



Isolation and characterization of facultative mixotrophic ammonia-oxidizing bacteria from constructed wetlands

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Abstract

Autotrophic ammonia-oxidizing bacteria (AOB) have been widely studied in constructed wetlands systems, while mixotrophic AOB have been less thoroughly examined. Heterotrophic bacteria were isolated from wastewater and rhizospheres of macrophytes of constructed wetlands, and then cultivated in a mixotrophic medium containing ammonium and acetic acid. A molecular characterization was accomplished using ITS-PCR amplification, and phylogenetic analysis based on 16S rRNA gene sequences. Results showed the presence of 35 bacteria, among 400 initially heterotrophic isolates, that were able to remove ammonia. These 35 isolates were classified into 10 genetically different groups based on ITS pattern. Then, a collection of 10 isolates were selected because of their relatively high ammonia removal efficiencies (ARE \geq 80%) and their phylogenetic diversity. In conditions of mixotrophy, these strains were shown to be able to grow (increase of optical density OD₆₆₀ during incubation with assimilation of nitrogen into cellular biomass) and to oxidize ammonia (important ammonia oxidation efficiencies, AOE between 79% and 87%). Among these facultative mixotrophic AOB, four isolates were genetically related to Firmicutes (*Bacillus* and *Exiguobacterium*), three isolates were affiliated to Actinobacteria (*Arthrobacter*) and three other isolates were associated with Proteobacteria (*Pseudomonas*, *Ochrobactrum* and *Bordetella*).

Key words: ammonia-oxidizing bacteria; mixotroph; constructed wetlands; ammonia oxidation; macrophytes' rhizosphere

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Introduction

In engineered systems such as wastewater treatment plants and constructed wetlands, the coupled nitrification and denitrification processes are regarded as the major mechanisms of nitrogen removal (You et al., 2009). Nitrification involves ammonia oxidation followed by nitrite oxidation. Ammonia oxidation is considered the limiting step of nitrification in several environments and is therefore critical to wastewater nitrogen removal (Choi and Hu, 2008). Ammonia-oxidizing bacteria (AOB) predominant in wastewater treatment plants suggest that the type of wastewater, as well as characteristics of the treatment plants, may establish significantly different environments and therefore select for different types of AOB (Park and Noguera, 2004). AOB are generally known to be autotrophic and have been widely studied (Herrmann et al., 2008; Okano et al., 2004; Wang et al., 2010).

However, some heterotrophic bacteria are also capable of ammonia oxidation (Taylor et al., 2009). Additionally, Féray and Montuelle (2003) reported that several nitrifying strains are able to grow under mixotrophic conditions in response to environmental changes, particularly the nature of the food source (Eiler, 2006). Mixotrophy was defined by Crane and Grover (2010) as the ability to use both heterotrophic and autotrophic modes of nutrition simultaneously. This metabolic type is particularly controlled by limiting nutritional factors. Mixotrophs grow using organic and inorganic compounds as carbon and energy sources (Pelmont, 1993). These bacteria may dominate aquatic environments due to their capability to use more resources than either photoautotrophic or organoheterotrophic bacteria (Eiler, 2006). They are expected to have important effects on the trophic dynamics of ecosystems, but the exact nature of these effects is unknown (Stickney et al., 2000). Also, the best-known and most widely-distributed mixotrophs are relatively few in number (Crane and Grover, 2010).

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AOB have a high ecological importance and have been detected in different ecological systems, such as soil (Wang et al., 2009), the rhizosphere (Herrmann et al., 2008), freshwater (Chen et al., 2009), and wastewater treatment systems (Qin et al., 2008; Wang et al., 2010). However, AOB have been less investigated in constructed wetlands and published studies have generally focused on autotrophic AOB (Allen et al., 2010; Gorra et al., 2007). Moreover, only a few studies have been interested in rhizospheric AOB within constructed wetland (CW) systems and, to the best of our knowledge, little is known about mixotrophic AOB in this particular ecological system.

In Tunisia, the Joogar constructed wetlands plant achieves good removal of carbon pollution, as opposed to nitrogen pollutants, mainly ammonia, that remain elevated in the treated wastewater (Kouki et al., 2009). Moreover, wastewaters flowing within this system contain both organic carbon and ammonia-nitrogen. Knowing that nitrification failure is a frequent occurrence in wastewater treatment plants (Qin et al., 2008) because of the high sensitivity of AOB to several environmental and engineering factors (Wang et al., 2010) and that heterotrophic bacteria, able to oxidize large amounts of ammonium, appear to be interesting with respect to *in situ* nitrification (De Boer and Kowalchuk, 2001), it seems to be of great interest to investigate mixotrophic AOB. Thus, the aims of this study were: (1) to isolate heterotrophic bacteria, characterized by their facultative mixotrophy and their ability to oxidize ammonia, from wastewater and rhizosphere of constructed wetlands and (2) to identify these isolates using the 16S rRNA gene sequencing method. This work was applied for

the first time in Tunisia to a constructed wetlands system.

1 Materials and methods

1.1 CWs plant description

The constructed wetlands plant of the rural community of Joogar is located in the North East of Tunisia. It treats the domestic wastewater of almost 1000 inhabitants. Influent wastewaters pass through a primary treatment pond followed by a vertical flow CW (VFCW) and finally a horizontal flow CW (HFCW) (Fig. 1). The two subsurface flow constructed wetlands contain gravel filter media planted with two types of emergent macrophytes: reeds (*Phragmites communis*) and cattails (*Typha latifolia*). The entire system is operated under gravity feed and flow of water. Purified wastewaters are discharged into a local brook.

