The *Shigella* ProU System is Required for Osmotic Tolerance and Virulence

Running title: *Shigella* ProU in osmotic tolerance and virulence

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Abstract

To cope with hyperosmotic stress encountered in the environments and in the host, the 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 a *Galleria mellonella* moth larvae model. In response to osmotic stress by either NaCl or KCl, *S. sonnei* highly up-regulates transcription of *pro* 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 *pro* genes 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 *ex vivo*, and for infection.

Keywords: *Shigella sonnei*, ProU, RNAi, osmotic tolerance, osmoprotectants

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 four 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 year 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 to protect themselves against turgor; 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 methyl-amines (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 colonization in the pathogen in *Listeria monocytogenes* [13, 14]. In *Salmonella enteric* 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 three 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 [28], analyzed genomic expression of *S. flexneri* during infection by DNA microarray; *pro* genes were found highly up-regulated in both epithelial HeLa and macrophage-like U937 cells; in particular, *proV* was up-regulated by 57-fold in U937 cells

[28]. Besides, it was 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 up-regulated 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], which involved two steps. In the first step, a Kanamycin cassette replaced the wild type *proV* gene via homologous recombination. In the second step, the Kanamycin gene was looped out using pCP20 plasmid, leaving a scar of 102 bp (Fig. S1A). For complementation of the resultant Δ*proV* mutant strain, the entire *proV* coding sequence was amplified with primers (c & 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 transformed into the Δ*proV* mutant (Fig.S1B). All *proV* constructs for mutagenesis as well as complementation were confirmed by PCR and agarose gel electrophoresis. All PCR products were further confirmed by DNA sequencing using primers c & 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 in vitro*. We first measured growth of the wild type, Δ*proV* and the complemented *ΔproV/*pProV strains in M9 medium supplemented with 0.3 M NaCl or 0.3 M KCl by measuring the optimal density (OD_{600nm}) each one hour till the late stationary phase (10 hours). While the wild type *S. sonnei* grew well, the *ΔproV* strain struggled to grow in the presence of 0.3 M of NaCl or KCl (Fig. 1A and 1B). Obviously, 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 *ΔproV* 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 glutamine or K^+, available in the medium to facilitate bacterial growth (Fig. 1A and 1B). We further tested the growth of wild type and the Δ*proV* strains in M9 medium supplemented with 0.2 M NaCl or 0.2 M KCl. Under these milder conditions, the *ΔproV* strain grew equally well as the wild type strain up to 6 hours. However, the growth of the Δ*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 Δ*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 *ΔproV* 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 *ΔproV* mutant in utilizing betaine under hyperosmotic conditions. In M9 medium containing 0.2 M NaCl or 0.2 M KCl, supplementation of betaine (500 µM) significantly elevated the growth of the wild type (Fig. 1C & 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 defect of the wild type induced by high concentrations of NaCl and KCl. In contrast, the growth of the *ΔproV* 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 *ΔproV* 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 (39), 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 three 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 *ΔproV* 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, and prompted us to construct mutants with a

deletion of the whole *proVWX* operon or in *proX* or *proW*. However, despite repeated efforts we were unable to obtain a *ΔproVWX, or ΔproX* or *ΔproW* 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). This suggested that ProU is rather important to maintain the genome stability due to its role in transporting important osmoprotectants or other yet unraveled functions. *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 [34] described an RNAi methodology to knockdown gene expression in *E. coli*. According to their work [34] Mirlon (which has the inverted sequence of the gene of interest), is the most potent in silencing the target genes compared to two other constructs (Paralon and Antilon). We adopted described Mirlon approach to knockdown ProU system in *S. sonnei*. We created a piece of dsDNA, by annealing two oligonucleotides of 87 bases each, which spand 45 and 42 base pairs of the promoter and 5'-end of *proV* coding sequence, respectively (Table S1). This piece of dsDNA was cloned into pGEM-T-Easy (ampR), with the 3'-end of the *proV* coding sequence facing the *lacZ* promoter, which would drive the transcription of RNA molecule with inverted sequences to the *proV* mRNA, hence termed MirproV RNA [34]. 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 & l; m & n respectively, Table S1). The house-keeping gene, *cysG*, was used as an internal control using primers (o & p, Table S1) [35]. Figure 2A shows the results of qRT-PCR after normalisation 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 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 with 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 normalisation. 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 three secretion system (TTSS)

