Phenotypic and transcriptional analysis of the osmotic regulator OmpR in *Yersinia pestis*

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**Abstract**

**Background:** The osmotic regulator OmpR in *Escherichia coli* regulates differentially the expression of major porin proteins OmpF and OmpC. In *Yersinia enterocolitica* and *Y. pseudotuberculosis*, OmpR is required for both virulence and survival within macrophages. However, the phenotypic and regulatory roles of OmpR in *Y. pestis* are not yet fully understood.

**Results:** *Y. pestis* OmpR is involved in building resistance against phagocytosis and controls the adaptation to various stressful conditions met in macrophages. The *ompR* mutation likely did not affect the virulence of *Y. pestis* strain 201 that was a human-avirulent enzootic strain. The microarray-based comparative transcriptome analysis disclosed a set of 224 genes whose expressions were affected by the *ompR* mutation, indicating the global regulatory role of OmpR in *Y. pestis*. Real-time RT-PCR or *lacZ* fusion reporter assay further validated 16 OmpR-dependent genes, for which OmpR consensus-like sequences were found within their upstream DNA regions. *ompC*, *F*, *X*, and *R* were up-regulated dramatically with the increase of medium osmolarity, which was mediated by OmpR occupying the target promoter regions in a tandem manner.

**Conclusion:** OmpR contributes to the resistance against phagocytosis or survival within macrophages, which is conserved in the pathogenic yersiniae. *Y. pestis* OmpR regulates *ompC, F, X,* and *R* directly through OmpR-promoter DNA association. There is an inducible expressions of the pore-forming proteins OmpF, C, and X at high osmolarity in *Y. pestis*, in contrast to the reciprocal regulation of them in *E. coli*. The main difference is that *ompF* expression is not repressed at high osmolarity in *Y. pestis*, which is likely due to the absence of a promoter-distal OmpR-binding site for *ompF*.

**Background**

The *ompB* operon consists of the *ompR* and *envZ* genes, whose coding regions overlap by several base pairs; this genetic structure is highly conserved in *Enterobacteriaceae* [1,2]. The inner membrane EnvZ, a histidine kinase, acts as a sensor responding to the elevation of medium osmolarity and undergoes trans-autophosphorylation. The high energy of phosphoryl group is subsequently transferred to the cytoplasmic protein OmpR. The phosphorylated OmpR (OmpR-P) acts as a DNA-binding transcription factor to regulate its target genes. EnvZ also possesses the phosphatase activity to dephosphorylate itself. Osmotic signals regulate the ratio of kinase/phosphatase activity of EnvZ to modulate the cellular OmpR-P level [1,2]. At low medium osmolarity, OmpR-P levels are also low due to the decreased kinase/phosphatase ratio of EnvZ; on the other hand, at high osmolarity, an elevated OmpR-P level results from the ratio increase. The *ompR* transcription is induced directly by its own gene product in *Salmonella enterica* [3]. OmpR consensus-like sequences are found in the upstream region of *ompR* in *Escherichia coli*, although there are still no reported experimental data for its autoregulation in this bacterium. Upon the elevation of medium osmolarity, cellular OmpR-P levels are likely enhanced by two distinct mechanisms, namely, post-translational
phosphorylation/dephosphorylation by EnvZ and transcriptional auto-stimulation.

*Enterobacteriaceae* express at least two major outer membrane (OM) porins, namely, OmpF and OmpC, both of which form transmembrane pore structures and function as ion channel [4-6]. OmpF and OmpC in the cell of *E. coli* form water-filled pores that are poorly selective to cations (so-called non-specific porins), thereby allowing the diffusion of low-molecular-weight polar compounds (not over 600 daltons) into the cell to maintain cell permeability. They exist as homotrimers in the OM. The basic structural element of the porin monomer is an ellipsoid in the section cylinder consisting of 16 transmembrane β-strands (so-called β-barrel) connected by short periplasmic and longer ‘external’ loops [7].

*E. coli* OmpX contains 8-stranded β-barrel, with polar residues on the inside and hydrophobic residues on the outside facing the membrane environment [8]. *Enterobacter aerogenes* OmpX is the smallest known channel protein with a markedly cationic selectivity [6,9,10]. Although several experiments have demonstrated that OmpX plays roles that are similar to those of porin [6,9-12], it is not yet clear whether or not OmpX forms pores on the cell membrane. *E. aerogenes* OmpX forms channels in the lipid bilayer [6]; however, the NMR and crystal structures of OmpX do not show pores [8,13]. The ompX expression in *E. coli* [12] or *E. aerogenes* [6] is enhanced during early exposure to environmental perturbations, such as high osmolarity, antibiotics and toxic compounds, that are accompanied by the repressed expression of non-specific porins (OmpF and/or OmpC). Over-expression of OmpX, with a channel structure that is much smaller than that of OmpF and OmpC [6], may stabilize cell OM and balance the decreased expression of the two non-specific porins for the exclusion of small harmful molecules. It is interesting to further investigate the roles of OmpX in modulating OM permeability and adaptability. OmpR consensus-like sequences have been found within the *ompX* upstream region in *E. coli* and *E. aerogenes* [6]; however, the regulation of *ompX* by OmpR has not yet been established experimentally in any bacterium.

