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PO Box 117
221 00 Lund
+46 46-222 00 00

Influence of activated charcoal, porcine gastric mucin and β -cyclodextrin on the morphology and growth of intestinal and gastric *Helicobacter* spp.

Jalal Taneera,¹ Anthony P. Moran,² Sean O. Hynes,¹ Hans-Olof Nilsson,¹ Waleed abu Al-Soud¹ and Torkel Wadström¹

Author for correspondence: Anthony P. Moran. Tel: +353 91 524411 ext 3163. Fax: +353 91 525700.
e-mail: anthony.moran@nuigalway.ie

¹ Department of Medical Microbiology, Dermatology and Infection, Lund University, Lund Sölvegatan 23, SE-22362, Sweden

² Department of Microbiology, National University of Ireland, Galway, Ireland

Bile-tolerant *Helicobacter* spp. are emerging human and animal pathogens. However, due to their fastidious nature, which requires nutrient-rich complex media to grow, infection with these bacteria may be underestimated. The accumulation of toxic metabolites in cultures may be one of the main obstacles for successful culture of these organisms. The present study examined various potential growth-enhancing substances for *Helicobacter* spp. and, furthermore, how they may affect spiral to coccoid conversion. Five *Helicobacter* spp. were cultured on agar and in broth media supplemented with activated charcoal, β -cyclodextrin, or porcine gastric mucin. Growth was determined by estimating the numbers of colony-forming units and colony diameter, as well as bacterial cell mass. Coccoid transformation was estimated every 24 h by both Gram and acridine-orange staining. Activated charcoal was superior in supporting growth and increased cell mass on agar and in broth media. β -Cyclodextrin delayed spiral to coccoid conversion by *Helicobacter pylori* and *Helicobacter canis*, whereas activated charcoal delayed the conversion to coccoid forms of *Helicobacter hepaticus* and *Helicobacter bilis*. The progression to coccoid forms by *Helicobacter pullorum* on agar media was not influenced by any growth supplement. The spiral to coccoid conversion was more rapid in broth media than on agar media. The growth enhancement observed is probably related to the capacity of activated charcoal to remove toxic compounds in culture media.

Keywords: *Helicobacter pylori*, coccoid forms, coccoid conversion, culture media

INTRODUCTION

Helicobacter pylori is known to be the causative agent of chronic gastritis and peptic ulcer disease (Goodwin *et al.*, 1997), as well as being associated with the development of gastric cancer (Forman, 1996). Since the original isolation of *H. pylori* in 1982, the genus *Helicobacter* has been expanded to include more than 20 identified species isolated from the gastrointestinal tract and liver of man and various animals (Versalovic & Fox, 2001), including dogs (Fox *et al.*, 1996; Jalava *et al.*, 1997), cats (Norris *et al.*, 1999), mice (Ward *et al.*, 1994; Fox *et al.*, 1995), rodents (Lee *et al.*, 1992), poultry (Stanley *et al.*, 1994), hamsters (Franklin *et al.*, 1996) and

cheetahs (Eaton *et al.*, 1993). These novel helicobacters have been associated with the development of various gastrointestinal disorders in their respective hosts.

However, despite being isolated from an ever-increasing number of hosts, most *Helicobacter* spp. remain difficult to culture from clinical samples. Various solid and broth culture media have been designed to optimize growth for the production of various antigens (Shahamat *et al.*, 1991; Marchini *et al.*, 1995; Walsh & Moran, 1997), to facilitate metabolic and enzyme studies, and to prevent accumulation of toxic metabolites that inhibit growth in the stationary phase (Hazell *et al.*, 1989). However, culture media for *Helicobacter* spp. remain poorly developed with the exception of *H. pylori*.

