Mahmoud, Rasha Y. and Li, Wenqin and Eldomany, Ramadan A. and Emara, Mohamed and Yu, Jun (2016) The Shigella ProU system is required for osmotic tolerance and virulence : Shigella ProU in osmotic tolerance and virulence. Virulence. pp. 1-27. ISSN 2150-5608 (In Press) ,

This version is available at https://strathprints.strath.ac.uk/57462/

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

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

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24 **Abstract**

25   To cope with hyperosmotic stress encountered in the environments and in the host, the

26 pathogenic microbes use diverse transport systems to obtain osmoprotectants. To study the

27 role of *Shigella sonnei* ProU system in response to hyperosmotic stress and virulence, we

28 constructed deletion and complementation strains of *proV* and used an RNAi approach to

29 silence the whole ProU operon. We compared the response between wild type and the

30 mutants to the hyperosmotic pressure *in vitro*, and assessed virulence properties of the

31 mutants using gentamicin protection assay as well as a *Galleria mellonella* moth larvae model*.*

32 In response to osmotic stress by either NaCl or KCl, *S. sonnei* highly up-regulates transcription

33 of *pro* genes. Supplementation of betaine greatly elevates the growth of the wild type *S. sonnei*

34 but not the *proV* mutants in M9 medium containing 0.2 M NaCl or 0.2 M KCl. The *proV* mutants

35 are also defective in intracellular growth compared with the wild type. The moth larvae model

36 of *G. mellonella* shows that either deletion of *proV* gene or knockdown of *pro* genes transcripts

37 by RNAi significantly attenuates virulence. ProU system in *S. sonnei* is required to cope with

38 osmotic stress for survival and multiplication *in vitro* and *ex vivo*, and for infection.

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

40

41 **Introduction**

42   *Shigella* is a facultative intracellular Gram-negative pathogen, known as the etiologic

43 agent of bacillary dysentery since the 1890s. Although *Shigella* was defined a genus with four

44 species *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* in the 1950s [1], it has become clear that

45 they are pathogenic lineages of *Escherichia coli* of multiple origins [2]. The primary transmission

46 is the fecal-oral route, so it is life threatening in developing countries because of poor

47 sanitation. *Shigella* strains are among the most prevalent causative agents of moderate-to-

48 severe diarrhea, and especially affect children under 5 year old in developing countries [3]. The

49 widespread of multiple antibiotic resistant strains, has made *Shigella* treatment increasingly

50 difficult and there is urgent need for vaccine development [4]. *Shigella* is highly invasive to the

51 colon and the rectum and it has the ability to proliferate in the cell cytoplasm and trigger the

52 host pro-inflammatory response. It causes variable clinical manifestations ranging from short

53 term illness, typically watery diarrhea, to a long lasting one manifesting with fever, bloody

54 diarrhea with intestinal cramps and mucopurulent feces [5].

55   Keeping a stable osmotic balance between the cell cytoplasm and the outer environment

56 is an important challenge to all cell types, especially the unicellular organisms. For bacteria, the

57 high surface area to total volume ratios makes them vulnerable when they encounter osmotic

58 stress; bacteria could tolerate the osmolarity changes in the environment through either

59 solutes efflux or water movement across the cytoplasmic membrane [6]. The external osmolarity

60 changes are translated by the microorganisms to an adaptation process to protect themselves

61 against turgor; this happens by a rapid K^+ ion influx through specific transporters, and at the

62 same time, microorganisms produce counter ions like glutamate [7]. However, high intracellular

63 concentration of K^+ and glutamate only support microbial adaptation to moderately high

64 osmolarity. At very high osmolarity, further accumulation of K^+ and glutamate becomes

65 impossible for growth, and therefore, bacteria exploit less deleterious compounds called

66 osmoprotectants, like polyoles (trehalose), amino acids (proline), and methyl-amines (glycine

67 betaine) [7]. Osmoprotectants can accumulate intracellularly via cellular uptake or synthesis

68 from their precursors. Glycine betaine is one of the most important osmoprotectants for

69 bacteria [7].

70 For pathogenic bacteria, osmoregulation is a very important factor for establishing

71 infection. For example, *Staphylococcus aureus* has a PutP proline transport system that helps in

72 host tissue colonization [8-10]. Other studies have found a link between osmotic stress and the

73 expression of virulence genes in *Pseudomonas aeruginosa* [11, 12] and between the transport of

74 compatible solutes and colonization in the pathogen in *Listeria monocytogenes* [13, 14]. In

75 *Salmonella enteric* serovar typhimurium and *E. coli* K-12, osmoprotectants are mainly

76 accumulated through the ProP and ProU transport systems [15-19].

77 ProP is a member of the major facilitator superfamily of permeases, and it is known as a

78 symporter [20]. ProP is of low affinity for proline and glycine betaine, it has Km of $\approx$ 0.1 mM for

79 both [20]. ProU system efficiently scavenges glycine betaine [21], Km of $\approx$ 1 μM [16], as well as proline

80 betaine for bacteria to cope extreme osmotic stress [22, 23]. It is composed of three proteins, i.e.,

81 ProV, ProW, and ProX, which are encoded by an operon *proVWX* [18], of which ProV belongs to

82 the ATP-binding cassette (ABC) superfamily [24, 25]. ProX is a periplasmic soluble substrate-

83 binding protein [19, 26, 27] which could bind and deliver glycine betaine to the inner membrane

84 protein ProW, whereas ProV hydrolyses ATP providing energy for transporting substrates

85 against the concentration gradient [24, 25].

86 In 2005, Lucchini and his colleagues [28], analyzed genomic expression of *S. flexneri* during

87 infection by DNA microarray; *pro* genes were found highly up-regulated in both epithelial HeLa

88 and macrophage-like U937 cells; in particular, *proV* was up-regulated by 57-fold in U937 cells

89   [28]. Besides, it was found that the level of ProU transcription in *E. coli* is induced upon exposure

90   to hyperosmotic stress [29]. These findings suggested that *Shigella* faced extreme osmotic stress

91   in the host cell cytosol and up-regulation of ProU was necessary for *Shigella* to cope with this

92   hostile cellular niche, allowing *Shigella* intracellular survival and growth to establish infection.

