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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

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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 porins 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 $\Delta ompR$ was verified by PCR.

A PCR-generated DNA fragment containing the *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 $\Delta ompR$, producing the complemented mutant strain *C-ompR*.

Bacterial growth and RNA isolation

Overnight cultures (an OD₆₂₀ of about 1.0) of WT or $\Delta ompR$ 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 OD₆₂₀ 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 RNAprotect 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 $\Delta ompR$ using a *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 µg of RNA and 3 µg 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 (ΔCt) of each gene using the classic ΔCt 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 µg of total RNA from each strain was annealed with 1 pmol of [γ -³²P] 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 β -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 *Y. pestis* 201 genome DNA as the template. PCR fragments were then cloned directionally into the *EcoRI* and *BamHI* sites of plasmid pRW50, which harbors a tetracycline resistance gene and a promoterless *lacZ* reporter gene [27]. Correct cloning was verified through DNA sequencing. *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. β -galactosidase activity was measured on cellular extracts using the β -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 §	
			Position§	Sequence	Score	LacZ	Footprinting
YPO1222	<i>ompC</i>	+	D-110...-91	ATAAATACTTGTGCAATTT	7.06	-379...+130	-245...+31
YPO1411	<i>ompF</i>	+	R-99...-80	TTTACATTTTGTAAACACATA	11.57	-328...+143	-389...+69
YPO2506	<i>ompX</i>	+	R-82...-63	GAAATTCITTTGTACATGAA	6.03	-374...+123	-191...+89
YPO0136	<i>ompR</i>	+	D-81...-62	AATAAGCTTTGTAAACAATTT	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 *Bam*HI and *Hind*III sites of plasmid pET28a. This was later verified through DNA sequencing. The recombinant plasmid encoding a His-protein was transformed into BL21λDE3 cells. Over-expression of His-OmpR in the LB medium was induced by adding 1 mM isopropyl-β-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), which was confirmed by SDS-PAGE for purity. The purified 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).

Computational promoter analysis

The 300 bp promoter regions upstream of the start codon of each indicated gene were retrieved with the 'retrieve-seq' program [28]. The 'matrices-paster' tool [28] was used to match the relevant position-specific scoring matrix (PSSM) within the above promoter regions.

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 Δ*ompR* 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 ($\Delta ompR$). Real-time RT-PCR was performed to assess the *ompR* mRNA levels in WT, $\Delta ompR$, and *C-ompR* (the complemented mutant). The *ompR* transcript was lacking in $\Delta ompR$, 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, $\Delta ompR$ 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 $\Delta ompR$ 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 nonpolar.

Phenotypes of $\Delta ompR$

The $\Delta ompR$ 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, $\Delta ompR$ was significantly more sensitive to high salt, high osmolarity,

