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The Shigella ProU system is required for osmotic tolerance and virulence

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ABSTRACT

To cope with hyperosmotic stress encountered in the environments and in the host, the pathogenic as well as non-pathogenic microbes use diverse transport systems to obtain osmoprotectants. To study the role of *Shigella sonnei* ProU system in response to hyperosmotic stress and virulence, we constructed deletion and complementation strains of *proV* and used an RNAi approach to silence the whole ProU operon. We compared the response between wild type and the mutants to the hyperosmotic pressure *in vitro*, and assessed virulence properties of the mutants using gentamicin protection assay as well as *Galleria mellonella* moth larvae model. In response to osmotic stress by either NaCl or KCl, *S. sonnei* highly up-regulates transcription of *proVWX* genes. Supplementation of betaine greatly elevates the growth of the wild type *S. sonnei* but not the *proV* mutants in M9 medium containing 0.2 M NaCl or 0.2 M KCl. The *proV* mutants are also defective in intracellular growth compared with the wild type. The moth larvae model of *G. mellonella* shows that either deletion of *proV* gene or knockdown of *proVWX* transcripts by RNAi significantly attenuates virulence. ProU system in *S. sonnei* is required to cope with osmotic stress for survival and multiplication *in vitro*, and for infection.

Introduction

Shigella is a facultative intracellular Gram-negative pathogen, known as the etiologic agent of bacillary dysentery since the 1890s. Although *Shigella* was defined a genus with 4 species *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* in the 1950s,[1] it has become clear that they are pathogenic lineages of *Escherichia coli* of multiple origins.[2] The primary transmission is the fecal-oral route, so it is life threatening in developing countries because of poor sanitation. *Shigella* strains are among the most prevalent causative agents of moderate-to-severe diarrhea, and especially affect children under 5 y old in developing countries.[3] The widespread of multiple antibiotic resistant strains, has made *Shigella* treatment increasingly difficult and there is urgent need for vaccine development.[4] *Shigella* is highly invasive to the colon and the rectum and it has the ability to proliferate in the cell cytoplasm and trigger the host pro-inflammatory response. It causes variable clinical manifestations ranging from short term illness, typically watery diarrhea, to a long lasting one manifesting with fever, bloody diarrhea with intestinal cramps and mucopurulent feces.[5]

Keeping a stable osmotic balance between the cell cytoplasm and the outer environment is an important challenge to all cell types, especially the unicellular organisms. For bacteria, the high surface area to total volume ratios makes them vulnerable when they encounter osmotic stress; bacteria could tolerate the osmolarity changes in the environment through either solutes efflux or water movement across the cytoplasmic membrane.[6] The external osmolarity changes are translated by the microorganisms to an adaptation process; this happens by a rapid K^+ ion influx through specific transporters, and at the same time, microorganisms produce counter ions like glutamate.[7] However, high intracellular concentration of K^+ and glutamate only support microbial adaptation to moderately high osmolarity. At very high osmolarity, further accumulation of K^+ and glutamate becomes impossible for growth, and therefore, bacteria exploit less deleterious compounds called osmoprotectants, like polyoles (trehalose), amino acids (proline), and methylamines (glycine betaine).[7] Osmoprotectants can accumulate intracellularly via cellular uptake or synthesis from their precursors. Glycine betaine is one of the most important osmoprotectants for bacteria.[7]

For pathogenic bacteria, osmoregulation is a very important factor for establishing infection. For example, *Staphylococcus aureus* has a PutP proline transport system that helps in host tissue colonization.[8-10] Other studies have found a link between osmotic stress and the expression of virulence genes in *Pseudomonas aeruginosa* [11,12] and between the transport of compatible solutes and pathogen colonization in *Listeria monocytogenes*.[13,14] In *Salmonella enterica* serovar typhimurium and *E. coli* K-12, osmoprotectants are mainly accumulated through the ProP and ProU transport systems.[15-19]

ProP is a member of the major facilitator superfamily of permeases, and it is known as a symporter.[20] ProP is of low affinity for proline and glycine betaine, it has Km of ≈ 0.1 mM for both.[20] ProU system efficiently scavenges glycine betaine,[21] Km of ≈ 1 μM,[16] as well as proline betaine for bacteria to cope extreme osmotic stress.[22,23] It is composed of 3 proteins, i.e., ProV, ProW, and ProX, which are encoded by an operon *proVWX*,[18] of which ProV belongs to the ATP-binding cassette (ABC) superfamily.[24,25] ProX is a periplasmic soluble substrate-binding protein[19,26,27] which could bind and deliver glycine betaine to the inner membrane protein ProW, whereas ProV hydrolyses ATP providing energy for transporting substrates against the concentration gradient.[24,25]

In 2005, Lucchini and his colleagues analyzed genomic expression of *S. flexneri* during infection by DNA microarray.[28] *proVWX* genes were found highly up-regulated in both epithelial HeLa and macrophage-like U937 cells; in particular, *proV* was upregulated by 57-fold in U937 cells.[28] Besides, previous studies found that the level of ProU transcription in *E. coli* is induced upon exposure to hyperosmotic stress.[29] These findings suggested that *Shigella* faced extreme osmotic stress in the host cell cytosol and up-regulation of ProU was necessary for *Shigella* to cope with this hostile cellular niche, allowing *Shigella* intracellular survival and growth to establish infection. Additionally, the orthologous ProXVWZ system in *Mycobacterium tuberculosis* has been shown to actively transport glycine betaine into macrophages, which contributed to early steps in colonization of the cellular niche.[30] Hence, we have investigated the impact of the ProU system on osmotic stress response and pathogenesis of *S. sonnei*. Our data have shown that the ProU transport system is important for *S. sonnei* to cope with hyperosmotic stress in the host cell cytoplasm, and for rapid intracellular proliferation to establish infection.

Results

proV *deletion and complementation*

In order to study the function of ProU we decided to construct a *proV* deletion mutant since *proV* is the most highly upregulated gene inside host cells and the first gene in the *proVWX* operon.[28] *ProV* deletion was constructed in wild type *S. sonnei* strain (20071599) [31] using the phage λ Red recombination system.[32] All *proV* constructs for mutagenesis as well as complementation were confirmed by PCR and agarose gel electrophoresis (Fig. S1). All PCR products were further confirmed by DNA sequencing using primers c and d (Table S1).

