Environmental Microbiology and Environmental Microbiology Reports

Decision Letter (EMI-2015-1518.R1)

From: kntimmis@gmail.com

To: kbrzostek@biol.uw.edu.pl

CC: paul@sfam.org.uk

Subject: Environmental Microbiology and Environmental Microbiology Reports - Decision on Manuscript ID EMI-2015-1518.R1

Body: 29-Nov-2015

Dear Prof. Brzostek:

I am pleased to inform you that your manuscript entitled "Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR" has been accepted for publication in Environmental Microbiology and Environmental Microbiology Reports.

From this point on, please direct all enquiries related to the production of your paper to the Production Editor:

Production Editor
Environmental Microbiology/Environmental Microbiology Reports
SPi Building, Pascor Drive
Sto. Niño, Parañaque City 1700
Manila, Philippines

Tel +63 2 855 8790
Fax +63 2 325 0768
Email: jrapay@wiley.com

If your manuscript has colour displays, in order to complete the acceptance process, it is necessary for you to provide Wiley immediately with a completed Colour Work Agreement form, which can be downloaded from: http://www.blackwellpublishing.com/pdf/SN_Sub2000_F_CoW.pdf

Please post the completed and signed form to:

Customer Services (OPI)
John Wiley & Sons Ltd, European Distribution Centre
New Era Estate
Oldlands Way
Bognor Regis
West Sussex
PO22 9NQ

Moreover, your article cannot be published until you have signed the appropriate license agreement. Within the next few days you will receive an email from Wiley's Author Services system which will ask you to log in and will present you with the appropriate license for completion.

Authors of accepted articles may choose to pay a fee in order for their published article to become open access (i.e. free for all to view and download) via the OnlineOpen service of the Wiley Online Library website. Further information can be found at:

http://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms.
Any authors wishing their paper to become open access should complete the payment form available at:
https://authorservices.wiley.com/bauthor/onlineopen_order.asp

Production status tracking - You can now track your article via the publisher's Author Services. Once your paper is with the Production Editor, you will receive an e-mail with a unique code that automatically adds your article to the system when you register. With Author Services you can check the status of your article online and choose to receive automated e-mails at key stages of production. Therefore, please ensure that we have your complete e-mail address. There may be a short delay whilst the article is sent to the Production Editor and logged into the production tracking system. Additional services will be added to the website soon.

Proofing your manuscript – EMI/EMIR uses a new electronic service, "e-proofing". You will receive an e-mail from the typesetter when your article is ready for proofing with instructions on how to download your paper and how to return your corrections. Your e-mail address is needed for this vital step, too. If you anticipate the possibility of being absent during this period, please arrange for a colleague to access your e-mail to retrieve the proofs.

OnlineEarly – EMI/EMIR operates a system called OnlineEarly, whereby articles are published online ahead of assignment to an issue and publication in print. You can track the progress of your article and learn when it is published online by registering for Author Services. The Wiley Online Library Website for Environmental Microbiology is http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1462-2920, and the site for Environmental Microbiology Reports is http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1758-2229. If you register in Author Services you will receive free access to your article.

Reprints and Offprints - You will receive instructions for ordering offprints when you are notified that your proofs are ready for review.

Environmental Microbiology and Environmental Microbiology Reports support the European Consortium of Microbial Resource Centres (EMbaRC) project that seeks to develop a strategy to enhance post-publication deposition of prokaryotic strains covered in the scientific literature. We would be grateful if you could consider taking a minute to complete their very short survey:
https://www.surveymonkey.com/s/EnvironmentalMicrobiology.

Sincerely,

Prof. Ken Timmis
Editor, Environmental Microbiology and Environmental Microbiology Reports
kntimmis@gmail.com

Date Sent: 29-Nov-2015

environmental microbiology
environmental microbiology reports

Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR

Journal:	*Environmental Microbiology and Environmental Microbiology Reports*
Manuscript ID	EMI-2015-1518.R1
Manuscript Type:	EMI - Research article
Journal:	Environmental Microbiology
Date Submitted by the Author:	26-Nov-2015

Complete List of Authors:	Nieckarz, Marta; Institute of Microbiology, Faculty of Biology, University of Warsaw, Department of Applied Microbiology Raczkowska, Adrianna; Institute of Microbiology, Faculty of Biology, University of Warsaw, Department of Applied Microbiology Dębski, Janusz; Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Mass Spectrometry Laboratory Kistowski, Michał; Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Mass Spectrometry Laboratory Dadlez, Michał; Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Mass Spectrometry Laboratory; Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Heesemann, Jürgen; Ludwig Maximilians University, Max von Pettenkofer Institute for Hygiene and Medical Microbiology Rossier, Ombeline; Ludwig Maximilians University, Max von Pettenkofer Institute for Hygiene and Medical Microbiology Brzostek, Katarzyna; Institute of Microbiology, Faculty of Biology, University of Warsaw, Department of Applied Microbiology
Keywords:	bacteria, gene expression/regulation, environmental signal/stress responses, microbe:higher organism interactions, microbial ecology, microbial genetics, pathogen ecology

Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR

Marta Nieckarz[1], Adrianna Raczkowska[1], Janusz Dębski[2], Michał Kistowski[2], Michał Dadlez[2,3], Jürgen Heesemann[4], Ombeline Rossier[4], Katarzyna Brzostek[1*]

[1]Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Warsaw, Poland

[2] Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

[3]Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland

[4]Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig Maximilians University, Munich, Germany

∗ correspondence: e-mail kbrzostek@biol.uw.edu.pl; tel. +48 22 55 41 305;

fax. +48 22 55 41 402

Short title

YadA and HemR, novel members of the OmpR regulon

Present addresses of authors

[1]Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Ilji Miecznikowa 1, 02-096 Warsaw, Poland.

[2]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland.

[4]Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig Maximilians University, Pettenkoferstrasse 9a, 80336 Munich, Germany.

Summary

Enteropathogenic *Yersinia enterocolitica* is able to grow within or outside the mammalian host. Previous transcriptomic studies have indicated that the regulator OmpR plays a role in the expression of hundreds of genes in enterobacteria. Here, we have examined the impact of OmpR on the production of *Y. enterocolitica* membrane proteins upon changes in temperature, osmolarity and pH. Proteomic analysis indicated that the loss of OmpR affects the production of 120 proteins, a third of which are involved in uptake/transport, including several that participate in iron or heme acquisition. A set of proteins associated with virulence was also affected. The influence of OmpR on the abundance of adhesin YadA and heme receptor HemR was examined in more detail. OmpR was found to repress YadA production and bind to the *yadA* promoter, suggesting a direct regulatory effect. In contrast, the repression of *hemR* expression by OmpR appears to be indirect. These findings provide new insights into the role of OmpR in remodeling the cell surface and the adaptation of *Y. enterocolitica* to different environmental niches, including the host.

Introduction

The enteropathogen *Yersinia enterocolitica* is a member of the genus *Yersinia*, which includes two other human pathogens: the plague bacillus *Y. pestis* and *Y. pseudotuberculosis*, a gastrointestinal pathogen (Bottone, 1997; Francis, 2013). *Y. enterocolitica* is a heterogeneous species classified into 60 serotypes and six biotypes that vary in pathogenicity (Thomson et al., 2006). Based on genomic sequence differences, *Y. enterocolitica* has been divided into two subspecies: *enterocolitica* and *palearctica* (Neubauer et al., 2000). Due to its ability to grow both outside and inside mammalian hosts, *Y. enterocolitica* experiences diverse environmental conditions. As a free-living enteric bacterium it exhibits features that are

expressed at ambient temperature, but only weakly or not at all at 37°C (mammalian body temperature), including motility, smooth lipopolysaccharide (LPS) production, invasin expression and some metabolic properties (Straley and Perry, 1995). Significantly, some of these characteristics are required in the early stages of infection of the human body (Pepe and Miller, 1993). *Y. enterocolitica* synthesizes numerous virulence factors that appear progressively during the process of pathogenesis and whose expression is altered in response to changes in growth conditions in the varied niches within the human body, and through the combined effects of bacterial colonization and the host response. In particular, changes in osmolarity and pH combined with temperature variation appear to have a considerable impact on the ability of yersiniae to survive and promote successful pathogenesis (Straley and Perry, 1995). *Y. enterocolitica* synthesizes many virulence factors, including the outer membrane (OM) adhesins YadA, Ail, Inv and Myf, which allow the bacterial cells to adhere to and invade the intestinal epithelium and/or to colonize the peripheral tissues (Bottone, 1997; Bialas et al., 2012). *Y. enterocolitica* also possesses a complex protein secretion machinery that spans both the inner and outer membranes – the Ysc Type 3 Secretion System (T3SS). This secretion apparatus enables the translocation of anti-host effector proteins known as Yops (Yersinia outer proteins) into host cells. The Ysc-Yop T3SS is required for full virulence in the late stages of the process of pathogenesis (Cornelis et al., 1998; Cornelis, 2002). The Yop proteins are responsible for the inhibition of host defense reactions and permit the multiplication of bacterial cells in the reticuloendothelial system (Viboud and Bliska, 2005). Iron acquisition and storage systems also play an essential role in *Y. enterocolitica* physiology and virulence by allowing the bacterium to adapt to specific niches outside and inside the human body where iron is limited (Heesemann et al., 1993; Perry, 1993).

Two-component regulatory systems (TCSs) constitute an important regulatory mechanism in bacterial cells that mediate a variety of adaptive responses to changes in environmental cues (Stock et al., 1989; Hoch and Silhavy, 1995). TCSs are found in saprophytic and pathogenic bacteria and the archetype is EnvZ/OmpR. This system was initially characterized in its role in the osmoregulation of OmpC and OmpF porin expression in non-pathogenic *Escherichia coli* K-12 (Russo and Silhavy, 1990; Hoch and Silhavy, 1995). The TCS consists of the sensor protein EnvZ, which has dual kinase/phosphatase activity, and regulatory protein OmpR, which acts to regulate transcription (Kenney, 2002). In response to environmental changes, EnvZ modulates the phosphorylation and thereby the transcriptional activity of OmpR. Besides its partner kinase EnvZ, OmpR is likely to be available for phosphorylation by small phospho-donors, e.g. acetyl-P, which may cause activation (Shin and Park 1995; Head et al. 1998). Recent studies in *Salmonella* have provided evidence that the binding of OmpR to the regulatory regions of target genes may result not only from phosphorylation by EnvZ, but also as an effect of DNA relaxation in response to acid stress that allows OmpR to cooperate with the altered topology to modulate transcription (Cameron and Dorman, 2012; Quinn et al., 2014).

A considerable body of research indicates that OmpR is involved in the control of various cellular processes and functions in *E. coli* (Higashitani et al., 1993; Shin and Park, 1995; Yamamoto et al., 2000, Hirakawa et al., 2003; Goh et al., 2004; Jubelin et al., 2005). These findings have prompted many laboratories to examine the role of the EnvZ/OmpR system in the physiology of pathogenic bacteria. This TCS has been identified in a number of pathogens, including pathogenic *E. coli*, and bacteria of the genera *Shigella* and *Salmonella*, where it participates in the regulation of target genes in response to changes in osmolarity and pH, and is also involved in virulence (Bernardini et al., 1990; Bang et al., 2000; Lee et al., 2000; Bang *et al* 2002; Schwan et al., 2002; Rentschler et al., 2013; Chakraborty et al., 2015).

Microarray studies have revealed that OmpR influences the expression of as many as 125 genes in *E. coli* (Oshima et al., 2002) and 208 genes in *S. enterica* serovar Typhi (Perkins et al., 2013). Moreover, it has been shown that although the OmpR proteins of *E. coli* and *S.* Typhimurium are identical, the OmpR regulons in these enteric bacteria are divergent, with only 15 genes in common (Quinn et al., 2014).

The EnvZ/OmpR system also operates in bacteria of the genus *Yersinia*, where it may serve a variety of functions. Some members of the *Yersinia* OmpR regulon have been identified by comparing gene expression in null *ompR* mutants with that in wild-type strains. The physiological consequences of the loss of the OmpR protein in *Y. enterocolitica* were studied by monitoring the growth and survival of cells subjected to various environmental stresses (Dorrell et al., 1998; Brzostek et al. 2003). These experiments provided evidence that OmpR is involved in the adaptation of *Y. enterocolitica* to high osmolarity, oxidative stress and low pH. OmpR was also found to be required for adaptation to osmotic upshifts and low pH in *Y. pseudotuberculosis* (Flamez et al., 2008; Zhang et al., 2013) and *Y. pestis* (Gao et al., 2011). These data confirmed that apart from its well-known role in the molecular response to changes in osmolarity, OmpR influences the expression of other environmental stress response genes, especially those encoding acid-induced proteins. OmpR of *Y. pseudotuberculosis* has been shown to positively regulate urease production, conferring the ability to resist acid stress conditions (Hu et al., 2009a). Studies on the role of OmpR in *Y. pseudotuberculosis* uncovered its function in the regulation of a Type VI secretion system that promotes resistance to low pH (Gueguen et al., 2013). OmpR is also involved in the positive regulation of flagella synthesis in *Y. enterocolitica* and *Y. pseudotuberculosis*, which contrasts with its negative role in *E. coli* (Hu et al., 2009b; Raczkowska et al., 2011a). Our laboratory has previously shown that OmpR inhibits transcription of the invasin gene *inv* in *Y. enterocolitica* (Brzostek et al., 2007). In a recent study we also observed a correlation

between serum resistance of *Y. enterocolitica* and the activity of OmpR, suggesting that OmpR-dependent changes in outer membrane proteins (OMPs) and surface-anchored components might be responsible for this phenomenon, which could assist this bacterium in switching between distinct niches within and outside the host body (Skorek et al., 2013).

Environmental factors like temperature, and calcium and ferric ion concentrations have a considerable impact on the production of membrane proteins, including virulence factors, in pathogenic *Yersiniae* (Straley and Perry, 1995). Proteomic studies on *Y. pestis* have examined changes in membrane or soluble proteins in response to temperature and calcium (Chromy et al., 2005; Pieper et al., 2009a; Pieper et al., 2009b). However, the effect of environmental signals and the influence of the EnvZ/OmpR pathway, OmpR alone or other TCSs on the membrane proteome composition has yet to be studied in *Yersinia* spp.

In this study, alterations in the outer membrane protein profile of *Y. enterocolitica* in response to the level of OmpR and varying temperature, pH or osmolarity were examined by comparative proteomic analysis. The shotgun proteomic analysis method was applied to permit quantification of any observed differences in the membrane proteome. Principle components analysis (PCA) of the LC-MS/MS expression list was used to evaluate distinct abundance patterns among the analyzed groups. Our results indicate that the loss of OmpR affects the production of 120 proteins, both positively and negatively. The impact of OmpR on the expression of the adhesin YadA and the HemR heme uptake receptor – identified as new OmpR-regulated targets – was studied in more detail.

Results and discussion

Proteomic analysis of outer membrane-enriched sarkosyl-insoluble fractions of *Y. enterocolitica* strains

To investigate the role of the response regulator OmpR in modulating the OM composition of *Y. enterocolitica*, we performed a proteomic analysis of outer membrane-enriched sarkosyl-insoluble membrane fractions (OMsl) of wild-type strain Ye9 and isogenic *ompR* null mutant AR4, cultured at 26°C or 37°C in standard LB medium (86 mM NaCl, pH 7.0), or in LB supplemented with NaCl (386 mM, pH 7.0) or adjusted to pH 5.0. Shotgun label-free quantitative LC-MS/MS analysis of all OMsl samples produced a dataset of 543 proteins identified by at least two peptides. Among these proteins the majority are annotated in the databases as cell envelope proteins, i.e. inner membrane (IM; 52%) and integral OM and OM-associated proteins (20%). The membrane proteins account for approximately 67% (OM) and 38% (IM) of the predicted respective membrane proteomes of *Y. enterocolitica*. We also identified periplasmic and cytoplasmic proteins, and proteins of unknown localization within the samples. Contamination by proteins localized outside the cell envelope is unavoidable because the lysis of bacterial cells leads to aggregation of the cellular contents. The presented data confirm the enrichment of OM proteins in the samples and support the validity of the procedure applied to isolate the OMsl fractions.

The proteomes of strains grown under different conditions were further compared to produce differential OMsl proteome lists (Tables S3 and S4).

Effect of temperature, osmolarity and pH on the membrane proteome of the wild-type *Y. enterocolitica* strain Ye9

As a first step in our differential analysis of the OMsl proteome of *Y. enterocolitica*, samples from wild-type strain Ye9 grown under different osmolarity and pH conditions at 26°C or 37°C were qualitatively and quantitatively compared (Table S3). Proteomic analysis revealed 76 differentially expressed proteins accepted for quantification (q-value $\leq$ 0.05, at least 2 peptides per protein) following growth of *Y. enterocolitica* under the different conditions.

The greatest impact on the OM proteome was observed in response to pH (44 proteins whose abundance changed at pH 5.0) followed by temperature (39 changes) and osmolarity (26 changes), with several proteins affected by more than one physico-chemical condition.

Temperature affected several proteins, particularly those involved in virulence. For example, the major *Y. enterocolitica* adhesin YadA (Skurnik and Toivanen, 1992) and components of the *Yersinia* Ysc-Yop T3SS (Lambert de Rouvroit et al., 1992; Akopyan et al., 2011) were more abundant at the higher temperature, in agreement with previous reports. The group of osmoregulated proteins included porin OmpC, in agreement with previous reports for *Y. enterocolitica* (Brzostek et al., 1989) and also *E. coli* (Russo and Silhavy, 1990). The proteins upregulated by low pH included the urease components UreA and UreG and the OM usher protein MyfC involved in *Y. enterocolitica* Myf fimbrial assembly, confirming previous reports (Hu et al., 2009a; Iriarte and Cornelis, 1995). In summary, this analysis detected several changes in protein abundance known to occur in response to different growth conditions which confirmed the ability of this method to identify temperature, osmo- and acid-regulated cellular components. Together, the changes detected in the OMsl proteome of *Y. enterocolitica* may reflect physiological adaptations necessary for growth of yersiniae in highly variable environments.

