Attraction of *Brachyspira pilosicoli* to mucin

Ram Naresh and David J. Hampson

Correspondence

David J. Hampson
d.hampson@murdoch.edu.au

Animal Research Institute, School of Veterinary and Biomedical Science, Murdoch University,
Murdoch, Western Australia 6150, Australia

The anaerobic intestinal spirochaete *Brachyspira pilosicoli* colonizes the large intestine of various species, including humans. In the colon this spirochaete can penetrate the overlying mucus layer, attach by one cell end to the underlying enterocytes, and initiate localized colitis and diarrhoea. The aim of this study was to investigate whether, as part of the colonization process, *B. pilosicoli* is attracted to mucin. Fifteen *B. pilosicoli* strains isolated from humans, pigs, chickens and dogs, and a control strain of *Brachyspira hyodysenteriae*, were analysed for their ability to enter solutions of hog gastric mucin in an *in vitro* capillary tube assay. No significant attraction was detected with 1 % mucin, but some strains started to enter a 2 % solution, and attraction then increased with increasing concentrations to peak at around 6–8 % mucin. A similar increase was seen with *B. hyodysenteriae*, although this activity peaked at 6 % mucin and then declined, suggesting that the two species have different affinities for mucin. These mucin concentrations were much higher than those used in previous experimental studies with *Brachyspira* species. The viscosities of the 6–8 % mucin solutions were around 7–12 mPa s, which were similar to the measured viscosities of the mucus layer overlying the epithelium in the caecum and colon of experimental pigs. The strains varied in their motility, as assessed by their ability to enter tubes containing chemotaxis buffer, but there was no significant relationship between this motility and the extent of their ability to enter the mucin solutions. Different strains also had different propensities to enter the mucin solutions, but there were no consistent differences according to the host species of origin. *B. pilosicoli* strain 95/1000 was attracted towards a solution of D-serine, suggesting that chemotaxis was involved in the attraction to mucin; however, 95/1000 was also attracted to viscous solutions of polyvinylpyrrolidone (PVP), in a manner mirroring the response to mucin, and hence suggesting the involvement of viscotaxis in the attraction to mucin. *B. hyodysenteriae* B204 showed a similar viscotaxis to PVP. Further studies are required to determine whether the *in vitro* interaction of a given strain with mucin is a useful indicator of its *in vivo* colonization ability, and hence could be used as a potential marker for virulence.

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INTRODUCTION

The anaerobic intestinal spirochaete *Brachyspira pilosicoli* colonizes the large intestine of various species of animals and birds, as well as human beings (Hampson *et al.*, 2006). A feature of the colonization is the intimate 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, in which the spirochaete is considered to be an important enteric pathogen (Stephens & Hampson, 2001; Hampson & Duhamel, 2006), but it is also common in people living in crowded and unhygienic conditions in developing countries (Trott *et al.*, 1997a; Margawani *et al.*, 2004; Munshi *et al.*, 2004), and in homosexual males and HIV patients in developed countries (Law *et al.*, 1994;

Trivett-Moore *et al.*, 1998). Based on the apparent panmictic population structure of the species, and the distribution of *B. pilosicoli* strains isolated from different host species on dendrograms produced from multilocus enzyme electrophoresis data, it has been suggested that there is unlikely to be any major barrier to zoonotic transmission of *B. pilosicoli* strains (Trott *et al.*, 1998; Hampson *et al.*, 2006).

In order to be able to colonize and attach to colonic enterocytes, *B. pilosicoli* cells need to penetrate and move through the overlying mucus layer. In mice, and presumably other species, the mucus gel overlying colonic enterocytes forms two layers, with the innermost layer being more densely packed than the outer layer, and devoid of the commensal bacteria that are normally found in the outer layer (Johansson *et al.*, 2008). Spirochaete cells have a corkscrew-like motility, allowing them to advance through highly viscous substances such as mucus (Li *et al.*, 2000). It

Abbreviations: PVP, polyvinylpyrrolidone; R_{att} , attraction ratio.