In addition, the related phenomenon of spiral to coccoid conversion has been investigated in *H. pylori* (Donelli *et*

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Abbreviation: CCUG, Culture Collection of the University of Gothenburg.

al., 1998; Worku *et al.*, 1999) but not in other helicobacters. *H. pylori* cells may exist in two morphological variants (Benaissa *et al.*, 1996): a bacillary form with a spiral or helical shape and a coccoid form which usually appears after several days of culture. This conversion generally occurs after 6–10 days of culture in stationary phase (Chan *et al.*, 1994; Benaissa *et al.*, 1996). Several factors may influence the spiral to coccoid conversion by *H. pylori*, such as acid pH stress, oxygen, temperature, nutritional starvation (West *et al.*, 1990; Cellini *et al.*, 1994; Donelli *et al.*, 1998; Worku *et al.*, 1999) and, thus, potentially culture media composition may influence the rate of spiral to coccoid conversion by *Helicobacter* spp. It has been speculated that the conversion to coccoid forms may be important for maintaining viability and survival of *H. pylori* outside the host (West *et al.*, 1990), as well as in recrudescence of infection and treatment failure of patients with peptic ulcer disease (Benaissa *et al.*, 1996). On the other hand, other microaerophilic bacteria produce non-culturable coccoid forms resembling those of helicobacters (Moran, 1997) and these have been shown to be a degenerate cell form which is undergoing cellular degradation (Moran & Upton, 1986) in response to oxygen toxicity and metabolites in culture media (Moran & Upton, 1987a, b). Likewise, compared to spiral forms, it has been shown that coccoid forms of *H. pylori* have impaired genomic DNA, total amounts of DNA and RNA are reduced, there is loss of membrane potential, and levels of intracellular ATP, indicative of metabolic state, are significantly reduced, collectively indicating a process of cellular degeneration (Kusters *et al.*, 1997; Moran, 1997; Narikawa *et al.*, 1997).

The present study was undertaken to establish optimized culture media for the growth of *Helicobacter hepaticus*, *Helicobacter bilis*, *Helicobacter pullorum* and *Helicobacter canis* through the addition of β -cyclodextrin, porcine mucin and activated charcoal, using *H. pylori* for comparison. Improvements in the ability to culture these emerging pathogens would allow a more precise diagnosis of infection. Furthermore, we also examined the effect of culture conditions on the conversion of spiral to non-culturable coccoid forms.

METHODS

Bacterial strains. A number of helicobacters were examined in the present study, including two *H. pylori* clinical isolates (BH43 and G50) from the Clinical Bacteriology Laboratory, University Hospital, Lund, Sweden. The *H. pylori* type strain (CCUG 17874), *H. pullorum* (CCUG 33838), and *H. bilis* (CCUG 38995) were purchased from the Culture Collection of the University of Gothenburg (CCUG), Gothenburg, Sweden, whereas *H. canis* (NCTC 12739) and *H. hepaticus* (NCTC 12886) were from the National Collection of Type Cultures, Colindale, UK. All bacteria were maintained at -70°C in tryptic soy broth supplemented with 15% (v/v) glycerol.

Agar media composition and culture conditions. Two types of agar media were used in the present study: (i) GAB-Camp agar (GCA) for culturing *H. pylori* (Soltesz *et al.*, 1992), and (ii) Brucella agar (BA) (Becton Dickinson) to culture *H.*

hepaticus, *H. pullorum*, *H. canis* and *H. bilis*. These media were modified by supplementation with 5% (v/v) sheep blood, 5% (v/v) inactivated horse serum (Gibco-BRL), 1% (v/v) IsoVitalax (Becton Dickinson) and 1% (v/v) haemin. Each strain was cultured on GCA or BA without antibiotics as indicated above at 37°C for 2–4 days under microaerobic conditions (5% O_2 , 5% CO_2 , 4% H_2 and 81% N_2 ; or 10% O_2 , 5% CO_2 and 85% N_2) and subcultured under the same conditions. Colonies were harvested, suspended in normal saline and adjusted to an OD_{540} of 1.0, equivalent to a concentration of 5×10^8 c.f.u. ml^{-1} , which was subsequently diluted, to a density of 10^4 c.f.u. ml^{-1} for each strain. An aliquot of this dilution (100 μl) was applied to agar media containing different supplements. These included various concentrations of 2,6-di-O-methylcyclodextrin (Cyclolab, Budapest, Hungary), porcine gastric mucin (type III from porcine stomach) (Sigma) and two types of activated charcoal added, with particle sizes of 1.2 mm (Sigma) and 2–4 mm (Rudolph Grave-Stockholm, Sweden) (Table 1). The 2,6-di-O-methylcyclodextrin was filter-sterilized before addition to sterile media, whereas activated charcoal and porcine mucin were added prior to autoclaving at 121°C for 20 min. The suitability of each medium for each *Helicobacter* spp. was evaluated by measuring both the number of c.f.u. in triplicate and the mean diameters of the colonies. All inoculations were performed in duplicate and evaluations of each medium were repeated.