Differences in protein abundance between *ompR* mutant and parental strains

We next focused our analysis on proteins within the OMsl fraction that showed significant differences in abundance in the *ompR* mutant AR4 compared to the wild-type Ye9, cultured at 26°C or 37°C under different osmolarity and pH conditions. Statistical analysis of the quantitative results of the MS analysis revealed 120 proteins (q-value ≤ 0.05, identified by at least two peptides) showing differential abundance in the *ompR* mutant compared to the wild-type strain under at least one of the tested conditions (ratio ≤ 0.67 or ≥ 1.5, Table S4).

Notably, differences between these two strains in the abundance of particular proteins were observed upon growth in all three media, i.e. standard conditions, high osmolarity and low pH (36 proteins), while for other proteins, differences were evident only at high osmolarity and/or low pH (Fig. S1). Similarly, some differences were seen only at 26°C or 37°C (Fig. S1). To assess the variation in protein abundance patterns in both strains under the tested growth conditions (confirmed by independent biological repetition) we performed the multivariate statistical test Principal Components Analysis (PCA; Fig. S2; Friedman et al., 2006; Friedman et al., 2007). First, two principle components indicated that temperature was a larger source of variation within the dataset than the *ompR* mutation (Fig. S2A). The different relative orientations of the group analyses at 26°C and 37°C under standard conditions (Fig. S2B), high osmolarity (Fig. S2C) or low pH (Fig. S2D) demonstrated high reproducibility between replicate samples and most likely indicated that a different subset of proteins was expressed. The PCAs confirmed the major differences between the protein expression patterns of the wild-type Ye9 and mutant AR4 at both temperatures under the different conditions of osmolarity and pH.

Of the 120 OmpR-dependent *Y. enterocolitica* proteins identified by proteomic analysis (Table 1 and Table S4), the majority are annotated in the Swiss-Prot database as integral OM and OM-associated proteins (38%). Proteins from the inner membrane (37%), periplasm (7%), cytoplasm (12%) and those of unknown localization (6%), were also identified. Some of the OmpR-dependent proteins recognized in this study are specific to the pathogenic Yersiniae (Ysc-Yop T3SS), others are present in different enteropathogenic Yersiniae, i.e. *Y. pseudotuberculosis* and *Y. enterocolitica* (Inv, YadA), while a few are found only in *Y. enterocolitica* serotype O:9 (O-Antigen biosynthesis enzymes WbcV, WbcU, WbcT).

The proteins regulated by OmpR (positively or negatively) were grouped into several Gene Ontology (GO) categories according to the biological processes in which they participate (Fig. 1, Table 1). About one-third of the OmpR-dependent proteins are involved in transport across membranes, i.e. transporter activity (26%) and porin activity (7%). The next most abundant category corresponds to proteins involved in pathogenesis (17%), followed by proteins participating in cell envelope organization (12%) [including outer membrane assembly (5%), cell wall organization (4%) and LPS-associated O-antigen biosynthesis (3%)]. Proteins facilitating iron ion homeostasis were also identified (5%), as well as some involved in resistance to stress (5%). Thus, many OmpR-dependent proteins appear to play a role in the interaction of *Y. enterocolitica* with its surroundings.

The relatively large number of differentially expressed proteins identified by this proteomic analysis supports the previously suggested involvement of OmpR in global gene regulation in enterobacteria (Oshima et al., 2002; Perkins et al., 2013; Quinn et al., 2014). The assembled panel of proteins is likely to include some whose differential abundance results from direct regulation by OmpR, i.e. binding of this factor to promoter regions of the corresponding genes, while the expression of others might be affected indirectly through the influence of OmpR on other transcriptional regulators, post-transcriptional regulators (e.g. small RNAs) or even proteases. OmpR regulates multiple genes in the *Enterobacteriaceae* by binding to sites in their promoter regions that have similar but not identical sequences (Maede et al., 1991; Harlocker et al., 1995; Huang and Igo, 1996; Yoshida et al., 2006; Rhee et al., 2008; Perkins et al., 2013). Binding site degeneracy makes the identification of new OmpR regulon members difficult. Nevertheless, we used the *E. coli* OmpR consensus sequence [TTTTACTTTTT**G**(A/T)A**AC**ATAT] (Fig. 2A) (Maeda et al., 1991) to search for candidate genes of *Y. enterocolitica* regulated by OmpR among those encoding proteins identified by our proteomic analysis. Moreover, we also compared these promoters with a *Yersinia*

consensus motif that was defined using sequences experimentally shown to bind OmpR (Fig. 2B). The predicted OmpR-DNA binding sites in the promoters of the indicated *Y. enterocolitica* genes (with highest similarity to the *E. coli* and *Yersinia* spp. consensus sequences) are listed in Fig. 2C. The fold change in the abundance of the identified OmpR-dependent proteins is shown graphically in Fig. 2D. Below, we describe the experimental testing of two of the putative elements identified by this *in silico* analysis using an *in vitro* DNA binding assay. In future it will be necessary to verify that the other genes with putative OmpR binding sequences are indeed the object of direct transcriptional control by this regulator. In the following sections we give a more detailed description of some of the identified OmpR-dependent proteins and provide some insights into the impact of this regulator on the adaptive abilities of *Y. enterocolitica.*

OmpR influences the production of general and substrate-specific porins

Eight proteins affected by OmpR were classified as porins (Table 1). They are homologs of general (i.e. non-specific) and substrate-specific porins from *E. coli* that form water-filled channels which permit the diffusion of hydrophilic solutes across the outer membrane (Nikaido, 2003). These proteins include the general porins OmpC and OmpF involved in the passive diffusion of small molecules (< 600 Da). We previously showed that both porins form hydrophilic diffusion channels across the OM of *Y. enterocolitica* and that their absence reduces the permeability of the OM for β-lactam compounds (Brzostek and Nichols, 1990). In the present analysis, these two proteins were found to be less abundant in the *ompR* mutant compared to the wild-type (Table 1), which confirms our previous finding (Brzostek and Raczkowska, 2007) and support the notion that OmpR is required for the activation of these genes in both *E. coli* and *Y. pestis* (Gao et al., 2011; Russo and Silhavy, 1990). The levels of OmpC and OmpF in the OM of enterobacteria vary depending on the osmolarity of the

medium. In the *E. coli* model, the osmoregulation of both porins is mediated by EnvZ/OmpR so that OmpC (the narrow porin) levels increase in media of high osmolarity, while those of OmpF (the wider porin) decrease (Forst and Inouye, 1988). It is thought that this alteration in membrane protein composition may limit the diffusion of harmful compounds into cells growing within a mammalian host. The observed osmoregulation has been correlated with the strength of OmpR binding to three and four consensus-like sequences identified in the DNA regions upstream of the *E. coli ompC* and *ompF* ORFs, respectively. In the wild-type *Y. enterocolitica* strain Ye9, we found an increased level of OmpC at high osmolarity, while OmpF abundance was not affected (Table S3). Notably, three consensus-like OmpR binding site sequences were identified within the regulatory regions of the *Y. enterocolitica ompC* and *ompF* genes (Fig. 2), as was also the case in pathogenic *Yersinia* (Gao et al., 2011). Thus, the lack of osmoregulation of the *ompF* gene in *Y. enterocolitica* Ye9 might be correlated with the absence of a distal fourth OmpR-binding site in the promoter. The pattern of porin osmoregulation in *Y. enterocolitica* is clearly different from that of *E. coli*, but is shared by *S.* Typhi and *Y. pestis* (Puente et al., 1991; Gao et al., 2011). Together, these results indicate that some features of the regulation of *ompC* and *ompF* expression, such as dependence on OmpR, appear to be common to these bacteria, although the osmoregulatory mechanism seems to be different. Such variations in porin regulation among different enterobacteria might reflect the varied function of these proteins in bacteria growing in different environmental niches. Since the OmpC porin seems to play some role in the adhesion properties of *Y. enterocolitica* (Raczkowska et al., 2011b), the increased level of this protein at high osmolarity could be beneficial to cells residing in the ileum.

The third general porin upregulated by OmpR is similar to the anion-specific phosphoporin PhoE induced by phosphate deprivation in *E. coli* (Nikaido, 2003). To our knowledge, a link between PhoE and OmpR has not previously been identified in *E. coli* and

thus might reflect a specific adaptation of *Y. enterocolitica* physiology, especially at low ambient temperature. However, we were unable to identify a consensus OmpR binding site in the *phoE* promoter. OmpR also influenced the production of OmpX, a porin of undefined function, in agreement with previous data demonstrating the positive regulation of *ompX* expression by OmpR in *Y. enterocolitica* (Skorek et al., 2013) and *Y. pestis* (Gao et al., 2011). Inspection of the regulatory region of *ompX* showed two putative OmpR binding sites with 45 and 60% identity to the *E. coli* consensus sequence, and 45 and 50% identity to the *Yersinia* spp. consensus sequence, respectively (Fig. 2). The panel of OmpR-dependent porins also included a sucrose-specific porin related to enterobacterial ScrY (Schmid *et al.*, 1991) and OmpW, a small porin of the OmpW/AlkL family present in all Gram-negative bacteria, which might be involved in the response to different stresses, e.g. osmotic and oxidative stress (Hong et al. 2006). Putative OmpR-binding sites were identified in the promoter regions of the genes encoding these proteins (Fig. 2).

A major impact of OmpR on the proteomic profile of *Y. enterocolitica* was its effect on the abundance of porin KdgM2 related to KdgM and KdgN oligogalacturonate-specific porins in *Dickeya dadantii* (Blot et al., 2002). Upon growth at 26°C, the OmpR-negative strain exhibited a more than 100-fold increase in the level of this protein (Table 1), implying a major role for OmpR in repressing KdgM2 production. A putative OmpR-binding motif was recognized in the promoter region of *kdgM2* (Fig. 2). In the pectinolytic bacterium *D. dadantii* KdgM and KdgN porins overlap functionally, and their expression is subject to reciprocal OmpR regulation, although the direct involvement of OmpR in this process was not verified (Condemine and Ghazi, 2007).

Transporters affected by OmpR

As stated above, the majority (24%) of proteins identified as OmpR-regulated belong to the GO category of proteins with "Transporter activity" (Fig. 1, Table 1). Strikingly, OmpR appears to exert a negative influence on amino acid uptake (seven proteins upregulated in the *ompR* mutant compared to the wild type) while promoting peptide uptake (downregulation of TppB, OppA, OppD and OppF in the *ompR*-negative strain). The D-alanine/D-serine/glycine permease CycA deserves a special mention since this protein exhibited a more than 30-fold increase in *ompR* mutant cells. Since D-alanine is a central molecule in peptidoglycan assembly and cross-linking (Walsh, 1989), the OmpR-dependent negative regulation of a D-alanine/D-serine/glycine permease might be relevant for the cell wall metabolism of *Y. enterocolitica*. Conversely, the tripeptide permease TppB was less abundant (~15-fold) in the *ompR* cells compared to the wild type strain (Table 1), which is in agreement with data obtained in *S.* Typhimurium and *E. coli* showing that OmpR is involved in the positive regulation of *tppB* (Gibson et al., 1987, Goh et al., 2004). *In silico* analysis identified putative OmpR-binding sites in the promoters of both the *cycA* and *tppB* genes of *Y. enterocolitica* (Fig. 2).

We also found that the transport of exogenous long chain fatty acids (LCFAs) across the *Y. enterocolitica* cell envelope could be modulated by OmpR, since the OM transporter FadL was 4- to 7-fold more abundant in the *ompR* mutant than in the parental strain. This suggests that OmpR exerts a negative effect on FadL production, which is in agreement with a study that reported the inhibition of *fadL* transcription by OmpR in *E. coli* (Higashitani et al., 1993). Putative OmpR-binding sites were identified in the promoter region of the *Y. enterocolitica fadL* gene (Fig. 2). The OmpR-dependent modulation of LCFA uptake from the environment may be important for several cellular processes in *Y. enterocolitica*, including lipid metabolism.

Another transporter whose abundance was decreased (11- to 16-fold) in the *ompR* mutant is DcuA, an inner membrane C_4-dicarboxylate transporter (antiporter for aspartate and fumarate) (Table 1). The *dcuA* and *dcuB* genes of *Escherichia coli* encode homologous proteins that appear to function as independent C_4-dicarboxylate transporters under different growth conditions (Golby et al., 1998). The predicted OmpR-binding site in *Y. enterocolitica dcuA* is shown in Fig. 2.

Finally, the abundance of some efflux transporters was also altered in the *ompR* mutant (Table 1). For example, AcrA a component of the AcrAB-TolC multidrug efflux pump was less abundant in the *ompR*-negative strain AR4 compared to the parental strain Ye9. This efflux pump belongs to the RND family, some members of which confer drug resistance in Gram-negative bacteria (Blair and Piddock, 2009). A putative OmpR-binding site found in the *acrA* promoter region is shown in Fig. 2.

In summary, our results suggest that OmpR influences the expression of nutrient transporters to promote the uptake of peptides (while repressing amino acid uptake) and reduce the uptake of long chain fatty acids. In addition, alterations in the membrane protein composition mediated by OmpR may promote the excretion of toxic compounds, thereby mitigating their harmful effects. Some of the differentially expressed proteins are encoded by genes that have not previously been considered members of the OmpR regulon and further work is required to identify those that are directly regulated by OmpR.

Outer membrane assembly: OMPs and LPS

Our proteomic analysis characterized the impact of OmpR on proteins belonging to the GO category “Gram-negative-bacterium-type cell outer membrane assembly”. Importantly, three proteins of the Bam complex, i.e. BamA, BamC and BamD were less abundant in the *ompR* mutant AR4 compared to the wild-type strain Ye9 (Table 1). In *E. coli*, BamABCD is a multi-

subunit complex in the outer membrane that is responsible for folding and inserting OMPs in a beta-barrel conformation (Rigel and Silhavy, 2012). The observed OmpR-dependent regulation of Bam proteins in *Y. enterocolitica* indicates a role for OmpR in modulating the protein composition of the outer membrane. In addition, we observed a 2- to 3-fold decrease in the proteins WbcV, WbcU and WbcT in the *ompR* mutant (Table 1). These proteins are involved in the synthesis of the unique serotype O:9 O-polysaccharide (OP) present in the LPS of *Y. enterocolitica* Ye9 (Skurnik et al., 2007). In a previous study we revealed that the loss of OmpR correlates with a reduced LPS/OP content in the OM of *Y. enterocolitica* serotype O:9 (Skorek et al., 2013). Thus, OmpR could modulate the LPS status of *Y. enterocolitica* through its influence on WbcV, WbcU and WbcT. Since we were unable to identify OmpR-binding sites in the regulatory regions of the *bam* and *wbc* genes, the role of OmpR in modulating the production of these proteins is probably indirect.

Pathogenesis

The second most abundant GO category of OmpR-dependent proteins is related to pathogenesis (Fig. 1, Table 1). Almost all proteins in this category were downregulated by OmpR, with the notable exception of the OM usher protein MyfC, involved in *Y. enterocolitica* Myf fimbrial assembly. OmpR promoted the production of MyfC upon growth at 37°C and acid pH (Table 1), in agreement with the environmental parameters known to induce Myf antigen synthesis (Iriarte and Cornelis, 1995). The predicted OmpR binding site in the *myfC* promoter region is shown in Fig. 2. Other interesting exceptions were components of urease, whose abundance was modulated either positively or negatively depending on the temperature. Urease is a multisubunit metalloenzyme that is crucial for resistance to low pH and promotes the survival of *Y. enterocolitica* in the presence of stomach acid (De Koning-Ward and Robins-Browne, 1995). Our results revealed the positive impact of OmpR on urease

391 expression in *Y. enterocolitica* grown at 37°C and the opposite effect in cells cultured at 26°C,
392 i.e. negative OmpR-dependent regulation of UreA, UreC and UreG at the lower temperature.
393 Urease genes were previously shown to be directly and positively regulated by OmpR in *Y.*
394 *pseudotuberculosis* at 37°C, but lower temperatures were not assessed (Hu et al., 2009a). It is
395 noteworthy that direct binding of OmpR to the promoter regions of urease genes has been
396 demonstrated in *Y. pseudotuberculosis* (Hu et al., 2009a), but we were unable to identify
397 consensus OmpR-binding sites in the regulatory regions of the three *ure* transcriptional units
398 of *Y. enterocolitica* (*ureABC, ureEF* and *ureGD*). We speculate that during infection of the
399 host (at 37°C), especially via the gastrointestinal route where the bacteria encounter gastric
400 acid, OmpR promotes the production of urease to facilitate survival in the stomach and
401 persistence in environmental niches of low pH in the later stages of pathogenesis.

402 The majority of the identified proteins in the Pathogenesis GO category are part of the
403 Ysc-Yop T3SS (Table 1) and are encoded by virulence plasmid pYV. These include structural
404 components of the *Yersinia* injectisome, the regulatory elements, the secreted effectors and
405 translocators (Dewoody et al., 2013). Increased levels of all these proteins were detected in
406 the *ompR* mutant at 37°C in at least one of the tested growth media (~3-70-fold). The
407 differences in the levels of Ysc-Yop proteins related to the presence of OmpR were confirmed
408 by Western blot analysis (data not shown). The regulation of Ysc-Yop expression in
409 pathogenic *Yersinia* is highly complex and tightly connected with the secretion process, which
410 is triggered at a temperature of 37°C in calcium-deficient medium and modulated by T3SS
411 regulatory proteins and certain host signals *in vivo* (Straley et al., 1993; Li et al., 2014).
412 However, since the growth conditions employed for this proteomic analysis were not designed
413 to optimize Yop secretion (the growth medium was not depleted of calcium), it is unclear
414 whether the observed alterations in the abundance of these proteins resulted from (i) a direct
415 effect of OmpR on *ysc-yop* gene expression, (ii) some indirect effect due to changes in the cell

envelope, or (iii) disturbance of the secretion process leading to accumulation of secreted proteins in the envelope. Definition of the precise role of OmpR in the regulation of *ysc-yop* gene expression will be the subject of future investigations. Finally, our proteomic analysis revealed that the loss of the OmpR regulator caused a 5- to 10-fold increase in YadA, a pYV-encoded, multifunctional OM protein (Table 1). Since YadA represents a major adhesin and serum resistance factor of *Y. enterocolitica* (El Tahir and Skurnik, 2001, Mikula et al., 2013), we decided to investigate the mechanism of OmpR-mediated downregulation of *yadA* expression in *Y. enterocolitica*.

