Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans

Colin Dale† and Ian Maudlin

Author for correspondence: Colin Dale. Tel: +44 1904 434383. Fax: +44 1904 434383.
e-mail: cd15@york.ac.uk

Division of Molecular
Genetics, University of
Glasgow, Glasgow G11 6NU,
UK

A secondary intracellular symbiotic bacterium was isolated from the haemolymph of the tsetse fly *Glossina morsitans morsitans* and cultured in *Aedes albopictus* cell line C6/36. Pure-culture isolation of this bacterium was achieved through the use of solid-phase culture under a microaerobic atmosphere. After isolation of strain M1^T, a range of tests was performed to determine the phenotypic properties of this bacterium. Considering the results of these tests, along with the phylogenetic position of this micro-organism, it is proposed that this intracellular symbiont from *G. m. morsitans* should be classified in a new genus *Sodalis* gen. nov., as *Sodalis glossinidius* gen. nov., sp. nov. Strain M1^T is the type strain for this new species.

Keywords: *Sodalis glossinidius* gen. nov., sp. nov., insect endosymbiont, tsetse, microaerophile

INTRODUCTION

Endosymbiotic bacteria are found in a wide range of insect taxa, where they provide supplementary nutritional components essential for the survival of insects that feed on a restricted diet such as plant sap or blood (Buchner, 1965; Douglas, 1989). Many of these micro-organisms have proved reluctant to yield to conventional culture techniques; currently a trivial nomenclature system is in use to provide a distinction between different types of insect endosymbiont. This distinction is recognized on the basis of host tissue distribution, with 'primary (P) endosymbionts' residing exclusively within a single (specialized) cell type and 'secondary (S) endosymbionts' residing in multiple host tissues. While phylogenetic analysis has proved useful for determining the taxonomic position of several insect endosymbionts (Aksoy, 1995; Beard *et al.*, 1993b; Campbell *et al.*, 1992; Clark *et al.*, 1992; Hypsa & Dale, 1997; Hypsa & Aksoy, 1997; Munson *et al.*, 1991; O'Neill *et al.*, 1992, 1993; Unterman *et al.*, 1989) these fastidious bacteria have proved refractory to conventional culture techniques and consequently

only a handful of specimens have been assigned correct nomenclature (Aksoy, 1995; Hypsa & Dale, 1997; Munson *et al.*, 1991).

In the tsetse fly (*Glossina* spp.) P- and S-endosymbionts coexist in the gut lumen, with P-endosymbionts occupying specialized mycetocyte cells in the anterior portion of the insect gut and S-endosymbionts occupying midgut epithelial cells (Huebner & Davey, 1974; Pinnock & Hess, 1974). While the role of each micro-organism has not been clearly defined, collectively their presence is known to be essential for egg production and larval development in the insect (Nogge, 1981). Elimination of the bacterial endosymbionts with antibiotics, lysozyme and specific antibodies leads to reproductive abnormalities and growth retardation in the aposymbiotic host (Hill & Campbell, 1973; Nogge, 1976, 1978; Pinnock & Hess, 1974; Southwood *et al.*, 1975). While the P- and S-endosymbionts of tsetse flies are both members of the family *Enterobacteriaceae* (γ subclass of the class *Proteobacteria*), each endosymbiont forms a distinct lineage within the family *Enterobacteriaceae*, sharing high 16S rDNA sequence identity with P- and S-endosymbionts found in other insects (Aksoy *et al.*, 1995). Only the P-endosymbionts show concordant evolution with their insect host, having 16S rDNA sequences which mirror the evolution of the *Glossina*

† **Present address:** Department of Biology, University of York, Heslington, York YO1 5YW, UK.

Abbreviations: MM, Mitsunashi–Maramorosch; P, primary; S, secondary.

complex (Aksoy *et al.*, 1997). Tsetse S-endosymbionts isolated from different *Glossina* spp. share almost identical 16S rDNA sequences, suggesting recent acquisition of these organisms and possible horizontal symbiont transfer (Aksoy *et al.*, 1997).

The multi-tissue S-endosymbionts of tsetse flies were the first true insect endosymbionts to be cultivated *in vitro*. This was achieved through the use of a mosquito (*Aedes albopictus*) feeder cell culture system (Welburn *et al.*, 1987), which has recently been used for the cultivation of other fastidious insect bacteria (Hypsa & Dale, 1997; O'Neill *et al.*, 1997). Tsetse fly S-endosymbionts have recently been cultivated in cell-free media, although solid-phase culture and pure culture has not been achieved (Beard *et al.*, 1993b). The cultivation of tsetse fly S-endosymbionts has provoked interest in the use of these micro-organisms as potential tools for engineering refractory arthropod disease vectors, incapable of transmitting parasitic trypanosomes (Aldhous, 1993; Beard *et al.*, 1993a). Since tsetse fly S-endosymbionts are located in the insect gut where incoming parasites arrive, they may provide an excellent platform for anti-parasite gene expression. In addition, the S-endosymbionts of tsetse flies have been proposed to play an important role in the acquisition of trypanosome infections in tsetse flies, with their presence in certain fly lines promoting susceptibility to trypanosome infection (Maudlin & Ellis, 1985).