ProU is required to cope with hyperosmotic stress *in vitro*

Because the ProU system is known to be required for *E. coli* to cope with extreme osmotic stress,[22,23] we tested whether this was also the case for *S. sonnei*. We first measured growth of the wild type, $\Delta proV$ and the complemented $\Delta proV$/pProV strains in M9 medium supplemented with 0.3 M NaCl or 0.3 M KCl by measuring the optical density (OD_{600nm}) each hour untill the late stationary phase (10 hours). While the wild type *S. sonnei* grew well, the growth of $\Delta proV$ strain was impaired in the presence of 0.3 M of NaCl or KCl (Fig. 1A and 1B). All the tested strains were able to grow properly in M9 medium without any supplements (Fig. S2). Overexpression of ProV *in trans* not only reversed the growth defect of the $\Delta proV$ strain, but also made the strain grow faster than the wild type. This suggested that deletion of *proV* is solely responsible for the slow growth phenotype of the mutant and also suggested that excess ProV made the system more effective and that ProU may also transport osmoprotectants other than betaine, such as K^+, available in the medium to facilitate bacterial growth (Fig. 1A and 1B). We further tested the growth of wild type and the $\Delta proV$ strains in M9 medium supplemented with 0.2 M NaCl or 0.2 M KCl. Under these milder conditions, the $\Delta proV$ strain grew equally well as the wild type strain up to 6 hours. However, the growth of the $\Delta proV$ strain ceased at 6 hours and remained flat to 10 hours. In contrast, the wild type grew steadily, reaching $OD_{600nm} = 0.7$ and 0.8 in M9 supplemented with 0.2 M NaCl and 0.2 M KCl, respectively (Fig. 1C and 1D). Thus, in the presence of 0.2 M NaCl, growth of $\Delta proV$ was more than 1.5-fold reduced (OD_{600nm} 0.4 vs. 0.7) compared to the wild type at the stationary phase 10 hours (Fig. 1C). In the presence of 0.2 M KCl, there was a 2-fold reduction of $\Delta proV$ growth (OD_{600nm} 0.4 vs. 0.8) compared to the wild type at the stationary phase (Fig. 1D).

Betaine is a well-known compatible solute that is a preferable osmoprotectant for many organisms.[33] We therefore tested the ability of both wild type and the $\Delta proV$ mutant in utilizing betaine under hyperosmotic conditions. In M9 medium containing 0.2 M
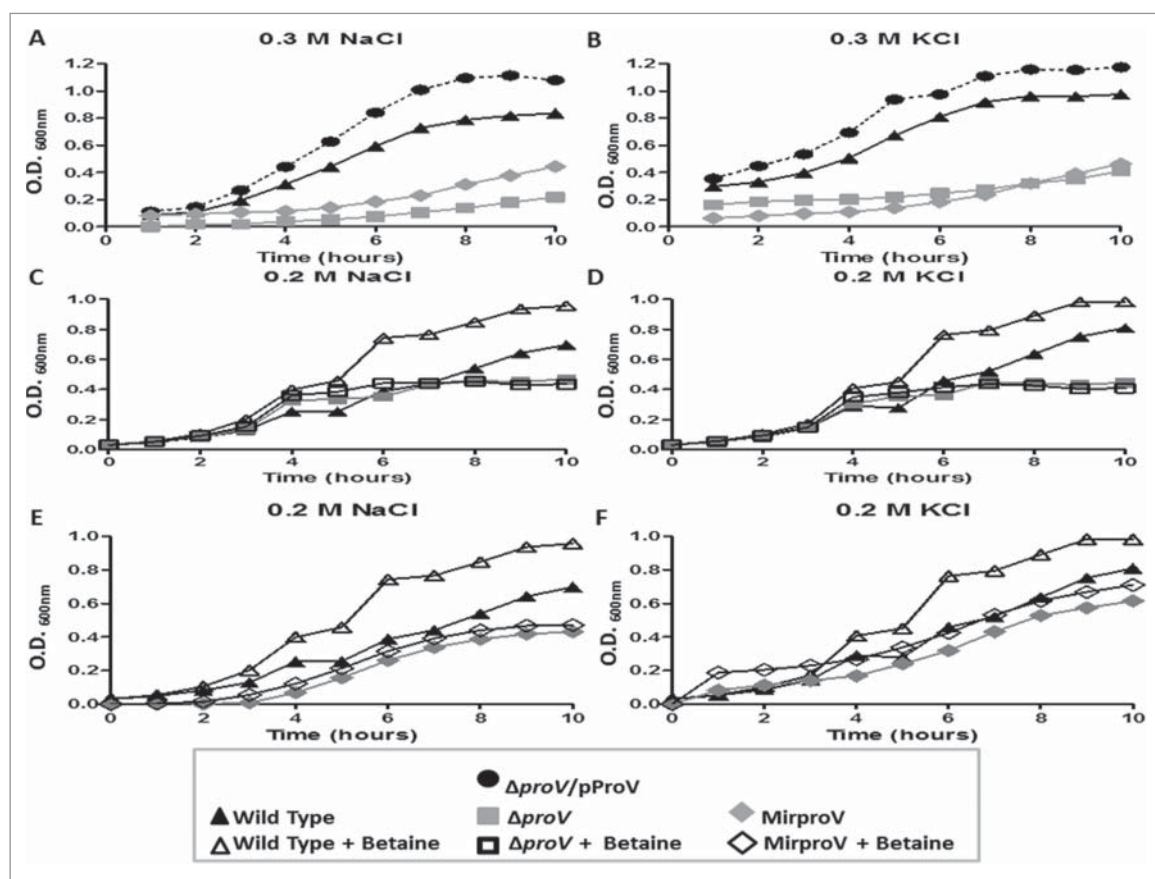
Figure 1. *S. sonnei* growth under various osmotic conditions. Growth curves of wild type (triangle), *proV* mutant (gray square), MirproV (RNAi proV strain) (gray trigonal) and Δ*proV* /pProV strains (circle) in M9 medium supplemented with 0.3 M NaCl (A), 0.3 M KCl (B), 0.2 M NaCl (C, E) and 0.2 M KCl (D, F) in the presence (open symbols) or absence (closed symbols) of 500 μM betaine (C, D, E, F). The results are means of 3 successive wells $\pm$ standard deviation (n $=$ 3).

