast, the tree topology shown in Fig. 1 was obtained by a ML analysis with ti/tv, base frequencies, proportion of invariable sites and gamma shape parameter estimated via ML, and with addition of taxa by the "as-is" mode.

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