In this study we describe the isolation and pure culture of an S-endosymbiont from the haemolymph of *G. m. morsitans*. This was achieved by culture on a semi-defined solid medium under microaerobic conditions. In addition, we present the results of the phenotypic tests that are required for the valid description of this new bacterial taxon and species. Considering the phylogenetic position and the results of the phenotypic tests we formally propose that the S-endosymbiont of *G. m. morsitans* should be classified in a new genus, *Sodalis* gen. nov., and in a new species, *Sodalis glossinidius* gen. nov., sp. nov.

METHODS

Isolation and cultivation of the *G. m. morsitans* S-endosymbiont in an insect cell line. The *G. m. morsitans* S-endosymbiont culture used for this study was isolated from a laboratory colony of *G. m. morsitans* (Tsetse Research Group, University of Glasgow, UK). *Aedes albopictus* cell line C6/36 (Igarashi, 1978) was maintained in Mitsuhashi-Maramorosch (MM) medium (ICN Biomedicals) supplemented with 20% (v/v) heat-inactivated foetal calf serum at 25 °C by passaging the cells every 10 d with a 1:10 split into fresh MM medium. Five adult *G. m. morsitans* were surface sterilized by immersion in Alcide LD (Life Science Laboratories) for 5 min and rinsed twice in sterile water. Haemolymph was collected in a sterile capillary tube after puncturing each fly in the fons region with a sterile needle. Immediately after collection, the haemolymph was mixed with 200 µl MM medium. The coverslip technique (Welburn *et al.*, 1987) was used to enhance infection of the confluent *Aedes albopictus* cell culture by centrifugation as follows. After mixing the 200 µl MM/haemolymph with 5 ml 48 h-

old C6/36 cells (cultivated on a coverslip in a flat-bottomed centrifuge tube), the cultures was centrifuged (1500 g, 5 min, 25 °C) to promote bacterial colonization of the insect cells. Infected cultures were maintained at 25 °C and passaged every 3 d by inoculating a fresh C6/36 culture with a 1/10 volume of symbiont-infected C6/36 cells. The Gimenez staining procedure (Gimenez, 1964) was used to examine the morphology of the cultured S-endosymbiont.

Solid-phase culture of the S-endosymbiont on agar media containing aerotolerance-enhancing supplements. Basal MM agar was prepared from serum-free MM medium by the addition of bacto-agar (Difco) to 1% (w/v). After autoclaving and cooling to 50 °C, aliquots of basal MM agar were supplemented with the following aerotolerance-enhancing supplements: Activated charcoal (10 mg ml⁻¹; Sigma), bovine catalase (10, 100 and 1000 U ml⁻¹; Sigma), FeSO₄ (5, 10 and 50 mg ml⁻¹; Sigma), dithioerythritol (1, 5 and 10 mg ml⁻¹; Sigma) and fresh horse red blood cells (2 ml packed red cells mixed with 8 ml MM medium; Tissue Culture Services). Plates containing supplemented MM agar were inoculated with 200 µl symbiont-infected C6/36 culture fluid and maintained at 25 °C.

Solid-phase culture of the S-endosymbiont on MM agar under different atmospheres. Basal MM agar plates were inoculated with 200 µl symbiont-infected C6/36 culture fluid and maintained at 25 °C under a range of different atmospheres in gas jars (Oxoid) at 25 °C. Anaerobic conditions were created by the action of an anaerobic catalyst (Oxoid), which was activated and maintained in a sealed jar along with inoculated MM agar plates. Microaerobic and aerobic conditions were created by flushing sealed gas jars (containing inoculated plates) with 100 vols appropriate gas mixture. The gas mixtures employed in the experiment were 5, 10, 15 and 20% oxygen balanced with either carbon dioxide or nitrogen. As a control treatment, a single inoculated plate was maintained in a gas jar filled with air.

Isolation of strain M1^T, a pure culture isolate of S-endosymbiont. Strain M1^T was obtained by the isolation of a single S-endosymbiont colony from an MM agar plate after 7 d growth in a microaerobic atmosphere (5% O₂, balanced with CO₂) at 25 °C. Strain M1^T was used for all subsequent experiments described in this study.

Detection of the S-endosymbiont by PCR. Primers GP01F (5' TGAGAGGTTTCGTCAATGA 3') and GP01R (5' ACGCTGCGTGACCATTC 3') were used for the detection of S-endosymbionts, as these primers are known to amplify a 1.2 kb fragment of an abundant extrachromosomal element found in this bacterium (O'Neill *et al.*, 1993). The single colony used for the isolation of strain M1^T was resuspended in 50 µl MM medium and 5 µl of this cell suspension was used as template for PCR with primers GP01F and GP01R. Samples (1 µl) of culture fluid from S-endosymbiont-infected and uninfected C6/36 cell cultures were used as template in positive and negative control PCRs (respectively) using the same primer set (GP01F/R). PCRs were carried out in 50 µl thermostable polymerase buffer (Promega) containing 1.5 mM MgCl₂, 0.2 mM dNTPs, 20 pmol each primer and 1 U *Taq* DNA polymerase (Promega). Reaction conditions consisted of an initial 5 min denaturation step (94 °C) followed by 35 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min).

