Atopococcus tabaci gen. nov., sp. nov., a novel Gram-positive, catalase-negative, coccus-shaped bacterium isolated from tobacco

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A novel Gram-positive, aerobic, catalase-negative, coccus-shaped organism originating from tobacco was characterized using phenotypic and molecular taxonomic methods. The organism contained a cell wall murein based on L-lysine (variation A4 α , type L-lysine–L-glutamic acid), synthesized long-chain cellular fatty acids of the straight-chain saturated and monounsaturated types (with C_{16:1} ω 9, C_{16:0} and C_{18:1} ω 9 predominating) and possessed a DNA G + C content of 46 mol%. Based on morphological, biochemical and chemical characteristics, the coccus-shaped organism did not conform to any presently recognized taxon. Comparative 16S rRNA gene sequencing studies confirmed the distinctiveness of the unknown coccus, with the bacterium displaying sequence divergence values of greater than 7 % with other recognized Gram-positive taxa. Treeing analysis reinforced its distinctiveness, with the unidentified organism forming a relatively long subline branching at the periphery of an rRNA gene sequence cluster which encompasses the genera *Alloiococcus*, *Allofustis*, *Alkalibacterium*, *Atopostipes*, *Dolosigranulum* and *Marinilactibacillus*. Based on phenotypic and molecular phylogenetic evidence, it is proposed that the unknown organism from tobacco be classified as a new genus and species, *Atopococcus tabaci* gen. nov., sp. nov. The type strain of *Atopococcus tabaci* is CCUG 48253^T (=CIP 108502^T).

The Gram-positive, catalase-negative, cocci embrace a phenotypically diverse range of organisms within the *Clostridium* subphylum. Since the mid-1980s, knowledge of the taxonomy of these organisms has improved greatly and there has been a dramatic increase in the number of recognized genera and species (Facklam & Elliot, 1995). Some of the newly described genera have arisen due to taxonomic revision of existing genera (e.g. *Abiotrophia*, *Enterococcus* and *Lactococcus* from *Streptococcus*; *Oenococcus* from *Leuconostoc*) but the majority of new genera have come to light due to improved taxonomic methodologies used for characterizing and identifying these organisms (e.g. Aguirre & Collins, 1992; Aguirre *et al.*, 1993; Collins *et al.*, 1992, 1997, 1999a, b). Today, over 15 genera of Gram-positive, catalase-negative cocci are recognized. However, despite the rapid increase in the number of recognized genera of Gram-positive, catalase-negative cocci, it is evident that much

diversity remains to be discovered. In the course of an ongoing study exploring the diversity of taxonomically problematic catalase-negative cocci, we have characterized a hitherto unknown organism from tobacco which does not correspond to any recognized genus. Based on the results of a polyphasic taxonomic study, we describe yet another new genus and species, *Atopococcus tabaci*, within the Gram-positive, catalase-negative cocci.

An unknown coccus-shaped organism (designated CCUG 48253^T) originating from moist snuff tobacco was received by CCUG, Sweden, for identification. The unidentified coccus was cultured on Columbia blood agar base supplemented with 5 % horse blood at 37 °C under aerobic conditions. The organism was biochemically characterized using the API Rapid ID 32Strep, API Coryne and API ZYM test systems according to the manufacturer's instructions (bioMérieux). The G + C content of DNA was determined by HPLC according to Mesbah *et al.* (1989). Cell-wall murein was prepared by the mechanical disruption of cells and the analysis of acid hydrolysates as described by Schleifer & Kandler (1972), Schleifer (1985) and MacKenzie (1987). For long-chain cellular fatty acid analysis, the

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organism was grown statically in BHI broth and fatty acids were analysed using the MIDI system). For isoprenoid quinone analysis, the organism was grown statically in BHI broth and in BHI broth with shaking. Isoprenoid quinones were extracted as described by Collins *et al.* (1977) and analysed by TLC and HPLC as described by Collins (1994) and Groth *et al.* (1997). For phylogenetic analysis, 16S rRNA gene sequences were amplified by PCR and directly sequenced 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 new isolate were determined by performing database searches using the FASTA program (Pearson & Lipman, 1985). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequence using the SEQtools program (Rasmussen, 2002). The resulting multiple sequence alignment was corrected manually using the GeneDoc program (Nicholas *et al.*, 1997) and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the programs SEQtools and TREEVIEW (Page, 1996). The stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same programs.

The unidentified coccus-shaped organism originating as a contaminant of tobacco stained Gram-positive and was non-motile. It grew aerobically, but not under strictly anaerobic conditions. The organism was catalase-negative. Using the API Rapid ID 32Strep system, the organism produced acid from lactose, maltose, ribose, sucrose and trehalose, but not from any of the other carbohydrates in this test gallery. The organism hydrolysed hippurate and was Voges–Proskauer negative. It displayed activity for β -glucosidase, β -galactosidase, pyroglutamic acid arylamidase and *N*-acetyl- β -glucosaminidase. All other enzyme tests included in this system were negative. Using the API ZYM test system, the organism showed activity for alkaline phosphatase, acid phosphatase, esterase C4, α -glucosidase, leucine arylamidase and valine arylamidase. All other enzyme tests in the API ZYM system were negative. Employing the API Coryne test kit, the organism produced acid from glucose, maltose, lactose, ribose (weak reaction) and sucrose. It hydrolysed aesculin and displayed activity for β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase and pyrrolydonyl arylamidase. The long-chain cellular fatty acids of the organism were found to be primarily of the unbranched saturated and monounsaturated types, with iso- and anteiso-methyl-branched forms present in very small amounts. The quantitative fatty acid data corresponded to: C_{10:0} (0.7%), C_{12:0} (2.6%), C_{14:0} (8.3%), C_{14:1} (4.5%), C_{16:1} ω 9c (41.9%), C_{16:0} (15.8%), iso-C_{17:1} (5.3%), C_{18:1} ω 9c (9%) and iso-C_{19:1} (1.9%). No respiratory quinones were detected using the methods employed. Analysis of the cell wall murein of isolate CCUG 48253^T showed the presence of an A4 α murein based on L-lysine type, L-Lys–L-Glu. The amino acids lysine, alanine and glutamic acid were detected in approximate