93   Additionally, the orthologous ProXVWZ system in *Mycobacterium tuberculosis* has been shown

94   to actively transport glycine betaine into macrophages, which contributed to early steps in

95   colonization of the cellular niche [30]. Hence, we have investigated the impact of the ProU

96   system on osmotic stress response and pathogenesis of *S. sonnei*. Our data have shown that

97   the ProU transport system is important for *S. sonnei* to cope with hyperosmotic stress in the

98   host cell cytoplasm, and for rapid intracellular proliferation to establish infection.

99   **Results**

100   ***proV* deletion and complementation**

101   In order to study the function of ProU we decided to construct a *proV* deletion mutant

102   since *proV* is the most highly up-regulated gene inside host cells and the first gene in the

103   *proVWX* operon [28]. *ProV* deletion was constructed in wild type *S. sonnei* strain (20071599) [31]

104   using the phage λ Red recombination system [32], which involved two steps. In the first step, a

105   Kanamycin cassette replaced the wild type *proV* gene via homologous recombination. In the

106   second step, the Kanamycin gene was looped out using pCP20 plasmid, leaving a scar of 102 bp

107   (Fig. S1A). For complementation of the resultant Δ*proV* mutant strain, the entire *proV* coding

108   sequence was amplified with primers (c & d Table S1), and cloned into pGEM-T-Easy (ampR),

109   with the 5'-end of the *proV* coding sequence facing the *lacZ* promoter. The resultant clone was

110   transformed into the Δ*proV* mutant (Fig.S1B). All *proV* constructs for mutagenesis as well as

111   complementation were confirmed by PCR and agarose gel electrophoresis. All PCR products

112   were further confirmed by DNA sequencing using primers c & d (Table S1).

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

114   Because the ProU system is known to be required for *E. coli* to cope with extreme

115   osmotic stress [22, 23], we tested whether this was also the case for *S. sonnei in vitro*. We first

116   measured growth of the wild type, Δ*proV* and the complemented Δ*proV/*pProV strains in M9

117   medium supplemented with 0.3 M NaCl or 0.3 M KCl by measuring the optimal density

118   (OD$_{600nm}$) each one hour till the late stationary phase (10 hours). While the wild type *S. sonnei*

119   grew well, the Δ*proV* strain struggled to grow in the presence of 0.3 M of NaCl or KCl (Fig. 1A

120   and 1B). Obviously, all the tested strains were able to grow properly in M9 medium without

121   any supplements (Fig. S2). Overexpression of ProV *in trans* not only reversed the growth defect

122   of the Δ*proV* strain, but also made the strain grow faster than the wild type. This suggested

123   that deletion of *proV* is solely responsible for the slow growth phenotype of the mutant and

124   also suggested that excess ProV made the system more effective and that ProU may also

125   transport osmoprotectants other than betaine, such as glutamine or K$^+$, available in the

126   medium to facilitate bacterial growth (Fig. 1A and 1B). We further tested the growth of wild

127   type and the Δ*proV* strains in M9 medium supplemented with 0.2 M NaCl or 0.2 M KCl. Under

128   these milder conditions, the Δ*proV* strain grew equally well as the wild type strain up to 6

129   hours. However, the growth of the Δ*proV* strain ceased at 6 hours and remained flat to 10

130   hours. In contrast, the wild type grew steadily, reaching OD$_{600nm}$ = 0.7 and 0.8 in M9

131   supplemented with 0.2 M NaCl and 0.2 M KCl, respectively (Fig. 1C and 1D). Thus, in the

132   presence of 0.2 M NaCl, growth of Δ*proV* was more than 1.5-fold reduced (OD$_{600nm}$ 0.4 vs. 0.7)

133   compared to the wild type at the stationary phase 10 hours (Fig. 1C). In the presence of 0.2 M

134   KCl, there was a 2-fold reduction of Δ*proV* growth (OD$_{600nm}$ 0.4 vs. 0.8) compared to the wild

135   type at the stationary phase (Fig. 1D).

136   Betaine is a well-known compatible solute that is a preferable osmoprotectant for many

137   organisms [33]. We therefore tested the ability of both wild type and the *ΔproV* mutant in

138   utilizing betaine under hyperosmotic conditions. In M9 medium containing 0.2 M NaCl or 0.2 M

139   KCl, supplementation of betaine (500 µM) significantly elevated the growth of the wild type

140   (Fig. 1C & 1D, S3). In contrast, the growth suppression of the *ΔproV* mutant persisted in the

141   presence of 500 µM betaine (Fig. 1C, 1D, S3). Taken together, these data demonstrated that a

142   functional ProU system is required to transport betaine for *S. sonnei,* and that betaine can

143   correct the growth defect of the wild type induced by high concentrations of NaCl and KCl. In

144   contrast, the growth of the *ΔproV* mutant remained severely impaired under high osmotic

145   stress, and could not be corrected by addition of betaine in the medium (Fig. 1C, 1D).

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

147   As shown in Fig. 1C and 1D, the *ΔproV* strain grew equally well as the wild type strain in the

148   first 6 hours albeit its growth was ceased thereafter. This result suggested that the ProU

149   system was functional at least in the first 6 hours when *proV* is removed. Given the fact that

150   *proV* encodes an ATPase and all ATPase of the ABC superfamily share highly conserved

151   sequence and structure (39), we reasoned that ATPase from other transporting systems may

152   compensate for the loss of *proV*. Using ProV as query sequence we identified putative 25

153   ATPases (Table S3), belonging to other transport systems in the *S. sonnei* SSO46 genome

154   (http://www.mgc.ac.cn/ShiBASE/Search.htm). We cloned three of them in random, *oppF*, *glnQ*

155   and *malK*, which are responsible for transporting oligopeptides, glutamate, and maltose,

156   respectively. Over-expressing each of them was able to complement the *ΔproV* strain for

157   better growth in LB broth supplemented with 0.2 M NaCl although OppF appeared less

158   effective than GlnQ and MalK (Fig. S5). These data support our hypothesis that removal of *proV*

159   only rendered ProU system partially inactive, and prompted us to construct mutants with a

160   deletion of the whole *proVWX* operon or in *proX* or *proW*. However, despite repeated efforts

161   we were unable to obtain a *ΔproVWX, or ΔproX* or *ΔproW* strain with intact virulence plasmid,

162   which would allow formation of small and smooth red colonies on Congo red agar. All strains

163   harboring these deletions formed large pale and rough colonies (data not shown). This

164   suggested that ProU is rather important to maintain the genome stability due to its role in

165   transporting important osmoprotectants or other yet unraveled functions. *S. sonnei* must

166   rearrange its genome when completely loosing ProU. Hence, we searched for an alternative

167   approach for knockdown of the ProU system.