OmpR downregulates expression of the major adhesin gene *yadA*

The proteomic data showed an increased amount of YadA in the *ompR* mutant strain AR4 compared to the wild-type strain Ye9 under all tested growth conditions (Table 1). The abundance of YadA protein in the OM of *Y. enterocolitica* cells grown at 26°C and 37°C under different osmolarity and pH conditions was evaluated further by Western blotting using a YadA-specific antibody (Fig. 3A and B). It has been shown previously that YadA is a trimeric protein exhibiting heat stability, with only slight denaturation during heating in Laemmli buffer (Mack et al., 1994; Schutz et al., 2010). To assess any differences in the levels of the oligomeric and monomeric forms of YadA between the wild-type strain Ye9 and the *ompR* mutant strain AR4, the OMsl samples were untreated or treated with 8 M urea to disrupt protein trimers. As shown in Fig. 3A OMsl samples from strains grown at 37°C, boiled in Laemmli buffer and examined by Western blotting, gave a YadA band of approximately 200 kDa and several bands of intermediate size. Moreover, the sample prepared from the *ompR* mutant AR4 showed an increased amount of YadA oligomers compared to the samples from wild-type strain Ye9. Western blot analysis of the OMsl fractions demonstrated that the quantity of the monomeric form of YadA resulting from urea

denaturation was higher in the *ompR* mutant that the wild-type strain (Fig. 3B). In both analyses (with and without urea) the differences in the level of YadA between the two strains were observed independently of the osmolarity and pH conditions. YadA production was negligible in both strains cultured at 26°C, confirming the temperature-inducible nature of YadA and suggesting that the observed thermoregulation of this protein is OmpR-independent.

To obtain further evidence that OmpR regulates YadA expression, experiments were performed using plasmid pFX-yadA, which carries the *yadA* promoter driving the expression of a translational fusion of the first 16 codons of *yadA* with the gene encoding GFP. Plasmid pFX-0 carrying the promoterless *gfp* gene was used as a negative control (Schmidtke et al., 2013). Both plasmids were introduced into the wild-type and mutant *Y. enterocolitica* strains, and following growth under different temperature, pH and osmolarity conditions, bacterial fluorescence was measured by flow cytometry (Fig. 4). The cells carrying the control plasmid pFX-0 gave a low fluorescence signal (data not shown), in contrast to those transformed with pFX-yadA, encoding the YadA'-'GFP fusion. Higher fluorescence was observed in the *ompR* mutant compared to the wild-type Ye9 in cells grown to stationary phase at 37°C and 27°C (Fig. 4). Interestingly, the increase in YadA'-'GFP expression in the mutant strain was much greater than in the wild-type, especially at 37°C, in response to high osmolarity stress (3-fold increase) (Fig. 4B). To confirm that the lack of OmpR resulted in derepression of *yadA*, plasmid pompR carrying the wild-type *ompR* allele was used to complement the mutation in strain AR4. Complementation caused reduced fluorescence in cultures grown in LB medium at both 27°C and 37°C (Fig. 4A and B), indicating that OmpR negatively regulates *yadA*. The complementation effect was not as clear in cells grown under high osmolarity and low pH.

A putative binding site for OmpR was identified 57-bp downstream of the transcription start of *yadA* by *in silico* analysis (Y1 site), suggesting that OmpR might directly

repress *yadA* transcription (Fig. 2, Fig. 5A). To more precisely define the OmpR binding site, three fragments (F1, F2, F3) from the *Y. enterocolitica yadA* regulatory region were amplified by PCR (Fig. 5B; Table S2) and used in an electrophoretic mobility shift assay (EMSA) with increasing concentrations of phosphorylated OmpR (OmpR-P). A PCR-amplified 304-bp fragment of 16S rDNA was included in each binding reaction as a negative control (Fig. 5C; Table S2). Specific OmpR-P binding caused a shift in the migration of the 392-bp fragment F1 that encompasses the OmpR-binding site indicated by *in silico* analysis (Fig. 5C). OmpR-P was unable to bind the control 16S rDNA fragment. Moreover, OmpR-P did not shift the migration of either the upstream regulatory region fragment F2 or the downstream fragment F3, both of which lack the 20-bp OmpR-binding site (Fig. 5C), implying that OmpR binds at the predicted position in fragment F1.

These results demonstrated that OmpR can specifically bind to the *yadA* promoter region, which suggests that expression is inhibited by a direct mechanism. In conclusion, our genetic studies identified *yadA* as a new member of the OmpR regulon. OmpR may modulate the production of YadA in response to environmental signals experienced by *Y. enterocolitica* in different niches during the infection process. Down-regulation of YadA might enhance the survival of *Y. enterocolitica* by preventing binding of the bacteria to host cells, thus favoring further dissemination to deeper tissues.

OmpR-dependent production of proteins involved in iron homeostasis

Another group of OmpR-dependent proteins identified in our proteomic analysis belong to the "Iron ion homeostasis" GO category (Table 1). Proteins of this category included three OM active transporters (also called TonB-dependent transporters): HemR, a receptor involved in heme/hemoprotein uptake (Stojiljkovic and Hantke, 1992), FepA, an iron-enterobactin receptor, and the FecA receptor responsible for dicitrate-mediated iron assimilation (Andrews

et al., 2003). These proteins were described previously as iron-regulated receptors whose expression is controlled by the regulator Fur in *Yersinia* spp. (Jacobi et al., 2001; Gao et al., 2008). When the intracellular iron concentration is high, Fur binds iron (Fe^{2+}-Fur) and represses the expression of genes involved in iron/heme acquisition and transport (Hantke, 2001; Troxell and Hassan, 2013). Both FecA and HemR were more abundant (2-fold increase) in the *ompR* mutant than in wild-type cells. FecA was affected under almost all conditions, while differences in the level of HemR were detected mainly at 37°C (Table 1). On the other hand, the level of the receptor FepA was slightly decreased in the *ompR* mutant cells (~1.5-fold), but only when cultured at 26°C in standard LB medium. The impact of OmpR on receptors of the siderophore and heme uptake systems, underscores the role of this regulator in the iron metabolism of *Y. enterocolitica.* Putative OmpR-binding sites were identified in the promoters of the genes *hemR*, *fepA* and *fecA* (Fig. 2).

Insights into the role of OmpR in the repression of *hemR*

Given our long-standing interest in *Y. enterocolitica hemR*, we further investigated the relationship between OmpR and HemR. HemR is a unique OM receptor in *Y. enterocolitica* which can bind heme or multiple host hemoproteins (hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, heme-albumin, myoglobin) (Bracken et al., 1999; Runyen-Janecky, 2013). Following binding to HemR, heme is transported through the periplasm and across the inner membrane via the TonB/ExbB/ExbD transport system (Stojiljkovic and Hantke, 1992). The regulation of *hemR* expression *in vitr*o and *in vivo* was previously elucidated in our laboratory using mouse-virulent *Y. enterocolitica* bio/serotype 1B/O:8 (Jacobi et al., 2001). However, the role of the OmpR regulator in the control of *hemR* expression has never been investigated. Since our proteomic analysis showed that the level of HemR receptor is elevated in the OM of the *ompR* mutant strain at 37°C, we first attempted to verify this result by Western blotting

using an antibody specific for HemR (Fig. 6A). Parental strain Ye9 and the *ompR* mutant AR4 were grown at 37°C in standard LB medium, LB medium at high osmolarity (386 mM NaCl) or low pH (pH 5.0). As expected, HemR was not visible in the wild-type Ye9 grown in LB medium, and could only be detected in this strain following growth under iron-derepressed conditions (LBD). This result confirmed the iron-regulated status of HemR in agreement with the previously established Fur-mediated repression of the *Y. enterocolitica hemR* gene (Stojiljkovic and Hantke, 1992). In contrast to the wild-type, HemR was detected in the *ompR* mutant AR4 grown in standard LB (under iron-repressed conditions), suggesting that the production of HemR is derepressed in the strain lacking OmpR (Fig. 6A). When the wild-type allele of *ompR* was introduced into mutant AR4 *in trans* on plasmid pompR, the production of HemR in LB medium decreased to the wild-type level, i.e. it was no longer detectable (Fig. 6A). Moreover, HemR was upregulated in the *ompR* mutant in all tested conditions, even in low iron medium (LBD) (Fig. 6A). Interestingly, in the *ompR* mutant grown in LB under high osmolarity conditions (386 mM NaCl), HemR was more abundant than in the same strain grown in standard LB (Fig. 6A). This effect was not observed in LB at low pH. This finding might indicate that in the absence of OmpR another regulatory mechanism operates to increase the HemR level in response to high osmolarity.

We next tested whether the expression of *hemR* is under the control of OmpR (Fig. 6B) by constructing *hemR-lacZYA'* chromosomal transcriptional fusions in the wild-type strain and the *ompR* mutant derivative (strains Ye9H and AR4H, respectively). Based on measurements of β-galactosidase activity we found higher *hemR* expression in strain Ye9H grown in LBD (under iron-starvation conditions) than in LB medium at 26°C (~ 22-fold) and at 37°C (~ 3-fold), confirming the iron-repressible nature of the *hemR* promoter (Fig. 6B). In the *ompR* mutant AR4H, *hemR* expression was upregulated 2- to 3-fold in standard LB medium compared to the isogenic wild-type strain Ye9H. This upregulation still occurred in

the mutant strain transformed with vector pBBR1MCS-5, but was absent following complementation with the wild-type *ompR* allele on plasmid pompR (Fig. 6B).

Increased *hemR* expression in the *ompR* mutant grown in LB (repressed conditions) might be caused by derepression of *hemR* expression directly and/or by the alleviation of transcriptional repression by the iron-responsive repressor Fur. To separate these two effects, we examined OmpR-mediated regulation of *hemR* expression under derepressed conditions (released from Fur repression in LBD) at 26°C and 37°C (Fig. 6B). The absence of iron in the medium resulted in an increase in the expression of *hemR* in the *ompR* mutant AR4H, almost to the wild-type level, i.e. OmpR-dependent regulation of *hemR* is lost. This finding suggested that OmpR could regulate *hemR* indirectly, presumably through an effect on *fur* expression.

Interestingly, while the OmpR-dependent regulation of *hemR* transcription disappeared under derepressed conditions (LBD medium), the effect of OmpR on the HemR protein (as judged by immunoblotting) was still observed, suggesting the involvement of OmpR in posttranscriptional regulation of *hemR*. Intriguingly, in *E. coli*, OmpR activates the expression of two small RNAs, OmrA and OmrB, which repress several iron receptor genes (*fepA*, *fecA* and *cirA*) (Guillier and Gottesman, 2006). Only the sRNA OmrA is present in *Y. enterocolitica* and as in *E. coli*, its expression is positively regulated by OmpR (our unpublished observation). Future studies will investigate the role of OmrA in post-transcriptional regulation of iron/heme receptors in *Y. enterocolitica*.

To test whether OmpR directly and/or indirectly regulates *hemR* transcription, we examined the ability of OmpR to bind to the *hemR* promoter region *in vitro*. Previous reports have shown that the *hemR* ORF is located downstream of the *hemP* ORF and that the expression of *hemR* is repressed by iron, suggesting that it is regulated by Fur (Stojiljkovic and Hantke, 1992; Jacobi et al., 2001). Using BPROM software, we identified two putative promoters for the *Y. enterocolitica hemR* gene (Fig. 7A). The first is located upstream of the

563 *hemP* ORF and might govern expression of both *hemP* and *hemR*. A well conserved Fur box
564 was identified 412 nucleotides from the beginning of the HemR coding region (Stojiljkovic
565 and Hantke, 1992). The second possible *hemR* promoter is located 73 bp upstream of the
566 *hemR* ORF. One potential OmpR-binding site (H1, located between nucleotides -179 and -199
567 bp upstream of the *hemR* ATG) was recognized in this second potential promoter region. This
568 20-bp element contains the conserved **G**XX**AC** motif, but it exhibits only 30% identity to the
569 *E. coli* and *Yersinia* spp. consensus OmpR-binding site sequences.

570 The binding of OmpR to the putative promoter region of *hemR* was examined in an
571 EMSA (Fig. 7B). Different amounts of phosphorylated OmpR (OmpR-P) were incubated with
572 a 385-bp DNA fragment of the *hemR* gene containing the predicted OmpR-binding site. As
573 shown in Figure 7B, OmpR-P was unable to bind the putative regulatory region of *hemR*. This
574 result suggested that OmpR indirectly regulates the transcription of *hemR*. Based on our
575 findings, we hypothesize that OmpR might cause repression of *hemR* expression indirectly by
576 its positive influence on Fur expression. Consistent with this hypothesis, four putative OmpR-
577 binding sites were identified in the *fur* regulatory region by *in silico* analysis (data not
578 shown). Detailed studies on the OmpR-dependent regulation of the *fur* gene are currently
579 being performed to verify this hypothesis.

580 The results of our proteomic analysis raised questions concerning the adaptive role of
581 OmpR associated with the modulation of iron/heme receptor levels. *Y. enterocolitica* exhibits
582 a dual lifestyle, existing as both a non-pathogenic saprophyte and a pathogen residing inside
583 the host body. The localization influences the nature of the iron available as well as its
584 dedicated transport mechanisms. In the saprophytic lifestyle, *Y. enterocolitica* may exploit
585 receptors for iron-bound siderophores to acquire iron from the surrounding environment. In
586 the host tissues the majority of iron is found within the heme molecule (free or in
587 hemoproteins). The acquisition of heme by *Y. enterocolitica* occurs via a dedicated HemR-

based heme transport system. Thus, the OmpR-mediated regulation of the appropriate OM receptors for iron/heme uptake, according to the local environment, may contribute to the fitness of *Y. enterocolitica.* In particular, regulation of the HemR receptor of the heme transport system by OmpR may be necessary to permit growth of *Y. enterocolitica* within the host. The tight negative regulation of HemR may prevent the acquisition of an excess of heme, which is toxic for bacteria (Anzaldi and Skaar, 2010). Finally, the regulation of the heme uptake system influences cellular levels of the heme moiety. Heme is the prosthetic group of cytochromes and catalase, and an essential cofactor for cellular respiration. Thus, the cellular level of heme may influence respiratory pathways and contribute to changes in the central metabolism of *Y. enterocolitica.* This regulatory network is likely to be significant for other yersiniae and members of the family Enterobacteriaceae that possess both the response regulator OmpR and a heme transport system based on homologs of HemR (Runyen-Janecky, 2013).

Conclusions

This study represents the first to examine the impact of high osmolarity and low pH on the proteome of *Y. enterocolitica* and, most significantly, constitutes the first proteomic analysis of the role of OmpR in this pathogen. Our results indicate that OmpR influences the production of a number of membrane proteins involved in the uptake and transport of compounds into the cell and in efflux or secretion processes. Thus, OmpR may have an impact on the passage of solutes across the cell envelope when yersiniae are exposed to the varied environmental conditions associated with different ecological niches. Moreover OmpR appears to influence *Y. enterocolitica* pathogenesis, by (1) modulating the expression of proteins that are likely to promote cellular survival in acidic pH and (2) repressing the expression of adhesin YadA, a major virulence factor. Finally, our results provide some novel

612 insights into the role of OmpR in the remodeling of the bacterial surface, a vital strategy
613 associated with growth/survival in niches outside and within the host organism, that vary in
614 osmolarity, pH and iron/heme content. These findings identify OmpR as the central integrator
615 of several cellular processes regulating the dual saprophytic and pathogenic lifestyles of *Y.*
616 *enterocolitica*.

617 **Experimental Procedures**

618 Bacterial strains and growth conditions

619 The strains and plasmids used in this study are listed in Table S1. Unless indicated, *Y.*
620 *enterocolitica* strains were cultured at 26°C in Luria-Bertani (LB) medium. *E. coli* strains
621 were grown at 37°C in LB medium. As required, media were supplemented with the
622 appropriate antibiotics: nalidixic acid (Nal) – 30 μg ml^{-1}, chloramphenicol (Cm) – 25 μg ml^{-1},
623 kanamycin (Km) – 50 μg ml^{-1}, gentamicin (Gm) – 40 μg ml^{-1}, spectinomycin (Sp) – 100 μg
624 ml^{-1}. For iron-derepressed growth conditions, yersiniae strains were cultured in LB medium
625 supplemented with 0.3 mM α,α'-dipirydyl to chelate iron ions (LBD medium). For proteomic
626 experiments, triplicate overnight cultures of *Y. enterocolitica* strains Ye9 and AR4 were
627 grown in LB, pH 7.0 at 26°C or 37°C to an OD_{600} of 1.0-1.3. The cultures were then
628 centrifuged (5000 x g, 10 min) and the cells resuspended to an OD_{600} of 1.0 in 25 ml of (i)
629 fresh LB at pH 7.0 with 86 mM NaCl (standard medium), (ii) LB adjusted to pH 5.0 by the
630 addition of 100 mM HOMOPIPES buffer [homopiperazine-N,N′-bis-2-(ethanesulfonic acid)],
631 or (iii) LB at pH 7.0 supplemented with NaCl to 386 mM. The pH of all LB media was
632 measured and found not to change significantly during subsequent growth of the cells.
633 Replicate cultures were incubated at 26°C or 37°C with shaking for 3 h, then 25 ml samples
634 were centrifuged (8000 x g, 20 min, 4°C), and the cell pellets flash frozen in liquid nitrogen
635 and stored at -80°C prior to fractionation for proteomic analysis.

