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Involvement of EnvZ–OmpR two-component system in virulence control of *Escherichia coli* in *Drosophila melanogaster*

Pattraporn Pukklay [a,1], Yoshinobu Nakanishi [a,b,c], Mao Nitta [c], Kaneyoshi Yamamoto [d], Akira Ishihama [d], Akiko Shiratsuchi [a,b,c,*]

[a] Graduate School of Natural Science & Technology, Kanazawa University, Kanazawa, Ishikawa, Japan
[b] Graduate School of Medical Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan
[c] School of Pharmacy, Kanazawa University, Kanazawa, Ishikawa, Japan
[d] Department of Frontier Bioscience, Hosei University, Koganei, Tokyo, Japan

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ABSTRACT

Bacteria adapt to environmental changes by altering gene expression patterns with the aid of signal transduction machinery called the two-component regulatory system (TCS), which consists of two protein components, a sensor kinase and response regulator. We examined the role of the TCS in bacterial adaptation to host environments using genetically tractable organisms, *Escherichia coli* as a pathogen and *Drosophila melanogaster* as a host. To determine the strength of the transcription promoters of TCS-encoding genes in *Drosophila*, adult flies were infected with a series of *E. coli* strains that expressed GFP driven by the promoters of genes coding for 27 sensor kinases and 32 response regulators of *E. coli* TCS followed by the measurement of fluorescence intensities. We further analyzed EnvZ–OmpR among the TCS encoded by genes having stronger promoters. A mutant *E. coli* strain lacking EnvZ–OmpR had a higher pathogenic effect on fly survival than that of the parental strain, and the forced expression of *envZ* and *ompR* in the mutant strain lowered its pathogenicity. The lack of EnvZ–OmpR did not affect the growth of *E. coli* in a culture medium as well as the level of colony-formable *E. coli* in flies. An increase in *E. coli* virulence with the loss of EnvZ–OmpR was observed in flies defective in an Imd-mediated humoral response, and both the mutant and parental strains were equally engulfed by hemocytes *in vitro*. These results suggest that EnvZ–OmpR mitigated the virulence of *E. coli* in *Drosophila* by a mechanism not accompanied by a change of bacterial burden. This behavior of *E. coli* is most likely a bacterial strategy to achieve persistent infection.

1. Introduction

The mechanism of infectious diseases caused by most pathogens remains to be elucidated. In particular, it is still unknown how diseases develop in the body upon infection with microorganisms that apparently do not produce toxins. Nevertheless, persistent infection with pathogens seems prerequisite to the development of diseases. It is presumed that host environments, such as temperature and humidity, are favorable for the survival and growth of microorganisms. However, the more microorganisms grow, the more likely they are to induce severe immune responses. Therefore, the best scenario for both the invader and host would be that the former continues to exist in the latter while the latter does not become sick. It is probable that bacteria may try to achieve such a situation by modifying the expression of their genes in the host.

Bactria alter gene transcription patterns using the two-component regulatory system (TCS)[2] to adapt to environmental changes. The TCS consists of a membrane-bound receptor called the sensor kinase and a transcription factor called the response regulator [1]. The sensor kinase is an enzyme that transfers phosphoryl groups to the response regulator at aspartate residues for activation [1,2]. The phosphorylated, and thus, activated response regulator binds to the *cis*-acting regulatory sequences of target genes resulting in the activation, or sometimes inactivation, of their transcription [1,3]. There appear to exist 30 sensor kinases and 34 response regulators in terms of the analysis of *Escherichia coli* genome although some of them have not been paired to constitute functional TCS [4]. These TCS can be categorized into several groups based on the consequence of their actions: control of metabolism, respiration, influx

Abbreviation: TCS, two-component regulatory system(s).

* Corresponding author. Address: Graduate School of Medical Sciences, Kanazawa University, Shizenken, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan. Fax: +81 76 234 4480.
E-mail address: ashira@staff.kanazawa-u.ac.jp (A. Shiratsuchi).
[1] Present address: Faculty of Sciences, MaeJo University Phrae Campus, 17 Moo 3 RongKwang, Phrae, Thailand.

