The microbiome of the cloacal openings of the urogenital and anal tracts of the tammar wallaby, *Macropus eugenii*

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The bacterial diversity of the openings of the urogenital and anal tracts of the adult female tammar wallaby, *Macropus eugenii*, was determined in order to ascertain whether the physical proximity of the openings of these tracts within the cloaca affected the two populations of bacteria. Terminal restriction fragment length polymorphism (T-RFLP) analyses of 42 wallabies identified 81 different terminal fragments, indicative of diverse and complex microbiomes at these anatomical locations. Subsequent amplified rDNA restriction analysis (ARDRA) identified 72 phylotypes from the urogenital tract and 50 from the anal tract. Twenty-two of these phylotypes were common to both tracts. Phylogenetic analysis of sequenced 16S rDNA showed that 83 % of the phylotypes were unidentified species based on the premise that any sequence possessing <97 % homology to a known bacterial species or phylotype was novel. Thus, despite the close proximity of the openings of the urogenital and anal tracts within the cloaca, the two sites retained a diverse range of distinct bacteria, with only a small percentage of overlapping species.

INTRODUCTION

Metatherian (marsupial) mammals offer a unique biological opportunity to investigate the relationship between the microflora of the urogenital and anal tracts. In contrast to eutherian (placental) mammals, which possess separate openings of the digestive and urogenital systems, most marsupials possess a common posterior chamber for these systems – the cloaca (Tyndale-Briscoe, 2005; Fig. 1). This structural feature might imply that bacteria from one tract have easy access to the other. To date, the bacteria associated with these two sites have not been studied in marsupials, although in humans it has been noted that the bacteria of the urogenital tract may vary and can be

influenced by bacteria growing in the anal tract and thus occasionally can cause urogenital disease (Reid *et al.*, 2001; Tannock, 1999).

The tammar wallaby, *Macropus eugenii*, is regarded as a model marsupial (Tyndale-Briscoe, 2005) with more than 30 years of fundamental research into its basic biology. Despite this, little is known about the bacterial populations of this animal, all published studies having used culture-based methods that are known to be biased in favour of a small percentage of the total microbiome (Hume, 1999; Lentle *et al.*, 2006; Old & Deane, 1998; Yadav *et al.*, 1972).

In this study we used molecular-based methods targeting the 16S rDNA gene to document the bacteria at the openings of the urogenital and anal tracts of 42 female tammar wallabies and undertook a comprehensive comparison of the major phylotypes and their diversity. This is believed to be the first extensive report of the bacterial species within the openings of these two tracts in any marsupial. The results showed that despite the close anatomical proximity of the openings of these tracts, each opening possessed a diverse and complex bacterial population comprising mainly unidentified species.

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Abbreviations: ARDRA, amplified rDNA restriction analysis; T-RFLP, terminal restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the DNA sequences of the species or phylotypes identified in this study are EU289036–EU289066 and EU289069–EU289139.

A supplementary table of phylotypes isolated from the urogenital and anal tracts of the tammar wallaby is available with the online version of this paper.

Fig. 1. Photograph of an everted cloaca from a juvenile female tammar wallaby showing the urogenital opening (a) and anal opening (b).

METHODS

Animals and sample collection. The tammar wallabies used in this study were from two captive breeding populations maintained in large natural enclosures. One colony was located at Macquarie University, Sydney, Australia, and the other was at the CSIRO Sustainable Ecosystems, Canberra, Australia. All research involving animal handling and sample collection was carried out in a manner approved by Macquarie University's Animal Ethics Committee (reference number 2004/015) or the CSIRO Sustainable Ecosystems Animal Ethics Committee (reference number 04-05-26). Sterile cotton-wool-tipped wooden swabs dipped in sterile PBS containing 15% (v/v) glycerol were used to collect samples from the openings of the anal and the urogenital tracts of 42 adult female tammar wallabies, 36 of which were carrying pouch young of varying ages (0–60 days). After swabbing, the cotton wool ends of the wooden swabs were broken off into sterile 1.5 ml microfuge tubes containing 300 µl sterile PBS with 15% (v/v) glycerol and kept at 4 °C until all sampling was completed (<5 h) and then transferred to a –80 °C freezer in the laboratory prior to processing.

