Lactobacillus salivarius modulates cytokine induction and virulence factor gene expression in *Helicobacter pylori*

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Human infection by the gastric pathogen *Helicobacter pylori* is characterized by a robust immune response which rarely prevents persistent *H. pylori* colonization. Emerging evidence suggests that lactobacilli may reduce *H. pylori* infection rates and associated inflammation. In this study, we measured the ability of two model strains of *Lactobacillus salivarius* (UCC118 and UCC119) to modulate gastric epithelial cell chemokine responses to *H. pylori* infection. Pre-treatment of AGS cells with either *L. salivarius* strain significantly decreased interleukin-8 (IL-8) production upon exposure to *H. pylori*, but not in cells stimulated with TNF- α . The production of the chemokines CCL20 and IP-10 by AGS cells infected with *H. pylori* was also altered following pre-treatment with UCC118 and UCC119. We showed that a greater reduction in IL-8 production with UCC119 was due to the production of more acid by this strain. Furthermore, UV-killed cells of both lactobacillus strains were still able to reduce *H. pylori*-induced IL-8 in the absence of acid production, indicating the action of a second anti-inflammatory mechanism. This immunomodulatory activity was not dependent on adhesion to epithelial cells or bacteriocin production. Real-time RT-PCR analysis showed that expression of eight of twelve Cag pathogenicity island genes tested was downregulated by exposure to *L. salivarius*, but not by cells of four other lactobacillus species. CagA accumulated in *H. pylori* cells following exposure to *L. salivarius* presumably as a result of loss of functionality of the Cag secretion system. These data identified a new mechanism whereby some probiotic bacteria have a positive effect on *H. pylori*-associated inflammation without clearing the infection.

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INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral bacterium that colonizes the human gastric mucosa. Despite advances in our understanding of this pathogen and the development of treatment strategies, *H. pylori* remains a significant human health problem, and it still infects about 50 % of the world's population (De Vries *et al.*, 2008). *H. pylori* is a causative agent of a range of human diseases (Uemura *et al.*, 2001). Although *H. pylori* elicits strong local and systemic immune responses in infected individuals, most hosts are unable to clear the infection and can remain infected for their lifetime (Robinson *et al.*, 2007).

The chemokine interleukin-8 (IL-8) is an important mediator of the chronic inflammation that characterizes *H. pylori* infection. Cells of the gastric epithelium are

induced to secrete IL-8 only following their injection with the CagA (cytotoxin associated gene A) protein or peptidoglycan from *H. pylori*. CagA is translocated into host epithelial cells via a type IV secretion system, where it is phosphorylated by the host cells' own machinery (Higashi *et al.*, 2002; Stein *et al.*, 2000). Phosphorylated CagA induces changes in epithelial cell morphology which are thought to allow the bacterium to adhere at higher levels (Bourzac *et al.*, 2007; Su *et al.*, 2003).

In recent years, increases in the rates of *H. pylori* resistance to antibiotics have been widely reported (Graham & Shiotani, 2008; Ryan *et al.*, 2001; Vakil & Megraud, 2007). Furthermore, due to the cost involved, antibiotic treatment for *H. pylori* infection is not freely available in developing countries where infection rates are highest (Magalhaes Queiroz & Luzzi, 2006). These factors, along with the many side effects commonly associated with antibiotic consumption, have prompted a search for alternative

Abbreviations: CBA, cytometric bead array; IL-8, interleukin-8; PAI, pathogenicity island.

treatments or treatment adjuncts for *H. pylori* infection. A number of independent research groups have shown that probiotic organisms can have beneficial effects in the treatment of *H. pylori* infection (recently reviewed by Franceschi *et al.*, 2007; Lesbros-Pantoflickova *et al.*, 2007). It has also been shown that various probiotics can alter the immune response to *Helicobacter* infection by influencing cytokine release by the epithelium (Pena *et al.*, 2005; Rokka *et al.*, 2008; Sgouras *et al.*, 2005). To date, studies on immune modulation by probiotic organisms have focused primarily on signal transduction events in the host and not on the bacterial factors which may facilitate these changes (O'Hara *et al.*, 2006; Tamura *et al.*, 2006; Zhou *et al.*, 2008). As a result, the mechanisms whereby probiotic bacteria may effect such changes are very poorly understood.

