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 ΔompR) 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 ΔompR 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 ΔompR and urease activity was significantly induced by acid in YpIII but not in ΔompR. 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 ΔompR 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.

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 σs) 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...
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 (YLBa) 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 DompR) and its complemented strain (DompR-com) grown in YLB medium at pH 7.0 were diluted (10° 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 YLBa, and colonies were counted after 20 h growth at 37 °C. Percentage survival was calculated as follows: ([c.f.u. ml−1 at 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 DompR 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 phenylmethylsulfonyl fluoride, 350 mM Tris base and 5 mM EDTA. Cells were lysed by sonication and centrifuged at 10 000 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 Protein 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 DompR 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-SE/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 DureC 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-SE/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 pDNA-ompR and pDNA-ureCm, and subsequently transformed into E. coli S17-1(Δpir), Transconjugation was performed as described by Atkinson et al. (1999) to obtain strains DompR and DureC, 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
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<tr>
<td>S17-1(Δ-pir)</td>
<td>λ-pir lysogen of S17-1, thi pro hsdR hsdM⁺ recA RP4 2-Tc::Mu-Km::Tn7</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Host for expression vector pET32a</td>
<td>Novagen</td>
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<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
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<tr>
<td>YpIII</td>
<td>Wild-type Y. pseudotuberculosis plBl, Nal'</td>
<td>Rosqvist et al. (1988)</td>
</tr>
<tr>
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<td>ompR gene deleted in YpIII, Nal'</td>
<td>This study</td>
</tr>
<tr>
<td>ΔompR-com</td>
<td>ΔompR carrying plasmid pKT-ompR, Nal' Kan'</td>
<td>This study</td>
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<tr>
<td>ΔureC</td>
<td>ureC gene deleted in YpIII, Nal'</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pKT100</td>
<td>Cloning vector, p15A replica, Kan'</td>
<td>This study</td>
</tr>
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<td>pKT-ompR</td>
<td>ompR under promoter of chloramphenicol resistance gene in plasmid pKT100, Kan'</td>
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<td>Expression vector for His-bind fusion proteins, Amp'</td>
<td>Novagen</td>
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<td>pET32a carrying ompR coding region, Amp'</td>
<td>This study</td>
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<td>pDM4</td>
<td>Suicide vector, mobRK2 oriR6K (pir requiring) sacBR of Bacillus subtilis, Cm'</td>
<td>O'Toole et al. (1996)</td>
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<td>pDM4-ompRm</td>
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<td>This study</td>
</tr>
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<td>pDM4-ureCm</td>
<td>pDM4 carrying two DNA fragments up- and downstream of ureC, Cm'</td>
<td>This study</td>
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<td>pDM4 carrying lacZ, used for lacZ gene fusion construction, Cm'</td>
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<td>pDM4-1137Z</td>
<td>469 bp DNA fragment before ureI stop codon inserted into pDM4-lacZ, Cm'</td>
<td>This study</td>
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*Nal', Cm', Kan' and Amp' represent resistance to nalidixic acid, chloramphenicol, kanamycin and ampicillin at 15, 30, 50 and 100 μg ml⁻¹, respectively.

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 309–352 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 into the SalI and SpeI 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(Δ-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 ΔompR 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 \(1000 \times (A_{420}−1.75A_{660})/OD_{600} \times \text{culture volume (ml)} \times \text{reaction time (min)}\).

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 and P1136R and P1137R (Table S1) following RNase-free DNase I (Promega) treatment. Primers P1133F, P1132F and P1131F were paired with P1133R, P1135R and P1137R 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 BamHI and HindIII 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 Histagged 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) andFd (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 µl 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 ΔompR-com strains was 62.3 % and 73.6 %, respectively, but only 4.5 % of the ΔompR cells remained viable (Fig. 1). Survival of YpIII and ΔompR-com at pH 4.0 was also much higher than that of ΔompR. 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 ΔompR 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 ΔompR (Table S2). These six proteins were examined by MALDI-TOF MS; spots 20 and 21 were identified as the ɑ 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 ΔompR were tested. As shown in Fig. 3(a), increasing the concentration of urea enhanced the survival of the YpIII parent and ΔompR-com at pH 4.5 but failed to increase the survival of ΔompR and ΔureC (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 ΔompR 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, ΔompR and ΔompR-com. Urease activities increased in YpIII and ΔompR-com with decreasing pH, but there was no obvious increase in urease activity in ΔompR or ΔureC (Fig. 3b). Moreover, ΔompR 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 (ɑ, 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 ΔompR. 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 ΔompR (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 ΔompR, which indicates that OmpR plays a key role in the acid-induction of urease components (Fig. 4b).
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
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
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 and downregulated in the DompR strain (Fig. 2). Our data strongly suggest that urease, which has been reported to be related 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 & Robins-Browne, 1995). 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, ureAB, ureA'B', ureI and ure'FGH, in H. pylori (Akada et
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 ΔompR 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 (ΔureC) 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 ΔureC (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 ΔompR was decreased and was lower than that of ΔureC (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 OmFC and OmF 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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