Cloning and sequencing of 16S rDNA from strain M1^T. One microlitre of strain M1^T cell suspension was used as template for a PCR with conserved primers 5' GCTTAACAC-ATGCAAG 3' and 5' ACGGGCAGTGTGTACAAGACC

3', which are known to amplify a 1361 bp fragment of 16S rDNA (O'Neill *et al.*, 1993). PCRs with the 16S rDNA consensus primers (including two negative-control reactions with no template) were carried out as outlined above for the GP01F/R primer set, using identical cycling parameters and reaction components, except for the primers. To facilitate A-tailing and subsequent cloning, the PCR program included an additional final 10 min extension step (72 °C). The 1361 bp PCR product obtained from strain M1^T was cloned into T-tailed pBS SK+ (Stratagene) and recombinant plasmids were sequenced by the chain-termination method on an Applied Biosystems automated sequencer.

Identification of strain M1^T by extrachromosomal DNA profile analysis. To confirm the identity of strain M1^T, the remaining 45 µl of the single colony isolate suspension was inoculated into 25 ml MM medium and incubated at 25 °C. After 7 d growth, 20 ml culture medium was removed and the bacteria were pelleted by centrifugation (5000 g, 5 min). Extrachromosomal DNA was prepared using a Wizard Minipreps plasmid DNA purification kit (Promega) according to the manufacturer's instructions with the following modifications: plasmid DNA was eluted from the column with 50 µl sterile water, which had been heated to 80 °C in a water bath prior to application onto the column. After elution into an Eppendorf, the aqueous DNA solution was combined with loading buffer and absorbed onto small squares of sterile Whatman No. 1 paper, to prevent shearing prior to electrophoresis. Extrachromosomal DNA was also prepared from S-endosymbionts cultivated in C6/36 cells following an identical procedure after removal of the C6/36 cells by low speed centrifugation (500 g, 10 min). Both extrachromosomal DNA preparations were resolved by overnight electrophoresis (1 V cm⁻¹) through a 0.6% (w/v) agarose gel.

Phenotypic tests. The remaining 5 ml of the M1^T culture used for extrachromosomal DNA preparation was streaked onto MM agar plates and incubated under a microaerobic atmosphere to provide material for a range of phenotypic tests. Phenotypic tests were conducted using diagnostic tablets (Rosco Diagnostica) following the manufacturer's instructions with the exception that the enzymic reactions were incubated at the optimum growth temperature for S-endosymbionts (25 °C). Reactions were permitted to continue for twice the normal time before scoring to account for reduced enzyme activity at this temperature. Where diagnostic tablets were not applicable, tests were conducted according to established procedures (Cowan, 1974), again at 25 °C using an extended incubation period. To ensure the validity of the test procedures control tests were conducted under identical conditions using *Escherichia coli* K12, *Citrobacter freundii* ATCC 10787 and *Xenorhabdus nematophilus* ATCC 19061. All phenotypic tests were conducted under both aerobic and microaerophilic conditions.

Carbon substrate assimilation tests. Since no minimal medium has been reported for the culture of *Glossina* S-endosymbionts, strain M1^T carbon substrate assimilation tests were conducted during culture in the semi-defined MM medium prepared without the addition of glucose. For testing, 10 µl of a suspension of strain M1 in glucose-free MM medium was inoculated into culture tubes containing 10 ml MM medium supplemented (individually) to 2% (w/v) with the carbon sources outlined in Table 2. For comparison, 10 µl of the same suspension of strain M1^T was inoculated into 10 ml glucose-free MM medium without any carbon supplement present. To assess carbon source utili-

zation, the increase in turbidity of each culture was determined by spectrophotometry after 7 d incubation at 25 °C by measuring OD₆₀₀ using a Corning spectrophotometer. Acid production was detected by repeating the carbon substrate assimilation tests with identical culture media containing the indicators phenol red (for detection of weak acid production) and bromocresol green (for detection of strong acid production). All tests were conducted under microaerobic conditions.

RESULTS AND DISCUSSION

Culture of the S-endosymbiont in *Aedes albopictus* cell line C6/36

Pleiomorphic, rod-shaped bacteria were visible in *Aedes albopictus* C6/36 cells at 24 h following inoculation with insect haemolymph. At this time, most of the bacteria observed were bound to the surface of C6/36 cells, forming small nests of infection. As the infection progressed, bacteria were observed penetrating the surface of the insect cells and dividing within the cell cytosol (48 h post-inoculation). By 72 h post-inoculation, bacterial infection was widespread with many insect cells displaying extensive intracellular infection (Fig. 1). The course and timing of the bacterial infection cycle was identical to that observed previously for *Glossina* S-endosymbionts cultivated in C6/36 cells (Welburn *et al.*, 1987).

Microaerobic culture of the S-endosymbiont

A solid-phase MM agar culture system was used to investigate the aerotolerance of the cultured S-endosymbiont. Chemical and enzyme supplements, known

Fig. 1. Light micrograph of *Aedes albopictus* C6/36 cells, 72 h after inoculation of haemolymph from *G. m. morsitans*. At this stage the insect cells display extensive intracellular infection. Bar, 5 µm.

Table 1. Aerotolerance of the *G. m. morsitans* S-endosymbiont

–, No growth; w, colony diameter <0.5 mm; +, colony diameter 0.5–1 mm; ++, colony diameter >1 mm.

