An observational study of the microbiome of the maternal pouch and saliva of the tammar wallaby, *Macropus eugenii*, and of the gastrointestinal tract of the pouch young

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Marsupial mammals, born in an extremely altricial state with no functional immune system, offer a unique opportunity to investigate both the developing microbiome and its relationship to that of the mother and the potential influence of this microbiome upon the development of the immune system. In this study we used a well-established marsupial model animal, *Macropus eugenii*, the tammar wallaby, to document the microbiome of three related sites: the maternal pouch and saliva, and the gastrointestinal tract (GIT) of the young animal. We used molecular-based methods, targeting the 16S rDNA gene to determine the bacterial diversity at these study sites. In the maternal pouch, 41 unique phylotypes, predominantly belonging to the phylum Actinobacteria, were detected, while in the saliva, 48 unique phylotypes were found that predominantly belonged to the phylum Proteobacteria. The GIT of the pouch young had a complex microbiome of 53 unique phylotypes, even though the pouch young were still permanently attached to the teat and had only been exposed to the external environment for a few minutes immediately after birth while making their way from the birth canal to the maternal pouch. Of these 53 phylotypes, only nine were detected at maternal sites. Overall, the majority of bacteria isolated were novel species (<97 % identity to known 16S rDNA sequences), and each study site (i.e. maternal pouch and saliva, and the GIT of the pouch young) possessed its own unique microbiome.

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INTRODUCTION

Metatherian mammals or marsupials are characterized by short gestation periods ranging from only 11 days in the

Abbreviations: ARDRA, amplified rDNA restriction analysis; GIT, gastrointestinal tract.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA phylotype sequences determined in this study are GQ358790–GQ358918.

Three supplementary tables, showing phylotypes found in the pouch of three tammar wallabies and their closest identity to 16S rDNA sequences in GenBank, phylotypes found in the saliva of three adult tammar wallabies and their closest 16S rDNA identity to 16S rDNA sequences in GenBank, and phylotypes found in the GIT of two pouch young and their closest 16S rDNA identity to 16S rDNA sequences in GenBank, are available with the online version of this paper.

stripe-faced dunnart, *Sminthopsis macroura* (Renfree, 2006), to 35 days in the koala, *Phascolartos cinereus* (Tyndale-Biscoe & Renfree, 1987). At birth, many of the major body systems, including the immune system, are immature, yet the young animal has well-developed forelimbs, mouth and tongue, features which allow it to make its way from the birth canal and into the maternal pouch. Here the young animal attaches itself to a teat and subsequently undergoes the major processes of development and maturation (Fig. 1) (Tyndale-Biscoe & Janssen, 1988; Tyndale-Biscoe, 2005).

Despite an immature immune system, the pouch young is able to survive in the pouch environment, which is known to contain a range of micro-organisms (Deakin & Cooper, 2004; Old & Deane, 1998). It has been presumed that a

Fig. 1. A day-old pouch young attached to its mother's teat inside the pouch.

range of maternal defence strategies protect the young animal against potentially pathogenic microbial infections. These strategies include immunoglobulins, lysozyme and antimicrobials found in the yolk sac, pouch secretions, milk and saliva (Ambatipudi *et al.*, 2008; Deane *et al.*, 1990; Joss *et al.*, 2009).

To date, the possible contribution of maternally derived micro-organisms to the protection of the young, possibly via competitive exclusion (Cross, 2002; Martin *et al.*, 2007; Savage, 1977), has not been investigated. These micro-organisms could arise from a number of maternal sites. Prior to birth of the young marsupial, females groom and lick their pouch, a process which presumably leads to deposition of salivary micro-organisms (Ambatipudi *et al.*, 2008; Tyndale-Biscoe, 2005; Yadav *et al.*, 1972). At birth, the young is exposed to the microbiome of the birth canal and the adjacent urogenital and anal area (Chhour *et al.*, 2008). Subsequently, as the young animal traverses the maternal fur it will also be exposed to a further array of micro-organisms. All of these micro-organisms have the potential to be of benefit or harm to the young animal, and all represent a potential source of the subsequent microbiome of the developing animal.