In the present study, we investigated the ability of two well-characterized *Lactobacillus salivarius* strains to alter chemokine responses by gastric epithelial cells upon *H. pylori* infection and the mechanisms behind these phenomena. Strain UCC118 was originally isolated from the human ileal-caecal region and strain UCC119 was isolated from the chicken intestine (Ryan *et al.*, 2008a). A number of differences have previously been noted between these strains, including the ability to inhibit growth of *H. pylori* (Ryan *et al.*, 2008a).

METHODS

Cell lines and bacterial strains. AGS gastric epithelial cells were maintained in Ham's F-12 medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Sigma) at 37 °C in 5% CO₂.

H. pylori strain CCUG 17874 (Culture Collection, University of Göteborg, Sweden; identical to NCTC 11637, the type strain of *H. pylori*) was used in this study. This strain is a high-level IL-8 inducer strain (Brandt *et al.*, 2005). Bacteria were routinely cultured on chocolate blood agar plates (Columbia agar base; Merck) supplemented with 10% (v/v) heat-inactivated whole horse blood (Charles River Laboratories) for 48 h at 37 °C in an atmosphere containing 5% CO₂. Lactobacilli were cultured for 24 h in Man-Rogosa-Sharpe (MRS) broth (Oxoid) at 37 °C in 5% CO₂. *L. salivarius* UCC118 was isolated from the human ileal-caecal region (Dunne *et al.*, 2001) and *L. salivarius* UCC119 was isolated from the chicken intestine (Ryan *et al.*, 2008a). *Lactobacillus reuteri* DSM 2016 was obtained from the German Collection of Micro-organisms and Cell Cultures; *Lactobacillus acidophilus* ATCC 4356 was from the American Type Culture Collection; *Lactobacillus plantarum* NCIMB 8826 was from the National Collection of Industrial, Marine and Food Bacteria, UK; and *Lactobacillus sakei* LMG 2313 was from the Laboratory of Microbial Gene Technology, Norway (Corsetti *et al.*, 2004).

To prepare cell envelope and cytoplasmic fractions of *L. salivarius* UCC118, cells were washed in PBS, and then resuspended in Ham's F-12 medium. Cells were then lysed at 2000 p.s.i. using a French Press cell disrupter (Thermo Scientific). The cell lysate was centrifuged for 5 min at 12 000 g, after which the supernatant (cytoplasmic fraction) was removed. The pellet (cell envelope fraction) was resuspended in an identical volume of Ham's F-12 medium.

Co-culture assay. AGS cells were seeded into a 24-well plate at a concentration of 1×10^5 per well or into a 6-well plate at a

concentration of 1×10^6 per well and grown overnight. An overnight culture of lactobacillus was washed twice in sterile PBS and resuspended in Ham's F-12 medium. AGS cell culture medium was changed to serum-free Ham's F-12 and the lactobacillus cells were added at the required concentration. AGS cells and lactobacilli were cultured together for 2 h before the addition of *H. pylori*. *H. pylori* cells were removed from a 48 h chocolate blood agar plate into 1 ml BHI broth. Cells were washed twice in sterile PBS, resuspended in Ham's F-12 and added to the co-culture plate at the required concentration. After 4 h further incubation, the supernatants were collected.

To investigate the specificity of anti-inflammatory effects, 5 ng tumour necrosis factor alpha (TNF- α) ml⁻¹ (R&D Systems) was used in the assay instead of *H. pylori* to induce IL-8 secretion.

IL-8/CCL20 ELISA. Cell supernatants were collected from *H. pylori*-lactobacillus co-cultures and stored at -20 °C until processing. Following thawing, supernatants were tested for IL-8 protein using the DuoSet kit (R&D Systems) or for CCL20 using the Quantikine kit (R&D Systems), both according to the manufacturer's instructions.

Cytokine cytometric bead array (CBA) assay. The levels of CCL5 (RANTES), CXCL9 (monokine induced by interferon- γ), CCL2 (monocyte chemoattractant protein-1) and CXCL10 in cell-culture supernatants were measured simultaneously by CBA using the human chemokine CBA kit I (BD Biosciences). In CBA, bead populations with distinct fluorescence intensities and coated with capturing antibodies specific for each chemokine were incubated with cell-culture supernatant (10 μ l) according to the manufacturer's instructions. Following incubation with a mixture of phycoerythrin-conjugated antibodies to the test chemokines, the samples were analysed using a four-colour FACSCalibur flow cytometer (BD Biosciences). The concentration of each chemokine in the culture supernatants was interpolated from a standard curve, which was generated with each recombinant chemokine, using FCAP Array software (BD Biosciences). The assay sensitivities of CCL5, CXCL9, CCL2 or CXCL10 were 1.0, 2.5, 2.7 or 2.8 pg ml⁻¹, respectively.

