Exposure to norepinephrine enhances Brachyspira pilosicoli growth, attraction to mucin and attachment to Caco-2 cells

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Brachyspira pilosicoli is an anaerobic intestinal spirochaete that colonizes the large intestine of many species of birds and mammals, including human beings. The spirochaete is found in the colon of pigs and chickens and also colonizes the rectum of humans, where it may induce ‘intestinal spirochaetosis’, a condition characterized by diarrhoea and reduced growth (Hampson & Duhamel, 2007; Cogan et al., 2007; Doherty et al., 2009). Colonization is also common among people living in crowded and unhygienic conditions in developing countries (Trott et al., 1997; Margawani et al., 2004; Nelson et al., 2009), as well as in homosexual males and HIV-positive individuals in developed countries (Law et al., 1994; Trivett-Moore et al., 1998). Colonization has also been found to be significantly associated with chronic diarrhoea, failure to thrive and being underweight (Brooke et al., 2006).

Catecholamines, including norepinephrine (NE), are known to have important effects on the growth and behaviour of a range of pathogenic bacterial species (Bansal et al., 2007; Cogan et al., 2007; Doherty et al., 2009). NE is present in the intestinal lumen, where it arrives due to diffusion down a concentration gradient from the blood (Lyte & Bailey, 1997). Consequently, B. pilosicoli is likely to be exposed to NE in the colon. The aim of the current study was to investigate whether NE exposure can influence B. pilosicoli in its in vitro growth rate, attraction to mucin and attachment to Caco-2 cell monolayers. These in vitro activities were chosen for study as they are likely to reflect the capacity of the spirochaete to colonize in vivo. Strain 95/1000 was used because its genome has been sequenced (Wanchanthuek et al., 2010) and it has been used in a number of published studies, including studies of motility and chemotaxis (Naresh & Hampson, 2010), and attachment to Caco-2 cells (Naresh et al., 2009).
METHODS

Preparation of NE stock solution. Stock solutions (0.01 M) of norepinephrine bitartrate salt (NE) (Sigma-Aldrich) were prepared in PBS and sterilized by filtration. The stock solutions were prepared just before the start of each experiment and were held in a dark glass vessel to avoid exposure to light.

Spirochaete strain and cultivation. B. pilosicoli strain 95/1000, which was originally isolated from a pig with porcine intestinal spirochaetosis in a Western Australian herd, was obtained as frozen stock from the culture collection held at the Australian Reference Centre for Intestine Spirochaetes, School of Veterinary and Biomedical Sciences, Murdoch University. The cells were thawed and grown at 39°C in Kunke’s pre-reduced anaerobic broth containing 2% (v/v) fetal bovine serum and 1% (v/v) ethanolic hydrochloride solution (Kunkle et al., 1986). The growth of the spirochaete and absence of contamination were monitored by examining aliquots under a phase-contrast microscope. The cultures were harvested in early exponential-phase, when the spirochaetes were actively motile, and were enumerated by direct counting in a counting chamber under a phase-contrast microscope. For counting, duplicate preparations were used, and spirochaetes were counted in 48 squares by one operator.

Effect of NE on the growth of B. pilosicoli 95/1000. A set of 20 ml glass tubes each containing 9 ml Kunke’s anaerobic broth medium were prepared, wrapped with aluminium foil to keep them dark, and each was seeded with 0.5 ml broth culture of B. pilosicoli 95/1000 at a concentration of 10⁷ cells ml⁻¹. A fresh stock solution of NE was prepared and added to the tubes to achieve concentrations of 0.05, 0.1, 0.5 and 1 mM NE. An equivalent volume of sterile PBS was added to the control tubes. Six replicates of each NE concentration and the NE-free control broths were used in each test. The tubes were incubated on a rocking platform at 39°C for 4 days, and then aliquots were removed and the spirochaetes were counted. Six biological replicates were used.

Chemotaxis assays. Chemotaxis assays were undertaken using glass haematocrit capillary tubes filled with either chemotaxis buffer [0.01 M potassium phosphate buffer (pH 7.0), 0.2 mM l-cysteine hydrochloride] or 4% porcine gastric mucin type II (Sigma Aldrich) prepared in chemotaxis buffer, as described previously (Naresh & Hampson, 2010).

