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ORIGINAL ARTICLE

In situ proteo-metabolomics reveals metabolite secretion by the acid mine drainage bio-indicator, *Euglena mutabilis*

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Euglena mutabilis **is a photosynthetic protist found in acidic aquatic environments such as peat bogs, volcanic lakes and acid mine drainages (AMDs). Through its photosynthetic metabolism, this protist is supposed to have an important role in primary production in such oligotrophic ecosystems. Nevertheless, the exact contribution of** ***E. mutabilis*** **in organic matter synthesis remains unclear and no evidence of metabolite secretion by this protist has been established so far. Here we combined** ***in situ*** **proteo-metabolomic approaches to determine the nature of the metabolites accumulated by this protist or potentially secreted into an AMD. Our results revealed that the secreted metabolites are represented by a large number of amino acids, polyamine compounds, urea and some sugars but no fatty acids, suggesting a selective organic matter contribution in this ecosystem. Such a production may have a crucial impact on the bacterial community present on the study site, as it has been suggested previously that prokaryotes transport and recycle** ***in situ*** **most of the metabolites secreted by** ***E. mutabilis*****. Consequently, this protist may have an indirect but important role in AMD ecosystems but also in other ecological niches often described as nitrogen-limited.**

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Introduction

Euglena mutabilis is a photosynthetic protist widespread in acidic environments all around the world. It has been described as a part of the eukaryotic microbial community in peat bogs (Pentecost, 1982), volcanic lakes (Sittenfeld *et al.*, 2002) and acid mine drainages (AMDs) (Casiot *et al.*, 2004a), and is considered as a bio-indicator of these latter ecosystems (Brake *et al.*, 2001a, 2001b). AMDs exposed environments are usually considered very toxic to biota as they are characterized, besides low pH values, by very high concentrations of metallic compounds (Leblanc *et al.*, 1996). Such drainages are observed in ancient mining exploitations where oxidation of the extruded rocks leads to the production of sulfuric acid and mobilization of elements such as iron, copper, aluminum and arsenic into the percolating waters (Leblanc *et al.*, 1996; Baker *et al.*, 2004; Canovas *et al.*, 2008). This process leads to a persistent contamination of downstream aquatic environments and is a major concern for environmental and public health in many countries.

The extreme physico-chemical characteristics of AMDs make these environments hostile for most of the living cells (Leblanc *et al.*, 1996; Baker *et al.*, 2009).

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Nevertheless, it is now well established that the microbial communities they harbor are well adapted and can have a decisive impact on the evolution of such ecosystems (Tichy *et al.*, 1998; Das *et al.*, 2009). For instance, the bacterial community of the Carnoulès AMD (Gard, France) has been extensively studied and appears to have an active role in bio-attenuation processes. Bacteria related to *Acidithiobacillus ferrooxidans* and *Thiomonas* spp. have been shown to oxidize Fe(II) and As(III), respectively, leading to co-precipitation of less bio-available As–Fe complexes (Bruneel *et al.*, 2003; Duquesne *et al.*, 2003; Bertin *et al.*, 2011). Besides these two bacteria, the Carnoulès AMD prokaryotic community has also been shown to be dominated by five other bacteria, of which two are related to a novel phylum (Bertin *et al.*, 2011). Interestingly, several of these dominant bacteria, including those having a role in bio-attenuation processes, express *in situ* biological functions involved in metabolite transport and recycling (Bertin *et al.*, 2011). Such an observation suggests that the organic matter present at the study site may be crucial for the activities of the prokaryotic community as a whole.

E. mutabilis has been identified previously in the Carnoulès AMD where it forms easily observable green mats at the sediment/water interface (Casiot *et al.*, 2004a). Owing to its photosynthetic metabolism, this protist is supposed to have an important role in primary production in such an oligotrophic environment, which could influence the composition and the metabolism of the whole microbial community (Johnson and Hallberg, 2003; Das *et al.*, 2009). Nevertheless, no evidence of such a metabolic contribution has been established so far. To address this question, the synthesis of organic compounds by *E. mutabilis* was investigated by identifying proteins and metabolites accumulated by the cells *in situ* and compared to the metabolites identified in the water at the sampling site. In addition, both the synthesis and secretion of metabolites were investigated under laboratory conditions after isolation of this protist. Metabolites accumulated within cells, that is, endo-metabolome, and those liberated into the extracellular medium, that is, exo-metabolome, were characterized. Taken together, the results allowed us to draw a model of the metabolic contribution of *E. mutabilis* in the AMD of Carnoulès.

Materials and methods

Sampling and chemical analyses

The Carnoulès AMD is localized in the south of France. Abandoned in 1960, the mining exploitation led to accumulation of 1.5 MT of rocks, containing high amounts of heavy metals and metalloids present in sulfide minerals. Oxidation of these rocks led to the production of an AMD, which pours into the Reigous creek presenting high levels of dissolved metals and metalloids (LeBlanc *et al.*, 2002). Samples were collected in this creek in October 2008 at the station called COWG and located 30 m downstream from the spring (Bruneel *et al.*, 2003). Two phases were distinguished at this sampling site: 6-cm deep white sediments covering the bottom of the creek where *E. mutabilis* forms green mats, and a thin column (that is, less than 10 cm) of running water covering these sediments.

The main physico-chemical parameters (pH, dissolved oxygen concentration, conductivity, total dissolved solid) were determined in the field at the sampling site. Oxygen measurements were performed at the sediment/water interface using a microsensor (Unisense). Sediments were collected in triplicates using a sterile bottle and the running water was filtered (300 ml) through sterile 0.22-μm Nuclepore filters, which were then transferred into a collection tube, frozen in liquid nitrogen and stored at −80 °C until further analysis. Samples for total iron and arsenic determination were acidified to pH 1 with HNO_3 (14.5 M) and stored at 4 °C in polyethylene bottles. Samples for Fe(II) determination were stabilized with 1,10-phenanthroline chloride in acetate buffer (pH 4.5) (Casiot *et al.*, 2003). Samples for arsenic speciation were preserved by addition of 5% (v/v) 0.25 M EDTA solution (Bednar *et al.*, 2002). Samples for arsenic speciation, Fe(II) and sulfate determination were stored in the dark and analyzed within 24 h.