Broth media and culture conditions. Two types of broth media were used in the present investigation. Gonococcal broth (GB) originally described for culturing *Neisseria gonorrhoeae* (Soltesz & Mårdh, 1980), was used to culture *H. pylori*, whereas brain heart infusion broth (BHI) was used for the culture of *H. bilis*, *H. canis*, *H. hepaticus* and *H. pullorum*. Standard broth media contained 7% (v/v) inactivated horse serum, 1% (v/v) IsoVitalax, 1% (v/v) haemin and 0.6% (w/v) yeast extract. All broth media were inoculated using two volumes of cells (5 μl each) harvested from solid media using a loop with a 5 mm diameter (Sarstedt) and deposited into a 100 ml Erlenmeyer flask containing 50 ml GB or BHI. The flasks containing media were incubated at 37°C in anaerobic jars on a rotary shaker (150 r.p.m.) under microaerobic conditions generated by an AnaerocultC envelope without palladium catalyst, which produced a final atmosphere containing 5–6% O_2 and 8–10% CO_2 (Merck). After incubation for 2 days, 1 ml of each culture was subcultured into GB or BHI supplemented with different concentrations of activated charcoal, porcine gastric mucin or β -cyclodextrin (see Table 1) under the same conditions as described above. To determine the number of c.f.u. ml^{-1} , cultures were serially diluted in 0.15 M PBS pH 7.2, with strains enumerated in triplicate, by plating onto GCA or BA medium under microaerobic conditions as described above. All inoculations were performed in duplicate and evaluations of each medium were repeated.

Calculation of percentage of coccoid forms. Bacterial cells from both agar and broth media were examined by Gram and acridine-orange staining at a magnification of $1000\times$, at 24 h intervals. Duplicate sets of inoculated media were examined. The percentage of cells with spiral or coccoid morphology was estimated microscopically by counting 100 cells in two samples of each bacterial suspension (all data are shown as the mean percentage). Bacterial cells were stained using standard Gram-staining techniques and also acridine-orange staining. Prior to either staining, cells were suspended to a standard OD_{540} of 1.0. For acridine-orange staining, cells (1 μl) were mixed with 9 μl acridine orange (1.0 mg PBS ml^{-1} , pH 7.2) on a microscope

Table 1. Solid culture media composition and their effect on five *Helicobacter* spp.

Results shown are mean values \pm standard deviation from duplicate experiments whose determinations were repeated in triplicate.

Growth indicator	<i>Helicobacter</i> sp.	Supplements						
		β -Cyclodextrin		Charcoal*		Charcoal†	Mucin‡	Control media§
		0.1% (w/v)	0.2% (w/v)	0.1% (w/v)	0.2% (w/v)	0.1% (w/v)	0.1% (v/v)	
Colony numbers (c.f.u. ml ⁻¹)	<i>H. pylori</i> (CCUG 17874)	60 \pm 11¶	52 \pm 7	70 \pm 11¶	60 \pm 11¶	63 \pm 10¶	50 \pm 9	50 \pm 11
	<i>H. pylori</i> (BH43)	55 \pm 11	46 \pm 6	63 \pm 10¶	56 \pm 9¶	58 \pm 9¶	43 \pm 8	47 \pm 11
	<i>H. pylori</i> (G50)	40 \pm 8	50 \pm 5	50 \pm 8¶	41 \pm 7¶	45 \pm 7¶	35 \pm 6	31 \pm 7
	<i>H. canis</i> (NCTC 12739)	90 \pm 5	98 \pm 8	120 \pm 6¶	112 \pm 6¶	110 \pm 3¶	110 \pm 17	100 \pm 6
	<i>H. hepaticus</i> (NCTC 12886)¶	+	+	+	+	+	+	+
	<i>H. pullorum</i> (CCUG 33838)	75 \pm 14	75 \pm 20	93 \pm 10¶	86 \pm 9¶	89 \pm 1¶	75 \pm 7	75 \pm 7
	<i>H. bilis</i> (CCUG 38995)	60 \pm 5	59 \pm 8	78 \pm 5¶	75 \pm 3	75 \pm 1	63 \pm 8	65 \pm 9
Colony diameter (mm)	<i>H. pylori</i> (CCUG 17874)	1.2	1.2	1.4	1.4	1.4	1.0	1.0
	<i>H. pylori</i> (BH43)	1.2	1.2	1.4	1.4	1.4	1.0	1.0
	<i>H. pylori</i> (G50)	1.0	1.0	1.5	1.5	1.5	1.2	1.0
	<i>H. canis</i> (NCTC 12739)	0.8	0.8	1.0	0.8	0.8	0.8	0.8
	<i>H. pullorum</i> (CCUG 33838)	0.8	0.8	1.0	1.0	1.0	0.8	0.8
	<i>H. bilis</i> (CCUG 38995)	0.8	0.8	1.0	1.0	1.0	0.8	0.8

