Colonization of the neonatal rat intestinal tract from environmental exposure to the anaerobic bacterium Oxalobacter formigenes

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Oxalobacter formigenes, an anaerobic bacterium that inhabits the mammalian gastrointestinal tract, has an important symbiotic relationship with its vertebrate hosts by regulating oxalic acid homeostasis. Epidemiological studies of O. formigenes colonization in man have shown that colonization occurs in young children, that every child can become colonized naturally, that >20% lose colonization during adolescence or as adults and that stable colonization can be disrupted by antibiotic use or changes in diet, greatly affecting subsequent health. As O. formigenes is a fastidious anaerobe that seldom re-colonizes adults, the question arises as to how initial colonization occurs. To investigate this question, non-colonized female laboratory rats were placed on diets high in oxalate and were colonized by oesophageal gavage with O. formigenes either before or after being impregnated. Faecal specimens from their offspring were tested for the presence of O. formigenes. Although the bacterium was first detected in a few neonates as early as 7 days post-partum, colonization of all the offspring did not occur until after weaning. In each case, the offspring were colonized with the bacterial strain carried by their mothers. To determine whether O. formigenes colonization occurs vertically or horizontally, newborn rats were placed with foster mothers that were either non-colonized or colonized with an O. formigenes strain different from that of their natural mothers. Colonization occurred temporally in a manner similar to natural colonization but all offspring became colonized only with the O. formigenes strain of the foster mothers. These data indicate that intestinal colonization occurs horizontally, but does not answer the question of how O. formigenes survives the aerobic environment in order to be transmitted.

INTRODUCTION

The gastrointestinal (GI) tract of the newborn infant is normally sterile but, soon after birth, becomes colonized rapidly with many different bacterial species (Long & Swenson, 1977). Several factors influence colonization including diet, environment, mode of delivery and the availability of an energy source and nutrients for the bacteria (Kirjavainen & Gibson, 1999; Grönlund et al., 1999; Dai & Walker, 1999). With conventional delivery, the newborn’s intestine is first colonized with bacteria contacted during birth, i.e. bacteria that are part of the mother’s normal vaginal and intestinal microflora. In the less-contaminated environment of a Caesarean delivery or a premature birth where the infant is kept in isolation, intestinal colonization is somewhat delayed with fewer strict anaerobes present than after vaginal delivery (Long & Swenson, 1977; Neut et al., 1987). Rodents are also born without an intestinal bacterial microflora and have a pattern of colonization similar to that seen in man (Krishnan & Ramakrishna, 1998).

Oxalobacter formigenes is an obligately anaerobic bacterium that colonizes the GI tracts of most vertebrates, including man (Allison et al., 1985). O. formigenes utilizes oxalic acid (or its anion, oxalate), a toxic by-product of metabolism and a common constituent of most diets, as its sole source of energy. There is evidence that this permits O. formigenes to maintain an important symbiotic relationship with its host by helping to regulate oxalic acid absorption in the intestine (especially the colon) as well as oxalic acid levels in the plasma (Allison et al., 1986; Hatch & Freel, 1996). Several studies (Goldkind et al., 1985; Kleinschmidt et al., 1994; Han et al., 1995; Sidhu et al., 1998, 1999) have shown that O. formigenes may influence a variety of oxalate-related diseases, the most notable being a direct relationship between the absence of the bacterium and increased episodes of recurrent kidney stone disease.

Previous studies (Sidhu et al., 1997b) have shown that natural colonization of the intestinal tract with O. formigenes...
can occur in children as early as 1 year of age but that it takes up to 8 years before colonization approaches 100%. The onset of colonization with *O. formigenes* is delayed compared with other intestinal bacteria, possibly due to the lack of sufficient oxalate in the intestinal environment to support growth. Because of this delay in intestinal colonization, it seems unlikely that transmission of *O. formigenes* is directly from the mother at birth (vertical transmission). The more probable route of transmission is from the environment (horizontal transmission) even though *O. formigenes*, as a fastidious obligate anaerobe, is highly sensitive to oxygen and thought to be incapable of survival in air for any length of time. Experiments reported here were designed to determine whether colonization of offspring with *O. formigenes* comes directly from the mother or indirectly via the environment.