Total dissolved arsenic was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Thermo X7 Series). Highly concentrated As samples were diluted and no interference owing to ArCl was detected. ICP-MS was calibrated using peak intensity, acquired in peak jump mode, by using standard solutions. Indium-115 was used as an internal standard. Analyses of arsenic species (As(III), As(V)) were performed by anion-exchange chromatography (25 cm × 4.1-mm inner diameter Hamilton PRP-X100 column) using a Spectra System SCM 1000 solvent delivery pump coupled to ICP-MS. The detection limit was 2 nM for As(III) and 4 nM for As(V), with a precision better than 5%. Total dissolved iron was measured by Flame Atomic Absorption Spectrometry. Fe(II) concentration was determined by reading absorbance at 510 nm after complexation with 1,10-phenanthroline chloride solution in buffered samples (pH 4.5) (detection limit: 0.2 μM, precision better than 5%). Sulfate concentration was determined after precipitation of $BaSO_4$ with $BaCl_2$ and by reading absorbance at 650 nm (Casiot *et al.*, 2003).

Eukaryotic microbial community analysis

Eukaryotic microbial diversity was analyzed by capillary electrophoresis coupled with single-strand conformation polymorphism, as described previously (Quéméneur *et al.*, 2010). DNA was extracted from sediments using the UltraClean Soil DNA

Isolation kit according to the recommendations of the manufacturer (MoBio Laboratories Inc, Carlsbad, CA, USA). All extracted genomic DNA samples were stored at −20 °C until further processing. 18S rRNA genes of the eukaryotic microbial community were amplified using primers Euk1A and Euk516r (Scanlan and Marchesi, 2008). Three independent amplifications were performed as follows: denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min, amplification at 72 °C for 1 min and repeated 35 times. 16S rRNA genes were amplified as described previously (Bruneel *et al.*, 2006).

E. mutabilis *cell recovery and 18S/16S rRNA analyses*

To recover *Euglena* cells, 10 g of sediments stored at 4 °C were homogenized for 30 s in 10 ml of saline buffer (NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.15%, KH_2PO_4 0.02%). After 10-min decantation, 7.5 ml of supernatant were added without mixing to 17.5 ml of 65% (w/v) Nycodenz solution (Axis-Shield, Dundee, Scotland), and then centrifuged for 1 h at 14 000 *g*. The Nycodenz gradient separated two distinct phases composed of *E. mutabilis* cells (green upper phase) and other microorganisms (brownish lower phase). The upper phase corresponding to *E. mutabilis* cells was recovered by pipetting, washed by adding 2 volumes of NaCl 0.9% and centrifuged (30 s, 1000 *g* at 4 °C). Genomic DNA of these cells was extracted using the Wizard Genomic extraction kit (Promega, Madison, WI, USA) and used as template for 18S rRNA and 16S rRNA amplifications as described above. A part of these *Euglena* cells was plated on minimal solid agar medium (pH 3.2) (Olaveson and Stokes, 1989) and cultivated by at least five successive streakings on this solid medium to ensure purity (25 °C, 16/8-h light/dark photoperiod/45 $\mu mol\, m^{-2}\, s^{-1}$ photon flux density) (Halter *et al.*, 2011). In order to demonstrate that the *Euglena* culture was axenic, cells were observed by fluorescence microscopy after DAPI (4',6-diamidino-2-phenylindole) staining. These observations were performed on samples conserved at −20 °C at a magnification of ×1000 under oil immersion using a Leica DM 4000 B epifluorescence microscope equipped with a Leica DFC300 FX digital camera (Leica Microsystems, Wetzlar, Germany). Images were recorded at an excitation wavelength of 358 nm and emission wavelengths from 450 to 500 nm (Supplementary Figure 4).

Protein extraction, mass spectrometric analyses and protein identification

Proteins were extracted from *Euglena* cells using cell lysis buffer (urea 6 M, sodium dodecyl sulfate 2%, dithiothreitol 2 mM, glycerol 4%, Tris-HCl (pH 6.8) 0.05 M and Bromophenol Blue 0.05%) and heated for 1 min at 100 °C. After centrifugation (5 min, 4 °C, 16 000 *g*), the supernatant was recovered and proteins were separated by one-dimensional sodium dodecyl sulfate-PAGE (Laemmli, 1970) using a 12% gradient slab gel (PROTEAN II; Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed at 60 mA per gel. Proteins were stained with Coomassie brilliant blue R-250. Bands were systematically excised from the gels and stored at −20 °C before mass spectrometric analysis. In-gel digestion of gel bands was performed as described previously (Weiss *et al.*, 2009). The peptide extracts were analyzed by nanoLC-MS/MS using a nanoACQUITY Ultra-Performance-LC (UPLC; Waters, Milford, MA, USA) coupled to a SYNAPT hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Waters). The capillary voltage was set at 3500 V and the cone voltage at 35 V. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides, preferably doubly and triply charged ions, were selected on each MS spectrum for further isolation and collision-induced dissociation (CID) fragmentation with two energies set using collision energy profiles. The complete system was fully controlled by MassLynx 4.1 (SCN 566; Waters). Raw data collected during nanoLC-MS/MS analyses were processed and converted using ProteinLynx Browser 2.3 (23; Waters) into the .pkl peak list format.

The MS/MS data were analyzed using the MASCOT 2.2.0 algorithm and searched against two in-house generated databases. The first database included expressed sequence tags of *Euglena* downloaded from http://www.ncbi.nlm.nih.gov and the second database was composed of the protein sequences downloaded from http://www.uniprot.org/. For estimation of the false positive rate in protein identification, a target–decoy database search was performed (Elias and Gygi, 2007). In this approach, peptides were matched against a database consisting of the native protein sequences found in the database (target) and the sequence-reversed entries (decoy). Spectra were searched, allowing a maximum of one missed cleavage, with a mass tolerance of 30 p.p.m. for MS and 0.1 Da for MS/MS data, and with carbamidomethylation of cysteines and oxidation of methionines specified as variable modifications.