168   **Silencing the *proVWX* operon using RNAi**

169   Tchurikov and his colleagues [34] described an RNAi methodology to knockdown gene

170   expression in *E. coli*. According to their work [34] Mirlon (which has the inverted sequence of the

171   gene of interest), is the most potent in silencing the target genes compared to two other

172   constructs (Paralon and Antilon). We adopted described Mirlon approach to knockdown ProU

173   system in *S. sonnei*. We created a piece of dsDNA, by annealing two oligonucleotides of 87

174   bases each, which span 45 and 42 base pairs of the promoter and 5'-end of *proV* coding

175   sequence, respectively (Table S1). This piece of dsDNA was cloned into pGEM-T-Easy (ampR),

176   with the 3'-end of the *proV* coding sequence facing the *lacZ* promoter, which would drive the

177   transcription of RNA molecule with inverted sequences to the *proV* mRNA, hence termed

178   MirproV RNA [34]. We tested the impact of MirproV on the expression of *proV* and *proX,* which

179   are the first and last genes, respectively, in the ProU operon, by qRT-PCR using primers (k & l;

180   m & n respectively, Table S1). The house-keeping gene, *cysG*, was used as an internal control

181   using primers (o & p, Table S1) [35]. Figure 2A shows the results of qRT-PCR after normalisation

182   with transcripts of *cysG* and using transcripts from wild type strain as calibrator. The transcripts

183   of both *proV* and *proX* were significantly reduced as a result of MirproV RNA expression, which

184   indicated that the RNAi construct in MirproV was successful in ProU attenuation.

185   Similar to the *proV* mutant, the MirproV strain grew well as the wild type in M9 without

186   supplements (Fig. S2) but became intolerant to hyperosmolarity; its growth was severely

187   compromised in M9 medium containing either 0.3 M NaCl or 0.3 M KCl (Fig. 1A, 1B). In M9

188   supplemented with 0.2 M NaCl, the MirproV strain had a long lag phase of 4 hours (Fig. 1E).

189   This was in contrast with the Δ*proV* strain, which grew equally well as the wild type strain

190   during this phase of growth under this condition (Fig. 1C). Thus, MirproV was more effective in

191   inactivating the ProU system. Moreover, addition of betaine to M9 medium containing 0.2 M

192   NaCl or 0.2 M KCl failed to rescue growth of the MirproV strain (Fig. 1E, 1F, S3). Thus,

193   expression of MirproV is effective in blocking ProU function and betaine transport.

194   By linear regression analysis, the significance of inhibition of wild type, *proV* and MirproV

195   mutants growth by 0.2 M NaCl and 0.2 M KCl supplements was identified (Fig. S3).

196   Furthermore, paired *t-test* was performed to compare optical density (OD_{600nm}) at the

197   stationary for wild type, Δ*proV* and MirproV in presence and absence of betaine (Fig. S4). The

198   results showed that betaine supplementation significantly increased the optical density for

199   wild type but not for either mutant strains (Fig. S4).

200   To gain insight into the transcriptional regulation of ProU and its inactivation by MirproV,

201   we isolated total RNA from wild type and the MirproV strain from M9 cultures containing

202   either 0.3M NaCl or 0.3M KCl and performed qRT-PCR for transcripts of *proV* and *proX*. Again

203   the house-keeping gene *cysG* was used as an internal control for normalisation. As expected,

204   the wild type responded to the osmotic stress of NaCl or KCl by massive upregulation of *proV*

205   and *proX* transcripts. The MirproV strain also responded, but at a significantly lower level (Fig.

206   2B). These data demonstrated that the ProU system was inducible under high osmotic stress

207 and that MirproV RNA effectively knocked down ProU transcripts which in turn rendered *S.*

208 *sonnei* intolerant to hyperosmolarity.

209 **Inactivation of ProU does not disrupt the type three secretion system (TTSS)**

210   *Shigella* strains possess a type III secretion system (TTSS), which is essential for cell-

211 invasion, phagosome escape and intracellular replication [36]. Therefore, we ought to separate

212 the roles of TTSS and ProU with regard to cell invasion and intracellular growth. We first

213 wanted to confirm that the mutant strains that we were going to test for virulence maintained

214 an intact TTSS. The presence of TTSS genes in the *ΔproV* strain was analyzed by PCR using

215 primers e & f to amplify *ipaB* and primers i & j to amplify *mxiD* (Table S1); both *ipaB* and *mxiD*

216 were found intact in wild type and the mutant strain (Fig. S6). Further, the production and

217 secretion of IpaB and IpaC proteins were investigated by Western blotting using Congo red as

218 an environmental cue *in vitro* as described previously [37]. Because it is known that MxiD is

219 required for type III secretion, we constructed a *ΔmxiD* mutant as a negative control for Ipa

220 secretion, using the same approach as for *ΔproV* construction with primers (g & h; Table S1).