NaCl or 0.2 M KCl, supplementation of betaine (500 μM) significantly elevated the growth of the wild type (Fig. 1C and 1D, S3). In contrast, the growth suppression of the Δ*proV* mutant persisted in the presence of 500 μM betaine (Fig. 1C, 1D, S3). Taken together, these data demonstrated that a functional ProU system is required to transport betaine for *S. sonnei*, and that betaine can correct the growth defects of the wild type induced by high concentrations of NaCl and KCl. In contrast, the growth of the Δ*proV* mutant remained severely impaired under high osmotic stress, and could not be corrected by addition of betaine in the medium (Fig. 1C, 1D).

proV *paralogues are able to compensate the loss of* proV

As shown in Fig. 1C and 1D, the Δ*proV* strain grew equally well as the wild type strain in the first 6 hours albeit its growth was ceased thereafter. This result suggested that the ProU system was functional at least in the first 6 hours when *proV* is removed. Given the fact that

proV encodes an ATPase and all ATPase of the ABC superfamily share highly conserved sequence and structure,[34] we reasoned that ATPase from other transporting systems may compensate for the loss of *proV*. Using ProV as query sequence we identified putative 25 ATPases (Table S3), belonging to other transport systems in the *S. sonnei* SSO46 genome (http://www.mgc.ac.cn/ShiBASE/Search.htm). We cloned 3 of them in random, *oppF, glnQ* and *malK*, which are responsible for transporting oligopeptides, glutamate, and maltose, respectively. Over-expressing each of them was able to complement the Δ*proV* strain for better growth in LB broth supplemented with 0.2 M NaCl although OppF appeared less effective than GlnQ and MalK (Fig. S5). These data support our hypothesis that removal of *proV* only rendered ProU system partially inactive. Since the *proV::Kan* intermediate strain used for producing Δ*proV* strain potentially had polar effect on expression of *proWX*, we performed complementation test for growth in L-broth. As seen in Fig. 1A and 1B, expression of *proV in trans* fully complemented the Δ*proV* strain but not the *proV::Kan* strain for growth in L-broth (Fig. S7).

Thus, ProU system alone is required for *S. sonnei* growth under the hyper osmotic condition used. To fully assess the ProU function and regulation, it was necessary to delete the whole operon. However, despite repeated efforts we were unable to obtain a Δ*proVWX, or* Δ*proX* or Δ*proW* strain with intact virulence plasmid, which would allow formation of small and smooth red colonies on Congo red agar. All strains harboring these deletions formed large pale and rough colonies (data not shown). Thus, *S. sonnei* must rearrange its genome when completely loosing ProU. Hence, we searched for an alternative approach for knockdown of the ProU system.

Silencing the proVWX *operon using RNAi*

Tchurikov and his colleagues[35] described an RNAi methodology to knockdown gene expression in *E. coli*. According to their work [35] Mirlon (which expresses the inverted sequence of the gene to be knocked down, *lon*), is the most potent in silencing the target genes. We adopted described Mirlon approach to knockdown ProU system in *S. sonnei*, hence termed MirproV RNA.[35] We tested the impact of MirproV on the expression of *proV* and *proX*, which are the first and last genes, respectively, in the ProU operon, by qRT-PCR using primers (k and l; m and n respectively, Table S1). The house-keeping gene, *cysG*, was used as an internal control using primers (o and p, Table S1).[36] Figure 2A shows the results of qRT-PCR after normalization with transcripts of *cysG* and using transcripts from wild type strain as calibrator. The transcripts of both *proV* and *proX* were significantly reduced as a result of MirproV RNA expression, which indicated that the RNAi construct in MirproV was successful in ProU attenuation.

Similar to the *proV* mutant, the MirproV strain grew as well as the wild type in M9 without supplements (Fig. S2) but became intolerant to hyperosmolarity; its growth was severely compromised in M9 medium containing either 0.3 M NaCl or 0.3 M KCl (Fig. 1A, 1B). In M9 supplemented with 0.2 M NaCl, the MirproV strain had a long lag phase of 4 hours (Fig. 1E). This was in contrast to the Δ*proV* strain, which grew equally well as the wild type strain during this phase of growth under this condition (Fig. 1C). Thus, MirproV was more effective in inactivating the ProU system. Moreover, addition of betaine to M9 medium containing 0.2 M NaCl or 0.2 M KCl failed to rescue growth of the MirproV strain (Fig. 1E, 1F, S3). Thus, expression of MirproV is effective in blocking ProU function and betaine transport.

By linear regression analysis, the significance of inhibition of wild type, *proV* and MirproV mutants growth by 0.2 M NaCl and 0.2 M KCl supplements was identified (Fig. S3). Furthermore, paired *t-test* was performed to compare optical density (OD_{600nm}) at the stationary for wild type, Δ*proV* and MirproV in presence and absence of betaine (Fig. S4). The results showed that betaine supplementation significantly increased the optical density for wild type but not for either mutant strains (Fig. S4).

To gain insight into the transcriptional regulation of ProU and its inactivation by MirproV, we isolated total RNA from wild type and the MirproV strain from M9 cultures containing either 0.3M NaCl or 0.3M KCl and performed qRT-PCR for transcripts of *proV* and *proX*. Again the house-keeping gene *cysG* was used as an internal control for normalization. As expected, the wild type responded to the osmotic stress of NaCl or KCl by massive upregulation of *proV* and *proX* transcripts. The MirproV strain also responded, but at a significantly lower level (Fig. 2B). These data demonstrated that the ProU system was inducible under high osmotic stress and that MirproV RNA effectively knocked down ProU transcripts which in turn rendered *S. sonnei* intolerant to hyperosmolarity.

Inactivation of ProU does not disrupt the type 3 secretion system (TTSS)

Shigella strains possess a type III secretion system (TTSS), which is essential for cell-invasion, phagosome escape and intracellular replication.[37] Therefore, we ought to separate the roles of TTSS and ProU with regard to cell invasion and intracellular growth. We first confirmed that all the mutant strains contained intact TTSS genes (*ipaB* and *mxiD*) using primers listed in Table S1. Further, the production and secretion of IpaB and IpaC proteins were investigated by Western blotting using Congo red as an environmental cue *in vitro* as described previously.[38] Because it is known that MxiD is required for type III secretion, we constructed a Δ*mxiD* mutant as a negative control for Ipa secretion, using the same approach as for Δ*proV* construction with primers (g and h; Table S1). Similar levels of IpaB and IpaC were detected in the cell lysates and supernatants of the wild type, the Δ*proV*, the MirproV and the complemented Δ*proV*/pProV strains whereas Ipa proteins were detected only in cell lysate but not supernatant of the Δ*mxiD* mutant (Fig. 3). We therefore conclude that removal of *proV* or knockdown of transcripts did not impair IpaB and IpaC production or secretion – TTSS is functional in the Δ*proV* and the MirproV strains.