DNA extraction. Samples were thawed on ice and vortexed for 2 min to achieve a homogeneous suspension. A 20 µl aliquot of each sample was taken for DNA extraction and mixed with 180 µl buffer consisting of 10 mM sodium phosphate pH 6.7 containing 400 µg lysozyme (Roche Applied Science), 200 U mutanolysin (Sigma Aldrich) and 400 µg proteinase K (Qiagen), with 20 mM diethyl pyrocarbonate (Sigma Aldrich) and incubated at 56 °C for 2 h. Bacterial cells were lysed with 1% (w/v) SDS and 200 µg RNase (Sigma Aldrich) added prior to further incubation at 37 °C for 10 min (Smith-Vaughan *et al.*, 2006). DNA was subsequently isolated and purified using a QIAamp DNA Mini kit (Qiagen), according to the manufacturer's protocol.

Terminal restriction fragment length polymorphism analysis. Terminal restriction fragment length polymorphism (T-RFLP) analysis (Marsh, 1999) was performed on the 16S rDNA extracted from bacteria present in the urogenital and anal swabs collected from 42 tammar

wallabies. Duplicate samples of bacterial 16S rRNA genes were amplified by PCR using AmpliTaq Gold with GeneAmp (Applied Biosystems) and the fluorescently labelled 5'-hexachlorofluorescein phosphoramidite (HEX) F27 (HEX-5'-AGAGTTTGATCMTGGCT-CAG-3') and the R1492 (5'-TACGGYTACCTGTTACGACTT-3') primers (Weisburg *et al.*, 1991). The PCR conditions were 94 °C for 10 min followed by 35 cycles of amplification at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s. PCR products were digested using *RsaI* (Promega Life Sciences) for 1 h and then deactivated at 65 °C for 15 min prior to T-RFLP analysis being performed on an Applied Biosystems 3130 × 1 Genetic Analyzer (Applied Biosystems) at the Macquarie University DNA Sequencing Facility.

Amplified rDNA restriction analysis and sequencing of 16S rRNA genes.

Urogenital and anal swabs from two female wallabies carrying a 1- or 4-day-old pouch young were found to generate the highest number of dissimilar terminal fragments following T-RFLP analysis and therefore were used to generate 16S rRNA gene libraries. Bacterial 16S rRNA genes were amplified by PCR as described above for T-RFLP analysis except that the forward primer, F27a, did not contain a fluorescent HEX tag and 25 amplification cycles were used. The PCR products were cloned into a T-tailed pGEM vector (Promega Life Sciences), heat shocked into *Escherichia coli* DH5α (Invitrogen) and the transformed *E. coli* grown on Luria–Bertani agar plates prior to 96 colonies being randomly selected from the urogenital and anal tracts of both wallabies to form a clone library. The inserts within the plasmid vectors were recovered by PCR using vector-specific primers pGEM-F (5'-GGCGGTCGCGGAATTCGATT-3') and pGEM-R (5'-GCCGCGAATTCAGTGTGATT-3') (Aislabie *et al.*, 2006) following lysis of the cells in 96-well plates at 94 °C for 30 min. The PCR conditions were 94 °C for 10 min followed by 25 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 90 s.

Amplified rDNA restriction analyses (ARDRA; Grimont & Grimont, 1986) of the PCR products made use of the restriction enzymes *RsaI* and *HaeIII* to identify unique restriction patterns within the cloned 16S rRNA genes. The restriction profiles were manually compared and all unique restriction profiles sequenced. Partial 16S rDNA gene sequences of approximately 1450 bp in length, representing a specific species or phylotype, were sequenced in both directions and compared with known sequences in GenBank (<http://www.ncbi.nlm.nih.gov>) using the BLAST search tool (Altschul *et al.*, 1997). Since the majority of the phylotypes were novel (<97% sequence similarity to any known 16S rDNA sequence), they were checked to determine whether they were chimaeras. This was done by first comparing short sections of each sequence to known sequences in the GenBank database as described by Ludwig *et al.* (1997), and secondly determining the position of each sequence on a phylogenetic tree (see below) with respect to all other phylotypes.

Phylogenetic analysis. The cloned 16S rDNA sequences from the anal and urogenital tracts were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) accessed through the Australian National Genome Information Service (ANGIS; <http://www.angis.org.au/>) and neighbourhood joining trees (Saitou & Nei, 1987) were constructed using the Jukes–Cantor parameter in the MEGA version 4 program (Tamura *et al.*, 2007). Bootstrap analyses of 1000 replicates were used to validate the branch points in the trees.

