Serratia glossinae sp. nov., isolated from the midgut of the tsetse fly *Glossina palpalis gambiensis*

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We report the isolation of a novel bacterium, strain C1^T, from the midgut of the tsetse fly *Glossina palpalis gambiensis*, one of the vector insects responsible for transmission of the trypanosomes that cause sleeping sickness in sub-Saharan African countries. Strain C1^T is a motile, facultatively anaerobic, rod-like bacterium (0.8–1.0 µm in diameter; 2–6 µm long) that grows as single cells or in chains. Optimum growth occurred at 25–35 °C, at pH 6.7–8.4 and in medium containing 5–20 g NaCl l⁻¹. The bacterium hydrolysed urea and used L-lysine, L-ornithine, citrate, pyruvate, D-glucose, D-mannitol, inositol, D-sorbitol, melibiose, amygdalin, L-arabinose, arbutin, aesculin, D-fructose, D-galactose, glycerol, maltose, D-mannose, raffinose, trehalose and D-xylose; it produced acetoin, reduced nitrate to nitrite and was positive for β-galactosidase and catalase. The DNA G + C content was 53.6 mol%. It was related phylogenetically to members of the genus *Serratia*, family *Enterobacteriaceae*, the type strain of *Serratia fonticola* being its closest relative (99% similarity between 16S rRNA gene sequences). However, DNA–DNA relatedness between strain C1^T and *S. fonticola* DSM 4576^T was only 37.15%. Therefore, on the basis of morphological, nutritional, physiological and fatty acid analysis and genetic criteria, strain C1^T is proposed to be assigned to a novel *Serratia* species, *Serratia glossinae* sp. nov. (type strain C1^T = DSM 22080^T = CCUG 57457^T).

Glossina species, the well-known tsetse flies, are the vectors of African trypanosomes, the causative agents of sleeping sickness in humans and nagana in animals. Despite progress in the understanding of the disease, there is no foreseeable progress towards producing mammalian vaccines or new effective and affordable drugs for chemotherapy, while drug resistance is increasing (De Koning, 2001; Mulugeta *et al.*, 1997). Therefore, novel strategies must be investigated. Several approaches are possible, including genetic modification of the insect (Robinson *et al.*, 2004) and also the use of the insect bacterial flora to control the insect host itself and/or the pathogen(s) it transmits (Beard *et al.*, 1993; Durvasula *et al.*, 1997). In this latter approach, bacteria that are highly adapted to the physiology of a particular insect are selected for their capacity to secrete naturally antiparasitic compounds in the insect gut, where both the bacteria and the parasite are harboured. The

isolation of the Gram-negative bacterium *Serratia marcescens* from the gut of *Rhodnius prolixus* (Azambuja *et al.*, 2004) illustrates this possibility: this bacterium naturally produces prodigiosin, a pigment that is toxic for the parasite *Trypanosoma cruzi* (Azambuja *et al.*, 2005). Bacteria genetically transformed to express compounds that can either block the transmission of pathogens (Durvasula *et al.*, 1997; Favia *et al.*, 2007) or harm the host insect directly (Chapco & Kellin, 1994; Tang *et al.*, 2004) can also be used.

Glossina species have been shown to harbour three different bacterial symbionts (Aksoy, 2000). One is a member of the *Rickettsiaceae* belonging to the genus *Wolbachia* (O'Neill *et al.*, 1993). It infects a broad range of insect species, causing a variety of reproductive abnormalities. The other two are members of the family *Enterobacteriaceae*. The first, *Wigglesworthia glossinidia* (Aksoy, 2000), is the primary nutritional symbiont of the tsetse, synthesizing B vitamins (Akman *et al.*, 2002), which are absent from the fly's diet of blood. The second, *Sodalis glossinidius*, is assumed to be involved in the vectorial competence of the fly (Welburn & Maudlin, 1999; Geiger *et al.*, 2007). Strategies that exploit

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C1^T is FJ790328.

