Bosea minatitlanensis sp. nov., a strictly aerobic bacterium isolated from an anaerobic digester

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A strictly aerobic, mesophilic bacterium, strain AMX 51^T, was isolated from anaerobic digester sludge. Cells were Gram-negative, motile, non-sporulating, straight to curved rods with one polar flagellum. The isolate had phenotypic traits of the genus *Bosea*, including cellular fatty acid and substrate utilization profiles. Physiological characteristics and antibiotic susceptibility were determined. Phylogenetic analysis revealed that strain AMX 51^T was a member of the α -*Proteobacteria*, most closely related to *Bosea thiooxidans* DSM 9653^T (similarity of 98.88%). *Methylobacterium organophilum* JCM 2833^T, *Methylobacterium mesophilicum* JCM 2829^T, *Afipia clevelandensis* DSM 7315^T, *Afipia felis* DSM 7326^T, *Afipia broomeae* DSM 7327^T, *Blastobacter denitrificans* LMG 8443^T and *Bradyrhizobium japonicum* DSM 30131^T showed significant 16S rRNA gene sequence similarities to strain AMX 51^T. The DNA G + C composition of strain AMX 51^T was 68.5 mol%. DNA–DNA hybridization analysis revealed 44.2 and 15.1 % relatedness between strain AMX 51^T and the respective type strains of *Bosea thiooxidans* and *A. felis*. Overall results suggest that strain AMX 51^T (=DSM 13099^T=ATCC 700918^T=CIP 106457^T) represents a novel species of the genus *Bosea*; the name *Bosea minatitlanensis* sp. nov. is proposed.

The genus *Bosea* is phylogenetically placed in the α -*Proteobacteria* (Das *et al.*, 1996; Stubner *et al.*, 1998). It was initially described with one species, *Bosea thiooxidans*, isolated from agricultural soils as a free-living micro-organism that was capable of oxidizing reduced inorganic sulfur compounds (Das *et al.*, 1996). Later, a strain designated 5Z2111, which was closely related to *B. thiooxidans* (97.3 % 16S rRNA sequence similarity), was isolated from rice field soil (Stubner *et al.*, 1998). Isolate 5Z2111 differs from the type strain of *B. thiooxidans* in its ability to grow autotrophically with CO₂ as a carbon source. Comparing all the characteristics of the type strain of *B. thiooxidans* and strain 5Z2111, the authors assumed that the latter probably represented a separate species and perhaps genus.

To our knowledge, the taxonomic status of strain 5Z2111 as a novel species of *Bosea* has not been established, and this strain is not available in any public collection.

During a study of the role of strictly aerobic bacteria in anaerobic digesters, enumeration and identification of these micro-organisms were performed using a laboratory-scale upflow anaerobic sludge blanket (UASB) reactor fed with the wastewater of a petrochemical company producing purified terephthalic acid. Eighty-five strains were isolated and subjected to identification by classical biochemical methods, analysis of the cellular fatty acids (CFA) and/or partial 16S rRNA gene sequence analysis. The taxonomic methods used could identify 87 % of the isolates accurately. Comparison of the CFA profiles of the unknown strains by unweighted arithmetic average clustering allowed separation of the strains into two groups. All of the strains in the first group were later shown to belong to one species, *Stenotrophomonas acidaminiphila* (Assih *et al.*, 2002). The second group was represented by a single isolate, strain AMX

Abbreviations: CFA, cellular fatty acid; UASB reactor, upflow anaerobic sludge blanket reactor.

The GenBank accession number for the 16S rRNA gene sequence of strain AMX 51^T is AF273081.

51^T (=DSM 13099^T=ATCC 700918^T=CIP 106457^T). This strain was then subjected to more-detailed taxonomic study. Its characterization is reported as the type strain of a novel species of the genus *Bosea*, *Bosea minatitlanensis* sp. nov.