636 Isolation of outer membrane-enriched sarkosyl-insoluble fractions for shotgun label-
637 free quantitative proteomic analysis

638 Each of the triplicate bacterial pellets from the different culture variants (36 samples, i.e. 2
639 strains x 2 temperatures x 3 media x 3 biological replicates) was resuspended in half the
640 original culture volume of buffer (200 mM Tris HCl pH 8.0, 0.5 M sucrose, 250 µg ml^{-1}
641 lysozyme, 1 mM EDTA), incubated for 1 h at 4°C and sonicated on ice for 18 cycles of 30 s,
642 separated by 30 s intervals, using a Sonics Vibra-Cell VCX 130 (Sonics & Materials, Inc.,
643 Newtown, CT, USA). After centrifugation (8000 x g, 10 min, 4°C) to remove unbroken cells
644 and debris, the supernatants were centrifuged at high speed (35,000 x g, 1.5 h, 4°C) to pellet
645 total membranes. Membrane pellets were then resuspended in 10 ml of 2% sodium lauryl
646 sarcosine (sarkosyl) in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
647 pH 7.4 and incubated for 1 h at 37°C with occasional shaking to solubilize the inner
648 membrane (Filip et al., 1973). The sarkosyl-insoluble outer membrane-enriched (OMsl)
649 fractions were pelleted by high speed centrifugation as described above.

650 Mass spectrometry

651 Sarkosyl-insoluble OM pellets were suspended in 40 µl of SDS/deoxycholate buffer (0.1%
652 SDS, 1% sodium deoxycholate, 20 mM DTT, 100 mM Tris HCl pH 8.5, in 25 mM
653 ammonium bicarbonate) and sonicated in a water bath (20 cycles of 30 s) to solubilize the
654 OM proteins. After clarification by centrifugation (15 min, 12,000 x g), protein concentrations
655 in the supernatant samples were estimated using a BCA assay (Pierce) and normalized by
656 dilution in 25 mM ammonium bicarbonate solution. The proteins were then reduced by
657 treatment with 50 mM DTT (30 min, 60°C), alkylated with 80 mM iodoacetic acid (IAA) (45
658 min at room temperature – RT) and 50 µg samples were digested overnight with trypsin
659 (sequencing Grade Modified Trypsin; Promega V5111).

660 The digestion reactions were quenched by acidifying the samples with 0.6% TFA
661 (trifluoroacetic acid) and precipitated sodium deoxycholate was removed by centrifugation.
662 Each peptide mixture was applied in turn to a RP-18 precolumn (nanoACQUITY Symmetry®
663 C18, Waters 186003514), using 0.1% TFA in water as the mobile phase, and then to a nano-
664 HPLC RP-18 column (nanoACQUITY BEH C18, Waters 186003545) using an acetonitrile
665 gradient (5-35% AcN over 180 min) in the presence of 0.05% formic acid, with a flow rate of
666 250 nl/min. The column outlet was directly coupled to the ion source of an Orbitrap Velos
667 mass spectrometer (Thermo Electron Corp., San Jose, CA) working in the regime of data
668 dependent MS to MS/MS switch. To prevent cross contamination by previous samples, each
669 analysis was preceded by a blank run. The raw data were processed using Mascot Distiller
670 followed by Mascot Search (Matrix Science, London, UK) to identify hits in the Swiss-Prot
671 database (20110124) restricted to *Yersinia* sequences. The following search parameters were
672 applied: precursor and product ion mass tolerances – 20 ppm and 0.4 Da, respectively;
673 enzyme specificity of trypsin – 1 missed cleavage site allowed; permitted modifications –
674 cysteine carbamidomethylation and methionine oxidation. To estimate the false-positive
675 discovery rate (FDR), the decoy search option was enabled. Peptides with a Mascot Score
676 exceeding the threshold value corresponding to < 1% FDR were considered to be positively
677 identified. Label-free quantitation was performed as described previously (Bakun et al., 2012;
678 Malinowska et al., 2012). Briefly, both qualitative and quantitative runs were performed for
679 each sample. From the qualitative run, peptide sequences, masses and retention times were
680 obtained, while from the quantitative run, peptide masses, retention times and intensities were
681 acquired. We used MS1 peak integration to obtain the intensities of individual peptides. The
682 protein intensity ratio between two groups or samples was calculated as the median of the
683 intensity ratios for all its peptides where the quantitative values are not missing. Using an in-
684 house software pipeline, data from these two measurements were integrated, resulting in a list

of identified peptides and their intensities for a given sample. These lists were then subjected to statistical analysis using in-house Diffprot software to identify differentially-expressed proteins. Details of the subsequent bioinformatic analyses are provided below.

Bioinformatic analyses

Following mass spectrometry, protein lists were generated by Mascot and further filtered using in-house Mscan software to select proteins with an FDR of < 1%, identified by at least 2 peptides. The obtained shortlist of selected peptides (SPL) was used to tag peptide peaks in 2D heat-maps generated on the basis of the MS profile data. In detail, LC-MS data obtained directly from the mass spectrometer were converted into 2D heat maps using an in-house MsConvert data conversion tool. This file format is recognized by Msparky, an in-house modification of the commonly used graphical NMR assignment and integration program Sparky NMR (http://www.cgl.ucsf.edu/home/sparky). Msparky displays LC-MS data as 2D peptide heat-maps (with peptide LC Rt and m/z as the vertical and horizontal axes, respectively). Overlaying qualitative data (SPL) on quantitative profile datasets (2D heat maps) was performed by MSparky, which matches the sequence information with intensity data for peptide signals of the same m/z and LC Rt, on the basis of m/z, Rt and isotopic profile fitting. The automatic labeling of peptide signals provided by Msparky was verified by manual data inspection, applying the following acceptance criteria: m/z value deviation – 20 ppm; LC retention time deviation – 10 min; envelope root mean squared error (deviation between the expected isotopic envelope of the peak heights and their experimental values) – 0.6. Qualitative and quantitative data were integrated and subjected to statistical analysis using in-house Diffprot software. Statistical significance values were calculated using the resampling test implemented in Diffprot (Malinowska et al., 2012). Diffprot processes obtained peptides lists by clustering proteins into families, removal non-unique peptides and

signal intensities normalization with Lowess method. In the next stage Diffprot employs a resampling-based statistics paired with FDR procedure for estimating the statistical significance of quantitative results, as well as a local-pooled-error-like procedure to deal with small number of biological replicates. Results are displayed in a table format with proteins segregated according to statistical relevance, supported by information on how many peptides were used for the analysis and observed ratio. All software used is accessible at http://proteom.ibb.waw.pl.

We ran our searches against entire *Yersinia* genus database, which contains 479355 sequences. Most identified peptides matched multiple protein sequences. To remove redundant orthologs we grouped protein sequences with highly similar sets of identified peptides (at least 90% cluster-coverage identical peptide sequences) into clusters. Then, assigned PSMs to sequence clusters, removing those with no unique assignment. Next we mapped each cluster to accession number of one of its members, preferably a sequence from our reference strain *Y. enterocolitica* subsp. *palearctica* 105.5R(r). If that has not been possible the other strains (i.e. *Y. enterocolitica* subsp. *palearctica* Y11), other subspecies (i.e. *Y. enterocolitica* subsp. *enterocolitica* 8081), or other species were chosen, in order.

Others bioinformatic analyses were based on the complete genome sequences of *Y. enterocolitica* subsp. *palearctica* 105.5R(r) and *Y. enterocolitica* subsp. *enterocolitica* 8081 (GenBank; http://www.ncbi.nlm.nih.gov). Gene ontology data were obtained from the UniProt databases (http://www.uniprot.org). Principal Components Analysis was performed using in-house software produced with scikit-learn (http://scikit-learn.org). Promoter prediction was conducted using the web-based software BPROM in the Softberry package (http://www.softberry.com/berry.phtml?topic=bprom; Solovyev and Salamov, 2011). Logo motif analysis to identify potential OmpR-binding sites within promoter regions was performed using WebLogo (Crooks et al., 2004; http://weblogo.berkeley.edu/logo.cgi).

Molecular biology techniques

All DNA manipulations, including the polymerase chain reaction (PCR), restriction digestions, ligations and DNA electrophoresis were performed as previously described (Sambrook and Russell, 2001). Plasmid and chromosomal DNA were isolated using a Plasmid Miniprep DNA Purification Kit and Bacterial & Yeast Genomic DNA Purification Kit, respectively (EURx, Gdańsk, Poland). Restriction enzymes were obtained from Thermo Scientific (Waltham, USA). PCR was routinely performed in 25 µl or 50 µl reaction mixtures for 35 cycles using Taq DNA polymerase or, when fragments were used for cloning, Phusion High-Fidelity DNA Polymerase (Thermo Scientific). DNA fragments amplified by PCR were purified with a PCR/DNA Clean-Up Purification Kit (EURx) before and after restriction digestion. All kits and reagents were used according to the recommendations of the supplier. Oligonucleotide primers used for PCR and sequencing were purchased from Genomed S.A. (Warsaw, Poland) and are listed in Table S2. Plasmids used in this study are described in Table S1. DNA sequencing was performed by Genomed S.A. (Warsaw, Poland).

Western blotting

The abundance of selected proteins in *Y. enterocolitica* cells was evaluated by Western blotting using the OMsl fractions prepared for proteomic analysis (YadA) or total bacterial protein extracts (HemR). The final protein concentrations in the OMsl samples were estimated using the RC-DC protein assay (Bio-Rad) and normalized by dilution in Laemmli buffer (Sambrook and Russell, 2001). For detection of YadA in the oligomeric form, the OMsl samples were resuspended in Laemmli buffer and boiled for 5 min prior to electrophoresis. For the detection of YadA in the form of monomers, the samples were resuspended in urea sample buffer (62.5 mM Tris/HCl, pH 6.8, 8 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue) and boiled for 10 min. To prepare total protein extracts for HemR analysis

the cultures were normalized to the same OD_{600} and after centrifugation the cell pellets were resuspended in Laemmli buffer and boiled for 5 min prior to electrophoresis. Equivalent samples were separated on 8% (for HemR) or 10% (for YadA) polyacrylamide gels by electrophoresis (SDS-PAGE), then transferred to nitrocellulose membrane (Amersham Protran Western blotting membrane, nitrocellulose, pore size 0.2 µM; GE Healthcare) using a wet electroblotting system (Bio-Rad; Hercules, USA). The blots were probed with rabbit antisera directed against HemR (1:8000) or YadA (1:5000). Both polyclonal antibodies were prepared at the Max von Pettenkofer Institute for Hygiene and Medical Microbiology (University of Munich). Goat anti-rabbit IgG, conjugated to alkaline phosphatase (Sigma-Aldrich; St. Louis, USA) was used as the secondary antibody (diluted 1:30,000). Positive immunoreaction was visualized using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT; Sigma-Aldrich). In each experiment, the loading of equivalent amounts of protein was controlled by Coomassie blue staining of an identical gel.

Construction of transcriptional *hemR- lacZYA'* reporter fusion

To construct a *hemR* promoter-*lacZYA*' fusion, a 385-bp fragment of the *hemR* promoter region was amplified from Ye9 chromosomal DNA using primers HemR1 and HemR2 (Table S2). The product was initially cloned into the cloning vector pDrive (Qiagen; Venlo; Netherlands) and then, following digestion with XbaI/SmaI, the released insert was subcloned into suicide plasmid pFUSE cleaved with the same enzymes to place them immediately upstream of a promoterless β-galactosidase gene (Baumler et al., 1996). The suicide vector construct containing the *hemR* fragment, verified by restriction digestion and DNA sequencing, was named pFH. This plasmid was used to transform *E. coli* S17 λ*pir* and then introduced into *Y. enterocolitica* Ye9N and the OmpR-deficient mutant strain AR4 by

biparental mating. Because pFUSE cannot replicate in *Y. enterocolitica* cells, all selected transconjugants carried the plasmid integrated into the genome. The conjugation between the donor and recipient strains was performed on LB agar plates for 18 h at RT. The Ye9N exconjugants were selected on LB agar plates containing chloramphenicol (25 μg ml^{-1}) and nalidixic acid (30 μg ml^{-1}), and the AR4 exconjugants on LB containing chloramphenicol (25 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). Single-crossover homologous recombination yielded genomic transcriptional fusion between the *hemR* promoter and the promoterless *lacZYA'* operon. The correct insertion of the suicide vector was verified by PCR using one primer (HemR3) located upstream of the homologous region used for recombination and another primer (lacZH991) within the *lacZ* gene, followed by sequencing of the amplified product. Strains carrying the desired transcriptional fusions were designated AR4H and Ye9H (*hemR-lacZYA'*).

Construction of plasmid pompR for complementation

To complement the *ompR* mutation, the *ompR* gene with the native ribosome binding site was amplified by PCR using Ye9 chromosomal DNA as the template with primers OmpB1 and OmpB2 (Table S2). The product was initially cloned into cloning vector pDrive (Qiagen) and then an EcoRI/BamH1 fragment was subcloned into plasmid pBBR1MCS-5 cleaved with the same enzymes (Kovach et al., 1995). The resulting construct, pompR was verified by DNA sequencing and used to transform *E. coli* S17 λ*pir*. This plasmid was then introduced into *ompR* mutant strain carrying the transcriptional *lacZYA'* reporter fusion (AR4H) by biparental conjugation. The exconjugants were selected on LB agar plates containing gentamicin (40 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). The parent vector pBBR1MCS-5 was introduced into the same strain as a negative control.

β-galactosidase assays

β-galactosidase assays were performed essentially as described by Thibodeau et al., (2004), using 96-well microtiter plates and a Sunrise plate reader (Tecan; Männedorf, Switzerland). Briefly, cultures were grown overnight and next were diluted into subcultures, which were then grown under various conditions in 96-well plates with shaking (250 rpm) to an OD_{600} of 0.3-0.5. 80 µl of each cell suspension was mixed with 10 µl of POPCulture Reagent (EMD Milipore Corp., Billerica, USA) and 4 units of lysozyme (Sigma-Aldrich), then incubated for 15 min to cause lysis. In the wells of a microtiter plate, 20 µl of each cell lysate were mixed with 130 µl Z-Buffer and 30 µl ONPG (4 mg ml^{-1}) as described by Miller (1992). For kinetic assays, the absorbance at 415 nm (relative to a blank) was measured at time intervals of 10 s, with 2 s of shaking before each reading. The assays were performed at 25°C and monitored for up to 20 min. Data were analyzed using Magellan data analysis software. The β-galactosidase activity was expressed in Miller units calculated as described previously (Thibodeau et al., 2004). Each assay was performed at least in triplicate.

Construction of GFP translational fusions with YadA

To measure transcriptional and post-transcriptional regulation of *yadA* expression, a translational fusion with GFP was constructed in plasmid pFX-P (Schmidtke et al., 2013) using the Golden Gate technique (Engler et al., 2008). A DNA fragment carrying the promoter, 5' untranslated region (5'UTR) and the first 16 codons of the *yadA* gene was amplified from Ye9 plasmid pYV DNA by PCR using primers YadA4 and YadA5 (Table S2). These primers contained BsaI sites and additional sequences designed to generate compatible ends with BsaI-cleaved pFX-P (Table S1). In a 20-µl Golden Gate cloning reaction, 40 fmol of vector were mixed with 40 fmol of PCR product, 5 units of BsaI (New England Biolabs, Frankfurt am Main, Germany) and 4.5 units of ligase (Thermo Scientific) in ligase buffer. The

reaction was incubated at 37°C for 1 h, 5 min at 50°C, followed by 5 min at 80°C, and then used to transform *E. coli* DH5α by electroporation. The recombinant fusion construct pFX-yadA and negative control plasmid pFX-0 (Schmidtke et al., 2013) were introduced into parental and *ompR*-negative *Y. enterocolitica* strains by electroporation.

Monitoring bacterial fluorescence by flow cytometry

Three independent overnight cultures of each strain grown from single colonies in LB medium supplemented with spectinomycin were diluted 1:20 in fresh medium and incubated at 27°C or 37°C. After 4 h and after approx. 22 h, the bacteria were diluted in sterile phosphate-buffered saline (PBS) to approx. 4-8 x 10^6 CFU ml^{-1}. For every sample, the mean fluorescence intensity of at least 20,000 bacterial cells was measured with a FACS Canto II flow cytometer (BD) using the FITC filter settings and analyzed with FACS Diva Software v6.1.2.

Construction of plasmid pETOmpR

To express OmpR as a fusion protein with an amino-terminal His_6 extension, a 725-bp fragment representing the entire *ompR* coding sequence was amplified from *Y. enterocolitica* chromosomal DNA with primers OmpRpET1 and OmpRpET2 (Table S2). The PCR product was digested with NheI and SalI and cloned into vector pET28a (Novagen) cleaved with the same enzymes. The resulting construct, pETOmpR, was verified by restriction digestion and sequencing and used to transform *E. coli* BL21(DE3).

Overproduction and purification of OmpR-His_6

The N-terminal His-tagged OmpR protein (OmpR-His_6, 29.78 kDa) was expressed and purified using Ni-NTA resin (Qiagen) as described in the manufacturer's standard protocol.