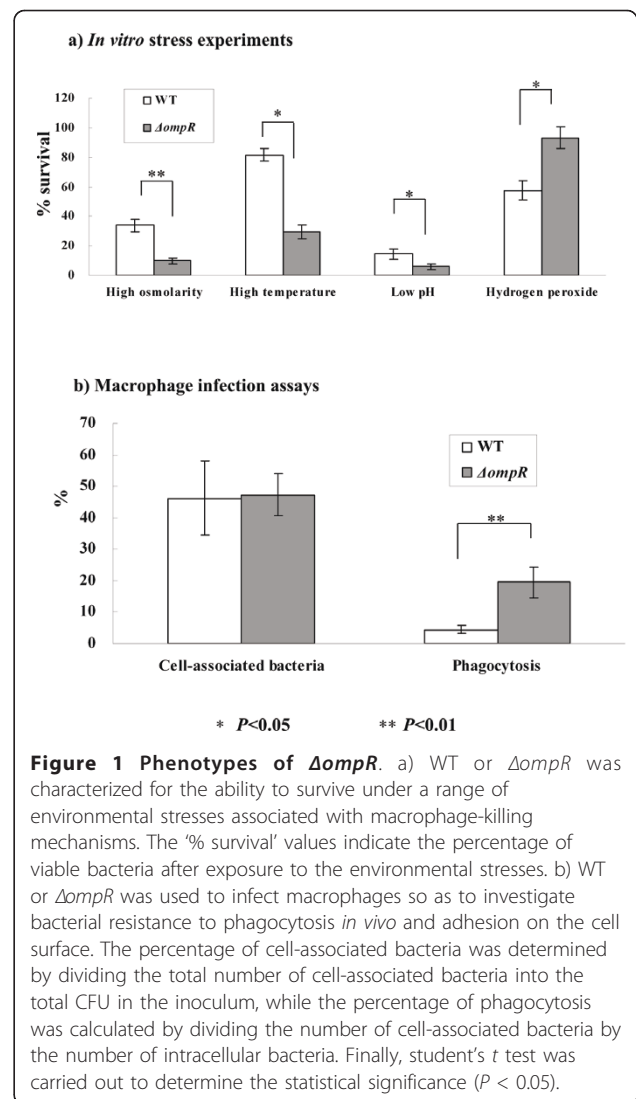


Figure 1 Phenotypes of $\Delta ompR$. a) WT or $\Delta ompR$ was characterized for the ability to survive under a range of environmental stresses associated with macrophage-killing mechanisms. The '% survival' values indicate the percentage of viable bacteria after exposure to the environmental stresses. b) WT or $\Delta ompR$ was used to infect macrophages so as to investigate bacterial resistance to phagocytosis *in vivo* and adhesion on the cell surface. The percentage of cell-associated bacteria was determined by dividing the total number of cell-associated bacteria into the total CFU in the inoculum, while the percentage of phagocytosis was calculated by dividing the number of cell-associated bacteria by the number of intracellular bacteria. Finally, student's *t* test was carried out to determine the statistical significance ($P < 0.05$).

and high temperature. Both WT and mutant strains were extremely sensitive to acid shock without any significant difference between them; in addition, $\Delta ompR$ 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 $\Delta ompR$ 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 $\Delta ompR$ 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 $\Delta ompR$ and WT. In accordance with microarray results, RT-PCR disclosed that all 14 genes were expressed differentially in $\Delta ompR$ 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 $\Delta ompR$ 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 $\Delta ompR$. 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 $\Delta ompR$ 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 $\Delta ompR$ relative to WT. Further *lacZ* fusion reporter assays demonstrated that the promoter activity of *ompC*, *F*, and *X* decreased significantly in $\Delta ompR$ relative to WT, thereby confirming the RT-PCR results. Primer extension experiments were further conducted for *ompC*, *F*, and *X* with $\Delta ompR$ 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 $\Delta ompR$ 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 $\Delta ompR$ 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 $\Delta ompR$ grown in the LB broth