Strain AMX 51^T was isolated from the anaerobic sludge of a laboratory-scale UASB reactor using R2A medium (Oxoid). Purification and culture procedures were described elsewhere (Assih *et al.*, 2002). *B. thiooxidans* DSM 9653^T and *Afiplia felis* DSM 7326^T were obtained from the DSMZ. Procedures for analytical techniques, determination of general phenotypic characteristics, antibiotic susceptibility, DNA G + C content and CFA composition, 16S rRNA sequencing and DNA–DNA hybridization are described elsewhere (Assih *et al.*, 2002). Thiosulfate oxidation was tested in basal medium (Assih *et al.*, 2002) with maltose or Casamino acids as substrate. Additional biochemical analysis was performed by inoculating Biotype 100 strips (bioMérieux) according to the manufacturer's instructions. Biotype medium 1 was used for inoculum preparation. For phylogeny, a non-redundant BLASTN search (Altschul *et al.*, 1997) of the full sequence through GenBank and EMBL (Benson *et al.*, 1999) identified its closest relatives. Sequence data were imported into the sequence editor BioEdit version 5.0.9 (Hall, 1999); the bases were examined and a contiguous consensus sequence was generated for each isolate. The full sequence was aligned using the RDP Sequence Aligner program (Maidak *et al.*, 2001). The consensus sequence was then adjusted manually to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 1999). Positions of sequence and alignment ambiguity were omitted and pairwise evolutionary distances based on 1350 unambiguous nucleotides (16S rRNA) were calculated using the method of Jukes & Cantor (1969). Dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined using 100 bootstrapped trees (Felsenstein, 1985).

After 2–10 days at 30 °C, strain AMX 51^T formed non-pigmented, circular colonies on trypticase soy or R2A agar. Growth was not accompanied by odour. Further attempts to obtain other colonies or strains with phenotypic characteristics similar to those of strain AMX 51^T were unsuccessful. Such results suggest that this bacterium was probably only a transient micro-organism in the reactor, which was fed with non-sterilized wastewater, from which it was isolated. Cells of isolate AMX 51^T were straight to curved rods, slowly motile and stained Gram-negative. Electron microscopy observations showed that the cell wall structure of the isolate was typical of Gram-negative bacteria and indicated a monotrichous polar flagellation type (data not shown). Spore formation was not observed. Cells occurred singly or in pairs and were 0.5 × 1.5–2.0 µm in size. Strain AMX 51^T was strictly aerobic, as shown by the absence of growth after 1 month

incubation at 35 °C in an anaerobic jar on R2A medium. Growth was observed between 15 and 42 °C, with optimum growth at 37 °C. No growth occurred at ≤4 or ≥45 °C. The pH range for growth was 5.0–8.5, with optimum growth at pH 6.0. The maximum growth rate determined in rich medium (Assih *et al.*, 2002) was approximately 0.237 h⁻¹ at 37 °C, pH 7.0, and 0.201 h⁻¹ at 35 °C, pH 6.0. Strain AMX 51^T was positive for oxidase, catalase, urease and amylase and weakly proteolytic. Tests for nitrite reductase, nitrate reductase, indole production, aesculin, Simmons' citrate, ONPG, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, DNase and Tween 80 esterase were negative. The substrate utilization tests performed on API 20NE and Biotype 100 strips or in defined basal liquid medium (Assih *et al.*, 2002) showed that strain AMX 51^T was able to use several amino acids, organic acids and methanol (for detailed list, see below). Discrepancies in the utilization of substrates were observed between the different tests. Whereas utilization of L-histidine, L-serine, L-tyrosine and succinate was positive in Biotype 100, these tests were negative in the defined medium, and vice versa for L-aspartate, fumarate, glycerol and propionate. Assimilation of all other substrates present on Biotype 100 (i.e. 74 out of 99) was negative. Negative results were also obtained for assimilation of choline, L-isoleucine, DL-leucine, L-methionine, D-ornithine, DL-threonine, DL-valine, oxalate and starch tested in basal liquid medium and adipate tested with API 20NE. With the API 50CH strips, none of the substrates was acidified after 9 days incubation. Thiosulfate oxidation was observed in the presence of succinate and Casamino acids. The striking feature of these results is that, under all culture conditions tested (API 20NE, API 50CH, Biotype 100, defined basal medium), none of the sugars tested could be used as a substrate by strain AMX 51^T.

Phenotypic similarities shared by *B. thiooxidans* DSM 9653^T and strain AMX 51^T included cell shape, the presence of catalase and oxidase, thiosulfate oxidation, utilization of L-glutamate, D-gluconate, L-malate, D-malate, L-proline, DL-alanine, L-arginine, L-cysteine, L-glutamine, L-asparagine, acetate and pyruvate and the absence of indole production and arginine dihydrolase. Up to 21 phenotypic differences could be observed between *B. thiooxidans* DSM 9653^T and strain AMX 51^T, including nitrate reductase activity, Simmons' citrate test (citrate assimilation), starch and gelatin hydrolysis, utilization of lactate, adipate, phenylacetate and sugars and growth above 41 °C (Table 1). Susceptibility of strain AMX 51^T to the following antibiotics was observed: imipenem (10 µg), cephalothin (30 µg), cefotaxime (30 µg), ticarcillin (75 µg), tobramycin (10 µg), amikacin (30 µg), gentamicin (10 IU), netilmicin (30 µg), colistin (300 IU), piperacillin/tazobactam (75/10 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). Isolate AMX 51^T was resistant to ceftazidime (30 µg), ofloxacin (5 µg) and ciprofloxacin (5 µg). Intermediate responses were observed with piperacillin (75 µg), amoxicillin (25 µg) and amoxicillin/clavulanic acid (25/10 µg).