RESULTS

T-RFLP analysis and cloning of 16S rDNA

Duplicate T-RFLP analyses, based on *RsaI* cleavage of the 16S rDNA of the bacteria isolated from 42 female tammar

wallabies, resulted in 84 T-RFLP population profiles from the urogenital and anal tracts of these animals. Two urogenital and two anal tract samples from two wallabies were found to possess terminal fragments within their T-RFLP profiles that together represented 77 of the 81 different terminal fragments identified within the T-RFLP profiles. These four samples were chosen to construct a 192 clone library for each tract. Following ARDRA analysis with *RsaI* and *HaeIII*, 105 different clones were identified. After elimination of five chimaeras, the remaining 100 near-full-length 16S rDNAs were sequenced. From these sequences, 72 different urogenital and 50 different anal phylotypes were identified (Supplementary Table S1, available with the online version of this article). Twenty-two common phylotypes were found in both tracts (Fig. 2; Supplementary Table S1).

As T-RFLP patterns based only on one restriction enzyme may be insufficient to accurately identify a unique phylotype (Liu *et al.*, 1997; Marsh, 1999; Osborn *et al.*, 2000), *in silico* terminal fragments from each of the 100 phylotypes were generated and compared with the 81 different terminal fragments observed in the 84 T-RFLP population profiles. This analysis identified 77 of the 81 terminal fragments as unique phylotypes. There were eight *in silico* phylotypes with no corresponding terminal fragments in the T-RFLP population profiles and four terminal fragments in these profiles with no corresponding *in silico* phylotype (Table 1).

On closer inspection, some of the phylotypes were found to have the same species identity. For example, clone 8837-D0-A-3C, isolated from the anal tract, and clone 8837-D0-C-12D, isolated from the urogenital tract, possessed 100 % and 99 % identity to the 16S rRNA of *Bacteroides denticanoris*. However, this 1 % difference in identity was sufficient for the two sequences to have different terminal fragment sizes of 456 bp and 461 bp, respectively (Supplementary Table S1), as well as different *HaeIII* and *RsaI* restriction patterns on ARDRA analysis (data not shown).

Phylogenetic and community analysis

The 100 different 16S rDNA sequences comprised five different phyla. These were the Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria and Bacteroidetes; their relative distribution in the two tracts is shown in Fig. 2. As a species is generally accepted to have ≥ 97 % homology within the 16S rRNA gene(s) (Stackebrandt & Goebel, 1994), 83 % of the clones isolated were considered to be novel species as they showed <97 % homology to any known species currently in GenBank (Supplementary Table S1).

The Firmicutes were the most diverse group; they constituted 54 % of the clones isolated from the urogenital tract and 43.9 % from the anal tract (Table 2). The Firmicutes could be further divided into two classes, the Clostridia and the Lactobacillales, with the Clostridia being the predominant class in both tracts (Table 2). Eight

phylotypes from the clostridial class were common to both tracts, with clone 8817-D4-A-7F representing a sizeable proportion (8.3 %) of the clones isolated from the anal tract, but only 0.6 % of the clones from the urogenital tract (Fig. 2). This phylotype was closely related to *Clostridium* sp. TO-931 isolated from human faecal samples and found capable of deconjugating bile acids (Wells *et al.*, 2003).

The Lactobacillales constituted 17.2 % of the clones from the urogenital tract and 14.2 % of those from the anal tract. The proportion of phylotypes belonging to the Lactobacillales was 19.2 % and 18 % from the urogenital and anal tracts, respectively, with six common phylotypes in both tracts. The major species in both tracts was *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Fig. 2).

The second major phylum was the Bacteroidetes, from the point of view of both the total number of clones isolated and the extent of diversity in both tracts (Table 2). The anal and urogenital tracts shared three common phylotypes, with clone 8817-D4-C-1B being the most predominant, representing 17.8 % of the clones isolated from the urogenital tract and 21.5 % from the anal tract (Fig. 2).