**METHODS**

**Laboratory rats.** Female and male Sprague–Dawley rats, weighing approximately 70 g each, were purchased from Harlan (Indianapolis, IN, USA) and maintained under conventional conditions in the Department of Animal Resources at the University of Florida, Gainesville. The rats were acclimatized for 2 or more days before the start of any experiment. Female rats were housed two to a cage in plastic cages with bedding and fed *ad libitum* with a semi-purified, low calcium (0.5%) low phosphate (0.4%) (lowCa/lowP) synthetic diet, TD 89222 (Harlan Teklad, Madison, WI, USA), supplemented with 2% (w/v) ammonium oxalate. Male rats were housed similarly but fed *ad libitum* regular laboratory food, except during periods of mating when they ate the same diet as the females. All rats maintained good health throughout the experiment with normal weight gains to about 350 g by the end of the experiments. The studies were approved by the Institutional Animal Care and Use Committee at the University of Florida, Gainesville.

**O. formigenes.** *O. formigenes* strain OxWR, originally isolated from wild rats and classified as subgroup I, and strain OxGP, originally isolated from guinea pig and classified as subgroup II, were a gift from Dr Milton J. Allison (Iowa State University, Ames, IA, USA) and were stored as frozen stock cultures. The bacteria were grown in medium B containing 60 mM oxalate as described by Allison et al. (1985). Bacteria isolated from guinea pigs were grown in medium B containing 60 mM oxalate as described by Allison et al. (1985). Bacteria from overnight cultures were collected by centrifugation and resuspended in culture medium containing 60 mM oxalate as described by Allison et al. (1985). Bacteria from overnight cultures were centrifuged and resuspended in culture medium containing 60 mM oxalate.

**Collection of faecal and urine samples.** To collect 24 h urine samples, rat or from the faecal cup in the metabolic cage. Analyses of faecal samples from rat pups younger than 15 days, pups were killed, and the intestinal and rectal contents were collected for analyses. After day 17, faecal samples were collected directly from the rectums of individual rats. A portion of each faecal sample was in the intestinal and rectal contents were collected for analyses.

**RESULTS AND DISCUSSION**

**Natural colonization of neonatal rats with *O. formigenes***

In an initial experiment, six timed-pregnant female Sprague–Dawley rats determined to be non-colonized with *O. formigenes* were placed on a lowCa/lowP diet, supplemented with 1.5% ammonium oxalate starting on day e-15. On days e-19 and e-20, each mother was inoculated by oesophageal gavage with 20 × 10⁸ bacteria from overnight cultures of *O. formigenes* strain OxWR. On day e-23, a total of 80 offspring were born. Starting at postnatal day 1 and repeated every 2 days until day 17, four pups were killed and the intestinal and rectal contents were collected for analyses. After day 17, faecal samples were collected directly from the rectums of individual rats. A portion of each faecal sample was inoculated anaerobically into culture vials containing 9 ml of medium B supplemented with 10 mM oxalate prepared anaerobically under high-grade CO₂ (Allison et al., 1985). The cultures were incubated at 37 °C for 10–12 days after which a sample of medium was removed and checked for the catabolic consumption of oxalate by CaCl₂ precipitation and spectrophotometric analysis. The disappearance of oxalate from the culture medium indicates the presence of an oxalic-acid-degrading micro-organism in the faecal sample, presumably *O. formigenes*.

**Detection of *O. formigenes* by anaerobic culture.** Approximately 20 mg of each faecal sample was inoculated anaerobically into culture vials containing 9 ml of medium B supplemented with 10 mM oxalate prepared anaerobically under high-grade CO₂ (Allison et al., 1985). The cultures were incubated at 37 °C for 10–12 days after which a sample of medium was removed and checked for the catabolic consumption of oxalate by CaCl₂ precipitation and spectrophotometric analysis. The disappearance of oxalate from the culture medium indicates the presence of an oxalic-acid-degrading micro-organism in the faecal sample, presumably *O. formigenes*.