As shown in *E. coli* as a model, OmpF and OmpC are reciprocally regulated by medium osmolarity. OmpC is predominant at high osmolarity, while the OmpF expression is repressed; in contrast, the reverse effect is observed at low osmolarity [14]. The reciprocal regulations of Omp36 and Omp35 (OmpF and OmpC-like, respectively) have been established in *E. aerogenes* as well [15]. Tight regulation of porin expression is crucial for bacterial adaptation to environments, which is mediated by a two-component system EnvZ/OmpR [2,16,17]. Likewise, four (tandem F1-F2-F3, and F4) and three (tandem C1-C2-C3) OmpR consensus-like sequences have been determined in the DNA regions upstream of *ompF* and *ompC* in *E. coli*, respectively. At low osmolarity, OmpR-P binds cooperatively to F1-F2 or F1-F2-F3 in order to activate the transcription of *ompF*; meanwhile, it only occupies C1, which is not sufficient to activate the transcription of *ompC*. At high osmolarity, C2-C3 becomes occupied by OmpR-P with the elevated cellular OmpR-P levels, resulting in the *ompC* expression. Moreover, OmpR-P also binds to F4, which is a weak OmpR-P-binding site located 260 bp upstream of F1-F2-F3 to form a loop. In turn, this interferes with the binding of OmpR-P to F1-F2-F3, so as to block the *ompF* transcription.

As a member of the *Enterobacteriaceae* family, the genus *Yersinia* includes three human-pathogenic species, namely, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. pestis* causes the deadly plague, while the latter two only cause non-fatal gastroenteric diseases [18]. *Y. pestis* has evolved recently (from the evolutionary point of view) from *Y. pseudotuberculosis* by a process combining gene acquisition, loss and inactivation, while *Y. enterocolitica* represents a far distinct evolutionary lineage [18]. *Yersinia ompF, C*, and *X* contains conservative amino acid residues or domains typical among porins [7,19-21]. However, regulation of porins in *Y. pestis* is not yet fully understood.

Data presented here disclose that OmpR is involved in the survival of *Y. pestis* within macrophages and in building resistance against various environmental perturbations including osmotic stress. DNA microarray and quantitative RT-PCR have been employed to identify a set of OmpR-dependent genes in *Y. pestis*. *Y. pestis* OmpR simulates *ompC, F, X*, and *R* directly by occupying the target promoter regions. Noticeably, there is an inducible expression of all of *ompF, C, X*, and *R* at high osmolarity in *Y. pestis*, in contrast to the reciprocal regulation of *OmpF* and *OmpC* in *E. coli*. The main difference is that *ompF* expression is not repressed at high osmolarity in *Y. pestis*, which is likely due to the absence of a promoter-distal OmpR-binding site for *ompF*.

**Methods**

**Bacterial strains**

The wild-type (WT) *Y. pestis* biovar microtus strain 201 is avirulent to humans but highly lethal to mice [22]. The 43 to 666 base pairs of *ompR* (720bp in total length) were replaced by the kanamycin resistance cassette using the one-step inactivation method based on the lambda Red phage recombination system, with the helper plasmid pKD46, to generate the *ompR* mutants of *Y. pestis* (designated as Δ*ompR*) [23]. Chromosomal integration of the mutagenic cassette was confirmed by PCR and sequencing using oligonucleotides external to
the integrated cassette (data not shown). The elimination of pKD46 in \(\Delta\text{ompR}\) was verified by PCR.

A PCR-generated DNA fragment containing the \(\text{ompR}\) coding region, together with its promoter-proximal region (~500 bp upstream the coding sequence) and transcriptional terminator (~300 bp downstream), was cloned into the pACYC184 vector harboring a chloramphenicol resistance gene (GenBank accession number X06403), and was then verified by DNA sequencing. The recombinant plasmid was subsequently introduced into \(\Delta\text{ompR}\), producing the complemented mutant strain \(\text{Y. pestis}\ C-\text{ompR}\).

**Bacterial growth and RNA isolation**

Overnight cultures (an \(\text{OD}_{620}\) of about 1.0) of WT or \(\Delta\text{ompR}\) in the chemically defined TMH medium [24] were diluted 1:20 into the fresh TMH. Bacterial cells were grown at 26°C to the middle exponential growth phase (an \(\text{OD}_{620}\) of about 1.0). To trigger the high osmolarity conditions in OmpR-related experiments, a final concentration of 0.5 M sorbitol was added, after which the cell cultures were allowed to grow for another 20 min.