is not known whether *B. pilosicoli* can disrupt the mucus layer, for example by enzymic degradation (Lidell *et al.*, 2006), but in any case the spirochaete presumably requires a high degree of motility and swimming efficacy to be able to penetrate the mucus layers. In addition to motility, chemotaxis is known to be an important virulence factor in many pathogenic spirochaete species, allowing them to target the relevant colonization sites (Lux *et al.*, 2000). Consistent with this, virulent strains of the related intestinal spirochaete *Brachyspira hyodysenteriae* (the aetiological agent of swine dysentery) have been reported to be chemotactic to mucin, the major component of the mucus gel, as well as to components of mucin such as L-serine (Kennedy & Yancey, 1996). On the other hand, avirulent strains of *B. hyodysenteriae* were found to have reduced chemotaxis to mucin compared to virulent strains such as B204 (Milner & Sellwood, 1994). In contrast, and unexpectedly, in the latter study analysis of five porcine *B. pilosicoli* strains showed that these were not attracted to mucin under the same test conditions as *B. hyodysenteriae* (Milner & Sellwood, 1994), despite *B. pilosicoli* clearly needing to be able to penetrate the mucous layers to access attachment sites. More recently, Witters & Duhamel (1999) investigated three *B. pilosicoli* strains, and found that one from a human being and one from a dog were slightly attracted to 1% porcine gastric mucin, whilst a third porcine strain that had also been examined by Milner & Sellwood (1994) was not attracted to mucin, but it was mildly attracted to 10 mM DL-serine.

The aim of the current study was to investigate the extent to which *B. pilosicoli* is attracted to mucin, and to determine whether this property differs amongst strains of the species. Comparisons were also made with the behaviour of *B. hyodysenteriae* strain B204, and the underlying basis of the strong attraction to mucin that was found was investigated.

METHODS

Animal ethics. Permission to use the pigs in this study was obtained from the Murdoch University Animal Ethics Committee.

Spirochaete strains and culture conditions. *Brachyspira* strains were obtained as frozen stock from the culture collection held at the Australian Reference Centre for Intestine Spirochaetes at Murdoch University. They included 15 strains of *B. pilosicoli* (five isolated from humans, four from pigs, and three each from chickens and dogs), and *Brachyspira hyodysenteriae* strain B204. Of these strains, *B. pilosicoli* porcine strain P43/6/78^T had previously been examined for chemotaxis to porcine gastric mucin by both Milner & Sellwood (1994) and Witters & Duhamel (1999), and canine strain 16242-94 had previously been examined by Witters & Duhamel (1999). *B. hyodysenteriae* strain B204 had been examined for chemotaxis to mucin by Milner & Sellwood (1994) and by Kennedy & Yancey (1996).

The spirochaete cells were thawed and grown in Kunkle's pre-reduced anaerobic broth, containing 2% (v/v) fetal bovine serum and 1% (v/v) ethanolic cholesterol solution (Kunkle *et al.*, 1986). Broth cultures were incubated at 39 °C on a rocking platform for 3–5 days,

and spirochaete growth and motility was monitored daily by examining aliquots under a phase-contrast microscope. Only actively motile mid-exponential-phase spirochaete cells were used in the assays. Cells were counted using a Neubauer counting chamber under the 40× objective of a phase-contrast microscope, and final dilutions to 10⁸ cells ml⁻¹ were made in freshly prepared pre-warmed anaerobic broth.

Capillary tube assays. The system used was based on the method of Milner & Sellwood (1994), with modifications. Porcine gastric mucin type II (Sigma Aldrich) was dissolved in chemotaxis buffer [0.01 M potassium phosphate buffer (pH 7.0), 0.2 mM L-cysteine hydrochloride] (Greenberg & Canale-Parola, 1977) to final concentrations of 10, 8, 6, 4, 3, 2, 1, 0.5 and 0.2% (w/v). D-Serine (Sigma) was similarly dissolved in chemotaxis buffer to make 100, 10, 1 and 0.1 mM solutions, and polyvinylpyrrolidone (PVP) (Sigma) was dissolved in chemotaxis buffer to make solutions of 4.0, 3.0, 2.5, 2.0, 1.5, 1.25, 0.75 and 0.25% (w/v).

