OmpR positively regulates urease expression to enhance acid survival of *Yersinia pseudotuberculosis*

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Yersinia pseudotuberculosis is an enteric bacterium which must overcome the acidic stress in host organs for successful colonization, but how this bacterium survives in acidic conditions remains largely unknown. In the present study, the importance of OmpR in acid survival of *Y. pseudotuberculosis* YpIII was confirmed by the fact that mutation of *ompR* (strain $\Delta ompR$) greatly reduced cell survival at pH 4.5 or lower. To characterize the regulatory role of OmpR in this acid survival process, proteomic analysis was carried out to compare YpIII at pH 7.0 and pH 4.5 with $\Delta ompR$ at pH 7.0, and urease components were revealed to be the main targets for OmpR regulation. Addition of urea to the culture medium also enhanced acid survival of YpIII but not $\Delta ompR$ and urease activity was significantly induced by acid in YpIII but not in $\Delta ompR$. Each of the seven components of the YpIII urease gene cluster was fused to a *lacZ* reporter and their expression was dramatically decreased in a $\Delta ompR$ background; this supports the notion that OmpR positively regulates urease expression. Furthermore, gel shift analysis revealed that OmpR binds to the deduced promoter regions of three polycistronic transcriptional units (*ureABC*, *ureEF* and *ureGD*) in the urease cluster, suggesting that the regulation of OmpR to urease components is direct. Taken together, these data strongly suggest that OmpR activates urease expression to enhance acid survival in *Y. pseudotuberculosis*.

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INTRODUCTION

The acidity of the stomach is a primary barrier which all food-borne microbial pathogens must overcome. Consequently, many food-borne pathogens have developed survival systems that protect against acidic conditions (Bearson *et al.*, 1997; Lin *et al.*, 1995). There are two types of acid survival systems currently described. The acid tolerance response (ATR), usually induced by sublethal environmental acid pH (pH 5.5 to 4.5), is a moderately effective system that protects bacteria such as *Salmonella enterica* at pH 3.0 or above (Boot *et al.*, 2002). However, some bacteria have more effective survival mechanisms known as acid resistance (AR) systems that will protect at pH 2.5 or below. AR systems have been extensively studied in *Helicobacter pylori*, *Salmonella typhimurium* and *Escherichia coli* (Bearson *et al.*, 1997; Lin *et al.*, 1995; Pflock *et al.*, 2006b). *E. coli* possesses at least three complex cellular systems involved in the regulation of cytoplasmic

pH (Foster, 2004; Richard & Foster, 2003), with RpoS (also known as σ^S) and CRP (cAMP receptor protein), GadABC and AdiAC (amino acid decarboxylases and cognate antiporters) playing key roles in these systems. In addition, many other genes which express metabolic enzymes, periplasmic proteins and regulators involved in AR in *E. coli* have also been examined by microarray and proteomic 2D-gel analyses (Blankenhorn *et al.*, 1999; Tucker *et al.*, 2002). Recently, the Lon protease, endoRNase RNase E and chaperone Hsp31 have been shown to be important in controlling AR systems in *E. coli* (Heuveling *et al.*, 2008; Mujacic & Baneyx, 2007; Takada *et al.*, 2007), and the roles of AraC-family regulators GadX and GadW and the multidrug resistance regulator MarA in AR systems have also been investigated (Ruiz *et al.*, 2008; Sayed *et al.*, 2007; Tramonti *et al.*, 2006). Nevertheless, despite advances in unravelling some of the regulatory networks involved in AR systems (Foster, 2004; Masuda & Church, 2003), it remains unclear how these factors function together to promote cell survival in low pH conditions.

In addition to resistance to extremely acidic pH, food-borne pathogens also develop ATR systems to survive

Abbreviations: AR, acid resistance; ATR, acid tolerance response; YLB, *Yersinia-Luria-Bertani*.

Two supplementary tables and two supplementary figures are available with the online version of this paper.

weakly acidic environments in acidic foods, animal feeds, food-processing treatments, macrophage phagolysosomes and intestines (Castillo *et al.*, 1999; Leyer *et al.*, 1995; Young *et al.*, 1996). The ATR has been extensively studied in *S. enterica* serovar Typhimurium, revealing a number of acid-shock proteins which are required in this process (Bang *et al.*, 2000; Lee *et al.*, 1994). Urease, a nickel metalloenzyme catalysing the hydrolysis of urea to form carbon dioxide and ammonia, was shown to enhance acid tolerance in *Yersinia enterocolitica* (De Koning-Ward & Robins-Browne, 1995; Young *et al.*, 1996) and the AR in *H. pylori* (Marshall *et al.*, 1990). It has been proposed that urease hydrolyses urea in animal organs and produces sufficient ammonia to raise the pH to levels at which the bacteria remain viable (Stingl & De Reuse, 2005).