Protein identifications using the expressed sequence tag database were validated when at least two peptides with high-quality MS/MS spectra (less than 12 points below Mascot's threshold score of identity at 95% confidence level) were detected. In the case of one-peptide hits, the score of the unique peptide had to be greater (minimal 'difference score' of 10) than the 95% significance Mascot threshold. Protein identifications using the UniProt database were validated when at least two peptides with a minimal Mascot ion score of 30 were detected. In the case of one-peptide hits, the score of the unique peptide had to be greater (minimal 'difference score' of 16) than the 95% significance Mascot threshold.

All identifications were included into the 'InPact' proteomic database developed in our laboratory (http://inpact.u-strasbg.fr/~db/) (Bertin *et al.*, 2008).

Metabolite extraction, identification and GC-TOF analyses
Metabolite extraction was performed using a methanol/water solvent (80/20 v/v). Metabolite derivatization, GC (gas chromatography)-TOF (time-of-flight)-MS, data processing and profile analyses were performed as described (Noctor *et al.*, 2007). Metabolites were extracted either from *E. mutabilis* cell pellets recovered from sediments or directly from Carnoulès water. In the latter case, 0.9% NaCl (2 ml) was added to 10 g of water/sediment sample and gently mixed for 5 min at room temperature. After 2-min decantation, the upper phase was recovered and filtered first through a 10-μm membrane (Millipore, Billerica, MA, USA) and then through a 0.22-μm pore membrane to remove microbial cells. Lyophilized samples were then stored at −20 °C for further analysis. For *in vitro* analysis, metabolites were extracted from *Euglena* cell pellets recovered by centrifugation after 1- and 7 days of incubation in inorganic liquid media. The supernatant was collected in parallel, filtered through a 0.22-μm pore membrane to remove microbial cells, lyophilized and stored at −20 °C.

Each metabolite was identified by comparison of spectra with those obtained using the corresponding pure metabolite and its relative amount was calculated on the basis of the corresponding peak area compared with an internal standard (ribitol) and taking into account the initial amount of material. Integrated peak areas were obtained after deconvolution by using the LECO PEGASUS III software. Covariance of the metabolites was evaluated by hierarchical classification using the Mev algorithm.

Chromatography and MS for metabolite quantification
Each sample was analyzed using a UPLC coupled to tandem MS (UPLC-MS/MS) at the MS mode. The analysis was performed using a Waters Quattro Premier XE (Waters) equipped with an Electrospray (ESI) source and coupled to an Acquity UPLC system (Waters) with a diode array detector. UV spectra were recorded from 190 to 500 nm. Chromatographic separation was performed using a Zic—Phihic SeQuant column (150 × 2.1 mm, 5 μm; AIT, Houilles, France). The mobile phases selected were water with 0.01% formic acid (solvent-A) and methanol with 0.01% formic acid (solvent-B). The separation started with 20% A maintained for 4 min, followed by 2-min gradient to reach 50% A. It was followed by a gradient to reach initial conditions (20% A, in 2 min), maintained for 2 min before the next run. Total run time was 10 min. The column was operated at 40 °C with a flow rate of 0.4 ml min^{-1}, with a sample injection volume of 4 μl. Nitrogen generated from pressurized air in an N2G nitrogen generator served as both the drying gas and nebulizing gas (Mistral; Schmidlin-dbs-AG, Geneva, Switzerland). The parameters involving mass spectrometric detection and ESI ionization were as follows: nebulizer gas flow was set to approximately 50 l h^{-1} and desolvatation gas flow to 900 l h^{-1}.The interface temperature was set at 400 °C and the source temperature at 135 °C. The capillary voltage was set at 3.4 kV, and the cone voltage and ionization mode (positive and negative) were optimized for each molecule. The cone voltage parameters were as follows: for lactate, cone 22 V; for fructose, cone 25 V; for urea, cone 19 V. Mass spectra of the different metabolites were acquired with a scan range of m/z range 40–200 AMU. The selected ion recording (SIR) MS mode was then used to determine the parent mass transition of lactate $[M-H]^- = 89$; fructose $[M+Na]^+ = 203$; and urea $[M+H]^+ = 61$. The combination of chromatography retention time (t) and parent mass pattern was used to selectively monitor the different metabolites. The different samples were spiked with internal standard molecules to evaluate the level of analysis reproducibility. Data acquisition and analysis were performed using the MassLynx software (ver.5.1) running under Windows XP professional on a Pentium PC. The different metabolites from the different samples were quantified by reporting MS peak areas to calibration curves made with standard molecules of lactate, fructose and urea at different concentrations. As ionization yields in pure metabolite solutions may differ from those measured in a complex matrix, an estimation of the ionization yield in the sample was performed by adding 10 μg of fructose, lactate or urea to 1 ml of interstitial water. A correction factor corresponding to the ratio between the experimentally measured value and the theoretical value obtained from the calibration curve was applied to the metabolite quantification results.

Results

Characterization of the Carnoulès AMD
To characterize the natural environment of *E. mutabilis*, the physical, chemical and biochemical parameters of the Carnoulès AMD were determined. These analyses were performed at the sampling site called COWG, located 30 m downstream from the spring, where *E. mutabilis* forms abundant green mats and where the bacterial community has been recently characterized (Bertin *et al.*, 2011). The physico-chemistry of the running water covering the sediments revealed a low pH (3.1), a high dissolved oxygen content at the sediment/water interface (5.72 mg l^{-1}) and a high concentration of sulfate (3409 (± 341) mg l^{-1}). High conductivity (4900 μS cm^{-1}) and total dissolved solid values (3630 mg l^{-1}) were also measured, suggesting occurrence of osmotic stress. Moreover, our analysis

Table 1 Exo-metabolites identified in the interstitial water of the sampling site