Only *B. pilosicoli* strain 95/1000 and *B. hyodysenteriae* strain B204 were tested in 8 and 10% mucin, whilst all strains were tested in the other seven dilutions of mucin. Strain 95/1000 was also tested in PBS (per litre: 10 g NaCl, 0.25 g KCl, 0.1438 g Na₂HPO₄, 0.25 g KH₂PO₄; pH 7.2), in chemotaxis buffer and in the D-serine solutions, and it and B204 were tested in the various dilutions of PVP, with 6% mucin used as a positive control. A suspension of strain 95/1000 that had been inactivated overnight in 4% formalin was also tested with chemotaxis buffer and 4% mucin. For each spirochaete strain to be tested, six 75 mm haematocrit capillary tubes (75 µl volume; Hirschmann Laborgerate) were filled with the appropriate concentration of mucin or the various other solutions, and six paired tubes were filled with chemotaxis buffer. The top ends of the tubes were sealed with Plasticine, and then the tubes were hung vertically with their lower ends submerged in fresh bacterial broth culture (10⁸ cells ml⁻¹) in 48-well round-bottomed tissue culture plates. These were incubated at 39 °C in a CO₂ incubator. An appropriate incubation time was determined by testing *B. pilosicoli* strain 95/1000 in 4% mucin in chemotaxis buffer with incubation times of 15, 30, 60, 90 and 120 min. Based on the results, a 90 min incubation time was selected for testing all the strains. After incubation, each tube was carefully removed, the outside wiped dry with a sterile tissue, and then placed upright into a 200 µl Eppendorf tube. The top of the capillary tube was gently broken and the contents were collected in the tube. The collected solution was serially diluted in PBS and the spirochaetes were counted under a phase-contrast microscope, as described above.

Viscosity measurements. Six healthy pigs of ~30 kg body weight, that had been fed on a commercial antimicrobial-free Australian grower diet based on wheat and lupins, were killed using a captive bolt pistol and exsanguination, and their large intestines were removed and opened. The intestinal contents were expelled manually, the mucosa rinsed with PBS, patted dry with paper tissue, and approximately 1 g quantities of the mucus layer immediately overlying the epithelium in both the mid-caecum and proximal colon were carefully collected by gently scraping with sterile scalpel blades. The viscosities of these samples were measured in a Brookfield LVDV-II+ cone plate (CP40) rotational viscometer (Brookfield Engineering Laboratories), as previously described (Hopwood *et al.*, 2002). Briefly, the material was diluted 1:1 (v/v) with distilled water, mixed and centrifuged at 12 000 g for 8 min. The viscosities of 0.5 ml fractions held at 39 °C in a water bath were measured, applying a shear rate of 60 s⁻¹. The viscosities of the dilutions of porcine gastric mucin and PVP used in the capillary tube assays were similarly tested.

Statistical analysis. For each replicate of each mucin concentration or other solution tested, attraction was expressed as the ratio (*R*_{att}) of

the number of spirochaetes in the tube containing the test solution compared to the number in the paired tube containing chemotaxis buffer. The mean and standard deviation of the six replicates for each concentration were calculated. One-way analysis of variance (ANOVA) was used to compare the R_{att} for each of the 15 strains across the different mucin concentrations, to compare the R_{att} for each of the 15 strains at each of the seven mucin concentrations, and to compare the R_{att} at each of the mucin concentrations for the sets of isolates from humans, pigs, chickens and dogs. R_{att} values of >2 were considered significant (Moulton & Montie, 1979). R_{att} values of zero were obtained when no spirochaetes were found in the mucin solution, but spirochaetes were present in the buffer tubes. Comparison of the number of spirochaetes in the capillary tubes containing only chemotaxis buffer for the 15 strains were similarly analysed by ANOVA. Regression analyses were made across all the strains for the numbers of spirochaetes in the tubes containing chemotaxis buffer and the R_{att} values in 4 % and in 6 % mucin.