**Detection of *O. formigenes* by DNA analysis.** Genomic DNA was isolated from rat faecal samples, as described elsewhere (Sidhu et al., 1997a). In brief, bacterial DNA was prepared using the DNeasy™ Tissue Kit (Qiagen). The isolated DNA was used as the template in PCR amplification with primers specific for the *oxc* gene of *O. formigenes*. All PCR products were verified by Southern blot analysis with hybridization to internal probes, either a genus-specific probe for *Oxalobacter* or a subgroup-specific probe for Group I (OxWR) and Group II (OxGP) strains (Allison et al., 2004).
(n = 2 rats per group). On days 10 and 11 after the diet change, they were inoculated by oesophageal gavage with $2 \times 10^8$ bacteria from overnight cultures of *O. formigenes* as follows: Group I rats received OxWR, Group II rats received OxGP and Group III rats received a mixture of OxWR and OxGP. Analyses of faecal samples collected on day 19 confirmed that all female rats were colonized with *Oxalo-bacter*. Because Southern blot analyses with the subgroup-specific probe for OxGP were negative or very weak at this time, rats in Groups II and III were given two additional doses of OxGP bacteria ($2 \times 10^8$ bacteria per rat). Subsequent Southern blot analyses with the subgroup-specific probes showed that Group I rats were positive for OxWR, Group II rats were positive for OxGP and Group III rats were positive for both OxWR and OxGP (data not shown).

Once it was determined that the female rats were stably colonized, they were mated by placing a different non-colonized male with each pair of females per group. The males were removed once the females were noted to be pregnant. Individual mothers, together with their offspring, were housed in separate cages to prevent cross-contamination between litters of neonates. Starting on day 9 post-partum, and every 4–5 days thereafter, faecal specimens were collected from the offspring for analysis by PCR and Southern blot hybridizations with Group-specific probes. As predicted by the first experiment, *O. formigenes* was not detected in a faecal specimen until the second week of age and, at time of weaning (day 23 post-partum), about 70% of specimens were positive. After weaning, however, the number of specimens positive for *O. formigenes* rapidly approached 100% (Table 1). Offspring of mothers inoculated with strain OxWR (Group I) showed colonization with only OxWR, while offspring of mothers inoculated with strain OxGP (Group II) showed colonization with only OxGP. Interestingly, offspring of mothers inoculated with both OxWR and OxGP often became colonized with both strains, indicating that two strains of *O. formigenes* can inhabit the GI tract simultaneously.

### Vertical versus horizontal colonization of offspring

A second set of experiments was designed to determine if the offspring become colonized with *O. formigenes* by vertical or horizontal transmission. Timed-pregnant female Sprague–Dawley rats (n = 4) were housed individually under conventional conditions. Initially, all rats were found to be non-colonized with *O. formigenes* by DNA analysis. Two days later, the rats were placed on the lowCa/lowP synthetic diet supplemented with 1% (w/w) ammonium oxalate and fed *ad libitum* throughout the experiment. On days 3 and 4 after the start of the diet regimen, each rat was inoculated by oesophageal gavage with $2 \times 10^8$ bacteria from an overnight culture of *O. formigenes*. Two rats were given OxWR (subgroup I) and two were given OxGP (subgroup II).