Y. pseudotuberculosis, a Gram-negative gastrointestinal pathogenic bacterium, causes self-limiting gastroenteritis and mesenteric lymphadenitis. Transmission between animals and humans is normally through the ingestion of contaminated food or faecal material (Nagano *et al.*, 1997). Survival in acidic environments is important for infection, but the *Y. pseudotuberculosis* acid response is not well understood. Although several homologues of genes such as *rpoS*, *crp* and *ureABC*, which are important for acid survival in other pathogenic bacteria (Castanie-Cornet *et al.*, 1999; De Koning-Ward & Robins-Browne, 1995), are present in *Y. pseudotuberculosis*, it remains unclear which of these genes are involved in acid survival in this bacterium. PhoP (regulator of the PhoP-PhoQ system) has been shown to play a key role in survival in macrophages in *Y. pseudotuberculosis* (Grabenstein *et al.*, 2004) and the two-component system regulon assay also showed that mutations of key regulator genes such as *ompR* (regulator of the EnvZ-OmpR system), *phoP* and *pmrA* (regulator of the PmrA-PmrB system) altered its acid survival (Flamez *et al.*, 2008). Among these regulators, OmpR is the most important two-component regulator in the acid response since mutation of *ompR* decreased the acid survival of *Y. pseudotuberculosis* 32777 compared to other mutants, such as *phoP* and *pmrA* mutants (Flamez *et al.*, 2008). Since the details of the regulatory role of OmpR in this process are unclear, we constructed an *ompR* mutant in *Y. pseudotuberculosis* to investigate the role of OmpR in acid survival. We then sought to investigate by proteomic analysis how OmpR regulates other proteins to increase acid survival in *Y. pseudotuberculosis* and revealed that urease components are the main targets for OmpR regulation in acid survival. Transcriptional analysis and gel retardation assays indicate that the urease locus is divided into three transcriptional units and that OmpR directly binds to the promoter regions upstream of each of these transcriptional units. We propose that this binding activates urease expression and enhances the acid survival response of *Y. pseudotuberculosis*.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli*

cultures were routinely grown in Luria-Bertani (LB) medium at 37 °C. *Y. pseudotuberculosis* (YpIII) was grown in Yersinia-Luria-Bertani (YLB) broth or on YLB agar (YLB_A) plates (Lennox, 1955) at 28 °C or 37 °C with the appropriate antibiotics where required.

Acid survival assays. Stationary-phase overnight cultures of YpIII, the *ompR* mutant (strain $\Delta ompR$) and its complemented strain ($\Delta ompR$ -com) grown in YLB medium at pH 7.0 were diluted (10^6 c.f.u. ml⁻¹) into fresh YLB at different pH values (pH 5.5–3.0) and incubated at 37 °C for 2 h. The cultures were serially diluted and plated onto YLB_A, and colonies were counted after 20 h growth at 37 °C. Percentage survival was calculated as follows: [(c.f.u. ml⁻¹ after challenge at different pH values)/(c.f.u. ml⁻¹ at pH 7.0 before acid challenge)] × 100.

2D-gel analysis and protein identification. Overnight cultures (stationary phase) of YpIII or $\Delta ompR$ grown at pH 7.0 at 28 °C were shifted to 37 °C for a further 2 h incubation; or for acid challenge, YpIII cultures diluted into YLB at pH 4.5 were incubated at 37 °C for a further 2 h. These cells were then centrifuged at 5000 g for 5 min and washed in 10 mM Tris (pH 7.0) and 0.4 M sucrose. After centrifugation at 5000 g at 4 °C for 5 min, cell pellets were resuspended in lysis buffer containing 8 M urea, 2 M thiourea, 4 % (w/v) 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 1 % (w/v) dithiothreitol, 0.8 % (v/v) ampholytes (pH 4.0–7.0, Amersham), 1 mM phenylmethanesulfonyl fluoride, 350 mM Tris base and 5 mM EDTA. Cells were lysed by sonication and centrifuged at 10000 g at 4 °C for 10 min to remove unbroken cells. The total protein concentration in the supernatant was determined by the method of Bradford (1976), using BSA as a standard. For 2D-gel analysis a Multiphor II electrophoresis unit (Amersham) was used in the first-dimension IEF, and a Bio-Rad Protean II Xi unit (Bio-Rad) in the second-dimension electrophoresis. Each sample was prepared and analysed in triplicate. For comparison of spot densities between different strains and growth conditions, gels were stained with colloidal CBB G-250 and scanned with a PowerLook 1000 (UMAX Technologies). PDQuest version 7.3.0 (Bio-Rad) was used for image analysis. Proteins with densities which increased or decreased ≥2-fold in all three experiments were excised and digested with trypsin, and identified by MALDI-TOF MS.

Mutation and complementation of *ompR*. To construct the $\Delta ompR$ mutant, two DNA fragments (1006 bp and 996 bp) up- and downstream of the *ompR* gene, which omitted 453 bp downstream from the ATG initiation codon in *ompR*, were amplified using two pairs of primers, Pompr-up-SF/Pompr-up-XR and Pompr-down-XF/Pompr-down-BR (primer sequences are listed in Supplementary Table S1, available with the online version of this paper). To construct the $\Delta ureC$ mutant, two fragments (509 bp and 565 bp) up- and downstream of the *ureC* gene, which omitted 510 bp downstream from the ATG initiation codon in *ureC*, were amplified using two pairs of primers, PureC-up-SF/PureC-up-ER and PureC-down-EF/PureC-down-BR (Table S1). For each mutant the PCR products were digested with the appropriate restriction enzymes and inserted into similarly digested pDM4 to obtain pDM4-*ompRm* and pDM4-*ureCm*, and subsequently transformed into *E. coli* S17-1(λ -pir). Transconjugation was performed as described by Atkinson *et al.* (1999) to obtain strains $\Delta ompR$ and $\Delta ureC$, respectively.

