Yersinia pestis Requires the 2-Component Regulatory System OmpR-EnvZ to Resist Innate Immunity During the Early and Late Stages of Plague

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Plague is transmitted by fleas or contaminated aerosols. To successfully produce disease, the causal agent (Yersinia pestis) must rapidly sense and respond to rapid variations in its environment. Here, we investigated the role of 2-component regulatory systems (2CSs) in plague because the latter are known to be key players in bacterial adaptation to environmental change. Along with the previously studied PhoP-PhoQ system, OmpR-EnvZ was the only one of Y. pestis’ 23 other 2CSs required for production of bubonic, septicemic, and pneumonic plague. In vitro, OmpR-EnvZ was needed to counter serum complement and leukocytes but was not required for the secretion of antiphagocyte exotoxins. In vivo, Y. pestis lacking OmpR-EnvZ did not induce an early immune response in the skin and was fully virulent in neutropenic mice. We conclude that, throughout the course of Y. pestis infection, OmpR-EnvZ is required to counter toxic effectors secreted by polymorphonuclear leukocytes in the tissues.

Keywords. Yersiniosis; plague; Yersinia; innate immunity; two-component systems; polymorphonuclear leukocyte.

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The plague bacillus (Yersinia pestis) is usually transmitted to a mammalian host during the blood meal of an infected flea [1, 2]. When regurgitated into the skin, the bacillus disseminates into the local draining lymph nodes, which becomes swollen, edematous, necrotic, and hemorrhagic. Swollen lymph nodes (called buboes) are a characteristic clinical feature of bubonic plague [3]. Y. pestis is subsequently seeded from the bubo into the blood and produces a fulminant, systemic infection. In some instances, Y. pestis is deposited directly into a dermal blood vessel; by bypassing the lymph nodes, the bacterium causes fatal septicemia without forming buboes [4, 5]. Sporadically, bacteria having invaded the lung after blood dissemination destroy respiratory function prior to production of an overwhelming episode of septicemia [6]. A patient developing pneumonia expectorates infectious aerosols, which pass on the disease once they are inhaled by another individual.

After the fleabite, production of plague in the mammalian host may depend on Y. pestis’ ability to replicate within phagocytes during the initial step in skin colonization [7, 8]. Within its intracellular shelter, Y. pestis may produce a pseudocapsule, a type 3 secretion system (T3SS), and its cognate exotoxins (so-called Yersinia outer proteins; Yops) to subvert phagocytosis and kill phagocytes encountered once the bacterium leaves its hiding place [9, 10]. Extracellular Y. pestis probably moves freely from the skin into the local lymph node via lymphatic vessels, and the bacillus appears to multiply in extracellular sites throughout the course of the infection [11]. To do so, Y. pestis must counteract a
number of toxic effectors released by the polymorphonuclear leukocytes (PMNs) located in infected tissues [12, 13]. Furthermore, Y. pestis must resist complement-dependent killing mechanisms [14, 15]. Last, a number of virulence factors (such as the T3SS) are needed throughout the infection, whereas other virulence factors are involved in the initiation of colonization only after intradermal, intranasal, or intravenous inoculation [16–18]. In summary, Y. pestis encounters a variety of environments and must rapidly sense and respond to a wide range of external stimuli if it is to produce a successful infection.

Y. pestis' ability to produce plague may rely on phospho-signaling transduction pathways, such as the regulatory 2-component systems (2CSs). Indeed, there is a large body of evidence to show that 2CSs play a key role in the bacterial adaptation to the vagaries of their external environment [19]. The 2CSs are canonically composed of a receptor (the sensor) and an effector (the response regulator). In response to specific environmental cues, the transmembrane sensor phosphorylates itself and then passes the phosphoryl group to its cognate cytoplasmic regulator; this process modulates gene expression so that the bacteria can adapt to the external medium. In addition to the orthodox 2CSs described above, many systems have additional regulatory partners, and some systems are interconnected through connector proteins [20]. The Y. pestis genome encodes 24 2CSs, although some (BaeRS, UhpAB, EvgAS, and RcsABCD) are presumed to be nonfunctional in some strains as a result of frameshift mutations [21]. With the exception of the PhoP-PhoQ system, the Y. pestis 2CSs' roles in the development of bubonic and pneumatic plague have yet to be defined [22]. We therefore attempted to (1) identify the 2CSs that are important for the production of bubonic and pneumatic plague and (2) further characterize the role of one key 2CS in bubonic plague.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**