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and efflux, chemotaxis, and stress response [4]. We hypothesized that bacteria used the TCS upon entering hosts for the expression of new genes in order to adapt to and get along with the host. Using genetically tractable model organisms, *E. coli* as a bacterium and the fruit fly *Drosophila melanogaster* as a host, we identified EnvZ–OmpR as this kind of TCS.

2. Materials and methods

2.1. Fly stocks, E. coli strains, and E. coli genes

Oregon R (Kyorin-Fly; Kyorin University, Tokyo, Japan), as the wild-type *Drosophila* line, and *imd*[1], a mutant fly line deficient in the Imd pathway [5], were used. The *E. coli* K-12 strain BW25113 (parental strain) and its derivative BW26424 lacking EnvZ–OmpR (operationally named ΔenvZ–ompR) were from Keio Collection, which is distributed through the National BioResource Project (National Institute of Genetics, Shizuoka, Japan). All bacteria strains were cultured with Luria–Bertani medium at 37 °C in the presence of antibiotics when necessary, harvested at full-growth, washed with PBS, and used in the experiments. The plasmid pAT224 with an insert of a 5.3 kbp fragment of *E. coli* genomic DNA, which includes the entire *envZ–ompR* operon, in pBR322 [6] was introduced into Δ*envZ–ompR* for gene complementation.

2.2. Bacterial infection, and assays for pathogenicity, colony formation, and phagocytosis of bacteria

Adult male flies were injected with given numbers (as indicated in the figure captions) of live bacteria in the abdomen, reminiscent of septic infection, according to established procedures [7]. Briefly, flies 3–7 days after eclosion (15–20 flies per vial, and 1–3 vials in each experiment) were anesthetized with CO_2 and injected with bacteria suspended in PBS (50–100 nl) using a nitrogen gas-operated microinjector (IM300; Narishige, Tokyo, Japan). The pathogenic effect of bacteria was determined based on the ratio of live flies at the given time points after the injection with bacteria. To determine the level of colony-formable bacteria, live adult flies (five chosen from 20 flies originally used) injected with bacteria were homogenized, and the resulting lysates were inoculated on Luria–Bertani agar-medium at serial dilutions followed by the measurement of colonies after overnight incubation at 37 °C, as described previously [8]. An assay for the phagocytosis of bacteria *in vitro* was carried out according to established procedures [9] with modifications [8]. Briefly, hemocytes were isolated from the hemolymph of wandering third-instar larvae and incubated with fluorescence-labeled bacteria (hemocytes:bacteria = 1:100) for 10–30 min at 25 °C. Samples were then washed with PBS, treated with trypan blue to quench the fluorescence of bacteria residing outside the hemocytes, and examined by fluorescence microscopy. The ratio of hemocytes containing bacteria and the number of bacteria contained in 100 hemocytes were determined.

2.3. Analysis of promoter strength of TCS-encoding genes

DNA fragments spanning a region from the translation start codon to approximately 300 bp upstream of the transcription initiation site of genes coding for *E. coli* TCS were inserted into the vector pGRP at a site upstream of the coding sequence of GFP so that the translation of GFP was initiated at its own start codon [10,11]. The resulting plasmid was used to transform the *E. coli* strain KP7600, a derivative of W3110. Adult males of *Oregon R* (more than 5 flies for each bacterium) were injected with these bacteria, which had been cultured in Luria–Bertani medium for 16–18 h at 37 °C to the stationary phase, and examined at the dorsal side by fluorescence microscopy (IX71; Olympus, Tokyo, Japan) after 30–50 min. The intensities of fluorescence derived from GFP were numerically determined using WinROOF 6.4 (Mitani Sangyo, Ishikawa, Japan).