The Actinobacteria and Proteobacteria phyla were represented by a similar number of clones irrespective of the tract from which they were isolated (Table 2). Three phylotypes belonging to Actinobacteria were found in both tracts, with *Arcanobacterium hippocoleae* constituting 10.4 % of the clones isolated from the urogenital tract and 3.4 % from the anal tract (Fig. 2). There were also two phylotypes belonging to the Proteobacteria that were found in both tracts, with *Campylobacter helveticus* (corresponding to clone 8817-D4-A-1C) being isolated in high numbers only from the anal tract (Fig. 2).

In contrast to the other phyla of bacteria, the Fusobacteria were only detected in the urogenital tract of the two wallabies used as the source of bacteria for ARDRA analysis, and then only at low frequency and diversity since the phylum was represented by only one clone from each of the genera *Fusobacterium* (clone 8817-D4-C-10F) and *Leptotrichia* (clone 8837-D0-C-11B; Fig. 2; Table 1). T-RFLP analysis of the 42 tammar wallabies, however, showed that these two phylotypes could potentially be isolated from both the urogenital and the anal openings within the cloaca. Clone 8817-D4-C-10F was isolated from 23.9 % of both openings while clone 8837-D0-C-11B was isolated from 76.2 % of the urogenital and 57.1 % of the anal openings, respectively.

DISCUSSION

As the identified phylotypes were based on the original T-RFLP analyses of 42 female wallabies, they represent a compilation of the major bacteria found in the two anatomical openings of the cloaca of the tammar wallaby. The results contrast strongly with previous cultivation-based studies, which suggested that there was little diversity

Fig. 2. Phylogenetic trees constructed from near-full-length (1442 bp) 16S rDNA sequences obtained from randomly cloned genes of bacteria isolated from the openings of the urogenital tract (left) and anal tract (right) of the tammar wallaby. Both trees were constructed with maximum-likelihood using the Jukes–Cantor parameter. The trees were bootstrapped 1000 times, and bootstrap values are shown at the branch points. Each phylum is defined by a different colour, with phylotypes common to both tracts highlighted in white ovals. The five most predominant phylotypes from the urogenital tract and the anal tract are highlighted, with the percentage of clones isolated from each site, respectively, shown in parentheses between the two trees. Where a phylotype possessed $\geq 97\%$ identity to a known species, the phylotype was given the species name, otherwise the clone name was used.

within the microbiome of marsupials at these sites (Hume, 1999; Lentle *et al.*, 2006; Yadav *et al.*, 1972). For example, only 15 different bacterial colony types were observed in the stomach of the quokka, *Setonix brachyurus* (Moir *et al.*, 1956), while direct visualization of smears from the vaginal cul-de-sac of the brushtail possum, *Trichosurus vulpecula*, detected only rod and coccid morphological types (Mahoney *et al.*, 2002). Furthermore, culture-based studies of the forestomach of the eastern grey kangaroo, *Macropus giganteus*, resulted in the identification of only 17 anaerobic bacterial species, all from the phylum Firmicutes, of which five were known species (Ouwkerk *et al.*, 2005). More pertinently, Lentle *et al.* (2006) recently found fewer than five bacterial species in the gut of the tammar wallaby pouch young. Unfortunately, the conclusions drawn from these culture-based studies are known to be biased, since such methods selectively isolate $\leq 1\%$ of the actual bacterial species present (Hugenholtz *et al.*, 1998).

Analysis of the 100 sequenced phylotypes in the current study showed that 83 were novel, based on the premise that their 16S rDNAs showed $<97\%$ sequence identity to known species or phylotypes. Most of the phylotypes belonged to two phyla, the Firmicutes and the Bacteroidetes, irrespective of the anatomical site from where they were isolated. This is consistent with the situation in humans, pigs and mice, where members of the phyla Firmicutes and the Bacteroidetes are also the two largest populations found in the distal gut (Tom-Petersen *et al.*, 2003; Xu *et al.*, 2007). In the tammar wallaby, the two classes of Firmicutes, the Clostridia and the Lactobacillales, represented the largest bacterial populations within the openings of the urogenital and anal tracts, from the point of view of both diversity of phylotypes and the number of clones isolated, a finding that might explain the observations of Ouwkerk *et al.* (2005), who cultured only Firmicutes from the forestomach of an eastern grey kangaroo.

