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ORIGINAL PAPER

Caldinitratiruptor microaerophilus, gen. nov., sp. nov. isolated from a French hot spring (Chaudes-Aigues, Massif Central): a novel cultivated facultative microaerophilic anaerobic thermophile pertaining to the *Symbiobacterium* branch within the *Firmicutes*

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Abstract A novel facultative microaerophilic nitrate-reducing bacterium designated CA62N^T was isolated from a thermal spring in France. Cells were non-motile rods (2–3 × 0.2 μm) and showed low cytoplasmic density when observed under a phase-contrast microscope. Strain CA62N^T grew at temperatures between 50 and 75°C (optimum 65°C) and at a pH between 6.3 and 7.9 (optimum 7.0). NaCl was not required for growth but was tolerated up to 10 gl^{-1}. Sulfate, thiosulfate, elemental sulfur, sulfite, and nitrite were not used as electron acceptors. Nitrate was reduced to nitrite. Strain CA62N^T used lactate, pyruvate, glucose, mannose, fructose, and casamino acids and some amino acids as electron donors only in the presence of nitrate as electron acceptor. None of these substrates was fermented. The main end-products of glucose oxidation were acetate, CO_2, and traces of H_2. The G + C content of the genomic DNA was 70.3 mol% (HPLC techniques). Phylogenetic analysis of the small-subunit (SSU) ribosomal RNA (rRNA) gene sequence indicated that strain CA62N^T was affiliated to the *Symbiobacterium* branch within the *Firmicutes* and had *Symbiobacterium thermophilum* and "*S. toebii*" as its closest phylogenetic relatives. On the basis of phylogenetical and physiological characteristics, strain CA62N^T is proposed to be the type strain for the novel species in the novel genus, *Caldinitratiruptor microaerophilus* gen. nov., sp. nov. (DSM 22660, JCM 16183).

Keywords *Caldinitratiruptor microaerophilus* · Thermophilic · Microaerophilic · Nitrate-reduction · Hot spring

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Introduction

Within the domain *Bacteria*, various thermophiles and hyperthermophiles belonging to the *Clostridia* have been isolated from hot springs. *Clostridia* members include a wide range of *Thermoanaerobacter* and *Thermoanaerobacterium* species, together with members of the genera *Moorella*, *Caldanaerobacter*, *Thermovenabulum*, order *Thermoanaerobacterales* (see Wagner and Wiegel 2008 for a more detailed review; Wiegel 2009) and species of genera *Carboxythermus*, *Thermincola*, *Thermoanaerovibrio, Anaerobranca*, *Caloramator*, or *Caldicellulosiruptor*, order *Clostridiales* (see Wagner and Wiegel 2008 for a more detailed review; Rayney 2009). All these microorganisms have been reported as strict anaerobes.

France contains a number of geothermal areas, many of which are found in the Massif Central region due to its volcanism. One such area located in the Southeast Cantal (Chaudes-Aigues) is characterized by an emergence zone of thermal springs with temperatures ranging from 40 to 82°C, making it one of the hottest spring waters in Europe.

This thermal spring was chosen as part of our microbial ecology of hot ecosystems research program to study the diversity of thermophilic anaerobic lithoautotrophic and heterotrophic microorganisms associated with the sulfur (sulfate-reducing microorganisms) and nitrogen cycles

(nitrate respiration). Hydrothermal samples were collected from this spring at different temperatures in 2004. Here, we describe the isolation and characterization of a facultative microaerophilic thermophilic nitrate-reducing bacterium within the *Clostridia* having phenotypic and phylogenetic characteristics consistent with its assignment to a new genus within the *Symbiobacterium* branch (Beppu and Ueda 2009). To date, representatives of this branch comprise two species, *S. thermophilum* and "*S. toebii*" (Ohno et al. 2000; Sung et al. 2003).

Materials and methods

Sources for sampling

Mixed water/sediment samples were collected from a terrestrial hot spring located at Chaudes-Aigues in the Massif Central, France. This area houses many hot springs closely associated with volcanism, with spring temperatures ranging from 40 to 82°C. At the sampling site (Source du Moulin du Ban), temperature was 63°C, pH was 6.2, and conductivity was 1,255 µs/cm.

