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The tsetse fly *Glossina fuscipes fuscipes* (Diptera: Glossina) harbours a surprising diversity of bacteria other than symbionts

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Abstract Three different bacterial species are regularly described from tsetse flies. However, no broad screens have been performed to investigate the existence of other bacteria in this medically and agriculturally important vector insect. Utilising both culture dependent and independent methods we show that Kenyan populations of *Glossina fuscipes fuscipes* harbour a surprising diversity of bacteria. Bacteria were isolated from 72% of flies with 23 different bacterial species identified. The Firmicutes phylum dominated with 16 species of which seven belong to the genus *Bacillus*. The tsetse fly primary symbiont, *Wigglesworthia glossinidia*, was identified by the culture independent pathway. However, neither the secondary symbiont *Sodalis* nor *Wolbachia* was detected with either of the methods used. Two other bacterial species were identified with the DNA based method, *Bacillus subtilis* and *Serratia marcescens*. Further studies are needed to determine how tsetse flies, which only ever feed on vertebrate blood, pick up bacteria and to investigate the possible impact of these bacteria on *Glossina* longevity and vector competence.

Introduction

Studies on the microbiota in insect guts have revealed that bacteria may affect their hosts in many different ways, from making a nutritional contribution to the insect to protecting it from pathogenic bacteria and the production of semiochemicals (Dillon and Dillon 2004). Several insects harbour symbionts (Baumann and Moran 1997). For instance, tsetse flies harbour two different symbiotic bacteria. First, the primary symbiont *Wigglesworthia glossinidia* which is an obligate mutualist and is postulated to help the fly with vitamin production (Akman et al. 2002). Second, the secondary symbiont *Sodalis glossinidius* which has been found to increase longevity and hence is described as beneficial to the fly (Dale and Welburn 2001). *S. glossinidius* has also been suggested to affect how efficient the flies are in transmitting the protozoan parasites of the genus *Trypanosoma* that cause sleeping sickness in humans and nagana in cattle (Geiger et al. 2007; Welburn and Maudlin 1999). In addition, tsetse flies may also harbour bacteria of the genus *Wolbachia* which are often described as pathogens in other insects although their function in tsetse flies remains to be determined. While *W. glossinidia*, as an obligate mutualist, is present in all tsetse flies the prevalence of *Sodalis* and *Wolbachia* varies considerably between fly

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populations. *S. glossinidius* has been detected in colony flies at high prevalence (Shaw and Moloo 1991), however, few studies have been performed on the prevalence of this species in field-caught flies. It has been shown that *S. glossinidius* is present in field populations of *Glossina palpalis palpalis* (9.3%), *Glossina nigrofusca* (85%) and *Glossina pallicera* (31%) in Liberia, however very few flies were examined for the last two species (Maudlin et al. 1990). In contrast, *S. glossinidius* was found in all *Glossina p. gambiensis* flies examined in Burkina Faso, however, in that study pupae from field caught females were investigated instead of screening the field caught flies directly (Geiger et al. 2007). *S. glossinidius* can be grown on nutrient agar plates under specialised conditions (Matthew et al. 2005), however neither *W. glossinidia* nor *Wolbachia* from tsetse flies have been grown in vitro. In fact, it has been suggested that the majority (80–99%) of all bacteria cannot grow under standard culturing conditions (Ward et al. 1990; Amann et al. 1995). DNA based techniques have revolutionised studies on bacterial diversity in nature, including studies on insect microbiota (Weisburg et al. 1991; Amann et al. 1995). The 16S ribosomal RNA gene is most frequently utilised to identify bacterial species in these studies. One technique utilised to screen environmental samples for bacteria is denaturing gradient gel electrophoresis (DGGE, Muyzer and Smalla 1998) which has proved to be very useful, especially since it allows for identification of the bacterial species (Dorigo et al. 2005; Nocker et al. 2007). Previous studies on insect microbiota have highlighted the need to combine culture dependent and culture independent methods since DNA based techniques also have limits (Forney et al. 2004; Wintzingerode et al. 1997; Baker et al. 2003). For example, several or most of the bacterial species isolated on culture plates in insect microbiota screens were not identified with the culture independent methods used, which in theory should detect all bacteria present (Lindh et al. 2005; Zahner et al. 2008).