RESULTS

Optimizing test conditions

The responses of *B. pilosicoli* strain 95/1000 and *B. hyodysenteriae* strain B204 to various mucin concentrations from 0.2 to 10 % are presented in Fig. 1. In both species the R_{att} value increased with increasing mucin concentrations, to peak at around 6–8 % mucin for 95/1000, and 6 % for B204, after which it showed a steep decline for the *B. hyodysenteriae* strain. Based on these results, all the *B. pilosicoli* strains were tested in mucin concentrations from 0.2 % to 6 %. The results of incubating strain 95/1000 in 4 % mucin for different time periods are shown in Fig. 2. Based on these findings, an incubation time of 90 min was selected for all further incubations.

Chemotaxis buffer supported the activity of *B. pilosicoli* better than did PBS, as significantly ($P<0.001$) more cells of strain 95/1000 were found in the tubes containing chemotaxis buffer than in those containing PBS (2.97 and 1.56×10^6 cells ml^{-1} , respectively). Cells of *B. pilosicoli* 95/1000 that had been inactivated in formalin did not enter the tubes containing chemotaxis buffer or mucin.

Fig. 1. R_{att} values (means $\pm$ SD) for *B. pilosicoli* strain 95/1000 (●) and *B. hyodysenteriae* strain B204 (○) incubated for 90 min in different concentrations of porcine gastric mucin in chemotaxis buffer.

Fig. 2. R_{att} values (means $\pm$ SD) for *B. pilosicoli* strain 95/1000 incubated for 15, 30, 60, 90, 120 and 180 min in 4 % mucin.

When analysing the *B. pilosicoli* numbers in the tubes containing only chemotaxis buffer after 90 min incubation, significant differences in counts were found for the different spirochaete strains ($P<0.001$; Table 1). The corresponding mean number of cells for *B. hyodysenteriae* B204 in chemotaxis buffer was $(2.09 \pm 0.28) \times 10^6$. When the numbers of *B. pilosicoli* cells in chemotaxis buffer were compared by linear regression with their R_{att} values for the same strains in 4 % and 6 % mucin, no significant associations were found ($P=0.738$ and $P=0.401$, respectively).

R_{att} values of different spirochaete strains with mucin

The means and standard deviations of the R_{att} values for the 15 *B. pilosicoli* strains at seven mucin concentrations are presented in Table 1. For each strain the different R_{att} values across the mucin concentrations all showed significant differences ($P<0.0001$). The results for individual strains varied considerably. Strain Cof-10, isolated from a pig, was not attracted to any concentration of mucin, and indeed it was unable to enter solutions of 1 % mucin or more. Strain 16242.94, isolated from a dog, showed the greatest attraction. All comparisons of the R_{att} for the 15 strains at all the mucin concentrations showed statistically significant differences ($P<0.0001$). Comparisons of sets of strains based on the four different species of origin revealed no significant differences at any mucin concentration. For mucin concentrations up to and including 1 %, no strains had a R_{att} value >2 . At mucin concentrations of 2 %, three human, one porcine and two canine strains of *B. pilosicoli* had R_{att} values >2 . At 3 % mucin all strains except Cof-10 and L72 had R_{att} values >2 , and at 4 % and 6 % mucin, only Cof-10 did not show evidence of attraction to mucin. For nearly all strains the attraction increased with increasing mucin concentrations, up to 6 %. The R_{att} values for some *B. pilosicoli* strains with 6 % mucin were extremely high, and exceeded those for *B. hyodysenteriae* B204 (Fig. 1).

R_{att} values of 95/1000 with D-serine

B. pilosicoli strain 95/1000 showed a significant R_{att} (2.58 ± 0.55) with the 100 mM solution of D-serine only.

Table 1. R_{att} values at seven concentrations of porcine gastric mucin, and the number of spirochaete cells in chemotaxis buffer tubes, for 15 strains of *B. pilosicoli* after 90 min incubationMeans $\pm$ SD are shown. Values indicating significant attraction to mucin ($R_{att} > 2$) are in bold.