Among the class Lactobacillales was a predominant phylotype represented by clone 8817-D4-C-11A (Fig. 2), the 16S-rDNA of which possessed 100% sequence identity to a *S. dysgalactiae* subsp. *dysgalactiae* strain isolated from swine (unpublished; GenBank Accession no. AB002504). Strains of this subspecies are known to be major pathogens in bovine mastitis (Denamiel *et al.*, 2005; Park *et al.*, 2002) and they have also recently been reported to cause bacteraemia in puppies (Vela *et al.*, 2006). The closely related bacterium, *S. dysgalactiae* subsp. *equisimilis* (99.8%

16S rDNA sequence identity), is also pathogenic in animals. This bacterium accounts for 5–8% of human streptococcal wound infections as well as causing toxic shock syndrome following misuse of tampons during menstruation (Horii *et al.*, 2006; Sachse *et al.*, 2002). As only certain strains of the two subspecies of *S. dysgalactiae* are pathogenic in placental mammals (Holt *et al.*, 1994), and as the tammar wallabies used in this study were all considered to be in good health despite the presence and colonization of their urogenital tracts with this bacterium, this may imply that this particular wallaby strain was not virulent, though this would require confirmation.

Surprisingly, no lactobacilli were detected in the opening of either the urogenital or the anal tract. This is in direct contrast to placental mammals, where lactobacilli are readily isolated from both tracts. Moreover, lactobacilli are predominant members of the resident urogenital microflora of healthy adult human females (Reid, 2000). Our observations, however, do not preclude lactobacilli being present in the urogenital tract of the tammar wallaby, as the chance of detecting a cloned 16S rDNA with 95% confidence using a sample size of 96 (as was the case in this study) requires a species to be present at a level $\geq 3\%$ of the population (Chhour *et al.*, 2005). The observation simply means that lactobacilli are not the predominant bacteria found in the urogenital tract of the female wallaby. However, as metatherian and eutherian mammals diverged from a common ancestor approximately 130 million years ago (Marshall Graves & Westerman, 2002), it is not surprising that the bacteria found in the urogenital tract of the tammar wallaby are different from those found in humans or other placental mammals. Provided a bacterial population contains the appropriate complement of genes for survival in a particular habitat, that population will proliferate independently of the presence of a particular species (Xu *et al.*, 2007). Thus, while the bacteria found in the urogenital tract of the tammar wallaby may be different from those of placental mammals, the various roles they play and the complement of genes they possess may be similar to those of eutherian mammals. In support of this hypothesis is the recent recognition that the greatest impact on the diversity of symbiotic bacteria within different hosts is the nature of the host itself, as up to 70% of the bacterial diversity at the species level is unique to a given host (Ley *et al.*, 2006).

Next to the Firmicutes, members of the Bacteroidetes were the second most abundant phylum found in the openings to the urogenital and anal tracts of the tammar wallaby.

Table 1. Phylotype or species corresponding to the terminal fragment length of cloned 16S rDNA from tammar wallabies following T-RFLP analysis with *RsaI*