Total RNA of bacterial cells was extracted using the TRIzol Reagent (Invitrogen) without the DNA removal step (for RT-PCR and primer extension) or by using MasterPure™ RNA Purification kit (Epicenter) with the removal of contaminated DNA (for microarray). Immediately before harvesting, bacterial cultures were mixed with RNAsprotect Bacteria Reagent (Qiagen) to minimize RNA degradation. RNA quality was monitored by agarose gel electrophoresis, and RNA quantity was determined using a spectrophotometer.

**Microarray expression analysis**

Gene expression profiles were compared between WT and \(\Delta\text{ompR}\) using a \(\text{Y. pestis}\) whole-genome cDNA microarray as described in a previous work [25]. RNA samples were isolated from four individual bacterial cultures as biological replicates for each strain. The dual-fluorescently (Cy3 or Cy5 dye) labeled cDNA probes, for which the incorporated dye was reversed, were synthesized from the RNA samples. These were then hybridized to 4 separated microarray slides. A ratio of mRNA levels was calculated for each gene. Significant changes of gene expression were identified using the SAM software [26]. After the SAM analysis, only genes with at least two-fold changes in expression were collected for further analysis.

**Real-time RT-PCR**

Gene-specific primers were designed to produce a 150 to 200 bp amplicon for each gene (all the primers used in this study were listed in the Additional file 1). The contaminated DNAs in the RNA samples were further removed using the Amibion’s DNA-free™ Kit. cDNAs were generated using 5 \(\mu\)g of RNA and 3 \(\mu\)g of random hexamer primers. Using 3 independent cultures and RNA preparations, real-time RT-PCR was performed in triplicate as described previously through the LightCycler system (Roche), together with the SYBR Green master mix [23]. On the basis of the standard curves of 16 S rRNA expression, the relative mRNA level was determined by calculating the threshold cycle (\(\Delta\text{Ct}\)) of each gene using the classic \(\Delta\text{Ct}\) method. Negative controls were performed using ‘cDNA’ generated without reverse transcriptase as templates. Reactions containing primer pairs without templates were also included as blank controls. The 16 S rRNA gene was used as an internal control to normalize all the other genes. The transcriptional variation between the WT and mutant strains was calculated for each gene. A mean ratio of 2 was taken as the cutoff of statistical significance.

**Primer extension assay**

For the primer extension assay [23], about 10 \(\mu\)g of total RNA from each strain was annealed with 1 pmol of [\(\gamma-^{32}\text{P}\)] end-labeled reverse primer. The extended reverse transcripts were generated as described in the protocol for Primer Extension System-AMV Reverse Transcriptase (Promega). The yield of each primer extension product indicates the mRNA expression level of the corresponding gene in each strain, which can then be used to map the 5′ terminus of RNA transcript for each gene. The same labeled primer was also used for sequencing with the fmol™ DNA Cycle Sequencing System (Promega). The primer extension products and sequencing materials were concentrated and analyzed by 8 M urea-6% polyacrylamide gel electrophoresis. The result was detected by autoradiography (Kodak film).

**LacZ reporter fusion and \(\beta\)-galactosidase assay**

The 500 to 600 bp upstream DNA region of each indicated gene (Table 1) was obtained by PCR with the ExTaq™ DNA polymerase (Takara) using \(\text{Y. pestis}\) 201 genome DNA as the template. PCR fragments were then cloned directionally into the \(\text{EcoRI}\) and \(\text{BamHI}\) sites of plasmid pRW50, which harbors a tetracycline resistance gene and a promoterless \(\text{lacz}\) reporter gene [27]. Correct cloning was verified through DNA sequencing. \(\text{Y. pestis}\) was then transformed with the recombinant plasmids and grown as described in microarray analysis. The empty plasmid pRW50 was also introduced into both strains as negative control. \(\beta\)-galactosidase activity was measured on cellular extracts using the \(\beta\)-Galactosidase Enzyme Assay System (Promega) [23]. Assays were performed in triplicate. A mean value of fold change was taken as the cutoff of statistical significance.
Table 1 Genes tested in both computational and biochemical assays

| Gene ID | Gene | Regulation | Computational matching of regulatory consensus | Position of DNA fragment used |
|---------|------|------------|-----------------------------------------------|-------------------------------|
| YPO1222 | ompC | +          | D-110...-91, ATAAATCTTTGTTGCAATTT             | Position§ Score LacZ Footprinting |
| YPO1411 | ompF | +          | R-99...-80, TTATCTTTGTTAACAACA              | 11.57 -328...+143 -389...+69 |
| YPO2506 | ompX | +          | R-82...-63, GAAATCCTTTGTTACATGAA            | 6.03 -374...+123 -191...+89 |
| YPO0136 | ompR | +          | D-81...-62, ATAAAGCCTTTGATAA            | 10.34 -409...+83 -238...+14 |

§, The numbers indicate the nucleotide positions upstream of the transcription start sites.
+, positive and direct regulation.
-, negative and direct regulation.