Metabolite	*Quantity (AU)*	± *s.d.*
Glycerol	11.079	2.699
Urea	4.130	0.947
Palmitic acid	1.909	0.597
Lactic acid	1.600	0.375
Glycolic acid	0.901	0.121
Putrescine	0.747	0.122
Stearic acid	0.477	0.160
Tetradecanoic acid	0.314	0.056
Lauric acid	0.246	0.043
Glycine	0.171	0.043
Octanoic acid	0.103	0.006
Tyramine	0.096	0.026
p-Hydroxybenzoic acid	0.084	0.016
Pipecolic acid	0.072	0.025
Decanoic acid	0.071	0.003
2-Furancarboxylic acid	0.054	0.009
Sucrose	0.052	0.050
Levoglucosan	0.050	0.011
Maleic acid	0.047	0.014
Glucose	0.046	0.009
Galactosylglycerol	0.042	0.017
Fructose	0.034	0.005
Mannitol	0.031	0.004
Succinic acid	0.030	0.006
Trehalose	0.030	0.007
Fructose	0.029	0.007
Threonine	0.026	0.029

Abbreviation: AU, arbitrary unit.
Each metabolite was quantified in triplicates by using ribitol as an internal standard.

revealed the presence of both the oxidized and reduced form of arsenic (20.5 (±0.6) mg l^{-1} As(V) and 133 (±2) mg l^{-1} As(III)) and the reduced form of iron (1 220 (±61) mg l^{-1} Fe(II)). Finally, the biochemical analysis at the sampling site led to the identification of 26 metabolites in the water. These compounds were mainly glycerol, urea, amino acids or derivatives, unsaturated fatty acids, sugars and organic acids (Table 1 and Supplementary Table 1). Taken together, these results confirmed the extreme environmental conditions prevailing in this AMD ecosystem and revealed the presence of organic compounds in the water that may be synthesized by the microbial community.

Euglena *cell recovery*

To investigate the role of *E. mutabilis* in primary production, this protist was recovered by a cultivation-independent step using density-gradient separation. This experimental procedure allowed separation of *Euglena* cells from the remaining part of the microbial community according to their specific density (Figure 1). Optical microscopic observations performed on the *Euglena* cell fraction revealed both the existence of a homogenous population of *E. mutabilis* and the absence of other microbial cells (data not shown).

To determine the efficiency of this fractionation procedure, both 18S rRNA and 16S rRNA genes

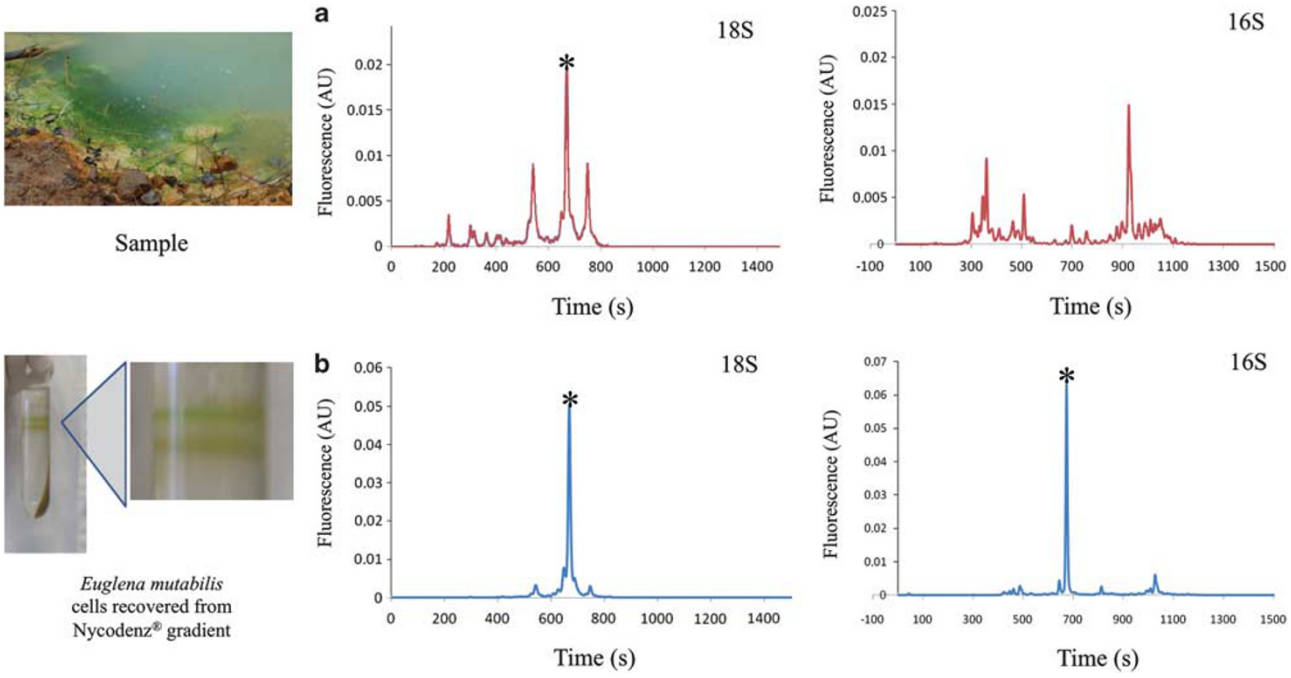

Figure 1 Diversity of the eukaryotic and prokaryotic microbial communities based on CE-SSCP 18S/16S rRNA analysis. The microbial community at the sampling site presented a low diversity (**a**) and seemed to be dominated by *E. mutabilis* (marked by *). Regarding 18S rRNA amplification, the signal specific to the *Euglena* 18S rRNA sequence increased from 40% of total fluorescence before the fractionation step (**a**) to more than 83% after the density-gradient fractionation step (**b**). Regarding the 16S rRNA sequence, these values were less than 0.1% (**a**) to more than 60% (**b**), respectively.

were amplified from DNA extracted from the green phase corresponding to *E. mutabilis*. The profiles of the amplified sequences were determined by CE-SSCP and compared to the CE-SSCP profiles of the 16S rRNA and 18S rRNA sequences amplified from DNA directly extracted from sediments (Figure 1). These fingerprinting profiles revealed the presence of a low eukaryotic and prokaryotic diversity in the sediments. A single highly dominant peak for both 18S rRNA and 16S rRNA sequences was observed in the *E. mutabilis* cell fraction, which was shown by sequencing to correspond to the *E. mutabilis* 18S rRNA gene and to the chloroplast 16 rRNA gene, respectively (data not shown). Although we cannot rule out that the *E. mutabilis* cells represent different ecotypes, they were not distinguished from each other according to morphological observations and 18S rRNA sequencing, and were consequently considered homogenous. These results suggest that the eukaryotic microbial community present in the Carnoulès sediments is dominated by *E. mutabilis* and that the fractionation procedure allowed us to efficiently recover these cells without any cultivation step.