Real-time PCR. Bacteria were collected from *H. pylori*-lactobacillus co-cultures by centrifugation for 15 s, and then placed in RNA Bacteria Protect Reagent (Qiagen). Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol for Gram-negative bacteria, and then treated for 2 h with TURBO DNA-free DNase (Ambion). RNA yield was estimated by Nanodrop (Thermo Scientific). A minimum of 0.2 μ g RNA was reverse-transcribed using 20 ng random primer and the Improm-II reverse transcriptase enzyme (both from Promega) as per the manufacturer's protocol. Real-time PCR primers for 10 Cag pathogenicity island (PAI) genes and *vacA* were designed using the Primer3 online software package (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are listed in Table 1. qRT-PCR was performed using an AB7000 thermocycler (Applied Biosystems). Each 12.5 μ l reaction contained 50 nM of each primer, 6.25 μ l 2 $\times$ Master Mix and 1/60 000 SYBR Green I (both from Biogene). Individual amplification reactions were first established and optimized for single-band specificity, verified by running pilot reaction products on gel, and monitoring of dissociation curves of all subsequent test reactions. Reactions were performed in triplicate and crossing threshold (ct) values were averaged. Fold change in expression was calculated according to the standard formula $2^{(En-Rn)-(Et-Rt)}$, where *En* is the ct of the experimental gene in the normal (*H. pylori* alone) sample, *Rn* is the ct of the reference gene in the normal sample, *Et* is the ct of the experimental gene in the treated (*H. pylori* and lactobacillus) sample and *Rt* is the ct of the reference gene in the treated sample. qRT-PCRs were repeated on three different biological replicates and fold expression changes were averaged.

Table 1. PCR primers used in this study

Gene (TIGR designation)	Forward primer (5'→3')	Reverse primer (5'→3')
HP0520	TGA AGC AAC TCA TGA AAC AAA AA	CCC TGC TCC AAT GAG ACC TA
HP0524 (TraG-like protein)	TTG ATT CTG CCA ATC CAT GA	TTC TGC CAT AAT CCC TAC CG
HP0527 (VirB10 homologue)	GTT GCG GAT TGT TTG GCT AT	TGC TTG ATG CAA CCT TTC TG
HP0528 (VirB9 homologue)	TGC GCT CAA ACA AAT TGA AG	TGG AGT TAT CCT CAG GGC TTT
HP0531	GCA GAT AGG ACA AGC CAA GA	TCC CAC CCA TAC ACA ATC CT
HP0534	TGC CAT TGC AGA GAA TCC TA	TGG GAG CTT AGT GCC ATA CA
HP0535	AAC CAA AGC AGA TCC CAT GT	AAC CAA AGC AGA TCC CAT GT
HP0538	CAG CAT TAT TGC CGA GTG AA	TTG CTC TGC TCT TGC CCT AT
HP0539	CTG AGC AAC AAT GCG GAA TA	AAG CGT CTG TGA AGC AGT GA
HP0543	CAT AAG CGA GGA CAT GCA GA	CCA TGA TCA AAT GCG TGA AG
HP0546 (VirB2 homologue)	AGT CCT GCA GAA GGC GTT AC	TAT CCG CTA TGC CTT TGA CC
HP0547 (<i>cagA</i>)	TAG CCC TGA ACC CAT TTA CG	TGA TTC CTT GAT CGC CCT AC
HP0887 (<i>vacA</i>)	CAC AAC CGT GAT CAT TCC AG	TAT TGG CTT CTT CGG CTT GT

Western immunoblotting. Cells were collected from the *H. pylori*-lactobacillus co-cultures by centrifugation. Residual medium was removed from the cell pellet, and the cells were resuspended in sterile water, boiled for 10 min and stored at -70°C . Standard conditions were employed for immunoblotting and SDS-PAGE, in gels containing 10% acrylamide. Separated proteins were transferred from SDS-PAGE gels to nitrocellulose paper by the methanol Tris-glycine system described by Towbin *et al.* (1979). CagA was detected using a rabbit polyclonal antibody (Santa Cruz Biotech). Bound antibodies were detected using horseradish-peroxidase-coupled goat anti-rabbit immunoglobulin (Dako), with hydrogen peroxide and 4-chloro-1-naphthol (Sigma) as chromogenic reagents.