To complement the *ompR* mutation, primers P25-SSF and P25A-R (Table S1) were used to amplify a fragment from the multiple cloning site to the p15A replicon of pKT25 (Karimova *et al.*, 1998), and primers PACYC-F and PACYC-p-SR were used to amplify a fragment from the p15A replicon to the initiation codon of the chloramphenicol resistance gene from pACYC184 (Rose, 1988). Overlap PCR was carried out using primers P25-SSF and PACYC-p-SR to obtain the full-length DNA fragment containing the multiple cloning site, p15A

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference
<i>E. coli</i>		
S17-1(λ -pir)	λ -pir lysogen of S17-1, <i>thi pro hsdR hsdM</i> ⁺ <i>recA</i> RP4 2-Tc::Mu-Km::Tn7	Simon <i>et al.</i> (1983)
BL21(DE3)	Host for expression vector pET32a	Novagen
<i>Y. pseudotuberculosis</i>		
YpIII	Wild-type <i>Y. pseudotuberculosis</i> pIB1, Nal ^r	Rosqvist <i>et al.</i> (1988)
$\Delta ompR$	<i>ompR</i> gene deleted in YpIII, Nal ^r	This study
$\Delta ompR$ -com	$\Delta ompR$ carrying plasmid pKT- <i>ompR</i> , Nal ^r Kan ^r	This study
$\Delta ureC$	<i>ureC</i> gene deleted in YpIII, Nal ^r	This study
Plasmids		
pKT100	Cloning vector, p15A replicon, Kan ^r	This study
pKT- <i>ompR</i>	<i>ompR</i> under promoter of chloramphenicol resistance gene in plasmid pKT100, Kan ^r	This study
pET32a	Expression vector for His-bind fusion proteins, Amp ^r	Novagen
pET32a-OmpR	pET32a carrying <i>ompR</i> coding region, Amp ^r	This study
pDM4	Suicide vector, <i>mobRK2 oriR6K</i> (<i>pir</i> requiring) <i>sacBR</i> of <i>Bacillus subtilis</i> , Cm ^r	O'Toole <i>et al.</i> (1996)
pDM4- <i>ompR</i> m	pDM4 carrying two DNA fragments up- and downstream of <i>ompR</i> , Cm ^r	This study
pDM4- <i>ureC</i> m	pDM4 carrying two DNA fragments up- and downstream of <i>ureC</i> , Cm ^r	This study
pDM4- <i>lacZ</i>	pDM4 carrying <i>lacZ</i> , used for <i>lacZ</i> gene fusion construction, Cm ^r	This study
pDM4-1131Z	309 bp DNA fragment before <i>ureA</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study
pDM4-1132Z	483 bp DNA fragment before <i>ureB</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study
pDM4-1133Z	461 bp DNA fragment before <i>ureC</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study
pDM4-1134Z	508 bp DNA fragment before <i>ureE</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study
pDM4-1135Z	532 bp DNA fragment before <i>ureF</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study
pDM4-1136Z	526 bp DNA fragment before <i>ureG</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study
pDM4-1137Z	469 bp DNA fragment before <i>ureD</i> stop codon inserted into pDM4- <i>lacZ</i> , Cm ^r	This study

*Nal^r, Cm^r, Kan^r and Amp^r represent resistance to naladixic acid, chloramphenicol, kanamycin and ampicillin at 15, 30, 50 and 100 μ g ml⁻¹, respectively.

replicon, kanamycin resistance gene and the promoter region of the chloramphenicol resistance gene, which was digested with *Sph*I and ligated to obtain pKT100. *ompR* was amplified using primers PompRo-SF and PompRo-SR and inserted into pKT100 to obtain pKT-*ompR*, which was then introduced into $\Delta ompR$ to obtain the complemented strain ($\Delta ompR$ -com).

Urease activity assay. Urease activity was quantified by determining the rate of ammonia produced from the hydrolysis of urea as described by Young *et al.* (1996). Assays were done in duplicate for at least two time points to determine the rate of ammonia produced per minute. The protein concentration was determined by the method of Bradford (1976), using BSA as a standard. Urease activity was expressed as micromoles ammonia produced per min per mg protein.

Construction of *lacZ* fusions. To construct plasmids containing the genes of the *ureA–D* cluster fused to *lacZ*, each primer pair was designed such that the reverse primer incorporated the 3'-end of the gene (omitting the stop codon, which allows for the continued translation of *lacZ*), and the forward primer of each gene was designed 309–532 bp upstream of the stop codon (all primers are listed in Table S1). Each PCR product (the length of each DNA fragment is listed in Table 1) was inserted between the *Sal*I and *Spe*I sites of pDM4-*lacZ* to generate a series of plasmids named pDM4-1131Z to pDM4-1137Z (Table 1) before being transformed into *E. coli* S17-1(λ -pir). Homologous recombination was carried out as described by Atkinson *et al.* (1999), with the successful reporter insertions being selected on Yersinia selective agar (Oxoid) containing nalidixic acid and chloramphenicol.

β -Galactosidase assay. YpIII or $\Delta ompR$ transformants carrying plasmids pDM4-1131Z to pDM4-1137Z (Table 1) were cultured overnight at 28 °C in YLB and then transferred into fresh acidic YLB at the appropriate pH. After incubation at 37 °C for 2 h, cells were collected and transferred into YLB (pH 7.0). β -Galactosidase activity was determined as described by Miller (1992) and calculated using the formula $[1000 \times (A_{420} - 1.75A_{550})]/[OD_{600} \times \text{culture volume (ml)} \times \text{reaction time (min)}]$.