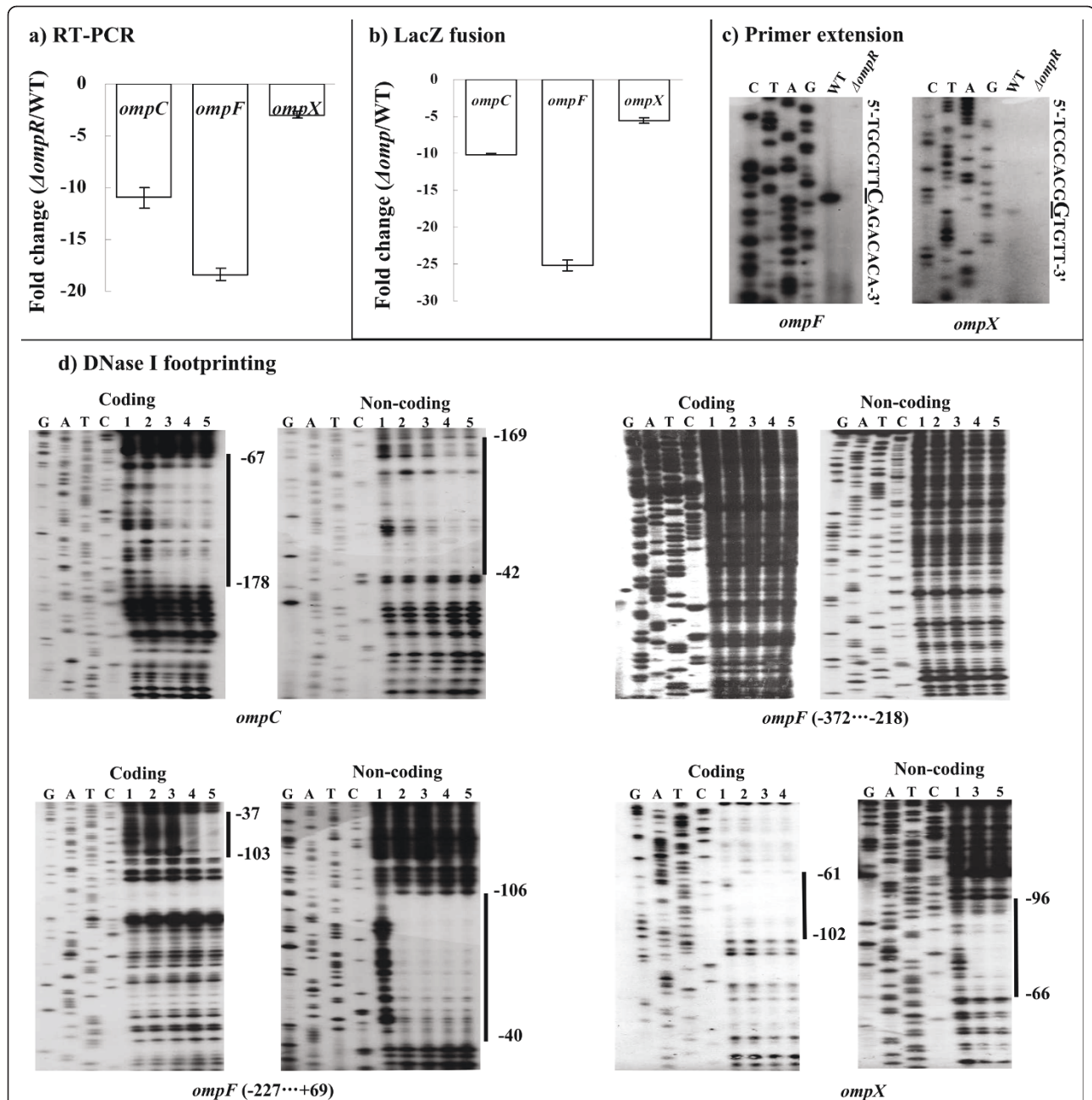


Figure 2 Regulation of *ompC*, *F* and *X* by OmpR. a) Real-time RT-PCR. The mRNA levels of each indicated gene were compared between $\Delta ompR$ and WT. This figure shows the increased (positive number) or decreased (minus one) mean fold for each gene in $\Delta ompR$ relative to WT. b) LacZ fusion reporter. A promoter-proximal region of each indicated gene was cloned into pRW50 containing a promoterless *lacZ* reporter gene, and transformed into WT or $\Delta ompR$ to determine the promoter activity (β -galactosidase activity in cellular extracts). The empty plasmid was also introduced into each strain as 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 $\Delta ompR$ 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 $\Delta ompR$. 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.