Preparation of media, isolation, and characterization

The basal medium contained (per liter of distilled water): 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 1 g NaCl, 20 mM $NaNO_3$, 0.1 g KCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g $MgCl_2 \cdot 6H_2O$, 1 g yeast extract (Difco Laboratories), and 10 ml trace mineral element solution Balch et al. (1979). pH was adjusted to 7.2 with 10 M KOH solution. The medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Aliquots of 5 ml were dispensed into Hungate tubes, degassed under N_2–CO_2 (80:20%, v/v) and subsequently sterilized by autoclaving at 110°C for 45 min. Prior to culture inoculation, 0.1 ml of 10% (w/v) $NaHCO_3$, and 20 mM glucose were injected into the tubes from sterile stock solutions. The Hungate technique (1969) was used throughout.

Growth experiments were performed in duplicate using Hungate tubes containing basal medium. pH, temperature, and NaCl concentration ranges for growth were determined using basal medium supplemented with 20 mM glucose. The pH of the medium was adjusted by injecting Hungate tubes with aliquots of anaerobic stock solutions of 1 M HCl (acidic pHs), 10% $NaHCO_3$ or Na_2CO_3 (basic pHs). Water baths were used to incubate bacterial cultures at 50°C and up to 80°C. For studies of NaCl requirements, NaCl was weighed directly in the tubes before the medium was dispensed. The strain was subcultured under the same experimental conditions before growth rates were determined. Each substrate was tested in basal medium at a final concentration of 20 mM for sugars and organic acids and 1 gl^{-1} for casaminoacids and aminoacids. Elemental sulfur (1% w/v), sulfate (20 mM), thiosulfate (20 mM), sulfite (2 mM), nitrate (10 mM), and nitrite (2 mM) were tested as terminal electron acceptors. The effect of O_2 on growth was determined in Hungate tubes containing anaerobic basal medium supplemented with 20 mM glucose. Tubes were inoculated and various amounts of sterile air were added to the gas phase. The final oxygen concentrations tested were 1, 2, 5, and 21%. The cultures were incubated at 60°C with agitation at 150 r.p.m. The presence of spores was checked by microscopic observation of cultures and pasteurization tests performed at 80, 90, and 100°C for 10 and 20 min. End-products of metabolism were measured by high-pressure liquid chromatography after 2-week incubation at 60°C (Fardeau et al. 2000).

Analytical techniques

Bacterial growth was monitored by measuring the increase in turbidity at 580 nm by insertion of Hungate tubes into the cuvette holder of a spectrophotometer (Cary 50, Varian). H_2S production was determined photometrically as colloidal CuS as described by Cord-Ruwisch (1985). Nitrite was measured by the Quantifix test (Macherey–Nagel), whereas NO and N_2O were detected by using CPG techniques as previously described (Bonilla Salinas et al. 2004). The presence of *meso*-diaminopimelic acid has been established using the solvent system of Rhuland et al. (1955) by DSMZ.

Light and electron microscopy

Morphological characteristics and purity were checked under an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as previously described (Fardeau et al. 1997).

Determination of G + C content

G + C content of DNA was determined at DSMZ via HPLC as described by Mesbah et al. (1989).

DNA extraction, PCR amplification, and DNA sequencing

The genomic DNA of CA62N^T was extracted using the Wizard Genomic DNA Purification kit according to the manufacturer's protocol (Promega). The universal primers Fd1 (5′-CAGAGTTTGATCCTGGCTCAG-3′), Rd1 (5′-AAGGAGGTGATCCAGCC-3′), and R6 (5′-TACGGTTA

CCTTGTTACGAC-3′) were used to amplify the SSU rRNA gene. Direct sequencing of PCR product was performed by GATC (Konstanz, Germany). The nucleotide sequence was manually aligned with reference sequences of various bacteria belonging to the *Bacilli*/*Clostridia* group using the BioEdit sequence alignment editor (Hall 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak et al. 2001) and Genbank databases (Benson et al. 1999). Sequence position and alignment uncertainties were omitted from analysis.