Tsetse flies (Diptera: Glossinidae) are vectors of several species of African trypanosomes pathogenic to both man and his domesticated animals. The diseases they cause continue to have a devastating impact at the level of individual lives and the development of the entire African continent (Waage 1979). *Glossina fuscipes fuscipes*, which is investigated in this study, is implicated as the vector of about 90% of all current sleeping sickness cases in Africa. Tsetse flies are obligate haematophages, feeding on nothing but vertebrate blood throughout their entire lives (Walshe et al. 2009). As such the fly midgut might be expected to have a very limited or biased microbiota. This merits investigation, not only because this is a very unusual niche, but also because in mosquitoes the midgut microbiota have been shown to profoundly influence susceptibility to malaria parasites (*Plasmodium* spp., Dong et al. 2009) and the same may be true for tsetse, where many of the most important trypanosome species first become established in the fly midgut. Recently, Geiger et al. (2009) identified bacteria other than the two previously described symbionts and *Wolbachia* in tsetse flies utilising a culture dependent method. The study reported here is the first screen for bacteria in tsetse-flies using both a culture dependent and a culture independent pathway.

Materials and methods

Culture dependent pathway

Flies and bacterial isolates

Glossina fuscipes fuscipes were caught in biconical traps on Chamaunga Island in Lake Victoria, western Kenya (0°25′S, 34°13′E) during 4 separate days in October and November, 2008. The flies, 39 males and 25 females, were killed at −20°C, washed in a series of three baths containing: sterile water, 70% ethanol (approx. 3 min) and sterile water again. The flies were then mashed in 200 µl of sterile saline, vortexed briefly, after which 50 µl of the solution were spread on LA-plates (Luria Broth Base, Invitrogen). Two types of controls were performed each time bacterial cultivation was performed. First, a sterility control, in which materials were treated as for the other samples, but in the absence of a fly. Second a surface bacteria control where a fly was placed in 200 µl of sterile saline and vortexed briefly (without first mashing the fly) after which 50 µl of the saline was spread on LA-plates. All colonies with different colony morphologies were re-streaked on LA-plates after approx. 24 and 48 h incubation at room temperature and re-incubated under the same conditions. The bacterial isolates were stored as glycerol stocks at −20°C for approximately

2 months before transport to the laboratory in Liverpool, UK. Once there, isolates were re-streaked and new glycerol stocks were made which were then stored at −70°C. Brief morphological descriptions of colony size, shape and colour and bacteria cell size, shape and motility were noted for each isolate. These descriptions were later used together with the DGGE data to group bacterial isolates of the same species.