1.2 Sample collection and rhizosphere suspension preparation

Sampling was conducted at different compartments in the constructed wetlands plant during autumn 2007. Wastewater samples were collected consecutively at the following points: the output of the VFCW (A) and the output of the HFCW (B) (Fig. 1). Sampling from the rhizosphere of macrophytes was practiced for reeds (C) and cattails (D) of the VFCW and for reeds (E) and cattails (F) of the HFCW. Rhizosphere samples were collected from each wetland at the entrance, middle, and exit at a depth of approximately 30 cm under the gravel surface. The obtained fragments were immediately placed in black

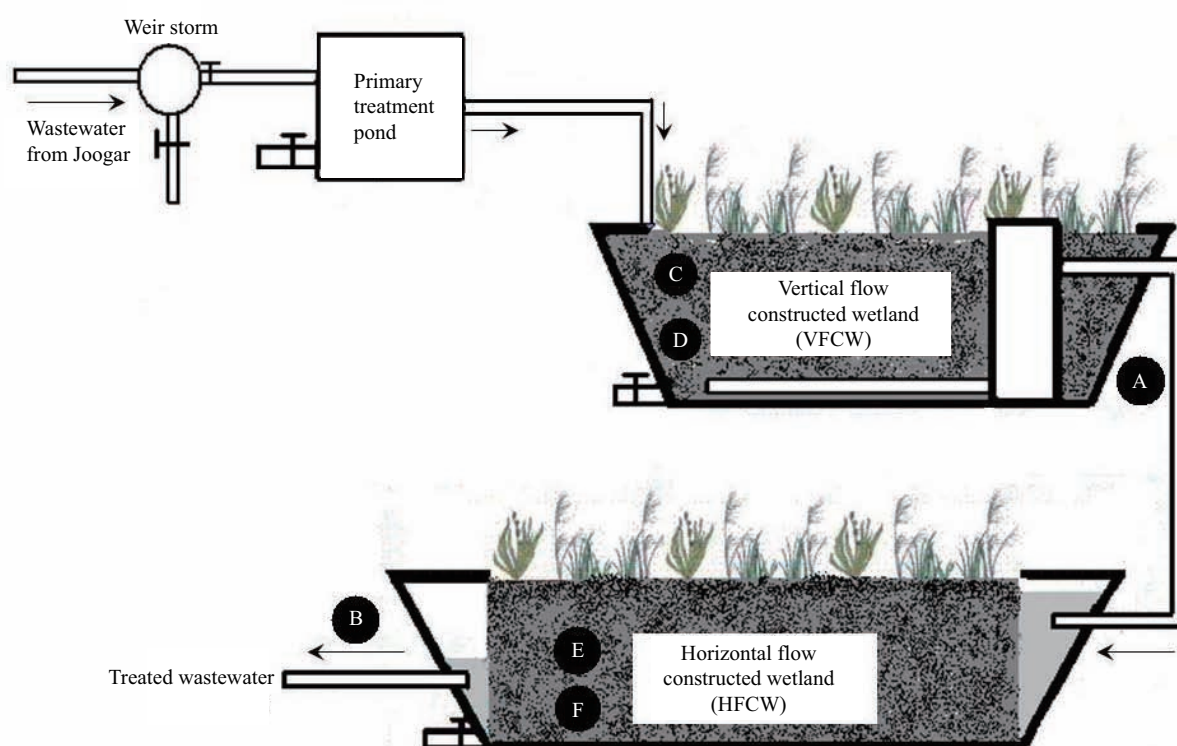


Fig. 1 Diagram of the constructed wetlands plant and location of sampling points. A, B: wastewater sampling points; C, D, E and F: sampling points of the rhizosphere of macrophytes.

plastic bags. All samples were stored at 4°C during transfer to the laboratory. Sampling was performed in triplicates. Rhizosphere suspensions (suspensions containing cells liberated from the rhizosphere) were prepared by extraction of the thin layer of sludge adsorbed to the roots. These roots were separated from the rhizomes and cut into 1 cm fragments after hand-shaking in order to separate off sludge particles not adhering tightly to the roots. The obtained fragments were introduced into a phosphate buffer solution (pH 7; 1:10, W/V) and then mechanically shaken at a speed of 350 r/min during 2 hr at room temperature (20°C) to release the roots' adsorbed biomass.

1.3 Pre-selection of mixotrophic strains

Different types of bacterial colonies were obtained after spreading 0.5 mL of water or rhizosphere suspensions on trypticase-soy-agar (TSA) plates and incubation at 25°C for 24–48 hr. This organic medium was used without adding autotrophic nitrification inhibitor N-serve (2-chloro-6-trichloromethyl pyridine (Goring, 1962). Then, colonies were sub-cultured in trypticase-soy-broth (TSB) at 25°C for 24–48 hr with shaking at 250 r/min. Thereafter, 0.5 mL of each bacterial culture was inoculated into Erlenmeyer flasks containing 50 mL of a mixotrophic medium composed by the Schmidt and Belser (1994) mineral medium supplemented with acetic acid according to the following composition in g/L: (NH₄)₂SO₄ 0.5; MgSO₄·7H₂O 0.04; KH₂PO₄ 0.203; CaCl₂·2H₂O 0.013; iron chelate 0.01; trace elements 0.01 and acetic acid 1. Acetic acid was added as an organic carbon source. The pH of the culture medium was adjusted to 7.2 using 1 mol/L NaOH. This mixotrophic medium reproduces the characteristics of the effluent of the Joogar CWs plant, which is overloaded with ammonia-nitrogen and contains residual organic carbon. Incubation was carried out at 25°C during 30 days in the dark with mechanical agitation at 200 r/min. This step of pre-culture involves the adaptation of previously heterotrophically isolated bacteria to the mixotrophic medium.