221 Similar levels of IpaB and IpaC were detected in the cell lysates and supernatants of the wild

222 type, the *ΔproV*, the MirproV and the complemented *ΔproV/*pProV strains whereas Ipa

223 proteins were detected only in cell lysate but not supernatant of the *ΔmxiD* mutant (Fig. 3). We

224 therefore conclude that removal of *proV* or knockdown of transcripts did not impair IpaB and

225 IpaC production or secretion – TTSS is functional in the *ΔproV* and the MirproV strains.

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

227   Evidence via DNA microarray analysis indicated that the host cell cytoplasm is a hostile

228 environment which exposes bacteria to hyperosmotic stress [28]. We therefore hypothesized that

229 removal of *proV* or reduction of *proVWX* transcription by RNAi would adversely influence *S.*

230 *sonnei* intracellular growth. To test our hypothesis, the HEK293 cell line, a good model for

231   testing *Shigella* invasion [38], was used as a host to perform gentamicin protection assay to test

232   intracellular growth of the wild type, the *ΔproV* and the MirproV strains. As anticipated, we

233   observed a significant drop in intracellular bacterial burden with the *ΔproV* mutant compared

234   to the wild type 2 hours post infection (Fig. 4A). Noticeably, the MirproV strain had a

235   significantly reduced intracellular bacterial burden, compared to the *ΔproV* mutant. This

236   suggested that suppression of *proVWX* operon was more effective than mutating *proV* gene

237   alone because ProV paralogues may compensate the loss of ProV (Fig. S5). As anticipated,

238   expressing ProV *in trans* restored intracellular growth of the *ΔproV* mutant, demonstrating that

239   the deletion of *proV* had no polar effect on the expression of *proW* and *proX* downstream;

240   removal of *proV* was solely responsible for the defective intracellular growth observed with the

241   *ΔproV* mutant.

242   To further test if the ProU system is required for intracellular growth not invasion per se,

243   we performed a time course analysis of intracellular growth after 1, 2, 3, and 4 hours using

244   gentamicin protection assay. At each time point, cells were lysed with Triton X-100, and cell-

245   lysates were plated out on LB agar, and incubated at 37 $^{\circ}$C overnight and enumerated by

246   colony count. The wild type grew rapidly with a doubling time of approximately 38 min from 1

247   hour onwards whereas the *ΔproV* and the MirproV strains had a doubling time of 60 and 90

248   min, respectively (Fig. 4B, Fig S7).

249   To gather further evidence for ProU requirement for intracellular growth we exploited

250   flow cytometry. We transformed all strains with a plasmid that expressed EGFP, and infected

251   HEK293 cells with these green bacteria. As shown in Figure 4C, 38% of the host cells infected

252   with wild type strain emitted green fluorescence 4 hours post infection, indicating that green

253   bacteria were metabolically active inside host cells at this time point. Noticeably, some cells

254   had very strong EGFP signals (fluorescence intensity above 10^2 units), demonstrating that these

255 cells harbored large numbers of green bacteria as a result of rapid bacterial intracellular

256 growth. In contrast to cells infected with wild type, there were less than 4% of cells infected

257 with the Δ*proV* mutant emitted green fluorescence (Fig. 4C), indicating impaired intracellular

258 growth. We left cell infection overnight and repeated flow cytometry and this showed that

259 43% of cells infected with wild type emitted EGFP signals, (Fig. 4D vs 4C). It was noticeable that

260 a smaller percentage of cells could emit high fluorescent above 10^2 scale compared to that of 4

261 hours post infection (Fig. 4D vs. 4C). Presumably, host cells harboring large numbers of green

262 bacteria were dead following the overnight infection. Consistent with this was the notion that

263 more cells were emitting low fluorescence (intensity of 10^1 to 10^2 units), suggesting that cells

264 harboring lower bacteria burden survived overnight infection. Noticeably also, there was an

265 increase in green fluorescence in host cells that have been infected with the Δ*proV* mutant

266 overnight, suggesting limited remaining intracellular growth of this strain (Fig. 4D). Taken

267 together, ProU is required for intracellular growth of *S. sonnei* in host cells.

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

269   The great moth *G. mellonella* larvae have become a popular *in vivo* model for assessing

270 bacterial virulence [39, 40]. We recently adopted this model to assess *Shigella* virulence and found

271 it comparable with the widely accepted Sereny test [41].

272   Here we exploited this model to compare the virulence of Δ*proV* and MirproV strains

273 with the wild type *S. sonnei*. Larvae were challenged with 10^5 CFU of each strain; 10 larvae per

274 group. Mock-infection was done using the same volume of saline. All groups of larvae were

275 observed daily for five days and dead larvae suffered from melanization and loss of motility. As

276 shown in Figure 5A, wild type and the complemented strain (Δ*proV/*pProV) were able to kill

277 90% and 80% of larvae, respectively, in 1 day and the remaining larvae died at the third day.

278 Using the same dose of the Δ*proV* strain, only 40% of larvae died at the first day, and 30% died

279   at the second day while the rest died at the third day. With the MirproV strain, a dose of 10^5

280   CFU only caused 20% of death at the first day, 10% at the second day and other 10% at the

281   fourth day, while 60% of the whole population survived to the end of the study (Fig. 5A). These

282   data were consistent with those of Figure 4 that MirproV was more effective than *proV*

283   deletion in inactivating the ProU system.

284   Our recent study has demonstrated that infection of larvae hemocytes is an important

285   mechanism for larvae-killing by *S. sonnei* [41]. Therefore, we infected moth larvae with EGFP-

286   expressing strains (Table S2) and isolated hemocytes from infected and mock-infected larvae

287   for flow cytometry. Larvae were infected with 10^6 CFU of each strain, and mock-infection was

288   done with saline. Hemocytes were isolated from larvae 4 hours post infection. We used

289   hemocytes from mock-infected larvae to set gates defining EGFP negative and positive

290   populations (Fig. 5B). It was apparent that wild type strain colonized more hemocytes giving

291   rise to two populations of hemocytes with low and high EGFP intensity, whereas the Δ*proV*

292   strain colonized less numbers of hemocytes with one population of high-EGFP expressing

293   hemocytes at this time point.