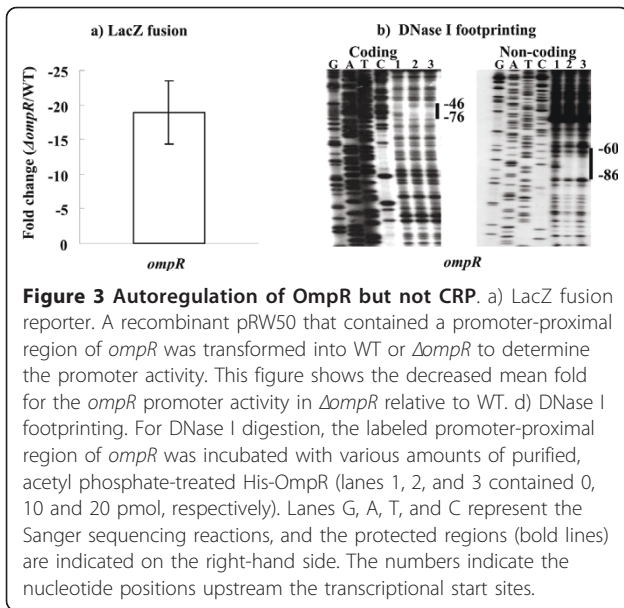


Figure 3 Autoregulation of OmpR but not CRP. a) LacZ fusion reporter. A recombinant pRW50 that contained a promoter-proximal region of *ompR* was transformed into WT or $\Delta ompR$ to determine the promoter activity. This figure shows the decreased mean fold for the *ompR* promoter activity in $\Delta ompR$ relative to WT. d) DNase I footprinting. For DNase I digestion, the labeled promoter-proximal region of *ompR* was incubated with various amounts of purified, acetyl phosphate-treated His-OmpR (lanes 1, 2, and 3 contained 0, 10 and 20 pmol, respectively). 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 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

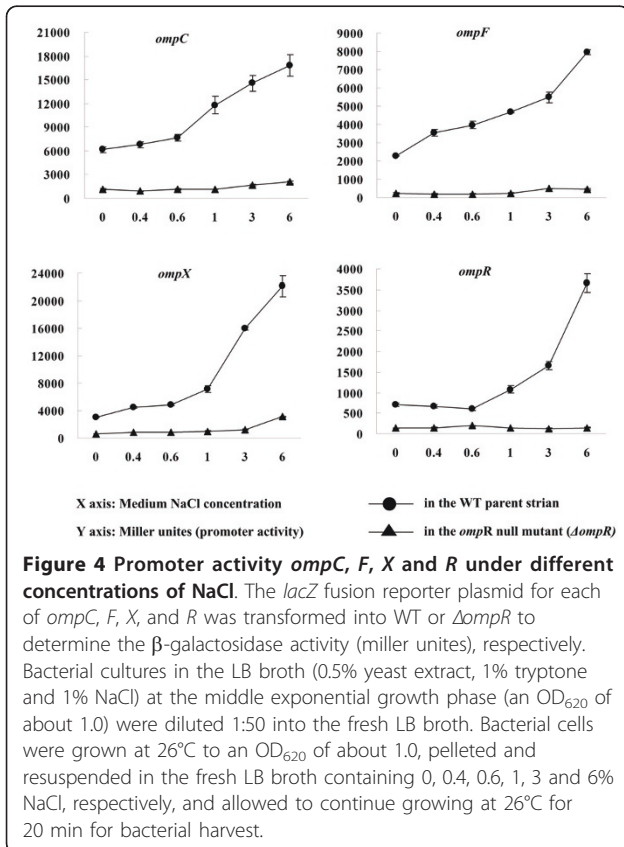


Figure 4 Promoter activity *ompC*, *F*, *X* and *R* under different concentrations of NaCl. The *lacZ* fusion reporter plasmid for each of *ompC*, *F*, *X*, and *R* was transformed into WT or $\Delta ompR$ to determine the β -galactosidase activity (miller units), respectively. Bacterial cultures in the LB broth (0.5% yeast extract, 1% tryptone and 1% NaCl) at the middle exponential growth phase (an OD_{620} of about 1.0) were diluted 1:50 into the fresh LB broth. Bacterial cells were grown at 26°C to an OD_{620} of about 1.0, pelleted and resuspended in the fresh LB broth containing 0, 0.4, 0.6, 1, 3 and 6% NaCl, respectively, and allowed to continue growing at 26°C for 20 min for bacterial harvest.

with the increasing of NaCl concentration (i.e., medium osmolarity) in WT. However, this effect almost disappeared in the $\Delta ompR$ 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

repression by H-NS [3]. In conclusion, transcription from the *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 *E. coli* vary depending on the medium osmolarity, and their relative levels fluctuate in a reciprocal manner; in addition, the *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, *ompX*, *C*, and *F* were up-regulated dramatically upon the increase of medium osmolarity in *Y. pestis*. This is in stark contrast to the classic reciprocal regulation of these same proteins. OmpF is over-expressed at low osmolarity in *E. coli*, while it is likely no longer employed by *Y. pestis*. How *Y. pestis* express porins during the transition from mammalian blood or lymph into the flea gut remains

unclear. Nevertheless, we could postulate that *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 *Y. pestis*, or conserved in *Y. pseudotuberculosis* with a life transitioning from free-living environments into mammalian gut (e.g., *E. coli* and *S. enterica*). A comparison between porin regulation in *Y. pestis* and *Y. pseudotuberculosis* may provide first insights into possible evolutionary forces selecting for altered gene regulation.