Supplement/atmosphere Days post inoculation ...	Growth									
	1	2	3	4	5	6	7	8	9	10
Control (air)	–	–	–	–	–	–	–	–	–	–
10 mg charcoal ml ⁻¹	–	–	–	–	–	–	–	–	–	–
10 U catalase ml ⁻¹	–	–	–	–	–	w	w	w	w	w
100 U catalase ml ⁻¹	–	–	–	w	+	+	+	+	+	+
1000 U catalase ml ⁻¹	–	–	–	w	+	+	+	+	+	+
5 mg FeSO ₄ ml ⁻¹	–	–	–	–	–	–	–	–	–	–
10 mg FeSO ₄ ml ⁻¹	–	–	–	–	–	–	–	–	–	–
50 mg FeSO ₄ ml ⁻¹	–	–	–	–	–	–	–	–	–	–
2 mg KMnO ₄ ml ⁻¹	–	–	–	–	–	–	–	–	–	–
10 mg KMnO ₄ ml ⁻¹	–	–	–	–	–	–	–	–	–	–
20 mg KMnO ₄ ml ⁻¹	–	–	–	–	–	–	–	–	–	–
1 mg dithioerythritol ml ⁻¹	–	–	–	–	–	–	–	–	–	–
5 mg dithioerythritol ml ⁻¹	–	–	–	–	–	–	–	–	–	–
10 mg dithioerythritol ml ⁻¹	–	–	–	–	–	–	–	–	–	–
20% packed horse red blood cells	–	–	–	w	+	++	++	++	++	++
20% O ₂ /80% N ₂	–	–	–	–	–	–	–	–	–	–
15% O ₂ /85% N ₂	–	–	–	–	–	–	–	–	–	–
10% O ₂ /90% N ₂	–	–	–	w	w	w	w	w	w	w
5% O ₂ /95% N ₂	–	–	–	w	+	+	+	+	++	++
20% O ₂ /80% CO ₂	–	–	–	–	–	–	–	–	–	–
15% O ₂ /85% CO ₂	–	–	–	–	–	–	–	–	–	–
10% O ₂ /90% CO ₂	–	–	–	w	+	+	+	+	++	++
5% O ₂ /95% CO ₂	–	–	–	+	++	++	++	++	++	++
Anaerobic catalyst	–	–	–	–	–	–	w	w	w	w

to enhance the growth of microaerophilic bacteria (Krieg & Hoffman, 1986), were incorporated into test media to examine the effects of oxygen toxicity (Table 1). After 5 d growth at 25 °C, colonies were observed only on MM agar plates containing either catalase or fresh horse blood. Fresh blood is known to be an excellent source of the enzyme catalase, which catalyses the breakdown of toxic hydrogen peroxide (Bolton *et al.*, 1984). In this experiment the S-endosymbiont grew most efficiently on media supplemented with fresh horse red blood cells. Phenotypic tests show that the S-endosymbiont, unlike most members of the Family *Enterobacteriaceae*, does not produce catalase. It seems likely that the absence of this enzyme is responsible, at least in part, for the restricted aerotolerance of this micro-organism. While growth was observed on MM agar plates supplemented with blood or catalase, colony size was irregular. At the beginning of the streak, where the inoculum was heavy, growth was dense and confluent with large colonies developing and merging. At the end of a streak, where the inoculum was thin, growth was sparse with colonies only attaining a small diameter. The subculture of individual colonies onto fresh plates

showed that colony morphology did not breed true, indicating that mutation or phase-variation were unlikely to be responsible for the irregular colony sizes observed. Population-dependent growth has been observed with a number of other microaerophilic bacteria whose growth rate often increases with total respiratory capacity (Krieg & Hoffman, 1986).

Synthetic gas mixtures were used to determine the effect of atmospheric oxygen and carbon dioxide availability on the solid-phase culture of the S-endosymbiont (Table 1). After 5 d at 25 °C, bacterial growth was observed only on MM agar plates incubated in atmospheres containing <10% oxygen, confirming that atmospheric oxygen inhibits the growth of the S-endosymbiont on solid media. While these bacteria grew very poorly in an anaerobic atmosphere, the presence of 5% oxygen greatly stimulated growth. In addition, growth was enhanced by the presence of carbon dioxide, a gas known to enhance the growth of a number of other microaerophiles, including *Campylobacter* spp. (Krieg & Hoffman, 1986). Carbon dioxide utilization is a feature of bacteria which employ an anapleurotic cycle for the

enzymic replenishment of tricarboxylic acid cycle intermediates that are used up during biosynthetic reactions (Moat & Foster, 1988). This is of some interest in the context of insect–bacterial symbioses where certain insect symbionts are known to assist their insect host through the provision of essential dietary factors lacking in the host's natural diet (Douglas, 1989). While there is no direct evidence of a biosynthetic capability in *Glossina* S-endosymbionts, it is known that the P-endosymbionts of aphids (including *Buchnera aphidicola*) supplement the insect host via the provision of essential amino acids which are lacking in the host's natural diet of plant sap (Douglas, 1997). In such a system, carbon dioxide fixation and assimilation by the anapleurotic cycle would certainly provide a means of replacing intermediates used up during the biosynthesis of nitrogenous compounds.

To our knowledge, this is the first example of successful insect S-endosymbiont culture on an agar-based medium. With increasing interest in the possibility of using insect symbionts to express anti-parasitic gene products in insect disease vectors (Aldhous, 1993), this new culture technique will permit the application of modern microbial genetic procedures requiring a solid-phase culture system. In addition a solid-phase culture system may prove useful for the isolation and cultivation of symbiotic bacteria from other insects.

Isolation of strain M1^T, a pure culture isolate of the S-endosymbiont

Bacterial strain M1^T was obtained by the isolation of a single S-endosymbiont colony from an MM agar plate after 7 d growth in a microaerobic atmosphere (5% O₂ balanced with CO₂) at 25 °C. Strain M1^T has been maintained in our laboratory for two years and was used for all of the subsequent experiments described in this study.