Statistical analyses. Data were expressed as the mean $\pm$ SEM unless otherwise stated. Values were compared using a paired *t*-test for parametric data and considered significant if *P*-values were <0.05 .

RESULTS AND DISCUSSION

L. salivarius UCC118 inhibits *H. pylori*-induced IL-8 in a dose-dependent manner

Probiotic bacteria are defined as commensals that, when administered to humans, have an inherent benefit over and above nutrition (Guarner & Schaafsma, 1998). Some probiotics, including lactobacilli, have previously been shown to decrease inflammatory markers in *H. pylori* infection models both *in vitro* and *in vivo* (Johnson-Henry *et al.*, 2004; Tamura *et al.*, 2006). We studied *L. salivarius* strain UCC118 for this property *in vitro* because this is a well-characterized strain (Claesson *et al.*, 2006) which has also been demonstrated to have anti-infective ability in murine and porcine models (Casey *et al.*, 2007; Corr *et al.*, 2007). Furthermore, this strain has previously been shown to downregulate IL-8 expression in intestinal epithelial cells (HT29) at baseline, and to suppress IL-8 production triggered by *Salmonella* (O'Hara *et al.*, 2006). Having previously established that *L. salivarius* UCC118 does not inhibit growth of *H. pylori* (Ryan *et al.*, 2008a), we now proceeded to investigate whether this strain could inhibit *H. pylori*-induced IL-8 secretion by AGS gastric epithelial

cells. AGS cells were pre-treated with an m.o.i. of either 10:1, 50:1 or 100:1 cells of UCC118 and incubated for 2 h. *H. pylori* was then added at the same concentrations and cells were co-incubated for a further 4 h. IL-8 secretion by AGS cells decreased in a dose-dependent manner (Fig. 1a); however, this decrease was only significant at the highest concentrations of UCC118 (m.o.i. 100:1). m.o.i. values of 50:1 *H. pylori* to AGS cells and 100:1 UCC118 to AGS cells were used in all subsequent experiments.

To further investigate whether this phenomenon was specific to induction by *H. pylori*, we treated AGS cells with TNF- α , which also induces gastric epithelial cells to secrete IL-8. Addition of 5 ng TNF- α ml $^{-1}$ induced significant IL-8 secretion by AGS cells but the presence of UCC118 even up to an m.o.i. of 100:1 did not have any effect on cytokine release (Fig. 1b).

We repeated these initial experiments using a different strain of *L. salivarius*, UCC119. Unlike UCC118, this strain had previously been shown to inhibit growth of *H. pylori* (Ryan *et al.*, 2008a). Exposure of AGS cells to UCC119 caused a significantly greater reduction in IL-8 secretion in the presence of *H. pylori* than the decrease caused by UCC118 (Fig. 1c).

The reduction in *H. pylori*-stimulated IL-8 production levels in AGS cells was statistically significant only with the highest numbers of lactobacillus cells tested. However, we would argue that this does not necessarily prove that clinically significant levels of reduction in inflammation are unachievable by naturally present or artificially introduced lactobacillus cells. The data we present here may explain some of the previous successful *H. pylori*-probiotic *in vivo* trials (Franceschi *et al.*, 2007; Lesbros-Pantoflickova *et al.*, 2007). A more modest reduction in inflammation caused by lactobacilli *in vivo* could be clinically significant in the case of chronic inflammation – assuming that lactobacilli with relevant properties could persist in the stomach. Recent work suggests that this is the case (Bik *et al.*, 2006; Roos *et al.*, 2005; Ryan *et al.*, 2008b).

Fig. 1. Lactobacilli inhibit *H. pylori*-induced IL-8 secretion by AGS gastric epithelial cells. (a) *L. salivarius* UCC118 downregulates *H. pylori*-induced IL-8 secretion by AGS cells in a dose-dependent manner. (b) UCC118 does not inhibit TNF- α -induced IL-8 secretion by AGS cells. (c) UCC118 and UCC119 alone do not induce IL-8 secretion and UCC119 downregulates *H. pylori*-induced IL-8 secretion by AGS cells more than UCC118. Data shown are from at least three independent experiments carried out in duplicate. *, *P*-value < 0.05 compared to AGS cells treated with *H. pylori* alone. Untx, untreated.