1.4 Ammonia removal assay and bacterial classification

The bacterial cells were recovered by 4000 r/min centrifugation for 10 min and then transferred back into the same mixotrophic medium under the same culture conditions mentioned in Section 1.3. This medium, containing 51.3 mg/L of ammonium as the sole nitrogen source, was used for detection of the bacterial capacity to remove ammonium under aerobic conditions. Subsequently, aliquots from each culture were used for a residual ammonium assay. For this, 2 mL of Seignette's salt solution (potassium sodium tartrate) and 2 mL of Nessler reagent were incorporated with 1 mL of bacterial culture. The standard solution was prepared from a concentrated NH₄Cl solution (10 mmol/L). Ammonium rates were assessed spectrophotometrically at a wavelength of 420 nm. Then, ammonium removal efficiency (ARE, %) was calculated by Eq. (1):

$$\text{ARE} = ((C_0 - C_a)/C_0) \times 100\% \quad (1)$$

where, C₀ (mg/L) and C_a (mg/L) are the initial and the final ammonium concentrations, respectively. ARE were used for bacterial classification using the SPSS for Windows Program, version 10, SPSS Inc., USA.

1.5 Test of mixotrophic growth and ammonia oxidation

A collection of 10 selected isolates were cultured again in the mixotrophic medium (51.3 mg/L of ammonium) (Section 1.3) and their capacity to grow and to oxidize ammonium was assessed. Sodium chlorate (1 mmol/L) was added to the culture medium to inhibit the oxidation of nitrite to nitrate (Belser and Mays, 1982). In fact, some heterotrophic bacteria, such as *Arthrobacter* sp. grown in a mixotrophic culture medium containing ammonium and acetate, are able to oxidize both ammonia and nitrite (Verstraete and Alexander, 1972). On the other hand, some heterotrophic bacteria could achieve heterotrophic nitrification and aerobic denitrification, accordingly oxidizing ammonia to nitrate, through nitrite, and reducing nitrate to nitrogen gas (Matsuzaka et al., 2003). Bacterial growth was assessed in 1 mL culture samples via the measure of optical density (OD₆₆₀) by a spectrophotometer (Philips PU 8620 series UV/Visible, Cambridge, UK) at 660 nm. Measurements were performed at 5, 15, and 25 days of incubation.

At the end of incubation period, aliquots from each culture were used for determination of biomass total nitrogen, ammonium and nitrite concentrations. Biomass total nitrogen measurement was accomplished using the Kjeldahl method modified by Hiller et al. (1948) and expressed as µg/g dry weight (dw). The dw was evaluated applying the method described by Wade (1952). Ammonium content was quantified as described in Section 1.4. Produced nitrite was assessed using the diazotization method. For this, 0.1 mL of the diazotization reagent (sulfanilic acid: 0.26 g; N1-Naphtyl-ethylenediamine dihydrochloride: 0.6 g) was incorporated with 5 mL of bacterial culture, mixed and left standing for 10 min. The standard solution was prepared from a concentrated NaNO₂ solution (10 mmol/L). Nitrite concentrations were assessed spectrophotometrically at 543 nm. Ammonia oxidation efficiency (AOE, %) was calculated by Eq. (2):

$$\text{AOE} = (C_n/C_0) \times 100\% \quad (2)$$

where, C_n (mg/L) is the final nitrite concentration and C₀ (mg/L) is the initial ammonium concentration.

1.6 DNA extraction, PCR amplification and bacterial grouping

Bacterial DNA was extracted according to the protocols developed by Chen and Kuo (1993) for Gram-negative bacteria and by Sohail (1998) for Gram-positive bacteria. The purified DNA was precipitated with ethanol, resuspended in Tris-EDTA-RNase buffer (10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, pH 8.0, RNase 10 µg/mL) and stored at -20°C.

ITS PCR amplification targeting ITS fragments was performed using the universal primers: ITSr (5'-CAAGGCATCCACCGT-3') and ITSf (5'-

GTCGTAACAAGGTAGCCGTA-3') (Alm et al., 1996). ITS fragments correspond to intergenic 16S–23S internally transcribed spacer sequences. PCR amplification was carried out in 25 μ L of reaction mixture. The reaction mixture was prepared in 1 \times buffer, 1.5 mmol/L MgCl₂, 2 μ L template DNA, 1 μ mol/L primers, 0.25 mmol/L dNTP and 1 U *Taq* DNA polymerase. The thermal profiles included an initial denaturing step consisting of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec and elongation at 72°C for 1 min. The cycle was ended by a final elongation step at 72°C for 7 min. Aliquots of the amplification products were analyzed using agarose gel (1.5%) electrophoresis.

Bacterial grouping based on ITS patterns was performed using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) algorithm of MVSP 3.131 software to produce a dendrogram of the genetic relationships between bacterial isolates.

1.7 16S rRNA gene sequencing and nucleotide sequence accession numbers

Bacterial isolates with the highest ARE were selected for phylogenetic analysis. For this, 16S rRNA genes of selected strains were PCR amplified using two universal primers: 16SR (5'-CTACGGCTACCTTGTTACGA-3') and 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') (Alm et al., 1996). PCR conditions were applied as described for ITS-PCR amplification.