PCR-denaturing gradient gel electrophoresis

A region of the 16S rRNA gene of the bacterial isolates was amplified with the following PCR primers 357f gc (5′-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G*CC TAC GGG AGG CAG CAG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′). A GC-clamp was attached to one of the primers (357f gc, in italics) to prevent complete strand dissociation during electrophoresis (Sheffield et al. 1989). To prepare template for PCR a single colony from a LA-plate was placed with the aid of a sterile tooth pick into a 1.5 ml Eppendorf tube with 50 μl of sterile water. The tube was placed on a heating block (95°C) for 10 min and then centrifuged (14,000 rpm) for 5 min. One μl of the supernatant was added as template to a 0.25 μl PCR reaction with 10 pmol of each primer using Ready-To-Go PCR beads, (Amersham Pharmacia Biotech). The PCR program utilised, which was optimised in a similar screen of *Anopheles gambiae* midgut bacteria (Lindh et al. 2005), was as follows: 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 58–48°C for 30 s (temperature decreased by 1°C every cycle for 10 cycles and then held at 48°C for 20 cycles), 72°C for 1 min followed by a final extension step at 72°C for 5 min. PCR fragments were analysed by electrophoresis on agarose gels (1%) before DGGE was performed on the samples. During DGGE (performed with equipment from BioRad), PCR fragments were separated with the aid of a polyacrylamide and denaturing gradient gel from 6% acrylamide/30% denaturing to 12% acrylamide/60% denaturing where 100% denaturing gel contains 7 M Urea and 40% (v/v) formamide. The gels were run in 1× TAE buffer at 200 V for 5 h at a constant temperature of 60°C. The gels were stained for 20 min with ethidium-bromide (1 mg/ml, 1× TAE), rinsed for 20 min (1× TAE) and then visualized on a UV trans-illumination table.

Sequencing and sequence analysis

Based on DGGE and morphology results (data not shown), the isolates were grouped and a few isolates from each group were selected for sequencing. The 16S rRNA genes from the selected isolates were amplified by PCR using the forward primer 8f (5′-AGA GTT TGA TNN TGG CTC AG-3′) and the reverse primer 1401r (5′-CGG TGT GTA CAA GAC CC-3′). Template preparation, PCR conditions and the PCR program were as above. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced at Macrogen, Korea on an ABI 3700 automatic DNA sequencer with primers 8f and 800r (5′-TAC CAG GGT ATC TAA TCC-3′). Preliminary sequence analyses were performed utilizing the Ribosomal Database Project II (RDP II, http://rdp.cme.msu.edu, Cole et al. 2005) and Blastn (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Further sequence analysis was performed with selected sequences from closely related species from RDPII using MEGA (Tamura et al. 2007).

Culture independent pathway

Flies and DNA extraction

G. f. fuscipes were caught in biconical traps on Chamaunga Island, in August and September, 2007. The flies were killed at −20°C, stored in acetone and transported to Liverpool, UK. The legs and wings were removed before the flies were mashed in 180 μl of PBS. DNA extractions were then performed according to the manufacturer's instructions for animal tissue DNA extraction with a DNeasy Blood and Tissue kit (Qiagen). The DNA was eluted in 200 μl of buffer AE. In total, 30 males and 45 females were processed. No fly was stored in acetone for more than five months before DNA extraction.

PCR–DGGE, sequencing and analysis

The eluted DNA (1–2 μl) was used as a template in PCR reactions with the same primers and conditions as for the bacterial isolates above. Genomic DNA from *G. m. morsitans* and *G. p. palpalis* colony flies were used as controls. After determining that the 357f–518r primer pair amplifies a region of the

Glossina 18S rRNA gene another reverse primer was utilised: 800r (5′-TAC CAG GGT ATC TAA TCC-3′) together with the same forward primer. All fly DNA samples were screened with both primer pairs and analysed with DGGE. Agarose electrophoresis and DGGE conditions were as above. Interesting bands from the DGGE gels were excised with the aid of a sterile pipette tip. The round gel pieces were incubated at 4°C overnight to elute the DNA. The eluted DNA was then used as template in PCR reactions. The PCR program, PCR purification, sequencing and sequence analysis were performed as above with the exception that the sequencing primers utilised were the same primers as originally used to amplify the fragment for DGGE analysis but without the gc clamp.

Detection limits for *S. glossinidius* and *B. subtilis* when extracted with *G. f. fuscipes* genomic DNA

A *B. subtilis* strain isolated during this study was grown in LB media to $OD_{600} = 0.5$ which according to viable counts performed on LA plates correspond to 3.9×10^8 colony forming units/ml. Bacterial DNA was prepared using the blood and tissue culture kit (Qiagen) from 5 ml of the bacterial suspension following the protocol for Gram positive bacteria. *S. glossinidius* DNA from a known number of bacterial cells were kindly provided by Lee Haines, LSTM, Liverpool, UK. DNA from the two bacterial species were serially diluted in tsetse DNA from two *G. f. fuscipes* flies and then used in PCR–DGGE protocols with the 357f gc–518r and the 357f gc–800r primer pairs as above.