Transcription unit analysis. YpIII grown to stationary phase was transferred into fresh YLB at pH 4.5 for 2 h at 37 °C. Total RNA was isolated using the E.Z.N.A. Bacterial RNA kit (Omega Bio-Tek). The cDNA was synthesized with the reverse primers P1133R, P1135R, P1136R and P1137R (Table S1) following RNase-free DNase I (Promega) treatment. Primers P1133F, P1132F and P1131F were paired with P1133R to test the transcription of *ureABC*. Similar primer pairs were used in *ureEF* and *ureGD* transcription. Total RNA and genomic DNA were used as templates for negative and positive controls, respectively.

OmpR expression and purification. The coding region of *ompR* was amplified using primers PompR-BF and PompR-HR (Table S1), digested with *Bam*HI and *Hind*III and inserted into similarly digested pET32a (Novagen) to obtain pET32a-OmpR. Following transformation into *E. coli* BL21(DE3) OmpR expression was induced by adding 1 mM IPTG to cultures at an OD₆₀₀ of 0.6 and incubating at 28 °C for 4 h. Ni-NTA resin (Novagen) was used to purify His₆-OmpR as described by the manufacturer.

Gel-retardation assay. To investigate whether OmpR would interact with promoter regions derived from the urease cluster, a

modification of the method described by Huang *et al.* (2006) was performed. DNA fragments named Fa (251 bp), Fe (280 bp), Fg (227 bp) and Fd (248 bp) (see Fig. 4a) were amplified from the urease cluster using primer pairs Pfa-F/Pfa-R, Pfe-F/Pfe-R, Pfg-F/Pfg-R and Pfd-F/Pfd-R (Table S1). Samples (200 ng) of DNA were incubated with various amounts of purified OmpR protein in 20 μ l binding buffer containing 20 mM Tris/HCl (pH 7.4), 4 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 10 % glycerol and 100 ng BSA. For OmpR phosphorylation, 100 mM acetyl phosphate (Sigma) was added to the reaction mixture. OmpR phosphorylation and probe binding were performed at 37 °C for 1 h. The samples were then loaded onto a 6.5 % native polyacrylamide gel. Electrophoresis was performed in 0.5 × TBE buffer containing 45 mM Tris, 45 mM boric acid and 1 mM EDTA on ice for 4 h. The gel was stained with ethidium bromide for 20 min and scanned using a Syngene GeneGenius gel documentation system.

RESULTS

OmpR is essential for survival of YpIII at acidic pH

After incubation at pH 4.5 for 2 h, the survival of the YpIII parent and $\Delta ompR$ -com strains was 62.3 % and 73.6 %, respectively, but only 4.5 % of the $\Delta ompR$ cells remained viable (Fig. 1). Survival of YpIII and $\Delta ompR$ -com at pH 4.0 was also much higher than that of $\Delta ompR$. None of the strains were able to tolerate pH below 4.0, with little or no survival once challenged (Fig. 1). This result underlines the importance of OmpR in acid survival of YpIII at pH 4.0–5.0.

Identification of OmpR-regulated proteins related to acid survival by 2D-gels

Since OmpR appears to be important for acid survival of YpIII, proteomic 2D-gel analysis was performed to identify OmpR-regulated proteins which might be involved in this response. Global proteins isolated from YpIII grown at pH 4.5 and pH 7.0 and $\Delta ompR$ at pH 7.0 were compared to find proteins regulated by both the acid response and OmpR. 2D-gel analysis revealed that in the YpIII parent 21 spots showed a >2-fold change at pH 4.5 compared to

pH 7.0. Six of these spots were found to be OmpR-regulated since their expression was increased at pH 4.5 compared to pH 7.0 in YpIII but decreased at pH 7.0 in $\Delta ompR$ (Table S2). These six proteins were examined by MALDI-TOF MS; spots 20 and 21 were identified as the α subunit of urease (UreC) and spot 6 was identified as the urease accessory protein, UreG (Fig. 2). Both UreC and UreG have been reported to participate in acid tolerance in *Y. enterocolitica* (De Koning-Ward & Robins-Browne, 1995), indicating that urease is a major candidate for OmpR-regulated proteins related to acid survival in YpIII.

OmpR controls urease activity

To test if urease is important for OmpR-controlled acid survival, the effects of urea on cell survival of YpIII and $\Delta ompR$ were tested. As shown in Fig. 3(a), increasing the concentration of urea enhanced the survival of the YpIII parent and $\Delta ompR$ -com at pH 4.5 but failed to increase the survival of $\Delta ompR$ and $\Delta ureC$ (used as a control in this assay), even in the presence of 20 mM urea. These data are consistent with the results presented in Fig. 2, which show that $\Delta ompR$ poorly expresses urease components. Similar results were also obtained at pH 5.0 and pH 4.0 (data not shown). These results suggest that urease is important for OmpR-controlled acid survival in YpIII.

A quantitative urease activity assay was also performed to compare urease activities between YpIII, $\Delta ompR$ and $\Delta ompR$ -com. Urease activities increased in YpIII and $\Delta ompR$ -com with decreasing pH, but there was no obvious increase in urease activity in $\Delta ompR$ or $\Delta ureC$ (Fig. 3b). Moreover, $\Delta ompR$ showed lower urease activity than YpIII at each pH, and the differences were more pronounced at acidic pH (Fig. 3b). Taken together, these two results demonstrate that OmpR positively controls urease activity in a pH-dependent manner.