16S rDNA sequence analysis of strain M1^T

To confirm the identity of M1^T, a 1361 bp fragment of the 16S rDNA locus was amplified by PCR using consensus primers (data not shown). Sequencing revealed that the partial 16S rDNA sequence obtained from strain M1^T was identical to that obtained in a previous study from the S-endosymbiont of *G. m. morsitans* (Beard *et al.*, 1993b; GenBank accession number M99060). This data confirms that bacterial strain M1^T, isolated on solid media, is the S-endosymbiont found in *G. m. morsitans*.

PCR using primer set GP01F/R

PCR using the *Glossina* S-endosymbiont-specific primer set GP01F/R was used to detect strain M1^T, isolated after solid-phase culture of the S-endosymbiont. The single expected 1.2 kb PCR product

Fig. 2. PCR analysis of strain M1^T using primer set GP01F/R. Lane 1, *Hind*III-digested lambda DNA markers of 2027 (upper small arrow) and 564 bp (lower small arrow); lane 2, PCR product obtained with strain M1^T cells used as template; lane 3, PCR carried out with C6/36–*G. m. morsitans* S-endosymbiont culture fluid used as template; lane 4, PCR product obtained with uninfected C6/36 culture fluid used as template. The large arrow indicates the expected 1.2 kb PCR product.

Fig. 3. Extrachromosomal DNA prepared from strain M1^T and the *G. m. morsitans* S-endosymbiont cultured with *Ae. albopictus* C6/36 cells. Lane 1, *Hind*III-digested lambda DNA markers of 23130 (upper arrow), 9416 (middle arrow) and 6557 bp (lower arrow); lane 2, extrachromosomal DNA prepared from strain M1^T; lane 3, extrachromosomal DNA prepared from the *G. m. morsitans* S-endosymbiont cultured with *Ae. albopictus* C6/36 cells.

(Fig. 2) was observed in reactions containing cells from strain M1^T and the original S-endosymbiont-infected C6/36 culture (depicted in Fig. 1). No product was observed when uninfected C6/36 culture material was used as template for the PCR.

Extrachromosomal DNA analysis of strain M1^T

Extrachromosomal DNA was prepared from strain M1^T and the original S-endosymbiont C6/36 culture (depicted in Fig. 1). Electrophoresis revealed that the extrachromosomal DNA profiles were identical (Fig. 3), in addition these profiles matched those obtained in a previous study (Dale, 1997). These individual profiles comprise a number of large extrachromosomal elements and have proved to be diagnostic for S-endosymbionts, permitting differentiation between isolates obtained from different *Glossina* spp. (Dale, 1997). It is clear that the array of extrachromosomal DNA molecules, which may prove important for

future symbiont engineering, are maintained through the microaerobic-culture procedure.

Colony morphology of strain M1^T

On MM agar plates incubated under a 5% oxygen, 95% carbon dioxide atmosphere, strain M1^T displayed uniform colony size and morphology with colonies appearing shiny, off-white and concave with entire edges.

Phenotypic features of strain M1^T

The results of the phenotypic tests were found to be identical under aerobic and microaerophilic conditions, and they reveal that strain M1^T is characterized by an inactive biochemical profile in comparison with other members of the family *Enterobacteriaceae*. Strain M1^T was non-motile and negative for catalase, oxidase, deoxyribonuclease, gelatinase, urease, nitrate reductase, indole production, hippurate hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, starch hydrolysis, α -fucosidase, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase and β -xylosidase. Strain M1^T did produce α -galactosidase and β -N-acetylglucosaminidase. The validity of the test results was confirmed by results obtained with the control bacterial strains (*Escherichia coli* K12, *Citrobacter freundii* ATCC 10787 and *Xenorhabdus nematophilus* ATCC 19061) with all results (data not shown) conforming to those published in Bergey's Manual of Determinative Bacteriology (9th edn). The choice of phenotypic tests for this study was based upon features which are commonly used to differentiate members of the family *Enterobacteriaceae* (consisting mainly of genera which have been isolated and investigated because of their intrinsic association with animals and humans). Phenotypic testing has so far only been conducted on a few members of the family *Enterobacteriaceae* known to be associated with insects and this can be attributed to the fastidious nature of these organisms, many of which have not been isolated in pure culture. Of the insect-associated members of the *Enterobacteriaceae* which are known, phenotypic testing has only been undertaken with members of the genera *Xenorhabdus* and *Photorhabdus*, and *Arsenophonus nasoniae*. In common with strain M1^T, these three genera all share a relatively inactive biochemical profile in comparison with other members of the family *Enterobacteriaceae*. However unlike *Xenorhabdus* spp. and *Photorhabdus* spp., which are both motile, and *Arsenophonus nasoniae*, which hydrolyses gelatin, strain M1 is both non-motile and does not hydrolyse gelatin. Unlike most members of the family *Enterobacteriaceae*, including *Arsenophonus nasoniae* and *Xenorhabdus luminescens*, strain M1^T does not produce catalase, accounting for the microaerophilic nature of this bacterium. It is known from a previous study that the S-endosymbiont produces at least one

Table 2. Carbon substrate assimilation and acid production tests.

