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

Ram Naresh and David J. Hampson*

Animal Research Institute, School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Murdoch, Western Australia 6150, Australia

Author for correspondence: David J. Hampson. Tel.: + 61 89360 2287. Fax: + 61 89310 4144. e-mail: d.hampson@murdoch.edu.au

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Summary

Brachyspira pilosicoli **is an anaerobic intestinal spirochaete that colonises the large intestine of a variety of species of birds and mammals, including human beings. Colonisation 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* colonisation 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 four 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 concentrations of 0.1, 0.5 and 1.0 mM than to 0.05 mM, 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 its rate of attachment to cultured enterocytes. These activities are likely to enhance the ability of *B. pilosicoli* to colonise, 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 colonises 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" (IS). 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). Colonisation 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 colonisation 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 (eg 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). Cconsequently, *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 colonise *in vivo.* Strain 95/1000 was used because its genome has been sequenced (Wanchanthuck *et al*., 2010) and it has been used in a number of published

71 studies, including studies of motility and chemotaxis (Naresh & Hampson 2010), and
72 attachment to Caco-2 cells (Naresh *et al*., 2009).
73
74 **METHODS**
75 **Preparation of norepinephrine stock solution.** Stock solutions (0.01 M) of norepinephrine
76 bitartrate salt (NE) (Sigma-Aldrich, St. Louis, USA) were prepared in phosphate buffered
77 saline (PBS) and were sterilised by filtration. The stock solutions were prepared just before
78 the start of each experiment, and were held in a dark glass vessel to avoid exposure to light.
79
80 **Spirochaete strain and cultivation.** *Brachyspira pilosicoli* strain 95/1000, which was
81 originally isolated from a pig with porcine intestinal spirochaetosis in a Western Australian
82 herd, was obtained as frozen stock from the culture collection held at the Australian
83 Reference Centre for Intestine Spirochaetes, School of Veterinary and Biomedical Sciences,
84 Murdoch University. The cells were thawed, and grown at 39^{o}C in Kunkle's pre-reduced
85 anaerobic broth containing 2% (v/v) foetal bovine serum and 1% (v/v) ethanolic cholesterol
86 solution (Kunkle *et al*., 1986). The growth of the spirochaete and absence of contamination
87 were monitored by examining aliquots under a phase contrast microscope. The cultures were
88 harvested in early log-phase, when the spirochaetes were actively motile, and were
89 enumerated by direct counting in a counting chamber under a phase contrast microscope. For
90 counting, duplicate preparations were used, and spirochaetes were counted in 48 squares by
91 one operator.
92
93 **Effect of NE on the growth of *B. pilosicoli* 95/1000.** A set of 20 mL glass tubes each
94 containing nine mL of Kunkle's anaerobic broth medium were prepared, wrapped with

aluminium foil to keep them dark, and each was seeded with 0.5 mL of a broth culture of *B. pilosicoli* 95/1000 at a concentration of 10^7 cells per mL. 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 four 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 either with 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 previously described (Naresh & Hampson, 2010).

NE added to the broth culture. NE was added to active cultures of *B. pilosicoli* 95/1000 (10^8/mL) 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 minutes in a CO_2 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 previously described (Naresh *et al*., 2009), with three biological replicates. Briefly, two-week-old confluent Caco-2 cell monolayers were grown on 10 mm round glass coverslips in 48 well plates at 37^{o}C. A fresh mid-log phase broth culture of *B. pilosicoli* strain 95/1000 (10^{8}cells/mL) was harvested and NE was added to an aliquot of the culture to give a final dilution of 0.05 mM. One mL volumes 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 previously described (Naresh *et al.,* 2009).

Analysis of data. *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 SEM was recorded subjectively.

RESULTS AND DISCUSSION

Effect of NE on growth of *B. pilosicoli*

143 The addition of NE to the *B. pilosicoli* culture resulted in a significant ($P > 0.002$) increase in
144 growth only with 0.05 mM NE (Fig. 1). With this concentration the number of spirochaetes
145 was just over 8 x 10^7 per mL compared to approximately 5 x 10^7 per mL for the non-exposed
146 culture. The number of bacteria in the latter cultures had increased approximately ten-fold
147 during the four-day incubation. The number of bacteria also was higher with the 0.1 mM NE
148 concentration than with the non-exposed control culture, but the difference was not
149 statistically significant. The number of bacteria in the two remaining NE concentrations did
150 not differ significantly from the control.