OmpR positively regulates urease gene expression

The chromosomally located *ure* locus of YpIII, which is responsible for the production of urease components, was analysed; this revealed that the genes encoding three urease subunits (α , β and γ) and four accessory proteins (UreDEFG) are arranged as reported for *Y. enterocolitica* (De Koning-Ward *et al.*, 1994) (Fig. 4a). In order to test which of these genes were regulated by OmpR, each was fused to *lacZ* and β -galactosidase activity was measured at pH 7.0, 5.5, 5.0 and 4.5 in YpIII and $\Delta ompR$. Fig. 4(b) shows that the expression of *ureA–ureD* decreased in the *ompR* mutant compared to the YpIII parent. This result supports our 2D-gel data showing that the expression of UreC and UreG decreases in $\Delta ompR$ (Fig. 2). In addition, an increase in the expression of all of the urease components at acidic pH was observed in YpIII but not in $\Delta ompR$, which indicates that OmpR plays a key role in the acid-induction of urease components (Fig. 4b).

Fig. 1. Survival of YpIII, $\Delta ompR$ and its complemented strain ($\Delta ompR$ -com) at different pH values.

Fig. 2. Proteomic comparison of YpIII at pH 4.5 and pH 7.0 with $\Delta ompR$ at pH 7.0 using 2D-gel analysis. Arrows indicate proteins whose expression was increased at pH 4.5 compared to pH 7.0 in YpIII but decreased at pH 7.0 in $\Delta ompR$.

OmpR directly binds to the urease cluster

To determine whether OmpR directly regulates urease expression, gel-retardation assays were undertaken to analyse the interaction of OmpR with the promoter regions of components of the urease cluster. Since the transcriptional organization of the urease cluster is unclear in YpIII it was first necessary to identify the promoters in this region. Inverse PCR was applied to confirm which of these genes were in the same transcriptional unit. RNA was first amplified using the seven primer pairs designed to the urease genes to confirm that there was no genomic DNA contamination after DNase digestion (Fig. 5b, lane R, as an example and data not shown) and then cDNA was synthesized as described in Methods. Fig. 5(b) reveals that *ureABC* were amplified together from the same cDNA (Fig. 5b, lane 3), indicating that they are in same transcriptional unit. *ureEF* were also amplified together, indicating that they also form a distinct transcriptional unit (Fig. 5b, lane 5) as do *ureGD* (Fig. 5b, lane 10). Because *ureCEF* could not be amplified from the same cDNA we propose that *ureEF* and *ureABC* are transcribed separately (Fig. 5b, lane 6). Similarly, *ureEF* and *ureGD* are two independent transcription units because *ureFG* could not be amplified from the same cDNA (Fig. 5b, lane 8). Taken together, these results indicate that there are three transcriptional units (*ureABC*, *ureEF* and *ureGD*) in the urease cluster with a promoter region located upstream of each unit in YpIII. In accordance with this cluster organization, the three transcriptional units are separated by two intergenic regions (296 bp and 127 bp) in the YpIII chromosome (Fig. 4a). In gel-retardation experiments, three different DNA fragments of 251, 227 and 280 bp upstream of *ureA*, *ureE* and *ureG* named Fa, Fe, Fg respectively (Fig. 4a) were amplified and used as the targets for OmpR binding in gel retardation assays. A fourth fragment of 248 bp (Fd; Fig. 4a), which is located upstream of *ureD*, was used as a control. OmpR interacts with Fa, Fe and Fg since the bound fragments (B) are clearly visible (Fig. 6a, b, c) while

Fig. 3. Effect of urea on (a) acid survival and (b) urease activity of YpIII, $\Delta ompR$, $\Delta ompR-com$ and $\Delta ureC$ at different pH values.

Fig. 4. (a) Organization of the genes constituting the urease locus of YpIII. Fragments upstream of *ureA*, *ureE*, *ureG* and *ureD* (Fa, Fe, Fg and Fd) were amplified by PCR and used in OmpR binding activity assays. (b) Expression of the urease cluster genes in YpIII and $\Delta ompR$ at different pH values.

the control DNA fragment Fd showed no interaction with OmpR (Fig. 6d). This result helps to confirm that OmpR binds directly to the promoter regions in the urease cluster to activate the expression of urease components.

DISCUSSION

Enteric pathogens must pass through the stomach at pH < 3.0 for up to 2 h before colonizing the intestinal tract (Giannella *et al.*, 1972). In the present study, over 50 % of

Fig. 5. (a) Transcriptional unit analysis of the urease cluster. DNA fragments that can be amplified from one cDNA are shown as solid lines and '+' and fragments that cannot be amplified are shown as dashed lines and '-'. The transcriptional units identified are labelled 'Tran' and deduced promoters are indicated as 'P'. (b) Inverse PCR. Lanes 1–10 correspond to the row numbers in (a). Lanes R and G are PCR products using RNA and genomic DNA isolated from YpIII as templates for primers P1136F and P1136R, respectively. Lane M, size standards.

Fig. 6. Interaction of OmpR with the promoter regions in the urease cluster studied by gel-retardation assays. The promoter regions of *ureABC* (Fa) (a), *ureEF* (Fe) (b) and *ureGD* (Fg) (c) were incubated with different amounts of OmpR protein (shown at the top of each lane). The positions of bound (B) and free (F) fragments are shown to the right of each panel. A 248 bp DNA fragment upstream of *ureD* named Fd was amplified and used as a negative control (d).