2.4. Determination of TCS mRNA levels

Messenger RNA levels of the *E. coli* genes *ompR*, *envZ*, *rpoA*, and *rpoB* as well as of *Drosophila Rp49*, which codes for a ribosomal protein and was analyzed as a housekeeping gene of the host, were determined in semi-quantitative reverse transcription-mediated PCR. Total RNA extracted from *E. coli* or adult flies injected with bacteria by the acid phenol method [12] was used as a template in reverse transcription with a 6-base random primer, and the resulting cDNA was then used as a template for PCR. PCR products were separated on a 6% (w/v) polyacrylamide gel followed by staining with ethidium bromide. The DNA oligomers used as primers in PCR were: 5′-ATCGCCTGCTGACTCGTGA-3′ (forward) and 5′-AGGTTAAGTTTGAACTTACCGA-3′ (reverse) for OmpR mRNA; 5′-ACCTTGCTGTTCGCCAGCC-3′ (forward) and 5′-CGTACCCAGATATTGGGCGA-3′ (reverse) for EnvZ mRNA; 5′-ATGCAGGGTTCTGTGACAGA-3′ (forward) and 5′-AACGCCTTCTTTGGTGCTGT-3′ (reverse) for RpoA mRNA; 5′-GTTCTGGATGTACCTTATCTC-3′ (forward) and 5′-CGCTTCGCGCTCATAGATCA-3′ (reverse) for RpoB mRNA; and 5′-AGATCGTGAAGAAGCGCACCAAG-3′ (forward) and 5′-CACCAGGAACTTCTTGAATCCGG-3′ (reverse) for Rp49 mRNA.

2.5. Data processing

Results from quantitative analyses are expressed as the mean and error of the data from at least two independent experiments. Other data are representative of at least two independent experiments that yielded similar results. Statistical analyses were preformed using the two-tailed Student's *t*-test. *p* values less than 0.05 were considered significant, and the data significantly different from controls are marked with asterisks.

3. Results

3.1. Identification of genes coding for E. coli TCS that possess promoters active in Drosophila

To examine the involvement of the TCS in the control of the pathogenic effect of *E. coli* to host organisms, we first determined which *E. coli* TCS, about 30 in total [4], were actively expressed in *Drosophila*. We analyzed the strength of the transcription promoters of genes coding for 27 sensor kinases and 32 response regulators. Adult flies were abdominally injected with a series of *E. coli* harboring the plasmid that expressed GFP driven by the promoters of each TCS-encoding gene, and examined by fluorescence microscopy. We found that the promoters of genes coding for the components of many TCS were active as was that of *lacUV5* used as a positive control (Fig. 1A), which indicated the effectiveness of this experimental system in determining the promoter activity of TCS-encoding genes. We then numerically analyzed fluorescence levels and compared them among TCS that were categorized based on known or expected functions [4] (Fig. 1B). The results indicated that genes coding for the components of *E. coli* TCS were differentially expressed in adult flies, and that the expression levels of TCS were not related to their functions. In addition, the activity of the promoter was not always consistent between the sensor kinase and response regulator constituting each TCS. We further analyzed EnvZ–OmpR for the involvement in bacterial adaptation to host environments because the promoter of *ompR* was the most active among others that code for response regulators, including *phoB*, *arcA*, *uvrY*, *evgA*, and *cheB*.