Shigella strains possess a type III secretion system (TTSS), which is essential for cell-invasion, phagosome escape and intracellular replication [36]. Therefore, we ought to separate the roles of TTSS and ProU with regard to cell invasion and intracellular growth. We first wanted to confirm that the mutant strains that we were going to test for virulence maintained an intact TTSS. The presence of TTSS genes in the *ΔproV* strain was analyzed by PCR using primers e & f to amplify *ipaB* and primers i & j to amplify *mxiD* (Table S1); both *ipaB* and *mxiD* were found intact in wild type and the mutant strain (Fig. S6). 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 [37]. Because it is known that MxiD is required for type III secretion, we constructed a *ΔmxiD* mutant as a negative control for Ipa secretion, using the same approach as for Δ*proV* construction with primers (g & h; Table S1). Similar levels of IpaB and IpaC were detected in the cell lysates and supernatants of the wild type, the *ΔproV*, the MirproV and the complemented *ΔproV/*pProV strains whereas Ipa proteins were detected only in cell lysate but not supernatant of the *ΔmxiD* 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 *ΔproV* 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 *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 [38], was used as a host to perform gentamicin protection assay to test intracellular growth of the wild type, the *ΔproV* and the MirproV strains. As anticipated, we observed a significant drop in intracellular bacterial burden with the *ΔproV* 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; 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 ^{o}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 *ΔproV* and the MirproV strains had a doubling time 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 Figure 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 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 *ΔproV* 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 [39, 40]. We recently adopted this model to assess *Shigella* virulence and found it comparable with the widely accepted Sereny test [41].

Here we exploited this model to compare the virulence of *ΔproV* 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 five days and dead larvae suffered from melanization and loss of motility. As shown in Figure 5A, wild type and the complemented strain (*ΔproV/*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 *ΔproV* 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 Figure 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* [41]. 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 with 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 two 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], the *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

303 importance of this system in osmotolerance *in vitro* and during early host cell colonization [30].
304 Here, we have presented compelling evidence for the first time that the *Shigella* ProU system
305 is required for coping with hyperosmolarity both *in vitro* (Fig. 1) and *ex vivo* (Fig. 4), and for
306 virulence in a moth larvae infection model (Fig. 5). We have also demonstrated that ProU is
307 highly up-regulated under hyperosmotic conditions (Fig. 2). This is consistent with the findings
308 for *S. flexneri* by Lucchini and co-workers [28]; *proV* is up-regulated up to 57-fold inside the host
309 cell cytoplasm compared to growth in LB broth. Lucchini suggested that the overall different
310 ionic composition in the host cell cytosol is the cue that triggers this up-regulation, because the
311 host cell cytosol should have similar osmolarity as LB broth [28]. Supporting evidence for this
312 hypothesis is the notion that transcription of the *phoRB* regulon and *mgtA* is also up-regulated
313 inside host cells compared to their transcription in LB broth. *phoRB* is required for transporting
314 phosphates and *mgtA* is required for transporting Mg^{2+}; their up-regulation indicates shortages
315 of phosphates and Mg^{2+} in the host cell cytosol. *M. tuberculosis* colonizes and modifies
316 phagosome by inhibiting acidification [42]. The exact cue for up-regulation of the *M. tuberculosis*
317 ProXVWZ system has not been identified so far [30]. Nevertheless, it is clear that both the
318 phagosome and the host cell cytosol impose osmotic stress on invading microbes ([30] and this
319 study). In both these cellular niches, the microbes use the ProU system to transport glycine
320 betaine to survive and multiply. Glycine betaine is an important free cytoplasmic constituent of
321 eukaryotic cells [43], and it is present at 20 to 60 µM in human serum [44]. Hence, it is not
322 surprising that pathogens like *M. tuberculosis* [45] and *S. sonnei* exploit glycine betaine as an
323 osmoprotectant during growth within the host cells.

324 *ProV* encodes an ATPase of the ProU system. Deletion of *proV* caused significantly slowed
325 growth in hyperosmotic media, and complementation by expressing ProV *in trans* restored
326 wild type rate of growth (Fig. 1A, 1B). These data reinforce that ProU is an energy dependent

transport system, and *ΔproV* is solely responsible for the slow growth phenotype of the deletion mutant. However, the *ΔproV* 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 three 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).

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 [46]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.