Briefly, *E. coli* BL21(DE3) carrying plasmid pETOmpR was grown to mid-logarithmic phase, IPTG was added to a final concentration of 0.8 mM, and the culture incubated for a further 4 h at 37°C. The cells were then pelleted by centrifugation, resuspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 55 µM PMSF, 5 mM imidazole and 10 mM 2-mercaptoethanol, and disrupted by sonication. After centrifuging the cell lysate to remove unbroken cells, the supernatant was passed through a Ni-NTA agarose column. The column was washed with 5 volumes of 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and then bound protein was eluted using a gradient of imidazole buffer. The fractions were analyzed by SDS-PAGE and those containing the purified OmpR-His_6 protein were loaded into a Slide-A-Lyzer Dialysis Cassette (10K MWCO; Thermo Scientific) and dialyzed at 4°C in 20 mM HEPES (pH 7.9) buffer containing 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 20% glycerol (Fernandez-Mora et al., 2004). The concentration of the purified OmpR protein was estimated using the RC DC protein assay (Bio-Rad).

Electrophoretic mobility shift assays (EMSAs)

The interaction between phosphorylated OmpR protein (OmpR-P) and the promoters of selected genes was examined essentially as described previously (Raczkowska et al., 2011a). The primers listed in Table S2 were used in PCRs with *Y. enterocolitica* genomic DNA to amplify fragments comprising the regulatory regions of the genes *yadA* and *hemR*. Purified OmpR-His6 was phosphorylated *in vitro* by incubation for 30 min at RT in phosphorylation buffer [50 mM Tris pH 8.0, 20 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 5% glycerol containing 20 mM acetyl phosphate (lithium potassium acetyl phosphate; Sigma-Aldrich)]. The purified DNA fragments (0.3 pmol in 20 µl) were then incubated with different amounts of OmpR-His_6 at RT for 30 min. The reactions were analyzed by electrophoresis on 5% native polyacrylamide gels (29:1 acrylamide/bis acrylamide) in 0.5x Tris-borate-EDTA buffer for

0.5 h at 90 V and 2.5 h at 130 V at 4°C. As a negative control, a 304-bp fragment of the *Y. enterocolitica* 16S rRNA gene amplified by PCR (Table S2) was included in the binding reactions. Ethidium bromide (Sigma-Aldrich) was used to stain the DNA bands in the gels which were visualized on a UV transilluminator.

Acknowledgements

This work was supported by a grant from the National Science Center, Poland (grant no. 2011/01/B/NZ6/01845). AR obtained a short term fellowship at the Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie der Ludwig Maximilians, Universität München from the Human Capital Operational Programme under Task 9 of the project: "Chemistry, physics, and biology for the XXI century society: New interdisciplinary studies of the first, second, and third cycles"

Competing interests

The authors declare that they have no competing interests.

References

Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist R, Fahlgren A,Wolf-Watz H, and Fallman, M. (2011) Translocation of surface-localized effectors in type III secretion. Proc Natl Acad Sci USA 108: 1639-1644.

Andrews, S.C. (1998) Iron storage in bacteria. Adv Microb Physiol 40: 281-351.

Andrews, S.C., Robinson, A.K., and Rodríguez-Quiñones, F. (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27: 215-237.

Anzaldi, L.L. and Skaar, E.P. (2010) Overcoming the heme paradox: Heme toxicity and tolerance in bacterial pathogens. Infect Immun. 78: 4977-4989.

Bakun, M., Niemczyk, M., Domanski, D., Jazwiec, R., Perzanowska, A., Niemczyk, S., et al. (2012) Urine proteome of autosomal dominant polycystic kidney disease patients. Clin Proteomics 9: 1-13.

Bang, I.S., Kim, B.H., Foster, J.W., and Park, Y.K. (2000) OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar typhimurium. J Bacteriol 182: 2245-2252.

Bang, I.S., Audia, J.P., Park, Y.K., and Foster, J.W. (2002) Autoinduction of the ompR response regulator by acid shock and control of the *Salmonella enterica* acid tolerance response. Mol Microbiol 44: 1235-1250.

Baumler, A.J., Tsolis, R.M., van der Velden, A.W.M., Stojiljkovic, I., Anic, S., and Heffron, F. (1996) Identification of a new iron regulated locus of *Salmonella typhi*. Gene 183: 207-213.

Bernardini, M.J., Fontaine, A., and Sansonetti, P.J. (1990) The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. J Bacteriol 172: 6274-6281.

Bialas, N., Kasperkiewicz, K., Radziejewska-Lebrecht. J., and Skurnik, M. (2012) Bacterial cell surface structures in *Yersinia enterocolitica*. Arch Immunol Ther Exp 60: 199-209.

Blair, J.M., Piddock, L.J. (2009) Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Curr Opin Microbiol 12: 512-519.

Blot, N., Berrier, C., Hugouvieux-Cotte-Pattat, N., Ghazi, A., and Condemine, G. (2002) The oligogalacturonate specific porin KdgM of *Erwinia chrysanthemi* belongs to a new porin family. J Biol Chem 277: 7936-7944.

Bottone, E.J. (1997) *Yersinia enterocolitica*: the charisma continues. Clin Microbiol Rev 10: 257-276.

Bracken, C.S., Baer, M.T., Abdur-Rashid, A., Helms, W., and Stojiljkovic, I. (1999) Use of heme-protein complexes by the *Yersinia enterocolitica* HemR receptor: histidine residues are essential for receptor function. J Bacteriol 181: 6063-6072.

Brzostek, K., Hrebenda, J., Benz, R., and Boos, W. (1989) The OmpC protein of *Yersinia enterocolitica*: purification and properties. Res Microbiol 140: 599-614.

Brzostek, K., Nichols, W. W. (1990) Outer membrane permeability and porin proteins of *Yersinia enterocolitica*. FEMS Microbiol Lett. 70: 275-278.

Brzostek, K., Raczkowska, A., and Zasada, A. (2003) The osmotic regulator OmpR is involved in the response of *Yersinia enterocolitica* O:9 to environmental stresses and survival within macrophages. FEMS Microbiol Lett 228: 265-271.

Brzostek, K., Brzóstkowska, M., Bukowska, I., Karwicka, E., and Raczkowska, A. (2007) OmpR negatively regulates expression of invasin in *Yersinia enterocolitica*. Microbiol 153: 2416-2425.

Brzostek, K. and Raczkowska, A. (2007) The YompC protein of *Yersinia enterocolitica*: molecular and physiological characterization. Folia Microbiol 52: 73-80.

Cameron, A.D., and Dorman, C.J. (2012) A fundamental regulatory mechanism operating through OmpR and DNA topology controls expression of *Salmonella* pathogenicity islands SPI-1 and SPI-2. Plos Genet 8(3):e1002615.

Chakraborty, S., Mizusaki, H., and Kenney, L.J. (2015) A FRET-based DNA biosensor tracks OmpR-dependent acidification of *Salmonella* during macrophage infection. Plos Biol 13(4):e1002116. doi: 10.1371/journal.pbio.1002116.

Chromy, B.A., Choi, M.W., Murphy, G.A., Gonzales, A.D., Corzett, C.H., Chang, B.C., et al. (2005) Proteomic characterization of *Yersinia pestis* virulence. J Bacteriol 187: 8172-8180.

Condemine, G., and Ghazi, A. (2007) Differential regulation of two oligogalacturonate outer membrane channels, KdgN and KdgM, of *Dickeya dadantii* (*Erwinia chrysanthemi*). J Bacteriol 189: 5955-5962.

Cornelis, G.R., Boland, A., Boyd, A.P., Geuijen, C., Iriarte, M., Neyt, C., et al. (1998) The virulence plasmid of *Yersinia*, an antihost genome. Microbiol Mol Biol Rev 62: 1315-1352.

Cornelis, G.R. (2002) *Yersinia* type III secretion: send in the effectors. J Cell Biol 158: 401-408.

Crooks, G., and Hon, G. (2004) WebLogo: a sequence logo generator. Genome Res 14: 1188-1190.

De Koning-Ward, T.F., and Robins-Browne, R.M. (1995) Contribution of urease to acid tolerance in *Yersinia enterocolitica.* Infect Immun 63: 3790-3795.

Dewoody, R.S., Merritt, P.M., and Marketon, M.M. (2013) Regulation of the *Yersinia* type III secretion system: traffic control. Front Cell Infect Microbiol 3: 1-13.

Dorrell, N., Li, S.R., Everest, P.H., Dougan, G., and Wren, B.W. (1998) Construction and characterisation of a *Yersinia enterocolitica* O:8 ompR mutant. FEMS Microbiol Lett 165: 145-151.

El Tahir, Y., and Skurnik, M. (2001) YadA, the multifaceted *Yersinia* adhesin. Int J Med Microbiol 291: 209-218.

Engler, C., Kandzia, R., and Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS One 3(11):e3647. doi: 10.1371/journal.pone.0003647.

Fernandez-Mora, M., Puente, J.L., and Calva, E. (2004) OmpR and LeuO positively regulate the *Salmonella enterica* serovar Typhi *ompS2* porin gene. J Bacteriol 186: 2909-2920.

Filip, C., Fletcher, G., Wulff, J.L., and Earhart, C.F. (1973) Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J Bacteriol 115: 717-722.

Flamez, C., Ricard, I., Arafah, S., Simonet, M., and Marceau, M. (2008) Phenotypic analysis of *Yersinia pseudotuberculosis* 32777 response regulator mutants: new insights into two-component system regulon plasticity in bacteria. Int J Med Microbiol 298: 193-207.

Francis, M.S. (2013) The pathogenic Yersiniae-advances in the understanding of physiology and virulence. Front Cell Infect Microbiol 3: 1-2.

Friedman, D.B., Stauff, D.L., Pishchany, G., Whitwell, C.W., Torres, V.J., and Skaar, E.P. (2006) *Staphylococcus aureus* redirects central metabolism to increase iron availability. Plos Pathog 2(8):e87.

Friedman, D.B., Wang, S.E., Whitwell, C.W., Caprioli, R.M., and Arteaga, C.L. (2007) Multivariable difference gel electrophoresis and mass spectrometry: a case study on transforming growth factor-beta and ERBB2 signaling. Mol Cell Proteomics 6: 150-169.

Gao, H., Zhou, D., Li, Y., Guo, Z., Han, Y., Song, Y., et al. (2008) The iron-responsive Fur regulon in *Yersinia pestis*. J Bacteriol 190: 3063-3075.

Gao, H., Zhang, Y., Han, Y., Yang, L., Liu, X., Guo, Z., et al. (2011) Phenotypic and transcriptional analysis of the osmotic regulator OmpR in *Yersinia pestis*. BMC Microbiol 23: 11-39.

Goh, E.B., Siino, D.F., and Igo, M.M. (2004) The *Escherichia coli tppB* (*ydgR*) gene represents a new class of OmpR-regulated genes. J Bacteriol 186: 4019-4024.

Golby, P., D. J. Kelly, J. R. Guest, and S. C. Andrews. 1998. Transcriptional regulation and organization of the *dcuA* and *dcuB* genes, encoding homologous anaerobic C4-dicarboxylate transporters in *Escherichia coli*. J. Bacteriol 180:6586-6596.

Gueguen, E., Durand, E., Zhang, X.Y., d'Amalric, Q., Journet, L., and Cascales, E. (2013) Expression of a *Yersinia pseudotuberculosis* type VI secretion system is responsive to envelope stresses through the OmpR transcriptional activator. PLoS ONE 19;8(6):e66615.

Guillier, M., and Gottesman, S. (2006) Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. Mol Microbiol 59: 231-247.

Gibson, M.M., Ellis, E.M., Graeme-Cook, K.A., and Higgins, C.F. (1987) OmpR and EnvZ are pleiotropic regulatory proteins: positive regulation of the tripeptide permease (*tppB*) of *Salmonella* typhimurium. Mol Gen Genet 207:120-129.

Hantke, K. (2001) Iron and metal regulation in bacteria. Curr Opin Microbiol 4: 172-177.

Harlocker, S.L., Bergstrom, L., and Inouye, M. (1995) Tandem binding of six OmpR proteins to the *ompF* upstream regulatory sequence of *Escherichia coli*. J Biol Chem 45: 26849-26856.

Head, C.G., Tardy, A., and Kenney, L.J. (1998) Relative binding affinities of OmpR and OmpR-phosphate at the *ompF* and *ompC* regulatory sites. J Mol Biol 281: 857-870.

Heesemann, J., Hantke, K., Vocke, T., Saken, E., Rakin, A., Stojiljkovic, I., and Berner, R. (1993) Virulence of *Yersinia enterocolitica* is closely associated with siderophore production, expression of an iron repressible outer membrane protein of 65000 Da and pesticin sensitivity. Mol Microbiol 8: 397-408.

Higashitani, A., Nishimura, Y., Hara, H., Aiba, H., Mizuno, T., and Horiuchi, K. (1993) Osmoregulation of the fatty acid receptor gene *fadL* in *Escherichia coli*. Mol Gen Genet 240: 339-347.

Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. (2003) Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. J Bacteriol 185: 1851-1856.

Hoch, J.A., and Silhavy, T.J. (1995) Two-component signal transduction. Washington, USA: American Society of Microbiology Press

Hong, H., Patel, D.R., Tamm, L.K., and van den Berg, B. (2006) The outer membrane protein OmpW forms an eight-stranded β-barrel with a hydrophobic channel. J Biol Chem 281: 7568-7577.

Hu, Y., Lu, P., Wang, Y., Ding, L., Atkinson, S., and Chen, S. (2009a) OmpR positively regulates urease expression to enhance acid survival of Yersinia pseudotuberculosis. Microbiol 155: 2522-2531.

Hu, Y., Wang, Y., Ding, L., Lu, P., Atkinson, S., and Chen, S. (2009b) Positive regulation of *flhDC* expression by OmpR in *Yersinia pseudotuberculosis*. Microbiol 155: 3622-3631.

Huang, K.J., and Igo, M.M. (1996) Identification of the bases in the ompF regulatory region, which interact with the transcription factor OmpR. J Mol Biol 262: 615-628.

Iriarte, M., and Cornelis, G.R. (1995) MyfF, an element of the network regulating the synthesis of fibrillae in *Yersinia enterocolitica*. J Bacteriol 177: 738-744

Jacobi, C.A., Gregor, S., Rakin, A., and Heesemann, J. (2001) Expression analysis of the yersiniabactin receptor gene *fyuA* and the heme receptor *hemR* of *Yersinia enterocolitica* in

vitro and in vivo using the reporter genes for green fluorescent protein and luciferase. Infect Immun 69: 7772-7782.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J.M., Lazzaroni, J.C., Lejeune, P., and Dorel, C. (2005) CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. J Bacteriol 187: 2038-2049.

Kenney, L.J. (2002) Structure/function relationships in OmpR and other winged-helix transcription factors. Curr Opin Microbiol 5: 135-141.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175-176.

Lambert de Rouvroit, C., Sluiters, C., and Cornelis, G.R. (1992) Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. Mol Microbiol 6: 395-409.

Lee, A.K., Detweiler, C.S., and Falkow, S. (2000) OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. J Bacteriol 182: 771-781.

Mack, D., Heesemann, J., and Laufs, R. (1994) Characterization of different oligomeric species of the *Yersinia enterocolitica* outer membrane protein YadA. Med Microbiol Immunol 183: 217-227.

Malinowska, A., Kistowski, M., Bakun, M., Rubel, T., Tkaczyk, M., Mierzejewska, J., and Dadlez, M. (2012) Diffprot – software for non-parametric statistical analysis of differential proteomics data. J Proteomics 75: 4062-4073.

Maeda, S., Takayanagi, K., Nishimura, Y., Maruyama, T., Sato, K., and Mizuno, T. (1991) Activation of the osmoregulated *ompC* gene by the OmpR protein in *Escherichia coli*: a study involving synthetic OmpR-binding sequences. J Biochem 110:324-327.

Miller, J.H. (1992) A short course in bacterial genetic: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Habor, NY, USA: Cold Spring Harbor Laboratory Press.

Mikula, K.M., Kolodziejczyk, R., and Goldman, A. (2013) *Yersinia* infection tools – characterization of structure and function of adhesins. Front Cell Infect Microbiol 2: 1-14.

Neubauer, H., Aleksic, S., Hensel, A., Finke, E.J., and Meyer, H. (2000) *Yersinia enterocolitica 16S rRNA* gene types belong to the same genospecies but form three homology groups. Int J Med Microbiol 290: 61-64.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67: 593-656.

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B.L., et al. (2002) Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol Microbiol 46: 281-291.

Pepe, J.C., and Miller, V.L. (1993) *Yersinia enterocolitica* invasin: a primary role in the initiation of infection. Proc Natl Acad Sci USA 90: 6473-6477.

Perkins, T.T., Davies, M.R., Klemm, E.J., Rowley, G., Wileman, T., James, K., et al. (2013) ChIP-seq and transcriptome analysis of the OmpR regulon of *Salmonella enterica* serovars Typhi and Typhimurium reveals accessory genes implicated in host colonization. Mol Microbiol 87: 526-538.

Perry, R.D. (1993) Acquisition and storage of inorganic iron and hemin by the yersiniae. Trends Microbiol 4: 142-147.

Pieper, R., Huang, S.T., Clark, D.J., Robinson, J.M., Alami, H., Parmar, P.P., et al. (2009a) Integral and peripheral association of proteins and protein complexes with *Yersinia pestis* inner and outer membranes. Proteome Sci 7: 1-16.

Pieper, R., Huang, S.T., Robinson, J.M., Clark, D.J., Alami, H., Parmar, P.P., et al. (2009b) Temperature and growth phase influence the outer-membrane proteome and the expression of a type VI secretion system in *Yersinia pestis*. Microbiol 155: 498-512.

Puente, J.L., Verdugo-Rodriguez, A., and Calva, E. (1991) Expression of *Salmonella typhi* and *Escherichia coli* OmpC is influenced differently by medium osmolarity; dependence on *Escherichia coli* OmpR. Mol Microbiol 5: 1205-1210.

Quinn, H.J., Cameron, A.D., and Dorman, C.J. (2014) Bacterial regulon evolution: distinct responses and roles for the identical OmpR proteins of *Salmonella* Typhimurium and *Escherichia coli* in the acid stress response. PLoS Genet 6;10(3):e1004215. doi: 10.1371/journal.pgen.1004215.