Table 1. Characteristics that differentiate strain AMX 51^T from *B. thiooxidans* DSM 9653^T

Data for *B. thiooxidans* from Das *et al.* (1996). Characteristics are scored as: + or –, test is positive/negative, substrate is used/not used; w, weakly positive.

Characteristic	Strain AMX 51 ^T	<i>B. thiooxidans</i> DSM 9653 ^T
Growth requirements	Casamino acids	Glutamate + yeast extract
Nitrate reduction	–	+
Simmons' citrate	–	+
Hydrolysis of starch and gelatin	w	–
Utilization of phenylalanine, DL-lactate, methanol	+	–
Utilization of sugars, N-acetylglucosamine, adipate, phenylacetate*	–	+
Growth below 20 °C and at 42 °C	+	–
Optimal temperature for growth (°C)	37	30–32
Optimal pH for growth	6.0	7.5–8.0

*Sugars tested were D-glucose, D-fructose, D-galactose, L-sorbose, L-arabinose, D-xylose, D-ribose, L-rhamnose, D-mannose and maltose.

The predominant CFA found in strain AMX 51^T were C_{18:1}ω7c (56.56%), C_{19:0} cycloω8c (13.27%), C_{16:0} (9.64%), C_{16:0} 3-OH (4.64%) and C_{18:1}ω7c 11-methyl (4.55%). This CFA pattern is similar to that of *B. thiooxidans* DSM 9653^T (Table 2). Notable differences in the their CFA profiles are in levels of C_{18:1}ω7c 11-methyl, C_{17:1}ω8c and C_{17:0} (Table 2). The Euclidian distance of 12 between the CFA profiles of isolate AMX 51^T and *B. thiooxidans* DSM 9653^T suggests that they are linked at the genus level, but not at the species level. The CFA profile of the novel isolate was significantly different from that of *A. felis*, which can be considered as a representative

of the genus *Afipia* (data not shown). The Euclidian distance found during CFA analysis between strain AMX 51^T, *B. thiooxidans* and *A. felis* was about 50–52. Such results confirm that these species cannot belong to the same genus, which is consistent with other phenotypic data.

The G + C content of isolate AMX 51^T was 68.5 ± 0.4 mol% (three determinations). This value is close to that reported for *B. thiooxidans* DSM 9653^T (68.2 mol%) (Das *et al.*, 1996) and a closely related bacterium, strain 5Z2111 (66.45 ± 0.05%) (Stubner *et al.*, 1998). A total of 1482 positions of its 16S rRNA gene was sequenced. Phylogenetic analysis revealed that strain AMX 51^T is member of the α-2 subgroup of *Proteobacteria*. Among species with validly published names, its closest relatives were *B. thiooxidans* DSM 9653^T, *Methylobacterium organophilum* JCM 2833^T, *Methylobacterium mesophilicum* JCM 2829^T, *Afipia clevelandensis* DSM 7315^T and *A. felis* DSM 7326^T, with respective similarity levels of 98.88, 92.70, 92.61, 91.67 and 91.59% (Fig. 1). As isolate AMX 51^T shares 91–93% 16S rRNA sequence similarity with *M. organophilum* JCM 2833^T, *M. mesophilicum* JCM 2829^T, *A. clevelandensis* DSM 7315^T and *A. felis* DSM 7326^T, it cannot be member of any of these species (Stackebrandt & Goebel, 1994). The phenotypic differences observed between the isolate and the above-mentioned species are sufficient to exclude its affiliation to the genera *Methylobacterium* and *Afipia*; this result correlates with the phylogeny results. The high 16S rRNA sequence similarity (98.88%) found between strain AMX 51^T and *B. thiooxidans* DSM 9653^T correlates with the similarities observed in phenotypic traits and CFA profiles. In accordance with this, it is proposed that strain AMX 51^T should be affiliated to the genus *Bosea*. Since the relatively high 16S rRNA sequence similarity (98.88%) did not allow discrimination of strain AMX 51^T from *B. thiooxidans* DSM 9653^T at the species level (Stackebrandt & Goebel, 1994), DNA–DNA hybridization analysis was used to resolve the genomic relationships. DNA–DNA hybridization revealed 44.2% similarity with *B. thiooxidans*

Table 2. Fatty acid profiles of strain AMX 51^T and *B. thiooxidans* DSM 9653^T

Values are percentages of total fatty acids. Data were determined in this study.