T-RFLP terminal fragment size (bp)	Percentage of female wallabies (<i>n</i> =42) possessing bacteria with a given terminal fragment size*		Corresponding species or phylotype(s)†
	Urogenital tract	Anal tract	
33	—	—	<i>Morganella morganii</i>
65	—	—	<i>Bacteroides fragilis</i>
70	—	—	8837-D0-C-9H
71	—	—	8837-D0-A-6D
76	—	—	8837-D0-A-1G, 8837-D0-A-9E
77	—	—	8817-D4-C-4H
78	—	—	8837-D0-C-2E
80	—	—	8837-D0-C-3A, 8817-D4-A-5G
81	—	—	8837-D0-A-10E
89	—	—	8817-D4-C-12D
114	—	—	8837-D0-C-11D
115	—	—	<i>Streptococcus gallolyticus</i>
118	—	—	8837-D0-C-11E
119	—	—	8817-D4-A-4H
126	0.0	50.0	8837-D0-A-8A
129	73.8	0.0	8837-D0-C-2A
142	83.0	81.0	8837-D0-C-9D
155			8837-D0-C-10B‡
157			8837-D0-A-7A‡
160			8837-D0-C-5G‡
170–173§	52.4	40.5	8817-D4-C-11D, 8817-D4-C-4A, 8817-D4-C-12A
174–175§	37.1	52.4	8817-D4-A-1F, 8817-D4-A-1B
181	0.0	2.4	8837-D0-A-1E
193	19.0	14.3	8837-D0-A-8H
242			8817-D4-C-9A‡
251	0.0	2.4	8837-D0-A-9C
276–278§	9.5	0.0	8817-D4-C-7D, 8817-D4-A-2E
279	0.0	14.3	8837-D0-A-3G
310	0.0	7.1	Not found
321	4.7	9.5	<i>Porphyromonas gulae</i>
414			8837-D0-A-2G‡
422–424§	4.7	16.7	<i>Morganella morganii</i> , <i>Escherichia coli</i>
430	4.7	4.7	Not found
434	52.4	47.6	8837-D0-A-7E
440	16.7	11.9	8837-D0-C-11G
443	23.9	23.9	8817-D4-C-10F
453	23.9	45.2	8837-D0-C-9C
454–456§	76.2	57.1	<i>Campylobacter helveticus</i> , Uncultured Bacterium clone J2-29, <i>Bacteroides denticanoris</i> , 8837-D0-C-1E, 8817-D4-C-2B
460–461§	78.6	52.4	8817-D4-C-10D, <i>Bacteroides denticanoris</i>
463–464§	50.0	61.9	8837-D0-C-4C, 8837-D0-C-12F, <i>C. helveticus</i> , 8817-D4-C-7C, 8817-D4-C-9G, 8817-D4-A-6B, 8837-D0-A-1F, 8817-D4-A-12G
465–466§	50.0	61.9	8837-D0-A-7B, 8837-D0-A-6A
467–468§	52.4	37.1	8837-D0-C-8B, 8817-D4-C-1H, 8837-D0-C-2B, 8837-D0-C-10C, 8837-D0-C-9F
475–476§	11.9	11.9	8817-D4-C-8F, 8817-D4-C-9D, 8817-D4-C-8C
479–481§	47.6	59.5	8817-D4-C-8H, 8817-D4-A-3G, 8817-D4-A-1A, 8817-D4-C-6B, 8817-D4-C-1B
483			8817-D4-A-9D‡
493	9.5	0.0	8817-D4-C-11C
500	28.6	45.2	Not found
550			8837-D0-A-10D‡

Table 1. cont.

T-RFLP terminal fragment size (bp)	Percentage of female wallabies (n=42) possessing bacteria with a given terminal fragment size*		Corresponding species or phylotype(s)†
	Urogenital tract	Anal tract	
556–563§	83.0	69.0	8837-D0-C-2F, 8837-D0-A-7G, 8837-D0-C-8A, 8817-D4-C-1C, 8837-D0-C-7F, 8817-D4-C-7F
571			<i>Streptococcus dysgalactiae</i> ‡
618–623§	2.4	0.0	<i>Mobiluncus curtisii</i> , <i>Actinomyces hyovaginalis</i>
628	11.9	38.1	<i>Actinomyces hyovaginalis</i> / <i>Streptococcus dysgalactiae</i>
631	14.3	7.1	<i>Streptococcus suis</i>
653	9.5	7.1	8817-D4-C-2C
657–660§	21.4	31.0	8817-D4-C-11F, 8837-D0-C-3E
733–736§	37.1	40.5	8817-D4-C-3D, 8837-D0-A-9F
743			8817-D4-C-5D‡
840	9.5	0.0	8837-D0-C-2D
844	0.0	7.1	8837-D0-C-5H
860	11.9	28.6	Not found
867	45.3	61.9	8837-D0-A-3B
883	19.0	38.1	8837-D0-C-6H/8837-D0-C-9G
886	50.0	37.1	<i>Streptococcus gallolyticus</i>
889	38.1	42.9	<i>Streptococcus gallolyticus</i>
893	50.0	40.5	8837-D0-A-8F
899	31.0	45.2	<i>Enterococcus faecalis</i>

*–, 16S rDNA with terminal fragment lengths that could not be accurately determined by T-RFLP analysis due to high background noise. The corresponding phylotypes may be present in the urogenital and anal tracts of a number of tammar wallabies, but the exact percentage could not be accurately determined.

†Where subsequent sequencing of a phylotype led to $\geq 97\%$ 16S rDNA identity to a known species, the species name is quoted. Where more than one species/phylotype is listed, each species/phylotype possessed the same terminal fragment size following restriction digestion of 16S rDNA with *RsaI*. 'Not found' indicates that no matching phylotypes were subsequently isolated despite the terminal fragment being identified in the original T-RFLP analysis. These phylotypes statistically represent $\leq 3\%$ of the total population in any given wallaby (see text).