Proteins expressed in situ *by* E. mutabilis

To identify the main biological functions expressed by *E. mutabilis in situ*, total proteins were extracted from *Euglena* cells. These extracted proteins were then separated according to their molecular weight by one-dimensional sodium dodecyl sulfate-PAGE (Supplementary Figure 1). NanoLC-MS/MS analyses of all gel bands led to the identification of 189 unique proteins using two databases. The first one, an expressed sequence tags database of *E. gracilis*, was used to identify proteins already described in *Euglena* species and led to the identification of 162 proteins. The second one, a UniProt subset database, was used to exhaustively identify proteins, including those not yet described in *Euglena* species. This second analysis led to the identification of 27 additional proteins, including 18 proteins identified in species either phylogenetically or functionally related to *Euglena*, that is, photosynthetic organisms (Figure 2, and Supplementary Tables 2 and 3). Even though some bacterial protein contaminants, that is, less than 5%, were still present, these results confirmed that the cellular fraction obtained by density-gradient separation was highly enriched in *Euglena* cells.

The majority of the identified *Euglena* proteins are known to be involved in photosynthesis, that is, PSI and PSII systems, ATP synthase and the cytochrome b_6–f complex, highlighting the active role of *E. mutabilis* in photosynthetic oxygen production. Moreover, proteins associated with cell division, transcription and protein turnover were also identified, suggesting that *Euglena* cells are physiologically active and able to grow at the study site. Phototactism-related proteins, that is, photoactivated adenylyl cyclase, and proteins involved in oxidative stress defense mechanisms, that is, thioredoxin peroxidase, ascorbate peroxidase,

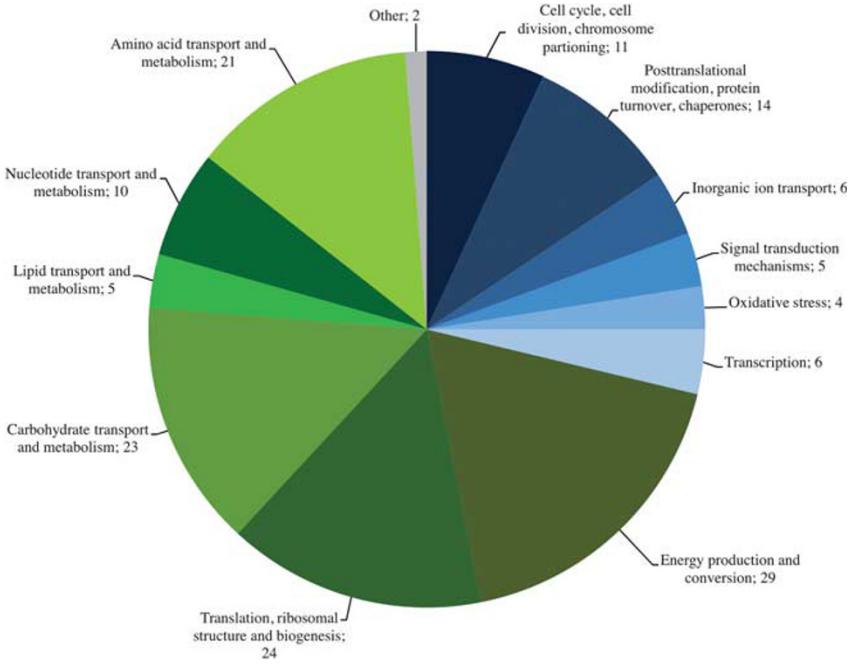

Figure 2 Functional categories of the proteins identified *in situ* in *E. mutabilis* cells. Proteins involved in primary production and organic matter biosynthesis are colored in green. The number following the functional categories represents the number of proteins identified within each of them. The list of the corresponding proteins is presented in Supplementary Tables 2 and 3.

superoxide dismutase and hydroperoxide reductase, were also accumulated suggesting existence of adaptation processes to environmental stresses (Lengfelder and Elstner, 1979; Shigeoka *et al.*, 1980; Seaver and Imlay, 2001). Similarly, a membrane-associated, ATP-dependent H^+ transporter was found, presumably reflecting an acclimation to the low-pH conditions prevailing in the Carnoulès AMD.

Most of the proteins accumulated by *Euglena* cells, that is, approximately 70%, are known to be involved in energy production and/or anabolic pathways (Figure 2). For instance, a large amount of enzymes involved in carbohydrate metabolism (Calvin cycle, glycolysis and pentose phosphate pathway); amino-acid, nucleotide and fatty acid biosynthesis; or nitrogen metabolism (urea cycle and ammonium assimilation) were identified, suggesting that *E. mutabilis* expressed functions possibly leading to specific organic matter production.

Metabolite identification in E. mutabilis *cells* in situ

To determine which metabolites were preferentially accumulated in *E. mutabilis* cells *in situ*, a metabolomic approach was used and allowed to identify 57 metabolites, mainly fatty acids (C:8, C:10, C:12, C:14, C:16, C:18, C:18(2), C:18(3)), sugars (arabinose, fructose, glucose, galactose, ribose, trehalose, xylose), amino acids (excepted leucine and histidine), organic acids (ascorbic, benzoic, lactic, malic, nicotinic, hydroxybenoic, pipecolic, pyroglutamic, succinic and sinapinic acid) and other metabolites such as urea, spermidine, putrescine, ornithine, phytol, myo-inositol, mannitol, glycerol derivatives, GABA and ethanolamine (Supplementary Table 1). These results correlated with the expression of the anabolic pathways highlighted by the proteomic approach, which may have led to the accumulation of multiple metabolites in *Euglena* cells. Interestingly, among the 26 metabolites identified in the water (Table 1), 22 were also found in the protist, that is, all those metabolites except octanoic acid, decanoic acid, levoglucosan and 2-furancarboxilic acid (Supplementary Table 1). The metabolites identified in *E. mutabilis* cells may have been accumulated from the environment. Alternatively, this protist may be able to synthesize and secrete them into the AMD water.