* Media supplemented with activated charcoal having a diameter of 1.2 mm.

† Media supplemented with activated charcoal having a diameter of 2–4 mm.

‡ Media supplemented with porcine gastric mucin.

§ Control media were without any additional supplements: GCA for *H. pylori* or BA for bile-tolerant species.

¶ *H. hepaticus* was plated semi-quantitatively and assessed as: –, no growth; +, poor growth; ++, good growth; + + +, very good growth.

¶ $P < 0.05$ and considered significantly different from control media, using the Mann–Whitney *U*-test.

slide, and immediately examined under a fluorescent microscope (Zeiss Axioskop G 42-110e).

Cell lysate. The protein content of cells harvested from broth media, supplemented with activated charcoal, β -cyclodextrin, or porcine gastric mucin, was examined as an indication of growth enhancement. Cells (5 ml) grown in broth media were collected following 72 h culture, washed in PBS and lysed by sonication in an ice bath, with 50% of pulse at 30 s intervals for 4 min (Labsonic U, B. Braun Diessel Biotech Inc.). Following centrifugation (12000 g, 20 min, 4 °C), the protein content of the cell-free fractions was determined using a Coomassie brilliant blue protein assay (G250 dye reagent, Bio-Rad). Duplicate sets of broth media were inoculated and, hence, determinations of protein content were made on two independent samples.

Electron microscopy. Cells were prefixed by application of 2.5% glutaraldehyde directly to agar plates. Subsequently, the cells on the plates were rinsed three times with Millonig phosphate buffer. A portion of growth equivalent to 1 cm² was then removed from the agar plate and transferred to a sterile container. The colonies were dehydrated with 25%, 50%, 75% and 98% ethanol (2 \times 20 min) respectively, followed by an overnight dehydration step with absolute ethanol, and were subsequently freeze-dried. The cells were mounted on metal stubs and sputter coated using gold/palladium. The samples were examined in a Philips 515 scanning electron microscope.

Statistical analysis. For analysis of quantitative growth in various media and comparison of independent groups of data, the Mann–Whitney *U*-test was used. The Tukey–Kramer multiple comparisons test was applied when comparing the level of protein content in supplemented and control media. A value of $P < 0.05$ was considered significant.

RESULTS

Growth and coccoid forms of *H. pylori* on agar media

All *H. pylori* strains were able to grow in the presence or absence of hydrogen gas. However, in the presence of 4% hydrogen, growth was visible after 48 h compared to 60 h in its absence. The highest c.f.u. numbers and the largest colony diameter (1.4 mm) for *H. pylori* after 4 days of growth were observed on the GCA medium supplemented only with 0.1% (w/v) activated charcoal (diameter 1.2 mm), whereas the lowest c.f.u. numbers and the smallest colony diameter (0.5 mm) were observed on GCA blood-free media, compared to 1.0 mm on control media (Table 1). Moreover, the numbers of c.f.u. on charcoal-supplemented media for the three *H. pylori* strains were significantly higher ($P < 0.05$) and those on blood-free media were significantly lower ($P < 0.05$) than on control media. GCA supplemented with porcine gastric mucin did not affect growth of *H. pylori*, compared to control media. The colony diameter was increased when cells began to switch to coccoid forms after 8–9 days of growth. As shown in Fig. 1(a), the spiral to coccoid conversion by *H. pylori* started on day 7, independent of the growth supplement used, with the exception of cells grown on GCA blood-free agar, where the conversion started on day 8. Microscopic examination revealed a complete conversion to coccoid forms by day 11 on both control media and GCA supplemented with porcine gastric mucin. However, a complete spiral to coccoid conversion on GCA supplemented with activated charcoal occurred on day 13 and

with β -cyclodextrin on day 14. On blood-free agar, the coccoid morphology was dominant following 16 days of culture.