294   **Discussion**

295   In pathogenic bacteria, osmoregulation is a very important mechanism not only for

296   survival during environmental osmotic stress, but also in establishment of infection. Examples

297   of osmoregulatory systems important for virulence include the *Staphylococcus aureus* PutP [8-10],

298   the *Pseudomonas aeruginosa* PlcH [11, 12], and *Listeria monocytogenes* OpuC [13, 14].

299   The ProU transport system is widely distributed in bacteria. Previous studies have firmly

300   established its role in environmental survival under hyperosmolarity in *E. coli*, but whether it is

301   required in the context of infection remained unknown [7]. By studying the orthologous

302   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

327   transport system, and *ΔproV* is solely responsible for the slow growth phenotype of the

328   deletion mutant. However, the *ΔproV* mutant grew well in early phase in the presence of 0.2 M

329   NaCl and 0.2 M KCl (Fig. 1C, 1D). This result suggested that the ProU system is, at least in part,

330   still functional when *proV* is removed. By overexpression of three paralogues of *proV in trans*,

331   we have demonstrated that ATPases from other transport systems are able to compensate for

332   the loss of ProV for better growth (Fig. S5).

333   The MirproV strain does not significantly respond to betaine supplementation in M9

334   medium with 0.2 M NaCl or 0.2 M KCl for better growth (Fig. 1E, 1F). These data suggest that

335   MirproV could effectively attenuate ProU system. Even though the MirproV strain produced

336   more transcripts of *proV* and *proX* in response to 0.3 M NaCl and 0.3 M KCl (Fig. 2B) compared

337   to its transcripts in M9 medium without any supplements (Fig. 2A), these levels of responses

338   were not sufficient to reverse the growth defect (Fig. 1E, 1F; S3, S4). The effectiveness of

339   MirproV was also demonstrated in gentamicin protection assay; the MirproV strain was more

340   severely defective than the *ΔproV* mutant in the intracellular growth (Fig. 4A, 4B). Moreover,

341   the possibility of 'off-target' effects by MirproV is low; there are only 4 low score hits when the

342   42 bp *proV* sequence is used as query to blast the *S. sonnei* SSO46 genome (Table S4).

343   Noticeably, *mxiJ* is one of the hits; *mxiJ* encodes an essential component of TTSS [46]However,

344   like the *ΔproV* strain, the MirproV strain produces and secretes Ipa proteins (Fig. 3), suggesting

345   that any 'off-target' hits, if they occur, do not significantly change the strain's osmotolerance

346   or virulence.

347   Our inability to obtain strains with intact virulence plasmid with deletion of the whole

348   *proVWX* operon, or *proX* or *proW* strongly suggests the ProU system plays an important role in

349   *Shigella* genome stability. Previous studies have shown that *S. sonnei* could frequently become

350   avirulent by losing its 120-megadalton virulence plasmid [47]. Besides, it is well known that both

351 ProU and the TTSS genes are controlled by the same negative regulator: the H-NS protein and

352 this regulatory link provided an additional rational for exploring the possibility of altered TTSS

353 expression upon ProU attenuation [48, 49]. So, it was very important to test the stability of this

354 plasmid in wild type and the mutant strains under investigation (Δ*proV*, MirproV and the

355 complemented Δ*proV/*pProV). Our data show *ΔproV* strain possesses key plasmid borne

356 virulence genes, *ipaB* and *mxiD*. Furthermore, all strains produce and secrete IpaB and IpaC

357 proteins, except *ΔmxiD* strain that produces but does not secrete Ipa proteins (Fig. 3). Hence,

358 we can conclude that inactivation of the ProU system by either deletion of *proV* or RNAi

359 approach does not impair genetic stability and function of the virulence plasmid.

360 Finally, we have once again demonstrated the usefulness of the moth larvae model in

361 assessing bacterial virulence, which enabled us to establish a link between osmotolerance and

362 virulence.

363 **Conclusions**

364 Altogether, we conclude that the ProU system is important for *S. sonnei* to tolerate

365 hyperosmotic stress *in vitro,* as well as for survival and proliferation of the bacteria in the

366 stressful intracellular niche. Silencing of the whole *proVWX* operon is more effective than

367 deleting *proV* alone because *proV* paralogues may compensate for the loss of *proV*. Last but

368 not least, the *G. mellonella* larvae model is a cost-effective and good model for studying

369 *Shigella* virulence, and reflects results of more established models such as the Sereny test.

370 **Materials and Methods**

371 **Bacterial strains and growth conditions**

372 The wild type *Shigella* strain used in this study was *S. sonnei* strain 20071599 [31] and

373 mutants thereof. Bacteria were routinely grown on Congo red TSA plates or in liquid LB at 37

374 ^{o}C. To obtain EGFP expressing bacteria, strains were transformed with pGEMT-Easy (Amp[R])

16

375   containing EGFP. Afterwards, 100 µg/ml ampicillin was added for selection of plasmid

376   containing strains. All primers and strains used in this study are listed in Tables S1 and S2

377   respectively.

378   **Genetic engineering**

379   Construction of the mutant bearing in-frame deletion in *proV* gene was done using phage

380   λ Red recombination system [32]. Construction of the mutant bearing in-frame deletion in *proV*

381   gene was done using phage λ Red recombination system [50]. Both IpaB and IpaC were detected

382   using the monoclonal antibodies H16 (anti-IpaB) and J22 (anti- IpaC), respectively [50], followed

383   by incubation with Alexa Fluor 680 goat anti-mouse IgG (H+L), and the images were visualized

384   using a UV scanner at 700 nm.

385   *In vitro* **osmotolerance test**

386   M9 medium was prepared, autoclaved and supplemented with filtered 200 µl/100ml of 1

387   M $MgSO_4$, 4% glucose, 12.5 µg/ml of Nicotinic acid, 45 µg/ml of L-methionine, and 20 µg/ml of

388   L-Tryptophan. Routinely, all strains were grown on M9 agar overnight, smooth colonies were

389   picked and grown in M9 broth 2 hours before the start of the experiment. Then, 96-well plates

390   were set for growth curve; each well contained 200 µl M9 medium with or without

391   supplements: 0.2 and 0.3 M NaCl or 0.2 and 0.3 M KCl in presence and absence of 500 µM

392   betaine. Each culture condition was tested in triplicates for each bacterial strain, and three

393   independent experiments were carried out. Afterwards, 10^7 CFU from each of the strains were

394   added to the 96-well plates. Wells without bacterial inocula were used as a blank, while wells

395   without supplements were used to study the growth curve of *Shigella* strains in M9 medium.