ProU is required for S. sonnei *intracellular growth (ex vivo)*

Evidence via DNA microarray analysis indicated that the host cell cytoplasm is a hostile environment which exposes bacteria to hyperosmotic stress.[28] We therefore hypothesized that removal of *proV* or reduction of
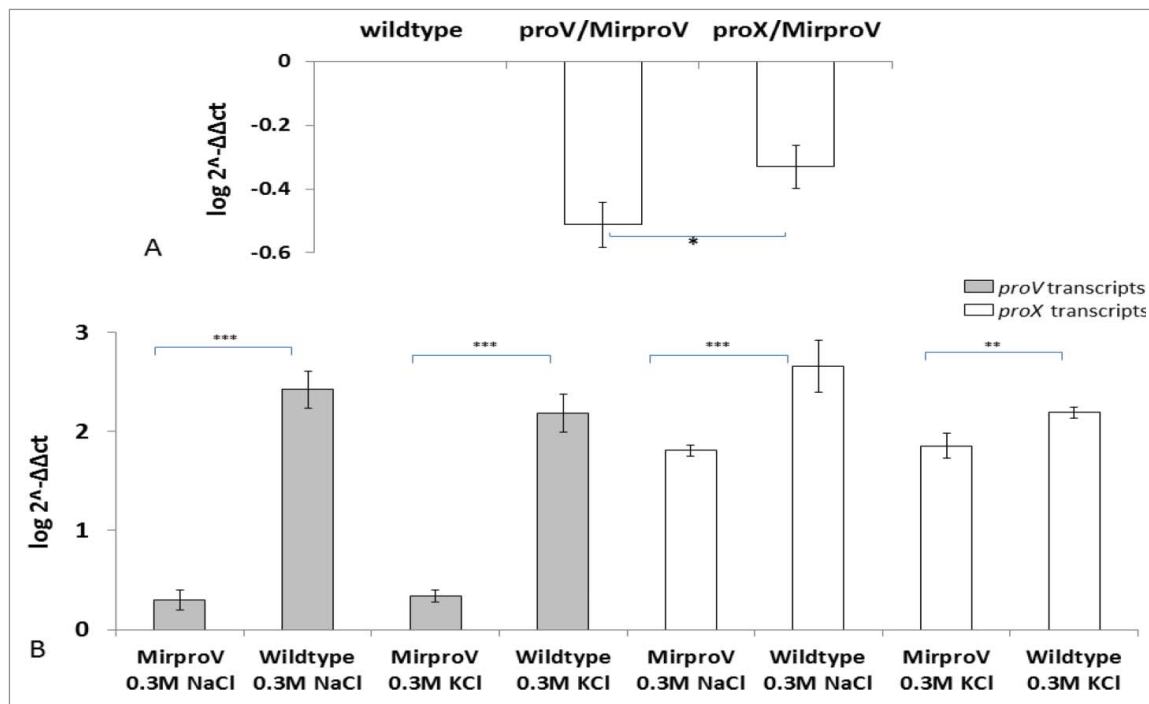
Figure 2. qRT-PCR analysis for both *proV* and *proX* transcripts in both wild type and MirproV. (A) qRT-PCR analysis of wild type and MirproV strains growth in M9 medium without supplements. Levels of *proV* and *proX* transcripts in wild type were set as calibrator (one) and levels of transcripts in MirproV strain were calculated using the $2^{-\Delta\Delta ct}$ method; significant drops of *proV* and *proX* transcripts were observed in MirproV strain. Levels of transcription between *proV* and *proX* also significantly differ in the MirproV strain (*p< 0.05). (B) qRT-PCR analysis for the expression of *proV* (gray columns) and *proX* (open columns) genes in both wild type and MirproV grown in hyperosmotic media. The differences in *proV* transcripts between wild type and MirproV strains in presence of both 0.3 M NaCl and KCl are highly significant (***$p < 0.0001$; gray columns); and the differences in *proX* transcripts between wild type and MirproV in the presence of 0.3 M NaCl or 0.3 M KCl are highly significant (***$p = 0.0007$, and **$p = 0.0025$, respectively). All the results are means of 3 successive groups ± standard deviation (n = 3).

proVWX transcription by RNAi would adversely influence *S. sonnei* intracellular growth. To test our hypothesis, the HEK293 cell line, a good model for testing *Shigella* invasion,[39] was used as a host to perform gentamicin protection assay to test intracellular growth of the wild type, the Δ*proV* and the MirproV strains. As anticipated, we observed a significant drop in intracellular bacterial burden with the Δ*proV* mutant compared to the wild type 2 hours post infection (Fig. 4A). Noticeably,

the MirproV strain had a significantly reduced intracellular bacterial burden, compared to the Δ*proV* mutant. This suggested that suppression of *proVWX* operon was more effective than mutating *proV* gene alone because ProV paralogues may compensate the loss of ProV (Fig. S5). As anticipated, expressing ProV *in trans* restored intracellular growth of the Δ*proV* mutant, demonstrating that the deletion of *proV* had no polar effect on the expression of *proW* and *proX* downstream;
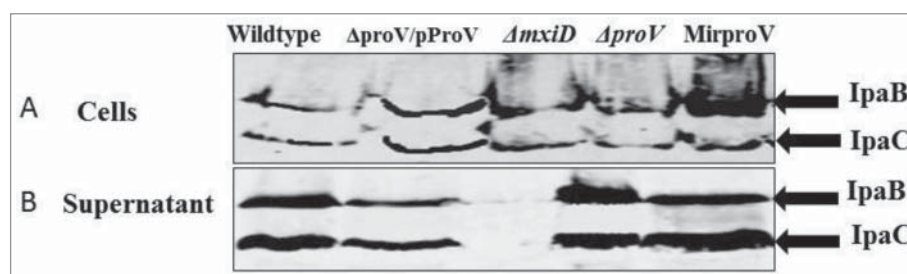

Figure 3. Deletion of *proV* doesn't disturb the function of TTSS. *S. sonnei* wild type, the complemented strain Δ*proV*/PproV, Δ*mxiD*, Δ*proV* and MirproV strains were grown to mid-log phase, and TTSS secretion was induced with Congo red. Total proteins from cell lysates (A) and culture supernatants (B) were separated on SDS-PAGE and IpaB and IpaC were detected with anti-IpB and anti-IpaC antibodies.

removal of *proV* was solely responsible for the defective intracellular growth observed with the Δ*proV* mutant.

To further test if the ProU system is required for intracellular growth not invasion per se, we performed a time course analysis of intracellular growth after 1, 2, 3, and 4 hours using gentamicin protection assay. At each time point, cells were lysed with Triton X-100, and cell-lysates were plated out on LB agar, and incubated at 37°C overnight and enumerated by colony count. The wild type grew rapidly with a doubling time of approximately 38 min from 1 hour onwards whereas the Δ*proV* and the MirproV strains had doubling times of 60 and 90 min, respectively (Fig. 4B, Fig S7).