The bacterial strains and plasmids used are presented in Supplementary Table 1. Mutants were generated using the lambda Red recombinase method and checked by polymerase chain reaction (PCR) analysis (as previously described [23]), using the primer sets described in Supplementary Table 1. The \( \text{ompR} \) mutant was complemented with the pBR322 cloning vector containing \( \text{ompR} \) under the control of its own promoter (plR4).

**Assessment of Virulence**

Groups of 8–9-week-old OF-1 female mice (Charles River) and brown Norway rats (Janvier) were inoculated with Y. pestis via the intradermal, intranasal, or intravenous routes, as previously described [12]. To study the virulence in neutropenic mice, animals were inoculated intraperitoneally 24 hours before and after bacterial challenge with phosphate-buffered saline (PBS) containing 100 \( \mu \)g of the antineutrophil antibody NIMPR-14 [24]. The time course of organ colonization was measured by colony-forming units (CFU) counting, as previously described [11]. All animal experiments were approved by the Nord-Pas-de-Calais regional Animal Care and Use Committee (reference CEEA-23012).

**Immune Response**

Skin-biopsy specimens from around the injection site were lysed (at 37°C in a 5% CO\(_2\) atmosphere) by incubation with 2.4 U of collagenase/dispose (Roche) in calcium- and magnesium-depleted PBS supplemented with 2% heat-inactivated fetal bovine serum (DPBS/FBS) for 90 minutes and then with 280 U of collagenase IV (Worthington) and 1000 U of DNase I (Roche) in DPBS/FBS for 60 minutes. The well contents were flushed several times and filtered through a 100-µm cell strainer, and cells attached to the wells were collected with DPBS supplemented with 0.01 M ethylenediaminetetraacetic acid (which was also used to rinse the cell strainer). The filtrates were washed twice in Roswell Park Memorial Institute medium supplemented with 5% FBS, washed once in DPBS/FBS, and resuspended in DPBS containing allophycocyanin-labeled mouse anti-CD11b and peridinin chlorophyll protein-Cy5.5-labeled anti-Ly6G antibodies (BD Biosciences). After 30 minutes of incubation, the cells were washed twice in DPBS/FBS and fixed in 4% paraformaldehyde. Last, cells were sorted by flow cytometry, and the data were analyzed using FlowJo software.

**Lipooligosaccharide (LOS) Analysis**

LOS from bacteria grown overnight in lysogeny broth (LB) was analyzed by sodium deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE), as previously described [25].

**Reverse-Transcription Quantitative PCR**

RNA was purified using RNeasy kit (Qiagen), treated with DNase by means of the DNA-free kit (Ambion), assessed for quality and quantity (with a Bioanalyzer [Agilent Technologies] and a NanoDrop [GE Healthcare], respectively), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was performed using SYBR Green–based technology. The primer sets GCCCTCTGGCAATAAAATGG and AGCATGGTGGTCTCGAAAATT (for \( \text{crr} \)) and GTTCGAGGAATCTGTTGACGG and GCCCCCAATAATCCATACAAGC (for \( \text{ail} \)) were used. The quantity of \( \text{ail} \) messenger RNA was determined relative to the \( \text{crr} \) transcript [13], and the relative fold-change was calculated using the \( \Delta \Delta CT \) method [26].