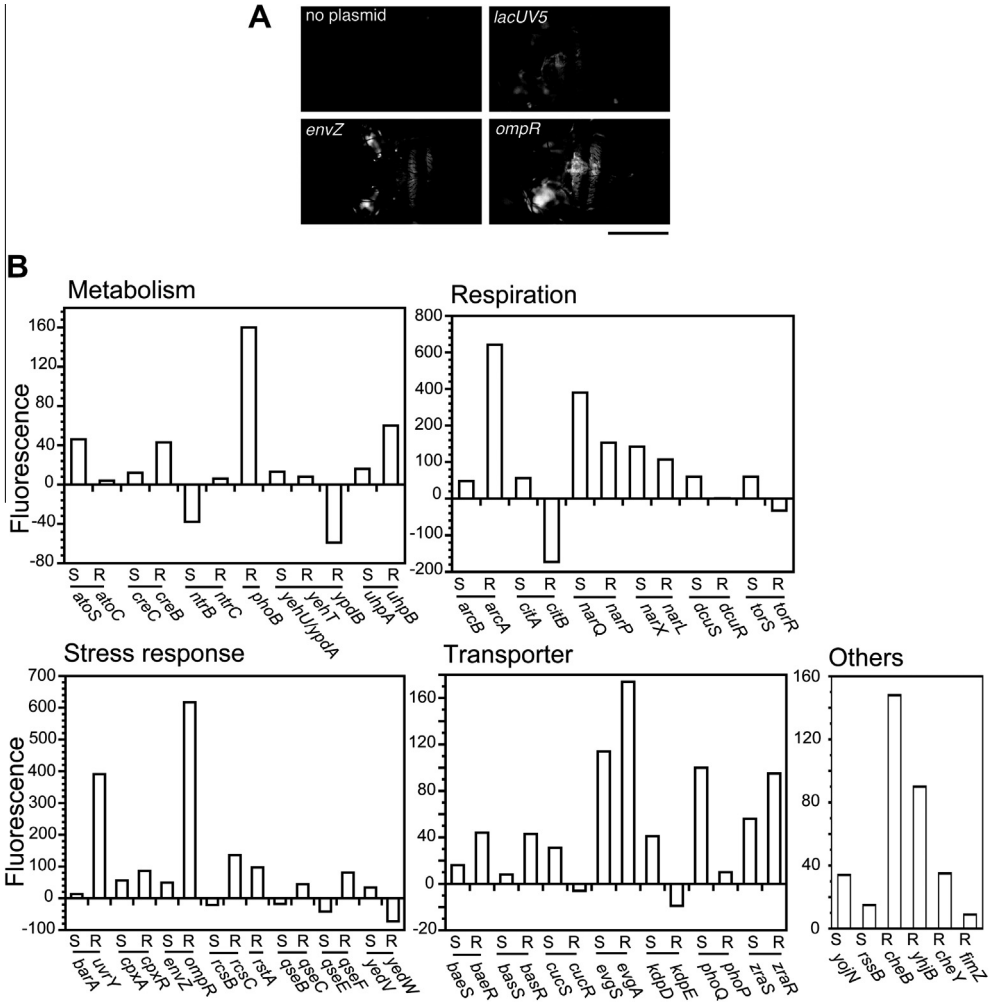

Fig. 1. Strength of transcription promoters of genes coding for components of *E. coli* TCS. A library of *E. coli* harboring a plasmid for the expression of GFP driven by the promoters of TCS-encoding genes was individually injected into the abdomen of *Oregon R* flies (6×10^6 per fly) followed by examination with a fluorescence microscope. (A) The micrographs show examples of the fluorescent views of flies with the head toward the left. The genes of which promoters were used to express GFP are indicated: *lacUV5* was examined as a positive control. Scale bar, 0.5 mm. (B) The fluorescence intensities were numerically analyzed and are shown as the means of the data from 2–3 independent experiments. TCS are categorized based on their known or expected functions [4]. S, sensor kinase; R, response regulator.

3.2. EnvZ–OmpR-mediated reduction in E. coli virulence

We examined possible changes in the pathogenic effect of *E. coli* on the survival of flies with the loss of EnvZ–OmpR. An *E. coli* strain lacking the expression of both *envZ* and *ompR* (Δ*envZ–ompR*) or its parental strain was injected into the hemocoel of adult flies, and the rate of fly deaths was determined. Under the condition in which the parental *E. coli* killed about 20% of flies, an injection of the same dosage of Δ*envZ–ompR* caused the earlier death of flies, with 50% being killed in 3 days (Fig. 2A). We then conducted a gene complementation experiment in which both *envZ* and *ompR* were forcedly expressed in the mutant *E. coli* using a plasmid, and examined possible changes in its virulence. The results showed that the pathogenic effect of Δ*envZ–ompR* harboring a plasmid for the expression of *envZ* and *ompR* was smaller than that of the same mutant strain possessing an empty vector (Fig. 2B), which confirmed a role for EnvZ–OmpR in the reduction of *E. coli* virulence.