Results

Culture dependent pathway

Bacteria were found in 46 (72%) out of the 64 flies screened with the culture dependent pathway. In total 25 different species were identified from 145 isolates of which three species were also found in the surface controls (Table 1), i.e. those three bacterial species may have originated from the outer cuticle as well as the interior organs of the flies. One species, *Bacillus megaterium*, was identified in one of the sterility

controls and data for this species have been excluded from the results. If the species identified in the surface controls are also excluded then bacteria were identified in 42% of the flies. The majority of the isolates identified ($n = 16$) belong to the phylum Firmicutes and the genus with most isolates was *Bacillus* with seven species (Table 1; Fig. 1). The other two phyla represented in this study were Proteobacteria, with eight isolates, and Actinobacteria, from which an *Arthrobacter* sp. was identified (Table 1). The maximum number of bacterial species isolated from one fly was five, two of these were also found in at least one of the surface controls. If the bacterial species isolated from the surface controls are excluded two flies harboured three different bacterial species, and 10 and 15 flies harboured two and one species, respectively.

Culture independent pathway

As expected, the primary symbiont *W. glossinidia* was found in all flies screened with the culture independent method (Fig. 2). However, neither *Wolbachia* nor *S. glossinidius* were identified in the field caught *G. f. fuscipes*. All three *Glossina* symbionts were detected in the *G. m. morsitans* (Genbank accession numbers: *Wolbachia* sp. GU361692, GU361701; *S. glossinidius* GU361693, GU361716: *W. glossinidia* GU361700, GU361714) and *S. glossinidius* (GU361699) and *W. glossinidia* (GU361694, GU361698, GU361715) were detected in the *G. palpalis* flies used as controls in this study (Fig. 2). Furthermore, an additional screen of 25 of the field caught *G. f. fuscipes* flies using *Sodalis* specific primers failed to detect *Sodalis* (data not shown). In addition to *W. glossinidia* (GU361695-97; GU361704-6; GU361708-9; GU361712-13), only *Glossina* 18S rRNA and *Serratia marcescens* (GU361 702-3) were identified by the culture independent method with the 357gcf-518r primer pair (Fig. 2). Since the reverse primer 518r has a perfect match with the tsetse 18S rRNA gene, this primer was changed (to primer 800r). The second primer pair did not amplify the 18S gene. However, this primer pair only detected one bacterial species in addition to the tsetse symbionts and *Serratia marcescens* (GU361710-11). This second non symbiotic bacterial species was *B. subtilis* (GU361707) which, in contrast to *S. marcescens*, was also isolated on plates.