**In Vitro Interaction With Macrophages**

RAW 264.7 mouse macrophage–like cells and bone marrow–derived macrophages (BMDMs) from C57BL/6 mouse were
produced and infected, as previously described [12]. Measurement of bacterial survival within macrophages was performed by CFU counting, again as previously described [12].

Cytotoxicity Assay
Regardless of the genetic background, the bacteria used in this assay contained the pEP1392 plasmid harboring the gene encoding green fluorescent protein (gfp) under the control of an isopropyl-beta-D-thiogalactopyranoside (IPTG)–inducible promoter. Bacteria diluted from an overnight culture at 28°C in LB medium supplemented with appropriate antibiotics were incubated at 37°C for 4 hours, washed in PBS, resuspended in DMEM supplemented with 10% FBS (and appropriate antibiotics), and added to 4 × 10⁵ HeLa cells (multiplicity of infection, 30) seeded in 96-well tissue culture plates. The plates were centrifuged at 30 × g at room temperature for 5 minutes and then incubated at 37°C in a 5% CO₂ atmosphere. IPTG (final concentration, 500 µM) and Syto-60 dye (final concentration, 5 µM) were added after 2 and 2.5 hours of incubation, respectively, to induce GFP synthesis and stain the HeLa cells’ cytoplasm and nucleus. Thirty minutes after addition of Syto-60 dye, cells were fixed in 4% paraformaldehyde for 30 minutes and then incubated for 5 minutes with 25 µL of 4’,6-diamidino-2-phenylindole (5 µM) to stain the nuclei. Last, each well was washed and refilled with PBS prior to image acquisition at a magnification of 20-fold, using the Opera QEHS confocal microscope (Perkin-Elmer). The proportion of dead cells was determined by counting the round-shaped cells with a shrunken cytoplasm (indicated by less expansive staining with Syto-60 dye) and dividing the number by the total cell count.

RESULTS
Along With the Previously Studied PhoP-PhoQ System, Only the OmpR-EnvZ System Modulates the Virulence of Y. pestis
To determine the role of the 2CSs systems in plague, we generated Y. pestis strains in which the locus for each 2CS had been replaced by an antibiotic resistance cassette (Supplementary Table 1). Next, OF-1 mice were inoculated intradermally or intranasally with a dose that consistently kills 75%–100% of animals (approximately 10 CFU and approximately 3 × 10⁵ CFU, respectively). Bacteria inoculated intradermally and those inoculated intranasally were grown at 21°C and 37°C, respectively, to mimic the natural infection conditions as closely as possible [13, 27, 28]. Regardless of the inoculation route, only the ΔompR-envZ mutant showed a significant alteration in virulence (Table 1 and Figure 1A and 1B).

The OmpR-EnvZ System Is Involved in Both Early and Late Stages of Bubonic Plague
To confirm the OmpR-EnvZ system’s role in bubonic plague, we constructed an OmpR-negative mutant expressing (or not) a copy of ompR (under the control of its own promoter) from a cloning vector and measured its virulence (Figure 1C). The ΔompR mutant showed virulence attenuation similar to that of the ΔompR-envZ mutant. In contrast, the complemented ΔompR mutant was fully virulent. To further investigate the role of the OmpR-EnvZ system in bubonic plague, we looked at whether or not the system responded to a stimulus encountered (1) at a specific stage of the infectious process or (2) throughout host colonization. To this end, the time course of organ colonization in mice inoculated intradermally with the ΔompR mutant was compared with that of the wild-type strain (Figure 1E). Relative to the wild type, the mutant was significantly slower in colonizing the skin and the draining lymph node and reached the blood and the spleen later, indicating that OmpR-EnvZ is required during the initial stage of skin colonization. However, these data did not rule out the involvement of OmpR-EnvZ during deep-tissue colonization. To investigate this possibility, the virulence of the mutant grown at 37°C (instead of 21°C) was measured in mice inoculated intravenously, to bypass the early steps of the infection cycle from skin colonization to blood dissemination (Figure 1D). When the intravenous inoculation route was used, the ΔompR mutant was less virulent than the wild-type strain. Last, OmpR-EnvZ’s role in bubonic plague was also assessed in rats, to determine whether this regulatory system was required in different animal species (Supplementary Figure 1). As had been seen in mice, the absence of OmpR-EnvZ or OmpR alone was associated with a significantly lower incidence of plague (median survival, ≥5.5 days for the mutants and 4 days for the wild type; mortality rate, 37.5% for the mutants and 12.5% for the wild type). Taken as a whole, our results suggest that the OmpR-EnvZ system is required during the early and late stages of bubonic plague in different animal species.