Carbon substrate	Assimilation*	Acid production†
N-acetyl-D-glucosamine	+	+
Glucose	+	w
Glycol chitosan	+	w
Mannitol	+	w
Raffinose	+	+
Sorbitol	+	w

* Positive assimilation indicates a final turbidity reading >0.1 OD₆₀₀ units. Assimilation was deemed to be negative when the final OD₆₀₀ reading was <0.05 . Strain M1^T was negative for acetic acid, adonitol, δ -amino valeric acid, L-arabinose, n-butanol, citric acid, dulcitol, ethanol, fructose, fumaric acid, galactose, glycerol, glycolic acid, histamine, *p*-hydroxy benzoic acid, α -ketoglutaric acid, lactose, D,L-malic acid, maltose, mannose, melibiose, methyl- α -D-glucopyranoside, *myo*-inositol, n-propanol, pyruvic acid, quinic acid, rhamnose, ribose, saccharic acid, salicin, sarcosine, sorbose, succinic acid, sucrose, starch, trehalose and xylose.

† w, weak acid production; +, strong acid production.

type of chitinase enzyme (Welburn *et al.*, 1993) and in this study we show that strain M1^T produces β -N-acetylglucosaminidase. Interestingly, chitinase production by this micro-organism has been postulated to account for an increase in trypanosome susceptibility in laboratory colonies of *G. m. morsitans* known to harbour large numbers of S-endosymbionts (Welburn & Maudlin, 1991). In addition to β -N-acetylglucosaminidase, strain M1^T also produced α -galactosidase.

Carbon substrate assimilation tests

Perhaps the most striking feature of strain M1^T was its unusual carbon substrate assimilation characteristics (Table 2). While strain M1^T did utilize and produce acid from glucose, both growth and acid production were more vigorous when either N-acetyl-D-glucosamine or raffinose was provided as a carbon source. These results are in agreement with the glycosidase detection tests, where it was found that strain M1^T produced α -galactosidase (involved in raffinose catabolism) and β -N-acetylglucosaminidase (involved in N-acetyl-D-glucosamine catabolism). Strain M1^T also used glycol chitosan, a polymer of N-acetyl-D-glucosamine, as a carbon source and this was accompanied by weak acid production. The sugar alcohols sorbitol and mannitol were also utilized by strain M1^T, and in both cases weak acid production was detected. This suggests that strain M1^T is devoted to the use of N-acetyl-D-glucosamine and raffinose as primary carbon sources. While polymerized N-acetyl-D-glucosamine (chitin) is known to form the exoskeleton and gut peritrophic membrane of tsetse flies (Lehane *et al.*, 1996; Wigglesworth, 1972), little is known about the

presence of raffinose in this insect. In the maize weevil, *Sitophilus zeamais*, an insect known to harbour closely related S-endosymbionts, raffinose is known to be an important dietary component (Baker, 1991). In addition, the S-endosymbiont-carrying aphid *Acyrtosiphon pisum* (Chen & Purcell, 1997; Unterman *et al.*, 1989) and whitefly *Bemisia tabaci* (Costa *et al.*, 1995) are known to produce honeydew rich in raffinose (Miller *et al.*, 1994). In the case of the silver leaf whitefly *Bemisia argentifolii*, experiments involving aposymbiotic strains have shown that endosymbionts are capable of influencing the composition of honeydew (Davidson *et al.*, 1994).

Taxonomic position of strain M1^T

Phylogenetic analysis based on the 16S rDNA locus has revealed that the *G. m. morsitans* S-endosymbiont is most closely related to the S-endosymbiont found in *Glossinia pallidipes*, sharing an identical 16S rDNA sequence over 1117 bp (Beard *et al.*, 1993b). In a more detailed study (Aksoy *et al.*, 1995) it was shown that the S-endosymbiont of *G. m. morsitans* is also closely related to the S-endosymbionts found in *Glossinia palpalis* (pairwise distance over 1026 bp, 1 bp), *Glossinia austeni* (pairwise distance over 1026 bp, 1 bp), *Glossinia fuscipes* (pairwise distance over 1026 bp, 2 bp) and *Glossinia brevipalpis* (pairwise distance over 1026 bp, 4 bp). In addition, it was shown that the S-endosymbiont of *G. m. morsitans* is also closely related to the S-endosymbionts of *Sitophilus zeamais* (pairwise distance over 940 bp, 21 bp) and *Acyrtosiphon pisum* (pairwise distance over 940 bp, 54 bp). These studies suggest that a distinct lineage of S-endosymbionts exists within the family *Enterobacteriaceae*. It is known that the P-endosymbionts of insects (including *G. m. morsitans*) form a separate distinct lineage within the *Enterobacteriaceae* and recently the P-endosymbiont of *G. m. morsitans* has been assigned correct nomenclature as *Wigglesworthia glossinidia* (Aksoy, 1995). On the basis of the phylogenetic analyses and the distinctions presented in this study we propose a new genus, *Sodalis* gen. nov., for the closely related secondary symbiotic bacteria found in *Glossinia* spp. In addition, we propose a new species, *Sodalis glossinidius*, for the S-endosymbiont found in *G. m. morsitans* and *G. pallidipes*, formerly known as the Rickettsia-like organism of tsetse. Phenotypic testing is required to determine the candidature of the other *Glossinia* spp. S-endosymbionts and the S-endosymbiont of *Sitophilus zeamais* within the genus *Sodalis*.

Description of *Sodalis* gen. nov.

Sodalis (So'da.lis. M.L. masc. n. *sodalis* a companion).