Our inability to obtain strains with intact virulence plasmid with deletion of the whole *proVWX* operon, or *proX* or *proW* strongly suggests the ProU system plays an important role in *Shigella* genome stability. Previous studies have shown that *S. sonnei* could frequently become avirulent by losing its 120-megadalton virulence plasmid [47]. 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 exploring the possibility of altered TTSS expression upon ProU attenuation [48, 49]. 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 *ΔproV* strain possesses key plasmid borne virulence genes, *ipaB* and *mxiD*. Furthermore, all strains produce and secrete IpaB and IpaC proteins, except *ΔmxiD* 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 liquid LB at 37 ^{o}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]. Construction of the mutant bearing in-frame deletion in *proV* gene was done using phage λ Red recombination system [50]. Both IpaB and IpaC were detected using the monoclonal antibodies H16 (anti-IpaB) and J22 (anti- IpaC), respectively [50], 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 $MgSO_4$, 4% 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 three independent experiments were carried out. Afterwards, 10^7 CFU from each of the 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 ^oC, and the OD_{600nm} was recorded each 1 hour.

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

399 Wild type and MirproV strains were grown in M9 minimal medium to mid-log phase.
400 Total RNA was isolated using RNA isolation kit (Bioline). The house-keeping gene, *cycG*, was
401 used as an internal control [35]. To establish standard curves for each gene primers k & l; m & n
402 and o & p (Table S1) were used to amplify *proV, proX* and *cycG* genes, respectively, and serial
403 dilutions (from 100 to 10^8 molecules/µl) of the genome DNA was used as templates using
404 SYBR-Green QRT-PCR kit on Rotor Gene 6000 (Qiagen). Triplicate RNA samples from triplicate
405 cultures (n = 3) were used to prepare cDNAs, which were quantified by the same PCR
406 procedure. The amplification curves of *proV* and *proX* were normalized with that of *cycG*, and
407 quantification was calculated using the standard curves. Changes in gene expression between
408 wild type (set as calibrator) and the MirproV strains were calculated using the $2^{-\Delta\Delta ct}$ method
409 and proprietary software in the Rotor Gene instrument (version 1.7.34) [51]. To analyze the
410 impact of high osmotic stress on ProU expression both wild type and MirproV strains were
411 grown in M9 supplemented with 0.3 M NaCl or 0.3 M KCl. The levels of transcripts from wild
412 type and MirproV grown in M9 without salt supplements were used as calibrators. The
413 transcripts of house-keeping gene, *cycG*, were again used for normalization to calculate the 2^{-}
414 $^{\Delta\Delta ct}$ [51].

415 **Gentamicin protection assay**

416 HEK293 (human embryonic kidney stem) cells were seeded and cultured until
417 approximately 80% confluence in 24-well plates and *S. sonnei* bacteria were added to the cell
418 monolayers at a multiplicity of infection (MOI) of 10. The plates were centrifuged at 700 xg for
419 10 min at 22 ^{o}C. The plates were incubated for 40 min at 37 ^{o}C under 5% CO_2 atmosphere to
420 allow bacterial invasion into host cells. Thereafter, cell monolayers were washed twice with
421 PBS, DMEM containing gentamicin (50 µg/ml) was added, and the plates were incubated for
422 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 ^{o}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 ^{o}C [52] and observed for 5 days post infection [41]. The experiments have been repeated three 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 two 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 [52]. 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 three 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.

Competing interests

The Authors declare no conflict of interest.

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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599 **Figures Legends**

600 **Figure 1.** *S. sonnei* growth under various osmotic conditions

601 Growth curves of wild type (triangle), *proV* mutant (square) , MirproV (trigonal) and Δ*proV*
602 /pProV strains (circle) in M9 medium supplemented with 0.3 M NaCl (A), 0.3 M KCl (B), 0.2 M
603 NaCl (C, E) and 0.2 M KCl (D, F) in the presence (open symbols) or absence (closed symbols) of
604 500 µM betaine (C, D, E, F). The results are means of three successive wells ± standard
605 deviation (n=3).

606 **Figure 2.** qRT-PCR analysis for both *proV* and *proX* transcripts in both wild type and MirproV

607 (A) qRT-PCR analysis of wild type and MirproV strains growth in M9 medium without
608 supplement. Levels of *proV* and *proX* transcripts in wild type was set as calibrator (zero) and
609 levels of transcripts in MirproV strain were calculated using the $2^{-\Delta\Delta ct}$ method; a significant
610 drop of *proV* and *proX* transcripts was observed in MirproV strains. Levels of transcription
611 between *proV* and *proX* also significantly differ in the MirproV strain (* $p < 0.05$).