There have been very few studies of bacterial populations associated with marsupials, and most of these have relied solely on culture-based protocols (Beighton & Miller, 1977; Deakin & Cooper, 2004; Lentle *et al.*, 2006; Old & Deane, 1998; Ouwerkerk *et al.*, 2005; Yadav *et al.*, 1972). It is well recognized that culture-based methods are highly biased, resulting in identification of only a small fraction of the actual microbial diversity (Hugenholtz *et al.*, 1998; Pace, 1997). Recently we used molecular-based methods to compare the bacterial diversity found within the openings of the urogenital and anal tracts of the female tammar wallaby, *Macropus eugenii*. We found an unexpected degree of bacterial diversity associated with each tract and, despite

their close anatomical proximity, each tract was able to maintain its own separate microbiome (Chhour *et al.*, 2008).

In this study we sought to document the microbiome of the maternal pouch and saliva and that of the gastrointestinal tract (GIT) of the pouch young. We used molecular-based methods targeting the 16S rDNA gene to investigate the bacteria present in the pouch and saliva from three different tammar wallabies, one carrying a 1-day-old pouch young, another carrying a 4-day-old pouch young and another with no pouch young (non-lactating). To investigate the bacterial diversity of the GIT of the pouch young we collected samples from two different pouch young of the same mothers at three GIT study sites: saliva from the tongue surface, the contents of the stomach cavity and the intestines. This study provides the first comprehensive insight into the bacterial ecology of the GIT of the pouch young and the maternal pouch and saliva, two keys sites of contact between mother and young.

METHODS

Animals and sample collection. Pouch and saliva swabs from three different female tammar wallabies, tammar 8837, carrying a 1-day-old pouch young, tammar 8817, carrying a 4-day-old pouch young, and tammar 1530, with no pouch young, were used in this study. The animals were sourced from two different captive populations maintained in large natural enclosures located at Macquarie University, Sydney, Australia (1530), and CSIRO Sustainable Ecosystems, Canberra, Australia (8837 and 8817). The animal handling and sample collection were 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 swabs dipped in PBS containing 15 % (v/v) glycerol were used to collect samples from the pouch and saliva of the three adult female tammar wallabies. The pouch sample was obtained by repeatedly rubbing a cotton swab around the entire surface of the pouch, whilst for collection of oral cavity and salivary bacteria the swab was retained in the animal's mouth for 3 s. The tips of the swabs were broken off into 1.5 ml microfuge tubes containing 300 μ l PBS and kept on dry ice until sampling was completed, whereupon the samples were stored at -80°C until processed.

Initial attempts to amplify 16S rDNA from the bacteria of the GIT samples obtained from pouch young at day 0 (from female 8837) and day 4 (from female 8817) at 25 cycles were unsuccessful. Samples were therefore obtained from older pouch young that were the offspring of the same two females (8817 and 8837). These samples were collected from two pouch young, 8817-PY male (40 days old), and 8837-PY female (56 days old), with the ages determined according to head length measurements (Poole *et al.*, 1991). It should be noted that at these ages (40 and 56 days post-partum) the young animals have been attached to the teat from the time of their entry into the pouch. For sampling, the young were removed from the maternal pouch and kept at 37°C in a sterile tissue culture incubator (<2 h) until sacrificed. The tongue and the intestines were removed and placed into sterile 1.5 ml microfuge tubes containing 300 μ l sterile PBS with 15 % (v/v) glycerol. The stomach and all its contents were placed in a sterile 1.5 ml microfuge tube, and all samples were kept on dry ice until sampling was completed and then stored at -80°C prior to processing. All reagents, swabs and tubes used in the collection protocol were sterilized prior to use.

DNA extraction. All samples were thawed on ice prior to DNA extraction. Maternal pouch and saliva samples were vortexed for 2 min to achieve a homogeneous suspension. For pouch young samples the tongue was vortexed vigorously for 2 min to wash off salivary micro-organisms and to achieve a homogeneous suspension with the storage buffer. The microbial contents of the intestine were obtained by initially disrupting the tissue using a sterile pipette and then subjecting the sample to repeated pipetting. The contents of the intestines were then vortexed vigorously for 2 min as described above. For the stomach samples a sterile pipette tip was used to pierce the stomach and 2 µl of the stomach contents was removed and mixed with 18 µl sterile water for DNA extraction. For all samples, with the exception of the stomach, a 20 µl aliquot was taken for DNA extraction and mixed with 180 µl buffer and incubated at 56 °C for 2 h. This buffer consisted of 10 mM sodium phosphate, pH 6.7, containing 400 mg lysozyme (Roche Applied Science), 200 U mutanolysin (Sigma Aldrich) and 400 mg proteinase K (Qiagen), with 20 mM diethyl pyrocarbonate (Sigma Aldrich). Bacterial cells were lysed with 1% (w/v) SDS and 200 mg 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.