Lactobacilli modulate the secretion of CCL20 and CXCL10

We assayed the supernatants from the co-culture assay for five additional chemokines using ELISA and CBA. CCL20 is a chemoattractant for immature dendritic cells, T cells and B cells (Schutyser *et al.*, 2003). It has been shown previously that *H. pylori* induces CCL20 secretion by gastric epithelial cells (Wu *et al.*, 2007). In our hands, *H. pylori* and both *L. salivarius* strains alone induced AGS cells to secrete a small amount of CCL20, but *H. pylori* together with *L. salivarius* UCC118 induced a significant increase in CCL20 secretion compared with either strain alone (Fig. 2a). Conversely, *L. salivarius* UCC119 together with *H. pylori* completely abolished CCL20 secretion by AGS cells (Fig. 2a). *L. salivarius* UCC118, *L. salivarius* UCC119 and *H. pylori* did not induce expression of the chemokines CCL5, CXCL9, CXCL10 or CCL2 (data not shown). However, a combination of *H. pylori* and either *L. salivarius* strain induced a significant increase in CXCL10 secretion by AGS cells (Fig. 2b). CXCL10 is primarily a chemoattractant for monocytes and T cells (Dufour *et al.*, 2002).

NF- κ B is a key mediator in the transcription of CCL20 and CXCL10 (Neville *et al.*, 1997; Tomimori *et al.*, 2007).

Although very little is known about the signalling pathways involved in inducing transcription of the genes that encode these chemokines, it is clear from the present data that the pathways involved differ from the well-described IL-8 NF- κ B signal transduction pathway as both lactobacilli on their own secreted CCL20 and CXCL10 but not IL-8.

Anti-inflammatory activity of *L. salivarius* UCC118 is not dependent on adhesion, bacteriocin production or cell viability

We carried out a number of experiments to investigate how *L. salivarius* UCC118 might inhibit *H. pylori*-induced IL-8 secretion. We first used two previously characterized mutant strains of *L. salivarius* UCC118 in our co-culture assay. One mutant, which is deficient in the sortase enzyme, has reduced adhesion to *intestinal* epithelial cells (van Pijkeren *et al.*, 2006). We first confirmed that this mutant strain similarly had a significant reduction in adhesion to *gastric* epithelial cells (Fig. 3a). The second mutant does not produce a functional Abp118 bacteriocin and cannot inhibit growth of *Listeria monocytogenes* (Corr *et al.*, 2007). Both mutant strains of UCC118 inhibited *H. pylori*-induced IL-8, and there was no difference between

Fig. 2. *L. salivarius* UCC118 and UCC119 differentially modulate the secretion of other chemokines by AGS cells. (a) UCC118 significantly increases CCL20 secretion by AGS cells in response to *H. pylori*. UCC119 significantly decreases CCL20 secretion by AGS cells in response to *H. pylori*. (b) Both UCC118 and UCC119 significantly increase CXCL10 secretion by AGS cells in response to *H. pylori* infection. Data shown are from three independent experiments carried out in duplicate. *, *P*-value < 0.05 compared to AGS cells treated with *H. pylori* alone.

the amount of inhibition observed with either strain compared to wild-type UCC118 (Fig. 3b). This indicates that the anti-inflammatory effect of UCC118 was not due to binding of UCC118 cells to AGS cells, nor was it mediated by a direct antibacterial activity of the UCC118 bacteriocin.

Because we had previously observed that live, viable cells were required for growth inhibition of *H. pylori* (Ryan *et al.*,

2008a), we next tested to see whether the anti-inflammatory properties of UCC118 also required the presence of whole, live and metabolically active cells. There was no difference between the abilities of live UCC118 cells and UCC118 cells killed by UV treatment for 15 min to diminish *H. pylori*-induced IL-8 (Fig. 3c). Thus killed UCC118 cells retained the same anti-inflammatory properties.