An electron micrograph of cells of strain C1^T and a comparison of its fatty acid profile with those of related *Serratia* type strains are available as supplementary material with the online version of this paper.

these symbioses to control vector-borne disease have been suggested (Beard *et al.*, 1993; Durvasula *et al.*, 1997; Rio *et al.*, 2004). It is possible that comparable strategies could be tested based on the use of other tsetse gut bacteria, if any. Thus, the major objective of our investigation was to isolate the micro-organisms of ecological significance within the tsetse gut microflora. Here, we report on a novel bacterial strain belonging to the genus *Serratia*, family *Enterobacteriaceae*, that was isolated from the midgut of insectary flies of the tsetse fly *Glossina palpalis gambiensis*.

G. palpalis gambiensis flies originated from individuals that had been field-collected in Burkina Faso (Africa). Pupae were collected from these flies. Following adult emergence, the population was maintained in a level 2 containment insectary at 23 °C and 80 % relative humidity (Geiger *et al.*, 2005) without any selection. Flies used in the present study for bacterial isolation were chosen randomly. Bacteria were isolated from 13 insectary-reared *G. palpalis gambiensis* flies. After surface sterilization with 1 % sodium hypochlorite and 70 % ethanol, the midgut of each fly was dissected and ground with a pestle.

Hungate tubes containing 2 ml liquid Mitsuhashi and Maramorosch insect culture medium (MMI; PromoCell) (Mitsuhashi & Maramorosch, 1964), which had previously been dispensed into the tubes under a N₂/CO₂ (80:20, v/v) gas stream and sterilized for 45 min at 110 °C, were inoculated with (20 %) fetal bovine serum (FBS; Gibco). The ground midguts of each fly were then transferred individually to a tube. After homogenizing, a 250 µl aliquot of the whole content was serially diluted in liquid medium to 10⁻¹⁰ to enrich only bacteria of ecological significance within the fly midgut: serial dilution in tubes was repeated at least twice. Tubes containing the highest dilutions (10⁻⁹, 10⁻¹⁰) were incubated without shaking under a micro-aerobic atmosphere providing 5 % O₂ in the gas phase at 26–27 °C for 36–72 h prior to isolation of bacteria by repeated use of the Hungate roll tube technique (Hungate, 1969) on MMI solid medium (MMI, 20 % FBS, 10 % horse blood, gelified with 1.6 % agar). MMI solid medium was prepared by autoclaving MMI with agar (Sigma-Aldrich). The culture medium was cooled to 50 °C in a water bath, and packed horse blood cells (Oxoid) and FBS were then added. The process of serial dilution in roll tubes was repeated at least twice in order to purify the cultures. Purity of the isolates was checked by microscopic observations.

Growth experiments were performed in duplicate, using Hungate tubes containing basal culture medium (MMI, 20 % FBS).

Serratia fonticola DSM 4576^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultivated according to the procedures recommended by the DSMZ. Additional reference strains *Serratia ficaria* CCUG 14507^T, *S. grimesii* CCUG 15721^T, *S. liquefaciens* CCUG 9285^T, *S. marcescens*

CCUG 1647^T, *S. plymuthica* CCUG 14509^T, *S. proteamaculans* CCUG 14510^T, *S. quinivorans* CCUG 14187^T, *S. ureilytica* CCUG 50595^T, *S. entomophila* CCUG 55496^T, *S. odorifera* CCUG 14508^T and *S. rubidaea* CCUG 9286^T were obtained from the CCUG and cultivated according to their recommended procedures. The morphological, nutritional, physiological and molecular characteristics of isolated strains were compared with those of the 12 *Serratia* type strains including *S. fonticola* DSM 4576^T.