396   The plates were incubated in a shaker incubator at 37 °C, and the OD_{600nm} was recorded each 1

397   hour.

398   **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 °C. The plates were incubated for 40 min at 37 °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

423   lysed with Triton X-100 (0.1 % in H_2O) for 10 min. Cell lysates were serially diluted, plated on LB

424   agar, incubated at 37 ^{o}C overnight and enumerated by colony count.

425   **Infection of *Galleria mellonella* larvae**

426   Smooth red colonies of *S. sonnei* strains were selected from Congo red TSA plates, grown

427   for 3 to 5 hours in LB broth to prepare bacterial suspensions. Each group of 10 healthy larvae

428   of approximately similar size were injected with 10 μl (10^5 CFU) of each bacterial suspension.

429   The mock-infection group received sterile PBS instead of bacteria. All larvae were incubated at

430   37 ^{o}C [52] and observed for 5 days post infection [41]. The experiments have been repeated three

431   times for results confirmation and the averages have been used in Kaplan-Meier survival

432   curves.

433   **Flow cytometry**

434   For HEK293 cells, infection was terminated at appropriate time intervals (either 4 hours

435   or overnight); cells were washed with saline twice and then trypsinized for 1 min after which

436   the DMEM medium was added. Cells were spun down and cell pellets were resuspended in

437   saline containing 4% (v/v) paraformaldehyde for fixation.

438   For the wax moth larvae, 4 hours post-infection with 10^6 CFU bacteria, hemocytes from

439   10 larvae were collected by incision between two segments near larvae tail to avoid gut

440   interruption. Hemocytes were collected in 1 ml of sterile PBS and the processing was within 10

441   min to prevent clotting [52]. Cells were centrifuged at 500 xg for 10 min at room temperature,

442   and were resuspended in 1 ml PBS containing 4% (v/v) paraformaldehyde for fixation.

443   Cells were vigorously mixed and then were used for the flow-cytometry. Lasers emitting

444   at 488 nm was set for the detecting signals in FITC channel, which overlap with EGFP

445   excitation. Data acquisition was performed using Kaluza™ software (Beckman Coulter, Inc). The

446   experiments have been repeated three times for results confirmation.

447   **Statistics**

448   The growth curves in Fig. 1 are x/y blotting using triplicates for each reading and the error bars

449   are for SD. In Fig 2, unpaired *t test* has been used to compare the expression of different genes

450   using $2^{-\Delta\Delta ct}$ method. In Fig 4, unpaired *t test* has been used to compare the % intracellular CFU

451   of different *Shigella* strains; the growth curve of intracellular growth of *Shigella* strains is x/y

452   blotting using triplicates for each reading and the error bars are for SD.

453   **Competing interests**

454   The Authors declare no conflict of interest.

455   **AUTHOR CONTRIBUTIONS**

456   Conceived and designed experiments: JY

457   Performed the experiments: RYM WL

458   Analyzed the data: RYM JY

459   Contributed reagents/materials/analysis tools: JY ME ERA

460   Wrote the paper: RYM JY

461

467

468   **References**

469   1. Hale TL. Genetic basis of virulence in *Shigella* species. Microbiol Rev 1991; 55:206-24.

470   2. Peng J, Zhang X, Yang J, Wang J, Yang E, Bin W, Wei C, Sun M, Jin Q. The use of comparative genomic

471   hybridization to characterize genome dynamics and diversity among the serotypes of *Shigella*. BMC

472   Genomics 2006; 7:218.

473   3. von Seidlein L, Kim DR, Ali M, Lee H, Wang X, Thiem VD, Canh do G, Chaicumpa W, Agtini MD,

474   Hossain A, et al. A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical

475   manifestations, and microbiology. PLoS Med 2006; 3:e353.

476   4. Sansonetti PJ. Slaying the Hydra all at once or head by head? Nat Med 1998; 4:499-500.

477   5. Sansonetti PJ. Microbes and microbial toxins: paradigms for microbial-mucosal interactions III.

478   Shigellosis: from symptoms to molecular pathogenesis. Am J Physiol Gastrointest Liver Physiol 2001;

479   280:G319-23.

480   6. Arakawa T, Timasheff SN. The stabilization of proteins by osmolytes. Biophys J 1985; 47:411-4.

481   7. Lucht JM, Bremer E. Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation

482   of the high-affinity glycine betaine transport system proU. FEMS Microbiol Rev 1994; 14:3-20.

483   8. Schwan WR, Coulter SN, Ng EY, Langhorne MH, Ritchie HD, Brody LL, Westbrock-Wadman S, Bayer

484   AS, Folger KR, Stover CK. Identification and characterization of the PutP proline permease that

485   contributes to *in vivo* survival of *Staphylococcus aureus* in animal models. Infect Immun 1998; 66:567-

486   72.

487   9. Bayer AS, Coulter SN, Stover CK, Schwan WR. Impact of the high-affinity proline permease gene

488   (putP) on the virulence of *Staphylococcus aureus* in experimental endocarditis. Infect Immun 1999;

489   67:740-4.

490   10. Schwan WR, Wetzel KJ, Gomez TS, Stiles MA, Beitlich BD, Grunwald S. Low-proline environments

491   impair growth, proline transport and *in vivo* survival of *Staphylococcus aureus* strain-specific putP

492   mutants. Microbiology 2004; 150:1055-61.

493   11. D'Souza-Ault MR, Smith LT, Smith GM. Roles of N-acetylglutaminylglutamine amide and glycine

494   betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. Appl Environ Microbiol 1993;

495   59:473-8.