Purified PCR products of 16S rRNA genes were then subjected to a sequence analysis. Sequence similarity searches were conducted using the BLAST network service of the GenBank database to identify the nearest relatives of the obtained sequences. Similarity levels among bacteria related to the correspondent sequences were performed based on the neighbour-joining method.

The studied sequences were deposited in the GenBank database under the following accession numbers: NC009848, NC009668, NC010556, NC008541, NC010322, NC008711, NC004604 and NC010170.

2 Results

2.1 Classification of bacterial isolates according to ammonia removal

A collection of 400 bacterial isolates, with different origins from the Joogar CWs plant and diverse phenotypic characteristics, were obtained on TSA plates. Then, the ammonia removal test was used to classify bacteria based on their ARE (Table 1). Bacterial isolates were subdivided into 5 bacterial classes (BC) corresponding to five intervals of ARE. We noted the absence of isolates achieving ARE in the range between 59% and 79%. Bacterial isolates of the class BC1 were inefficient at ammonia removal. They were predominant, representing a majority of all isolates with 359. The class BC2 showed relatively low ARE with values under 20%. However, the remaining 35 isolates belonging to classes BC3, BC4 and BC5 were

Table 1 Classification of bacterial isolates based on their ammonia removal efficiency (ARE)

ARE (%)	Bacterial class	Number of isolates
< 2	BC1	359
2–19	BC2	6
21–39	BC3	24
41–58	BC4	1
\geq 80	BC5	10

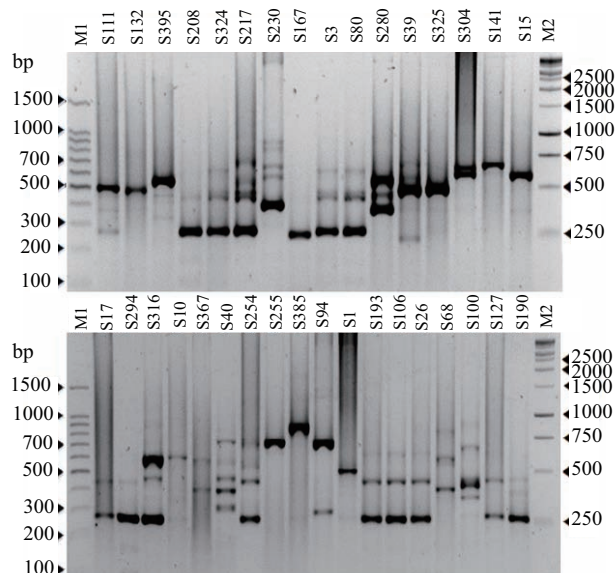


Fig. 2 PCR amplification of ITS fragments of the 35 bacterial isolates. M1: molecular mass marker 100 bp DNA ladder; M2: molecular mass marker 1 kb DNA ladder. The remaining lanes correspond, each one, to the bacterial isolates.

able to remove ammonium from the mixotrophic culture media with more or less considerable levels of ARE. The class BC5 contained 10 isolates which achieved the highest ARE, exceeding 80%.

2.2 Genetic grouping of ammonia removing bacteria

Thirty-five bacterial isolates occupying BC3, BC4 and BC5 classes (Table 1) were selected for molecular classification based on their genetic patterns after amplification of ITS fragments. Analysis of these patterns revealed heterogeneous bands in terms of size (Fig. 2). In general, amplicons ranged approximately from 250 to 800 bp. However, several isolates showed common haplotypes. For example, strains S324, S3, and S80 were each characterized by four bands of about 250, 420, 480 and 600 bp in size. Thereafter, ITS patterns were used to build a dendrogram for genetic classification of bacterial isolates (Fig. 3). Bacteria were classified into 10 different phylogenetic groups. We noted also that the 10 isolates of the class BC5 were each classified in a different group: isolates S385, S217 and S230 formed the groups G1, G4 and G8, respectively; S316, S338, S41, S255, S106, S94 and S325 belonged to G2, G3, G5, G6, G7, G9 and G10, respectively. Since they represent each group, the 10 bacterial isolates were selected for assessment of their mixotrophic growth and ammonia oxidation ability and then for sequencing.