‡Phylotypes with no matching terminal fragments in the original T-RFLP analysis.

§Terminal fragment sizes that could not be separated in the T-RFLP analysis.

Among the porphyromonads belonging to this phylum, 79.3 % of the clones were phylogenetically related to *Porphyromonas cangingivalis*, originally isolated from the subgingival plaque of dogs (Collins *et al.*, 1994). Although most known porphyromonads are regarded as oral pathogens (Domisch *et al.*, 2007; Holt *et al.*, 1994), the porphyromonads found in the microbiome of the tammar wallaby could possibly play a different role as they possess $\leq 91\%$ sequence identity to the 16S rDNA to *P. cangingivalis* (Supplementary Table S1). In humans, the genus *Bacteroides* accounts for approximately 25 % of the bacterial population of the gastrointestinal tract (Xu *et al.*, 2004), with *Bacteroides thetaiotaomicron* being regarded as a potential probiotic due to its role in regulating glycan metabolism (Xu *et al.*, 2004).

Clone 8837-D0-C-5C represented a phylotype that was also consistently isolated from the openings of the urogenital and anal tracts (Fig. 2; Supplementary Table S1). Its 16S rDNA sequence possessed 97 % identity to *Arcanobacterium hippocoleae* isolated from a horse with

vaginitis (Hoyles *et al.*, 2002). To our knowledge, there are only six validated species within the genus *Arcanobacterium* (Ohba *et al.*, 2007). Members of this genus have been isolated from both animal and human sources (Collins *et al.*, 1982) and include the known pathogens *Arcanobacterium pyogenes* and *Arcanobacterium haemolyticum*, and the putative opportunistic pathogen *Arcanobacterium bernardiae* (Billington & Jost, 2006; Funke *et al.*, 1995). Other species within the genus, such as *Arcanobacterium phocae*, are of unknown pathological significance (Hoyles *et al.*, 2002).

In the phylum Proteobacteria, the most readily isolated phylotype was represented by clone 8837-D0-A-1C, which possesses 98 % 16S rDNA identity to *Campylobacter helveticus* (Fig. 2; Supplementary Table S1). This phylotype was isolated only from the anal tract of the tammar wallaby. Species within the genus *Campylobacter*, such as *Campylobacter coli* and *Campylobacter jejuni*, are important pathogens in humans and animals (Gorkiewicz *et al.*, 2003). However, to the best of our knowledge, *C. helveticus*, which

Table 2. Total number of clones analysed by ARDRA and the corresponding proportion of unique phylotypes belonging to each phylum that were detected at the openings of the urogenital and anal tracts of the tammar wallaby

Bacterial phylum (class)	ARDRA clones (%)*		No. of phylotypes (%)†	
	Urogenital	Anal	Urogenital	Anal
Firmicutes (Clostridia)	36.8	29.7	42.5	42.0
Firmicutes (Lactobacillales)	17.2	14.2	19.2	18.0
Bacteroidetes	26.4	29.7	17.8	14.0
Actinobacteria	12.3	10.8	9.6	16.0
Proteobacteria	6.1	15.5	8.2	14.0
Fusobacteria	1.2	ND	2.3	ND

ND, not detected.

*The total number of ARDRA clones that were isolated was 162 from the urogenital tract and 148 from the anal tract.

†The number of different phylotypes within the bacterial population was 72 in the urogenital tract and 50 in the anal tract.

was originally isolated from cat and dog faeces (Stanley *et al.*, 1992), is not classified as a pathogen (Mandrell *et al.*, 2005). *C. helveticus* is closely related to *Campylobacter upsaliensis*, which, although being associated with human diseases such as abscess, gastroenteritis and bacteraemia, has an ill-defined mode of pathogenicity (Moser *et al.*, 2001).

In conclusion, the current molecular phylogenetic study showed that the bacterial populations found in the openings of the urogenital and anal tracts of the tammar wallaby are far more diverse and complex than previously reported for other anatomical sites in marsupials using simple culturing techniques. Although most of the bacteria represent potentially novel species, the majority belong to two predominant phyla, the Firmicutes and the Bacteroidetes, in a similar manner to that documented for placental mammals.

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