Possible involvement of the strain in the nitrogen cycle was investigated by PCR with primers amplifying marker genes for dissimilative nitrate reduction (*napA*, *narG*), denitrification (*nirS*, *nirK*, and *nosZ*), dinitrogen fixation (*nifH*), and nitrification (*amoA*). According to the literature, two membrane-associated dissimilative nitrate reductases can be encountered, one facing into the periplasm (Nap), the other facing into the cytoplasm (Nar). For *napA*, three different PCR primer sets were tested: two sets already published napAV66-napAV67, napV16-napAV17 (Flanagan et al. 1999) and the third one designed in this study according to the *napA* sequence from *E. coli* and *Symbiobacterium thermophilum* (napAF-symb GACGACATCGAGCAKGCYGA and napAR-symb GTCAGCAGGTGCAGGTTGTAGA). The presence of *narG* was tested with na3F–narG5′R (Goregues et al. 2005). Dissimilatory nitrite reductase could be encoded either by a *nirS* or a *nirK* type gene. Presence of both gene types was screened with cd3F-cd4R for *nirS* (Michotey et al. 2000) and nirKCF-nirKCR (Goregues et al. 2005) and F1aCu-R3Cu (Hallin and Lindgren 1999) for *nirK*. Presence of nitrous oxide reductase gene was screened with nosZ1211F-nosZ1897R (Rösch et al. 2002). Possible involvement of the strain in dinitrogen fixation was tested with nifHF-nifHR-amplifying dinitrogen reductase (Zehr and McReynolds 1989). For nitrification, presence of *amoA* encoding for ammonium monooxygenase was screened with AmoA-1F-AmoA-2R (Rotthauwe et al. 1997). PCR amplification was carried out with a 20 µl reaction mixture (20 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$ except for napAV16-napAV17) containing 0.2 mM of each deoxyribonucleoside triphosphate, 160 pmol of each primer, and 0.5 unit of Taq polymerase (Promega France). For PCR, 50 ng of template DNA was used in the reaction. Amplification was performed on a mini-cycler (MJ Research) for 30 cycles. PCR cycles were performed as previously described (Flanagan et al. 1999; Goregues et al. 2005; Michotey et al. 2000; Rösch et al. 2002; Rotthauwe et al. 1997; Zehr and McReynolds 1989). Amplification products were analyzed by electrophoresis on a 1% (wt/vol) agarose gel (Roche Diagnostics, Mannheim Germany).

Nucleotide sequence accession number

The GenBank accession number for the 16S rRNA gene sequence is GQ 405534.

Results

Enrichment and isolation

A 0.5-ml aliquot of sample was inoculated into Hungate tubes containing 5 ml of basal medium, $H_2 + CO_2$ (80/20%; 2 bars), and nitrate (20 mM) as terminal electron acceptor to look for hydrogen-oxidizing nitrate-reducing microorganisms. The tubes were then incubated at 60°C. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in roll tubes containing agar (2% w/v). Several colonies that developed after incubation at 60°C were picked separately. The serial dilution process was repeated several times until the isolates were deemed to be axenic. Several strains similar in morphology and phylogeny, using nitrate as electron acceptor and reducing it to nitrite were isolated. None of these strains was shown to oxidize hydrogen, suggesting that the primary source of energy in the enrichment medium was yeast extract and not hydrogen. All these isolates oxidized glucose in the presence of nitrate. A strain designated CA62N^T was selected and used for further characterization.

Morphology

Colonies were yellowish, flat, and circular with diameters ranging from 0.5 to 1.0 mm after 2–4 days incubation at 60°C. The cells stained Gram-negative. They were non-motile, non-spore-forming-rods, approximately 2–3 µm in length and about 0.2 µm in diameter. They generally occurred singly or in pairs and showed low cytoplasmic density when observed under a phase-contrast microscope (Fig. 1a).

Electron microscopy

Sections for electron microscopy revealed a multilayered cell wall outside the cytoplasmic membrane. The structure consisted of three electron-dense layers and two electron-transparent layers (Fig. 1b). The Gram-positive type of cell wall was confirmed by the presence of *meso*-diaminopimelic acid as diagnostic diamino acid of the peptidoglycan in strain CA62N^T.