molar ratios of 1.0 Lys:1.9 Ala:2.4 Glu. The partial hydrolysate contained the peptides L-Ala–D-Glu and L-Lys–D-Ala. Dinitrophenylation revealed that glutamic acid represented the N terminus of the interpeptide bridge. Since the peptide D-Ala–D-Glu was not detected, it is likely that the N terminal glutamic acid is of the L-configuration. The DNA G + C composition of the unidentified strain was found to be 46 mol%.

In order to investigate the phylogenetic affinities of the isolate, an almost complete 16S rRNA gene sequence (1498 nucleotide bases, from positions 37 to 1510, *Escherichia coli* numbering system) was determined. Sequence database searches revealed that the strain was a member of the low-G + C *Clostridium* subphylum of the Gram-positive bacteria. From the comparative 16S rRNA gene sequence analysis, it was evident that the unidentified, catalase-negative, coccus-shaped organism isolated from tobacco represented an unknown taxon. Phylogenetically, the unidentified isolate formed a distinct subline branching at the periphery of a distinct rRNA gene sequence cluster which embraced *Alkalibacterium olivoapovliticus*, *Alloiococcus otitis*, *Allofustis seminis*, *Atopostipes suicloacale*, *Dolosigranulum pigrum* and *Marinilactobacillus psychrotolerans*. The branching of the unknown organism at the base of this group was supported by a bootstrap resampling value of 95% (Fig. 1). Amongst the aforementioned taxa, the unidentified organism displayed the highest sequence similarity to *Alkalibacterium olivoapovliticus* (92.6%), *Alloiococcus otitis* (89.9%), *Allofustis seminis* (87.5%), *Atopostipes suicloacale* (90.4%), *Dolosigranulum pigrum* (86.8%) and *Marinilactobacillus psychrotolerans* (92.6%). The relatively high divergence values (>7%), together with the deep branching position of the unidentified organism, showed that the unidentified bacterium was only distantly related to these taxa and merited classification at a similar taxonomic rank (i.e., Genus).

Support for the separateness of the tobacco bacterium is also evident from phenotypic considerations, with the unknown organism being readily distinguished from all currently described Gram-positive taxa. In particular, it differed markedly from *Alkalibacterium olivoapovliticus* and *Marinilactobacillus psychrotolerans* in not being alkaliphilic and by its coccoid morphology. In addition, the unidentified organism possessed a cell wall based on L-lysine (type L-Lys–L-Glu) whereas the walls of *Alkalibacterium olivoapovliticus* and *Marinilactobacillus psychrotolerans* contain ornithine as their wall dibasic amino acid (types Orn–D-Asp and Orn–D-Glu, respectively) (Ishikawa *et al.*, 2003). Similarly, the unidentified isolate differed from *Alloiococcus otitis*, *Allofustis seminis* and *Dolosigranulum pigrum* in having a growth temperature optimum of 30 °C. *Alloiococcus otitis*, *Allofustis seminis* and *Dolosigranulum pigrum* all grow optimally at 37 °C (Aguirre & Collins, 1992; Aguirre *et al.*, 1993; Collins *et al.*, 2003). In addition, unlike the unidentified isolate, *Alloiococcus otitis* is nutritionally very fastidious. *Dolosigranulum pigrum* differed from the

Description of *Atopococcus* gen. nov.

Cells stain Gram-positive, are coccoid in shape and occur in pairs or short chains. Non-endospore-forming and non-motile. Aerobic. Catalase-negative. Acid is produced from

Description of *Atopococcus tabaci* sp. nov.

Displays the following properties in addition to those given in the genus description. α -Haemolytic on horse blood agar. Grows aerobically but not under anaerobic conditions. Optimal growth temperature on tryptic soy agar is 30 °C. Halotolerant, growing in 8–9 % NaCl. Using API test kits, acid is produced from glucose, lactose, maltose, ribose, sucrose and trehalose but not from L-arabinose, D-arabitol, cyclodextrin, glycogen, mannitol, melibiose, melizitose, methyl β -D-glucopyranoside, pullulan, raffinose, sorbitol, tagatose or D-xylose. Aesculin and hippurate are

hydrolysed but not gelatin. Activity is detected for acid phosphatase, esterase C4, β -glucosidase, β -galactosidase, leucine arylamidase, valine arylamidase, pyroglutamic acid arylamidase and *N*-acetyl- β -glucosaminidase. Activity may or may not be detected for alkaline phosphatase, α -glucosidase and urease. Alanine phenylalanine proline arylamidase, arginine dihydrolase, ester lipase C8, lipase C14, chymotrypsin, trypsin, phosphoamidase, cystine arylamidase, β -glucuronidase, glycyl tryptophan arylamidase, α -fucosidase, α -mannosidase and β -mannosidase are not detected. Nitrate is not reduced and the Voges–Proskauer test is negative. The predominant long-chain fatty acids are C_{14:0}, C_{16:1} ω 9c, C_{16:0} and C_{18:1} ω 9c.

The type strain, CCUG 48253^T (=CIP 108502^T), was isolated from moist powdered tobacco.

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