151 **[Fig. 1 about here]**

152 The increase in cell numbers that occurred following exposure to 0.05 mM NE was not large,
153 and other bacterial species have shown far greater increases in growth after NE exposure. For
154 example, *Campylobacter jejuni* showed a 50-fold increase in growth following NE exposure
155 (Cogan *et al*., 2007). Nevertheless, *B. pilosicoli* is a slow-growing anaerobe and any increase
156 in growth rate could enhance its capacity to colonise the large intestine.

157

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

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

164 **[Fig. 2 about here]**

165 Previously it has been shown that the attraction of *B. pilosicoli* 95/1000 to 4% mucin is likely
166 to involve elements of both chemotaxis and viscotaxis (Naresh & Hampson). 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 the NE acted as a chemoattractant for the spirochaete, with this activity being saturated at 0.01 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 the mucin appeared to be a stronger attractant than the NE.

[Fig. 3 about here]

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 4., panel D).

[Fig 4 about here]

Others have reported that NE can enhance attachment of bacteria species; for example, exposure to 0.05 mM NE enhanced the attachment of *E. 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 to 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 colonise the large intestine. It would be instructive to test whether cultures that are exposed to NE *in vitro* do colonise 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 colonisation (Hughes *et al.,* 2009; Pullinger *et al.,* 2010a; Reading *et al.,* 2010). Examination of transcriptomics 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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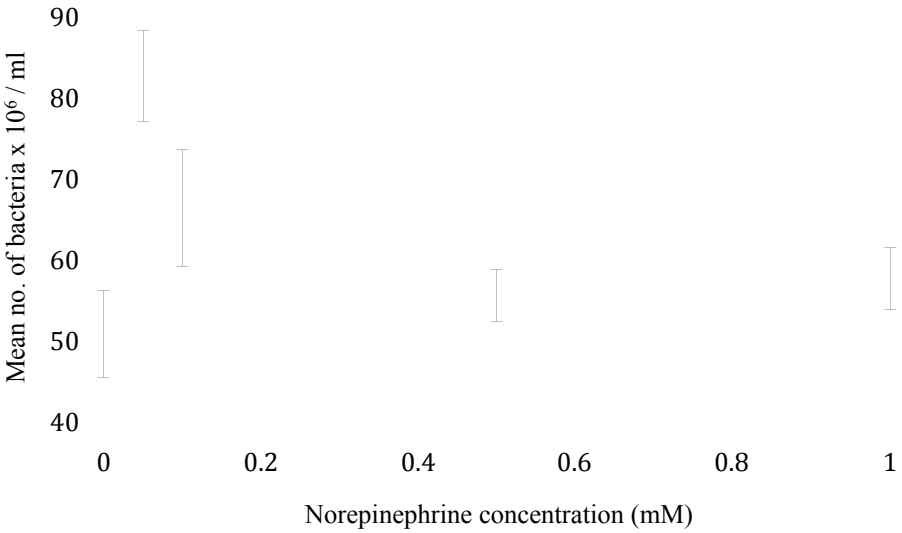
Figure legends