Amplified rDNA restriction and sequencing of 16S rDNA genes.

Bacterial 16S rDNA genes were amplified by PCR using AmpliTaq Gold with GeneAmp (Applied Biosystems) using the primer pairs F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGY-TACCTTGTACGACTT-3') (Weisburg *et al.*, 1991). These primers have been proven to amplify a wide range of bacterial taxa and to generate PCR amplicons of approximately 1500 bp. The conditions for the PCR were as previously described (Chhour *et al.*, 2008). The PCR products were visualized on a 1% (w/v) agarose gel, purified using a PCR Clean-Up kit (MO BIO) and cloned into a T-tailed pGEM vector (Promega Life Sciences), before being heat-shocked into *Escherichia coli* DH5α (Invitrogen). The transformed *E. coli* was then cultivated on Luria-Bertani agar plates, and 96 colonies were randomly selected from each of the samples from each of the three adult female wallaby collection sites (i.e. pouch and saliva) to produce two separate clone libraries. From each of the samples of the pouch young GIT (i.e. saliva from the tongue surface, stomach cavity and intestines), 96 colonies were randomly selected to produce a 576 clone library. The cloned inserts within the plasmids were recovered by PCR using vector-specific primers pGEM-F (5'-GGCGGTCGCGG-GAATTCGATT-3') and pGEM-R (5'-GCCGCGAATTCAC-TAGTGATT-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 90s.

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 rDNA genes, as ARDRA based on one enzyme is only able to resolve bacterial diversity to the genus level. The restriction profiles were manually compared and all 129 unique restriction profiles from the pouch, saliva and the GIT of the pouch young were sequenced. Partial 16S rDNA gene sequences of approximately 1450 bp in length, representing a specific species or phylotype, were 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 species (<97% sequence identity to any known 16S rDNA sequences), they were checked to determine whether they were chimaeras by first comparing short sections of each sequence with known sequences in GenBank (Ludwig *et al.*, 1997) and, subsequently, by determining the position of each sequence on a phylogenetic tree with respect to all other phylotypes (see below).

Phylogenetic analysis and diversity index. The cloned 16S rDNA sequences from the maternal pouch and saliva samples and the GIT pouch young were aligned using the CLUSTAL W program (Thompson *et al.*, 1994), accessed through BioManager provided by the Australian National Genome Information Service (ANGIS). Neighbour-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. The Shannon-Weiner diversity index (H') (Gray, 2000) was calculated for each sample.

RESULTS AND DISCUSSION

Maternal pouch bacteria

A near full-length 16S rDNA library containing 227 clones was generated from the pouch samples collected from three adult female wallabies (8837, 69 clones; 8817, 68 clones; 1530, 90 clones). Sequencing of the 16S rDNA genes showed that these clones represented 41 different phylotypes belonging to five different bacterial phyla: the Actinobacteria, Firmicutes, Deinococcus-Thermus, Proteobacteria and Bacteroidetes (Fig. 2, Supplementary Table S1). As a species is generally accepted to have $\geq 97\%$ sequence homology and a genus $\geq 95\%$ (Stackebrandt & Goebel, 1994), 30 of the 41 phylotypes were considered to be novel species and 10 of these could not be grouped into a currently recognized genus.

Bacterial diversity was lowest in the pouch of the female carrying a day-old pouch young, tammar wallaby 8837 with an H' of 1.51, with eight of the nine different phylotypes detected belonging to the phylum Actinobacteria (Supplementary Table S1). Tammar wallaby 8817, carrying a 4-day-old pouch young, possessed 19 different phylotypes and an H' of 2.43, while tammar wallaby 1530, with no pouch young, possessed the greatest diversity, with an H' of 2.46 and 23 phylotypes (Supplementary Table S1). These findings are consistent with those reported using cultivation protocols (Deakin & Cooper, 2004; Old & Deane, 1998; Yadav *et al.*, 1972), where a reduction in both diversity and number of bacteria in the pouch of various marsupials occurred at the time of birth. This suggests a need for further investigation into the bacteria found in the pouch at the time of birth of the young, as they may play a role in protection of the pouch young.