The four female rats delivered on day e-23. Approximately 3 h after birth, each litter of pups was taken from their mother and placed with a foster mother: all pups from OxWR-positive mothers were put with OxGP-positive mothers and all pups from OxGP-positive mothers were put with OxWR-positive mothers. By the following day, all mothers had adopted the new pups and were nursing. On days 8 and 15 post-partum, two pups from each litter were killed and the intestinal and rectal contents were collected for DNA analyses for the presence or absence of *O. formigenes* by PCR and Southern blot analysis. On days 23, 29 and 42 post-partum, faecal pellets were taken directly from the rectum of

### Table 1. Natural colonization of offspring matches environmental contamination with *O. formigenes*

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Age of offspring tested</th>
<th>No. of offspring</th>
<th>Colonization of offspring (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Group I</td>
</tr>
<tr>
<td>I (OxWR)</td>
<td>1–2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2–4</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>&gt;4</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>II (OxGP)</td>
<td>1–2</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2–4</td>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>&gt;4</td>
<td>6</td>
<td>88</td>
</tr>
<tr>
<td>III (OxWR+OxGP)</td>
<td>1–2</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2–4</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>&gt;4</td>
<td>5</td>
<td>80</td>
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*Detection of *O. formigenes* performed with Group-specific probes.

![Fig. 1. Temporal development of natural colonization of neonatal rats with *O. formigenes*.](http://jmm.sgmjournals.org)
each of the remaining pups and assayed for the presence of *O. formigenes*. Southern blot analyses with subgroup-specific hybridization probes revealed that pups born to mothers inoculated with Group I bacteria (OxWR) and fostered by mothers inoculated with Group II bacteria (OxGP) became colonized with Group II bacteria (shown for day 29 in Fig. 2a). In contrast, pups born to mothers inoculated with Group II bacteria (OxGP) and fostered by mothers inoculated with Group I bacteria (OxWR) became colonized with Group I bacteria (Fig. 2b).

In a separate experiment, two timed-pregnant female Sprague–Dawley rats were housed individually under conventional conditions. DNA analyses on faecal samples collected 1 day after their arrival showed that the rats were not colonized with *O. formigenes*. Beginning 2 days after their arrival, the rats were placed on the lowCa/lowP synthetic diet supplemented with 1% (w/w) ammonium oxalate and fed *ad libitum* throughout the experiment. On days 2 and 3 after the start of the diet regimen, one of the rats was inoculated by oesophageal gavage with $2 \times 10^8$ bacteria from an overnight culture of *O. formigenes* subgroup I strain OxWR. DNA analysis after the second inoculation of OxWR showed this rat to be colonized while the other rat remained negative.

Both rats delivered 12 pups on day 6 after their arrival (e-21). Approximately 3 h after birth, the pups were taken from their mothers and half of each litter was placed with foster mothers: half of the pups ($n=6$) from the OxWR-positive mother were put with the non-colonized mother and half of the pups ($n=6$) from the non-colonized mother were put with the OxWR-positive mother. The other half of each litter was returned to the original mother. Both mothers were maintained on the oxalate-containing diet. By the following day, the mothers had adopted the new pups and were nursing normally. DNA analyses on faecal samples collected from the offspring at weaning (day 23) showed that all rats nursed by the colonized mother were positive for *O. formigenes*, while those nursed by the non-colonized mother were negative (Fig. 3).

Observations from these two experiments indicate that the GI tract was colonized with the *O. formigenes* bacteria present in the environment during the pups’ development, not the bacterium colonizing the mother at time of birth. After weaning, the pups retained a robust colonization of *O. formigenes* as long as they were maintained on the low-calcium, high-oxalate diet. Once placed on a diet of normal rat food, colonization was lost within 1 week, irrespective of whether the normal rat food was supplemented with oxalate, confirming the importance of calcium in the diet to sequester oxalate and the maintenance of an adequate calcium-to-oxalate ratio to support *O. formigenes* colonization (unpublished results).

Laboratory rats, like many other animals raised under artificial conditions or in laboratory facilities and maintained

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**Fig. 2.** Southern blot analyses of faecal samples with Group-specific probes show that neonatal rats become colonized with the *O. formigenes* strain of their foster mothers. (a) Offspring of Group I-positive mothers fostered by Group II-positive mothers. (b) Offspring of Group II-positive mothers fostered by Group I-positive mothers.