Fatty acid	Strain AMX 51 ^T	<i>B. thiooxidans</i> DSM 9653 ^T
13:1ω1c	0.72	0.24
14:0	0.23	0.19
15:0	1.06	1.16
15:0 3-OH	0.43	0.35
16:0	9.64	6.60
16:0 3-OH	4.64	3.34
16:1ω7c/15:0 iso 2-OH	2.50	3.49
17:0	1.33	3.35
17:0 3-OH	0.27	0.59
17:0 cyclo	2.91	2.03
17:1ω8c	0.87	2.77
18:0	0.36	0.49
18:1ω7c	56.56	64.64
18:1ω7c 11-methyl	4.55	0.39
19:0 cycloω8c	13.27	9.67
19:0 10-methyl	0.63	0.72

Fig. 1. Phylogenetic dendrogram showing the position of strain AMX 51^T (=DSM 13099^T) among closely related species of *Proteobacteria*. Bar, 2 nt substitutions per 100 nt. Numbers at nodes correspond to bootstrap percentages based on 100 resamplings. Only values greater than 80% were considered significant and are therefore reported.

DSM 9653^T. This value is high enough to support affiliation of strain AMX 51^T to the genus *Bosea*. However, since the DNA–DNA hybridization value observed is significantly below 70%, it is concluded that strain AMX 51^T represents a separate species (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987). In contrast, the DNA–DNA hybridization values found between AMX 51^T and *A. felis* (15.1%) and between *B. thiooxidans* DSM 9653^T and *A. felis* (19.6%) are very low. Such results, combined with general phenotypic characteristics, CFA profiles and 16S rRNA sequence similarity values, support the conclusion that the novel isolate and *B. thiooxidans* are not members of the genus *Afipia*.

Description of *Bosea minatitlanensis* sp. nov.

Bosea minatitlanensis (mi.na.tit.lan.en'sis. N.L. *minatitlanensis* of Minatitlán, a town in the south of Veracruz state in Mexico, where the anaerobic sludge of a pilot-scale reactor was sampled to inoculate the lab-scale UASB reactor from which the type strain was isolated).

Cells are straight to curved rods, 0.5 × 1.5–2.0 µm. Gram-negative, non-sporulating, motile, strictly aerobic bacterium. Monotrichous polar flagellation. Circular, non-pigmented colonies on trypticase soy or R2A agar. Growth is not accompanied by odour. Thiosulfate oxidation is observed in presence of succinate or Casamino acids. Positive for oxidase, catalase and urease; weakly positive for amylase and proteolysis; negative for nitrite reductase, nitrate reductase, indole production, Simmons's citrate, ONPG, aesculin, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, DNase and Tween 80 esterase. All API 50CH tests are negative. Amino acids (Casamino acids, L-alanine, L-arginine, L-asparagine, L-cysteine, L-glutamate, L-glutamine, L-phenylalanine and L-proline) and organic acids (acetate, 4-aminobutyrate, crotonate, formate, D-galacturonate, D-gluconate, D-glucuronate, glutarate,

DL-glycerate, 3-hydroxybutyrate, 2-ketogluconate, 2-oxoglutarate, DL-lactate, D-malate, L-malate and pyruvate) are utilized as substrates, as well as methanol, but sugars are not. Casamino acids are required as nitrogen source. Susceptible to imipenem, cephalothin, cefotaxime, ticarcillin, tobramycin, amikacin, gentamicin, netilmicin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole and colistin. Resistant to ceftazidime, ofloxacin and ciprofloxacin. Intermediate responses are observed for piperacillin and amoxicillin/clavulanic acid. Growth is observed between 15 and 42 °C, with an optimum at 37 °C. No growth at ≤4 or ≥45 °C. The pH range for growth is 5.0–8.5; optimum pH 6.0. Predominant CFA are C_{18:1}ω7c, C_{19:0} cycloω8c, C_{16:0}, C_{16:0} 3-OH and C_{18:1}ω7c 11-methyl. The G+C content of the type strain is 68.5 ± 0.4 mol%.

The type strain, AMX 51^T (=DSM 13099^T=ATCC 700918^T=CIP 106457^T), is an environmental bacterium, probably originating from soil or water, that was isolated as a transient micro-organism from anaerobic sludge of a lab-scale UASB reactor treating the petrochemical wastewater of a purified terephthalic acid plant in Minatitlán (Mexico).

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