Species of origin	Strain	Mucin concentration (%)							$10^{-6} \times$ No of cells*
		0.2	0.5	1	2	3	4	6	
Human	WesB	1.06 $\pm$ 0.39	1.32 $\pm$ 0.12	0.63 $\pm$ 0.08	1.17 $\pm$ 0.28	4.94 $\pm$ 0.28	2.91 $\pm$ 0.94	22.6 $\pm$ 2.52	2.01 $\pm$ 0.65
	Karlton	0.78 $\pm$ 0.05	0.56 $\pm$ 0.15	0.38 $\pm$ 0.07	1.19 $\pm$ 0.05	3.59 $\pm$ 0.62	4.44 $\pm$ 0.85	22.64 $\pm$ 2.95	1.67 $\pm$ 0.30
	PE-90	1.08 $\pm$ 0.16	1.16 $\pm$ 0.17	1.31 $\pm$ 0.11	8.27 $\pm$ 0.75	16.01 $\pm$ 1.23	21.38 $\pm$ 1.77	40.34 $\pm$ 2.45	4.26 $\pm$ 0.66
	BR 81/80	1.1 $\pm$ 0.14	1.30 $\pm$ 0.1	1.5 $\pm$ 0.11	9.32 $\pm$ 1.57	20.62 $\pm$ 1.39	26.63 $\pm$ 1.03	28.96 $\pm$ 1.71	2.82 $\pm$ 0.55
	V2H5	1.12 $\pm$ 0.13	0.99 $\pm$ 0.10	1.06 $\pm$ 0.09	4.38 $\pm$ 0.33	10.97 $\pm$ 0.70	16.04 $\pm$ 2.26	32.75 $\pm$ 4.00	6.70 $\pm$ 0.23
Pig	P43/6/78 ^T	0.63 $\pm$ 0.17	0.52 $\pm$ 0.06	0.40 $\pm$ 0.07	1.8 $\pm$ 0.12	6.32 $\pm$ 1.08	12.79 $\pm$ 1.01	19.12 $\pm$ 1.12	7.39 $\pm$ 1.01
	95/1000	0.87 $\pm$ 0.16	1.01 $\pm$ 0.06	1.79 $\pm$ 0.28	5.22 $\pm$ 0.21	9.09 $\pm$ 0.81	14.95 $\pm$ 2.28	30.19 $\pm$ 1.32	2.97 $\pm$ 0.20
	L-72	0.73 $\pm$ 0.05	0.62 $\pm$ 0.04	0.53 $\pm$ 0.04	0.41 $\pm$ 0.05	1.91 $\pm$ 0.53	5.86 $\pm$ 1.10	29.84 $\pm$ 2.43	5.82 $\pm$ 0.15
	Cof-10	0.27 $\pm$ 0.08	0.13 $\pm$ 0.12	0	0	0	0	0	0.9 $\pm$ 0.09
Chicken	CSP-1	0.71 $\pm$ 0.58	0.58 $\pm$ 0.09	0.93 $\pm$ 0.05	1.43 $\pm$ 0.12	4.57 $\pm$ 0.37	9.52 $\pm$ 1.22	39.91 $\pm$ 1.41	7.62 $\pm$ 0.35
	98.0026	1.07 $\pm$ 0.17	0.78 $\pm$ 0.05	0.67 $\pm$ 0.04	0.75 $\pm$ 0.14	2.60 $\pm$ 0.61	2.33 $\pm$ 0.26	35.96 $\pm$ 1.42	3.22 $\pm$ 0.08
	97.006	1.07 $\pm$ 0.11	0.86 $\pm$ 0.05	0.59 $\pm$ 0.10	1.22 $\pm$ 0.05	5.23 $\pm$ 0.47	11.57 $\pm$ 0.46	27.91 $\pm$ 0.85	6.80 $\pm$ 0.35
Dog	Boof	0.73 $\pm$ 0.11	0.65 $\pm$ 0.07	0.98 $\pm$ 0.12	2.91 $\pm$ 0.12	4.49 $\pm$ 1.0	8.03 $\pm$ 1.51	39.99 $\pm$ 1.0	3.32 $\pm$ 0.85
	16242.94	0.52 $\pm$ 0.08	0.85 $\pm$ 0.33	0.93 $\pm$ 0.34	3.73 $\pm$ 0.37	13.52 $\pm$ 2.0	17.63 $\pm$ 1.17	51.24 $\pm$ 3.22	1.66 $\pm$ 0.15
	D 148	0.93 $\pm$ 0.03	0.44 $\pm$ 0.03	0.34 $\pm$ 0.11	0.45 $\pm$ 0.17	4.76 $\pm$ 2.2	16.31 $\pm$ 3.47	16.11 $\pm$ 0.44	2.79 $\pm$ 0.17