Table 1 Bacterial species isolated with the culture dependent method

Phylum/class[a]	Phylogenetic affiliation	Closest relative according to RDPII[b]	No. of flies	Genbank accession no. (GU3616xx)
Actinobacteria	*Arthrobacter* sp.	*Arthrobacter* sp. AF408952	4	51–53
Firmicutes	*Bacillus* a sp.[c]	*B. subtilis/B. polyfermenticus*	3	54–55
		B. pumilus[d]*/B. safensis*	25	43, 45, 56, 58
		B. pumilus[d]*/B. altitudines*		40, 46, 48, 49, 57
	Bacillus c sp.	*B. simplex*	8	38, 42, 59, 60
	Bacillus d sp.[c]	*B. cereus/B. thuringienisis/B. anthracis*	22	39, 41, 61–70
	Bacillus f sp.	*Bacillus* sp. FJ688409/*B. oleronius*	1	71
	Bacillus endophyticus	*B. endophyticus*	1	72
	Bacillus h sp.	*Bacillus* sp. AM950291/*B. arsenicus*	1	37
	Bacillaceae sp.	*Terribacillus* sp. AM931170/*Oceanobacillus picturae* AM237397	1	81–82
	Bacillales sp.	*Rummeliibacillus stabekisii* AB271737	1	76
	Planococcaceae sp.	*Solibacillus silvestris* AJ006086	1	73
	Planococcaceae sp.	*Lysinibacillus sphaericus* EU741101/*Bacillus* sp. AF169543	1	78
	Paenibacillus sp.	*Paenibacillus turicensis* AF378696	1	77
	Staphylococcus sp.[b]	*Staphylococcus* sp. AY940424	6	79–80
	Staphylococcus sp.	*Staphylococcus pasteuri* EF127830	1	47
	Staphylococcus epidermidis	Several strains of *Staphylococcus epidermidis* among them type strain D83363	1	44
	Exiguobacterium sp.	*Exiguobacterium acetylicum* FJ970034	2	74–75
Beta-proteobacteria	*Burkholderia* sp.	Several *Burkholderia fungorum* strain among them type strain AF215705	3	83–84
Gamma-proteobacteria	Enterobacteriaceae sp.	*Enterobacter* sp. AY683044	1	85
	Enterobacteriaceae sp.	*Pantoea oleae*	1	89
	Enterobacteriaceae sp.	*Erwinia soli/Pantoea* sp./*Flavobacterium* sp.	3	88
	Pantoea sp.	*Pantoea agglomerans*	1	91
	Morganella sp.	*Morganella morganii* subsp. *morganii*/ uncultured *Morganella*	3	50, 86
	Providencia sp.	*Providencia* sp.	1	87
	Pseudomonas sp.	*Pseudomonas* sp./iron reducing bacteria FJ269058	1	90

[a] Class is indicated for the proteobacteria phylum, [b] Pubmed accession numbers and further phylogenetic affiliation of species within the *Bacillus* genera and gamma-proteobacteria class see Figs. 1 and 4, respectively. [c] "*Bacillus* a sp." related to *B. pumilus*, "*Bacillus* d sp." and *Staphylococcus* sp. were also isolated from the surface bacteria controls. [d] The isolates related to *B. pumilus* form two different groups closest related to *B. pumilus/B. safensis* and *B. pumilus/B. altitudines*, respectively

The detection limit of bacterial DNA in the presence of tsetse DNA was investigated for one of the *Bacillus* species (*B. subtilis*) and for *S. glossinidius*. The detection limit was approximately the same for both primer pairs utilised: between 4×10^3 and 4×10^2 *B. subtilis* and 10^5 and 10^4 *S. glossinidius* bacterial cells could be detected (Fig. 3 and data not shown).
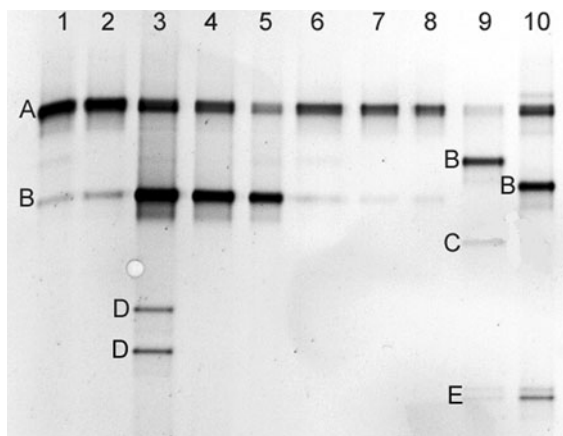
## Discussion

Culture dependent pathway

In this study Firmicutes and *Bacillus* were the dominant phylum and genus respectively. In contrast, during a recent study on the cultivable flora of *Glossina palpalis palpalis* 39 bacterial strains were