To gather further evidence for ProU requirement for intracellular growth we exploited flow cytometry. We transformed all strains with a plasmid that expressed EGFP, and infected HEK293 cells with these green bacteria. As shown in Fig. 4C, 38% of the host cells infected with wild type strain emitted green fluorescence 4 hours post infection, indicating that green bacteria were metabolically active inside host cells at this time point. Noticeably, some cells had very strong EGFP signals (fluorescence intensity above 10^2 units), demonstrating that these cells harbored large numbers of green bacteria as a result of rapid bacterial intracellular growth. In contrast to cells infected with wild type, there were less than 4% of cells infected with the Δ*proV* mutant emitted green fluorescence (Fig. 4C), indicating impaired intracellular growth. We left cell infection overnight and repeated flow cytometry and this showed that 43% of cells infected with wild type emitted EGFP signals, (Fig. 4D vs 4C). It was noticeable that a smaller
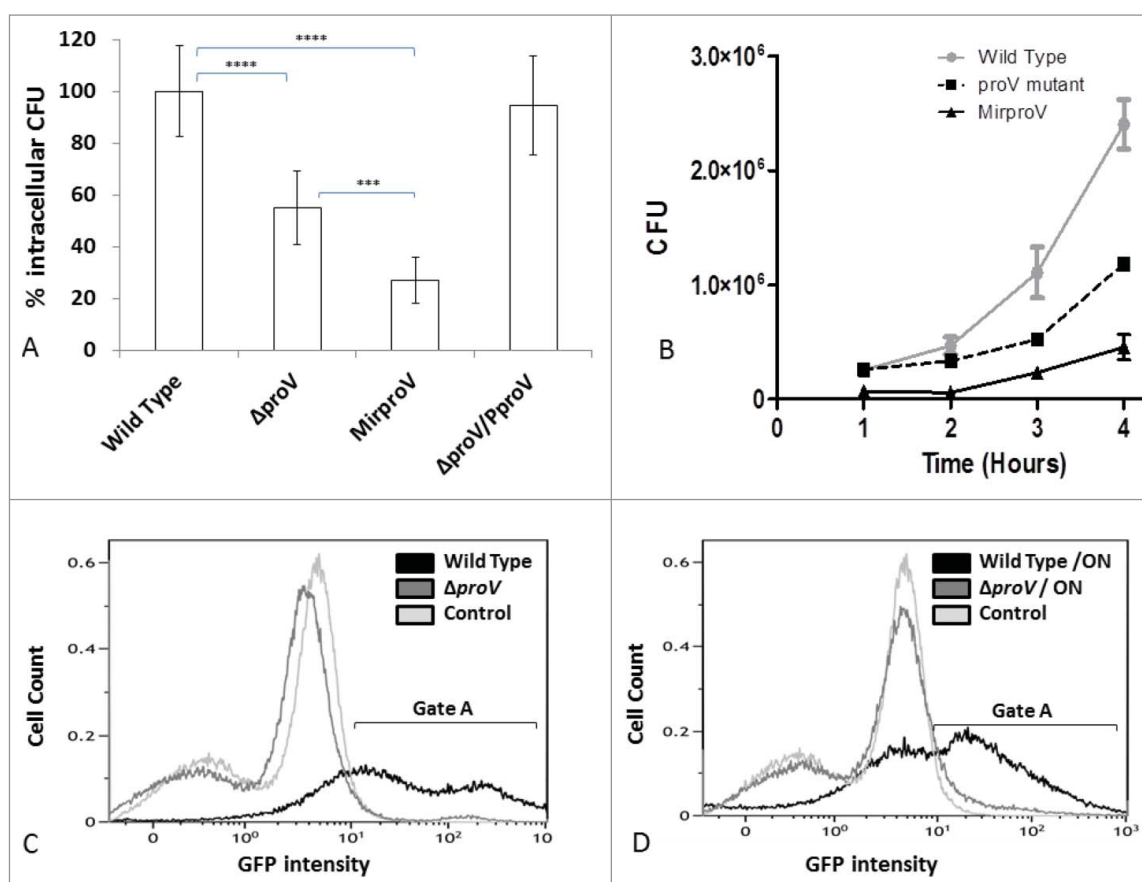

Figure 4. Testing the intracellular growth of *S. sonnei in vitro* using HEK293 cells. (A) Intracellular growth of *S. sonnei* 2 hours post infection (MOI of 10). Intracellular CFU of the wild type were taken as 100%, and intracellular CFU from strains Δ*proV*, MirproV and Δ*proV*/pProV were expressed as percentages to that of wild type. Each value is the mean of 3 independent determinations ± standard deviation. The levels of significance were determined using unpaired *t*-test, Asterisks (****) indicate *p*-values< 0.0001, (***) mean *p*-values = 0.0003. (B) Time course of intracellular growth of *S. sonnei* (MOI of 10). At indicated time interval post infection, cells were lysed and intracellular CFU were determined by plating on agar. Each value is the mean of triplicates ± standard deviation (n = 3). Doubling time for each strain is calculated by linear regression analysis (Fig. S6). (C, D) Overlay of the flow cytometry analysis of cells were infected with Δ*proV* (dark gray), wild type (black) or mock-infected (light gray). Both *S. sonnei* strains were expressing EGFP. Controls are cells mock-infected with saline. Analysis was done 4 hours post infection (C) or overnight (D). Gate A depicts populations of cells emit EGFP signals.
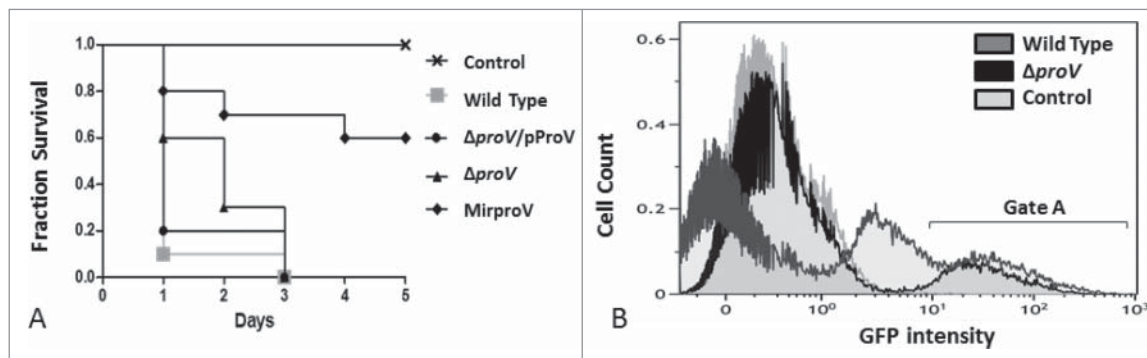
Figure 5. Testing *S. sonnei* virulence *in vivo* using *G. mellonella* larvae. (A) Fraction survival of *G. mellonella* larvae model challenged by 10^5 CFU of wild type *S. sonnei* strain 20071599 (gray square), the complemented strain (Δ*proV*/pProV) (black circle), Δ*proV* (black triangle) and the MirproV (black trigonal), using saline as a control (crosses). The observation lasted for 5 d. The results are means of 3 successive groups (n = 10 larvae). (B) Overlaid histogram of the flow cytometry analysis of hemocytes isolated from *G. mellonella* larvae mock-infected as a control (light gray), challenged by *S. sonnei* wild strain 20071599 (dark gray) or by Δ*proV* (black). Both *S. sonnei* strains were expressing EGFP. Hemocytes were isolated 4 hours post infection for analysis; gate A depicts hemocytes emit GFP signals.