Actinobacteria were the most commonly identified bacteria in the maternal pouch, representing 92.5% of the total number of clones (Table 1) and 82.9% of the phylotype diversity (Fig. 3). All six of the phylotypes that were represented by $\geq 5\%$ of the isolated clones were from this phylum. *Corynebacterium* was the most commonly isolated bacterial genus, with five of the six phylotypes belonging to this genus being represented by $\geq 5\%$ of the isolated clones (phylotypes 8837-D0-P-7C, 8837-D0-P-1C, 8817-D4-P-1B, 1530-P-2G and 8837-D0-P-1A; Fig. 2, Supplementary Table S1). The genus *Corynebacterium* comprises more than 60 species and is found in a variety

Fig. 2. Phylogenetic trees constructed from near full-length 16S rDNA sequences from randomly cloned genes of bacteria isolated from the pouch (left) and saliva (right) of three adult female tammar wallabies. Both trees were constructed by neighbour-joining using the Jukes–Cantor parameter. The trees were bootstrapped 1000 times, and bootstrap values are shown at branch points. Each phylum is defined by a different colour: Actinobacteria, green; Firmicutes, blue; Proteobacteria, red; Bacteroidetes, grey; Fusobacteria, yellow; Deinococcus–Thermus, pink. The phylotype that was found to be common to both the pouch and saliva of the tammar wallaby is highlighted by a white rectangle. The phylotypes which are represented by $\geq 5\%$ of the total clones in the pouch are highlighted by their own box in the centre of the figure giving the percentage of the number of clones. Where a phylotype possessed $\geq 97\%$ identity to a known species, the phylotype was given the species name, otherwise the clone name was used.

Table 1. Number of clones belonging to different phyla isolated from the pouch and saliva of adult tammar wallabies

ND, Not detected.

Phylum	Number of clones found in the pouch of three tammar wallabies (%) [*]				Number of clones found in the saliva of three tammar wallabies (%) [†]			
	1530	8837	8817	Total	1530	8837	8817	Total
Actinobacteria	95.6	95.7	85.3	92.5	ND	1.4	4.4	1.9
Proteobacteria	2.2	ND	ND	0.9	55.7	81.7	61.8	66.5
Firmicutes	1.1	4.3	1.5	2.2	31.4	14.1	22.0	22.5
Bacteroidetes	ND	ND	13.2	4.0	12.9	1.4	11.8	8.6
Fusobacteria	ND	ND	ND	ND	ND	1.4	ND	0.5
Deinococcus–Thermus	1.1	ND	ND	0.4	ND	ND	ND	ND

^{*}A total of 227 16S rDNA clones were isolated from the pouch of three adult tammar wallabies: tammar wallaby 1530 (no pouch young; 90 clones); tammar wallaby 8837 (carrying a day-old pouch young; 69 clones); tammar wallaby 8817 (carrying a 4-day-old pouch young; 68 clones).

[†]A total of 209 16S rDNA clones were isolated from the saliva of three adult tammar wallabies: tammar wallaby 1530 (no pouch young; 70 clones); tammar wallaby 8837 (carrying a day-old pouch young; 71 clones); tammar wallaby 8817 (carrying a 4-day-old pouch young; 68 clones).

of environments (Feurer *et al.*, 2004; Funke *et al.*, 1997; Khamis *et al.*, 2005). Several members of this genus are implicated in human disease (Feurer *et al.*, 2004; Van den Velde *et al.*, 2006) and are known to be present in intertriginous areas (Roth & James, 1988). Our observations of the abundance of *Corynebacterium* are consistent with culture-based studies of the bacterial flora of the tammar wallaby pouch (Old & Deane, 1998), but contrast with reported results on the pouch of the brushtail opossum, *Trichosurus vulpecula* (Deakin & Cooper, 2004), in which Proteobacteria and not Actinobacteria are the predominant culture isolates.

Phylotype 1530-P-1A, representing 8.8% of the total number of clones, grouped closest to *Brevibacterium otitidis* on the phylogenetic tree (Fig. 2). Members of the genus *Brevibacterium* have been isolated from human toe webs (Roth & James, 1988), and are regarded as opportunistic

pathogens (Pascual *et al.*, 1999; Wauters *et al.*, 2000). This close grouping may indicate that these two organisms share similar properties, particularly the capacity to colonize skin.