The pH, temperature and NaCl concentration for optimal growth of the type strains were determined using basal culture medium (MMI, 20 % FBS). The initial pH was adjusted to 5.0. The pH range was obtained by injecting in Hungate tubes suitable aliquots of anaerobic stock solutions of 1 M HCl (acidic pH range) or 10 % NaHCO₃ or Na₂CO₃ (basic pH range). Water baths ranging from 10 to 50 °C were used to test growth temperatures. To determine NaCl requirements, NaCl was weighed directly into the tubes before the medium was dispensed. Strain C1^T was subcultured under the same experimental conditions before growth rates were determined. The ability of the strains to metabolize various substrates was tested using API 20E and API 50CH profiles (API system; bioMérieux). Growth was monitored by measuring the OD₅₈₀ and by microscopic observations. Morphological characteristics and the purity of cultures were observed with an Optiphot phase-contrast microscope (Nikon). Anaerobic growth was tested in Hungate tubes containing anaerobic medium without shaking. Aerobic growth was determined in Hungate tubes filled with air in the gas phase and incubated at 26–27 °C under 150 r.p.m. agitation. Growth was monitored by measuring the OD₅₈₀ and by microscopic observations. All experiments were conducted in duplicate and repeated at least twice.

Fatty acid profiling was performed according to the MIDI-MIS protocol (details are given at <http://www.ccug.se/>).

Genomic DNA of the strains was extracted using the Wizard genomic DNA purification kit (Promega). The 16S rRNA gene (positions 8 to 1492, *Escherichia coli* numbering) was amplified by PCR with the eubacteria-specific primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GTCGTAACAAGGTAACCGTA-3'). The PCR product was purified using the Nucleo Spin Extract kit (Macherey Nagel) and cloned using the pGEM-T-easy cloning kit (Promega). A plasmid containing an insert of the correct length was purified with the Wizard Plus SV Minipreps DNA purification system (Promega) and sent for sequencing to GATC Biotech (Konstanz, Germany).

Prior to phylogenetic analysis, 1488 nucleotides of the 16S rRNA gene sequences of the tsetse fly bacterial isolates and reference sequences available in GenBank (Benson *et al.*, 1999) were aligned using the sequence aligner software from the Ribosomal Database Project II (Maidak *et al.*, 2001) and the sequence alignment editor BioEdit version 5.0.9 (Hall, 1999). The resulting alignment was used to construct a maximum-likelihood tree (Yang, 1997) using

the PHYLML program (Guindon & Gascuel, 2003). The robustness of the tree topology was tested by bootstrap analysis with 1000 resamplings (Felsenstein, 1985).

The G+C content of strain C1^T was determined at the DSMZ using HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization between C1^T and *S. fonticola* DSM 4576^T was performed in duplicate at the DSMZ as described previously (Miranda-Tello *et al.*, 2004).

The midguts of 13 *G. palpalis gambiensis* flies were dissected. Cultures from midgut bacteria were incubated at 26–27 °C for 3 days. White, circular colonies, 1 mm in diameter, appeared after 3 days of incubation at 26–27 °C in roll-tubes: 39 colonies were then cultured (three colonies per fly midgut). Only one type of cell was observed under the microscope. Individual bacteria were approximately 0.8–1 µm in diameter and 2–6 µm long. They occurred mainly as single cells (Supplementary Fig. S1, available in IJSEM Online).

The 16S rRNA gene sequences of the 39 bacterial isolates from fly midguts were amplified and sequenced as described previously (Maidak *et al.*, 2001; Miranda-Tello *et al.*, 2003; Weisburg *et al.*, 1991) and aligned using BioEdit. The 16S rRNA gene sequences of the 39 isolates were identical (represented by GenBank accession no. FJ790328), suggesting that they should be considered as members of the same species. These isolates showed less than 98 % 16S rRNA gene sequence similarity with the type strains of other *Serratia* species, except that of *S. fonticola*; *S. fonticola* DSM 4576^T was the closest phylogenetic relative of the novel isolates (99 % similarity) (Fig. 1). Strain C1^T was chosen for further characterization in comparison with *S. fonticola* DSM 4576^T. The G+C content of the DNA, as determined by HPLC, was 53.6 mol%, and was found to be identical to that of *S. fonticola* DSM 4576^T. However, DNA–DNA hybridization experiments revealed only

37.15 % relatedness (individual values 37.0 and 37.3 %) between strain C1^T and *S. fonticola* DSM 4576^T. Therefore, according to Wayne *et al.* (1987), strain C1^T cannot be affiliated with *S. fonticola* and should be considered as a member of a novel species of the genus *Serratia*.