612 (B) qRT-PCR analysis for the expression of *proV* (grey columns) and *proX* (open columns) genes
613 in both wild type and MirproV grown in hyperosmotic media. The difference in *proV* transcripts
614 between wild type and MirproV strains in presence of both 0.3 M NaCl and KCl is highly
615 significant (*** $p < 0.0001$; grey columns); and the difference in *proX* transcripts between wild
616 type and MirproV in the presence of 0.3 M NaCl or 0.3 M KCl are highly significant (*** $p =$
617 0.0007, and ** $p = 0.0025$, respectively).

618 All the results are means of three successive groups ± standard deviation (n=3).

619 **Figure 3.** Deletion of *proV* doesn't disturb the function of TTSS

620 *S. sonnei* wild type, the complemented strain Δ*proV/*PproV, *ΔmxiD*, Δ*proV* and MirproV strains
621 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.

Figure 4. Testing the intracellular growth of the two mutation approaches *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 three independent determinations ± standard deviation. The level of significance was determined using unpaired *t*-test, Asterisks (****) indicate *p*-values < 0.0001, (***) means *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. S7). (C, D) Overlay of the flow cytometry analysis of cells were infected with Δ*proV* (dark grey), wild type (black) or mock-infected (light grey). 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 the two mutation approaches *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 (grey 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 days. The results are means of three 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 grey), challenged by *S. sonnei* wild strain 20071599

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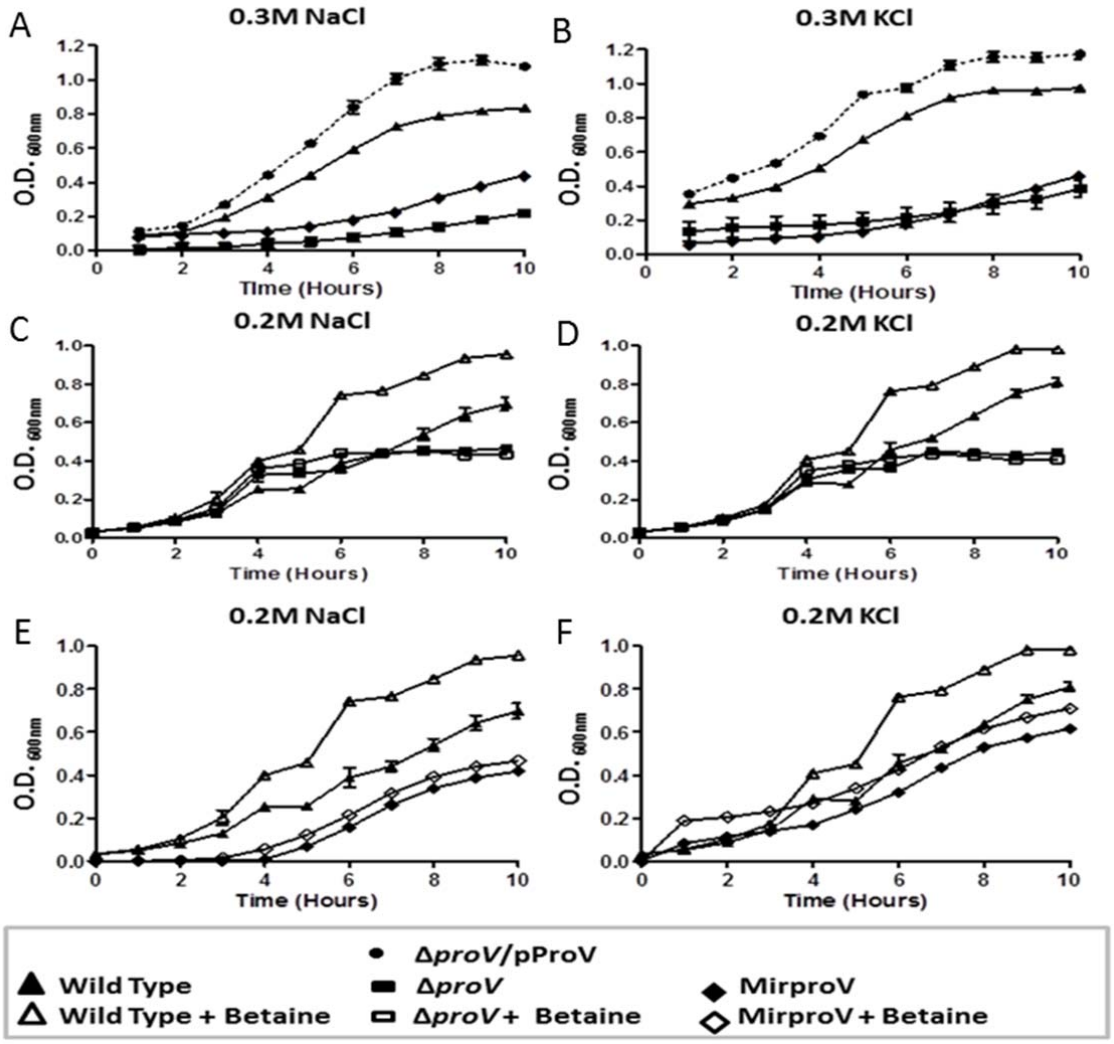

