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 a variety of species of birds and mammals, including human beings. Colonization may result in a mild colitis and diarrhoea in a condition known as ‘intestinal spirochaetosis’. The catecholamine norepinephrine (NE), which is known to influence the behaviour of many bacterial species, may be present in the colon. The purpose of the current study was to determine whether exposure of *B. pilosicoli* to NE would influence its *in vitro* behaviour in assays that may reflect *in vivo* colonization potential. *B. pilosicoli* strain 95/1000 was used in all the assays. Addition of NE at a concentration of 0.05 mM to *B. pilosicoli* growing in anaerobic broth significantly increased spirochaete numbers after 4 days incubation. The effect of higher concentrations of NE was not significant. Exposure to 0.05 mM NE, but not to higher concentrations, also resulted in significantly more spirochaete cells entering capillary tubes containing 4% porcine gastric mucin than occurred with untreated cultures. When NE was added to chemotaxis buffer in capillary tubes, significantly more spirochaetes were attracted to the buffer containing NE at 0.1, 0.5 and 1.0 mM than to buffer containing 0.05 mM NE, or when no NE was added. Exposure of *B. pilosicoli* cultures to 0.05 mM NE prior to incubation with Caco-2 monolayers resulted in more attachment to the monolayer than occurred with non-exposed cultures. These results show that at higher concentrations, NE acts as a chemoattractant for *B. pilosicoli*, and at 0.05 mM it increases the spirochaete’s growth rate, attraction to mucin and rate of attachment to cultured enterocytes. These activities are likely to enhance the ability of *B. pilosicoli* to colonize, and may be induced by conditions that increase NE concentrations in the intestinal tract, such as the stresses associated with crowding.

INTRODUCTION

*Brachyspira pilosicoli* is an anaerobic intestinal spirochaete that colonizes the large intestines of many species of birds and mammals, including human beings. A frequent feature of the colonization is the end-on attachment of spirochaete cells to the luminal surface of colonic and rectal epithelial cells, in a condition called ‘intestinal spirochaetosis’. Infections with *B. pilosicoli* are common amongst intensively farmed pigs and chickens, where they cause diarrhoea and reduced production (Hampson & Duhamel, 2006; Hampson & Swayne, 2008). Colonization also is common in 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). In some studies, colonization in humans has 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 driven by 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).

Abbreviation: NE, norepinephrine.
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 Kunkle’s pre-reduced anaerobic broth containing 2% (v/v) fetal bovine serum and 1% (v/v) ethanolic hydrochloride and grown at 39 °C, 20 ml glass tubes each containing 9 ml Kunkle’s anaerobic broth after 4 days incubation. Data shown are ± standard error of the mean (SEM) and the coverslips were removed and processed for scanning electron microscopy (SEM), as described previously (Naresh et al., 2009).

Effect of NE on the growth of B. pilosicoli 95/1000. A set of 20 ml glass tubes each containing 9 ml Kunkle’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 10⁵ 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).

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 (10⁶ 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...
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 viscostaxis (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...
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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**Fig. 4.** Attachment of *B. pilosicoli* 95/1000 to Caco-2 cell monolayers viewed with a scanning electron microscope. Attachment following incubation for 2 h (a, b) or 6 h (c, d) is shown. Spirochaetes in (a) and (c) were not exposed to NE; spirochaetes in (b) and (d) were exposed to 0.05 mM NE immediately before the assay. Bars, 5 μm.


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