Fig. 3. Anti-inflammatory activity of *L. salivarius* UCC118 is not dependent on adhesion, bacteriocin production or cell viability. (a) A UCC118 sortase mutant has reduced adhesion to AGS cells. (b) *H. pylori*-induced IL-8 secretion is downregulated equally by UCC118 wild-type cells, an adhesion mutant and a bacteriocin-deficient mutant. (c) *H. pylori*-induced IL-8 secretion is downregulated equally by wild-type live and UV-killed cells. (d) Cytoplasmic and envelope fractions of UCC118 do not downregulate *H. pylori*-induced IL-8. Data shown are from at least three independent experiments carried out in duplicate. *, *P*-value < 0.05 compared to wild-type cells (a) or AGS cells treated with *H. pylori* alone (b, c, d).

To investigate whether this anti-inflammatory activity of UCC118 could be localized to either the envelope or cytoplasm, fractions of the bacterium were prepared with an equivalent number of cells. Neither the cytoplasmic fraction nor the envelope fraction retained any statistically significant anti-IL-8 activity (Fig. 3d), suggesting that intact cells, either alive or killed, were required.

It is well established that dead *H. pylori* cells do not induce gastric epithelial cells to secrete significant amounts of IL-8 (Aihara *et al.*, 1997; Sharma *et al.*, 1995). As we have previously shown that UCC118 does not have any *in vitro* antimicrobial activity against *H. pylori* (Ryan *et al.*, 2008a), and a bacteriocin-deficient UCC118 isogenic mutant had the same anti-inflammatory cytokine phenotype as the wild-type, this indicates that the decrease in IL-8 observed upon exposure to lactobacilli was not simply due to a decrease in *H. pylori* numbers. It is worth noting that a previous study showing an anti-IL-8 effect of *Lactobacillus gasseri* in a similar *H. pylori* infection model did not control for the fact that if the probiotic can kill or retard growth of *H. pylori*, this will in turn lead to less activation of immune responses to the organism (Tamura *et al.*, 2006).

Acid inhibits IL-8 secretion by AGS cells

Live UCC119 cells inhibited *H. pylori*-induced IL-8 secretion by AGS cells to a greater degree than UCC118

(Fig. 1c). We tested whether this greater inhibitory activity was also present in UV-killed UCC119 cells. Although UV-killed UCC119 cells still inhibited *H. pylori*-induced IL-8, the decrease observed was significantly less than that seen for live UCC119 cells (Fig. 3c and Fig. 4a). The levels of IL-8 that we measured using UV-killed UCC119 cells were not significantly different to those seen with live or UV-killed UCC118 cells, suggesting that the source of the effect was possibly the same in both cases. It also suggested that UCC119 had a second viability-dependent mechanism for modulating IL-8 production. We had observed that strain UCC119 produced much more acid in culture than UCC118, so we hypothesized that this may be responsible for the IL-8 reduction difference observed between the strains. We grew UCC119 overnight in Ham's F-12 medium, centrifuged the culture and used the filtered cell-free supernatant instead of cells in the co-culture assay. This UCC119 spent medium (pH 4) completely abolished IL-8 secretion by AGS cells infected with *H. pylori* (Fig. 4b). In contrast, UCC119 spent medium adjusted to pH 7 (with filtered sodium hydroxide) did not have any effect on IL-8 secretion (Fig. 4b). We tested AGS cell viability using trypan blue staining and did not observe any differences in viability comparing cells treated with either UCC119 spent media or *H. pylori* alone (data not shown). Furthermore, although we observed a slight decrease in *H. pylori* viability at pH 3, the viability of *H. pylori* cells was not diminished at pH 4.

Fig. 4. Inhibition of *H. pylori*-induced IL-8 secretion by *L. salivarius* UCC119 is partly due to the production of acid. (a) UV-killed UCC119 cells do not inhibit *H. pylori*-induced IL-8 as well as live cells. (b) UCC119 cell supernatant inhibits *H. pylori*-induced IL-8; UCC119 cell supernatant adjusted to pH 7 does not. (c) In the absence of lactobacilli, acid alone can inhibit *H. pylori*-induced IL-8 secretion by AGS cells. Data shown are from at least three independent experiments carried out in duplicate. *, *P*-value < 0.05 compared to AGS cells treated with *H. pylori* alone. SN, supernatant.