OmpR-EnvZ Does Not Appear to Be Associated With Differences in Phagocyte Recruitment at the Skin Inoculation Site
The fulminant progression of plague is thought to result from the lack of a rapid innate immune response, in part because Y. pestis disables the host immune system [28, 29]. The ompR mutant’s time lag in colonization after intradermal inoculation may reflect the fact that this mutant induces more inflammation and thus a faster immune response. To test this hypothesis, the recruitment of macrophages and dendritic cells (both CD11⁺) and granulocytes (Ly6G⁺) at the skin inoculation site was determined in mice infected with the mutant strain or the wild type (Supplementary Figure 2). Regardless of the time point and the bacterial strain used, the numbers of CD11⁺ and Ly6G⁺ cells in the skin of inoculated mice were not significantly different.

The OmpR-EnvZ System Facilitates the Intracellular Survival of Y. pestis in Macrophages
Intracellular survival of Y. pestis within phagocytes is thought to be important for the initiation of infection at the dermal
inoculation site [8]. Hence, impaired survival within macrophages might explain why the OmpR-EnvZ-negative mutant did not multiply in the skin as quickly as the wild-type strain (Figure 1E). To test this hypothesis, we examined the survival of the wild-type strain, the ΔompR-envZ mutant, the ΔompR mutant, and the latter’s complemented derivative following uptake by RAW macrophages (Figure 2A). Ten hours after internalization, the count of viable ΔompR-envZ and ΔompR-negative bacteria was approximately 1 log lower than the corresponding value for the wild type. Poor survival of both mutants (relative to that of the wild type) was also observed in BMDMs (Figure 2A). Regardless of the macrophage cell line used, the trans-complemented OmpR-negative mutant survived to much the same extent as the wild type, supporting the hypothesis that OmpR-EnvZ is required for intracellular survival within macrophages.

The OmpR-EnvZ System Minimizes the Action of Complement and Enables Optimal Growth in Serum

The ΔompR-envZ mutant’s inability to efficiently counteract the action of complement could also explain its virulence attenuation, since complement activation is known to lyse gram-negative bacteria [30]. To test this hypothesis, we monitored the growth of the ΔompR-envZ strain, the ΔompR strain, and the ΔompR strain bearing ompR on a plasmid in both fresh and heat-inactivated serum (Figure 2B). In fresh serum, the 2 strains lacking OmpR protein grew more slowly than the wild-type strain. In heat-inactivated serum, the growth rates for the

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Abbreviations: d, days; NA, not applicable; ND, not determined; UD, undefined; WT, wild type.

<sup>a</sup> Virulence was determined after intradermal inoculation of approximately 10 colony-forming units. Groups of 8 animals per strain were used.

<sup>b</sup> A system was considered to be necessary (yes) or not (no) for virulence if the comparison of the survival curves drawn from animals infected with the mutant and WT strain revealed that the incidence of plague in animals infected with the mutant of interest was significantly reduced (P < .05, by the Gehan–Breslow–Wilcoxon test).

<sup>c</sup> Virulence was determined after intranasal inoculation of approximately 3 × 10<sup>8</sup> colony-forming units. Groups of 8 animals per strain were used.
strains and the wild type were not significantly different. Taken as a whole, these results indicate that the OmpR-EnvZ system is required for optimal growth in fresh serum, presumably because the regulatory system confers effective tolerance of complement.