Fig. 1 Evolutionary relationships of the *Bacillus* species. ▶ Lineages labelled bx where x is a number refer to the different bacterial isolates from tsetse flies that were sequenced. *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* are very hard to separate based on the 16S rRNA sequence (Ash et al. 1991). The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff 1966). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000, p. 128) with search level 3 (Felsenstein 1985; Nei and Kumar 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 667 positions in the final dataset, out of which 509 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007)

isolated out of which 95% were Gram negative and 82% belonged to the Gamma-proteobacteria class (Geiger et al. 2009). Several other insect microbiota screens have also reported Gamma-proteobacteria as the dominant class (Azambuja et al. 2005; Lindh et al. 2005; Zientz et al. 2001). Seven species from the Gamma-proteobacteria class were isolated in this study, four of these belong to the family Enterobacteriaceae (Table 1; Fig. 4), where many insect symbionts are found including *W. glossinidia* and *S. glossinidius*. Many of the bacterial species identified in this study have also been detected in other insect vector species (Azambuja et al. 2005; Lindh 2007), however not in the screen of *G. p. palpalis* (Geiger et al. 2009). The difference in bacterial flora between *G. f. fuscipes* and *G. p. palpalis* may reflect differences in blood-feeding patterns between the two species (Mayer and James 1969). However, it may also reflect the different culture media utilised in the two studies (Geiger et al. 2009). It is possible that an even more diverse bacterial flora would have been detected on culture plates in this study if several different culture media and conditions had been utilised.

The high prevalence and diversity of cultivatable bacteria in tsetse flies is surprising given that they are monophagous, only feeding on vertebrate blood throughout their life. In the main vertebrate blood is sterile. Hence the data from this study argues against