The other four phyla, Proteobacteria, Firmicutes, Bacteroidetes and Deinococcus–Thermus, were sparsely represented in samples from the pouches of the three tammar wallabies. In tammar wallaby 8817, the most significant of the four phyla was the Bacteroidetes, which represented 5.3% of the overall phylotype diversity and 13.2% of the total number of clones (Fig. 3, Table 1). The phylum Proteobacteria was only detected in tammar wallaby 1530 (with no pouch young), where it contributed 8.7% of the phylotype diversity and 2.2% of the total number of clones (Fig. 3, Table 1). The phylum Bacteroidetes was only detected in the pouch of tammar wallaby 8817, while the phylum Deinococcus–Thermus was only detected in tammar wallaby 1530 (Table 1).

Fig. 3. Histogram showing the diversity of phylotypes based on ARDRA in the different maternal sites compared with the diversity of phylotypes found in the pouch young GIT of the tammar wallaby. A, anal tract; U, urogenital tract; P, pouch; S, saliva; PY GIT, GIT of pouch young. Asterisks indicate that the data were sourced from Chhour *et al.* (2008).

Maternal salivary and mouth bacteria

The final clone library generated from the saliva samples of the three adult female tammar wallabies consisted of 209 clones (71 clones from 8837; 68 clones from 8817; 70 clones from 1530). These salivary clones are considered representative of those to be found in the maternal oral cavity. Tammar wallaby 8837 possessed 17 phylotypes and an H' of 2.31. Tammar wallaby 8817 had an H' of 2.81 and 26 phylotypes, while tammar wallaby 1530 had an H' of 2.59 and 22 phylotypes. In total, 48 different phylotypes were identified that belonged to five different phyla, the Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes and Proteobacteria (Fig. 2, Supplementary Table S2). Only one phylotype, 8837-D0-P-2G, with 95 % identity to *Devrisea agamarum*, was found in both the pouch and salivary samples (Fig. 2, Supplementary Tables S1 and S2). This phylotype represented 8.7 % of the total clones isolated from the pouch of tammar wallaby 8837 and 1.5 % of the total salivary clones isolated from tammar wallaby 8817.

The Proteobacteria were dominant in the saliva of all three wallabies, representing 54.2 % of the phylotype diversity and 66.5 % of the clones isolated (Fig. 3, Table 1). Four of the six phylotypes, 8817-D4-O-10C, 1530-O-11A, 8837-D0-O-2D and 8817-D4-O-10A, each represented ≥ 5 % of the total number of clones isolated (Fig. 2, Supplementary Table S2) and belonged to this phylum. 1530-O-11A was the most frequently detected phylotype and contributed 26.3 % of the total number of clones isolated. This phylotype possessed 98 % identity to *Moraxella cuniculi*, which has recently been isolated from the GIT of post-weaning piglets (Su *et al.*, 2008). However, this species was not detected in the GIT of the pouch young. This may simply reflect the level of sensitivity of the detection system rather than an absence per se. Beighton & Miller (1977) isolated species of *Moraxella* from the dental plaque of macropods, which most likely explains why this genus was found only in the saliva of the adult female wallabies. However, the role that this bacterium plays in the oral cavity of the tammar wallaby and other macropods remains unknown.

Phylotype 8837-D0-O-2D was found in the saliva of all three adult tammar wallabies. Due to its low sequence identity (90 %) to any known 16S rDNA, there is little that one can infer about its identity or the role it may play in the oral cavity of the tammar wallaby. On the other hand, phylotype 8817-D4-O-10A possessed 95 % identity to *Actinobacillus indolicus*, which has been isolated from pneumonia lesions and the brains of slaughtered pigs (Blackall *et al.*, 1994; Møller *et al.*, 1993). Phylotype 8817-D4-O-10C was found to possess a 96 % sequence identity to *Simonsiella crassa*. Members of this genus have been isolated from the oral cavity of dogs, cats, sheep and humans, and are considered to be non-pathogenic (Kuhn *et al.*, 1978; Xie & Yokota, 2005). The role of *Simonsiella* in any of these mammals, however, remains unknown.