The fatty acid profile of strain C1^T differed significantly from that of *S. fonticola* DSM 4576^T, notably in the content of 15:0 and 16:1ω7c (Supplementary Table S1). However, the most marked difference between the fatty acid profiles of the tested strains concerned 17:0 cyclo, which was absent from strain C1^T and made up 16.5 % of the profile of *S. fonticola* DSM 4576^T, confirming that strain C1^T does not belong to *S. fonticola*.

Both strain C1^T and *S. fonticola* DSM 4576^T were mesophilic (growing at 10–40 °C) and were able to grow under aerobic and anaerobic conditions and in the same ranges of pH (4.8–8.9) and NaCl concentration (0–50 g l⁻¹). Table 1 shows biochemical characteristics of the type strains of *Serratia* species. Both C1^T and *S. fonticola* DSM 4576^T were β-galactosidase-positive and used L-lysine, L-ornithine, citrate, inositol, D-sorbitol, melibiose, amygdalin, L-arabinose, arbutin, aesculin, D-galactose, glycerol, maltose, raffinose and D-xylose (Table 1), reduced nitrate to nitrite and were oxidase-negative. However, in contrast to *S. fonticola* DSM 4576^T, strain C1^T hydrolysed urea and produced acetoin, used pyruvate, did not use L-rhamnose and was found to be catalase-positive. Based on phenotypic, genetic and phylogenetic characteristics, we therefore propose that strain C1^T, isolated from the tsetse fly *G. palpalis gambiensis*, should be assigned to a novel species of the genus *Serratia*, family Enterobacteriaceae, for which we propose the name *Serratia glossinae* sp. nov.

Members of the genus *Serratia* are ubiquitous, and 13 species are currently recognized; *Serratia* species have been isolated from water, soil, animals (including humans) and plant surfaces (Grimont & Grimont, 1992). Some species are often associated with insects belonging to many orders (Grimont & Grimont, 1978). Results suggest that *Serratia* species in general have metabolic features of ecological significance in interactions with insects, and these may be beneficial for or deleterious to the physiology of these invertebrates (Grimont *et al.*, 1979; Oliver *et al.*, 2006; Lamelas *et al.*, 2008).

We are looking at other facets of the biology of the novel strain, including the role, if any, played by *S. glossinae* in the midgut of the tsetse fly. Another aspect that deserves to be investigated is the possibility of genetic engineering to produce strains that would express trypanolytic compounds. The selection and use of host-specific species to carry out such approaches should be favoured, since non-resident bacteria often cannot become established within a particular intestinal environment (Husseneder & Grace, 2005; Husseneder *et al.*, 2005; Thimm *et al.*, 1998). Moreover, the use of transgenic bacteria that are closely associated with a single host species may reduce undesirable impacts on non-target insects. In research into

Fig. 1. Maximum-likelihood phylogenetic tree based on comparative analysis of 16S rRNA gene sequence data indicating the position of strain C1^T among *Serratia* species. A sequence of *Buchnera aphidicola* was used as the outgroup and bootstrap values (percentages of 1000 tree replications) greater than 40 % are displayed at nodes. Sequence accession numbers are shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

Table 1. Biochemical characteristics of strain C1^T and *Serratia* type strains

Reference strains: 1, *S. fonticola* DSM 4576^T; 2, *S. ficaria* CCUG 14507^T; 3, *S. grimesii* CCUG 15721^T; 4, *S. liquefaciens* CCUG 9285^T; 5, *S. marcescens* CCUG 1647^T; 6, *S. plymuthica* CCUG 14509^T; 7, *S. proteamaculans* CCUG 14510^T; 8, *S. quinivorans* CCUG 14187^T; 9, *S. ureilytica* CCUG 50595^T; 10, *S. entomophila* CCUG 55496^T; 11, *S. odorifera* CCUG 14508^T; 12, *S. rubidaea* CCUG 9286^T. All strains were positive for utilization of D-glucose, D-mannitol, D-fructose, D-mannose and trehalose and were β -galactosidase-positive. All strains were negative for utilization of L-arginine, sodium thiosulfate, L-tryptophan, erythritol, glycogen and L-sorbose and were oxidase-negative. +, Positive; w, weakly positive; -, negative.