The type species is *Sodalis glossinidius*. The essential characteristics of this genus are given in the description of *S. glossinidius*.

Description of *Sodalis glossinidius* sp. nov.

Sodalis glossinidius (glos.sin.i'dus. M.L. *glossinidius* of the genus *Glossina*).

The cells are non motile, non spore-forming, filamentous, Gram-negative rods (2–12 µm in length, 1–1.5 µm in diameter), dividing by septation. The bacteria grow intracellularly in *Aedes albopictus* cell culture and can grow axenically in media containing enzymically digested proteins as nitrogen sources. They are microaerophilic and can only be cultivated on solid media with aerotolerance-enhancing supplements or under a reduced-oxygen atmosphere. Optimum atmospheric conditions for solid-phase culture are 5% oxygen, balanced with carbon dioxide. The optimum temperature for growth is 25 °C with little or no growth at temperatures exceeding 30 °C. Negative for catalase, oxidase, DNase, gelatinase, urease, nitrate reductase, indole production, hippurate hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine decarboxylase and starch hydrolysis. Produces α-galactosidase and β-N-acetylglucosaminidase. Do not produce α-fucosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, α-mannosidase and β-xylosidase. Utilizes N-acetyl-D-glucosamine and raffinose accompanied by a high level of acid production. Utilizes glucose, glycol chitosan, mannitol and sorbitol with accompanying weak acid production. No increase in growth is detected when any of the following carbon sources are incorporated in media: acetic acid, adonitol, δ-amino valeric acid, L-arabinose, n-butanol, citric acid, dulcitol, ethanol, fructose, fumaric acid, galactose, glycerol, glycolic acid, histamine, p-hydroxybenzoic acid, α-ketoglutaric acid, lactose, D,L-malic acid, maltose, mannose, melibiose, methyl-α-D-glucopyranoside, myo-inositol, n-propanol, pyruvic acid, quinic acid, rhamnose, ribose, saccharic acid, salicin, sarcosine, sorbose, succinic acid, sucrose, starch, trehalose or xylose. The type strain is M1^T, isolated from a laboratory colony of *Glossinia morsitans morsitans*. The bacterium is found in the midgut, fat body and haemolymph of *G. m. morsitans*. Strain M1^T has been deposited at the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) under deposition NCIMB 13495.

ACKNOWLEDGEMENTS

This document is an output from a project funded by the Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of DFID.