*Spirochaete cell numbers in tubes containing only chemotaxis buffer.

 R_{att} values of 95/1000 and B204 in relation to viscosity

The viscosities of the mucin and PVP dilutions are listed in Table 2. The R_{att} results in these solutions in relation to their viscosity for *B. pilosicoli* 95/1000 and *B. hyodysenteriae* B204 are shown in Fig. 3. For 95/1000, the R_{att} increased with increasing PVP concentrations to plateau from a viscosity of around 6 mPa s (Fig. 3a). These R_{att} values followed the same trend as seen with mucin at the same viscosities, but at a given viscosity were only about 20 % of the values obtained with mucin (Fig. 3b). For B204, the R_{att} continued to increase with increasing viscosities of the PVP solutions, although the R_{att} had declined in the mucin solutions at the (similar) high viscosities. As with 95/1000,

the R_{att} values obtained with B204 generally were much higher in the mucin solutions than in the PVP solutions; however, at the higher viscosities the R_{att} in mucin declined to values similar to those in the PVP solutions.

Fig. 3. R_{att} values obtained for *B. pilosicoli* 95/1000 (●) and *B. hyodysenteriae* B204 (○) in dilutions of PVP (a) and mucin (b), plotted against measured viscosities.**Table 2.** Viscosities of the dilutions of mucin and PVP used in the attraction assays

Mucin (%)	Viscosity (mPa s)	PVP (%)	Viscosity (mPa s)
0.2	1.2	0.25	1.5
0.5	1.3	0.75	2.1
1.0	1.4	1.25	3.5
2.0	1.6	1.5	4.3
3.0	1.9	2.0	5.8
4.0	2.9	2.5	7.8
6.0	7.2	3.0	10.8
8.0	12.4	4.0	16.9
10.0	13.6		

Viscosity of caecal and colonic mucus

The mucus layer overlying the epithelium in the porcine caecum and colon had viscosities of 7.53 ± 1.89 and 7.16 ± 2.21 mPa s, respectively. These values were similar to those of the 6 % mucin concentration (Table 2).

DISCUSSION

In optimizing the capillary tube assay, the chemotaxis buffer supported the motility of the spirochaetes better than did PBS, and this is in agreement with previous findings (Milner & Sellwood, 1994). On the other hand, an incubation period of 90 min was preferable to the incubation period of 60 min that has previously been used in similar experiments (Milner & Sellwood, 1994; Witters & Duhamel, 1999), and hence this was selected for use throughout the study. The use of a shorter incubation time by previous investigators might have underestimated the extent of the attraction to mucus that the previous workers recorded for a given strain.

A striking and important finding early in this study was the observation that the attraction of *B. pilosicoli* strain 95/1000 and *B. hyodysenteriae* strain B204 increased with increasing mucin concentrations. Previous studies on attraction to mucin by *B. pilosicoli* and *B. hyodysenteriae* have used 1 % mucin, and it is clear that this concentration is not optimal for assessing how the spirochaetes are attracted to mucin. Indeed, in the current study no strains showed significant attraction to 1 % mucin. These differences in the assay conditions help to explain the discrepancies in the results in the current study with those of the previous studies.