It is known that LOS and the invasin/adhesin Ail protect Y. pestis against the action of complement [14, 15, 31]. To establish whether a variation in the structure of LOS and/or the amount of Ail could explain the ΔompR-envZ mutant’s growth defect in fresh serum, we compared the LOS and ail expression profiles (after contact with serum) in the mutant and the wild-type strain by using DOC-PAGE and a SYBR Green assay, respectively. There was not a marked difference between the 2 strains in terms of LOS migration and ail expression (data not shown).

Figure 1. Effect of the OmpR-EnvZ regulatory system on Yersinia pestis virulence. A–D, Incidence of plague in OF-1 mice injected intradermally (A and C), intranasally (B), or intravenously (D) with approximately 10 colony-forming units (CFU; for intradermal and intravenous inoculation) and 3.10^3 CFU (for intranasal inoculation) of Y. pestis wild type (WT; black circles), ΔompR-envZ (white squares), ΔompR (white triangles), or ΔompR bacteria harboring the plasmid pBR322 (which contains a WT copy of ompR under the control of its own promoter; grey triangles). The ΔompR-envZ and ΔompR mutants were less virulent than the WT strain (P < .05), regardless of the infection route. In contrast, the complemented ΔompR mutant strain was fully virulent when inoculated intradermally (P > .38). P values were obtained using the Gehan-Breslow-Wilcoxon test. E, The time course of colonization of OF-1 mice inoculated intradermally with approximately 10 CFU of Y. pestis WT (black circles) or ΔompR (white triangles). The time course of colonization of the skin and the draining lymph node was significantly slower in mice infected with the ΔompR mutant than in mice infected with the WT strain (P < .05 in a 2-way analysis of variance). The difference was notable 48 hours after inoculation (P < .05 in a 2-way analysis of variance with a Bonferroni post hoc test). For each panel, data were obtained using 8 animals per group. *P < .05.

The OmpR-EnvZ System Is Involved in the Resistance of Y. pestis to PMNs In Vivo
The fact that OmpR-EnvZ mediates resistance to primary granule extracts from PMNs [32] provides an additional explanation for the virulence-attenuated phenotype of the ΔompR-envZ mutant. We therefore sought to determine the role of OmpR in resistance to PMNs in vivo by comparing the virulence of the OmpR-negative mutant and the wild-type strain in mice that had been rendered neutropenic by administration of an anti-neutrophil antibody (anti-NIMP-R14). Virulence was also assessed in control mice treated with an isotype HB12 antibody (Figure 2C). The results confirmed that the ΔompR mutant was significantly less virulent in immunocompetent mice but was fully virulent in neutropenic mice.
The research on Yersinia pestis' ability to confront different parts of the immune system's arsenal.

A-C, Differential colony-forming unit (CFU) counts at t₀ and then 10 hours after phagocytosis of bacteria by RAW 264.7 macrophage-like cells or bone marrow-derived macrophages (BMDMs; A) or after contact with normal or heat-inactivated serum from different healthy donors (B), and the incidence of plague in immunocompetent and neutropenic mice (C). Wild type (WT; black bars and black circles), a ΔompR-envZ mutant (white bars), a ΔompR mutant (grey bars and white triangles), and a ΔompR mutant harboring a WT copy of ompR under the control of its own promoter in pBR322 (grey hatched bars) were used. Shown are means ± standard errors of the mean of 3 independent experiments, for intracellular survival in macrophages; 5 independent experiments, for normal serum; or at least 2 independent experiments, for heat-inactivated serum. *P < .05 in a t test. The incidence of plague was determined in groups of 8 mice that were (dashed lines) or were not (solid lines) depleted of polymorphonuclear leukocytes (PMNs), using the anti-neutrophil NIMP-R14 antibody, and after intradermal inoculation with approximately 10 CFU. Depletion of PMNs restored the mutant's virulence to WT levels (P < .05 in a Gehan-Breslow-Wilcoxon test). Data were obtained using 8 mice per group.