NE added to the broth culture. NE was added to active cultures of B. pilosicoli 95/1000 (10⁷ cells ml⁻¹) to obtain final concentrations of 0.05, 0.1, 0.5 and 1 mM. For each concentration, six capillary tubes were allocated; these were filled with 4% mucin, the top ends were sealed with plasticine and they were hung vertically with their lower ends submerged in the appropriate B. pilosicoli 95/1000 culture in 48-well round bottomed tissue culture plates. These were incubated at 39°C for 90 min in a CO₂ incubator. The outside of each tube was then wiped dry with a sterile tissue and it was placed upright into a 200 μl Eppendorf tube. The top of the capillary tube was gently broken and the contents were collected. The solution was serially diluted in PBS and the spirochaetes were counted. Six biological replicates were used.

NE added to chemotaxis buffer. Fresh NE was added to chemotaxis buffer to final concentrations of 0.05, 0.1, 0.5 and 1 mM. Six capillary tubes were filled with buffer for each of the NE concentrations. The chemotaxis assay was conducted as described above, again with six biological replicates.

Effect of NE on B. pilosicoli attachment to Caco-2 cells. The attachment assays were conducted as described previously (Naresh et al., 2009), with three biological replicates. Briefly, 2-week-old confluent Caco-2 cell monolayers were grown on 10 mm round glass coverslips in 48-well plates at 37°C. A fresh mid-exponential-phase broth culture of B. pilosicoli strain 95/1000 (10⁸ cells ml⁻¹) was harvested and NE was added to an aliquot of the culture to give a final dilution of 0.05 mM. Aliquots (1 ml) of this culture or the culture without NE were pipetted into the wells and incubated for 2, 4 and 6 h. Three wells were allocated for each time interval. The wells then were washed three times with PBS to remove unattached spirochaetes and the coverslips were removed and processed for scanning electron microscopy (SEM), as described previously (Naresh et al., 2009).

Data analysis. B. pilosicoli growth in broth containing different concentrations of NE was compared by one way analysis of variance (ANOVA) using SPSS for Windows. ANOVA also was used to compare the numbers of spirochaete cells recovered from the capillary tubes in the chemotaxis assays. The degree of B. pilosicoli attachment to Caco-2 cells as observed under the scanning electron microscope was recorded subjectively.

RESULTS AND DISCUSSION

Effect of NE on growth of B. pilosicoli

The addition of NE to the B. pilosicoli culture resulted in a significant (P<0.002) increase in growth only with 0.05 mM NE (Fig. 1). With this concentration of NE, the number of spirochaetes was just over 8×10⁷ ml⁻¹ compared with approximately 5×10⁷ ml⁻¹ for the non-exposed culture. The number of bacteria in the latter cultures had increased approximately 10-fold during the 4 day incubation. The number of bacteria also was higher with the 0.1 mM NE concentration than with the non-exposed control culture, but the difference was not statistically significant. The number of bacteria in the two remaining NE concentrations did not differ significantly from the control.

The increase in cell numbers that occurred following exposure to 0.05 mM NE was not large, and other bacterial

![Fig. 1. The effect of exposure to different concentrations of NE on the number of B. pilosicoli 95/1000 cells grown in Kunke’s anaerobic broth after 4 days incubation. Data shown are mean ± SEM.](image-url)
species have shown far greater increases in growth after NE exposure. For example, *Campylobacter jejuni* showed a 50-fold increase in growth following NE exposure (Cogan *et al.*, 2007). Nevertheless, *B. pilosicoli* is a slow-growing anaerobe and any increase in growth rate could enhance its capacity to colonize the large intestine.

**Effect of NE on the attraction of *B. pilosicoli* to mucin**

The results of the assays in which *B. pilosicoli* was exposed to different concentrations of NE at the time they were added to the mucin attraction assay are summarized in Fig. 2. The culture exposed to the lowest NE concentration (0.05 mM) showed significantly (*P*<0.02) greater attraction to 4% mucin than the control that was not exposed to NE. No other differences were statistically significant.