OmpC is highly expressed in *S. typhi* independent of medium osmolarity, whereas OmpF is osmoregulated as it is in *E. coli* [41]. In addition, OmpC is always more abundant than OmpF in *S. typhi*, regardless of the growth conditions [42]. The lack of osmoregulation of OmpC expression in *S. typhi* is determined in part by the *ompB* operon, as well as by other unknown trans-acting regulators in *S. typhi* [42]. The evidenced differences in porin regulation (as seen in *Y. pestis*, *S. typhi*, and *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 *ompC*, *F*, *X*, and *R* to regulate the target promoter activity. We aligned OmpR-binding sites within relevant promoter regions from *E. coli* and the 3 pathogenic yersiniae (Figure 5). Then, 3 tandems of OmpR consensus-like sequences were detected for *ompC*

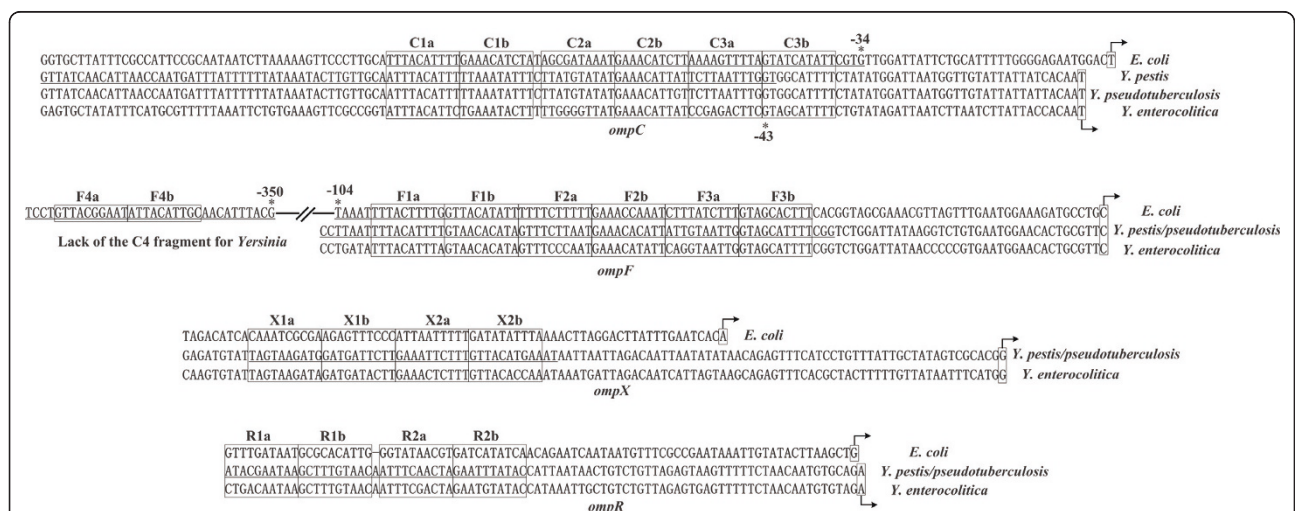


Figure 5 OmpR consensus-like sequences within the target promoter regions. The underlined segments are OmpR binding sites determined by DNase I footprinting in *Y. pestis*. The boxed areas represent the sub-elements of OmpR consensus-like sequence. This figure also shows the three (e.g., C1-C2-C3) or 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 \times 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, Δ *ompR* and *C-ompR*.

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

Additional file 4: The 16 verified OmpR-dependent genes.

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Authors' contributions

DZ and RY conceived the study and designed the experiments. HG and ZY 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.

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