percentage of cells could emit high fluorescent above 10^2 scale compared to that of 4 hours post infection (Fig. 4D vs. 4C). Presumably, host cells harboring large numbers of green bacteria were dead following the overnight infection. Consistent with this was the notion that more cells were emitting low fluorescence (intensity of 10^1 to 10^2 units), suggesting that cells harboring lower bacteria burden survived overnight infection. Noticeably also, there was an increase in green fluorescence in host cells that have been infected with the Δ*proV* mutant overnight, suggesting limited remaining intracellular growth of this strain (Fig. 4D). Taken together, ProU is required for intracellular growth of *S. sonnei* in host cells.

ProU is requried for virulence in the Galleria mellonella *larvae model (in vivo)*

The great moth *G. mellonella* larvae have become a popular *in vivo* model for assessing bacterial virulence.[40,41] We recently adopted this model to assess *Shigella* virulence and found it comparable with the widely accepted Sereny test.[42]

Here we exploited this model to compare the virulence of Δ*proV* and MirproV strains with the wild type *S. sonnei*. Larvae were challenged with 10^5 CFU of each strain; 10 larvae per group. Mock-infection was done using the same volume of saline. All groups of larvae were observed daily for 5 d and dead larvae suffered from melanization and loss of motility. As shown in Fig. 5A, wild type and the complemented strain (Δ*proV*/pProV) were able to kill 90% and 80% of larvae, respectively, in 1 day and the remaining larvae died at the third day. Using the same dose of the Δ*proV* strain, only 40% of larvae died at the first day, and 30% died at the second day while the rest died at the third day. With the MirproV strain, a dose of 10^5 CFU only caused 20% of

death at the first day, 10% at the second day and other 10% at the fourth day, while 60% of the whole population survived to the end of the study (Fig. 5A). These data were consistent with those of Fig. 4 that MirproV was more effective than *proV* deletion in inactivating the ProU system.

Our recent study has demonstrated that infection of larvae hemocytes is an important mechanism for larvae-killing by *S. sonnei*.[42] Therefore, we infected moth larvae with EGFP-expressing strains (Table S2) and isolated hemocytes from infected and mock-infected larvae for flow cytometry. Larvae were infected with 10^6 CFU of each strain, and mock-infection was done using saline. Hemocytes were isolated from larvae 4 hours post infection. We used hemocytes from mock-infected larvae to set gates defining EGFP negative and positive populations (Fig. 5B). It was apparent that wild type strain colonized more hemocytes giving rise to 2 populations of hemocytes with low and high EGFP intensity, whereas the Δ*proV* strain colonized less numbers of hemocytes with one population of high-EGFP expressing hemocytes at this time point.

Discussion

In pathogenic bacteria, osmoregulation is a very important mechanism not only for survival during environmental osmotic stress, but also in establishment of infection. Examples of osmoregulatory systems important for virulence include the *Staphylococcus aureus* PutP,[8-10] *Pseudomonas aeruginosa* PlcH,[11,12] and *Listeria monocytogenes* OpuC.[13,14]

The ProU transport system is widely distributed in bacteria. Previous studies have firmly established its role in environmental survival under hyperosmolarity in *E. coli*, but whether it is required in the context of infection remained unknown.[7] By studying the orthologous

ProXVWZ system in *M. tuberculosis*, Price and his colleagues have demonstrated the importance of this system in osmotolerance *in vitro* and during early host cell colonization.[30] Here, we have presented compelling evidence for the first time that *Shigella* ProU system is required for coping with hyperosmolarity both *in vitro* (Fig. 1) and *ex vivo* (Fig. 4), and for virulence in a moth larvae infection model (Fig. 5). We have also demonstrated that ProU is highly upregulated under hyperosmotic conditions (Fig. 2). This is consistent with the findings for *S. flexneri* by Lucchini and co-workers[28]; *proV* is up-regulated up to 57-fold inside the host cell cytoplasm compared to growth in LB broth. Lucchini suggested that the overall different ionic composition in the host cell cytosol is the cue that triggers this upregulation, because the host cell cytosol should have similar osmolarity as LB broth.[28] Supporting evidence for this hypothesis is the notion that transcription of the *phoRB* regulon and *mgtA* is also upregulated inside host cells compared to their transcription in LB broth.[28] *phoRB* is required for transporting phosphates and *mgtA* is required for transporting Mg^{2+}; their upregulation indicates shortages of phosphates and Mg^{2+} in the host cell cytosol. *M. tuberculosis* colonizes and modifies phagosome by inhibiting acidification.[43] The exact cue for upregulation of the *M. tuberculosis* ProXVWZ system has not been identified so far.[30] Nevertheless, it is clear that both the phagosome and the host cell cytosol impose osmotic stress on invading microbes ([30]and this study). In both these cellular niches, the microbes use the ProU system to transport glycine betaine to survive and multiply. Glycine betaine is an important free cytoplasmic constituent of eukaryotic cells,[44] and it is present at 20 to 60 μM in human serum.[45] Hence, it is not surprising that pathogens like *M. tuberculosis* [46] and *S. sonnei* exploit glycine betaine as an osmoprotectant during growth within the host cells.