Growth and coccoid forms of *H. pylori* in broth culture

H. pylori cultured in GB supplemented only with 0.1% (w/v) activated charcoal showed the highest numbers of c.f.u. ml⁻¹, whereas control media or GB supplemented with porcine gastric mucin yielded the lowest numbers of c.f.u. (Fig. 2a). Compared to control media, the number of c.f.u. in charcoal-supplemented broth was significantly higher ($P < 0.05$) at day 2 and on subsequent days of incubation, but was not significantly different ($P > 0.05$) in the other supplemented media. The conversion to coccoid forms began in all supplemented media on day 3; 10% of the total cell numbers were converted to coccoid forms on day 3, whereas on day 4 this had increased to approximately 30%. In control media or GB supplemented with porcine gastric mucin, approximately 80% of the cells were converted to coccoid forms on day 6, with a complete conversion recorded after 7 days of culture. Around 60% of bacterial cells grown in GB supplemented with 0.1% (w/v) activated charcoal or β -cyclodextrin were coccoid forms on day 6 of culture, and 80% and 75% by day 7, respectively. Complete conversion occurred on day 8 in GB supplemented with activated charcoal, and on day 9 in GB supplemented with β -cyclodextrin. As shown in Table 2, the results of the total protein assay revealed that *H. pylori* cultured in GB supplemented with activated charcoal had the highest protein content, when compared to other supplements. Furthermore, the protein content of *H. pylori* grown in charcoal-supplemented media was significantly higher ($P < 0.01$) than that grown in control media.

Growth and coccoid forms of bile-tolerant *Helicobacter* spp. on agar media

Several differences were noted in the growth characteristics of the *Helicobacter* spp. tested on agar media. In contrast to *H. hepaticus* and *H. pullorum*, which both demonstrated notable growth after 24 h of incubation, *H. pylori*, *H. canis* and *H. bilis* exhibited notable growth only after 48–72 h of incubation. Only *H. canis* was able to grow in the absence of hydrogen. At a low cell inoculum (10^{-7} , 10^{-8} dilution) no growth was obtained with *H. hepaticus*. In general, the lowest c.f.u., which differed significantly ($P < 0.05$) from the number of c.f.u. on control media, as well as the smallest colony diameters were obtained on BA blood-free agar and, moreover, no growth of *H. hepaticus* occurred on such media (Table 1). Supplementation of the blood-free media with 0.1% (w/v) activated charcoal or 0.1% (w/v) β -cyclodextrin did not influence growth (data not shown). Also, similar to *H. pylori*, supplementation with 0.1% (w/v) activated charcoal (diameter 1.2 mm) gave the highest c.f.u. and the largest colony diameter, compared to the other supplements of β -cyclodextrin or

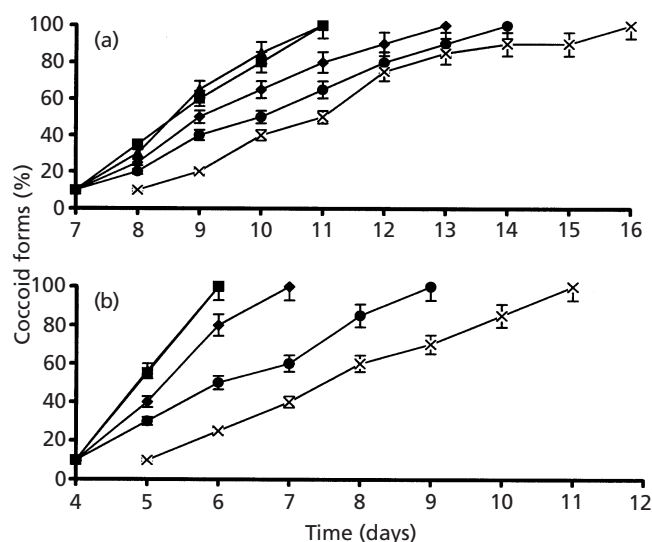


Fig. 1. Influence of various supplements in agar media on the conversion rate of spiral to coccoid forms by *H. pylori* CCUG 17874 (a) and *H. canis* NCTC 12739 (b) when grown on media supplemented with 0.1% (w/v) β -cyclodextrin (●), 0.1% (w/v) activated charcoal having a diameter of 1.2 mm (◆) or 0.1% (v/v) porcine gastric mucin (▲), compared to when grown without any of these supplements (■) and in blood-free agar media (x). Bars indicate standard deviations of the means.