Among salivary bacteria, the Firmicutes represented 18.8 % of the phylotype diversity and 22.5 % of the total number

of clones isolated (Fig. 3, Table 1). Two phylotypes each with >5 % of the total number of isolated clones, phylotypes 8837-D0-7B and 8837-D0-O-6G, belonged to the Firmicutes. Phylotype 8837-D0-O-6G, which represented 5.7 % of the total number of clones, possessed 98 % identity to *Streptococcus iniae* and was more frequently identified in tammar wallabies 1530 and 8817 than tammar wallaby 8837 (Supplementary Table S2). *S. iniae* is reportedly a fish pathogen (Eyngor *et al.*, 2007, 2008; Fuller *et al.*, 2001). Phylotype 8837-D0-O-7B possessed 96 % sequence identity to the 16S rDNA of *Streptococcus suis*, normally considered to be a swine pathogen (Manzin *et al.*, 2008; Wangsomboonsiri *et al.*, 2008), and was more evenly distributed in the saliva of the three animals (Supplementary Table S2). To what extent, if any, these phylotypes can be considered pathogens remains to be determined.

Members identified as belonging to the phylum Bacteroidetes possessed a phylotype diversity of 16.7 %, and represented 8.6 % of the total clones isolated from maternal saliva (Fig. 3, Table 1). Members of this phylum were mainly isolated from tammar wallaby 1530, with only three phylotypes detected in the saliva of the other female wallabies. Actinobacteria were only detected in the tammar wallabies carrying pouch young (8837 and 8817), and Fusobacteria were only detected in tammar wallaby 8837 (Supplementary Table S2).

GIT bacteria of the pouch young

A 526 clone library was constructed from samples obtained from the GIT of the two pouch young. This included 258 clones from the 40-day-old pouch young, 8817-PY (tongue surface 82 clones, H' 1.73; stomach contents 86 clones, H' 2.23; intestine 90 clones, H' 2.54) and 268 clones from the 56-day-old pouch young, 8837-PY (tongue 92 clones, H' 1.63; stomach 87 clones, H' 1.93; intestines 89 clones, H' 2.84). These clones represented 53 different phylotypes (Supplementary Table S3) belonging to four different phyla, the Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes (Fig. 4). These findings are in sharp contrast to those reported by Lentle *et al.* (2006), who found fewer than five different species of bacteria in the GIT of the pouch young. Their relative distribution within the GIT of the pouch young is shown in Table 2 and Fig. 4. Of the clones isolated, 43.3 % were considered to be novel species as they showed <97 % identity to any known species (Supplementary Table S3). Although the phylotypes found in this study were not always found throughout the GIT of the pouch young, all the phylotypes with >5 % of the total number of clones were widely distributed (Fig. 4, Supplementary Table S3).

The GIT of the pouch young was dominated by the Firmicutes, namely the genera *Enterococcus* and *Streptococcus* (Fig. 4, Supplementary Table S3). Phylotype 8837-D0-A-2C represented 20.9 % of the total number of clones isolated and was also found to contribute a

Fig. 4. Phylogenetic tree constructed from near full-length 16S rDNA sequences from randomly cloned genes of bacteria isolated from the tongue surface, stomach cavity and intestines of two tammar wallaby pouch young. The tree was constructed by neighbour-joining using the Jukes–Cantor parameter. The tree was bootstrapped 1000 times, and the bootstrap values are shown at the branch points. Each phylum is defined by a different colour. The phylotypes highlighted by a white rectangle represent the phylotypes that are also found from maternal sources. The shapes next to the phylotypes represent the location within the GIT at which the phylotype was found: ■, tongue surface; ●, stomach cavity; ▲, intestine. The phylotypes that represent $\geq 5\%$ of the total number of clones are highlighted and expressed as a percentage of the total number of clones found throughout the entire GIT.

significant number of clones in all sites (i.e. tongue surface 21.3%, stomach cavity 29.5% and intestines 12.3%; Supplementary Table S3). This phylotype possessed 99% identity to *Enterococcus faecalis* and was also found in the anal and urogenital tracts (Chhour *et al.*, 2008) and the pouch of adult female tammar wallabies. However, in the adult animal, it represented $<1\%$ of the total clones

isolated. These observations suggest that differences in the mucosal environment of the GIT, and the availability of nutrients from maternal milk to the developing pouch young, enable this species to proliferate in the GIT. Pertinent to the concept that these bacteria play a protective role in marsupials is the fact that strains of *Enterococcus faecalis* are known to reduce the colonization

Table 2. Combined number of clones belonging to different phyla isolated from the GIT of two pouch young

Phylum	Number of clones isolated from the GIT of two pouch young (%) [*]			
	Tongue surface	Stomach cavity	Intestines	Total
Actinobacteria	9.2	20.8	42.5	24.3
Proteobacteria	7.5	17.9	7.3	10.8
Firmicutes	82.2	58.4	44.7	61.6
Bacteroidetes	1.1	2.9	5.6	3.2