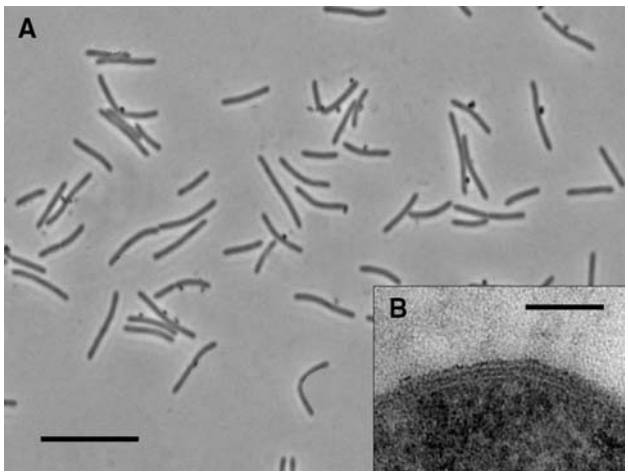

Fig. 1 **a** Phase-contrast micrograph of rod-shaped cells of strain CA62N^T using glucose as energy source and nitrate as terminal electron acceptor, showing low cytoplasmic density. *Bar* 10 μm; **b** Electron micrograph of fine sections of strain CA62N^T showing the multilayered cell wall. *Bar* 0.1 μm

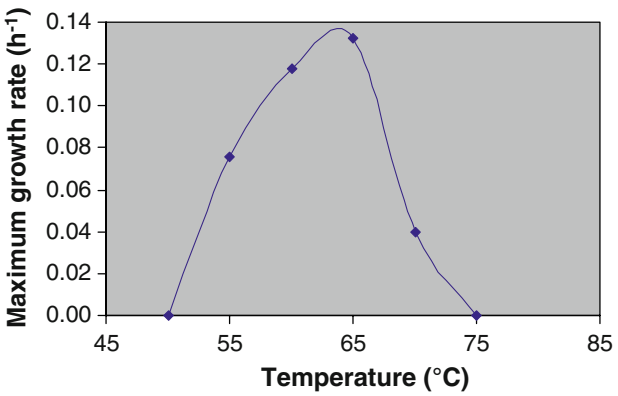

Fig. 2 Effect of temperature on the growth of strain CA62N^T

Metabolic properties

Strain CA62N^T was thermophilic with growth on glucose occurring under low partial pressure of O_2 up to 3–4% in the gas phase (optimum growth with 2% O_2) in the absence of nitrate. Nitrate was reduced into nitrite even in the presence of excess of electron donors (e.g. glucose). In these culture conditions, growth was inhibited in the presence of 5-mM nitrite. It grew at temperatures ranging from 50 to 75°C (no growth observed at 50°C and 75°C), with an optimum at 65°C (Fig. 2). The isolate grew in the absence of NaCl but could tolerate it up to 10 gl^{-1} (data not shown). The optimum pH range for growth was 7.0, and growth occurred between pH 6.3 and 7.9 (no growth observed at pH 6.0 and pH 8.0). Yeast extract (minimum 0.01%) was required for growth and could not be replaced by peptides (Tryptone, Panreac, Spain) or vitamins (Widdel and Bak 1992). Sulfate, thiosulfate, elemental sulfur, sulfite, and nitrite were not utilized as terminal electron acceptors. Strain CA62N^T grew on lactate, pyruvate, glucose, mannose, fructose, casamino acids, and single amino acids (serine, proline, glutamine, threonine, alanine, glycine, glutamic acid, isoleucine, leucine, asparagine, valine, methionine, and histidine) in the presence of nitrate as terminal electron acceptor. None of these substrates was fermented. The main end-products resulting from glucose oxidation were acetate, CO_2, with only trace amounts of hydrogen. Acetate was found as the main end-product of amino acid oxidation, with the exception of isoleucine, leucine, and valine which were converted to 2-methylbutyrate, isovalerate, and isobutyrate, respectively. The compounds that did not support growth in the presence of nitrate included saccharose, raffinose, xylose, cellobiose, arabinose, sorbose, maltose, galactose, formate, acetate, succinate, starch, gelatin, and H_2–CO_2. Under optimal growth conditions (glucose 20 mM, nitrate 10 mM), doubling time was 3 h.