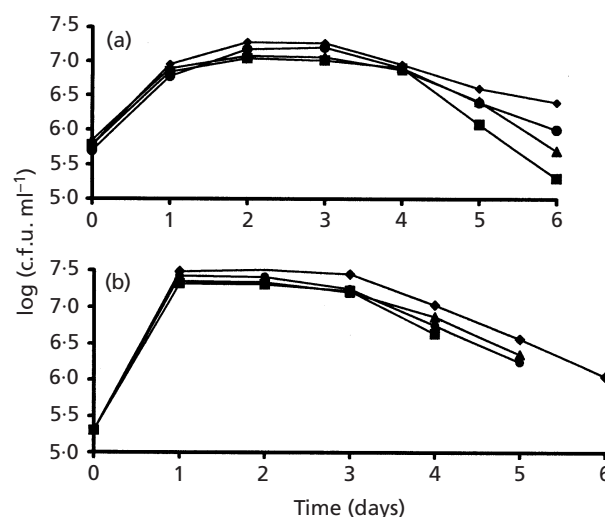


Fig. 2. Influence of various supplements on the growth kinetics in broth of *H. pylori* CCUG 17874 (a) and *H. canis* NCTC 12739 (b) when grown on media supplemented with 0.1% (w/v) β -cyclodextrin (●), 0.1% (w/v) activated charcoal having a diameter of 1.2 mm (◆) or 0.1% (v/v) porcine gastric mucin (▲), compared to when grown without any of these supplements (■).

porcine gastric mucin (Table 1). Furthermore, the number of c.f.u. on charcoal-supplemented media was significantly higher ($P < 0.05$) than on control media. The colony size was about 0.8–1.0 mm, except for colonies on BA blood-free agar media, where colonies

Table 2. Total protein content ($\mu\text{g ml}^{-1}$) extracted from *Helicobacter* cells after 48 h growth on media with various supplements

Results shown are mean values \pm standard deviation from duplicate experiments.

<i>Helicobacter</i> sp.	Supplements			
	0.1% (w/v) Cyclodextrin	0.1% (w/v) Charcoal*	0.1% (w/v) Mucin†	Control media‡
<i>H. pylori</i> CCUG 17874	184 \pm 3	206 \pm 7§	170 \pm 5	170 \pm 5
<i>H. pylori</i> BH43	175 \pm 5.5	196 \pm 5.5§	165 \pm 4.5	162 \pm 3.5
<i>H. pylori</i> G50	162 \pm 3.5	179 \pm 5§	162 \pm 3	136 \pm 5
<i>H. canis</i> NCTC 12739	175 \pm 5	201 \pm 8§	162 \pm 3.5	162 \pm 3.4
<i>H. bilis</i> CCUG 38995	180 \pm 5	199 \pm 4.5	177 \pm 4	175 \pm 5

* Media supplemented with activated charcoal having a diameter of 1.2 mm.

† Media supplemented with porcine gastric mucin.

‡ Control media were without any additional supplements: GCA for *H. pylori* or BA for bile-tolerant species.

§ $P < 0.01$ and considered significantly different from control media, using the Tukey–Kramer multiple comparisons test.

|| $P < 0.05$ and considered significantly different from control media, using the Tukey–Kramer multiple comparisons test.

had a diameter < 0.6 mm. The colony diameter of bile-tolerant *Helicobacter* spp. as well as *H. pylori* increased when spiral forms began to convert to coccoid forms.

As shown in Fig. 1(b), the conversion of *H. canis* started on day 4 on various agar media. On control media and BA supplemented with porcine gastric mucin, conversion to coccoid forms was complete after 6 days of culture, whereas the conversion was complete on BA supplemented with activated charcoal on day 7 and on BA supplemented with β -cyclodextrin on day 9. On the other hand, on BA blood-free agar, coccoid forms were dominant only at day 11.