Previously, it has been shown that the attraction of *B. pilosicoli* 95/1000 to 4% mucin is likely to involve elements of both chemotaxis and viscotaxis (Naresh & Hampson, 2010). The rapid change in the spirochaete’s responsiveness to the mucin following exposure to 0.05 mM NE may involve either increased sensitivity to chemotactic signals and/or an increased motility and motion efficiency that allowed it to enter the mucin solution more rapidly.

**Attraction of *B. pilosicoli* to NE in chemotaxis buffer**

The effect of addition of NE to the chemotaxis buffer on the number of *B. pilosicoli* cells entering the buffer is shown in Fig. 3. The 0.1, 0.5 and 1 mM NE concentrations attracted significantly more spirochaetes than the control chemotaxis buffer (*P*<0.004). Spirochaete numbers did not differ significantly at these three NE concentrations. The number of bacteria at the lowest NE level (0.05 mM) was significantly less (*P*<0.03) than in the other three NE concentrations, and did not differ significantly from the control without NE. The most likely explanation for these results was that NE acted as a chemoattractant for the spirochaete, with this activity being saturated at 0.1 mM NE. Similar chemoattractant responses to NE occur with other bacterial species (Bansal *et al.*, 2007; Bearson & Bearson, 2008). Interestingly, there was approximately one log fewer spirochaetes in the capillary tubes containing NE than in those tubes containing mucin where the spirochaetes had not been exposed to NE. Hence, mucin appeared to be a stronger attractant than NE.

**Attachment assays with a culture of *B. pilosicoli* exposed to NE**

The *B. pilosicoli* cultures that either had or had not been exposed to NE immediately prior to adding to the attachment assay both showed a time-dependent increase in attachment to the Caco-2 cells, but at all time points more of the NE-treated spirochaetes were observed to be attached (Fig. 4). The NE-exposed *B. pilosicoli* cells tended to be more clumped and tangled than the non-exposed cells (Fig. 4d).

Others have reported that NE can enhance attachment of bacterial species, for example exposure to 0.05 mM NE enhanced the attachment of *Escherichia coli* O157:H7 to HeLa cells (Bansal *et al.*, 2007). In future work it would be informative to investigate whether cultures of *B. pilosicoli* exposed to NE cause increased cytopathic effects in the Caco-2 cells compared with untreated cultures.

**Conclusions**

Exposure of *B. pilosicoli* to NE changed the behaviour of the spirochaete in a number of ways that appear likely to increase its capacity to colonize the large intestine. It would be instructive to test whether cultures that are exposed to NE *in vitro* do colonize better than non-exposed cultures. Under natural conditions, NE is present in the intestinal tract, and elevated levels are likely to occur in periods of stress. It has recently been shown in experimentally

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![Fig. 2](http://mic.sgmjournals.org)  
**Fig. 2.** The effect of exposure to different concentrations of NE on the number of *B. pilosicoli* 95/1000 cells entering capillary tubes containing 4% mucin. Data shown are mean ± SEM.

![Fig. 3](http://mic.sgmjournals.org)  
**Fig. 3.** The effect of having different NE concentrations in chemotaxis buffer on the number of *B. pilosicoli* 95/1000 cells entering the buffer. Data shown are mean ± SEM.
infected pigs that elevated plasma NE levels, such as those found in stressed animals, were associated with increased faecal excretion of *Salmonella Typhimurium* (Pullinger *et al.*, 2010b). Hence, it seems likely that the *in vitro* observations may translate to altered activity of the spirochaete *in vivo*.

The mechanisms involved in the change in *B. pilosicoli* behaviour require further study, but, by analogy with other Gram-negative enteric pathogenic bacteria, they are likely to involve mediation of iron acquisition to enhance growth, and/or alteration in gene expression by activation of sensor kinases that may increase motility or other activities required for colonization (Hughes *et al.*, 2009; Pullinger *et al.*, 2010a; Reading *et al.*, 2010). Examination of transcriptomic profiles of the spirochaete after addition of NE should help identify potential pathways involved in the observed changes, and this work will be assisted by the recent availability of a full genomic sequence for *B. pilosicoli* 95/1000 (Wanchanthuek *et al.*, 2010).

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**REFERENCES**


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