G + C content of DNA and phylogeny

The G + C content of strain CA62N^T was 70.3 mol%. Pairwise evolutionary distances based on 1,362 unambiguous nucleotides were computed by the Jukes and Cantor (1969) method. The phylogenetic tree obtained by the neighbor-joining method (Saitou and Nei 1987) is given in Fig. 3. Tree topology was re-examined by the bootstrap method (1,000 replications) of resampling (Felsenstein 1985). Its topology was also supported by using maximum parsimony and maximum likelihood algorithms. According to its phylogenetic position, the novel isolate is likely to represent a novel genus in the *Symbiobacterium* branch within the *Firmicutes*. Its closest relatives were *Symbiobacterium thermophilum* and "*S. toebii*" which shared 87.3% and 85.5% identity, respectively. These phylogenetic distances indicate that strain CA62N^T represents a new genus within the *Symbiobacterium* branch. The 16S rRNA gene sequences of strain CA62N^T have been deposited in GenBank under accession number GQ 405534.

Genes involved in the nitrogen cycle

Strain CA62N^T was negative for all the tested genes involved in the nitrogen cycle. The discrepancy between physiologic and phenotypic results concerns the nitrate reduction step, since the strain was able to reduce nitrate into nitrite but neither *narG* nor *napA* could be detected. Primers used to amplify *narG* have successfully amplified this gene from bacteria pertaining to different *phyla* (Goregues et al. 2005). Since *napA* primers from the literature were designed for γ proteobacteria, we designed a