Preparation of His-OmpR protein
The entire coding region of ompR was amplified from Y. pestis 201 and then cloned directionally into the respective BamH I and HindIII sites of plasmid pET28a. This was later verified through DNA sequencing. The recombinant plasmid encoding a His-protein was transformed into BL21 DE3 cells. Over-expression of His-OmpR in the LB medium was induced by adding 1 mM isopropyl-b-D-thiogalactoside. The over-expressed protein was purified under native conditions with nickel-loaded HiTrap Chelating Sepharose columns (Amersham). The purified and eluted protein was concentrated to a final concentration of 0.1 to 0.3 mg/ml with the Amicon Ultra-15 (Millipore), eluted protein was stored at -80°C.

DNase I footprinting
The promoter DNA regions (Table 1) were prepared by PCR amplification performed with the promoter-specific primer pairs (see Additional file 1 for primer sequences), including a 5'-32P-labeled primer (either forward or reverse) and its non-labeled counterpart. The PCR products were purified using QiaQuick cleanup columns (Qiagen).

Increasing amounts of purified His-protein were incubated with the labeled DNA fragment (2 to 5 pmol) for 30 min at room temperature in a binding buffer containing 10 mM Tris-HCl (pH7.4), 50 mM KCl, 0.5 mM DTT, 1 mM MgCl₂, 4% glycerol, 0.05 mg/ml BSA, 0.05 mg/ml shared salmon sperm DNA and 0.5 mM EDTA, with a final volume of 10 μl. Afterwards, 25 mM of fresh acetyl phosphate was added in the binding buffer and incubated with purified His-OmpR for 30 min to achieve the OmpR phosphorylation, after which the labeled DNA was added for additional incubation for 30 min. Prior to DNA digestion, 10 μl of Ca²⁺/Mg²⁺ solution (5 mM CaCl₂ and 10 mM MgCl₂) was added, followed by incubation for 1 min at room temperature. The optimized RQ1 RNase-Free DNase I (Promega) was then added to the reaction mixture, which was subsequently incubated at room temperature for 30 to 90 s. The cleavage reaction was stopped by adding 9 μl of the stop solution (200 mM NaCl, 30 mM EDTA and 1% SDS) followed by DNA extraction and precipitation. The partially digested DNA samples were then analyzed in a 6% polyacrylamide/8 M urea gel. Protected regions were identified by comparing these with the sequence ladders. For sequencing, the fmol® DNA Cycle Sequencing System (Promega) was used. The result was detected by autoradiography (Kodak film).

Environmental stress experiments
Y. pestis strain 201 inoculated into TMH was grown to the early logarithm phase at 26°C. To determine the effect of high osmolarity stress on Y. pestis, the log-phase cells were kept incubated at 26°C for 20 min in the presence of 1.5 M sorbitol. For high-temperature stress experiments, log-phase cells were transferred to pre-warmed 50°C tubes and incubated at 50°C for 5 min. For low pH stress experiments, log-phase cells were incubated at 37°C in TMH medium adjusted by adding 2 M HCl to pH 3.0 for 10 min. To test the effect of oxidative stress, the cells were incubated for 10 min in 220 mM H₂O₂. The bacterial viable count after exposure to the appropriate stresses was determined by pelleting the appropriate dilutions on the BHI agar plates, which were then incubated at 26°C for 36 h.

Macrophage infection assay
J774A.1 mouse macrophage cells (6 × 10⁵) were seeded in 24-well tissue culture plates (0.5 ml/well) and maintained in the minimum essential medium (MEM) containing the modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine until confluence was achieved at 37°C under 5% CO₂. WT and ΔompR were grown in TMH as described.
above. The cultures were collected and suspended in the MEM medium and then respectively added to cell monolayers in 24-well tissue culture plates at a multiplicity of infection generally of 20:1 (bacteria to macrophages). After incubation at 37°C for 1 h to permit phagocytosis, 6 wells of infected cell monolayers were washed thrice with 1× phosphate-buffered saline (PBS). Afterwards, the number of total macrophage cell-associated bacteria was determined. Cell-associated bacteria were determined by harvesting in 0.5 ml of 0.1% Triton X-100 in 1× PBS. After 10 min, infected cell lysates were collected serially and diluted 10-fold in PBS; on the other hand, viable bacterial CFU was determined as described above. A second set of 6 infected monolayer wells were washed twice with 1× PBS. MEM medium supplemented with 200 μg/ml gentamicin (Invitrogen) was added to these wells for 1 h to kill extracellular bacteria. The infected monolayers were then lysed and treated as described above to determine the number of intracellular bacteria. Each experiment was repeated three or four times on different days, and each bacteria sample was used to infect at least four wells of macrophage monolayers.