Metabolite synthesis and secretion by E. mutabilis in vitro

A metabolic profile was established *in vitro* from a pure culture to determine which metabolites found in the *E. mutabilis* cells may be synthesized by the protist itself and liberated into the water. *E. mutabilis* cells were grown in a synthetic medium mimicking the acidic conditions of the AMDs, and containing salts and cobalamine as the sole organic compound as this protist is an auxotroph for this cofactor (Olaveson and Stokes, 1989). After 2 weeks of growth on solid medium, *E. mutabilis* cells were transferred to liquid medium and incubated for 1 week. Metabolites accumulated within the cells, that is, the endo-metabolome, and those secreted into the culture medium, that is, the exo-metabolome, were analyzed after 1 day and 1 week of incubation. A total of 78 metabolites were identified from *E. mutabilis* cells grown *in vitro*, which is significantly higher than the 57 metabolites identified from *Euglena* cells recovered *in situ*. This observation may be explained, at least in part, by the amount of biological material, which was higher in the experiment performed under laboratory conditions. Only three metabolites were found in the *Euglena* cells *in situ* but not in the *Euglena* cells *in vitro*, that is, 5-oxopyrrolidine-2-carboxyamide, pipecolic acid and sinapinic acid. This result indicates that *E. mutabilis* is able to synthesize the majority of the metabolites found within the *Euglena* cells *in situ*.

To determine which metabolites may be liberated by *E. mutabilis*, the exo-metabolites were identified and compared to the endo-metabolites after 1 day and 1 week of incubation (Supplementary Table 1). The amount of each metabolite was quantified using ribitol as an internal standard. The metabolite variations in both the medium and in the *E. mutabilis* cells were analyzed by hierarchical classification. Variation patterns (P-value <0.05) were represented as a heat map highlighting the existence of three main groups of metabolites (Figure 3). The first one corresponds to metabolites, mainly fatty acids, accumulated within the cells after 7 days of incubation but not in the medium (I). The second group is represented by metabolites (glucose-6-phosphate and some downstream products such as organic acids and ascorbate) that disappeared from cells upon *in vitro* incubation (II). Surprisingly, fatty acids and some organic acids such as ascorbic acid, maleic acid and glyceric acid were not identified in the medium despite their accumulation in the cells. The third group is composed of metabolites accumulated in the external medium (III). These compounds were mainly amino acids (proline, isoleucine, alanine, valine, leucine, phenylalanine, glycine, asparagine, tyrosine, proline), sugars (glucose, arabinose, mannose, ribose, xylose, fructose, mannitol) and other compounds (putrescine, spermidine, glycerol, glycolic acid, galactosyl glycerol, hydroxybenzoic acid).

To quantify the observed accumulation or depletion patterns in both the extracellular medium and in the *Euglena* cells, we calculated variations in metabolite content (% mg^{-1} fresh weight (FW)) (Supplementary Figure 2). Fatty acids accumulated in *Euglena* cells (by up to sixfold) whereas putrescine appeared to be the most specifically excreted metabolite, with a 16-fold increase upon cell incubation. Sugars such as glucose and arabinose (10-fold increase), and amino acids (from 2- to 8-fold