A factor potentially influencing the viability of the spirochaetes and their attraction to mucin is the medium and conditions under which they are grown. For example, Witters & Duhamel (1999) found that two *B. pilosicoli* strains only showed attraction to mucin if they were grown in a defined medium containing either pig faecal extract or 0.1 % mucin. The influence of the culture medium was not investigated in the current study, as clear responses were obtained under the standard culture conditions that were used.

Another important finding in the study was that attraction to mucin varied considerably between *B. pilosicoli* strains, with one of the 15 strains (Cof-10) not showing any attraction. Furthermore, the spirochaete strains varied in their capacity to enter the chemotaxis buffer, and this was taken to indicate that they also varied in their intrinsic motility under a given set of environmental conditions. Although Cof-10 was the least motile strain, and it was not attracted to mucin, over all the strains greater or lesser 'basal' motility did not necessarily equate with greater or lesser attraction to mucin. *In vivo*, the capacity to efficiently colonize and cause disease presumably requires both motility and strong attraction to mucin. Cof-10 was isolated from an Australian pig as part of a prevalence survey, but its ability to cause disease has not been

investigated experimentally. On the other hand, *B. pilosicoli* strains WesB, P43/6/78^T, 95/1000 and CSP-1 as well as other strains have been used successfully to experimentally infect pigs and/or chickens (Taylor *et al.*, 1980; Trott *et al.*, 1995, 1996; Stephens & Hampson, 2002). These strains were all found to be motile and to be attracted to mucin in the current study, although they differed in the extent of this activity. Ideally, strains with known attraction and motility properties should be tested simultaneously in an animal model to determine whether the *in vitro* measurements equate with *in vivo* colonization capacity, and pathogenic potential. It was interesting that the human strains PE-90 and BR 81/80, isolated from the bloodstream of French patients (Trott *et al.*, 1997b), were strongly attracted to mucin. This attribute may have facilitated their colonization of the large intestine in these patients, and increased opportunities for their translocation through the colonic epithelium.

In this study there was no significant correlation between the species from which a given strain of *B. pilosicoli* was isolated and the extent of its attraction to mucin, or motility. This is consistent with previous observations that strains of *B. pilosicoli* from humans do not form a genetically distinct group from strains recovered from animals (Hampson *et al.*, 2006). Strains from all sources varied in their properties, and this supports the likelihood that cross-species transmission of *B. pilosicoli* strains can occur.

Mucin is a serine-threonine-proline-rich protein with covalently bound polysaccharides, and such amino acids and polysaccharides can bind to chemotaxis receptors on the surface of various bacteria, including spirochaetes (Lux *et al.*, 2000). Previously *B. pilosicoli* strain P43/6/78^T has been shown to be chemotactic to DL-serine (Witters & Duhamel, 1999), a component of mucin, and a similar response to D-serine was obtained in the current study with strain 95/1000. Hence it appears likely that the attraction to mucin by *B. pilosicoli* includes an element of chemotaxis. Other potential attractants that would be present in mucin and the mucous gel include a range of simple sugars, and further work is required to identify these. Previously *B. hyodysenteriae* has been shown to be chemotactic towards a variety of mucin components (Kennedy & Yancey, 1996), and *B. hyodysenteriae* is known to have a large number of genes encoding methyl-accepting chemotaxis proteins (46) and chemosensory transducer molecules (17) (Bellgard *et al.*, 2009). Less is known about *B. pilosicoli*, although one putative glucose-galactose chemoreceptor has been described (Zhang *et al.*, 2000). It is not clear how chemotactic molecules would be released from the mucin solutions that were used in the *in vitro* assay in the current study, although *B. pilosicoli* is known to possess membrane-associated serine proteases and other proteases that could be involved in their degradation and release (Dassanayake *et al.*, 2004).

