Arsenicicoccus bolidensis gen. nov., sp. nov., a novel actinomycete isolated from contaminated lake sediment

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An unknown Gram-positive, catalase-positive, facultatively anaerobic, non-spore-forming, coccus-shaped bacterium originating from sediment was characterized using phenotypic, molecular chemical and molecular phylogenetic methods. Chemical studies revealed the presence of a cell-wall murein based on LL-diaminopimelic acid (type LL-Dpm-glycine$_1$), a complex mixture of saturated, monounsaturated and iso- and anteiso-methyl-branched, non-hydroxylated, long-chain cellular fatty acids and tetrahydrogenated menaquinones with eight isoprene units [MK-8(H_4)] as the major respiratory lipoquinone. This combination of characteristics somewhat resembled members of the suborder *Micrococcineae*, but did not correspond to any currently described species. Comparative 16S rRNA gene sequencing confirmed that the unidentified coccus-shaped organism is a member of the *Actinobacteria* and represents a hitherto-unknown subline related to, albeit different from, a number of taxa including *Intrasporangium*, *Janibacter*, *Terrabacter*, *Terracoccus* and *Ornithinicoccus*. Based on phenotypic and phylogenetic considerations, it is proposed that the unknown bacterium originating from lake sediment be classified as a new genus and species, *Arsenicicoccus bolidensis* gen. nov., sp. nov. (type strain CCUG 47306^T = DSM 15745^T).

During the course of mining for base metals in the Boliden region in the Vasterbotten district of northern Sweden, millions of tons of mine tailings have been generated. Much of the area is badly affected by acid mine drainage (pH 3–4) and has quite limited vegetation cover. These tailings contain about 0·5 % arsenic (As), resulting in approximately 600 000 tons of arsenic exposed to weathering processes (Grip, 1973). Arsenic from mine tailings leaches out due to weathering and other biogeochemical processes, resulting in high As concentrations in ground water, surface sediments and soils (Jacks *et al.*, 2003). A site in Adak received the sulfidic tailings (with >4000 mg As kg^{-1}) from an ore-processing unit that was functional from 1945 to 1975. These tailings were mixed with till to restrict oxidation of pyrites and leaching of heavy metals. The tailings extend over a large area and drain into a man-made lake. During the course of an investigation into the microbiological flora of the aforementioned lake sediment, we isolated a Gram-positive, coccus-shaped organism of uncertain taxonomic position. In this article, we report the results of a polyphasic taxonomic study on the unknown coccus. Based on the findings presented, we propose that the unknown organism be assigned to a new genus, *Arsenicicoccus*, as *Arsenicicoccus bolidensis* gen. nov., sp. nov.

Strain CCUG 47306^T was isolated from sediment containing mine waste (Boliden, Sweden). In 2002, we collected several 30–45 cm long undisturbed sediment cores across the lake using a gravity corer. The cores were brought to the laboratory within 48 h and immediately sliced for various geochemical and microbiological assays. During slicing, the outer surface of the cores was pared with a sterile knife and samples stored in zip-lock plastic bags. Sediments were added to basal salts medium for enrichment, which contained the following constituents (l^{-1}): 0·25 g NH_4Cl,

Abbreviation: Dpm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCUG 47306^T is AJ558133.

0·14 g K_2HPO_4, 0·5 g KCl, 0·15 g $CaCl_2.2H_2O$, 1·0 g NaCl, 0·62 g $MgCl_2.6H_2O$ and 1 ml trace-elements solution [containing 0·1 mg $MnCl_2.4H_2O$, 1·5 mg $FeCl_2.4H_2O$, 0·12 mg $Co(NO_3)_2.6H_2O$, 0·07 mg $ZnCl_2$, 0·015 mg $CuCl_2.2H_2O$, 0·06 mg H_3BO_3, 0·025 mg $Na_2MoO_4.2H_2O$, 0·025 mg $Ni(NO_3)_2.6H_2O$ and 0·05 mg *p*-aminobenzoic acid]. Lactate (0·089 mM) was used as the sole carbon source in the medium. The medium was later spiked with 0·435 mM As to facilitate the isolation of As-resistant strains. As-enriched cultures were diluted 10-fold and 0·1 ml of the extract was spread onto tryptic soy agar plates spiked with 0·435 mM As. After 72 h of incubation at 22 °C, colonies were selected and replated on the same medium until pure cultures were obtained.