the theory that polyphagous insects have a higher prevalence of intestinal bacteria and a more diverse bacterial flora (Mrazek et al. 2008; Dillon et al. 2008). It is interesting to note that bacteria were isolated from 72% of the tsetse flies in this study while a study of bacteria in *Anopheles* mosquito midguts performed in the same area in 2005 only detected bacteria in 15% of the mosquitoes and only in 7% with the culture dependent method utilising the same type of culture plates as in this study (Lindh et al. 2005). The reason for the higher prevalence of bacteria in tsetse flies compared to mosquitoes may depend on one of two reasons or a combination of both. First, tsetse flies
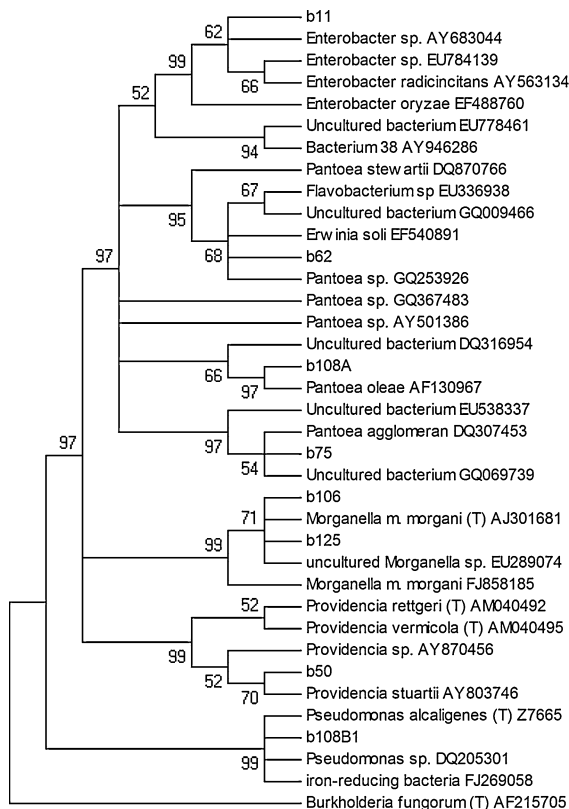
Fig. 2 DGGE gel visualising bacteria in *G. f. fuscipes* field flies and *G. m. morsitans* and *G. p. palpalis* controls PCR with primers 357f gc and 518r. *Lanes 1–8*: *G. f. f.* females, *lane 9*: *G. m. m.* colony fly, *lane 10*: *G. p. p.* colony fly. *Bands A*: *Glossina* 18S, *B*: *W. glossinidia*, *C*: *Wolbachia* sp. *D*: *S. marcescens E*: *S. glossinidius*. *Wigglesworthia* are species specific and hence the 16S sequences vary slightly between *Glossina* species. *S. marcescens* generates two bands
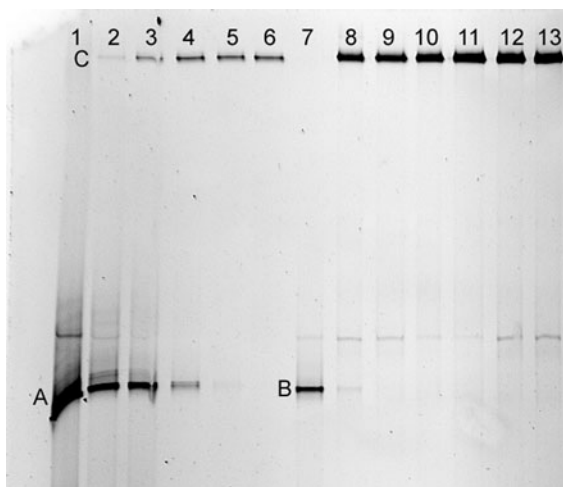
Fig. 3 DGGE gel visualising the detection limit of *B. subtilis* and *S. glossinidius* in tsetse DNA. PCR with primers 357f gc and 800r. *Lane 1*: *B. subtilis* DNA, lanes *2–6*: *B. subtilis* diluted in tsetse DNA, 4×10^6–4×10^2 bact./PCR react, $10\times$ dilution between each *line*, lanes *7–12*: *S. glossinidius* diluted in tsetse DNA, 10^6–10^1 bact./PCR react, $10\times$ dilution between each *line*, lane *13*: *G. f. f.* DNA used for bacterial DNA dilutions. *Bands A*: *B. subtilis*, *B*: *S. glossinidius*, *C*: *W. glossinidia*

Fig. 4 Evolutionary relationships of the gamma-proteobacteria species See legend for Fig. 1. There were a total of 653 positions in the final dataset, out of which 151 were parsimony informative. Genebank accession numbers GQ009466, GQ069739 and EU538337 belong to bacteria found on human skin (Grice et al. 2008, 2009)

might encounter and ingest more bacteria than mosquitoes. Given that adult mosquitoes feed on various sugar sources in the field in addition to blood

this seems improbable. Furthermore, larval mosquitoes live in water bodies with high loads of bacteria and the adults are capable of ingesting bacteria from that water after they emerge (Lindh et al. 2008). However, mosquitoes and tsetse flies have a different mode of blood-feeding and this may account for the differences seen. Mosquitoes cannulate capillaries and so suck blood from within the enclosed vertebrate blood system. In contrast, tsetse flies have much larger mouthparts which they use to drill into the skin of the vertebrate causing a sub-dermal haematoma from which they suck blood (Lehane 2005). The sub-dermal haematoma may well be contaminated with bacteria from the skin surface. This possibility is supported by the findings of Kaaya and Darji (1989) and Poinar et al. (1979) who showed that *G. m. morsitans* mortality was increased after feeding on rabbit ears that had been smeared with different

species of bacteria that were supposed to be harmful to the flies. Given this hypothesis it is interesting to note that two of the Enterobacteriaceae spp. isolated in this study are close relatives of bacteria found in human skin microbiota (Fig. 4, Grice et al. 2009). The second reason may be that tsetse flies have a weaker intestinal anti-bacterial immune system compared to *Anopheles* mosquitoes. *Anopheles* may need a stronger immune system since, as argued above, they imbibe a broader and more bacterially contaminated set of foods. Alternatively, tsetse flies may have an impaired anti-bacterial response to allow for symbiont survival. Yet another difference between mosquitoes and tsetse which may have a bearing here is length of adult life. Female tsetse can live for 100 days or more while adult mosquitoes rarely achieve 30 days of adult life. As both species feed every 2–3 days clearly a population of tsetse flies will have had a greater chance of contracting a bacterial load if this is associated with the feeding process.