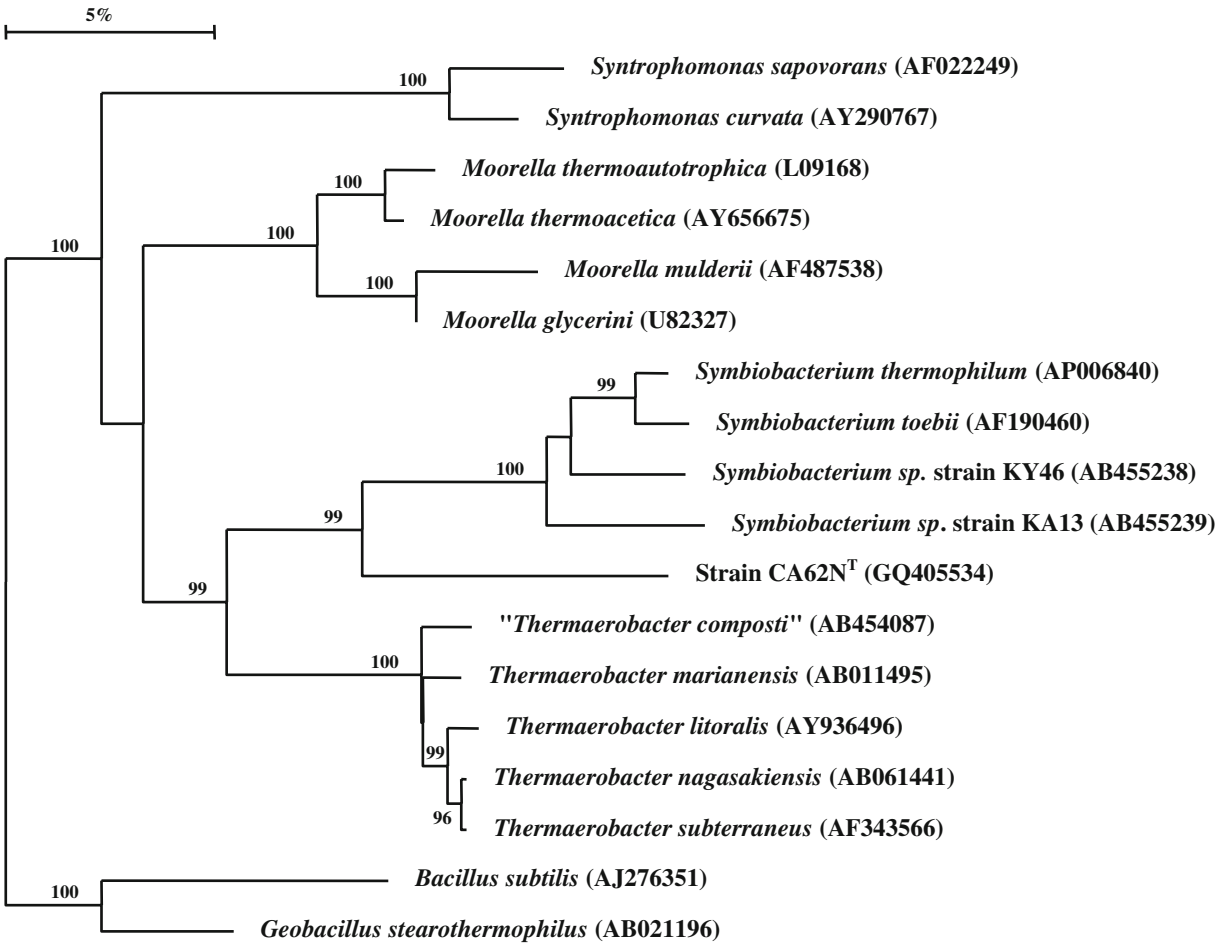

Fig. 3 Phylogenetic tree based on 16S rDNA sequence data indicating the position of strain CA62N^T among the *Symbiobacterium* branch and related bacteria. Accession numbers of 16S rDNA gene sequences of reference organisms are included in the dendrogram. *Bar* 5 nucleotide substitutions per 100 nucleotides

new set of primers that could amplify *napA* from *E. coli* but also the ORF annotated as *napA* on *Symbiobacterium thermophilum* genome, the bacterium which is the closest relative of strain CA62N^T according to 16S RNA phylogeny.

Discussion

The *Symbiobacterium* branch within the *Firmicutes* includes only two thermophilic species so far, *Symbiobacterium thermophilum* and "*S. toebii*" (Suzuki et al. 1988; Ohno et al. 2000; Sung et al. 2003; Rhee et al. 2002) which grow optimally in broth co-culture with a thermophilic *Geobacillus* sp. (Suzuki et al. 1988; Beppu and Ueda 2009). Indeed, crude extracts or culture supernatants of microorganisms pertaining to this latter genus significantly improved growth of both *Symbiobacterium* species. Interestingly, molecular techniques based on the analysis of genes encoding for the 16S rRNA have demonstrated that members of the *Symbiobacterium* branch, consisting of a large majority of uncultivated microorganisms (data not shown), have been recovered not only from compost as their primary source of enrichment but also from soil, animal feces, intestinal tract contents, and feeds (Ueda et al. 2004; Beppu and Ueda 2009). Here, we report on the isolation and characterization of a thermophilic member of this branch from a hot spring in France. Because *Symbiobacterium* species (*S. thermophilum* and "*S. toebii*"), considered as microaerophilic, have low growth yield in pure cultures (Suzuki et al. 1988; Ohno et al. 2000; Rhee et al. 2002), it has so far proven difficult to determine most of their phenotypic features (e.g. nutrient utilization, acid production from sugars, etc.), aside from their thermophilic nature. Consequently, for the reasons given above, their type of metabolism under anaerobic or microaerobic conditions has not been clearly elucidated so far. However, significant metabolism-related information has been provided with the genome sequence of *Symbiobacterium thermophilum* (Ueda et al. 2004). Those identified genes

for primary metabolism have indicated that *S. thermophilum* may (i) use sugars (e.g. glucose) and aminoacids (e.g. tyrosine, and tryptophan) and (ii) reduce nitrate into nitrite (Ueda et al. 2004). Interestingly, strain CA62N^T, despite being only distantly phylogenetically related to *Symbiobacterium thermophilum* (Fig. 3), was shown to be microaerophilic and to oxidize a wide range of substrates including sugars, peptides, and amino acids through nitrate respiration, with nitrate being reduced into nitrite. Therefore, based on phylogenetic and metabolic features of strain CA62N^T, here we confirm that this bacterium should belong to the *Symbiobacterium* branch and that members of this branch most probably share common metabolic traits, including a microaerobic, but also an anaerobic type of metabolism coupled to nitrate reduction. Surprisingly, the different primer sets, that we tested, did not allow us to amplify any gene from our isolate involved in nitrate reduction. Indeed, despite *nar* primers were successfully used to amplify in vitro the nitrate reductase gene from microorganisms pertaining to different *phyla*, *nar* gene was never found in strain CA62N^T. *Nap* gene, also, was not retrieved from this strain, although the primers designed in this study allowed us to detect *nap* gene from *E. coli* (in vitro and in silico) and *S. thermophilum* (in silico), the closest phylogenetic relative of strain CA62N^T. However, as *nar* and *nap* genes, in particular, are prime candidates for horizontal gene transfer (Stolz and Basu 2002), it cannot be ruled out that the PCR primers were inadequate for genes of a peculiar strain.