the YpIII parent challenged at pH 4.5 for 2 h survived but this decreased to less than 0.1% when the bacteria were challenged at pH 3.0 for 2 h (Fig. 1). These data do not correlate with a report that 3% of *Y. pseudotuberculosis* strain 32777 survived when challenged at pH 3.0 for 2 h (Flamez *et al.*, 2008). It has been reported that some *Y. pseudotuberculosis* isolates carry non-functional alleles of *phoP*, which is key for survival in the acidic environment in macrophages (Grabenstein *et al.*, 2004). Whether the difference in acid survival between strains 32777 and YpIII is due to the difference in PhoP function needs to be addressed. Moreover, differences in acid survival among different *S. enterica* strains have also been reported (Berk *et al.*, 2005) and it is therefore entirely possible that different strains of *Y. pseudotuberculosis* exhibit different acid survival abilities. Pre-adaptation of YpIII to pH 4.5 for 2 h greatly enhanced cell survival at pH 3.0 in the presence of urea (Fig. S1), indicating that an adaptive AR also exists in YpIII as reported for *S. typhimurium* (Bearson *et al.*, 1998) and that acid tolerance at sublethal pH is a key factor for survival of YpIII at extremely acidic pH. The mechanism by which YpIII withstands extreme acidic stress *in vivo* is currently under investigation.

The two-component regulator OmpR has long been considered a global regulator, but the majority of the data concerning OmpR-associated regulation concerns virulence factors and outer-membrane proteins (Brzostek *et al.*, 2007; Yoshida *et al.*, 2006). Although OmpR is known to participate in acid responses in *S. enterica* and *Y.*

pseudotuberculosis (Bang *et al.*, 2000; Flamez *et al.*, 2008), the mechanisms behind OmpR-mediated acid survival were not clear. In the present study, we used proteomics analysis to scan targets regulated by OmpR in the acid response and showed that the expression of two urease components, *ureC* and *ureG*, was increased in the parent YpIII strain at pH 4.5 and downregulated in the Δ *ompR* strain (Fig. 2). Our data strongly suggest that urease, which has been reported to be related to resistance to gastrointestinal stress in several bacteria in the presence of urea (Bandara *et al.*, 2007; Maroncle *et al.*, 2006), is regulated by OmpR during acid survival in YpIII. Studies with other bacterial species have shown that urease expression is activated by H-NS, UreR, NikR and ArsR (Coker *et al.*, 2000; Dattelbaum *et al.*, 2003; Delany *et al.*, 2005; Pflock *et al.*, 2005; Pflock *et al.*, 2006a), but to our knowledge OmpR-mediated urease expression has not been reported previously.

Upon fusing each of the genes in the urease cluster to *lacZ* we found that OmpR plays a positive regulatory role (Fig. 4), although how OmpR functions in this regulatory process was unclear. Urease is a multimer that contains two or three subunit complexes and several accessory proteins (De Koning-Ward *et al.*, 1994). Earlier studies on urease-mediated acid survival focused on mutation of the whole cluster (De Koning-Ward & Robins-Browne, 1995) rather than individual component genes, although Northern blot analysis highlighted five transcriptional units, *ureABI*, *ureAB*, *ureIE'*, *ureI* and *ure'FGH*, in *H. pylori* (Akada *et al.*

al., 2000). Our study indicates that there are three transcriptional units (*ureABC*, *ureEF* and *ureGD*) in the YpIII urease cluster and that OmpR binds to the region presumed to be the promoter region upstream of each transcriptional unit (Fig. 6). Upon comparing the interaction of OmpR and urease promoters in the presence and absence of acetyl phosphate, we observed that there was a weaker interaction when OmpR was not phosphorylated (Fig. S2), which suggests that the regulation of urease expression by OmpR is not only direct but also enhanced by OmpR phosphorylation. These data are consistent with a report showing that the regulatory link between OmpR and outer-membrane proteins is enhanced by OmpR phosphorylation (Yoshida *et al.*, 2006). It is noteworthy that the regulation of urease expression by OmpR was enhanced at acidic pH (Fig. 4b), although there were no differences in expression of OmpR in YpIII between neutral and acidic pH as determined by 2D-gel or *lacZ* fusion analyses (data not shown). These data lead us to speculate that either (i) the phosphorylation of OmpR is promoted by acid, more OmpR thereby binding to urease promoter regions to activate the expression of urease genes at acidic pH than at neutral pH, or (ii) an acid-induced protein mediates the regulation of urease expression by OmpR, whereby at neutral pH the absence of mediated protein leads to low expression of urease genes. The detailed mechanism is still largely unknown and needs to be further investigated.

The roles of urease and OmpR in acid survival in YpIII are complicated. Besides OmpR, other regulators also participate in controlling urease expression since urease activity in Δ *ompR* at pH 5.0 and 5.5 was higher than at pH 7.0 (Fig. 3b) and many of the urease gene fusions (most notably *ureB-lacZ*) are acid-inducible even in the absence of OmpR (Fig. 4b). Moreover, fusions with genes in the same transcriptional unit showed different β -galactosidase activities. For example, *ureD* was transcribed together with *ureG* (Fig. 5), but the expression of *ureD* was lower than that of *ureG* (Fig. 4). This suggests that some post-transcriptional regulation of urease production occurs during acid survival. A urease mutant (Δ *ureC*) also exhibited decreased acid survival compared to YpIII in YLB in the absence of added urea (Fig. 3a). To eliminate the effect of low-level urea production from bacterial nitrogen metabolism (Stingl & De Reuse, 2005), which may affect acid survival, we compared the survival of YpIII in defined M9 medium (without tryptone, yeast extract or other potential urea-containing materials) at pH 4.5; we found no obvious difference in cell survival rates between YpIII and Δ *ureC* (data not shown). This strongly suggests that the role of urease in acid survival is also urea-dependent. It is also interesting to note that even without adding urea to the culture medium the acid survival of Δ *ompR* was decreased and was lower than that of Δ *ureC* (Fig. 3a). These results indicate that the role of OmpR in the acid response is multi-faceted; this is also supported by our 2D-gel analyses, where we see several other OmpR-

associated proteins which could be related to the acid response (Table S2). Previous studies have shown that the OmpR-regulated porin proteins OmpC and OmpF were repressed by acid in *E. coli* O157:H7 (Allen *et al.*, 2008) and induced by the acid response regulator CadC in *S. enterica* (Lee *et al.*, 2007). However, our 2D-gel analysis revealed that *ompC* and *ompF* expression was similar between neutral and acidic pH in YpIII (data not shown), which indicates that the relationship between these two proteins and OmpR requires further investigation.