^{*}A combined total of 526 16S rDNA clones were isolated from three different segments of the GIT of two tammar wallaby pouch young aged 40 and 56 days. Tongue surface, 174 clones; stomach content, 173 clones; intestines, 179 clones.

of pathogens. For instance, strains of *Enterococcus faecalis* inhibit *Staphylococcus aureus* colonization of mucosal surfaces by production of bacteriocins or by lowering the pH (Alomar *et al.*, 2008a, b). Although strains of *Enterococcus faecalis* are also known to be opportunistic pathogens (Punj & John, 2008), the role that the strains of *Enterococcus faecalis* found in this study may play in the GIT of the pouch young is not known.

Phylotype 8817-D40-PY-O-1B possessed 99 % identity to *Enterococcus raffinosus*. This phylotype made up 14.8 % of the total clones isolated (Fig. 4, Supplementary Table S3). As with *Enterococcus faecalis*, *Enterococcus raffinosus* was found throughout the GIT of the pouch young. However, it was not isolated from the pouch, saliva or the cloaca (Chhour *et al.*, 2008) of the adult tammar wallabies. Phylotype 8837-D60-PY-O-3A showed a 99 % match to *Lactococcus garvieae*. This phylotype contributed 5.9 % of the total number of clones found in the GIT (Fig. 4, Supplementary Table S3).

Within the genus *Streptococcus*, phylotype 8837-D60-PY-O-1B showed a 99 % match to the 16S rDNA of *Streptococcus dysgalactiae* and represented 10.3 % of the total number of clones isolated. The bulk of these clones were from the surface of the tongue (29.3 %), with only two clones being isolated from the stomach and one from the intestines (Supplementary Table S3). Strains of *S. dysgalactiae* are associated with a variety of diseases (Denamiel *et al.*, 2005; Kawata *et al.*, 2003; Vela *et al.*, 2006), and strains of *S. dysgalactiae* have previously been reported in the cloaca of the adult female tammar wallaby (Chhour *et al.*, 2008). This species was also detected in the pouch of tammar wallaby 8837 carrying a day-old pouch young. However, none of the maternal phylotypes was found in the GIT of the pouch young.

The phylum Actinobacteria contributed 37.7 % of the phylotype diversity and 24.3 % of the total number of clones in the GIT of the pouch young (Fig. 3, Table 2).

Phylotype 8817-D40-PY-O-1C represented 7.4 % of the total number of clones and possessed 94 % identity to *Corynebacterium* sp. C3 (Supplementary Table S3). Phylotype 1530-P-4D represented 5.1 % of the isolated clones and was found throughout the GIT of both the pouch young. It possessed 96 % identity to *Devrisea agamarum* str. IMP2 (GenBank accession no. EU009865; Fig. 4, Supplementary Table S3). This phylotype was also found in the pouch of tammar wallaby 1530, but not in the two tammar wallabies carrying pouch young (Supplementary Table S1).

In contrast to the Firmicutes and the Actinobacteria, the phyla Proteobacteria and Bacteroidetes were poorly represented within the GIT of the pouch young (Fig. 4, Supplementary Table S3). The most numerous phylotype was 8817-D40-PY-O-1E, which possessed 99 % identity to *E. coli* and represented 9.3 % of the total number of clones (Fig. 4, Supplementary Table S3). Indigenous *E. coli* strains are known to prevent colonization and infection by enterotoxigenic strains of *E. coli* in several mouse and pig models (McFarland, 2000). The high presence of phylotype 8817-D40-PY-O-1E throughout the GIT of both pouch young bears further investigation, as this phylotype may serve its host in a similar manner.

The bacterial composition of the GIT of the two pouch young was remarkably similar in that each of the seven phylotypes that represented >5 % of the total number of clones isolated was found throughout the GIT. This may be due to the immaturity of the GIT of the pouch young at the age of sampling (Dra, 1986), yet it contrasts to what was observed in maternal samples. Here, the bacterial composition was found to be unique at all the sites sampled, which is consistent with recent observations in humans and other eutherian mammals (Fierer *et al.*, 2008; Ley *et al.*, 2006, 2008). One other interesting possibility that we have been unable to explore, given the very small (microlitre) volumes available, is that the bacterial flora of the GIT of the young arises from the maternal milk. Recently, Martin *et al.* (2007) reported that bacteria commonly isolated from human infant faeces, such as staphylococci, streptococci, micrococci, lactobacilli and enterococci, are also isolated from breast milk and are considered as part of the normal flora. This range of bacteria is similar to what we found in the GIT of the pouch young, where the enterococci were the most commonly isolated, followed by the streptococci. Interestingly, no lactobacilli have been detected at any site in either the adult animals or the pouch young (Supplementary Table S3; Chhour *et al.*, 2008).