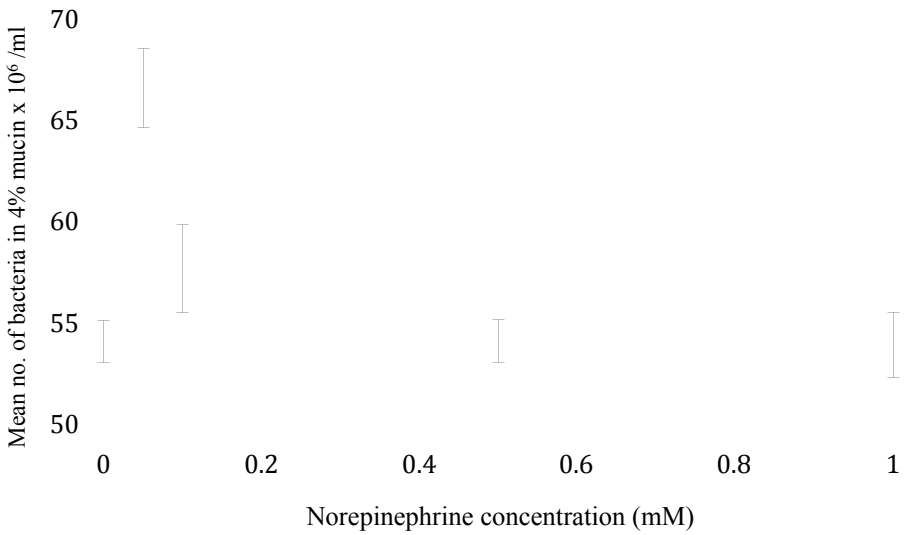
Fig 1. The effect of exposure to different concentrations of norepinephrine on the mean number (± standard error) of *B. pilosicoli* 95/1000 cells grown in Kunkle's anaerobic broth after four days incubation.

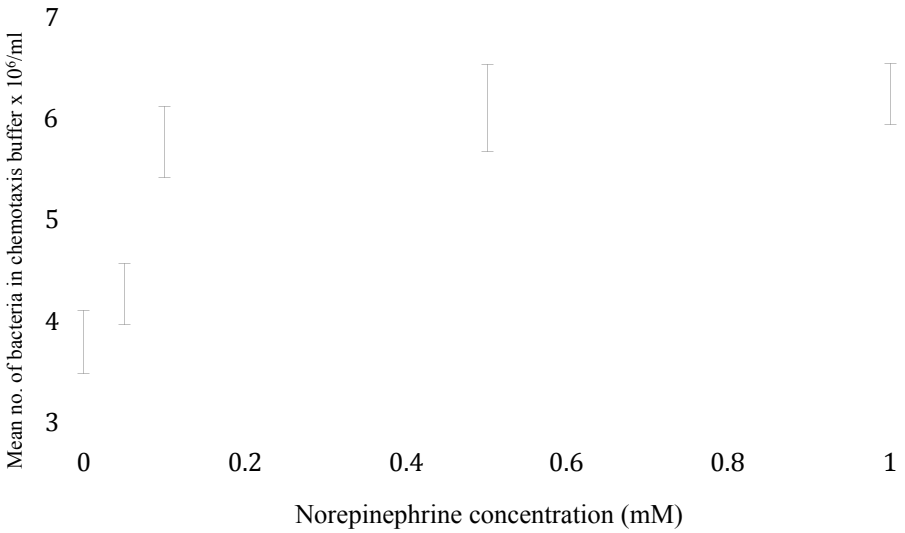
Fig 2. The effect of exposure to different concentrations of norepinephrine on the number (mean ± standard error) of *B. pilosicoli* 95/1000 cells entering capillary tubes containing 4% mucin.