The higher concentrations of mucin that were used here were highly viscous (Table 2), and these concentrations had

similar viscosities to those that were found in the mucus layer overlying the epithelium in the porcine caecum and colon. Hence, the behaviour of the strains at the higher mucin concentrations is likely to reflect their activities *in vivo*, when they encounter the mucus barrier. This relationship led to an investigation of the potential involvement of the viscosity of the mucin solutions in their attractiveness to the spirochaetes. Previously, a strain of the spirochaete *Leptospira interrogans* (biflex) was shown to possess a 'viscotaxis' response, whereby it was attracted to a viscosity gradient made up of different concentrations of the viscous linear polymer PVP (Petrino & Doetsch, 1978). When *B. pilosicoli* 95/1000 was tested against dilutions of PVP with different viscosities, it showed a response that resembled its response to mucin, although the R_{att} values were numerically considerably less than those achieved with mucin at a given viscosity. Hence, it appears that at least part of the response of *B. pilosicoli* to mucin is likely to be a form of viscotaxis. In future work it would be interesting to add chemotactic materials from mucin to the PVP solutions, to see whether the combinations would increase the overall R_{att} to levels similar to those achieved with mucin. It is not clear how a viscotaxis response could function, but *B. pilosicoli* strain NK1f has been shown to develop improved motion efficiency with increasing viscosity of a motility medium (Nakamura *et al.*, 2006). Hence it is possible that the spirochaete cells that entered the viscous solutions through normal motility developed improved motion efficiency in the solutions, and then travelled up the tubes. In this way, at least part of the observed 'viscotaxis' may be simply due to increases in motion efficiency in response to increasing viscosity of the medium.

It was interesting that *B. hyodysenteriae* strain B204 showed a somewhat different pattern of 'attraction' to mucin, with this phenomenon declining at higher mucin concentrations. In explanation, initially it was thought that *B. pilosicoli* and *B. hyodysenteriae* might differ in their changes in motion efficiency relative to the viscosity, perhaps related to the fact that *B. pilosicoli* strains have 8–12 periplasmic flagella per cell, whilst *B. hyodysenteriae* strains have 22–28 (Stanton, 2006). When B204 was tested with the PVP solutions, however, its R_{att} values continued to increase as the viscosity increased. It appears therefore that factors other than viscosity may affect the behaviour of *B. hyodysenteriae* in high concentrations of mucin. For example, at high mucin concentrations the response to the chemoattractant properties of mucin may be switched off, such that only the viscotaxis effects still operate. This would then explain why, at a viscosity of around 14 mPa s, the R_{att} for B204 had declined in mucin to a value similar to that found in PVP (Fig. 3).

The apparent viscotaxis of the spirochaetes was of practical interest and application, as different dietary ingredients and/or the addition of carboxymethylcellulose to experimental pig diets have been shown to influence the viscosity of the contents in the large intestine, with higher viscosities being associated with greater colonization by *B. pilosicoli*

(Hampson *et al.*, 2000; Hopwood *et al.*, 2002; Lindecrona *et al.*, 2004). Hence there may be a link between viscosity of the colonic contents and the propensity for colonization with *B. pilosicoli* and *B. hyodysenteriae*. Such a link would also be important in disease states, where the composition or viscosity of the mucus layer might change. For example, in swine dysentery there is a great outpouring of mucus from the colonic crypts in response to the presence of *B. hyodysenteriae*. Depending on the composition and viscosity of this material, it might either enhance or inhibit colonization by this spirochaete species. The differences in response to mucin by the two pathogenic species also may help to account for the different behaviours and colonization sites for the two species. *B. hyodysenteriae* is found in the lumen of the colon and deep in the colonic crypts, where it enters goblet cells. Although *B. pilosicoli* also can enter the crypts, unlike *B. hyodysenteriae* it specifically penetrates the dense mucus layer at the crypt shoulders, and attaches to the underlying colonic enterocytes (Jensen *et al.*, 2000). Presumably, as the mucus concentration increases towards the enterocyte surface, the attraction to mucin and the motion efficiency of *B. pilosicoli* would continue to increase, and this may be the means by which the subsequent specific attachment to the cell surface is facilitated. On the other hand, the densest part of the mucus barrier at the cell surface might be less attractive to *B. hyodysenteriae*, such that it does not undergo polar attachment.

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