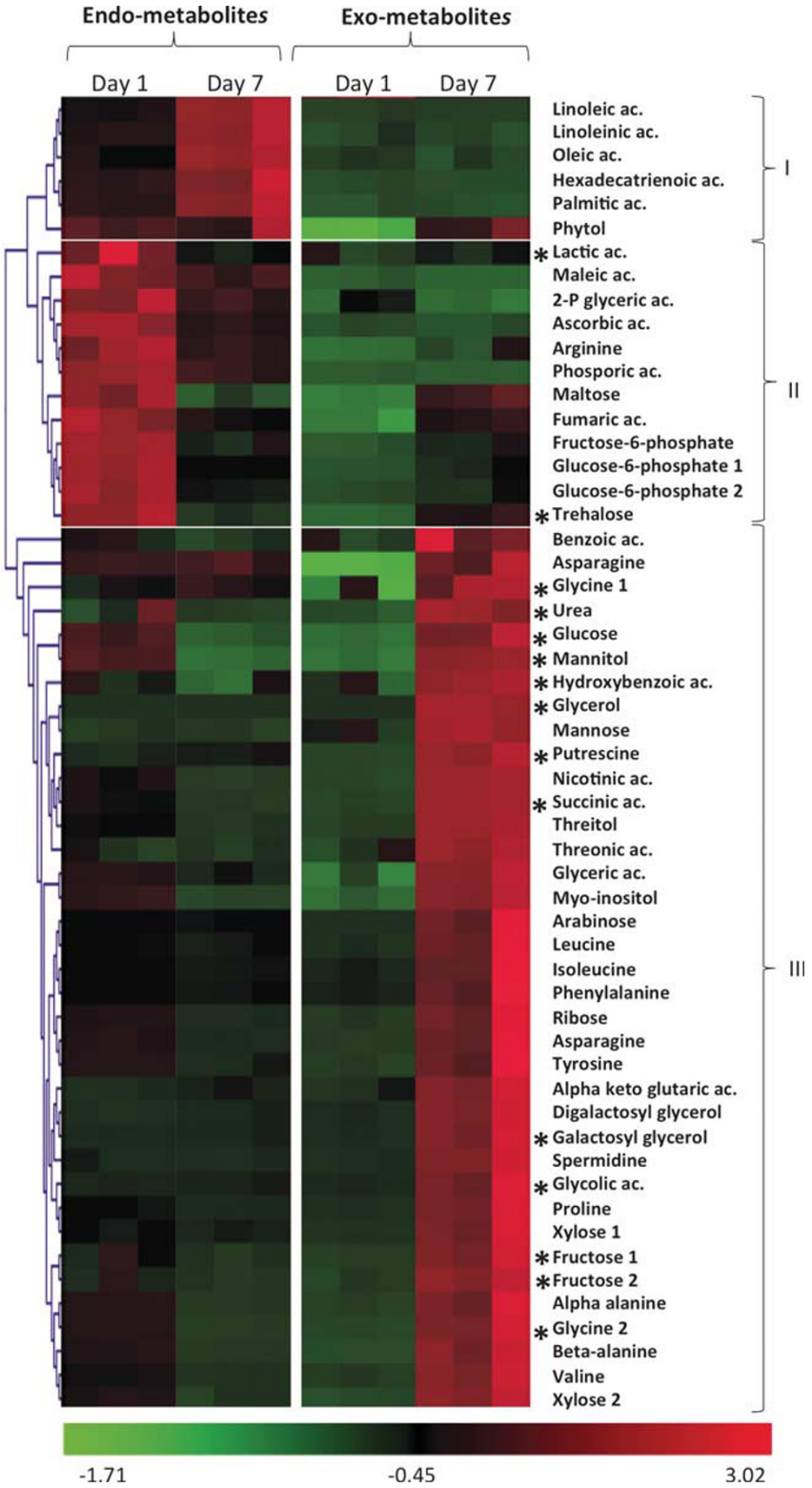

Figure 3 Heat map and hierarchical classification of the endo- and exo-metabolites synthesized and secreted by *E. mutabilis* after 1- or 7-day incubation in minimal liquid medium under laboratory conditions (ac. = acid). Only metabolites showing a significant (*P*-value <0.05) accumulation pattern after 7 days were represented. The analysis was performed in triplicates and the color of each cell indicates the relative content of the corresponding metabolite. The clustering analysis gathers metabolites with a similar variation pattern. Metabolites marked by * were identified in the water at the sampling site. The complete list of the metabolites identified is presented in Supplementary Table 1 and Supplementary Figure 2.

increase), were also shown to be abundantly excreted. These quantification patterns suggest that accumulation of some metabolites in the medium is not simply due to cell lysis but rather relies on a selective secretion by *Euglena* cells.

The exo-metabolites identified *in vitro* were compared to those identified at the sampling site (Figure 3). Of the 26 metabolites identified in AMD water, 15 were shown to be secreted under laboratory conditions by *E. mutabilis*. These metabolites were mainly sugars (mannitol, glucose, fructose), amino acids (glycine, threonine, tyrosine) and other compounds (glycerol, urea, lactic acid). An estimation of the concentration of some of these metabolites, that is, urea, lactate and fructose, revealed that they were present in a concentration of approximately $1\,mg\,l^{-1}$ in the interstitial water of the sampling site (Supplementary Figure 3), which is of the same order of magnitude as the total organic carbon concentration, that is, $2.5\,mg\,l^{-1}$, measured at this site (Casiot, unpublished). These results suggest that some metabolites shown to be specifically secreted by *E. mutabilis in vitro* may be synthesized by this protist *in situ*, allowing us to propose a model of the organic matter contribution of *E. mutabilis* at the Carnoulès site (Figure 4).

Discussion

Our proteo-metabolomic data revealed that *E. mutabilis* may have a crucial role on the functioning of the Carnoulès AMD ecosystem. Two main activities may be highlighted according to our results: the ability of *E. mutabilis* to perform photosynthetic metabolism on the one hand, and its specific organic matter synthesis and secretion on the other hand.

Adaptation of E. mutabilis *to AMD and photosynthetic metabolism*

AMD ecosystems are usually described as extreme environments for microbial communities. Our biochemical characterization of the Carnoulès ecosystem (Gard, France) supports the existence of multiple abiotic stresses in accordance with previous observations (Casiot *et al*., 2003, 2004b; Bruneel *et al*., 2011). In particular, osmotic stress, low pH and oxidative stress caused by heavy metals and metalloids may explain the reduction in eukaryotic and prokaryotic diversity observed in such ecosystems (Johnson and Hallberg, 2003). Despite these conditions, *E. mutabilis* is frequently