Results
Non-polar mutation of ompR
Given that the coding regions of ompR and envZ overlap in the ompB operon, a partial segment of the coding region of ompR was replaced by the kanamycin resistance cassette to generate the ompR mutant (ΔompR). Real-time RT-PCR was performed to assess the ompR mRNA levels in WT, ΔompR, and C-ompR (the complemented mutant). The ompR transcript was lacking in ΔompR, while it was restored in C-ompR relative to WT (data not shown), indicating successful mutation and complementation. To prove the non-polar mutation of ompR, we constructed the pRW50-harboring fusion promoter consisting of a promoter-proximal region of ompF and promoterless lacZ, and then transformed into WT, ΔompR and C-ompR, respectively (Additional file 2). The ompF gene was positively regulated by OmpR as determined by several distinct methods (see below). As expected, the ompF promoter activity (β-galactosidase activity) decreased significantly in ΔompR relative to WT grown at high medium osmolarity (0.5 M sorbitol); however, it showed almost no difference between WT and C-ompR, thereby confirming that the ompR mutation was non-polar.

Phenotypes of ΔompR
The ΔompR mutant was characterized for its ability to survive under a range of in vitro stress conditions associated with macrophage-killing mechanisms (Figure 1a). In comparison to its WT parent strain, ΔompR was significantly more sensitive to high salt, high osmolarity, and high temperature. Both WT and mutant strains were extremely sensitive to acid shock without any significant difference between them; in addition, ΔompR seemed more resistant to hydrogen peroxide. Therefore, OmpR should play roles in the regulation of the adaptation to well-documented hyperosmotic stress and additional environmental perturbations, such as heat and oxidative stresses.

Macrophage infection assay was performed to investigate the role of OmpR in the initiation of bacterial strategies against macrophages. A significant increase in the percentage of phagocytosis for ΔompR relative to WT (Figure 1b) suggested that the mutant was more susceptible to phagocytosis. For the percentage of cell-associated bacteria, no difference was observed between the WT and mutant strains, thereby suggesting that OmpR does not have a role in the bacterial adhesion to phagocytes (Figure 1b).
OmpR-dependent genes

By standard cDNA microarray experiments, the mRNA level of each gene was compared between ΔompR and WT grown at 0.5 M sorbitol. In all, 224 genes were affected by the ompR mutation. These genes represented more than 4% of total protein-encoding capacity of Y. pestis and were distributed in 24 functional categories according to the genome annotation of Y. pestis CO92 [29], indicating the global regulatory effect of OmpR. The microarray data (GSE26601) had been deposited in Gene Expression Omnibus (GEO).

Known OmpR-binding sites from S. enterica and E. coli were collected and aligned to generate an OmpR consensus that was a position-specific scoring matrix (PSSM) (Additional file 3), in which each row and column represents a position and a nucleotide, respectively. Given that the OmpR protein sequences were highly conserved among S. enterica, E. coli and Y. pestis (data not shown), this PSSM represents conserved signals for OmpR recognition of promoter DNA regions for all these bacteria. Thus, the PSSM generated from the pre-existing data in E. coli and S. enterica can be used to predict computationally the presence of OmpR consensus-like elements within a target promoter-proximal sequence of Y. pestis.

Accordingly, the 300 bp upstream promoter DNA regions of the 234 mpR-dependent genes that were disclosed by microarray were scanned using PSSM. This computational promoter analysis generated a weight score for each gene, and a higher score denoted the higher probability of OmpR binding. With a cutoff value of 7, only 14 genes gave predicted OmpR consensus-like elements (Additional file 4); these were then subjective to real-time RT-PCR analysis to compare their mRNA levels between ΔompR and WT. In accordance with microarray results, RT-PCR disclosed that all 14 genes were expressed differentially in ΔompR relative to WT.

In addition to these 14 genes, we still included 2 additional ones, namely, ompR and X, for further analysis. The OmpR-dependent expression of ompR could not be determined by microarray and RT-PCR since the coding region of ompR was deleted from the ΔompR mutant strain. The ompX gene was discarded by SAM in the microarray assay (which could be attributed to the fact that the repeatability of the 8 replicated data points of this gene were unacceptable by SAM), although it gave a more than 2-fold mean change of expression between WT and ΔompR. Further biochemical assays (see below) confirmed that OmpR did regulate these genes.

Altogether, we validated 16 genes whose transcriptions were OmpR-dependent (Additional file 4), including ompR, C, F, and X that were further characterized below (Table 1). All of these represented the candidates of direct OmpR targets (ompR, C, F, and X were confirmed below) since OmpR consensus-like sequences were predicted within their respective promoter-proximal regions.