Culture independent pathway

Only two bacterial species other than the tsetse symbiont *W. glossinidia* were identified from field caught *G. f. fuscipes* with the culture independent pathway utilising two different primer pairs. We speculated that the high copy number of *W. glossinidia* 16S and the tsetse 18S rRNA genes might have caused bacteria, if present in low copy number, to fail to be detected in the PCR. However, changing the reverse primer and thereby excluding amplification of the tsetse 18S rRNA gene only resulted in one more bacterial species being identified. DNA from both *B. subtilis* and *S. glossinidius* could be detected when diluted in tsetse DNA but only when present above a threshold. Hence, we cannot exclude that *Sodalis* and *Wolbachia*, in addition to other bacteria, are present in *G. f. fuscipes* flies in numbers lower than the detection limits for the PCR–DGGE protocol utilised here. The similar detection limits of the two primer pairs, where one set amplifies the 18S rRNA tsetse gene while the other does not, indicate that the amplification of the 18S rRNA tsetse gene did not affect the sensitivity of the PCR. However, the high copy number of the *Wigglesworthia* 16S rRNA gene might still explain the difference in prevalence and diversity of non-symbiotic bacteria identified with the

culture dependent and culture independent pathway utilised in this study. A solution might be to use phylum or class specific primers (Muhling et al. 2008) although this will not be feasible for species within the Gamma-proteobacteria since *W. glossinidia* belongs to this class.

Influence of bacteria on vector competence and longevity?

Previous studies have suggested that gut bacteria may influence parasite transmission by insects (Azambuja et al. 2005; Dong et al. 2009). For instance, Pumpuni et al. (1993) found that a Gram-negative bacteria inhibited oocyst formation of the malaria parasite *Plasmodium falciparum* in *Anopheles stephensi* mosquitoes. This observation was later confirmed by Gonzalez-Ceron et al. (2003) who reported a lower infection rate in *An. albimanus* when Gram-negative bacteria were co-infected with *P. vivax* parasites. In addition, *S. marcescens* isolated from *Rhodnius prolixus* was shown to lyse the parasite *Trypanosoma cruzi* in vitro (Azambuja et al. 2004). Moreover, *Pseudomonas fluorescens* strains have also been reported to be able to lyse *T. cruzi* (Mercado and Colon-Whitt 1982). As a contrast the secondary symbiont in tsetse *S. glossinidius* has been shown to increase susceptibility of tsetse flies to trypanosomes (Welburn et al. 1993) although the relationship appears complex (Geiger et al. 2007; Geiger et al. 2005). Bacteria have also been reported to affect *Glossina* mortality. A number of Gram-negative and Gram-positive bacteria, among them *S. marcescens*, *Providencia rettgeri* and several *Bacillus* spp. induced mortality of *G. m. morsitans* (Kaaya and Darji 1989). Furthermore, *S. marcescens* have been shown to cause increased mortality in *An. albimanus* mosquitoes and in *G. morsitans* and *G. pallidipes* flies (Poinar et al. 1979; Gonzalez-Ceron et al. 2003). Taken together, these studies clearly suggest that gut bacteria may influence vector competence and or longevity of disease transmitting insects. Hence, it would be interesting to investigate the impact the non-symbiotic bacteria isolated in this study may have on vector competence and mortality of tsetse flies, especially since several of the bacterial species investigated in the studies above correlate with species identified in this study.

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