Raczkowska, A., Skorek, K., Bielecki, J., and Brzostek, K. (2011a) OmpR controls *Yersinia enterocolitica* motility by positive regulation of *flhDC* expression. Antonie Van Leeuwenhoek 99: 381-394.

Raczkowska, A., Skorek, K., Łasińska, A., and Brzostek, K. (2011b) Pleiotropic effects of a *Yersinia enterocolitica ompR* mutation on adherent-invasive abilities and biofilm formation. FEMS Microbiol Lett 321: 43-49.

Raczkowska, A., Trzos, J., Lewandowska, O., Nieckarz, M., and Brzostek, K. (2015) Expression of the AcrAB components of the AcrAB-TolC multidrug efflux pump of *Yersinia enterocolitica* is subject to dual regulation by OmpR. PLos One 20;10(4):e0124248.

Rentschler, A.E., Lovrich, S.D., Fitton, R., Enos-Berlage, J., and Schwan, W.R. (2013) OmpR regulation of the uropathogenic *Escherichia coli fimB* gene in an acidic/high osmolality environment. Microbiol 159: 316-327.

Rhee, J.E.,Sheng, W., Morgan, L.K., Nolet, R., Liao, X., and Kenney, L.J. (2008) Amino acids important for DNA recognition by the response regulator OmpR. J Biol Chem 283: 8664-8677.

Rigel, N.W., and Silhavy, T.J. (2012) Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria. Curr Opin Microbiol 15: 189-193.

Rodionov, D.A., Gelfand, M.S., and Hugouvieux-Cotte-Pattat, N. (2004) Comparative genomics of the KdgR regulon in *Erwinia chrysanthemi* 3937 and other gamma-proteobacteria. Microbiol 150: 3571-3590.

Runyen-Janecky, L.J. (2013) Role and regulation of heme iron acquisition in gram-negative pathogens. Front Cell Infect Microbiol 3: 1-11.

Russo, F.D., and Silhavy, T.J. (1990) EnvZ controls the concentration of phosphoryled OmpR to mediate osmoregulation of the porin genes. J Mol Biol 222: 567-580.

Sambrook, J., and Russell, D.W. (2001) Molecular Cloning: a Laboratory Manual. 3rd ed. Cold Spring Habor, NY, USA: Cold Spring Harbor Laboratory Press.

Schmid, K., Ebner, R., Jahreis, K., Lengeler, J.W., and Titgemeyer, F. (1991) A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. Mol Microbiol 5: 941-950.

Schmidtke, C., Abendroth, U., Brock, J., Serrania, J., Becker, A., and Bonas, U. (2013) Small RNA sX13: a multifaceted regulator of virulence in the plant pathogen *Xanthomonas*. PLoS Pathog 9 (9):e1003626.

Schutz, M., Weiss, E.M., Schindler, M., Hallstrom, T., Zipfel, P.F., Linke, D., and Autenrieth, I.B. (2010) Trimer stability of YadA is critical for virulence of *Yersinia enterocolitica*. Infect Immun 78: 2677-2690.

Schwan, W.R., Lee, J.L., Lenard, F.A., Matthews, B.T., and Beck, M.T. (2002) Osmolarity and pH growth conditions regulate *fim* gene transcription and type 1 pilus expression in uropathogenic *Escherichia coli*. Infect Immun 70: 1391-1402.

Shin, S., and Park, C. (1995) Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. J Bacteriol 177: 4696-4702.

Skorek, K., Raczkowska, A., Dudek, B., Miętka, K., Guz-Regner, K., Pawlak, A. et al. (2013) Regulatory protein OmpR influences the serum resistance of *Yersinia enterocolitica* O:9 by modifying the structure of the outer membrane. PLoS One 19;8(11):e79525. doi: 10.1371/journal.pone.0079525.

Skurnik, M., and Wolf-Watz, H. (1989) Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol Microbiol 3: 517-529.

Skurnik, M., and Toivanen, P. (1992) LcrV is the temperature-regulated activator of the *yadA* gene of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. J Bacteriol 174: 2047-2051.

Skurnik, M., Biedzka-Sarek, M., Lubeck, P.S., Blom, T., Bengoechea, J.A., Perez-Gutierrez, C. et al. (2007) Characterization and biological role of the O-polysaccharide gene cluster of *Yersinia enterocolitica* serotype O:9. J Bacteriol 189: 7244-7253.

Solovyev, V., and Salamov, A. (2011) Automatic annotation of microbial genomes and metagenomic sequences. In metagenomics and its applications in agriculture, biomedicine and environmental studies (Ed. R.W. Li), Nova Science Publishers, pp. 61-78.

Stock, J.B., Ninfa, A.J., and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev 53: 450-490.

Stojiljkovic, I., and Hantke, K. (1992) Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J 11: 4359-4367.

Straley, S.C., Plano, G.V., Skrzypek, E., Haddix, P.L., and Fields, K.A. (1993) Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol Microbiol 8: 1005-1010.

Straley, S.C., and Perry, R.D. (1995) Environmental modulation of gene expression and pathogenesis in *Yersinia*. Trends Microbiol 3: 310-317.

Thibodeau, S.A., Fang, R., and Joung, J.K. (2004) High-throughput betagalactosidase assay for bacterial cell-based reporter systems. Biotechniques 36: 410-415.

Thompson, J.M., Jones, H.A., and Perry, R.D. (1999) Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. Infect Immun 67(8):3879-3892.

Thomson, N.R., Howard, S., Wren, B.W., Holden, M.T., Crossman, L., Challis, G.L, et al. (2006) The complete genome sequence and comparative genome analysis of the High Pathogenicity *Yersinia enterocolitica* strain 8081. PLoS Genet 15;2(12):e206.

Troxell, B., and Hassan, H.M. (2013) Transcriptional regulation by Ferric Uptake Regulator (Fur) in pathogenic bacteria. Front Cell Infect Microbiol 3: 1-13.

Viboud, G.I., and Bliska, J.B. (2005) *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol 59: 69-89.

Walsh, C.T. (1989) Enzymes in the D-alanine branch of bacterial cell wall peptidoglycan assembly. J Biol Chem 264: 2393-2396.

Yamamoto, K., Nagura, R., Tanabe, H., Fujita, N., Ishihama, A., and Utsumi, R. (2000) Negative regulation of the *bolA1p* of *Escherichia coli* K-12 by the transcription factor OmpR for osmolarity response genes. FEMS Microbiol Lett 186: 257-226.

Yoshida, T., Qin, L., Egger, L.A., and Inouye, M. (2006) Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. J Biol Chem 25: 17114-17123.

Zhang, W., Wang, Y., Song, Y., Wang, T., Xu. S., Peng. Z., et al. (2013) A type VI secretion system regulated by OmpR in *Yersinia pseudotuberculosis* functions to maintain intracellular pH homeostasis. Environ Microbiol.;15: 557-569.

Table 1. OMsl proteins differentially expressed in wild-type *Y. enterocolitica* strain Ye9 and isogenic *ompR* mutant AR4 cultured under different growth conditions.

Differentially expressed proteins		Regulation Ye9 vs AR4[b]					
		standard conditions		high osmolarity		low pH	
Accession	Protein description[a]	26°C	37°C	26°C	37°C	26°C	37°C
Porin activity GO:0015288							
ADZ43059	Putative outer membrane porin protein F, OmpF	11.6		8.03		8.16	3.3
ADZ43215	Outer membrane porin protein C, OmpC		3.86	7.39	1.97	10.2	2.98
ADZ42354	Outer membrane phosphoporin protein E, PhoE	7.28	2.08	7.07		5.64	
ADZ41941	Outer membrane protein X, OmpX					5.69	
ADZ44282	Oligogalacturonate-specific porin KdgM2	- 120.03	- 10.57	- 183.86	- 4.1	- 145.25	- 15.68
ADZ42758	Outer membrane protein W, OmpW				- 2.31		- 3.72
ADZ40635	Vitamin B12 transporter BtuB				- 2.03		
ADZ41063	Sucrose porin ScrY	2.76	- 8.44				- 5.33
Transporter activity GO:0005215							
ADZ42555	Dipeptide and tripeptide permease A, DtpA/TppB	15.81				16.4	
CBY28945	Anaerobic C4-dicarboxylate transporter DcuA	10.89		16.39		14.67	
ADZ44176	Nitrite transporter NirC				6.05		
ADZ42774	Periplasmic oligopeptide-binding protein OppA	5.36		2.8		4.27	
ADZ42770	Oligopeptide transport ATP-binding protein OppF			2.98			
ADZ42771	Oligopeptide transporter ATP-binding component OppD			2.53			
ADZ44050	Putative xanthine/uracil permease			2.13		2.32	
ADZ41508	Outer membrane efflux protein	2.78		1.8			
ADZ41657	Multidrug efflux protein AcrA				1.49*		2.13
ADZ41656	Multidrug efflux protein AcrB		1.24*				
ADZ43362	ABC transport system substrate-binding protein	2.91		3.46		2.71	
ADZ44078	Maltose ABC transporter periplasmic protein MalE	2.54				2.17	
ADZ44153	Putative sugar transferase		2.65				
ADZ42972	Glucose-specific PTS system IIBC components			1.85		1.83	
ADZ41295	Protein translocase subunit SecA		1.66		1.78		
ADZ42257	D-alanine/D-serine/glycine permease CycA	- 37.02	- 32.2	- 33.54		- 44.45	
ADZ41741	Putative glutamate/aspartate transport system permease			- 5.12		- 12.54	
ADZ42241	Proline permease		- 11.31				
ADZ43898	Serine/threonine transporter SstT	- 10.49		- 10.88			
ADZ40803	Cation/acetate symporter ActP	- 7.12		- 9.75		- 7.57	
EHB19555	Amino acid permease					- 9.39	
ADZ41742	Glutamate and aspartate transporter subunit	- 4.21		- 7.46		- 5.57	
ADZ41044	Arginine/ornithine antiporter	- 2.48				- 2.21	
ADZ42170	Mg(2+) transport ATPase protein B	- 8.3	-4.25	- 9.64		- 7.55	
ADZ43328	Long-chain fatty acid outer membrane transporter FadL		- 4.71	- 3.89	- 7.46	- 4.69	- 5.66
EOR82078	Putative phosphotransferase system protein		- 5.04		- 3.74		- 4.61
ADZ44370	PTS system, mannitol-specific IIABC component						- 1.81
ADZ43484	PTS system, glucitol/sorbitol-specific IIBC component	- 4.2	- 3.64				
ADZ41346	Chloride channel protein ClcA	- 2.23	- 4.18			- 2.06	
ADZ43615	Voltage-gated potassium channel	- 2.36					
ADZ41961	D-galactose-binding periplasmic protein MglB		- 3.37				
ADZ41046	RND family efflux transporter			- 1.88			
ADZ43857	Type I secretion outer membrane protein TolC	- 1.39*	- 1.65		- 1.61	- 1.62	- 1.57
ADZ41495	DL-methionine transporter substrate-binding subunit		2.38	-2	2.86		2.9
Gram-negative-bacterium-type cell outer membrane assembly GO:0043165							
ADZ43450	Outer membrane protein assembly factor BamC	2.72	1.78	2.07		2.44	1.8
ADZ41154	Outer membrane protein assembly factor BamD					2.01	
ADZ41474	Outer membrane protein assembly factor BamA	1.38*		1.35*		1.35*	
ADZ42323	Outer membrane lipoprotein LolB						-4.5
ADZ41736	LPS-assembly lipoprotein LptE				- 1.74		- 1.5
ADZ41135	LPS-assembly protein LptD		- 1.54	1.37*	- 1.79		- 1.43*
Biosynthetic process GO:0009058							
ADZ42004	WbcT protein	1.73		2.29	2.74	2.24	2.91
ADZ42006	WbcV protein		2.57				

ADZ42005	WbcU protein			1.93		1.95	
Cell wall organization GO:0071555							
ADZ42523	Murein L,D-transpeptidase		2.49				
ADZ42453	N-acetylmuramoyl-L-alanine amidase	1.82	2.13				1.81
EOR80052	Major outer membrane lipoprotein Lpp	1.79		1.47*	1.98	1.9	2.05
ADZ41339	Penicillin-binding protein 1b						1.93
ADZ41447	Membrane-bound lytic murein transglycosylase A			- 2.07			
Pathogenesis GO:0009405							
ADZ43157	Outer membrane usher protein MyfC						6.24
ADZ42189	Invasin, Inv	1.87	1.71	1.49*			
ADZ44444	Transmembrane effector protein YopB		- 48.12		- 42.4		- 75.73
ADZ44443	Translocator protein YopD		- 40.37		- 37.76		- 71.6
ADZ44440	Type III secretion system effector protein YopM		- 20.79		- 42.45		
ADZ44516	Protein kinase YopO		- 16.25		- 9.64		- 22.77
ADZ44518	Type III secretion system effector protein YopP		- 10.37		- 11.94		- 19.12
EOR65641	Type III secretion system effector protein YopE		- 5.77		- 8.41	- 3.96	- 15.03
ADZ44479	Tyrosine-protein phosphatase effector protein YopH		- 6.78		- 8.49		- 13.32
ADZ44435	Type III secretion system effector protein YopT		- 4.12				
ADZ44434	Type III secretion modulator of injection YopK/YopQ		- 18.54		- 21.11		- 33.89
ADZ44454	Type III secretion outer membrane protein YopN		- 14.62		- 14.4		- 17.46
ADZ44467	Type III secretion OM pore forming protein YscC	-6.63	- 7.48	- 16.2	- 6.37	- 10.1	- 7.66
ADZ44451	Type III secretion protein YscX		- 11.89				
ADZ44455	Type III secretion apparatus H+-transporting two-sector		- 2.87				- 3.23
ADZ44457	Type III secretion system needle length determinant YscP		- 2.78				
ADZ44497	Adhesin YadA	- 4.97	- 9.83	- 10.37	- 10.81	- 5.02	- 10.27
ADZ40701	Phospholipase A, YplA		- 2.07		- 2.37		- 2.06
ADZ43625	Urease subunit gamma UreA			- 2.44			2.2
ADZ43623	Urease subunit alpha UreC		2.43	- 2.03	2.89	- 1.28*	5.4
ADZ43620	Urease accessory protein UreG	- 2.5	1.99		2.05	- 1.86	2.65
Iron ion homeostasis GO:0055072							
ADZ43721	Ferric anguibactin-binding protein FatB				2.31		
ADZ41314	Outer membrane receptor FepA	1.68					
ADZ41093	Heme ABC exporter, ATP-binding protein CcmA		1.59				
ADZ41067	Iron transporter FecA	- 2.08	- 2.56	- 2.53		- 2.3	- 2.96
ADZ40857	Hemin receptor HemR		- 2.02				- 1.72
ADZ44135	Bacterioferritin Bfr		10.28		8.15	- 4.57	7.17
Response to stress GO:0006950							
ADZ42566	Phage shock protein PspA						2.36
ADZ41933	DNA protection during starvation protein			2.23			
ADZ41491	Copper homeostasis protein CutF			- 2.03			- 2.16
ADZ42722	Putative carbon starvation protein A, CstA		- 2.99	- 1.94	- 2.43	- 1.58	- 3.24
ADZ41113	Chaperone protein DnaK, Hsp70	- 1.56	- 2.01				- 1.64
ADZ42757	Osmotically-inducible protein Y			- 2.27			
ADZ43049	Paraquat-inducible protein B						1.9
Catalytic activity GO:0003824							
ADZ43177	Inner membrane protein YeiU	12.47					
ADZ41168	Signal recognition particle protein			1.83			
ADZ40865	Keto-acid formate acetyltransferase				1.61		
ADZ43088	Formate acetyltransferase 1		1.51				
ADZ42412	Long-chain-fatty-acid-CoA ligase FadD	- 11.73	- 4.32			- 5.15	- 4.44
ADZ40899	Protein HflC	- 3.28					
ADZ42794	Protease 4		- 1.67				
Cell motility GO:0048870							
ADZ42196	Flagellar hook protein FlgE						3.07
ADZ42168	Putative methyl-accepting chemotaxis protein	- 5.05					
ADZ42180	Methyl-accepting chemotaxis protein	- 2.32					
ADZ42216	Flagellar M-ring protein					- 1.71	
Cell redox homeostasis GO:0045454							
ADZ41797	Cytochrome D ubiquinol oxidase subunit II					2.56	
ADZ41796	Cytochrome D ubiquinol oxidase subunit I	1.48*		1.83		2.33	
ADZ42602	NAD(P) transhydrogenase subunit alpha			1.77			

ADZ41617	Cytochrome O ubiquinol oxidase subunit II			- 1.52		- 1.4*	- 1.51
Cell division GO:0051301							
ADZ43396	Cell division protein ZipA homolog	- 3.52		- 2.15			
ADZ41291	Cell division protein FtsZ	- 1.9					
Undefined GO term							
ADZ41569	Putative exported protein						14.98
ADZ40718	Putative membrane protein	7.34		5.61		7.44	
ADZ43361	Putative exported protein					2.53	
ADZ43548	Putative lipoprotein YfhG						2.47
ADZ44035	Outer membrane lipoprotein PcP						1.86
ADZ43116	Putative lipoprotein					1.51	
ADZ40804	Inner membrane protein YjcH	- 9.68	-35	- 10.62		- 12.42	- 37.22
ADZ41451	Lipoprotein			- 4.43			
ADZ43231	Outer membrane protein YfaZ				- 4.17		- 3.89
ADZ41832	Uncharacterized protein					- 3.97	
ADZ41163	Putative exported protein			- 3.28		- 2.61	
ADZ42504	Lipoprotein NlpC			- 3.22			
ADZ41640	Lipoprotein, YscW Superfamily			- 2.43		- 1.87	
ADZ43738	Putative outer membrane lipoprotein			- 2.1			
ADZ42938	Putative exported protein	1.75				2.12	- 2.16

[a]Description of the identified proteins of OMsl (outer membrane-enriched sarkosyl-insoluble fractions) according to their UniProt database or GenBank entries, or their similarity to homologous sequences identified using BLAST searches. Proteins were clustered based on Gene Ontology (biological process) terms.