496   12. Sage AE, Vasil ML. Osmoprotectant-dependent expression of plcH, encoding the hemolytic

497   phospholipase C, is subject to novel catabolite repression control in *Pseudomonas aeruginosa* PAO1. J

498   Bacteriol 1997; 179:4874-81.

499   13. Sleator RD, Wouters J, Gahan CG, Abee T, Hill C. Analysis of the role of OpuC, an osmolyte transport

500   system, in salt tolerance and virulence potential of *Listeria monocytogenes*. Appl Environ Microbiol

501   2001; 67:2692-8.

502   14. Wemekamp-Kamphuis HH, Wouters JA, Sleator RD, Gahan CG, Hill C, Abee T. Multiple deletions of

503   the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth

504   at high osmolarity. Appl Environ Microbiol 2002; 68:4710-6.

505   15. Frossard SM, Khan AA, Warrick EC, Gately JM, Hanson AD, Oldham ML, Sanders DA, Csonka LN.

506   Identification of a third osmoprotectant transport system, the osmU system, in *Salmonella enterica*. J

507   Bacteriol 2012; 194:3861-71.

508   16. Cairney J, Booth IR, Higgins CF. Osmoregulation of gene expression in *Salmonella typhimurium*: proU

509   encodes an osmotically induced betaine transport system. J Bacteriol 1985; 164:1224-32.

510   17. Dunlap VJ, Csonka LN. Osmotic regulation of L-proline transport in *Salmonella typhimurium*. J

511   Bacteriol 1985; 163:296-304.

512   18. Gowrishankar J. Nucleotide sequence of the osmoregulatory proU operon of *Escherichia coli*. J

513   Bacteriol 1989; 171:1923-31.

514   19. May G, Faatz E, Villarejo M, Bremer E. Binding protein dependent transport of glycine betaine and

515   its osmotic regulation in *Escherichia coli* K12. Mol Gen Genet 1986; 205:225-33.

516   20. MacMillan SV, Alexander DA, Culham DE, Kunte HJ, Marshall EV, Rochon D, Wood JM. The ion

517   coupling and organic substrate specificities of osmoregulatory transporter ProP in *Escherichia coli*.

518   Biochim Biophys Acta 1999; 1420:30-44.

519   21. Ko R, Smith LT, Smith GM. Glycine betaine confers enhanced osmotolerance and cryotolerance on

520   *Listeria monocytogenes.* J Bacteriol 1994; 176:426-31.

521   22. Gouesbet G, Jebbar M, Talibart R, Bernard T, Blanco C. Pipecolic acid is an osmoprotectant for

522   *Escherichia coli* taken up by the general osmoporters ProU and ProP. Microbiology 1994; 140 (Pt

523   9):2415-22.

524   23. Haardt M, Kempf B, Faatz E, Bremer E. The osmoprotectant proline betaine is a major substrate for

525   the binding-protein-dependent transport system ProU of *Escherichia coli* K-12. Mol Gen Genet 1995;

526   246:783-6.

527   24. Doige CA, Ames GF. ATP-dependent transport systems in bacteria and humans: relevance to cystic

528   fibrosis and multidrug resistance. Annu Rev Microbiol 1993; 47:291-319.

529   25. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol 1992; 8:67-113.

530   26. Barron A, Jung JU, Villarejo M. Purification and characterization of a glycine betaine binding protein

531   from *Escherichia coli*. J Biol Chem 1987; 262:11841-6.

532   27. Higgins CF, Sutherland L, Cairney J, Booth IR. The osmotically regulated proU locus of *Salmonella*

533   *typhimurium* encodes a periplasmic betaine-binding protein. J Gen Microbiol 1987; 133:305-10.

534   28. Lucchini S, Liu H, Jin Q, Hinton JC, Yu J. Transcriptional adaptation of *Shigella flexneri* during

535   infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial

536   pathogen. Infect Immun 2005; 73:88-102.

537   29. Gowrishankar J, Manna D. How is osmotic regulation of transcription of the *Escherichia coli* proU

538   operon achieved? A review and a model. Genetica 1996; 97:363-78.

539   30. Price CT, Bukka A, Cynamon M, Graham JE. Glycine betaine uptake by the ProXVWZ ABC transporter

540   contributes to the ability of *Mycobacterium tuberculosis* to initiate growth in human macrophages. J

541   Bacteriol 2008; 190:3955-61.

542   31. Xu D, Yang X, Wang D, Yu J, Wang Y. Surface display of the HPV L1 capsid protein by the

543   autotransporter *Shigella* IcsA. J Microbiol 2014; 52:77-82.

544   32. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using

545   PCR products. Proc Natl Acad Sci U S A 2000; 97:6640-5.

546   33. Haardt M, Bremer E. Use of phoA and lacZ fusions to study the membrane topology of ProW, a

547   component of the osmoregulated ProU transport system of *Escherichia coli*. J Bacteriol 1996; 178:5370-

548   81.

549   34. Tchurikov NA, Chistyakova LG, Zavilgelsky GB, Manukhov IV, Chernov BK, Golova YB. Gene-specific

550   silencing by expression of parallel complementary RNA in *Escherichia coli*. J Biol Chem 2000; 275:26523-

551   9.

552   35. Zhou K, Zhou L, Lim Q, Zou R, Stephanopoulos G, Too HP. Novel reference genes for quantifying

553   transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. BMC Mol

554   Biol 2011; 12:18.

555   36. Carayol N, Tran Van Nhieu G. Tips and tricks about *Shigella* invasion of epithelial cells. Curr Opin

556   Microbiol 2013; 16:32-7.

557   37. Bahrani FK, Sansonetti PJ, Parsot C. Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules

558   and kinetics of activation. Infection and immunity 1997; 65:4005-10.

559   38. Paciello I, Silipo A, Lembo-Fazio L, Curcuru L, Zumsteg A, Noel G, Ciancarella V, Sturiale L, Molinaro

560   A, Bernardini ML. Intracellular *Shigella* remodels its LPS to dampen the innate immune recognition and

561   evade inflammasome activation. Proc Natl Acad Sci U S A 2013; 110:E4345-54.