Direct regulation of ompC, F and X by OmpR

The mRNA levels of each of ompC, F, and X were compared between ΔompR and WT at 0.5 M sorbitol using real-time RT-PCR (Figure 2a). The results showed that the mRNA level of ompC, F, and X decreased significantly in ΔompR relative to WT. Further lacZ fusion reporter assays demonstrated that the promoter activity of ompC, F, and X decreased significantly in ΔompR relative to WT, thereby confirming the RT-PCR results. Primer extension experiments were further conducted for ompC, F, and X with ΔompR and WT at 0.5 M sorbitol (Figure 2c). A single primer extension product was detected for each of ompF and X, after which the 5' terminus of RNA transcript (transcription start site) for each gene was identified accordingly. The yield of primer extension product indicated the mRNA expression level for each gene in the corresponding strain. These results further verified the above RT-PCR data for ompF and X. However, we failed to detect the primer extension product for ompC in both ΔompR and WT after repeated efforts using different primers. This could be attributed to the failure to synthesize the primer extension product for ompC by polymerase.

Given that OmpR consensus-like sequences were found within the promoter regions of ompC, F and X (Table 1), DNase I footprinting experiments (Figure 2d) were subsequently performed with both coding and non-coding strands of the corresponding promoter-proximal DNA fragments. The purified His-OmpR-P protein protected a single distinct region (OmpR-binding site) within each target promoter region in a dose-dependent pattern. Taken together, the OmpR regulator stimulated the expression of ompC, F, and X through the process of OmpR-promoter DNA association.

Autoregulation of OmpR

According to the lacZ fusion reporter assay (Figure 3a); there was a more than 10-fold decrease of the ompR promoter activity in ΔompR relative to WT at 0.5 M sorbitol, thereby indicating that OmpR stimulated the promoter activity of its own gene. The subsequent DNase I footprinting experiments (Figure 3a) showed that His-OmpR-P protected a single region within the ompR promoter. Therefore, OmpR stimulated its own gene at the transcriptional level, which was mediated through the binding of OmpR-P to its own promoter.

Expression of ompC, F, X and R under different osmotic conditions

The promoter activities of ompC, F, X, and R were each determined in WT or ΔompR grown in the LB broth
Figure 2 Regulation of ompC, F and X by OmpR.

a) Real-time RT-PCR. The mRNA levels of each indicated gene were compared between ΔompR and WT. This figure shows the increased (positive number) or decreased (minus one) mean fold for each gene in ΔompR relative to WT.

b) LacZ fusion reporter. A promoter-proximal region of each indicated gene was cloned into pRW50 containing a promoterless lacZ reporter gene, and transformed into WT or ΔompR to determine the promoter activity (β-galactosidase activity in cellular extracts). The empty plasmid was also introduced into each strain as a negative control, which gave extremely low promoter activity (data not shown). Positive and minus numbers indicate the increased and decreased mean folds, respectively, for the detecting promoter activity in ΔompR relative to WT.

c) Primer extension. Primer extension assays were performed for each indicated gene using total RNAs isolated from the exponential-phase of WT or ΔompR. An oligonucleotide primer complementary to the RNA transcript of each gene was designed from a suitable position. The primer extension products were analyzed with 8 M urea-6% acrylamide sequencing gel. Lanes C, T, A, and G represent the Sanger sequencing reactions; on the right side, DNA sequences are shown from the bottom (5') to the top (3'), and the transcription start sites are underlined.

d) DNase I footprinting. The labeled DNA probe was incubated with various amounts of purified His-OmpR (lanes 1, 2, 3, 4, and 5 contained 0, 5, 10, 15 and 20 pmol, respectively) with the addition of acetyl phosphate, and subjected to DNase I footprinting assay. Lanes G, A, T, and C represent the Sanger sequencing reactions, and the protected regions (bold lines) are indicated on the right-hand side. The numbers indicate the nucleotide positions upstream of the transcriptional start sites.
using lacZ fusion reporter assay (Figure 4). The LB broth was used here instead of the TMH medium since it was convenient to modify the medium osmolarity in the LB medium by adding different concentrations of NaCl. The results demonstrated that the promoter activities of ompC, F, X, and R were enhanced dramatically with the increasing of NaCl concentration (i.e., medium osmolarity) in WT. However, this effect almost disappeared in the ΔompR mutant, suggesting that OmpR mediated the noticeably inducible transcription of these genes upon exposure to hyperosmotic stress.