We next examined the expression of *envZ* and *ompR* in *E. coli* before and after the infection of adult flies. The RNA extracted from the parental *E. coli* before injection was first analyzed by reverse transcription-mediated PCR. We found that signals derived from the mRNA of EnvZ and OmpR, together with those of the α (RpoA) and β (RpoB) subunits of *E. coli* RNA polymerase analyzed as internal controls, were detectable depending on reverse transcription (Fig. 2C, left panel). RNA prepared from Δ*envZ–ompR* did not show the signals corresponding to EnvZ and OmpR mRNA, while the same strain containing a plasmid for the expression of *envZ* and *ompR* gave these signals (Fig. 2C, middle panel), indicating the successful expression of *envZ* and *ompR* in the mutant strain. We then similarly analyzed RNA prepared from flies that had been infected with bacteria (Fig. 2C, right panel). RNA of flies injected with the parental strain or Δ*envZ–ompR* containing the expression plasmid gave the signals derived from the mRNA of EnvZ and OmpR as well as that from Rp49 mRNA examined as an internal control of *Drosophila* mRNA, indicating the expression of *envZ* and *ompR* after infection. In addition, the comparison of signal intensities between the mRNA of *Drosophila* Rp49 and *E. coli* RpoB suggested that adult flies received similar levels of bacterial load with all 3 *E. coli* strains analyzed.

From the results shown above, we concluded that EnvZ–OmpR acted to reduce the virulence of *E. coli* in adult flies.

3.3. Mode of actions of EnvZ–OmpR in the control of E. coli virulence

To characterize EnvZ–OmpR in terms of the control of *E. coli* virulence, we first examined the growth of Δ*envZ–ompR* relative to the parental strain in Luria–Bertani liquid medium. No significant differences were observed in growth rates between the two