REFERENCES

Aksoy, S. (1995). *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associ-

- ated, primary endosymbionts of tsetse flies. *Int J Syst Bacteriol* **45**, 848–851.
- Aksoy, S., Pourhosseini, A & Chow, A. (1995). Mycetome endosymbionts of tsetse flies constitute a distinct lineage related to *Enterobacteriaceae*. *Insect Mol Biol* **4**, 15–22.
- Aksoy, S., Chen, X. & Hypsa, V. (1997). Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: *Glossinidae*). *Insect Mol Biol* **6**, 183–190.
- Aldhous, P. (1993). Bacteria may provide access to the tsetse fly. *Science* **261**, 548.
- Baker, J. E. (1991). Properties of glycosidases from the maize weevil, *Sitophilus zeamais*. *Insect Biochem* **21**, 615–621.
- Beard, C. B., O'Neill, S. L., Tesh, R. B., Richards, F. F. & Aksoy, S. (1993a). Modification of arthropod vector competence via symbiotic bacteria. *Parasitol Today* **9**, 179–183.
- Beard, C. B., O'Neill, S. L., Mason, P., Mandelco, L., Woese, C. R., Tesh, R. B., Richards, F. F. & Aksoy, S. (1993b). Genetic transformation and phylogeny of bacterial symbionts from tsetse. *Insect Mol Biol* **1**, 123–131.
- Bolton, F. J., Coates, D. & Hutchinson, D. N. (1984). The ability of *Campylobacter* media supplements to neutralize photochemically induced toxicity and hydrogen peroxide. *J Appl Bacteriol* **56**, 151–7.
- Buchner, P. (1965). *Endosymbiosis of Animals with Plant Microorganisms*. New York: Interscience.
- Campbell, B. C., Bragg, T. S. & Turner, C. E. (1992). Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: *Curculionidae*) based on analysis of 16S ribosomal DNA. *Insect Biochem Mol Biol* **22**, 415–421.
- Chen, D. Q. & Purcell, A. H. (1997). Occurrence and transmission of facultative endosymbionts in aphids. *Curr Microbiol* **34**, 220–225.
- Clark, A., Baumann, L., Munson, M. A., Baumann, P., Campbell, B. C., Duffus, J. E., Osborne, L. S. & Moran, N. A. (1992). The eubacterial endosymbionts of whiteflies (Homoptera: *Aleyrodoidea*) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. *Curr Microbiol* **25**, 119–123.
- Costa, H. S., Westcot, D. M., Ullman, D. E., Rosell, R., Brown, J. K. & Johnson, M. W. (1995). Morphological variation in *Bemisia* endosymbionts. *Protoplasma* **189**, 194–202.
- Cowan, S. T. (1974). *Manual for the Identification of Medical Bacteria*, 2nd edn. Cambridge, UK: Cambridge University Press.
- Dale, C. (1997). *The secondary (S-) endosymbionts of Glossinia spp.* PhD thesis. University of Liverpool, UK.
- Davidson, E. W., Segura, B. J., Steele, T. & Hendrix, D. L. (1994). Microorganisms influence the composition of honeydew produced by the silverleaf whitefly, *Bemisia argentifolii*. *J Insect Physiol* **40**, 1069–1076.
- Douglas, A. E. (1989). Mycetocyte symbiosis in insects. *Biol Rev* **64**, 409–434.
- Douglas, A. E. (1997). Parallels and contrasts between symbiotic bacteria and bacterial-derived organelles: evidence from *Buchnera*, the bacterial symbiont of aphids. *FEMS Microbiol Ecol* **24**, 1–9.
- Gimenez, D. (1964). Staining *Rickettsiae* in yolk-sac cultures. *Stain Techn* **39**, 135–140.
- Hill, P. D. S. & Campbell, J. A. (1973). The production of symbiont-free *Glossina morsitans* and an associated loss of female fertility. *Trans R Soc Trop Med Hyg* **67**, 727–728.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (editors) (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore: Williams & Wilkins.
- Huebner, E. & Davey, K. G. (1974). Bacteroids in the ovaries of a tsetse fly. *Nature* **249**, 260–261.
- Hypsa, V. & Aksoy, S. (1997). Phylogenetic characterization of two transovarially transmitted endosymbionts of the bedbug *Cimex lectularius* (Heteroptera: *Cimicidae*). *Insect Mol Biol* **6**, 301–304.
- Hypsa, V. & Dale, C. (1997). *In vitro* culture and phylogenetic analysis of *Candidatus Arsenophonus triatominarum* an intracellular bacterium from the triatomine bug *Triatoma infestans*. *Int J Syst Bacteriol* **47**, 1140–1144.
- Igarashi, A. (1978). Isolation of Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *J Gen Virol* **40**, 531.
- Krieg, N. R. & Hoffman, P. S. (1986). Microaerophily and oxygen toxicity. *Annu Rev Microbiol* **40**, 107–130.
- Lehane, M. J., Allingham, P. G. & Weglicki, P. (1996). Composition of the peritrophic matrix of the tsetse fly, *Glossina morsitans morsitans*. *Cell Tissue Res* **283**, 375–384.
- Maudlin, I. & Ellis, D. S. (1985). Association between intracellular rickettsial-like infections of midgut cells and susceptibility to trypanosome infection in *Glossina* spp. *Z Parasitenkd* **71**, 683–687.
- Miller, W. B., Peralta, E., Ellis, D. R. & Perkins, H. H. (1994). Stickiness potential of individual insect honeydew carbohydrates on cotton lint. *Textile Res J* **64**, 344–350.
- Moat, A. G. & Foster J. W. (1988). *Microbial Physiology*. New York: Wiley.
- Munson, M. A., Baumann, P. & Kinsey, M. B. (1991). *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. *Int J Syst Bacteriol* **41**, 566–568.
- Nogge, G. (1976). Sterility in tsetse flies caused by loss of symbionts. *Experientia* **32**, 995.
- Nogge, G. (1978). Aposymbiotic tsetse flies, *Glossina morsitans morsitans*, obtained by feeding on rabbits immunised specifically with symbionts. *J Insect Physiol* **24**, 299–304.
- Nogge, G. (1981). Significance of symbionts for the maintenance of an optimal nutritional state of successful reproduction in hematophagous arthropods. *Parasitology* **82**, 101–104.
- O'Neill, S. L., Giordano, R., Colbert, A. M. E., Karr, L. & Robertson, H. M. (1992). 16S rRNA phylogenetic analysis of bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA* **89**, 2699–2702.
- O'Neill, S. L., Gooding, R. H. & Aksoy, S. (1993). Phylogenetically distant symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. *Med Vet Entomol* **7**, 377–383.
- O'Neill, S. L., Pettigrew, M. M., Sinkins, S. P., Braig, H. R., Andreadis, T. G. & Tesh, R. B. (1997). *In vitro* cultivation of *Wolbachia pipiensis* in an *Aedes albopictus* cell line. *Insect Mol Biol* **6**, 33–39.
- Pinnock, D. E. & Hess, R. T. (1974). The occurrence of intracellular rickettsia-like organisms in the tsetse flies, *Glossina morsitans*, *G. fuscipes*, *G. brevipalpis* and *G. pallidipes*. *Acta Trop* **31**, 70–79.
- Southwood, T. R. E., Khalaf, S. & Sinden, R. E. (1975). The microorganisms of tsetse flies. *Acta Trop* **32**, 259–266.
- Unterman, B. M., Baumann, P. & McLean, D. L. (1989). Pea aphid symbiont relationships established by analysis of 16S rRNAs. *J Bacteriol* **171**, 2970–2974.

Welburn, S. C. & Maudlin, I. (1991). Rickettsia-like organisms, puparial temperature and susceptibility to trypanosome infection in *Glossina morsitans*. *Parasitology* **102**, 201–206.

Welburn, S. C., Maudlin, I. & Ellis, D. S. (1987). *In vitro* cultivation of rickettsia-like organisms from *Glossina* spp. *Ann Trop Med Parasitol* **81**, 331–335.

Welburn, S. C., Arnold, K., Maudlin, I. & Gooday, G. W. (1993). Rickettsia-like organisms and chitinase production in relation to transmission of trypanosomes by tsetse flies. *Parasitology* **107**, 141–145.

Wigglesworth, V. B. (1972). *The Principles of Insect Physiology*, 7th edn. London: Chapman & Hall.