**Discussion**

**Conserved OmpR-dependent phenotypes among pathogenic yersiniae**

As shown in *Y. enterocolitica* [30,31], *Y. pseudotuberculosis* [32] and *Y. pestis* (the present work) in a conserved manner, OmpR is involved in the resistance to phagocytosis and/or survival within macrophages and controls the adaptation to various killing mechanisms used by macrophages against pathogens. The *ompR* mutants of both *Y. enterocolitica* [30] and *Y. pseudotuberculosis* [32] are attenuated in the mouse model. OmpR is a repressor of the *inv* gene, which encodes the major virulence determinant invasin in *Y. enterocolitica* [33]. In *Y. pseudotuberculosis*, OmpR regulates positively the urease expression to enhance acid survival [34], whereas it controls negatively the expression of FlhD and FlhC that form a heterohexameric transcriptional activator of the flagellar genes [35]. In this work, the *ompR* mutation likely had not affect on the virulence of *Y. pestis* 201, which was a human-attenuated enzootic strain in a mouse model after subcutaneous infection (data not shown). In this light, a further animal virulence test using a typical epidemic strain is hereby required.

**Global regulatory effect of OmpR in *Y. pestis***

The microarray expression analysis disclosed a set of 224 genes that were affected by the *ompR* mutation in *Y. pestis*. A similar global regulatory effect of OmpR has been observed in *E. coli* [36]. Real-time RT-PCR or lacZ fusion reporter assay further validated 16 OmpR-dependent genes, for which OmpR consensus-like sequences were found within their promoter regions. These 16 genes represent the candidates of direct OmpR targets in *Y. pestis*, of which *ompR, C, F,* and *X* were further characterized for the molecular mechanisms of regulation by OmpR.

**Transcriptional auto-stimulation of OmpR**

We confirmed the direct transcriptional auto-stimulation of *ompR* in *Y. pestis*. In addition, the *ompR* promoter activity was dramatically and persistently enhanced in *Y. pestis* with the increasing medium osmolarity, which was mediated by OmpR itself. The auto-stimulation of the *ompB* operon appears to be conserved in *Y. pestis, E. coli,* and *S. enterica* [3]. The histone-like protein HN-S is a negative regulator of *ompB* expression in both *E. coli* [37] and *S. enterica,* and the role of OmpR-P in autoinduction is to help to counteract it.
repression by H-NS [3]. In conclusion, transcription from the \textit{ompB} promoter is repressed by H-NS and requires OmpR-P for induction; in addition, EnvZ (as a sensor kinase) and acetyl phosphate collaborate to produce the optimum level of OmpR-P needed for autoinduction [3,37].

Osmotic regulation of porins

Previous works [38,39] have proposed that the shift in cellular porin levels reflects the adaptation of enteric bacteria to a transition between a life in the mammalian gut as ‘high osmolarity’ and a free-living aqueous state as ‘low osmolarity.’ OmpC expression is favored in the gut, while OmpF is predominately expressed in the aqueous habitats. Compared to OmpF, OmpC has smaller pore and, hence, slower flux [39]. The smaller pore size of OmpC can aid in excluding harmful molecules, such as bile salts, in the gut. In the external aqueous environment, the larger pore size of OmpF can assist in scavenging for scarce nutrients.

The amounts of OmpC and OmpF in the outer membrane of \textit{E. coli} vary depending on the medium osmolarity, and their relative levels fluctuate in a reciprocal manner; in addition, the \textit{ompX} expression is inducible upon early exposure to high osmolarity, which is accompanied by the repressed expression of OmpF and OmpC [12,14]. In this work, \textit{ompX}, \textit{C}, and \textit{F} were up-regulated dramatically upon the increase of medium osmolarity in \textit{Y. pestis}. This is in stark contrast to the classic reciprocal regulation of these same proteins. OmpF is over-expressed at low osmolarity in \textit{E. coli}, while it is likely no longer employed by \textit{Y. pestis}. How \textit{Y. pestis} express porins during the transition from mammalian blood or lymph into the flea gut remains unclear. Nevertheless, we could postulate that \textit{Y. pestis} has lost the mechanism of over-expressing the relevant porin at low osmolarity, since it always encounters high osmolarity environments in its life in mammalian blood or lymph and flea midgut, and has a rare chance of living in the environment [40]. Another issue involves whether or not the mechanism of porin regulation observed is specific for \textit{Y. pestis}, or conserved in \textit{Y. pseudotuberculosis} with a life transitioning from free-living environments into mammalian gut (e.g., \textit{E. coli} and \textit{S. enterica}). A comparison between porin regulation in \textit{Y. pestis} and \textit{Y. pseudotuberculosis} may provide first insights into possible evolutionary forces selecting for altered gene regulation.

OmpC is highly expressed in \textit{S. typhi} independent of medium osmolarity, whereas OmpF is osmoregulated as it is in \textit{E. coli} [41]. In addition, OmpC is always more abundant than OmpF in \textit{S. typhi}, regardless of the growth conditions [42]. The lack of osmoregulation of OmpC expression in \textit{S. typhi} is determined in part by the \textit{ompB} operon, as well as by other unknown trans-acting regulators in \textit{S. typhi} [42]. The evidenced differences in porin regulation (as seen in \textit{Y. pestis}, \textit{S. typhi}, and \textit{E. coli}) could possibly have an effect on how these bacteria survive in the environment or during pathogenesis.