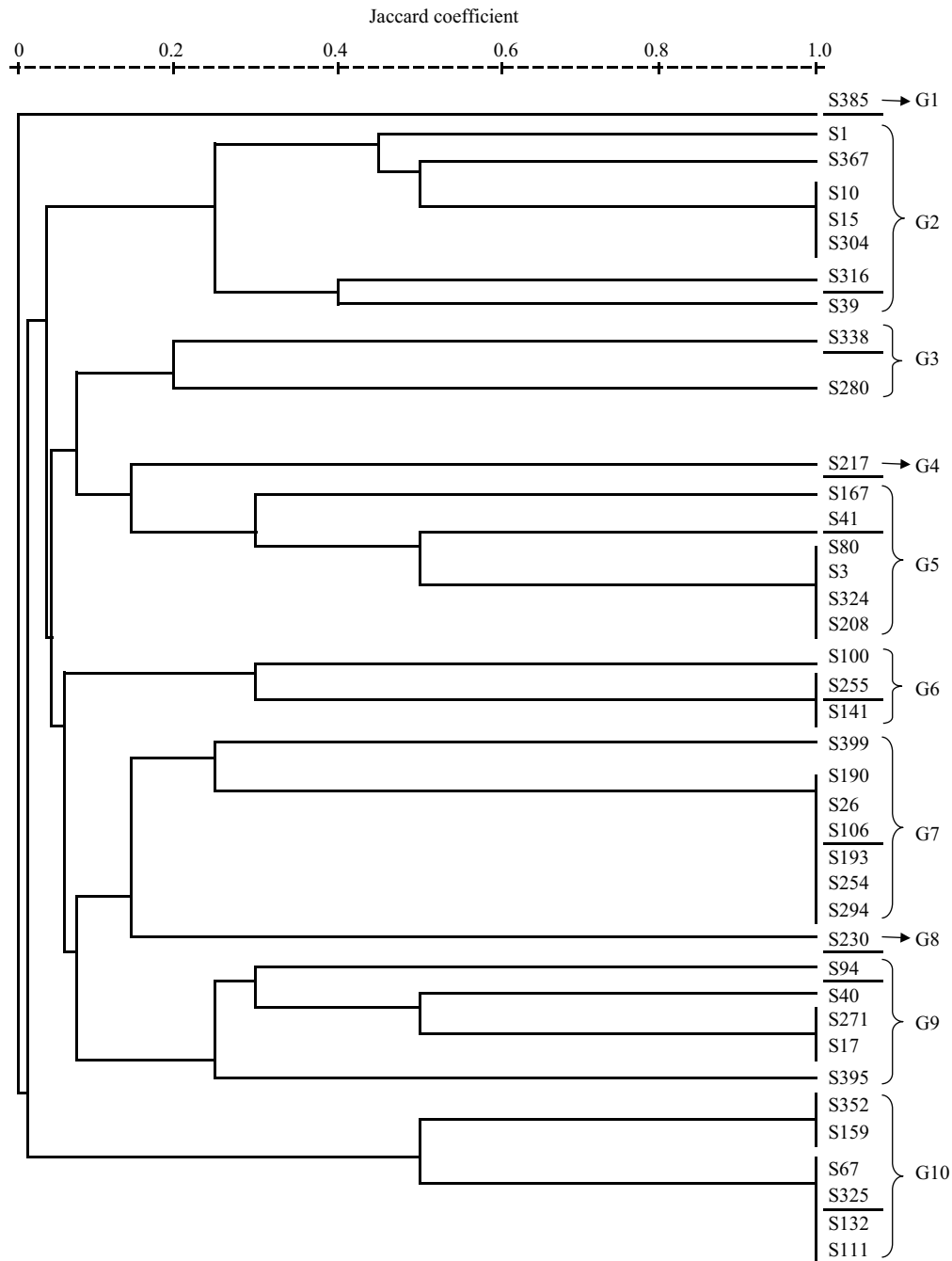


Fig. 3 Genetic grouping based on the comparative analysis of ITS fragments of the 35 bacterial isolates. Analysis was performed using the UPGMA method/Jaccard Coefficient (MVSP version 3.131; Kovach, 1995). The 10 underlined strains correspond to the bacterial class BC5. G: phylogenetic group.

2.3 Bacterial growth and ammonia oxidation capability

Table 2 presents OD_{660} , ammonium and nitrite concentrations and biomass total nitrogen content measured in bacterial cultures.

The resulting values showed that OD_{660} increased during the culture assay. Differences were noticed between strains demonstrating various growth rates. Meanwhile, evolution of OD_{660} corresponding to isolates S217, S106 and S316 showed close shapes over the time. As well, do OD_{660} of strains S94, S255 and S325. On the other hand, important concentrations of nitrite, versus low residual ammonium contents, were detected at the end of incubation period. Then, significant AOE ranging between 79% and

87% were achieved. Considerable total nitrogen contents were also obtained in isolates' biomass, with maximal value of $9.1 \mu\text{g/g dw}$ registered for strain S385.

2.4 Sequence analysis and phylogenetic assignment

The 16S rRNA gene sequences of the 10 selected bacterial isolates were determined. Phylogenetic analysis revealed that these bacteria were clustered within three phyla: Firmicutes, Actinobacteria and Proteobacteria (Fig. 4, Table 3). Figure 4 shows the result of neighbour-joining cluster analysis of mixotrophic bacterial isolates.

Strains belonging to Firmicutes were subdivided into two genera: *Bacillus* and *Exiguobacterium*. Indeed, isolates S106 and S217 appear to be phylogenetically

Table 2 Bacterial growth and ammonia oxidation assessment

Isolate	OD ₆₆₀			Nitrogen forms			AOE (%)
	5 days	15 days	25 days	BTN (µg/g dw)	NH ₄ ⁺ (mg/L)	NO ₂ ⁻ (mg/L)	
S217	0.16 ± 0.02	1.40 ± 0.33	2.10 ± 0.18	4.9 ± 0.4	4.2 ± 0.3	42.2 ± 0.6	82 ± 1
S106	0.21 ± 0.07	1.26 ± 0.12	1.93 ± 0.09	3.7 ± 0.2	4.8 ± 0.5	42.8 ± 0.2	84 ± 2
S316	0.18 ± 0.04	1.12 ± 0.20	2.16 ± 0.22	4.8 ± 0.3	4.1 ± 0.2	42.4 ± 0.4	83 ± 2
S94	0.05 ± 0.01	0.15 ± 0.04	0.23 ± 0.07	8.1 ± 0.2	1.5 ± 0.4	41.7 ± 0.7	81 ± 4
S255	0.06 ± 0.00	0.16 ± 0.02	0.24 ± 0.01	7.1 ± 0.2	2.9 ± 0.3	41.3 ± 0.4	81 ± 2
S325	0.06 ± 0.02	0.13 ± 0.01	0.22 ± 0.04	8.8 ± 0.2	2.1 ± 0.2	40.4 ± 0.5	79 ± 2
S41	0.41 ± 0.11	1.36 ± 0.22	2.09 ± 0.10	5.0 ± 0.4	1.9 ± 0.6	44.4 ± 0.3	87 ± 5
S338	0.12 ± 0.05	0.17 ± 0.06	0.19 ± 0.04	4.3 ± 0.1	2.8 ± 0.5	44.2 ± 0.2	86 ± 4
S230	1.15 ± 0.20	1.89 ± 0.19	1.93 ± 0.38	6.8 ± 0.4	1.7 ± 0.4	42.8 ± 0.3	83 ± 3
S385	0.48 ± 0.13	2.51 ± 0.23	3.60 ± 0.52	9.1 ± 0.3	1.2 ± 0.1	41.0 ± 0.7	80 ± 5