[b]Proteins whose abundance differed between the wild-type strain Ye9 and OmpR-deficient mutant AR4, according to MS analysis. Within each category, the proteins are sorted according the effect of OmpR: positive followed by negative (ranked from highest to lowest fold change). Standard conditions (LB medium); high osmolarity (LB supplemented with NaCl to 386 mM); low pH (LB adjusted to pH 5.0), at 26°C and 37°C; q-value ≤ 0.05; '-'protein more abundant in *ompR* mutant strain, fold changed is shown; * - value of fold change slightly different from the accepted threshold value of 1.5.

Figure Legends

Fig. 1. Functional characterization of *Y. enterocolitica* proteins whose abundance is regulated by OmpR. (A) Classification of proteins differentially expressed in the wild-type strain Ye9 compared to OmpR-deficient mutant AR4 under all tested conditions, using Gene Ontology (GO) biological process. The classification is based on BioCyc Database Collections, the Uniprot databases and literature data. (B) Classification of differentially-expressed proteins associated with biological transport processes. (C) Chart indicating the number of differentially-expressed proteins that are upregulated (more abundant) or downregulated (less abundant) in the *ompR* mutant AR4 compared to the wild type Ye9, divided according to biological process classification. (D) Chart indicating the number of differentially-expressed proteins associated with biological transport processes that are upregulated or downregulated in *ompR* mutant AR4 compared to the wild-type Ye9.

Fig. 2. Putative OmpR-binding sites identified in the promoter regions of *Y. enterocolitica* genes encoding OmpR-dependent proteins. (A) The consensus OmpR binding site of *E. coli* and logo motif defined based on analysis of OmpR binding elements in the *ompC* and *ompF* promoter regions (Maede et al., 1991). (B) The consensus OmpR binding site of *Yersinia* spp. and logo motif defined based on experimentally validated OmpR binding elements present in the promoter regions of *inv* (Brzostek et al., 2007), *flhDC* (Hu et al., 2009b; Raczkowska et al., 2011a), *acrR* and *acrAB* (Raczkowska et al., 2015) and *ompC*, *ompF*, *ompR* and *ompX* (Gao et al., 2011). WebLogo (http://weblogo.berkeley.edu/logo.cgi) was used to obtain consensus sequence logos in which the height of individual letters within the stack of letters represents the relative frequency of that particular nucleotide at a given position, and the number of letters in the stack indicates the degree of conservation at that position. (C) Sequences of putative OmpR-binding sites in the promoters of the indicated *Y. enterocolitica* genes determined based on similarity to the consensus sequences of *E. coli* and *Yersinia* spp. (percentage identity values are shown). The central motif **G**XX**AC** or **G**XXX**C** and the **AC** or **C** nucleotides usually located about 10 nt away from the **AC** elements of the central motif are marked. (D) Graphical representation of the fold change in the abundance of the identified OmpR-dependent proteins under the tested growth conditions. Proteins that are more or less abundant in the *ompR* mutant strain are indicated by the scale which shows pale to dark colors of red and blue, respectively.

Fig. 3. OmpR-dependent YadA expression. (A) Immunodetection of the oligomeric forms of YadA protein in the outer membrane-enriched sarkosyl-resistant fractions of wild-type Ye9 (wt) and OmpR-deficient mutant AR4 (*ompR*) strains of *Y. enterocolitica*. The analyzed samples were prepared from cells grown at 26°C in standard LB medium (std, 86 mM NaCl, pH 7.0), at 37°C in

standard LB, in LB with raised osmolarity (high osm., 386 mM NaCl, pH 7.0) or in LB of low pH (low pH, 86 mM NaCl, pH 5.0). Samples were boiled for 5 min in Laemmli buffer before electrophoresis in a 10% polyacrylamide gel (SDS-PAGE). The top panel shows the immunoblot probed with a polyclonal antibody against YadA (α-YadA) and the bottom panel shows the Coomassie blue-stained gel as a loading control. On the Western blot, oligomeric YadA gives a band of approximately 200 kDa and several others of intermediate size. M – molecular weight standards (PageRuler Prestained Protein Ladder; kDa). This result is representative of least three independent experiments. (B) Immunodetection of the monomeric form of YadA protein. To disrupt protein trimers, samples were boiled with urea sample buffer prior to loading the gel. The top panel shows the immunoblot and the bottom panel shows the Coomassie blue-stained gel. Wild-type strain Ye9 (wt) and OmpR-deficient mutant AR4 (*ompR*) were grown under the conditions described in part A. The band corresponding to YadA monomers on the Western blot (approx. 50 kDa) is shown. M – molecular weight standards (PageRuler Prestained Protein Ladder; kDa). This experiment was performed twice with similar results.

Fig. 4. Analysis of OmpR-dependent YadA expression using a YadA'-'GFP- translational fusion. Fluorescence intensity of Ye9 (wt), AR4 (*ompR*) and complemented strain AR4 (*ompR*/pompR) containing pFX-*yadA*, analyzed by flow cytometry. All strains were grown to stationary phase in LB medium (standard conditions), LB supplemented with NaCl (386 mM NaCl, pH 7.0; high osm.) or LB adjusted to pH 5.0 (low pH), at 27°C (A) or 37°C (B). In these experiments, the mean fluorescence intensity of strains carrying a promoterless *gfp* gene (plasmid pFX-0) was between 8 and 14. The data represent mean values with the standard deviation from at least two independent experiments, each performed using at least triplicate cultures of each strain. Significance was calculated using Student's unpaired *t*-test (*** – $p<0.001$, * – $p<0.05$).

Fig. 5. Interaction of OmpR with the *yadA* promoter region. (A) The promoter and 5'UTR of *yadA*. The experimentally verified -35 and -10 promoter elements (underlined) and the transcription start (asterisk) are indicated (Skurnik and Wolf-Watz, 1989). The sequence shaded gray (Y1) corresponds to the putative OmpR binding site. The *yadA* start codon (ATG) is shown in bold. Beneath the sequence, the putative binding site Y1 is compared with the consensus OmpR binding motifs of *E. coli* and *Yersinia* spp. The percentage identity to these sequences is shown. (B). Schematic representation of the *yadA* regulatory region showing the putative OmpR-binding site (Y1) revealed by *in silico* analysis and the position of the DNA fragments (F1, F2, F3) used in electrophoretic mobility shift assays (EMSAs) (C). EMSAs examining the binding of various concentrations of phosphorylated OmpR to fragments of the *yadA* regulatory region: F1 (392 bp) which contains the putative OmpR-binding site, and F2 (411 bp) and F3 (227 bp) which lack this

site. A fragment of 16S rDNA (304 bp) was included in each reaction mixture as a non-specific binding control. The binding reactions were comprised of the DNA fragments mixed with increasing concentrations of OmpR-P (0.168, 0.336, 0.504 μM; lanes 2-4), or with no added OmpR (lane 1). The identities of the bands resolved by electrophoresis on 5% native polyacrylamide gels are indicated.

Fig. 6. HemR expression in the wild-type (Ye9) and the *ompR* mutant (AR4) strains. (A) Immunodetection of the HemR protein in total cell extracts of *Y. enterocolitica.* The top panel shows the immunoblot probed with a polyclonal antibody against HemR (α-HemR) and the bottom panel shows the Coomassie blue-stained gel as a loading control. Wild-type strain Ye9 (wt), OmpR-deficient mutant AR4 (*ompR*) and AR4 complemented with a plasmid expressing OmpR (*ompR*/pompR) were grown overnight in LB medium at 37°C, then subcultured in LB medium (standard conditions, std), LB at pH 5.0 (LB, low pH), LB with 386 mM NaCl (LB, high osm.), or LB with 0.3 mM α,α'-dipirydyl (LBD), and incubated at 37°C for 3 h. Equivalent whole-cell lysate samples were loaded. The arrow indicates the HemR band, which is only visible in the parental strain grown under low iron conditions (LBD), but is detected in the *ompR* mutant under all tested growth conditions. M – molecular weight standards (PageRuler Prestained Protein Ladder; kDa). This experiment was repeated twice with similar results. (B) Analysis of *hemR* expression by measuring the β-galactosidase activity of strains carrying a chromosomal *hemR-lacZYA'* transcriptional fusion: wild-type Ye9 (Ye9H), *ompR* mutant AR4 (AR4H), complemented strain AR4H (AR4H/pompR) and AR4H transformed with empty vector pBBR1MCS-5 (AR4H/pBBR1MCS-5). All strains were grown to logarithmic phase in LB medium, with or without 0.3 mM α,α'-dipirydyl, at 26°C or 37°C and β-galactosidase activity was assayed. The data represent mean activity values (Miller units) with the standard deviation from three independent experiments, each performed using at least triplicate cultures of each strain. Significance was calculated using Student's unpaired *t*-test (**** – $p<0.0001$, *** – $p<0.001$, ** – $p<0.01$, ns – $p>0.05$).

Fig. 7. Interaction of OmpR with the *hemR* promoter region. (A) The *hemPR* and *hemR* promoters and 5'UTRs. The putative -35 and -10 promoter elements of *hemPR* and *hemR* are single and double underlined, respectively. The Fur binding site in the *hemP* ORF is boxed (R1). The sequence shaded gray (H1) corresponds to the putative OmpR binding site. The start codons (ATG) of *hemP* and *hemR*, and the stop codon of *hemP* are shown in bold (Stojiljkovic and Hantke, 1992; Thompson et al., 1999). Beneath the sequence, the putative OmpR and Fur binding sites are compared with the respective consensus binding motifs, and the percentage identities are shown. (B) Electrophoretic mobility shift assay of a *Y. enterocolitica hemR* promoter region fragment (385

bp) incubated with purified and *in vitro* phosphorylated OmpR protein. A fragment of 16S rDNA (304 bp) was included as a non-specific binding control. The binding reactions were comprised of the DNA fragments mixed with increasing concentrations of OmpR-P (0.38, 0.76, 1.14, 1.52, 3.04 μM (lanes 2-6) or with no added OmpR (lane 1). The identities of the bands resolved by electrophoresis on 5% native polyacrylamide gels are indicated.

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

Supporting information

Table S1. Strains and plasmids used in this study.

Table S2. Primers used in this study.

Table S3. Comparison of the patterns of OMsl proteins produced by *Y. enterocolitica* wild-type strain Ye9 grown under standard conditions (LB medium), high osmolarity (LB supplemented with NaCl to 386 mM) or low pH (LB adjusted to pH 5.0), at 26°C and 37°C. Differentially expressed proteins identified in the OMsl (outer membrane-enriched sarkosyl-insoluble fractions) are described according to their UniProt database or GenBank entries, or their similarity to homologous sequences identified using BLAST searches. Proteins were clustered based on Gene Ontology (biological process) terms. Significant changes in protein abundance (*q*-value ≤ 0.05) are defined by a ratio of ≤ 0.67 (protein more abundant at 37°C or at high osm. or low pH) or ≥ 1.5 (protein less abundant at 37°C or at high osm. or low pH). Values for the fold change in abundance and the number of identified peptides belonging to the proteins are indicated.

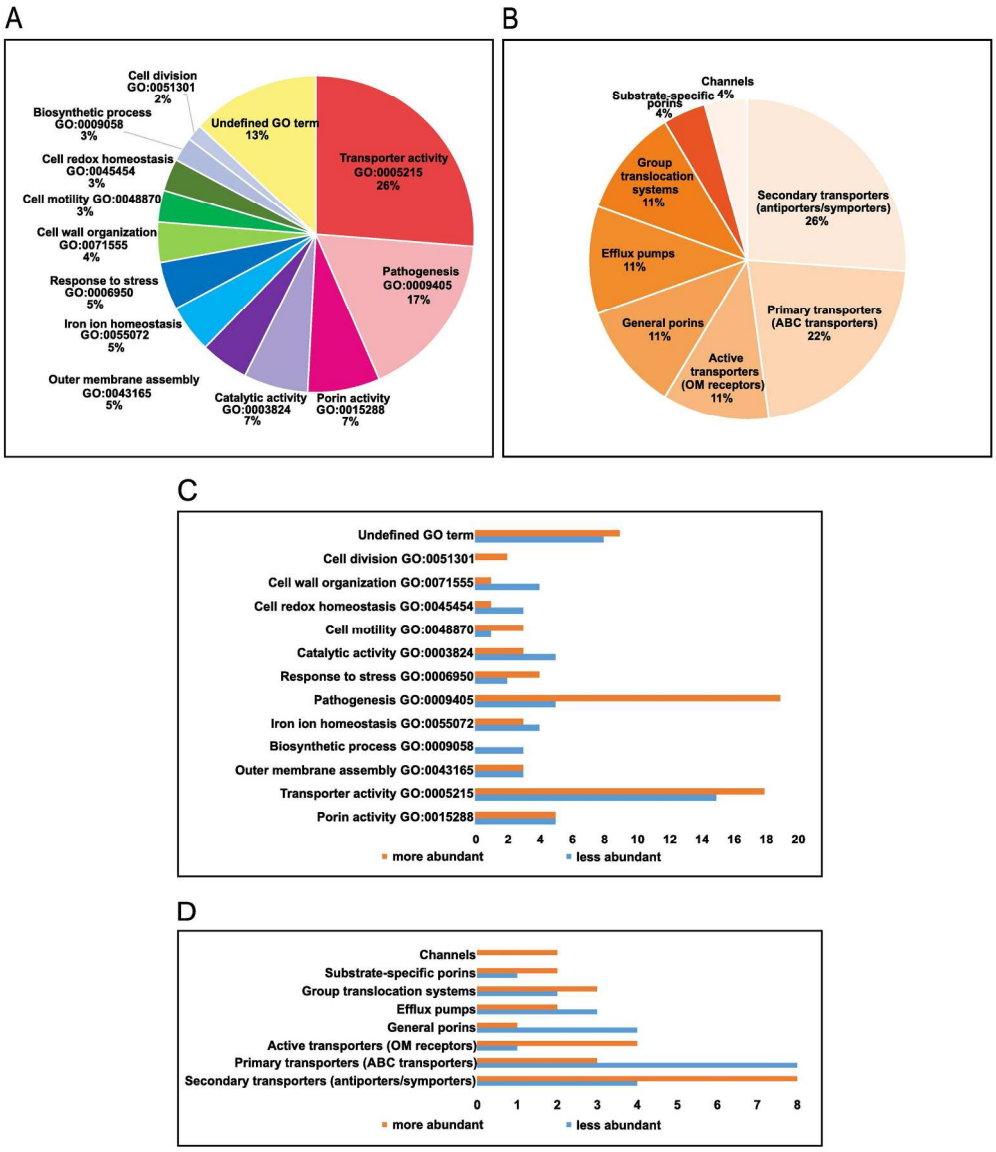
Word doc 1. Detailed description of the effect of temperature, osmolarity and pH on the membrane proteome of the wild-type *Y. enterocolitica* strain Ye9 presented in Table S3.

Table S4. Comparison of the patterns of OMsl proteins produced by *Y. enterocolitica* wild-type strain Ye9 and isogenic *ompR* mutant AR4 grown under standard conditions (LB medium), high osmolarity (LB supplemented with NaCl to 386 mM) or low pH (LB adjusted to pH 5.0), at 26°C and 37°C. Differentially expressed proteins identified in the OMsl are described according to their UniProt database or GenBank entries, or their similarity to homologous sequences identified using BLAST searches. Significant changes in protein abundance (*q*-value ≤ 0.05) are defined by a ratio of ≤ 0.67 (protein more abundant in *ompR* mutant strain) or ≥ 1.5

(protein less abundant in *ompR* mutant strain). Values for the fold change in abundance and the number of identified peptides belonging to the proteins are indicated.

Fig. S1. Summary of OmpR-dependent changes in protein production under the different growth conditions tested. Differences in the abundance of proteins in the OMsl of the *ompR* mutant (strain AR4) compared to the wild-type (strain Ye9) were studied in strains grown under standard conditions (LB), high osmolarity (LB supplemented with 386 mM NaCl) or low pH (LB, pH 5.0), at 26°C and 37°C. Significant changes in protein abundance (q-value ≤ 0.05) of ≥ 1.5 were accepted. (A) Venn diagram illustrating the total number of OmpR-dependent changes observed at 26°C and 37°C and their distribution between standard conditions, high osmolarity and low pH. (B) Venn diagrams showing the extent of the overlap between the proteins differentially expressed (less or more abundant) in the *ompR* mutant AR4 compared to the wild-type strain Ye9 at 26°C (left sets) vs. 37°C (right sets) under standard conditions, high osmolarity and low pH.

Fig. S2. Principle components analysis used to cluster the identified protein patterns according to OmpR status and growth conditions. The effect of temperature (26°C vs. 37°C), pH (pH 7.0 vs. pH 5.0) and osmolarity (86 mM vs. 386 mM NaCl) on the wild-type Ye9 and *ompR* mutant AR4 protein patterns is shown (A). Each point corresponds to a single replicate sample. The value of the principal components is not a measure of the magnitude of the variable. PCA is used to cluster the protein patterns at 26°C and 37°C produced by OmpR activity under standard growth conditions (B), high osmolarity (C) and pH 5.0 (D). Each point corresponds to the protein pattern of each replicate sample generated by the presence (wild-type Ye9) or absence (*ompR* mutant AR4) of OmpR under the particular growth conditions, projected onto a two-dimensional principal component space.