Organization of OmpR-recognized promoter regions

The present study confirmed that OmpR-P recognized the promoter regions of \textit{ompC}, \textit{F}, \textit{X}, and \textit{R} to regulate the target promoter activity. We aligned OmpR-binding sites within relevant promoter regions from \textit{E. coli} and the 3 pathogenic yersiniae (Figure 5). Then, 3 tandems of OmpR consensus-like sequences were detected for \textit{ompC}.

Figure 5 OmpR consensus-like sequences within the target promoter regions. The underlined segments are OmpR binding sites determined by DNase I footprinting in \textit{Y. pestis}. The boxed areas represent the sub-elements of OmpR consensus-like sequence. This figure also shows the three (e.g., C1-C2-C3) or 2 (e.g., X1-X2) tandems of OmpR consensus-like sequences, where each 20 bp tandem has been divided into two 10 bp sub-elements (boxed).
(C1-C2-C3) or ompF (F1-F2-F3), while 2 tandems were detected for ompR (R1-R2) or ompX (X1-X2) in yersiniae. As expected, each OmpR consensus-like element consisted of 20 base pairs that can be divided into two 10 bp sub-elements (e.g., X1a and X1b), providing a tandem binding site for 2 OmpR-P molecules [43]. These results confirmed that multiple OmpR proteins occupied the target promoter in a tandem manner to regulate its activity. Remarkably, F1-F2-F3 and C1-C2-C3 were detected for ompF and ompC, respectively, although F4 was absent for ompF. Given that OmpR-P binding to the promoter-distal F4 site at high osmolarity likely formed a loop that interacted with OmpR-P molecules binding to the promoter-proximal F1, F2, and F3 sites—thereby blocking the transcription of ompF—the absence of F4 in Y. pestis destroyed the above blocking mechanism. Indeed, ompF was up-regulated gradually in an OmpR-dependent manner upon the increase of medium osmolarity in Y. pestis.

**Regulation of ompX by OmpR**

OmpR still recognized the ompX promoter region and stimulated its transcription in Y. pestis. To our knowledge, this is the first report of ompX regulation by OmpR, although OmpR consensus-like sequences have also been found within the ompX upstream region in E. coli (data not shown) and E. aerogenes [6]. At the very least, the direct transcriptional regulation of ompX by OmpR is conserved in the above-mentioned bacteria.

**Conclusion**

The ompR mutation in Y. pestis strain 201 attenuated the resistance to phagocytosis as well as the adaptation to various stressful conditions met in macrophages; however, it had no effect on the virulence of this pathogen. Microarray expression analysis disclosed at least 232 genes whose transcription was affected by the OmpR-dependent in Y. pestis. Real-time RT-PCR or lacZ fusion reporter assays were then conducted to validate 16 OmpR-dependent genes, including ompC, F, X, and R. Notably, OmpR consensus-like sequences were found within the upstream DNA regions of these 16 genes, thereby representing the candidates of direct OmpR targets. ompC, F, X, and R were subsequently proven to be directly regulated by OmpR through OmpR-promoter DNA association. All of ompC, F, X, and R were up-regulated dramatically with the increase in medium osmolarity, which was mediated by OmpR that occupied the target promoter regions in a tandem manner. The inducible expressions of the pore-forming proteins OmpF, C, and X at high osmolarity in Y. pestis were in contrast to their reciprocal regulations in E. coli. The main difference was that ompF expression was not repressed at high osmolarity in Y. pestis, which was likely due to the absence of a promoter-distal OmpR-binding site for ompF.

**Additional material**

Additional file 1: Oligonucleotide primers used in this study

Additional file 2: Promoter activity ompF within WT, ΔompR and C-ompR

Additional file 3: Construction of the OmpR consensus (PSSM)

Additional file 4: The 16 verified OmpR-dependent genes

**Acknowledgements**

Financial support for this work came from the National Natural Science Foundation of China (30930003, 30900823 and 30771178) and the 973 Program (2009CB522600). The English writing of the manuscript was polished by EnPapers.

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**Authors’ contributions**

DZ and RY conceived the study and designed the experiments. HG and YZ performed all the experiments. YH contributed to phenotypic experiments. YH and DZ performed the microarray experiments. LY, XL, and ZG contributed to RT-PCR, primer extension assay, and DNA binding assays. ZG and YT participated in protein expression and purification. HG and DZ performed computational analysis and figure construction. The manuscript was written by HG and DZ, and revised by RY. All the authors read and approved the final manuscript.

**Received: 11 June 2010 Accepted: 23 February 2011**

Published: 23 February 2011

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