Figure 2. Effect of the OmpR-EnvZ system on Yersinia pestis' ability to confront different parts of the immune system's arsenal. A–C, Differential colony-forming unit (CFU) counts at t₀ and then 10 hours after phagocytosis of bacteria by RAW 264.7 macrophage-like cells or bone marrow-derived macrophages (BMDMs; A) or after contact with normal or heat-inactivated serum from different healthy donors (B), and the incidence of plague in immunocompetent and neutropenic mice (C). Wild type (WT; black bars and black circles), a ΔompR-envZ mutant (white bars), a ΔompR mutant (grey bars and white triangles), and a ΔompR mutant harboring a WT copy of ompR under the control of its own promoter in pBR322 (grey hatched bars) were used. Shown are means ± standard errors of the mean of 3 independent experiments, for intracellular survival in macrophages; 5 independent experiments, for normal serum; or at least 2 independent experiments, for heat-inactivated serum. *P < .05 in a t test. The incidence of plague was determined in groups of 8 mice that were (dashed lines) or were not (solid lines) depleted of polymorphonuclear leukocytes (PMNs), using the anti-neutrophil NIMP-R14 antibody, and after intradermal inoculation with approximately 10 CFU. Depletion of PMNs restored the mutant’s virulence to WT levels (P < .05 in a Gehan-Breslow-Wilcoxon test). Data were obtained using 8 mice per group.

The OmpR-EnvZ System Is Not Required for pYV-Mediated Cytotoxicity

The translocation of Yop effectors via a T3SS into host cells is a major virulence mechanism in Y. pestis [9]. The Yops disable phagocytosis and the production of proinflammatory cytokines and induce cell death. A default in the delivery of Yops to host cells might explain why OmpR was required to counter PMNs during infection (Figure 2C). To test this hypothesis, the pYV-mediated cytotoxicity of the ΔompR and ΔompR-complementated mutants was compared with that of a wild-type strain lacking (or not) the pYV Yersinia virulence plasmid (which encodes the T3SS and the Yops). After 3-hour incubation with HeLa cells, the 4 strains’ respective abilities to elicit a cytotoxicity phenotype was evaluated by quantifying round cells (Figure 3). As expected, the pYV-cured strain (used here as negative control) was not cytotoxic; the percentage of round cells was essentially the same for the pYV-cured strain and uninfected cells (approximately 15%). In contrast, the wild-type strain and the ΔompR mutant strain were both cytotoxic. Indeed, the ΔompR mutant was even more cytotoxic than the wild-type strain (75% vs 30%). Last, the complemented strain was as toxic as the wild-type strain, confirming that the loss of OmpR was responsible for the greater cytotoxicity.

DISCUSSION

Our present results showed that in addition to the previously studied PhoP-PhoQ system, only one of Y. pestis’ 23 other 2CSs (OmpR-EnvZ) is required for bubonic and pneumonic plague (Table 1 and Figure 1) [22]. Our data suggest that the bacterium encounters and/or responds to a limited number of stimuli in the mammalian host but could also reflect overlapping regulatory pathways among the 2CSs; this is possible, given that overlapping regulons are common in bacteria [33, 34]. The drawbacks of our animal model provide additional clues. Our model of bubonic plague poorly mimics the fleabite and the Y. pestis phenotype (biofilm) in the flea; in fact, these specific aspects are known to influence the virulence data obtained after needle inoculation of cultured bacteria [4, 5, 35, 36]. In other words, 2CSs other than PhoP-PhoQ and OmpR-EnvZ might be required for bubonic plague in the context of natural infection. However, the number of 2CSs required to produce plague after a fleabite might be low, given that passage of Y. pestis through the flea vector prepares the bacterium for colonization of the mammalian host [35]. In contrast to the bubonic plague model, the pneumonic plague model used here resembles a real-world infection scenario more closely. Pneumonic plague is transmitted from person to person; the bacteria inoculated artificially in our study were incubated at 37°C, a temperature that primes Y. pestis to resist the host immune system [37]. A preadapted phenotype might also explain why so few 2CSs were required for the production of pneumonic plague.