ProV encodes an ATPase of the ProU system. Deletion of *proV* caused significantly slow growth in hyperosmotic media, and complementation by expressing ProV *in trans* restored wild type rate of growth (Fig. 1A, 1B). These data reinforce that ProU is an energy dependent transport system, and Δ*proV* is solely responsible for the slow growth phenotype of the deletion mutant. However, the Δ*proV* mutant grew well in early phase in the presence of 0.2 M NaCl and 0.2 M KCl (Fig. 1C, 1D). This result suggested that the ProU system is, at least in part, still functional when *proV* is removed. By overexpression of 3 paralogues of *proV in trans*, we have demonstrated that ATPases from other transport systems are able to compensate for the loss of ProV for better growth (Fig. S5). According to complete genome sequence, *S. sonnei* has intact ProP, a low affinity transporter for proline and glycine betaine. But, the function of ProP

appeared insignificant because the growth defect of Δ*proV* strains cannot be reversed by supplementing betaine (Fig. 1C, 1D). Furthermore, *S. sonnei* also has K^+ specific transporters, Trk, Kdp, and Kup, according to complete genome sequence. The functions of these systems appeared also insignificant because Δ*proV* strain had impaired growth in M9 medium, which contained K^+ (Fig. 1).

The MirproV strain does not significantly respond to betaine supplementation in M9 medium with 0.2 M NaCl or 0.2 M KCl for better growth (Fig. 1E, 1F). These data suggest that MirproV could effectively attenuate ProU system. Even though the MirproV strain produced more transcripts of *proV* and *proX* in response to 0.3 M NaCl and 0.3 M KCl (Fig. 2B) compared to its transcripts in M9 medium without any supplements (Fig. 2A), these levels of responses were not sufficient to reverse the growth defect (Fig. 1E, 1F; S3, S4). The effectiveness of MirproV was also demonstrated in gentamicin protection assay; the MirproV strain was more severely defective than the Δ*proV* mutant in the intracellular growth (Fig. 4A, 4B). Moreover, the possibility of 'off-target' effects by MirproV is low; there are only 4 low score hits when the 42 bp *proV* sequence is used as query to blast the *S. sonnei* SSO46 genome (Table S4). Noticeably, *mxiJ* is one of the hits; *mxiJ* encodes an essential component of TTSS.[47] However, like the Δ*proV* strain, the MirproV strain produces and secretes Ipa proteins (Fig. 3), suggesting that any 'off-target' hits, if they occur, do not significantly change the strain's osmotolerance or virulence. However, we cannot categorically rule out a mild reduction of the MirproV strain in invasion as noted in Fig. 4B; the MirproV strain had reduced intracellular CFU at first hour postinfection.

Despite the fact that we were able to delete *proV* from a *proV::Kan* intermediate, which had completely inactivated ProU as expression of ProV *in trans* did not correct its impaired growth defect (Fig. S7). This *proV::Kan* strain formed smooth red colonies on Congo red agar, indicating it harbored intact virulence plasmid. However, it was a surprise that we were unable to obtain a strain with intact virulence plasmid when either the whole *proVWX* operon, or single gene (*proW* or *proX*) was removed. Previous studies have shown that *S. sonnei* could frequently become avirulent by losing its 120-megadalton virulence plasmid.[48] Besides, it is well known that both ProU and the TTSS genes are controlled by the same negative regulator: the H-NS protein and this regulatory link provided an additional rational for monitoring the possibility of altered TTSS expression upon ProU attenuation.[49,50] So, it was very important to test the stability of this plasmid in wild type and the mutant strains under investigation (Δ*proV*, MirproV and the complemented Δ*proV*/pProV). Our data show all the strains

possess key plasmid borne virulence genes, *ipaB* and *mxiD*. Furthermore, all strains produce and secrete IpaB and IpaC proteins, except Δ*mxiD* strain that produces but does not secrete Ipa proteins (Fig. 3). Hence, we can conclude that inactivation of the ProU system by either deletion of *proV* or RNAi approach does not impair genetic stability and function of the virulence plasmid.

Finally, we have once again demonstrated the usefulness of the moth larvae model in assessing bacterial virulence, which enabled us to establish a link between osmotolerance and virulence.

Conclusions

Altogether, we conclude that the ProU system is important for *S. sonnei* to tolerate hyperosmotic stress *in vitro*, as well as for survival and proliferation of the bacteria in the stressful intracellular niche. Silencing of the whole *proVWX* operon is more effective than deleting *proV* alone because *proV* paralogues may compensate for the loss of *proV*. Last but not least, the *G. mellonella* larvae model is a cost-effective and good model for studying *Shigella* virulence, and reflects results of more established models such as the Sereny test.

Materials and methods

Bacterial strains and growth conditions

The wild type *Shigella* strain used in this study was *S. sonnei* strain 20071599[31] and mutants thereof. Bacteria were routinely grown on Congo red TSA plates or in LB broth at 37°C. To obtain EGFP expressing bacteria, strains were transformed with pGEMT-Easy (Amp^R) containing EGFP. Afterwards, 100 μg/ml ampicillin was added for selection of plasmid containing strains. All primers and strains used in this study are listed in Tables S1 and S2 respectively.

Genetic engineering

Construction of the mutant bearing in-frame deletion in *proV* gene was done using phage λ Red recombination system.[32] Plasmid, pKD46, which carries the red lambda recombinase genes, was first transformed to strain 20071599. Primers (a and b; Table S1) were used to amplify kanamycin cassette using pKD4 plasmid as a template; the PCR product, Kan-cassette, contained the first and last 51 base pairs of *proV* coding sequences at the 5′- and 3′-ends, respectively. Introduction of the Kan-cassette into 20071599/pKD46 enabled homologous recombination; the wild type *proV* gene was replaced with the kan-cassette. Plasmid, pCP20, was then

transformed to the *proV*-Kan mutant for second crossover to loop out the Kan-cassette, leaving an in-frame deletion scar (102 bp) through the gene. EGFP was cloned using pGEM-T-Easy which was used to express EGFP in wild type and Δ*proV* strains. Entire *proV* coding sequence was PCR-amplified from the wild type *S. sonnei* using primers c and d (Table S1) and cloned into pGEM-T-Easy (ampR), with the 5′-end of the *proV* coding sequence facing the *lacZ* promoter. The resultant clone was used to complement the Δ*proV* mutant.

For RNAi experiments, 2 oligonucleotides were synthesized, which were complementary to each other and 87 bases in length each, they span 45 and 42 base pairs of the promoter and 5′-end of *proV* coding sequence, respectively (Table S1). The two oligonucleotides were adjusted to the concentration of 100 pmol each, mixed together and heated to 65°C (Tm = 71°C) in a heating block. The heating block was allowed to cool down to 37°C; then incubated at 37°C overnight. The double stranded DNA was cloned into pGEM®-T-Easy Vector and transformed into *E. coli* DH5α. The resultant clones were analyzed using restriction enzyme SspI and by DNA sequencing to ensure the *proV* coding sequence 3′-end facing the *lacZ* promoter, which produced RNA mirrored that of *proV* gene sense strand (hence called MirproV).