Phylotypes found both in the pouch young GIT and in maternal samples

Only nine phylotypes out of the 53 detected in the GIT of the pouch young matched those found in the pouch and saliva of the three adult female tammar wallabies and those found in the urogenital and anal openings of tammar wallabies 8837 and 8817 (Chhour *et al.*, 2008) (Table 3).

Table 3. Phylotypes common to both maternal sites and the GIT of pouch young

ND, Not detected.

Phylotype	Distribution of the phylotypes at maternal sites (%)				Distribution of the phylotypes in the GIT of pouch young (%)		
	Urogenital opening*	Anal opening*	Pouch	Saliva	Tongue surface	Stomach cavity	Intestine
8817-D4-C-2C	1.2	0.7†	ND	ND	0.6‡	0.6‡	ND
8817-D4-A-1F	0.6§	8.1§	ND	ND	ND	ND	0.6‡
8837-D0-A-2C	0.6†	1.4†	1.5§	ND	21.3‡	29.5‡	12.3‡
8837-D0-A-9C	ND	1.4	ND	ND	ND	ND	0.6
1530-P-1A	ND	ND	22.2¶	ND	ND	ND	0.6
1530-P-3F	ND	ND	4.4¶	ND	0.6	0.6	2.2
1530-P-4D	ND	ND	11.1¶	ND	1.7‡	4.6‡	8.9‡
8817-D4-P-1E	ND	ND	13.2§	ND	ND	1.2	ND
8817-D4-O-11C	ND	ND	ND	8.8§ 2.9¶	0.6‡	0.6‡	3.4‡

*Data from Chhour *et al.* (2008).

†Phylotype found in adult female tammar wallaby 8837.

‡Phylotype found in pouch young of tammar wallaby 8817.

§Phylotype found in adult female tammar wallaby 8817.

||Phylotype found in pouch young of tammar wallaby 8837.

¶Phylotype found in adult female tammar wallaby 1530.

Interestingly, these nine phylotypes were not necessarily detected in the mother of a given pouch young. For example, phylotype 1530-P-4D was isolated only from the tammar wallaby with no pouch young, yet was found in the GIT of both the pouch young. It may well be that the bacteria are present but in numbers that are below the detection capabilities of the methods used in this study, as the probability of detecting a cloned 16S rDNA with 95 % confidence using a sample size of 96 requires a species to be present at a level of ≥ 3 % of the population (Chhour *et al.*, 2005).

Phylotype 8837-D0-A-2C, which was isolated throughout the GIT of the pouch young, was found only sparsely in the maternal pouch and urogenital and anal openings, and was not detected in the saliva. In contrast, phylotype 1530-P-1A was found to contribute 22.2 % of the total number of clones isolated from the pouch of tammar wallaby 1530, yet represented only 0.6 % of the clones from the intestines of the pouch young (Table 3). Maternal phylotypes 1530-P-3F and 1530-P-4D were found throughout the GIT of the pouch young, while phylotype 8817-D4-P-1E was only found in the stomach cavity.

The only phylotype that was found in both the GIT of the pouch young and the maternal saliva was phylotype 8817-D4-O-11C, which possessed a 96 % 16S rDNA sequence identity to *Bergeyella* species clone g8498. This phylotype was found in adult tammar wallabies 8817 and 1530, but not in tammar wallaby 8837, the female carrying the 1-day-old pouch young. This phylotype was found throughout the GIT of the pouch young (Table 3, Fig. 4).

CONCLUSION

This study has documented the microbiome of the adult pouch and saliva of the tammar wallaby and that of the GIT of the pouch young, and has laid the foundation for further studies investigating the source of a young animal's microbiome. This study has highlighted the fact that the bacterial microbiome of the tammar wallaby is much more diverse than was previously thought based solely on cultivable bacteria, especially as far as the GIT of the pouch young is concerned. In addition, we observed that the maternal and pouch young microbiomes were able to maintain their uniqueness, despite their close proximity to one another.

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