On control media, 20% coccoid forms occurred after 2 days of culture of *H. pullorum* and spiral to coccoid conversion was complete on day 4. On supplemented media, the transformation from spiral to coccoid forms by *H. pullorum* appeared on day 4 with 15% coccoid forms; on days 5 and 6 the percentages of coccoid forms were 40% and 75%, respectively. The spiral to coccoid conversion was complete on day 7 on all supplemented agar media, and on BA blood-free agar 1 day later.

The conversion to coccoid forms by *H. bilis* on all agar media started on day 7 with 10% coccoid forms occurring. In BA control media, BA with activated charcoal, and BA blood-free agar, the spiral to coccoid conversion occurred at a conversion rate of 5% daily and was complete after 26 days of culture. Although the conversion on BA supplemented with β -cyclodextrin or porcine gastric mucin began similarly after 7 days of culture, the daily conversion rate was higher (7%) and was complete after 21 days of incubation. The spiral to

coccoid conversion by *H. hepaticus* occurred 1 day after appearance of visible colonies and was complete on control media and BA supplemented with β -cyclodextrin 2 days later. On BA supplemented with activated charcoal, complete conversion to coccoid forms occurred after one further day.

Growth and coccoid forms of bile-tolerant *Helicobacter* spp. in broth culture

Unlike the other helicobacters examined, *H. canis* was successfully subcultured from BHI. Moreover, *H. canis* grown on BHI supplemented with 1% (w/v) activated charcoal gave higher numbers of c.f.u. ml^{-1} (Fig. 2b) and the highest protein content (Table 2) compared to the other media. This protein content was significantly greater ($P < 0.01$) than that of cells grown on control media. In addition, compared to control media, the number of c.f.u. in charcoal-supplemented broth was significantly higher ($P < 0.05$) at day 2 and on subsequent days of incubation, but was not significantly different ($P > 0.05$) in the other supplemented media. Approximately 20% of the total bacterial cells examined in control media and BHI supplemented with porcine gastric mucin had converted completely to coccoid forms after 2 days of culture, and 1 day later the coccoid percentage had increased to 60% and 50%, respectively. Complete conversion occurred in both media by day 4. In BHI supplemented with activated charcoal, the spiral to coccoid conversion started after 3 days of incubation, when 20% coccoid forms occurred, and was complete on day 6. In BHI supplemented with β -cyclodextrin, the conversion started on day 3, when 20% coccoid forms

were present; 50% coccoid forms occurred on day 5, and conversion was complete after 7 days of culture.

H. pullorum and *H. hepaticus* began conversion with 20% coccoid forms after 2 days of culture; 60% coccoid forms were present at day 3 in all media, except that supplemented with 1% (w/v) activated charcoal, where 40% coccoid forms were present. Moreover, 1 day later, conversion to coccoid forms was complete in all media except for that supplemented with charcoal, where conversion to coccoids was complete on day 5. The spiral to coccoid conversion of *H. bilis* in BHI supplemented with β -cyclodextrin or porcine mucin was slow compared to the other species; it began on day 4 and was complete on day 8 of incubation. In control media and BHI supplemented with activated charcoal, coccoid forms predominated after 12 days, whereas in BHI supplemented with porcine mucin or β -cyclodextrin the spiral to coccoid conversion started on day 4 and finished on day 8. Moreover, compared to growth on other supplemented media, *H. bilis* cultured on charcoal-supplemented media had the highest protein content, and this was significantly higher ($P < 0.05$) than that obtained when *H. bilis* was cultured in control media (Table 2).