Western blot

Smooth red colonies of *S. sonnei* tested strains were picked from fresh overnight Congo red TSA plates and were grown into TSB to an OD_{600nm} of 0.7. Bacteria were spun down and pellets were washed once with sterile PBS, and resuspended in 500 μl PBS with 0.01 % Congo red. The bacterial suspensions were incubated at 37°C for 30 min. Cells were again spun down and supernatant was transferred to a fresh micro-tube. The pellets were washed once with PBS then 100 μl of sample buffer was added and boiled at 95°C for 5 min. Total proteins in the supernatant were precipitated using 10% TCA and samples were incubated on ice for 20 min before spinning the protein down at 20000 xg for 20 min at 4°C. The protein pellets were washed with 70% ethanol before re-suspension in 10 μl sample buffer and boiling at 95°C for 5 min[51]. Both IpaB and IpaC were detected using the monoclonal antibodies H16 (anti-IpaB) and J22 (anti-IpaC), respectively,[51] followed by incubation with Alexa Fluor 680 goat anti-mouse IgG (H+L), and the images were visualized using a UV scanner at 700 nm.

In vitro osmotolerance test

M9 medium was prepared, autoclaved and supplemented with filtered 200 μl/100ml of 1 M MgSO₄, 0.4g% glucose,

12.5 μg/ml of Nicotinic acid, 45 μg/ml of L-methionine, and 20 μg/ml of L-Tryptophan. Routinely, all strains were grown on M9 agar overnight, smooth colonies were picked and grown in M9 broth 2 hours before the start of the experiment. Then, 96-well plates were set for growth curve; each well contained 200 μl M9 medium with or without supplements: 0.2 and 0.3 M NaCl or 0.2 and 0.3 M KCl in presence and absence of 500 μM betaine. Each culture condition was tested in triplicates for each bacterial strain, and 3 independent experiments were carried out. Afterwards, 10^7 CFU from each of the tested strains were added to the 96-well plates. Wells without bacterial inocula were used as a blank, while wells without supplements were used to study the growth curve of *Shigella* strains in M9 medium. The plates were incubated in a shaker incubator at 37°C, and the OD_{600nm} was recorded each 1 hour.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Wild type and MirproV strains were grown in M9 minimal medium to mid-log phase. Total RNA was isolated using RNA isolation kit (Bioline). The house-keeping gene, *cycG*, was used as an internal control.[36] To establish standard curves for each gene primers k and l; m and n and o and p (Table S1) were used to amplify *proV, proX* and *cycG* genes, respectively, and serial dilutions (from 100 to 10^8 molecules/μl) of the genome DNA was used as templates using SYBR-Green qRT-PCR kit on Rotor Gene 6000 (Qiagen). Triplicate RNA samples from triplicate cultures (n = 3) were used to prepare cDNAs, which were quantified by the same PCR procedure. The amplification curves of *proV* and *proX* were normalized with that of *cycG*, and quantification was calculated using the standard curves. Changes in gene expression between wild type (set as calibrator) and the MirproV strains were calculated using the $2^{-\Delta\Delta ct}$ method and proprietary software in the Rotor Gene instrument (version 1.7.34).[52] To analyze the impact of high osmotic stress on ProU expression both wild type and MirproV strains were grown in M9 supplemented with 0.3 M NaCl or 0.3 M KCl. The levels of transcripts from wild type and MirproV grown in M9 without salt supplements were used as calibrators. The transcripts of house-keeping gene, *cycG*, were again used for normalization to calculate the $2^{-\Delta\Delta ct}$ [52].

Gentamicin protection assay

HEK293 (human embryonic kidney stem) cells were seeded and cultured until approximately 80% confluence in 24-well plates and *S. sonnei* bacteria were added to the cell monolayers at a multiplicity of infection (MOI) of 10. The plates were centrifuged at 700 xg for 10 min at 22°C. The plates were incubated for 40 min at 37°C under 5% CO_2 atmosphere to allow bacterial invasion into host cells. Thereafter, cell monolayers were washed twice with PBS, DMEM containing gentamicin (50 μg/ml) was added, and the plates were incubated for required time intervals before terminating infection. Cells were washed 3 times using PBS and lysed with Triton X-100 (0.1 % in H_2O) for 10 min. Cell lysates were serially diluted, plated on LB agar, incubated at 37°C overnight and enumerated by colony count.

Infection of Galleria mellonella *larvae*

Smooth red colonies of *S. sonnei* strains were selected from Congo red TSA plates, grown for 3 to 5 hours in LB broth to prepare bacterial suspensions. Each group of 10 healthy larvae of approximately similar size were injected with 10 μl (10^5 CFU) of each bacterial suspension. The mock-infection group received sterile PBS instead of bacteria. All larvae were incubated at 37°C[53] and observed for 5 d post infection.[42] The experiments have been repeated 3 times for results confirmation and the averages have been used in Kaplan-Meier survival curves.

Flow cytometry

For HEK293 cells, infection was terminated at appropriate time intervals (either 4 hours or overnight); cells were washed with saline twice and then trypsinized for 1 min after which the DMEM medium was added. Cells were spun down and cell pellets were resuspended in saline containing 4% (v/v) paraformaldehyde for fixation.

For the wax moth larvae, 4 hours post-infection with 10^6 CFU bacteria, hemocytes from 10 larvae were collected by incision between 2 segments near larvae tail to avoid gut interruption. Hemocytes were collected in 1 ml of sterile PBS and the processing was within 10 min to prevent clotting.[53] Cells were centrifuged at 500 xg for 10 min at room temperature, and were resuspended in 1 ml PBS containing 4% (v/v) paraformaldehyde for fixation.

Cells were vigorously mixed and then were used for the flow-cytometry. Lasers emitting at 488 nm was set for the detecting signals in FITC channel, which overlap with EGFP excitation. Data acquisition was performed using Kaluza™ software (Beckman Coulter, Inc.). The experiments have been repeated 3 times for results confirmation.

Statistics

The growth curves in Fig. 1 are x/y blotting using triplicates for each reading and the error bars are for SD. In Fig 2, unpaired *t test* has been used to compare the expression of different genes using $2^{-\Delta\Delta ct}$ method. In Fig 4, unpaired *t test* has been used to compare the % intracellular CFU of different *Shigella* strains; the growth curve of intracellular growth of *Shigella* strains is x/y blotting using triplicates for each reading and the error bars are for SD.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Author contributions

Conceived and designed experiments: JY
Performed the experiments: RYM WL
Analyzed the data: RYM JY
Contributed reagents/materials/analysis tools: JY ME ERA
Wrote the paper: RYM JY.

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