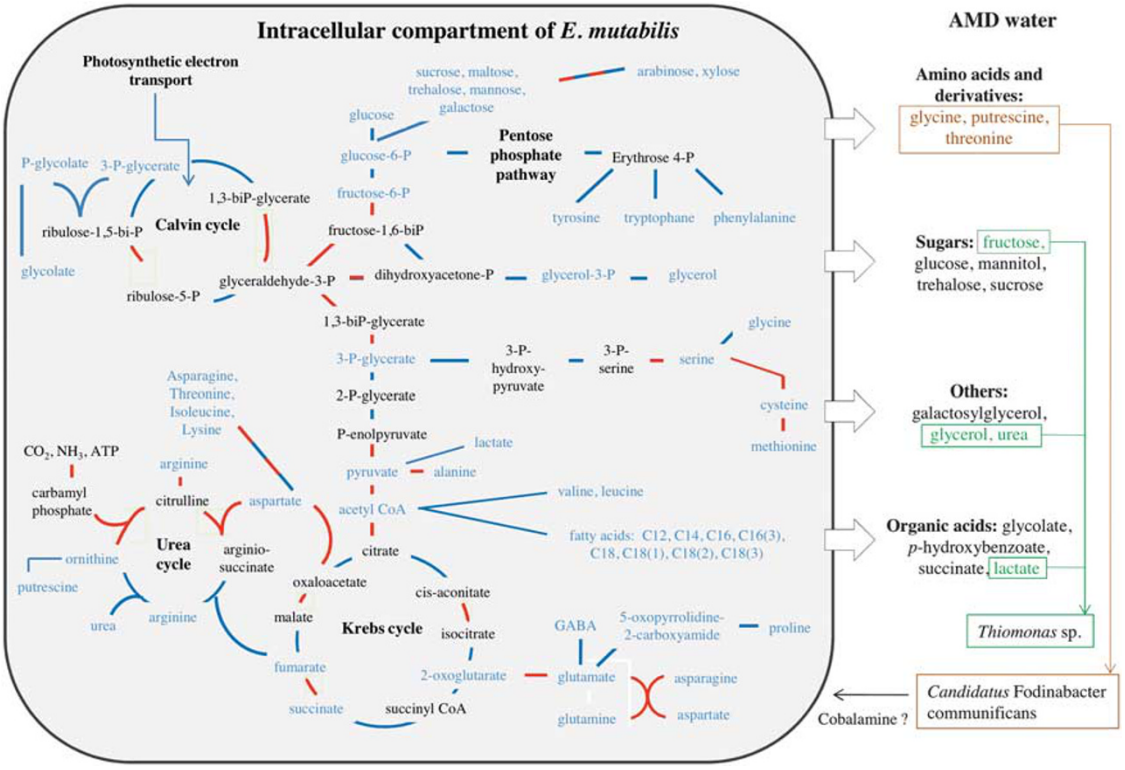

Figure 4 A functional overview of *E. mutabilis* main metabolic traits determined from proteomic and metabolomic data. For each metabolic pathway shown in the figure, examples of metabolites are indicated (blue). Enzymes involved in those metabolic pathways are represented by a red line when identified by the proteomic approach and a blue line otherwise. In the extracellular medium, only metabolites found in the water at the study site and shown to be secreted by *E. mutabilis in vitro* were represented. Those possibly used as nutrient by *Thiomonas* sp. and *Ca.* F. communificans according to our previous analysis are represented in green and in brown, respectively (Bertin *et al*., 2011).

observed in AMDs (Olaveson and Nalewajko, 2000), which suggests that this protist is able to cope with most of these environmental stresses. Indeed, our proteo-metabolomic approach highlighted the expression of multiple defense mechanisms expressed *in situ*. For instance, expression of a proton efflux system, accumulation of antioxidant proteins and metabolites (ascorbic acid), and synthesis of trehalose may help *E. mutabilis* to cope with acidic, oxidative and osmotic stresses, respectively.

In addition, the photosynthetic metabolism of *E. mutabilis* supported by our proteomic analysis may have an important impact on the Carnoulès ecosystem through several ways. First, it has been shown that the 'oversaturation' in dissolved oxygen (that is, >200% saturation with the atmosphere) facilitates inorganic co-precipitation of arsenic and iron colloids (Brake *et al.*, 2001a, 2001b). Second, an increased concentration in dissolved oxygen may favor the development of aerobic species such as *Thiomonas* or *Acidothiobacillus* spp., both known to be dominant and involved in the bio-attenuation processes at the Carnoulès AMD (Bruneel *et al.*, 2003; Duquesne *et al.*, 2003; Bertin *et al.*, 2011). Photosynthetic metabolism of *E. mutabilis* may therefore affect directly and indirectly the metal precipitation processes observed in AMDs. Interestingly, this metabolism allows the protist to produce organic compounds that may be substrates for the Carnoulès bacterial community.

Metabolic interactions between E. mutabilis *and the bacterial community*

The *in situ* functional genomics analyses highlighted the ability of *E. mutabilis* to grow, divide and metabolize under harsh environmental conditions. As this protist is an auxotroph for cobalamine, our observations suggest that *E. mutabilis* gets this cofactor from the AMD waters. A previous analysis performed on the prokaryotic community at the same study site revealed that bacteria belonging to the novel phylum *Candidatus* Fodinabacter communificans express *in situ* genes involved in cobalamine biosynthesis (Bertin *et al.*, 2011). It is therefore tempting to speculate that these bacteria may help *E. mutabilis*, at least in part, to colonize the Carnoulès ecosystem and that metabolic interactions may occur between the bacterial community and *E. mutabilis*.

Conversely, the comparison between metabolites found *in situ* and those secreted by the protist *in vitro* strongly suggests that *E. mutabilis* may be at the origin of the accumulation of primary metabolites in AMD waters. This assertion is supported by our proteomic data revealing that *Euglena* expressed *in situ* anabolic pathways involved in fatty acid, amino-acid, sugar and nucleotide biosynthesis. Surprisingly, the *in vitro* metabolite accumulation observed in the medium strongly differs according to their nature. Indeed, sugars, amino acids, urea and polyamine compounds (putrescine, ornithine) were among the more actively secreted, unlike some organic acids and fatty acids (Figure 3). Some of these organic compounds may be used as an energy source by other members of the microbial community. Indeed, *Thiomonas* spp. isolated from Carnoulès carry genes involved in transport and degradation of glucose, fructose, glycerol, lactate and several amino acids, and are able to use fructose as a unique carbon source (Arsène-Ploetze *et al.*, 2010; Slyemi *et al.*, 2011). Interestingly, most of the secreted compounds contained nitrogen, which may have a key role in the ecosystems where *E. mutabilis* is found. For example, acidic lakes and peat bogs both have very low available nitrogen concentrations, and this element has been shown to constitute the growth-limiting factor for the sulfate-reducing bacteria in AMDs (Waybrant *et al.*, 2002; Beamud *et al.*, 2010; Crowley and Bedford, 2011). In the Carnoulès ecosystem, some of the secreted compounds may therefore be used as a nitrogen source by bacteria. In agreement with this, our previous studies revealed that the genome of *Thiomonas* strains contains a complete operon involved in the degradation of urea (Arsène-Ploetze *et al.*, 2010), which was shown here to be secreted by *E. mutabilis*. Moreover, another dominant bacterial species present at this site, that is, *Ca.* F. communificans, has been shown to contain and express many genes involved in the transport and recycling of amino acids (Bertin *et al.*, 2011). *E. mutabilis* may thus participate in the feeding of these bacteria, which in turn produce cobalamine useful for the protist (Bertin *et al.*, 2011). These observations suggest that *E. mutabilis* may have an important role in the development of such strains in the Carnoulès AMD, and further support the existence of strong trophic interactions within this microbial community.

In conclusion, our metabolomic data revealed that *E. mutabilis* has a significant and selective role in organic matter production in the partly oligotrophic ecosystem of Carnoulès. Although we cannot rule out that other microorganisms are also involved in this process, the expression of multiple anabolic pathways detected in *E. mutabilis* by the proteomic approach further supports its role in the biosynthesis of organic compounds. Consequently, *E. mutabilis* may also indirectly contribute to the AMD bioattenuation by feeding bacterial strains and maintaining aerobic conditions via its photosynthetic metabolism. Such metabolic capacities may have an important impact in other acidic environments where *E. mutabilis* is found, especially in nitrogen-limited ecosystems such as peat bogs or volcanic lakes.

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