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REFERENCES

- Akada, J. K., Shirai, M., Takeuchi, H., Tsuda, M. & Nakazawa, T. (2000). Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. *Mol Microbiol* **36**, 1071–1084.
- Allen, K. J., Lepp, D., McKellar, R. C. & Griffiths, M. W. (2008). Examination of stress and virulence gene expression in *Escherichia coli* O157:H7 using targeted microarray analysis. *Foodborne Pathog Dis* **5**, 437–447.
- Atkinson, S., Throup, J. P., Stewart, G. S. & Williams, P. (1999). A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol Microbiol* **33**, 1267–1277.
- Bandara, A. B., Contreras, A., Contreras-Rodriguez, A., Martins, A. M., Dobrea, V., Poff-Reichow, S., Rajasekaran, P., Sriranganathan, N., Schurig, G. G. & Boyle, S. M. (2007). *Brucella suis* urease encoded by *ure1* but not *ure2* is necessary for intestinal infection of BALB/c mice. *BMC Microbiol* **7**, 57.
- Bang, I. S., Kim, B. H., Foster, J. W. & Park, Y. K. (2000). OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar typhimurium. *J Bacteriol* **182**, 2245–2252.
- Bearson, S., Bearson, B. & Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147**, 173–180.
- Bearson, B. L., Wilson, L. & Foster, J. W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J Bacteriol* **180**, 2409–2417.
- Berk, P. A., Jonge, R., Zwietering, M. H., Abee, T. & Kieboom, J. (2005). Acid resistance variability among isolates of *Salmonella enterica* serovar Typhimurium DT104. *J Appl Microbiol* **99**, 859–866.
- Blankenhorn, D., Phillips, J. & Slonczewski, J. L. (1999). Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* **181**, 2209–2216.
- Boot, I. R., Cash, P. & O'Byrne, C. (2002). Sensing and adapting to acid stress. *Antonie Van Leeuwenhoek* **81**, 33–42.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