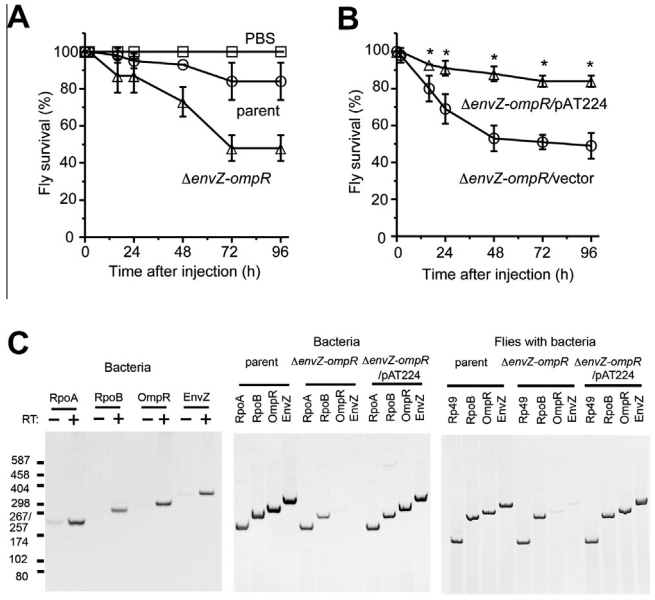

Fig. 2. Identification of EnvZ–OmpR as TCS that mitigates bacterial virulence. (A) *Oregon R* flies were injected with an *E. coli* strain lacking EnvZ–OmpR (Δ*envZ–ompR*) (2×10^6 per fly), the parental strain (parent) (2×10^6 per fly), or a vehicle alone (PBS), and the ratio of live flies was determined at the indicated time points. The data from two (PBS, Δ*envZ–ompR*) or three (parent) independent experiments are shown. (B) The rate of fly deaths was determined after infection with Δ*envZ–ompR* harboring a plasmid for the expression of *envZ* and *ompR* (Δ*envZ–ompR*/pAT224) or an empty vector (Δ*envZ–ompR*/vector) (6×10^6 per fly). The results from three independent experiments are presented. (C) The level of EnvZ and OmpR mRNA was determined by reverse transcription-mediated PCR together with the mRNA of *E. coli* RpoA and RpoB as well as *Drosophila* Rp49 as internal controls. PCR products separated on polyacrylamide gels were visualized by staining with ethidium bromide. In the left panel, RNA prepared from the parental strain was analyzed with and without the addition of reverse transcriptase (RT). The middle panel shows the analysis of RNA of Δ*envZ–ompR* with and without the forced expression of *envZ* and *ompR*. In the right panel, RNA prepared from adult flies that had been infected with the indicated *E. coli* strains (2×10^6 per fly) for 1 h was analyzed. The positions of size markers (*Hae*III-cleaved pUC19) are indicated on the left. Representative data from two independent experiments with similar results are shown.

E. coli strains (Fig. 3A, left panel). Also, the growth rate of Δ*envZ–ompR* did not change after the transfection with a plasmid for the expression of *envZ* and *ompR* or a vector alone (Fig. 3A, right panel). We next determined the pathogenic effect of *E. coli* in *imd*[1] flies, a fly line defective in the Imd-mediated production of antimicrobial peptides, and found that Δ*envZ–ompR* was more virulent than the parental strain (Fig. 3B), as observed using wild-type flies, which suggested no role for the Imd pathway in the EnvZ–OmpR control of *E. coli* virulence. We then determined the bacterial burden in flies injected with the parental *E. coli* and Δ*envZ–ompR*. Flies injected with bacteria were homogenized, and the resulting lysates were subjected to an assay for colony formation. We observed only a marginal difference between the two strains: the number of colony-formable bacteria decreased in the first 24 h and kept similar levels thereafter (Fig. 3C). Finally, the susceptibility of *E. coli* to phagocytosis by *Drosophila* hemocytes was determined *in vitro*. We found that Δ*envZ–ompR* and the parental strain were almost equally engulfed by larval hemocytes (Fig. 3D). These results suggested that the loss of EnvZ–OmpR did not cause significant changes in either the growth rate of *E. coli* both *in vitro* and *in vivo* or the susceptibility of *E. coli* to both humoral and cellular immune reactions.

4. Discussion

In the present study, we demonstrated that the TCS EnvZ–OmpR acted to mitigate the virulence of *E. coli* to *Drosophila*. Previous studies showed the involvement of other types of TCS in the control of bacterial pathogenicity. SsrA–SsrB appeared to contribute to the virulence of *Salmonella* by inducing the expression of a set of genes, which included those coding for components of the type III secretion system [13]. The PhoQ–PhoP of *Salmonella* [14,15] and *Pseudomonas* [16–19], PmrB–PmrA of *Salmonella* [20,21] and *Pseudomonas* [16,17], and CpxA–CpxR of *Salmonella* [22] confer bacterial resistance to cationic antimicrobial peptides through a change in the structure of lipopolysaccharide. Similar mechanisms appear to exist for Gram-positive bacteria: the GraS–GraR of *Staphylococcus aureus* induced the expression of genes coding for proteins that