The unidentified isolate was characterized biochemically using the API Rapid ID 32Staph, API Coryne and API ZYM systems according to the manufacturer's instructions. Cell-wall murein was prepared by mechanical disruption of cells and acid hydrolysates analysed as described by Schleifer & Kandler (1972), except that ascending TLC with cellulose sheets was used. Molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987). Long-chain cellular fatty acids were analysed as described by Kämpfer & Kroppenstedt (1996). Isoprenoid quinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC as described by Groth *et al.* (1997). The G+C content of DNA was determined by HPLC according to Mesbah *et al.* (1989). The 16S rRNA gene of the isolate was amplified by PCR and sequenced directly using a *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the novel isolate were determined by performing database searches. These sequences and those of other known related strains were retrieved from GenBank and aligned with the newly determined sequence using the program DNATools (Rasmussen, 1995). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated with the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR and the stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The unidentified organism recovered from the lake sediment consisted of Gram-positive-staining, coccoid-shaped cells. The organism was facultatively anaerobic and catalase-positive. Using the API Coryne system, the unknown organism produced acid from glucose, glycogen, mannitol, maltose, lactose, ribose, sucrose and D-xylose. The microorganism produced alkaline phosphatase, β-galactosidase, α-glucosidase, pyrazinamidase and pyrrolidonyl arylamidase and hydrolysed aesculin and gelatin. All other tests were negative with this kit. With the API ZYM system, the strain gave positive reactions for alkaline phosphatase, esterase C-4, ester lipase C8, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase, whereas reactions for acid phosphatase and phosphoamidase were either weakly positive or negative. All other tests were negative using the API ZYM gallery. Analysis of cell-wall murein hydrolysates of the unknown isolate revealed the presence of a wall based on LL-diaminopimelic acid (LL-Dpm). The molar amino acid ratio of murein was 1·5 alanine : 1·2 glycine : 1·0 glutamic acid : 1·1 LL-Dpm, which is consistent with a murein type LL-Dpm-glycine$_1$ (type A41.1; http://www.dsmz.de/species/murein.htm). Long-chain cellular fatty acid compositional analyses revealed a complex mixture of straight-chain saturated, monounsaturated, iso- and anteiso-methyl-branched acids in the unidentified coccus (percentages of total fatty acids: $C_{14:0}$, 6·1; $C_{15:0}$, 0·6; $C_{16:0}$, 4·8; $C_{18:0}$, 1·4; iso-$C_{13:0}$, 1·3; iso-$C_{14:0}$, 3·9; iso-$C_{15:0}$, 19·8; iso-$C_{16:0}$, 2·8; iso-$C_{17:0}$, 0·5; anteiso-$C_{13:0}$, 0·5; anteiso-$C_{15:0}$, 11·2; anteiso-$C_{17:0}$, 2·1; $C_{14:1}\omega5c$, 0·4; $C_{15:1}\omega6c$, 0·5; $C_{16:1}\omega7c$, 21·9; $C_{17:1}\omega8c$, 2·0; $C_{18:1}\omega9c$, 19·0; anteiso-$C_{17:1}\omega9c$, 1·1), whilst its major respiratory lipoquinone was MK-8(H_4). These chemical biomarkers were strongly indicative of a possible affinity between the unknown coccus and some actinobacterial genera, but the organism did not appear to correspond closely to any described taxon.

To investigate the taxonomic affinities of the unknown organism further, comparative 16S rRNA gene sequencing studies were conducted. Sequence database searches revealed that the unknown organism was indeed a member of the *Actinobacteria*, with highest sequence similarities being shared with *Terrabacter tumescens* (95·2 %), *Intrasporangium calvum* (94·9 %), *Ornithinicoccus hortensis* (94·8 %), *Janibacter* species (94–94·5 %) and *Terracoccus luteus* (94·1 %). Other *Actinobacteria* revealed significantly lower levels of sequence similarity to the unknown isolate (data not shown). A tree constructed using the neighbour-joining method showing the phylogenetic relationships of the unidentified coccus (Fig. 1) confirms the association of the isolate with *Intrasporangium*, *Ornithinicoccus*, *Janibacter*, *Terrabacter*, *Terracoccus* and related organisms.