Y. pestis is often viewed as a bacterium that circulates continuously between rodents and fleas. However, other factors may have a role in the ecology of plague: (1) long-term persistence in fleas, hibernating hosts, or soil; (2) ingestion of infected rodents and fleas by carnivores and insectivores; and (3) transmission by lice [38–40]. Hence, it is conceivable that 2CSs other
than PhoP-PhoQ and OmpR-EnvZ are involved in the maintenance of plague if they are required in 1 or more of the above-mentioned situations. It is also possible that some systems have been lost over the course of recent evolution. In contrast to its recent ancestor (Yersinia pseudotuberculosis, an enteropathogen found widely in the environment), Y. pestis is restricted to a few ecological niches by its 5 amino acid auxotrophies. Hence, one can expect Y. pestis to have jettisoned the 2CSs required for colonizing ecological niches that the bacterium no longer encounters. Comparative genome analysis has revealed that ancestral Y. pestis strains (Microtus) and recent strains (Antiqua, Medievalis, and Orientalis) encode an altered RcsCDB system. The recent strains also have impaired BaERs and UhpAB systems. Last, the EvgAS system is also impaired in Orientalis strains (the most recent ones). In the present study, we found that loss of functional RcsCDB, BaERs, UhpAB, and EvgAS systems in the Y. pestis CO92 Orientalis strain did not attenuate bacterial virulence (Table 1). Hence, our data provide another example showing that Y. pestis is in the process of genome reduction [41] because Y. pestis does not require the genes that Y. pseudotuberculosis presumably needed for survival under a variety of environmental conditions. However, we cannot yet completely rule out the possibility that the genetic changes in rcsD, uhpR, baeS, and evgS were required for adaptation to Y. pestis’ new life cycle.

OmpR-EnvZ is a global regulatory system that is required for the virulence of several bacteria, presumably because it is involved in the response to the variations in osmolarity, pH, and oxidative stress encountered during infection [42–49]. The ompR-envZ operon was one of the negatively selected genes in a transposon site hybridization screen in which BMDMs were infected with a Y. pestis Medievalis strain [50]. OmpR is also reportedly involved in macrophage resistance in a Y. pestis Microtus strain [34]. Accordingly, our Orientalis strain required OmpR-EnvZ for intracellular survival in macrophages (Figure 2A). Furthermore, the Orientalis strain used here needed OmpR-EnvZ to resist complement (Figure 2B). It is also known that a Medievalis strain’s ability to resist PMN alpha granules is OmpR-EnvZ dependent [32]. However, despite OmpR-EnvZ’s pleiotropic role, the system’s major role in vivo might be to protect Y. pestis against PMNs throughout the course of infection. Indeed, the ompR-envZ mutant is defective for colonization of both superficial and deep tissues (Figure 1) but is fully virulent in neutropenic mice after intradermal inoculation (Figure 2C). Notably, OmpR-EnvZ might be required for resistance to the antimicrobial peptides secreted by PMNs in infected tissues because (1) Y. pestis lacking this regulatory system is not defective for Yop translocation (Figure 3) but shows increased sensitivity to PMN alpha granules [32] and (2) it has been suggested that extracellular Y. pestis is nevertheless exposed to the toxic effectors secreted by PMNs in infected tissues [12, 13].

Last, the phoP-phoQ operon is the only validated OmpR-regulated gene known to be required for resistance to PMNs in vitro and for plague [22, 32, 34]. Hence, OmpR might regulate the production of the effectors required for PMN resistance (either directly, or indirectly through the induction of the PhoP-PhoQ regulatory system). It now remains for us to characterize the mechanism by which OmpR-EnvZ enables PMN resistance.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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Potential conflicts of interest. All authors: No reported conflicts.

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