We next assessed whether adjusting the pH of the tissue culture media alone would have any effect on *H. pylori*-induced IL-8 in the absence of UCC119 cells or media (Fig. 4c). AGS cells did not produce any IL-8 in response to *H. pylori* infection when the medium was adjusted to pH 4 or pH 3. *H. pylori*-induced IL-8 decreased at pH 5 although this was not significant, and there was no reduction at pH 6. Thus lowering the pH in the vicinity of gastric epithelial cells by lactobacillus fermentation activity could contribute to a reduction in inflammation.

A previous study by Choi *et al.* (2007) has shown, contradictory to our own findings, that IL-8 secretion by AGS cells in response to *H. pylori* infection is in fact enhanced at lower pH. One explanation for this difference might be due to the different properties of the acids used to adjust the pH of the media. The previous study used hydrochloric acid whereas we used lactic acid. Both this study and that of Choi *et al.* (2007) were performed in the absence of serum.

L. salivarius UCC118 alters expression and functionality of the Cag type IV secretion system

It is well established that proteins of the Cag PAI are largely responsible for inducing IL-8 secretion by gastric epithelial cells, both *in vivo* and *in vitro* (Brandt *et al.*, 2005; Fischer *et al.*, 2001; Sharma *et al.*, 1995). We therefore carried out qRT-PCR on 12 genes of the Cag PAI from *H. pylori* cells taken from the co-culture system to test for changes in the expression of these genes in the presence of *L. salivarius*. Strain UCC118 was used, since strain UCC119 has direct anti-*H. pylori* activity that would have confounded the assay (Ryan *et al.*, 2008a). *cag* genes spanning the entire PAI were chosen. When it appeared likely from bioinformatic analysis that a group of genes were co-transcribed, we endeavoured to include at least one gene from each transcriptional unit. Of the 12 genes tested, eight were

downregulated in the presence of UCC118 cells (Fig. 5), and of these, five were downregulated more than twofold (HP0520, HP0527, HP0534, HP0538 and HP0539). One gene, HP0528, was upregulated and there was no change in the expression of three genes. Expression of *cagA* (HP0547) was unchanged in the presence of UCC118.

We used Western immunoblotting to examine cellular levels of CagA protein in *H. pylori* in either the presence or absence of *L. salivarius* UCC118. Higher levels of CagA were observed in *H. pylori* cells in the presence of *L. salivarius* UCC118, indicating that the Cag secretion system was disrupted (Fig. 6). There was also an increase in the amount of the 40 kDa processed form of CagA (Moese *et al.*, 2001) in the presence of UCC118 (marked with an arrow). Cellular levels of the constitutively expressed protein Lpp20 (O'Toole *et al.*, 1995) were unchanged by exposure of *H. pylori* cells to *L. salivarius* (Fig. 6). We were unable to detect CagA protein in the cell supernatants (data not shown). This is not surprising since CagA is only translocated in significant amounts into host cells via the Cag secretion needle at later time points (>12 h) (Brandt *et al.*, 2005). Prior to this, Cag-mediated IL-8 induction is primarily through a peptidoglycan–Nod1-mediated pathway (Viala *et al.*, 2004). Our data, however, suggest that UCC118 may affect the virulence of *H. pylori* by decreasing the functionality of the secretion system at early time points.

Other lactobacilli modulate *cag* expression

To investigate whether the ability to downregulate *cag* genes was specific to *L. salivarius* UCC118, we tested the expression of three *cag* genes in the presence of four other lactobacilli (Fig. 7). These genes were chosen because they had previously been shown to be essential for maximal IL-8 induction (Fischer *et al.*, 2001) and also because they were downregulated in the presence of UCC118 (Fig. 5). Only

Fig. 5. The presence of *L. salivarius* UCC118 downregulates expression of genes of the *H. pylori* *cag* pathogenicity island. Schematic showing the gene orientation of the *H. pylori* Cag PAI (TIGR gene annotations are shown below the gene). Expression of boxed genes was measured in the presence or absence of *L. salivarius* UCC118. Genes in black were downregulated in the presence of UCC118, those in stripes showed no change, and those in grey were upregulated. Fold change in gene expression is shown above the gene (standard error of the mean is given in parentheses). Data shown are from three independent experiments. Genes marked with a dot are known to be essential for maximum IL-8 induction (see text).