From the polyphasic taxonomic study, it is evident that the unidentified coccus from contaminated lake sediment represents a hitherto-unknown species within the *Actinobacteria*. Comparative 16S rRNA gene sequence analysis showed that the unidentified organism was a neighbour taxon of the family *Intrasporangiaceae* and the genus *Ornithinicoccus*. However, the unknown coccus formed a distinct subline and did not display a statistically significant affinity with any described genus. Furthermore, sequence divergence values of approximately 5 % between the unidentified organism and its nearest relatives (*Terrabacter tumescens*, *Intrasporangium calvum*, *Ornithinicoccus hortensis*, *Janibacter* species and *Terracoccus luteus*) reinforced its distinctiveness. Although the relatively unusual respiratory quinone composition of the unknown organism [MK-8(H_4)] reinforced its affinity with most members of the

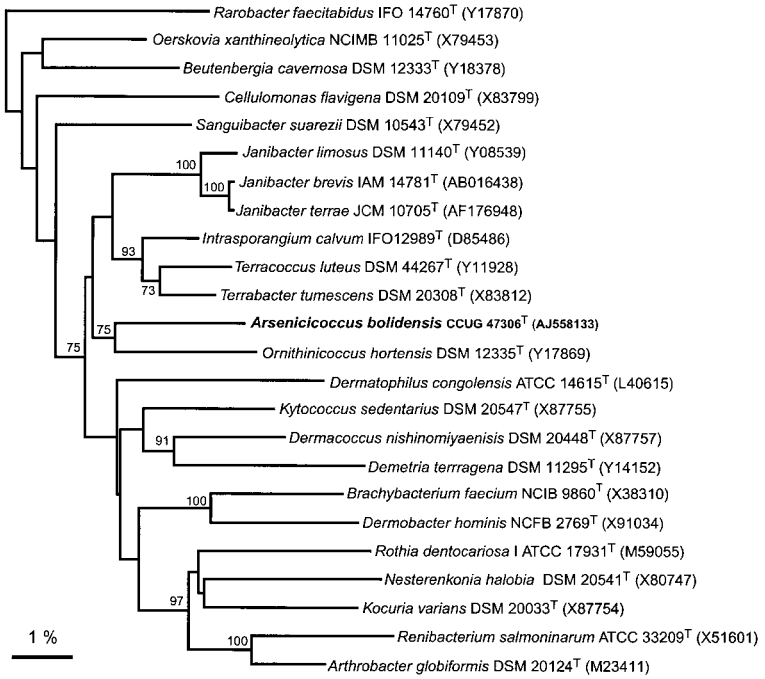

Fig. 1. Unrooted tree based on 16S rRNA showing the phylogenetic relationships of *Arsenicicoccus bolidensis* gen. nov., sp. nov. Bar, 1 % sequence divergence.

family *Intrasporangiaceae* (except for *Intrasporangium calvum*, which contains MK-8; Collins *et al.*, 1984) and the genus *Ornithinicoccus*, other chemotaxonomic features demonstrated its uniqueness. In particular, the presence of LL-Dpm in the wall of the unidentified coccus readily distinguishes it from the genera *Ornithinicoccus* and *Janibacter*, which respectively possess mureins based on L-ornithine (Groth *et al.*, 1999) and *meso*-Dpm (Martin *et al.*, 1997). Similarly, the presence of a single glycine residue within the murein interpeptide bridge of the unknown coccus serves to distinguish it from *Intrasporangium calvum*, *Terrabacter tumescens* and *Terrabacter luteus*, which possess a murein type LL-Dpm-glycine$_3$ (Prauser *et al.*, 1997). The long-chain cellular fatty acids of the unknown coccus were also quite distinct from those of its phylogenetic neighbours. The unknown bacterium was characterized by a complex mixture of straight-chain saturated, monounsaturated, iso- and anteiso-methyl-branched acids, with $C_{16:1}\omega7c$, iso-$C_{15:0}$ and $C_{18:1}\omega9c$ as the predominant acids. In contrast, members of *Ornithinicoccus* and *Terracoccus* are characterized by containing major amounts of anteiso-$C_{15:0}$ and iso-$C_{15:0}$ and much reduced levels of monounsaturated acids (Groth *et al.*, 1999; Prauser *et al.*, 1997), whereas *Terrabacter tumescens* and *Intrasporangium calvum* contain predominantly iso-methyl-branched acids (Collins *et al.*, 1983; Schumann *et al.*, 1997). *Janibacter* species also produce complex fatty acids profiles, but they differ markedly from that of the unknown coccus. In particular, *Janibacter* species produce significantly higher levels of iso-$C_{16:0}$ and do not synthesize major amounts of $C_{16:1}\omega7c$ (Martin *et al.*, 1997; Yoon *et al.*, 2000). Therefore, based on the distinct subline formed by the novel bacterium, in concert with its quite distinct chemotaxonomic characteristics, we are of the opinion that the unknown bacterium from sediment merits assignment to a new genus and species within the *Actinobacteria*, for which the name *Arsenicicoccus bolidensis* gen. nov., sp. nov. is proposed.