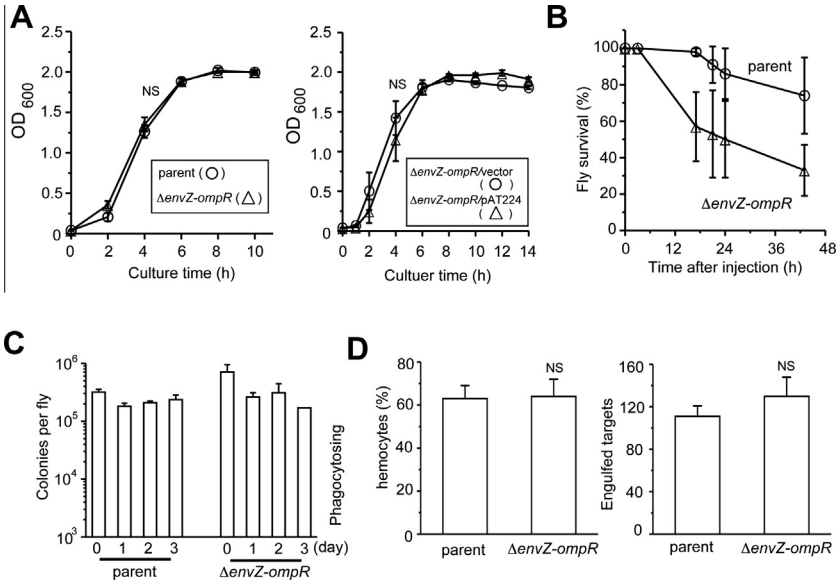

Fig. 3. Mode of actions of EnvZ–OmpR. (A) The rate of growth of the indicated *E. coli* strains in Luria–Bertani medium was determined. The data are from four (left) and three (right) independent experiments. NS, difference not significant. (B) The indicated bacterial strains (5×10^5 per fly) were examined for their pathogenic effect on the fly line *Imd*[1]. The results from two independent experiments are presented. (C) *Oregon R* flies injected with the indicated strains (2×10^6 per fly) were lyzed and subjected to an assay for colony formation. The data show the results in two independent experiments. (D) The indicated *E. coli* strains were subjected to an assay for phagocytosis using larval hemocytes of *Oregon R* as phagocytes. The data from three independent experiments are shown. NS, difference not significant.

add positively-charged amino acids to the cell membrane and cell wall, which made bacteria resistant to cationic antimicrobial peptides [23]. The TCS of *Mycobacterium tuberculosis* have been intensively studied for their involvement in the control of virulence both *in vitro* using culture cell lines and *in vivo* with rodents [24]. Mutations in most TCS of *M. tuberculosis*, including SenX3–RegX3 [25,26], PhoP–PhoR [27], MprA–MprB [28], DevR–DevS [29,30], and MtrA–MtrB [31], impaired persistent infection. The environmental factors that the above-described TCS sense to provoke a change in gene expression remain largely unknown, although there have been several reports on this issue [13,19,21,22].

As described above, most TCS analyzed were positively involved in the virulence and persistent infection of bacteria. However, our findings were different: the loss of EnvZ–OmpR led to an increase in the level of virulence of *E. coli* in adult flies. It is hard at present to explain the mode of EnvZ–OmpR actions to mitigate the virulence of *E. coli*: there is no indication of changes in bacterial burden in flies and the susceptibility of bacteria to phagocytosis by hemocytes or humoral immune responses mediated by the Imd pathway after the loss of this TCS. EnvZ–OmpR, one of the best-studied TCS, mostly senses a change in osmolarity and subsequently alters the level of transcription of over a dozen genes, called the OmpR regulon, including those coding for outer membrane proteins [32]. The OmpR regulon also includes genes that encode proteins related to the synthesis of curli (*csgDEFG*) [33] and flagella (*flhDC*). We speculate that EnvZ–OmpR may reduce the virulence of *E. coli* by altering the expression levels of genes involved in the synthesis of these extracellular structures and allow *E. coli* to get along with the host, resulting in persistent infection. We have interpreted this phenomenon as a host–pathogen interaction for both organisms to survive. It is of importance to identify and characterize the 'getting-along-with-host' genes of *E. coli* that are induced through the actions of EnvZ–OmpR as well as the presumed host factors that stimulate invading *E. coli* for the activation of EnvZ–OmpR.

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