Characteristic	C1 ^T	1	2	3	4	5	6	7	8	9	10	11	12
Urease	+	-	-	-	-	-	-	-	-	+	-	-	-
Catalase	+	-	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	+	-
Acetoin production	+	-	+	-	+	+	-	+	+	+	+	+	-
Utilization of:													
L-Lysine	+	+	-	+	+	+	-	+	+	+	-	+	-
L-Ornithine	+	+	-	+	+	+	-	+	+	+	-	+	-
Trisodium citrate	+	+	+	-	+	+	+	+	-	+	+	+	+
Gelatin	-	-	+	-	-	+	-	-	+	+	+	+	+
Pyruvate	+	-	+	-	+	+	-	+	+	+	+	+	-
Inositol	+	+	-	+	+	+	-	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	+	+	-	-	+	-	+	-
L-Rhamnose	-	+	+	-	-	-	-	+	-	-	-	+	-
Sucrose	-	-	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	-	+	-	-	+	+
Amygdalin	+	+	+	+	+	+	+	+	-	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	-	-	+	+
Arbutin	+	+	+	+	+	-	+	+	-	+	+	+	+
Cellobiose	-	-	w	+	-	-	+	-	+	+	w	+	+
Dextrin	w	-	-	-	-	-	-	w	+	+	-	w	-
Aesculin	+	+	+	+	+	+	+	+	-	+	+	+	+
D-Galactose	+	+	+	+	+	-	+	+	+	+	+	+	+
Glycerol	+	+	-	+	+	+	-	+	+	+	+	-	+
Inulin	-	-	+	-	-	-	-	-	w	+	-	-	-
Maltose	+	+	+	+	+	+	+	-	-	+	+	+	+
Melezitose	-	-	w	-	+	-	+	-	-	-	-	-	-
Raffinose	+	+	-	+	w	-	+	-	+	-	-	-	+
Starch	-	-	-	+	-	-	-	-	-	-	-	-	-
D-Xylose	+	+	+	+	+	-	+	+	+	-	-	+	+

vector-parasite interactions and the control of sleeping sickness, our findings may be of particular interest. Finally, as discussed above, with the isolation of *S. glossinae*, we extend our knowledge of the ecological significance of members of the family *Enterobacteriaceae* within the midgut of insects. In addition, many questions have arisen from this research that deserve further attention from health scientists in efforts to control the spread of insect-borne parasitic disease.

Description of *Serratia glossinae* sp. nov.

Serratia glossinae (glos.si'nae. N.L. gen. n. *glossinae* of *Glossina*, referring to the isolation of the type strain from the tsetse fly *Glossina palpalis gambiensis*).

Bacillus-like (0.8–1.0 μ m in diameter; 2–6 μ m long), motile, facultatively anaerobic bacterium that grows as single cells or in chains. Optimum growth occurs at 25–35 °C, at pH 6.7–8.4 and in medium containing 5–20 g NaCl l⁻¹. Able to hydrolyse urea and to use L-lysine, L-ornithine, citrate, pyruvate, D-glucose, D-mannitol, inositol, D-sorbitol, melibiose, amygdalin, L-arabinose, arbutin, aesculin, D-fructose, D-galactose, glycerol, maltose, D-mannose, raffinose, trehalose and D-xylose. Produces acetoin, reduces nitrate to nitrite and is positive for β -galactosidase and catalase. The DNA G+C content of the type strain is 53.6 mol% (HPLC).

The type strain is strain C1^T (=DSM 22080^T =CCUG 57457^T), isolated from the midgut of the tsetse fly *Glossina palpalis gambiensis*.

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