- Brzostek, K., Brzostkowska, M., Bukowska, I., Karwicka, E. & Raczkowska, A. (2007). OmpR negatively regulates expression of invasins in *Yersinia enterocolitica*. *Microbiology* **153**, 2416–2425.
- Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F. & Foster, J. W. (1999). Control of acid resistance in *Escherichia coli*. *J Bacteriol* **181**, 3525–3535.
- Castillo, A., Lucia, L. M., Goodson, K. J., Savell, J. W. & Acuff, G. R. (1999). Decontamination of beef carcass surface tissue by steam vacuuming alone and combined with hot water and lactic acid sprays. *J Food Prot* **62**, 146–151.
- Coker, C., Bakare, O. O. & Mobley, H. L. (2000). H-NS is a repressor of the *Proteus mirabilis* urease transcriptional activator gene *ureR*. *J Bacteriol* **182**, 2649–2653.
- Dattelbaum, J. D., Lockatell, C. V., Johnson, D. E. & Mobley, H. L. (2003). UreR, the transcriptional activator of the *Proteus mirabilis* urease gene cluster, is required for urease activity and virulence in experimental urinary tract infections. *Infect Immun* **71**, 1026–1030.
- De Koning-Ward, T. F. & Robins-Browne, R. M. (1995). Contribution of urease to acid tolerance in *Yersinia enterocolitica*. *Infect Immun* **63**, 3790–3795.
- De Koning-Ward, T. F., Ward, A. C. & Robins-Browne, R. M. (1994). Characterisation of the urease-encoding gene complex of *Yersinia enterocolitica*. *Gene* **145**, 25–32.
- Delany, I., Ieva, R., Soragni, A., Hilleringmann, M., Rappuoli, R. & Scarlato, V. (2005). In vitro analysis of protein-operator interactions of the NikR and Fur metal-responsive regulators of coregulated genes in *Helicobacter pylori*. *J Bacteriol* **187**, 7703–7715.
- Flamez, C., Ricard, I., Arafah, S., Simonet, M. & Marceau, M. (2008). Phenotypic analysis of *Yersinia pseudotuberculosis* 32777 response regulator mutants: new insights into two-component system regulon plasticity in bacteria. *Int J Med Microbiol* **298**, 193–207.
- Foster, J. W. (2004). *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat Rev Microbiol* **2**, 898–907.
- Giannella, R. A., Broitman, S. A. & Zamcheck, N. (1972). Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. *Gut* **13**, 251–256.
- Grabenstein, J. P., Marceau, M., Pujol, C., Simonet, M. & Bliska, J. B. (2004). The response regulator PhoP of *Yersinia pseudotuberculosis* is important for replication in macrophages and for virulence. *Infect Immun* **72**, 4973–4984.
- Heuveling, J., Possling, A. & Hengge, R. (2008). A role for Lon protease in the control of the acid resistance genes of *Escherichia coli*. *Mol Microbiol* **69**, 534–547.
- Huang, Y. Y., Deng, J. Y., Gu, J., Zhang, Z. P., Maxwell, A., Bi, L. J., Chen, Y. Y., Zhou, Y. F., Yu, Z. N. & Zhang, X. E. (2006). The key DNA-binding residues in the C-terminal domain of *Mycobacterium tuberculosis* DNA gyrase A subunit (GyrA). *Nucleic Acids Res* **34**, 5650–5659.
- Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A* **95**, 5752–5756.
- Lee, I. S., Slonczewski, J. L. & Foster, J. W. (1994). A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. *J Bacteriol* **176**, 1422–1426.
- Lee, Y. H., Kim, B. H., Kim, J. H., Yoon, W. S., Bang, S. H. & Park, Y. K. (2007). CadC has a global translational effect during acid adaptation in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **189**, 2417–2425.
- Lennox, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.
- Leyer, G. J., Wang, L. L. & Johnson, E. A. (1995). Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Appl Environ Microbiol* **61**, 3752–3755.
- Lin, J., Lee, I. S., Frey, J., Slonczewski, J. L. & Foster, J. W. (1995). Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. *J Bacteriol* **177**, 4097–4104.
- Maroncle, N., Rich, C. & Forestier, C. (2006). The role of *Klebsiella pneumoniae* urease in intestinal colonization and resistance to gastrointestinal stress. *Res Microbiol* **157**, 184–193.
- Marshall, B. J., Barrett, L. J., Prakash, C., McCallum, R. W. & Guerrant, R. L. (1990). Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. *Gastroenterology* **99**, 697–702.
- Masuda, N. & Church, G. M. (2003). Regulatory network of acid resistance genes in *Escherichia coli*. *Mol Microbiol* **48**, 699–712.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics: a Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Mujacic, M. & Baneyx, F. (2007). Chaperone Hsp31 contributes to acid resistance in stationary-phase *Escherichia coli*. *Appl Environ Microbiol* **73**, 1014–1018.
- Nagano, T., Kiyohara, T., Suzuki, K., Tsubokura, M. & Otsuki, K. (1997). Identification of pathogenic strains within serogroups of *Yersinia pseudotuberculosis* and the presence of non-pathogenic strains isolated from animals and the environment. *J Vet Med Sci* **59**, 153–158.
- O'Toole, R., Milton, D. L. & Wolf-Watz, H. (1996). Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. *Mol Microbiol* **19**, 625–637.
- Pflock, M., Kennard, S., Delany, I., Scarlato, V. & Beier, D. (2005). Acid-induced activation of the urease promoters is mediated directly by the ArsRS two-component system of *Helicobacter pylori*. *Infect Immun* **73**, 6437–6445.
- Pflock, M., Finsterer, N., Joseph, B., Mollenkopf, H., Meyer, T. F. & Beier, D. (2006a). Characterization of the ArsRS regulon of *Helicobacter pylori*, involved in acid adaptation. *J Bacteriol* **188**, 3449–3462.
- Pflock, M., Kennard, S., Finsterer, N. & Beier, D. (2006b). Acid-responsive gene regulation in the human pathogen *Helicobacter pylori*. *J Biotechnol* **126**, 52–60.
- Richard, H. T. & Foster, J. W. (2003). Acid resistance in *Escherichia coli*. *Adv Appl Microbiol* **52**, 167–186.
- Rose, R. E. (1988). The nucleotide sequence of pACYC184. *Nucleic Acids Res* **16**, 355.
- Rosqvist, R., Skurnik, M. & Wolf-Watz, H. (1988). Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature* **334**, 522–524.
- Ruiz, C., McMurry, L. M. & Levy, S. B. (2008). Role of the multidrug resistance regulator MarA in global regulation of the *hdeAB* acid resistance operon in *Escherichia coli*. *J Bacteriol* **190**, 1290–1297.
- Sayed, A. K., Odom, C. & Foster, J. W. (2007). The *Escherichia coli* AraC-family regulators GadX and GadW activate *gadE*, the central activator of glutamate-dependent acid resistance. *Microbiology* **153**, 2584–2592.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1**, 784–791.
- Stingl, K. & De Reuse, H. (2005). Staying alive overdosed: how does *Helicobacter pylori* control urease activity? *Int J Med Microbiol* **295**, 307–315.
- Takada, A., Umitsuki, G., Nagai, K. & Wachi, M. (2007). RNase E is required for induction of the glutamate-dependent acid

resistance system in *Escherichia coli*. *Biosci Biotechnol Biochem* **71**, 158–164.

Tramonti, A., De Canio, M., Delany, I., Scarlato, V. & De Biase, D. (2006). Mechanisms of transcription activation exerted by GadX and GadW at the *gadA* and *gadBC* gene promoters of the glutamate-based acid resistance system in *Escherichia coli*. *J Bacteriol* **188**, 8118–8127.

Tucker, D. L., Tucker, N. & Conway, T. (2002). Gene expression profiling of the pH response in *Escherichia coli*. *J Bacteriol* **184**, 6551–6558.

Yoshida, T., Qin, L., Egger, L. A. & Inouye, M. (2006). Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. *J Biol Chem* **281**, 17114–17123.

Young, G. M., Amid, D. & Miller, V. L. (1996). A bifunctional urease enhances survival of pathogenic *Yersinia enterocolitica* and *Morganella morganii* at low pH. *J Bacteriol* **178**, 6487–6495.

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