**Fig. 3.** Natural colonization with *O. formigenes* occurs only when neonatal rats are raised by a colonized mother regardless of the colonization status of the natural mother. (a) Offspring raised with non-colonized mother. (b) Offspring raised with colonized mother.
in clean animal housing, are generally not colonized with O. formigenes (Daniel et al., 1987; personal observations); this was re-confirmed in the present study. This probably results from the use of rodent and animal foods high in calcium content but lacking sufficient oxalate for growth of O. formigenes. With an absolute requirement for oxalate as a nutrient and energy source, O. formigenes cannot survive long-term and is eventually lost from the breeding colonies. Studies in both man (Sidhu et al., 1997a) and rats (Cornelius et al., 2000) have shown that a diet low in oxalate and/or high in calcium results in a rapid decrease in O. formigenes colonization. At times, colonization can fall below detectable levels, even though the bacteria remain viable. This permits subsequent recovery when oxalate becomes bio-available in the GI tract; however, there appears to be a limit to the length of time O. formigenes can retain the ability to recover.

The development of the normal microbiota of the intestine of a newborn is a gradual process that is influenced by many factors, including the mode of delivery, environment, degree of hygiene during delivery, use of medications (particularly antibiotics) and diet (e.g. breast feeding versus formula feeding) (Kirjavainen & Gibson, 1999; Grönlund et al., 1999; Dai & Walker, 1999; Orrhage & Nord, 1999). Organisms transmitted vertically from mother to newborn are usually the result of vaginal colonization in the mother (Harvey et al., 1995; Matsumiya et al., 2002). The results of the current studies suggest that natural colonization with O. formigenes in the rat model, as in man, does not occur in utero or at the time of birth, but is delayed until the neonatal rats begin exploring their environment. The fact that the neonatal rats became colonized with the strain of O. formigenes of their nursing mothers, whether natural or foster mothers, indicates that this bacterium is acquired from the immediate environment rather than directly from their natural mother. This is further supported by the observation that offspring born to colonized mothers but fostered with non-colonized mothers never became colonized.

It is possible that the newborn rats could pick up the bacteria when suckling, not from the mother’s milk but from organisms on the mother’s fur or skin. However, stable colonization probably occurs from coprophagy as it has been shown that young laboratory rats begin to ingest maternal faeces at about 2 weeks of age (Novakova & Babicky, 1989), corresponding with the time O. formigenes is first detected in the neonates. More often, though, stable colonization of the bacterium in the intestinal tract appeared to be delayed until an oxalate source became available.

A comparison of natural colonization in experimental laboratory rats, as seen in the present study, with previous studies in human children (Sidhu et al., 1997b) reveals similarities in the temporal appearance and loss of O. formigenes in faecal specimens. Colonization in rats first occurs at about 1–2 weeks of age, when the pups first begin exploration of their cages. Colonization in human infants is first detected at about 9–12 months of age when children begin crawling. For both species, there is a rapid increase in frequency of colonization until nearly 100 % of offspring are colonized (3–4 weeks of age in rats and 4–8 years of age in man). Interestingly, following puberty, there is some loss of colonization, which is seen in both species, but is more prevalent in man. This is probably due to external factors, such as antibiotic treatments and changes in diet. Nevertheless, considering the results obtained from the rat experiments, we would predict that children could easily be infected with strains of O. formigenes quite different from their parents and more representative of their environment. Thus, it would be interesting to determine the diversity of strains present within families or even communities.

A further question remains unanswered: how do neonate rats and children become colonized if O. formigenes must be picked up from the environment? The bacterium appears to be passed by faecal–oral transmission, but as a strict and obligate anaerobe, the bacterium is highly susceptible even to low levels of oxygen. The question as to whether O. formigenes can persist in the environment longer than currently suspected or has a dormant state permitting it to survive in the air needs to be addressed. Much is yet to be learned about this unique bacterium.

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REFERENCES