OD₆₆₀: optical density at 660 nm; BTN: biomass total nitrogen; dw: dry weight; AOE: ammonia oxidation efficiency. Data are expressed as mean ± standard deviation.

Table 3 Partial sequences analysis of 16S rRNA gene sequences of the mixotrophic bacterial isolates

Isolate	An	Most related organisms	Pg	Ss (%)	Sl (bp)	Origin*
S217	NC.009848	<i>Bacillus pumilus</i> SAFR-032	Firmicutes	100	711	RT_HFCW
S41	NC.009668	<i>Ochrobactrum anthropi</i> ATCC 49188	Proteobacteria	99	611	RT_VFCW
S338	NC.010556	<i>Exiguobacterium sibiricum</i> 255-15	Firmicutes	98	756	RT_VFCW
S106	NC.009848	<i>Bacillus pumilus</i> SAFR-032	Firmicutes	98	597	RR_VFCW
S94	NC.008541	<i>Arthrobacter</i> sp. FB24	Actinobacteria	97	703	RR_HFCW
S230	NC.010322	<i>Pseudomonas putida</i> GB-1	Proteobacteria	97	471	E_VFCW
S255	NC.008541	<i>Arthrobacter</i> sp. FB24	Actinobacteria	96	742	RT_VFCW
S325	NC.008711	<i>Arthrobacter aureescens</i> TC1	Actinobacteria	88	689	RT_HFCW
S316	NC.004604	<i>Bacillus megaterium</i> QMB1551	Firmicutes	87	683	E_HFCW
S385	NC.010170	<i>Bordetella petrii</i> DSM 12804	Proteobacteria	81	517	RR_HFCW

An: accession number; Pg: phylogenetic group; Ss: sequence similarity; Sl: sequence length.

* RT: rhizosphere of *Typha*; RR: rhizosphere of reeds; E: effluent; VFCW: vertical flow constructed wetland; HFCW: horizontal flow constructed wetland.

related to *Bacillus pumilus* with sequence similarity of 98% and 100%, respectively (Table 3). Strain S316 appeared to be genetically closer to *Bacillus megaterium* with 87% homology. Isolate S338 obtained from the rhizosphere of *Typha* shows sequence homology of around 98% with *Exiguobacterium sibiricum*. The three strains corresponding to Actinobacteria are related to species of the *Arthrobacter* genus. Indeed, isolates S94 and S255 showed relatedness degrees of 97% and 96% respectively with *Arthrobacter* sp. FB24. These two strains were isolated from the macrophytes rhizosphere of the HFCW. Strain S325 was identified as phylogenetically related to *Arthrobacter aureescens* with a similarity of around 88%. Concerning isolates S41, S230 and S385, they were detected to be genetically related to classes α -, β - and γ -Proteobacteria phylum. Bacterial strain S41 was related to *Ochrobactrum anthropi* of the Alphaproteobacteria class. This isolate originated from *Typha* roots of the VFCW. Strain S230 was phylogenetically related to *Pseudomonas putida*, belonging to the class Gammaproteobacteria. Finally, S385 isolated from the rhizosphere of HFCW reeds appeared to be genetically similar to *Bordetella petrii* of the Betaproteobacteria.

3 Discussion

This study focused on the test of ability of heterotrophically isolated bacteria to grow and to oxidize ammonia-nitrogen in a mixotrophic culture medium, containing ammonium as inorganic nitrogen and acetic acid

as organic carbon. Indeed, Hagopian and Riley (1998) stated that a mixotrophic medium contains both ammonia and organic matter. The particular use of acetate as an organic compound that can be metabolized by AOB in mixotrophic conditions was cited by several authors (Martiny and Koops, 1982; Wallace et al., 1970). Moreover, Wallace et al. (1970) reported that acetate is rapidly and highly assimilated by nitrifying bacteria. Then, a collection of heterotrophic bacteria were initially isolated from wastewaters and macrophytes' rhizosphere of the Joogar CWs plant. Mixotrophic ammonia oxidation test aimed to simulate the nutritional conditions of the Joogar constructed wetlands' wastewaters, containing inorganic nitrogen (ammonium) and organic carbon. A number of 35 isolates within the obtained bacterial collection (400 isolates), were selected because of their capacity to remove ammonia-nitrogen in the presence of acetate. These strains were characterized by various ARE and diverse haplotypes belonging to different phylogenetic groups. Among these bacteria, 10 isolates were able to achieve the highest ARE exceeding 80% in the mixotrophic culture medium.