An examination of the 16S rRNA sequence of *Arsenicicoccus bolidensis* CCUG 47306^T revealed a close similarity to other taxa within the family *Intrasporangiaceae*. In particular, *Arsenicicoccus bolidensis* CCUG 47306^T possessed 25 of 31 rDNA signature nucleotides used to define the family *Intrasporangiaceae* (Stackebrandt *et al.*, 1997). Therefore, it is also proposed that the new genus *Arsenicicoccus* be classified in the family *Intrasporangiaceae* within the suborder *Micrococcineae*.

Description of *Arsenicicoccus* gen. nov.

Arsenicicoccus (Ar.sen.i.ci.coc′cus. L. n. *arsenicum* arsenic; L. masc. n. *coccus* berry; N.L. masc. n. *Arsenicicoccus* arsenic coccus, because the type species was recovered from an arsenic enrichment).

Cells are Gram-positive, non-spore-forming cocci that occur in clusters. Facultatively anaerobic and catalase-positive. Acid is formed from glucose and some other carbohydrates. Nitrate is reduced. Voges–Proskauer negative. The major long-chain cellular fatty acids are a complex mixture of straight-chain saturated, monounsaturated, iso- and anteiso-methyl-branched acids. Hydroxy fatty acids are not present. The major respiratory quinone is MK-8(H_4). Cell-wall murein is based on LL-Dpm (type: LL-Dpm-glycine$_1$). The G+C content of genomic DNA of the

type species is 72·2 mol%. The type species is *Arsenicicoccus bolidensis*.

Description of *Arsenicicoccus bolidensis* sp. nov.

Arsenicicoccus bolidensis (bol.id.en′sis. N.L. masc. adj. *bolidensis* pertaining to the Boliden region in Vasterbotten district of northern Sweden, where the type strain was isolated).

Displays the following properties in addition to those given in the genus description. Using commercially available API kits, acid is formed from glucose, glycogen, fructose, mannitol, mannose, sucrose and D-xylose. Depending on the test kit, acid may or may not be produced from cellobiose, lactose, ribose and maltose. Using the API ZYM system, alkaline phosphatase, esterase C-4, ester lipase C8, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase are positive; acid phosphatase and phosphoamidase are either weakly positive or negative. Chymotrypsin, cystine arylamidase, α-fucosidase, β-glucuronidase, α-mannosidase, lipase C14, leucine arylamidase, *N*-acetyl-β-glucosaminidase, trypsin and valine arylamidase are not detected. Using the API Coryne test kit, alkaline phosphatase, β-galactosidase, α-glucosidase, pyrazinamidase and pyrrolidonyl arylamidase are detected but β-glucuronidase and *N*-acetyl-β-glucosaminidase are not. Aesculin and gelatin are hydrolysed but urea is not. Nitrate is reduced. Chemotaxonomic characteristics are given in the genus description. The G+C content of DNA is 72·2 mol%. Highly As-tolerant. Possesses As(V) reduction mechanisms that are coupled to respiration or to impart resistance to As toxicity.

The type strain is CCUG 47306^T (=DSM 15745^T).

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