DISCUSSION

Helicobacter spp. are capable of colonizing a range of environmental niches in the gastrointestinal tract of many mammalian species (Lee *et al.*, 1992; Stanley *et al.*, 1994; Franklin *et al.*, 1996; Norris *et al.*, 1999). Moreover, a number of species are known to infect the liver and bile tree (Ward *et al.*, 1994; Fox *et al.*, 1995; Versalovic & Fox, 2001) and others, such as *H. pullorum* and *H. canis*, may cause zoonotic infections in humans (Fox *et al.*, 1998; Nilsson *et al.*, 2000a, b). Since such bile-tolerant *Helicobacter* spp. are difficult to culture, we attempted in the present investigation to establish optimal conditions for culture. Four different formulations of agar and broth media were examined. In addition, these media were supplemented with activated charcoal, β -cyclodextrin, or porcine mucin. Our results demonstrated that activated charcoal was superior to β -cyclodextrin or porcine gastric mucin in stimulating growth of all the five species examined. Several studies have shown that activated charcoal promotes the growth of other pathogenic bacteria such as species of *Legionella* (Edelstein & Edelstein, 1993), *Brucella* (Issam *et al.*, 1990) and *Bordetella* (Hoppe & Schwaderer, 1989). Furthermore, activated charcoal has been reported to enhance production of the virulence factors of *Listeria monocytogenes in vitro* (Ermolaeva *et al.*, 1999).

Both activated charcoal and β -cyclodextrin have no nutrient value and therefore the effect could be related to their ability to remove toxic compounds present in cultures such as hydrogen peroxide and superoxide ions (Marchini *et al.*, 1995; Buck & Smith, 1987). Moreover, our results indicate that activated charcoal has a higher adsorption capacity than β -cyclodextrin. The adsorption

properties of activated charcoal depend on the number of fine pores giving a large inner surface area for adsorption. This may explain why greater growth enhancement was observed with activated charcoal having a 0.15 mm pore size than that with a 2–4 mm pore size.

Mucin preparations have been shown previously to enhance growth of several oral bacteria (Bradshaw *et al.*, 1994) and *Bacteroides fragilis* (Robertson & Stanley, 1982). Furthermore, *H. pylori* has surface mucin-binding proteins, in common with the bile-tolerant *Helicobacter* strains studied in this investigation (J. Taneera, S. Hynes & T. Wadström, unpublished data). However, in this study, in contrast to activated charcoal, porcine gastric mucin did not affect the growth kinetics of bile-tolerant *Helicobacter* spp. Although blood is the major iron source for some micro-organisms and enhances their growth, bile-tolerant *Helicobacter* spp., with the exception of *H. hepaticus*, were capable of growth on blood-free media. This indicates that blood is not essential for their growth, although it did enhance the growth of all these helicobacters, similar to previous observations with *H. pylori* (Walsh & Moran, 1997). Our data also indicated that hydrogen is an essential growth factor for bile-tolerant helicobacters, but the mechanism by which these organisms utilize hydrogen is poorly understood.

Spiral to coccoid conversion has been observed under certain stress conditions for several Gram-negative pathogens such as *Vibrio* spp., *Escherichia coli*, *Shigella* spp., *Salmonella enteritidis*, *Campylobacter* spp., *Aeromonas* spp. and *Legionella* spp. (Moran & Upton, 1987a; Barer *et al.*, 1993). Furthermore, culture media supplemented with activated charcoal or β -cyclodextrin delayed the conversion to coccoid forms by all the helicobacters we examined. In general, a spiral to coccoid conversion was more rapid in broth than on solid media, most likely due to the dissolved toxic substances enhancing the coccoid transformation (Moran & Upton, 1987b; Coudron & Statton, 1995). The ability to delay the spiral to coccoid conversion may be beneficial in the production of cell surface antigens for immunodiagnostic tests since such conversion has been shown to induce changes in the cell wall composition of *H. pylori* and other bacteria (Costa *et al.*, 1999; Signorello *et al.*, 2000).

Collectively, our data have shown that activated charcoal enhances growth of five *Helicobacter* spp. to a greater extent than β -cyclodextrin or porcine gastric mucin in various culture media and, furthermore, a delay in spiral to coccoid conversion was observed in media supplemented with either activated charcoal or β -cyclodextrin. Thus, addition of charcoal by increasing the growth yields and reducing conversion to coccoid forms could aid diagnostic laboratories in the ability to isolate and grow these helicobacters. Moreover, the ability to delay conversion to coccoid forms in helicobacters would be of benefit to various analyses carried out on these bacteria. Optimal growth conditions which

would allow routine culturing of pathogenic helicobacters from liver and bile specimens would be important for a diagnosis and correlation with disease which, at present, rely on molecular biology or serological techniques for detection. In addition to improved diagnostics, optimal conditions for subculture of intestinal helicobacters would allow far greater research in this area where, to date, low numbers of isolates from each species have been available for study.

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