Figure 1. *S. sonnei* growth under various osmotic conditions

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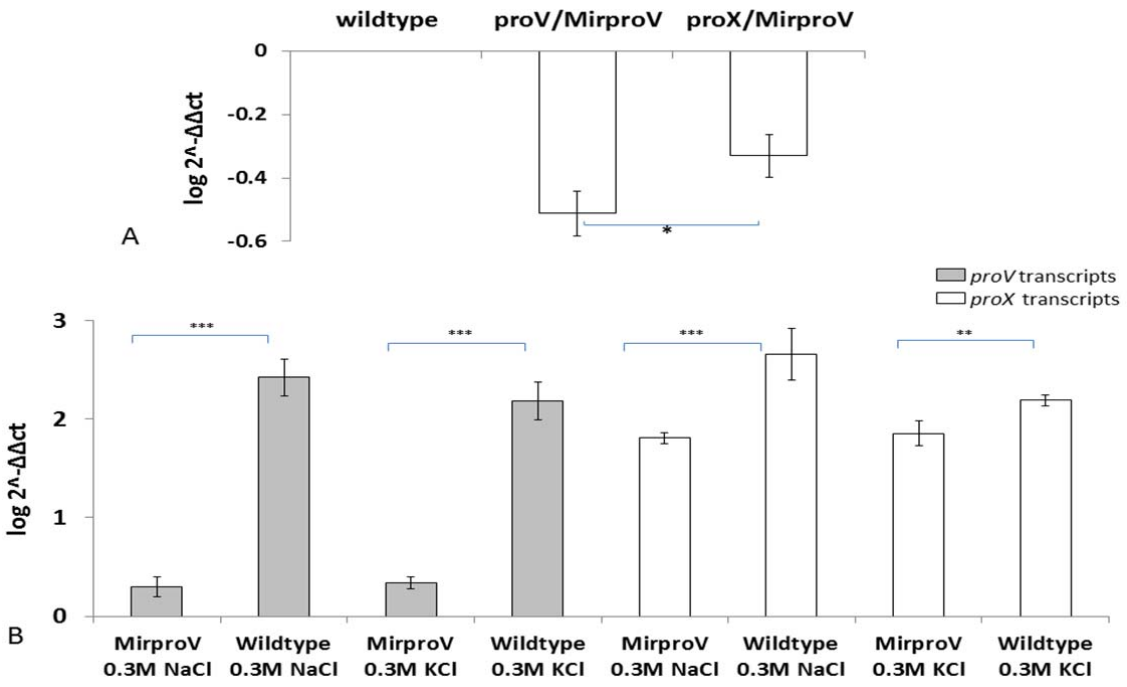

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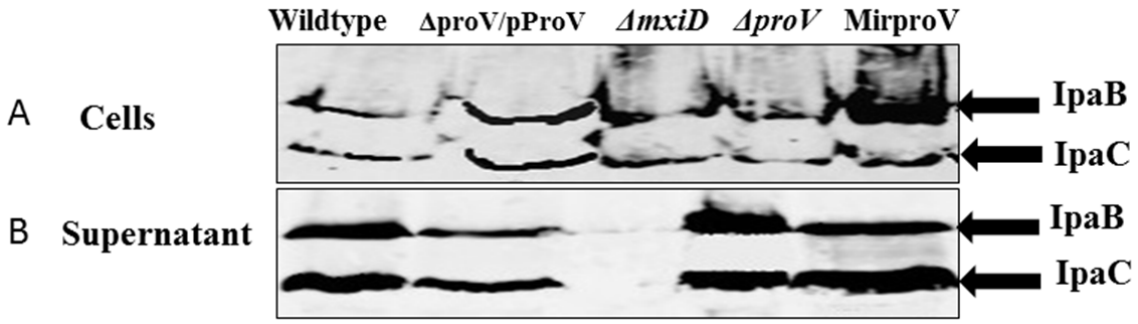