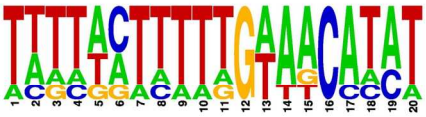


196x228mm (300 x 300 DPI)

A

The consensus OmpR binding site of *E. coli*

TTTTACTTTTTG(T/A)AACATAT



B

The consensus OmpR binding site of *Yersinia* spp.

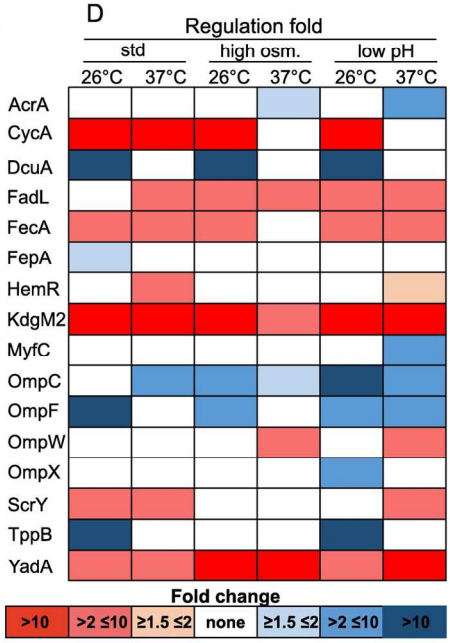
(A/T)TTTTCA(A/T)TTTGTAACATTT

C

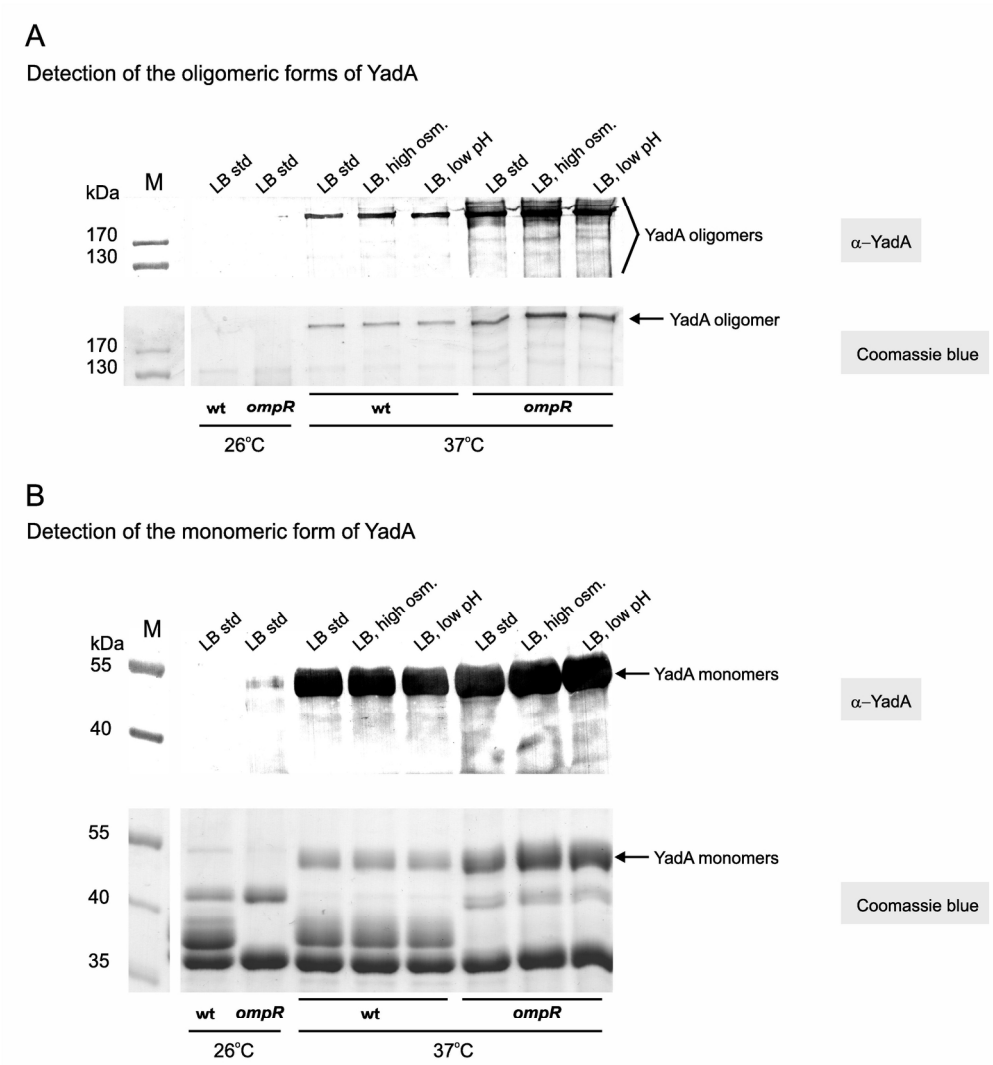
Putative OmpR-binding sites

Gene	OmpR motif	% identity to *E. coli* consensus	% identity to *Yersinia* spp. consensus
acrA	TTAAATGCGTCGTTACCCAA	45	35
cycA(1)	TTGTTAGTTATGTAACTTCA	60	65
cycA(2)	TACATCTGGTAGTTACGATT	45	50
dcuA	TCACCATAATGGTGACATCG	45	45
fadL(1)	AGGCCTATTAAGTGACAGAA	40	45
fadL(2)	AAAACACACTTGCAACACGC	35	45
fadL(3)	AAAACACACTTGCAACACGC	35	45
fecA	ATTTATCATATGAATCAATT	60	65
fepA	GTATTGATGTAGTAACAACA	50	60
hemR	GCAGGGAAACTGATACTGAC	30	30
kdgM2	CTTTGAACCTTGTCACATAA	60	60
myfC	TACTTTTATTTGATACTTCC	55	55
ompC(1)	ACTTACATTTTGAAATGCTT	65	70
ompC(2)	TTTTTGGTTATGAAACATTA	70	75
ompC(3)	TCCGAGACTTCGTAGCATTT	55	60
ompF(1)	ATTTACATTTAGTAACACAT	80	80
ompF(2)	AGTTTCCCAATGAAACATAT	65	65
ompF(3)	TCAGGTAATTGGTAGCATTT	50	65
ompW	TTATTTAAATTGTTACTAAA	50	60
ompX(1)	CACAAAAAGGAGTAACATAG	45	45
ompX(2)	TGAAACTCTTTGTTACACCA	60	50
scrY	GCTATCTTGCTGTATCAATA	50	55
tppB	GCATAACATTTGCAACATAG	60	55
yadA	AGATTACAAACGACGCATAT	40	45

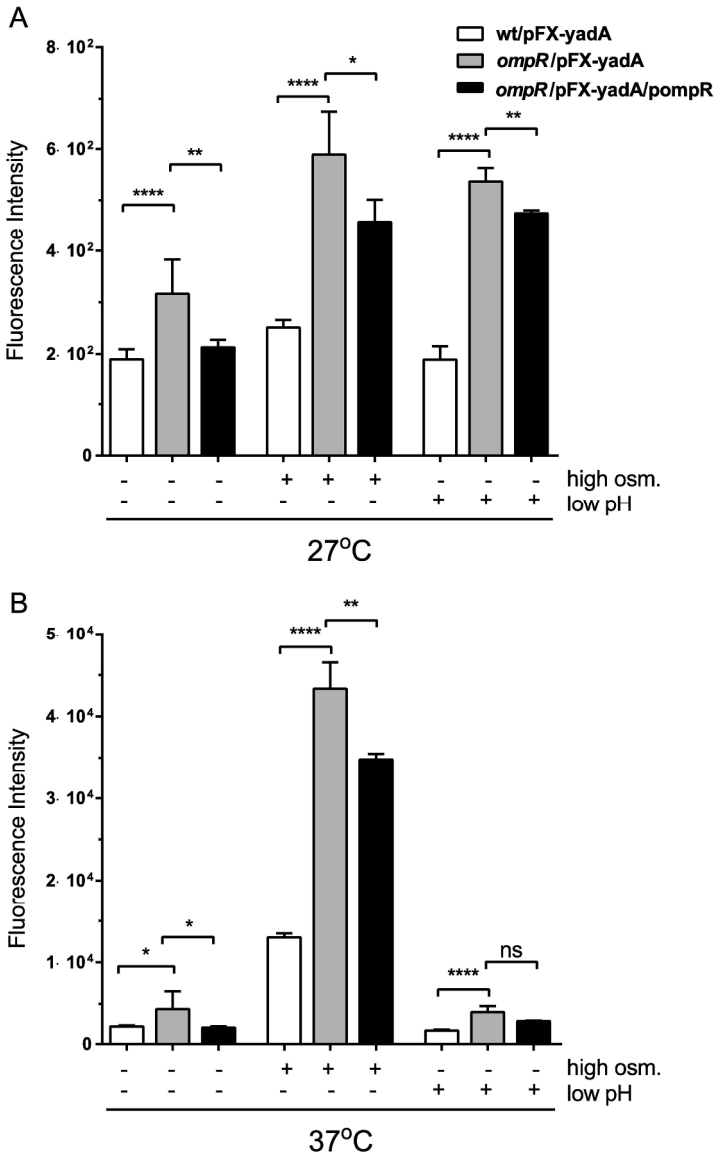
D



156x144mm (300 x 300 DPI)



184x206mm (300 x 300 DPI)

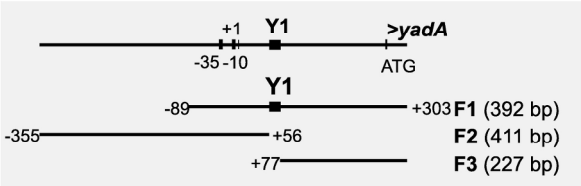


218x339mm (300 x 300 DPI)

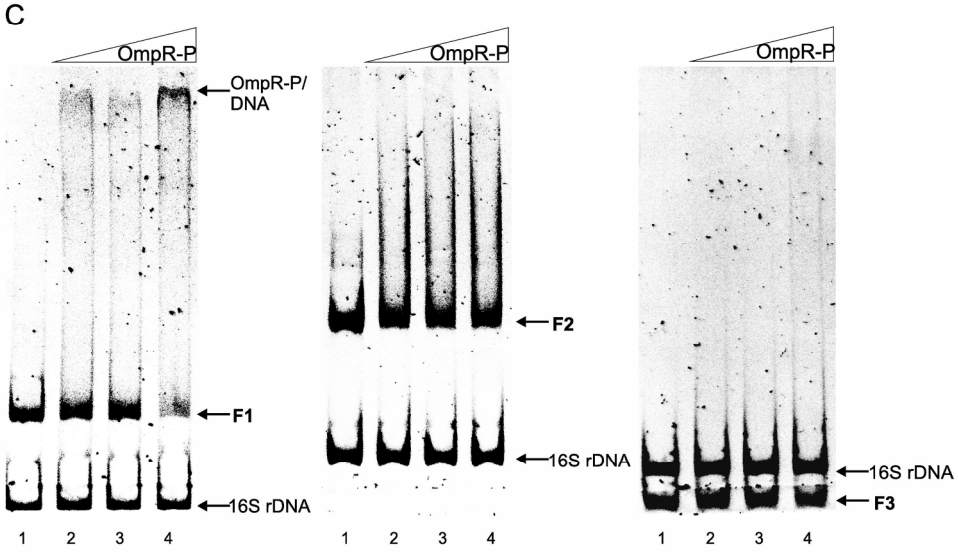
A

```
                                                *
GTTTGAGGTGAGGAGTTTTTTTTTATATTATCCGCATAACACTTTTCGTGTTATCTGAAAGTATTTTGTAGTGGGCTGACTC
                 Y1
CGACGATTAGAGATTACAAACGACGCATATACTCAGTAGTTAATCGATATATTTTTAAGATCGATTAGTGCTGTTTTTTGCA
TGATTATCAGAAAATAAGTCATAGATAATCCTATCCCTCTTCTATGGGAGGCGTCCGCTTTAATTAATATATTTCTCAGATG
                                                          >yadA
TTATAACTGAGCTTTTATTCACGGGAAATTAAAGAAATATAAAAGGTGCTTACAATG

OmpR consensus E. coli:      TTTTACTTTTTG(T/A)AACATAT
OmpR consensus Yersinia spp.:(A/T)TTTTCA(A/T)TTTGTAACATTT
Y1                            AGATTACAAACGACGCATAT

% identity to the E. coli consensus: 40%
% identity to the Yersinia spp. consensus: 45%
```

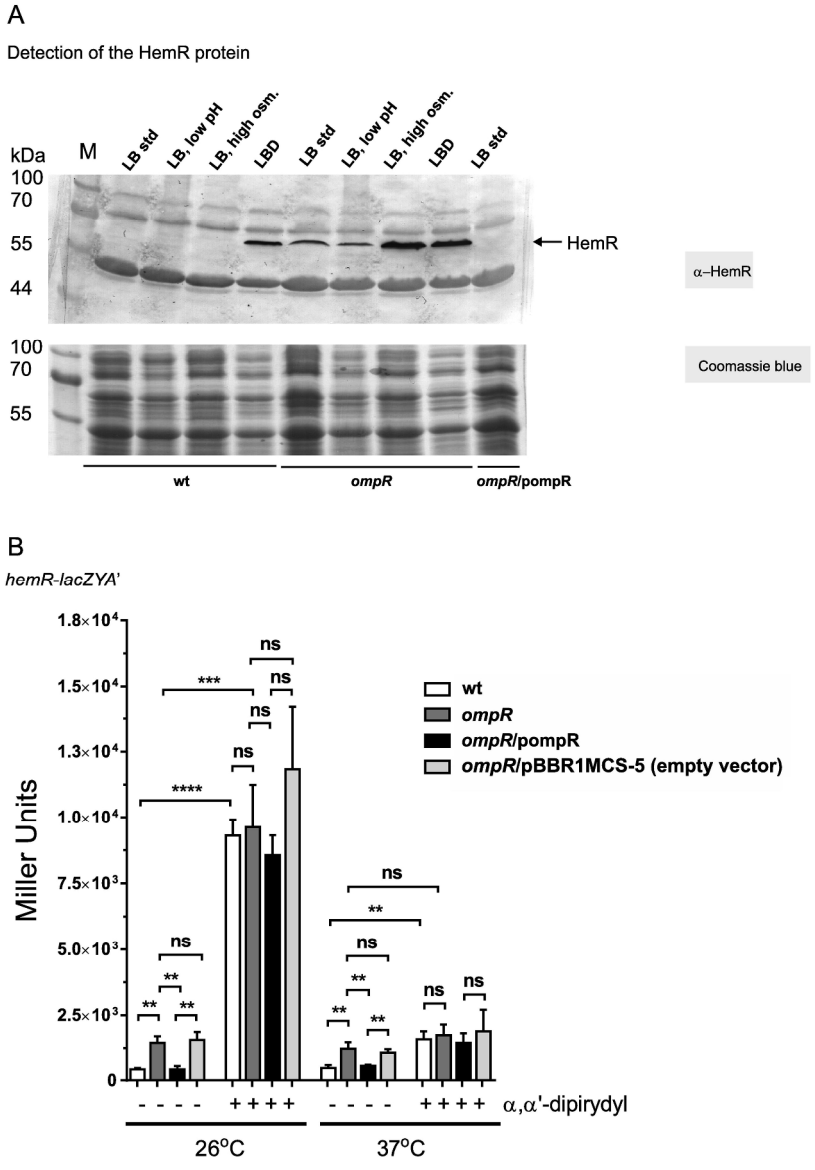
B



C



207x253mm (300 x 300 DPI)



234x325mm (300 x 300 DPI)

A

R1

TAAATTAACAATAAATATGTCTTGATGTAACACTGAATCATGATTGATAATGCTTATCATATTGATATCGGTTATCATTACC

>*hemP*

TTGTTTACAATATGGATAAACAGTTGAACAAAGCACCCACAATGAATGACGAGCCTGCAGCCAAACCTCCTGCGGGCAACAA

GCCCCTGTCTGTCTCCAGCGAGCAATTGCTGGGAGAGCATAGTGTCGCTTTTATCATCCATCAGGGCGAATGCTATCAACTG

H1

CGCCAGACCAAAGCAGGGAAACTGATACTGACTAAATAATAGCCCAATGCCAATGTCGTGACAGCAAGGTAGCGGTTCCCGC

TAGCACCGCTGTGGCAGGGGCAAAGGATACATCGCAAGCCACCCAGATTTTAGAATCAAGGCAGCCAGCAACCTATTTATTT

>*hemR*

GTTTTGCATATGATTTTTTTGCATAGAAAATATGGAGAATTGCCGACATG

OmpR *consensus E. coli*: TTTTACTTTTTG(T/A)AACATAT

OmpR *consensus Yersinia* spp.: (A/T)TTTTCA(A/T)TTTGTAACATTT

H1 GCAGGGAAACTGATACTGAC

% identity to the *E. coli consensus*: 30%

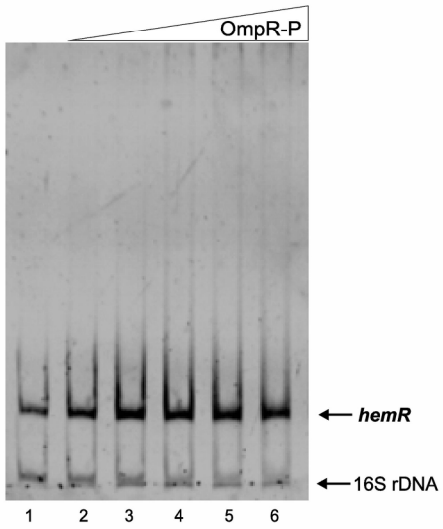
% identity to the *Yersinia* spp. *consensus*: 30%

Fur *consensus E. coli*: GATAATGATAATCATTATC

R1 GATAATGCTTATCATATTG

% identity to the *E. coli consensus*: 74%

B



192x220mm (300 x 300 DPI)