Nevertheless, with the isolation and detailed physiological characterization of strain CA62N^T from a French hot spring, we highlight other salient metabolic traits that may be also shared by members of the *Symbiobacterium* branch. They include the use of organic acids (e.g. lactate, and pyruvate) and numerous other amino acids (see Sect. Results), suggesting that growth of such bacteria in the environment may depend on the activity of proteolytic microorganisms, as already suggested for "*S. toebii*" (Rhee et al. 2002). On the basis of these results, it is clear that uncultivated members of this phylogenetic branch could be most probably enriched and isolated at high temperature, in particular by using media containing organic acids as energy sources and nitrate as terminal electron acceptor. From a phylogenetic point of view, strain CA62N^T, similarly to the two *Symbiobacterium* spp., should be related to the *Clostridia* within the *Firmicutes* (Fig. 3). The ability of strain CA62N^T together with *Symbiobacterium* species to reduce nitrate into nitrite makes them good candidates to play a significant ecological role in the nitrogen cycle of ecosystems that they inhabit. This is particularly true in composts, where *Symbiobacterium* species and related clones have frequently been retrieved (Suzuki et al. 1988; Rhee et al. 2002; Ueda et al. 2004; Beppu and Ueda 2009), but still remains questionable for strain CA62N^T which has been recovered for the first time from a thermal hot spring.

The isolation of strain CA62N^T as a member of the *Symbiobacterium* branch therefore provides significant physiological and metabolical information key to monitoring enrichments and isolation of other microorganisms in this branch. Furthermore, based on phenotypic and phylogenetic characteristics of this isolate, we propose to assign it as a novel species of novel genus of this branch within the *Firmicutes*, *Caldinitratiruptor microaerophilus*, gen. nov., sp. nov.

Description of *Caldinitratiruptor* gen. nov. (cal.di.ni.-tra.ti.rup'tor. L. adj. *caldus* hot; N.L. masc. n. *nitras-atis* nitrate; L. masc. n. *ruptor* breaker; N.L. masc. n. *Caldinitratiruptor* a hot nitrate-breaker (-reducer).

Cells stain Gram-negative, but have a Gram-positive type of cell wall. They are non-motile and non-sporulating straight rods. They are thermophilic and facultative microaerophilic. Anaerobic-type of metabolism with nitrate being used as terminal electron acceptor and reduced into nitrite; oxidizes sugars and organic acids, with acetate being the major end-product of sugar metabolism during nitrate respiration. The type species is *Caldinitratiruptor microaerophilus***.**

Description of *Caldinitratiruptor microaerophilus* sp. nov. (mi.cro.ae.ro'phi.lus. Gr. adj. *mikros* small, little; Gr. n. *aer-aeros* air; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*) loving; N.L. masc. adj. *microaerophilus* low-air loving).

Cells are facultative microaerophilic (growth up to 3–4% O_2 in the gas phase; optimum growth in the presence of 2% O_2), non-motile, non-spore-forming rods, approximately 2–3 μm in length and about 0.2 μm in diameter, generally occurring singly or in pairs. Nitrate can replace O_2 as electron acceptor and is reduced into nitrite. Stains Gram-negative, but has a Gram-positive type of cell wall. Cell cytoplasm has low density when observed under a phase-contrast microscope. Temperature range for growth is 50–75°C (optimum 65°C). NaCl is not required for growth, but is tolerated up to 10 gl^{-1}. Optimum pH is 7.0 (range 6.3–7.9). Uses lactate, pyruvate, glucose, mannose, fructose, and casamino acids and some amino acids as electron donors only in the presence of nitrate as terminal electron acceptor. None of these substrates is fermented. Glucose is oxidized to acetate, CO_2, and trace amounts of H_2 during nitrate respiration. Substrates that are not used include saccharose, raffinose, xylose, cellobiose, arabinose, sorbose, maltose, galactose, formate, acetate, succinate, starch, gelatin, and H_2. Sulfate, sulfite, thiosulfate, elemental sulfur, and nitrite are not used as terminal electron acceptors. The G + C content of DNA is 70.3 mol%.

The type strain is CA62N^T (=DSM 22660= JCM 16183) which was isolated from a thermal hot spring in the southeastern Cantal (Chaudes-Aigues, Massif Central, France).

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