Knowing that ammonia could be removed by both assimilation into cells, for cell growth, and oxidation by AOB (Harms et al., 2003), these processes were assessed in pure cultures of the 10 selected bacterial strains. Accordingly, bacterial growth in the cited mixotrophic medium was evaluated by measuring OD₆₆₀ during incubation. Increase of OD₆₆₀ confirmed that bacterial strains, isolated from CWs, were able to grow in the mixotrophic culture medium, after heterotrophically isolation. By the same way,

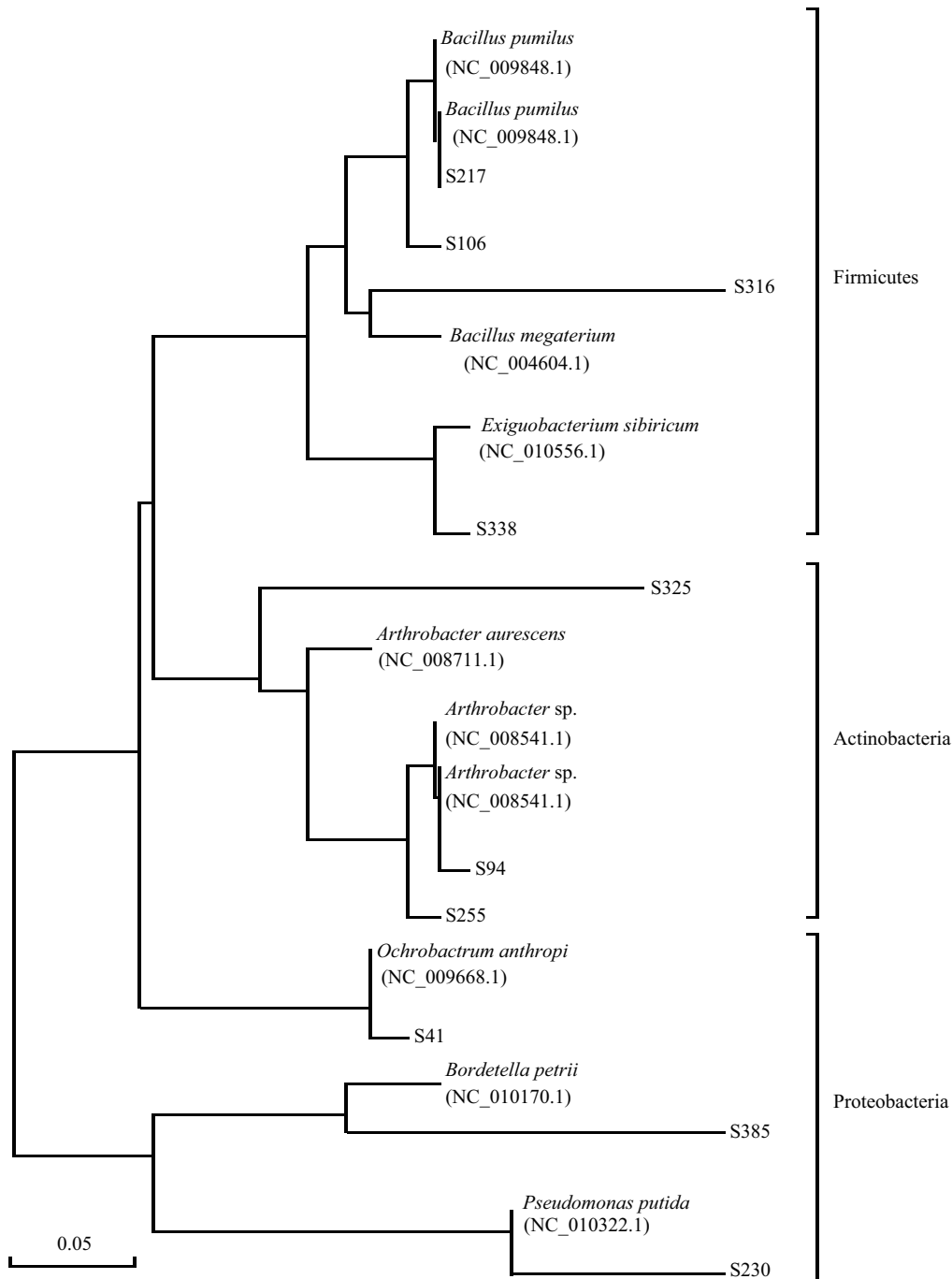


Fig. 4 Neighbour-joining tree representing the phylogenetic relationship of the 16S rRNA gene sequences of the mixotrophic AOB isolates to closely related sequences obtained from BLAST searches. The scale indicates genetic distance. The numbers between parentheses are GenBank accession numbers.

Prinčič et al. (1998) showed that heterotrophic nitrifiers are able to grow in culture media containing 50 mg/L ammonium. Thus, bacterial growth would be consistent with facultative mixotrophy of isolates. This metabolic form was likely involved by the presence of ammonium substituting organic nitrogen of the previous isolating medium (TSA). Likely, isolated strains switched to the use of this ammonium during their growth. According to Stoecker (1998), this metabolic switch might be the factor by which an organism can survive and reproduce effectively in dynamic systems like aquatic microbial communities. Growth of the 10 strains was further demonstrated by the considerable nitrogen assimilation within cell biomass.

Then, facultative mixotrophy of these bacterial isolates could be deduced.