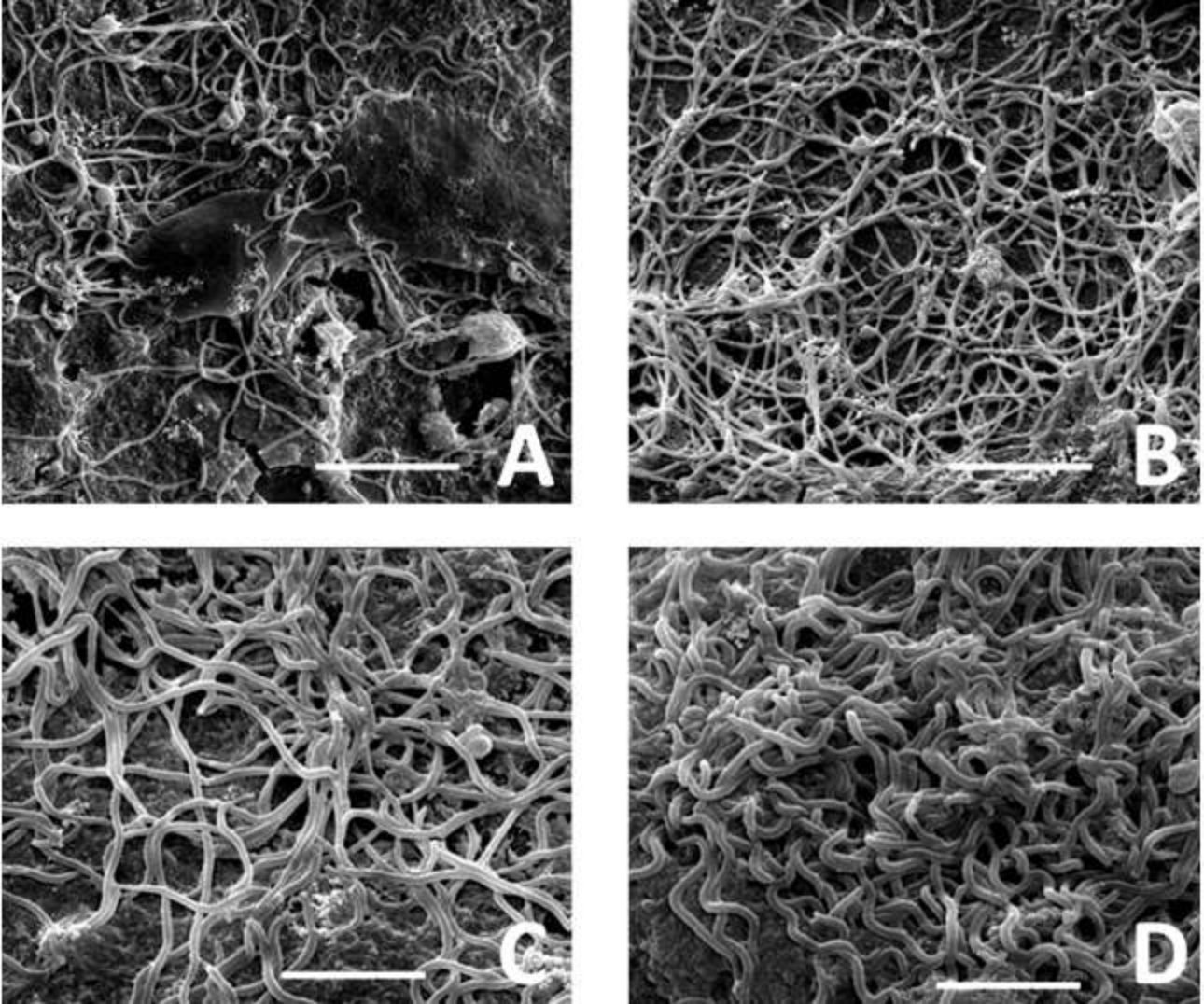
Fig 3. The effect of different norepinephrine concentrations in chemotaxis buffer on the number (mean ± standard error) of *B. pilosicoli* 95/1000 cells entering the buffer.

Fig 4. Attachment of *B. pilosicoli* 95/1000 to Caco-2 cell monolayers viewed with a scanning electron microscope. Panels A and B show attachment following incubation for 2 hours. Panels C and D show attachment following incubation for 6 hours. Panels A and C show spirochaetes that were not exposed to norepinephrine; panels B and D show spirochaetes that were exposed to 0.05 mM norepinephrine immediately before the assay.


90
80
70
60
50
40
Mean no. of bacteria x 10^6 / ml
0
0.2
0.4
0.6
0.8
1
Norepinephrine concentration (mM)


70
65
60
55
50
Mean no. of bacteria in 4% mucin x 10^6 /ml
0
0.2
0.4
0.6
0.8
1
Norepinephrine concentration (mM)


Mean no. of bacteria in chemotaxis buffer x 10⁶/ml
7
6
5
4
3
0
0.2
0.4
0.6
0.8
1
Norepinephrine concentration (mM)


A
B
C
D