Fig. 6. CagA protein accumulates in *H. pylori* cells cultured in the presence of *L. salivarius* UCC118 after 2 h and 4 h. Lanes: 1, *H. pylori* alone; 2, *H. pylori* alone 2 h; 3, *H. pylori* and *L. salivarius* UCC118, 2 h; 4, *H. pylori* alone 4 h; 5, *H. pylori* and *L. salivarius* UCC118 4 h. The blot shown is representative of three independent experiments. A densitometric analysis of the blot is also shown for comparison purposes. Black bars represent abundance of the 40 kDa form of CagA while the white bars represent abundance of the 120 kDa form, both normalized to abundance of Lpp20.

gene HP0539 in the presence of *L. sakei*, and HP0527 in the presence of *L. acidophilus*, were downregulated to statistically significant levels. There was no change in expression of most genes, with the exception of HP0527, which was upregulated in the case of 3/4 lactobacilli. It is interesting to note that *L. salivarius* UCC118 downregulated the expression of the *cag* genes examined more consistently than the other species studied.

Fig. 7. Comparison of expression of three essential *cag* genes in *H. pylori* cells cultured with *L. salivarius* UCC118 (*L. sal*) and four other lactobacilli as described in Methods: *L. sakei* (*L. sak*); *L. plantarum* (*L. plant*); *L. acidophilus* (*L. acid*); *L. reuteri* (*L. reut*).

Interestingly, recent studies on *E. coli* have shown that probiotics can downregulate virulence genes of the locus of enterocyte effacement (LEE) PAI (Jelcic *et al.*, 2008; Medellin-Pena *et al.*, 2007), possibly through a quorum sensing-mediated process (Jelcic *et al.*, 2008). In the present study, since whole UCC118 cells either live or UV-killed repressed *H. pylori*-induced IL-8 but envelope or cytoplasmic fractions did not, it is possible that we are observing a similar phenomenon. Furthermore, envelope fractions of UCC118 did not decrease expression of any of the 12 *cag* genes examined (data not shown). Moreover strain UCC118 was not able to decrease TNF- α -induced IL-8, suggesting that the bacteria were not a general anti-inflammatory stimulus to AGS cells. This is consistent with the evidence presented here that reduction in *cag* gene transcription and intracellular CagA accumulation is the basis for the anti-inflammatory effect seen with *L. salivarius* UCC118.

Concluding remarks

Direct inhibition of *H. pylori* by *L. salivarius* is strain-specific (Ryan *et al.*, 2008a) and thus a combination of variable lactobacillus populations in the stomach, and variable lactobacillus phenotypes (Fig. 8) – anti-*H. pylori* activity and modulation of inflammatory cytokine pro-

Fig. 8. Schematic diagram showing how *L. salivarius* might modulate the immune response of gastric epithelial cells to *H. pylori* by a number of different mechanisms.

duction – could add another dimension to the recognized dependence of clinical severity and outcome of *H. pylori* infection on *H. pylori* genotype and host genotype (reviewed by Gillen & McColl, 2005). As illustrated in Fig. 8, enhanced CCL20 and CXCL10 combined with decreased IL-8 secretion during *H. pylori* infection would skew the host immune system away from strongly neutrophil-mediated chronic inflammation (Robinson *et al.*, 2007) and towards a more cell-mediated immunity by increasing numbers of macrophages, B cells and T cells (Neville *et al.*, 1997; Schutyser *et al.*, 2003).

This work describes a novel mechanism for how probiotics can decrease inflammation during infection with the pathogen *H. pylori* and this could explain why some probiotic bacteria have been shown to have a positive effect on *H. pylori*-associated inflammation without clearing the infection. Preclinical studies have already shown that vaccines can clear *Helicobacter* infection, indicating that it may be possible to successfully exploit the host immune system to alter the outcome of infection (Velin *et al.*, 2005; Weltzin *et al.*, 1997). In addition to acid production, our data indicate a second mechanism for inhibition of IL-8 production by AGS cells that requires intact whole lactobacillus cells, but not necessarily live cells. Arguing for such a specific mechanism is the fact that several other lactobacillus species tested failed to impact on *cag* gene transcription, even though some, like *L. acidophilus*, also bind to human epithelial cells *in vitro* (Buck *et al.*, 2005). A candidate mechanism could be energy-dependent secretion of a preformed metabolite or protein that inhibits *cag* gene transcription. This could be a direct lactobacillus–*H. pylori* interaction effect, or driven by feedback from altered interaction of *H. pylori* cells with the AGS cells. Experiments are under way to explore these possibilities.

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