On the other hand, bacterial growth was accompanied by a significant decrease in ammonium concentrations in favour of nitrite production by the 10 isolates. Then, ammonia disappearance and nitrite production were assumed to be consequences of ammonia oxidation process, suggesting that bacterial isolates catalyzed ammonia oxidation to nitrite. By the same way, Young et al. (2010) showed that the reaction from ammonia to nitrite is catalyzed by bacterial metabolism. Also, this finding agrees with the result previously reported by Robertson and Kuenen (1988) who obtained ammonia loss from heterotrophic

bacteria grown in mixotrophic cultures with acetate and ammonia.

Based on their important ammonia oxidation ability, the 10 AOB were selected for molecular identification. Sequencing of 16S rRNA genes showed a genetic diversity of these facultative mixotrophic isolates. Species within the Firmicutes, the Actinobacteria, and the Proteobacteria phyla were identified. This result supports the existence of diverse mixotrophic AOB in the constructed wetlands' compartments. Similar classifying groups were also reported by Wang et al. (2007) for nearly 400 bacterial strains from the rhizosphere of macrophytes of estuarine marshes. Three isolates from wastewater and rhizospheres were affiliated with two species of *Bacillus* genus: *B. pumilus* and *B. megaterium*. Similarly, Borsodi et al. (2007) have isolated bacterial strains affiliated with these two *Bacillus* species which originated from a reed roots habitat. In fact, *B. megaterium* does not require the presence of an organic source to achieve its growth, being able to be satisfied by a minimum medium containing ammonium for its survival. Moreover, this species is characterized by its ability to grow in extreme environmental conditions due to its spore forming capacity (Dong and Reddy, 2010). Another bacterial isolate from the roots of *Typha* was identified as *Exiguobacterium sibiricum*. This is in accordance with results obtained by Sawayama (2006), who showed that some species of the genus *Exiguobacterium* are involved in the ammonium oxidation process. Recently, *E. sibiricum* was also isolated from the rhizosphere of tomato plants (Lioussanne et al., 2010). Furthermore, Fall et al. (2004) reported that the genera *Bacillus* and *Exiguobacterium*, as well as *Arthrobacter*, are characterized by resistance to environmental stress conditions.

Three other bacterial strains, those isolated from the rhizospheric zone of the constructed wetlands' macrophytes, were genetically identified as *Arthrobacter* species. The nitrification capacity of this genus was effectively observed in isolates from diverse environmental ecosystems including lake waters (Witzel and Overbeck, 1979), soils (Brierley and Wood, 2001), and sewage sludge (Kapley et al., 2007).

The remaining AOB isolates were affiliated with *Ochrobactrum*, *Pseudomonas* and *Bordetella* genera belonging to the Proteobacteria phylum. Recently, bacteria of these genera were also isolated from activated sludge (Kapley et al., 2007). This finding is consistent with that obtained by Teske et al. (1994) who reported that nitrifying bacteria are common among the large group of Proteobacteria. In other studies, the species *Ochrobactrum anthropi* has been isolated from soil samples, sewage, and wheat rhizospheres (Thoma et al., 2009) and has been involved in the ammonium-nitrogen removal process (Xia et al., 2008). Similarly, the role of *Pseudomonas putida* in nitrification in wastewater was previously reported (Xia et al., 2008). According to Gross et al. (2008), *Bordetella petrii* is the only naturally occurring species of *Bordetella* genus known to be pathogenic. Furthermore, this bacterium was found in different ecological niches such as

sewage, sediment, soil, and plant roots (Chowdhury et al., 2007; Gross et al., 2008; Von Wintzingerode et al., 2001). It was similarly isolated from reed roots in this study. Recently, *Bordetella* sp. has been reported as an arsenite-oxidizing bacterium in treatment wetlands planted with *Typha* treating acid mine drainage (Faulwetter et al., 2009). To our knowledge, this is the first time that the species *Bordetella petrii* has been isolated from the rhizosphere of macrophytes in a CW system as a mixotrophic AOB.

The majority of AOB isolates obtained in this work belonged to the rhizospheres of reeds and cattails. In fact, AOB play a major role in the N-cycle in the root environment (Briones et al., 2002). Furthermore, emergent aquatic plants are characterized by oxygen transfer into their roots, thus facilitating nitrification and the establishment of nitrifiers installation in the immediate vicinity of roots (Kadlec and Wallace, 2009). The release of oxygen by roots can influence the type of microorganisms present in the rhizosphere (Kantawanichkul et al., 2009). In particular, the rhizosphere of macrophytes appears to be the main site for ammonium oxidation. Gorra et al. (2007) found that ammonium oxidation rates were higher in the root zone versus the bulk soil in an ammonia-rich subsurface-flow treatment wetland. According to Osem et al. (2007), incorporation of plant roots into the wetland substrate may provide unique attachment sites for certain microbial populations. This also indicates the importance of the presence of macrophytes in CW systems, mainly in treatment processes such as ammonium removal.

4 Conclusions

In this study, 35 bacterial strains were isolated from different compartments of the Joogar constructed wetlands plant, based on their ammonia removal capability. Among them, a collection of 10 isolates were characterized by high ARE and different phylogenetic affiliations. These bacteria were shown to be facultative mixotrophic ammonia oxidizers. The 10 AOB were identified within the three phyla of Firmicutes (four isolates), Actinobacteria (three isolates) and Proteobacteria (three isolates). The majority of these isolates belonged to the rhizospheres of reeds and cattails, likely indicating their capacity to form protective biofilms. This work needs to be continued by studying the nitrification activity and biofilm-forming capacity of these isolates to understand their contribution to the treatment process in constructed wetland systems.

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