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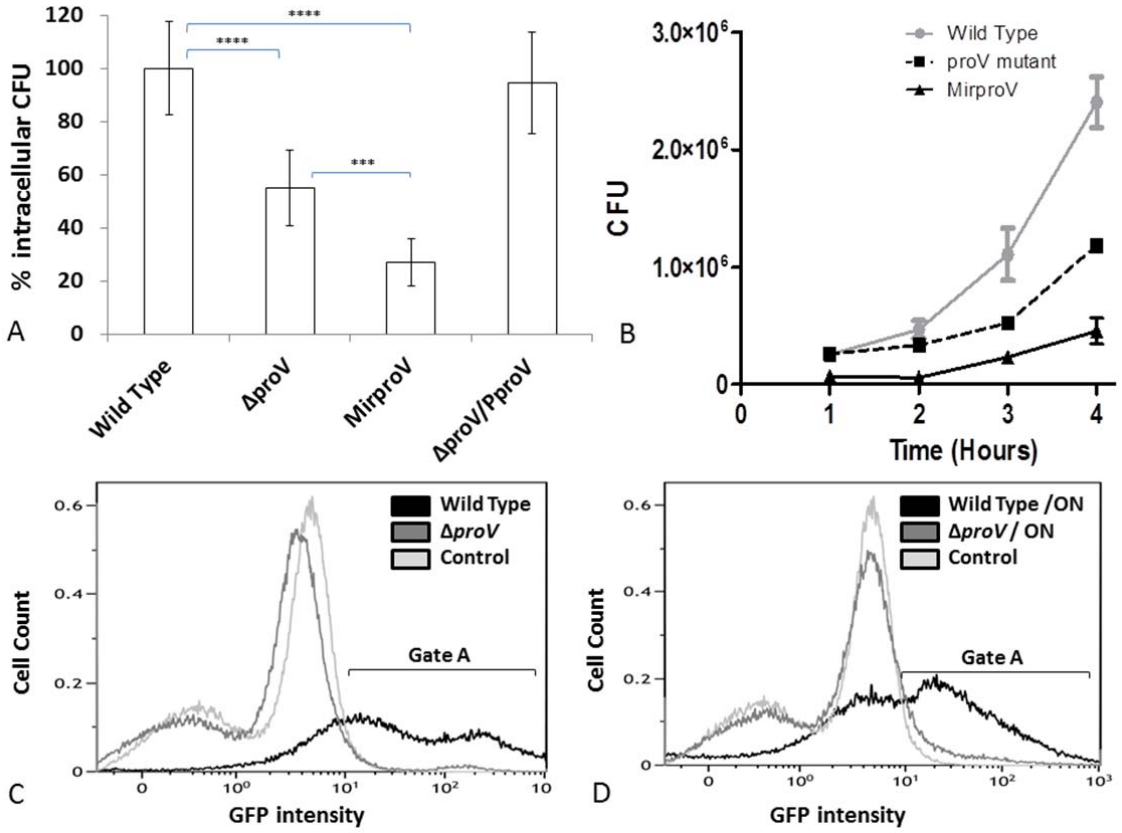

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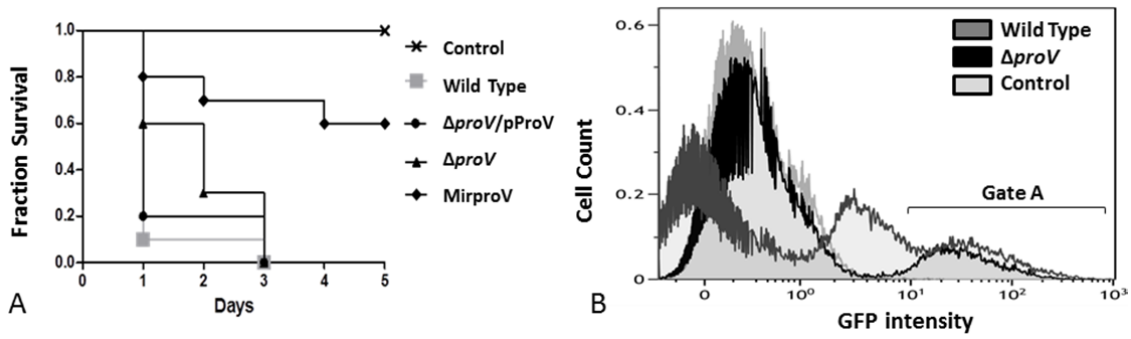

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