562   39. Leuko S, Raivio TL. Mutations that impact the enteropathogenic *Escherichia coli* Cpx envelope stress

563   response attenuate virulence in *Galleria mellonella*. Infect Immun 2012; 80:3077-85.

564   40. Viegas SC, Mil-Homens D, Fialho AM, Arraiano CM. The virulence of *Salmonella enterica* Serovar

565   Typhimurium in the insect model *Galleria mellonella* is impaired by mutations in RNase E and RNase III.

566   Appl Environ Microbiol 2013; 79:6124-33.

567   41. Mahmoud RY, Stones DH, Li W, Emara M, El-Domany RA, Wang D, Wang Y, Krachler AM, Yu J. The

568   Multivalent Adhesion Molecule SSO1327 plays a key role in *Shigella sonnei* pathogenesis. Mol Microbiol

569   2015; 99:658-73.

570   42. Wong D, Bach H, Sun J, Hmama Z, Av-Gay Y. *Mycobacterium tuberculosis* protein tyrosine

571   phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification. Proc Natl

572   Acad Sci U S A 2011; 108:19371-6.

573   43. Denkert C, Warskulat U, Hensel F, Haussinger D. Osmolyte strategy in human monocytes and

574   macrophages: involvement of p38MAPK in hyperosmotic induction of betaine and myoinositol

575   transporters. Arch Biochem Biophys 1998; 354:172-80.

576   44. Melse-Boonstra A, Holm PI, Ueland PM, Olthof M, Clarke R, Verhoef P. Betaine concentration as a

577   determinant of fasting total homocysteine concentrations and the effect of folic acid supplementation

578   on betaine concentrations. Am J Clin Nutr 2005; 81:1378-82.

579   45. Metris A, George SM, Mulholland F, Carter AT, Baranyi J. Metabolic shift of *Escherichia coli* under

580   salt stress in the presence of glycine betaine. Appl Environ Microbiol 2014; 80:4745-56.

581   46. Allaoui A, Sansonetti PJ, Parsot C. MxiJ, a lipoprotein involved in secretion of *Shigella* Ipa invasins, is

582   homologous to YscJ, a secretion factor of the *Yersinia* Yop proteins. J Bacteriol 1992; 174:7661-9.

583   47. Sansonetti PJ, Kopecko DJ, Formal SB. *Shigella sonnei* plasmids: evidence that a large plasmid is

584   necessary for virulence. Infect Immun 1981; 34:75-83.

585   48. Dagberg B, Uhlin BE. Regulation of virulence-associated plasmid genes in enteroinvasive *Escherichia*

586   *coli*. J Bacteriol 1992; 174:7606-12.

587   49. Kane KA, Dorman CJ. Rational design of an artificial genetic switch: Co-option of the H-NS-repressed

588   proU operon by the VirB virulence master regulator. J Bacteriol 2011; 193:5950-60.

589   50. Yu J, Edwards-Jones B, Neyrolles O, Kroll JS. Key role for DsbA in cell-to-cell spread of *Shigella*

590   *flexneri*, permitting secretion of Ipa proteins into interepithelial protrusions. Infect Immun 2000;

591   68:6449-56.

592   51. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW,

593   Shipley GL, et al. The MIQE guidelines: minimum information for publication of quantitative real-time

594   PCR experiments. Clin Chem 2009; 55:611-22.

595   52. Harding CR, Schroeder GN, Collins JW, Frankel G. Use of *Galleria mellonella* as a model organism to

596   study *Legionella pneumophila* infection. J Vis Exp 2013:e50964.

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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

622   from cell lysates (A) and culture supernatants (B) were separated on SDS-PAGE and IpaB and

623   IpaC were detected with anti-IpB and anti-IpaC antibodies.

624   **Figure 4**. Testing the intracellular growth of the two mutation approaches *in vitro* using

625   HEK293 cells

626   (A) Intracellular growth of *S. sonnei* 2 hours post infection (MOI of 10). Intracellular CFU of the

627   wild type were taken as 100%, and intracellular CFU from strains Δ*proV*, MirproV and

628   Δ*proV/*pProV were expressed as percentages to that of wild type. Each value is the mean of

629   three independent determinations ± standard deviation. The level of significance was

630   determined using unpaired *t*-test, Asterisks (****) indicate *p*-values < 0.0001, (***) means *p*-

631   values = 0.0003. (B) Time course of intracellular growth of *S. sonnei* (MOI of 10). At indicated

632   time interval post infection, cells were lysed and intracellular CFU were determined by plating

633   on agar. Each value is the mean of triplicates ± standard deviation (n=3). Doubling time for

634   each strain is calculated by linear regression analysis (Fig. S7). (C, D) Overlay of the flow

635   cytometry analysis of cells were infected with Δ*proV* (dark grey), wild type (black) or mock-

636   infected (light grey). Both *S. sonnei* strains were expressing EGFP. Controls are cells mock-

637   infected with saline. Analysis was done 4 hours post infection (C) or overnight (D). Gate A

638   depicts populations of cells emit EGFP signals.

639   **Figure 5.** Testing the two mutation approaches *in vivo* using *G. mellonella* larvae

640   (A) Fraction survival of *G. mellonella* larvae model challenged by 10^5 CFU of wild type *S. sonnei*

641   strain 20071599 (grey square), the complemented strain (Δ*proV/*pProV) (black circle), Δ*proV*

642   (black triangle) and the MirproV (black trigonal), using saline as a control (crosses). The

643   observation lasted for 5 days. The results are means of three successive groups (n=10 larvae).

644   (B) Overlaid histogram of the flow cytometry analysis of hemocytes isolated from *G. mellonella*

645   larvae mock-infected as a control (light grey), challenged by *S. sonnei* wild strain 20071599

646   (dark grey) or by Δ*proV* (black). Both *S. sonnei* strains were expressing EGFP. Hemocytes were

647   isolated 4 hours post infection for analysis; gate A depicts hemocytes emit GFP signals.
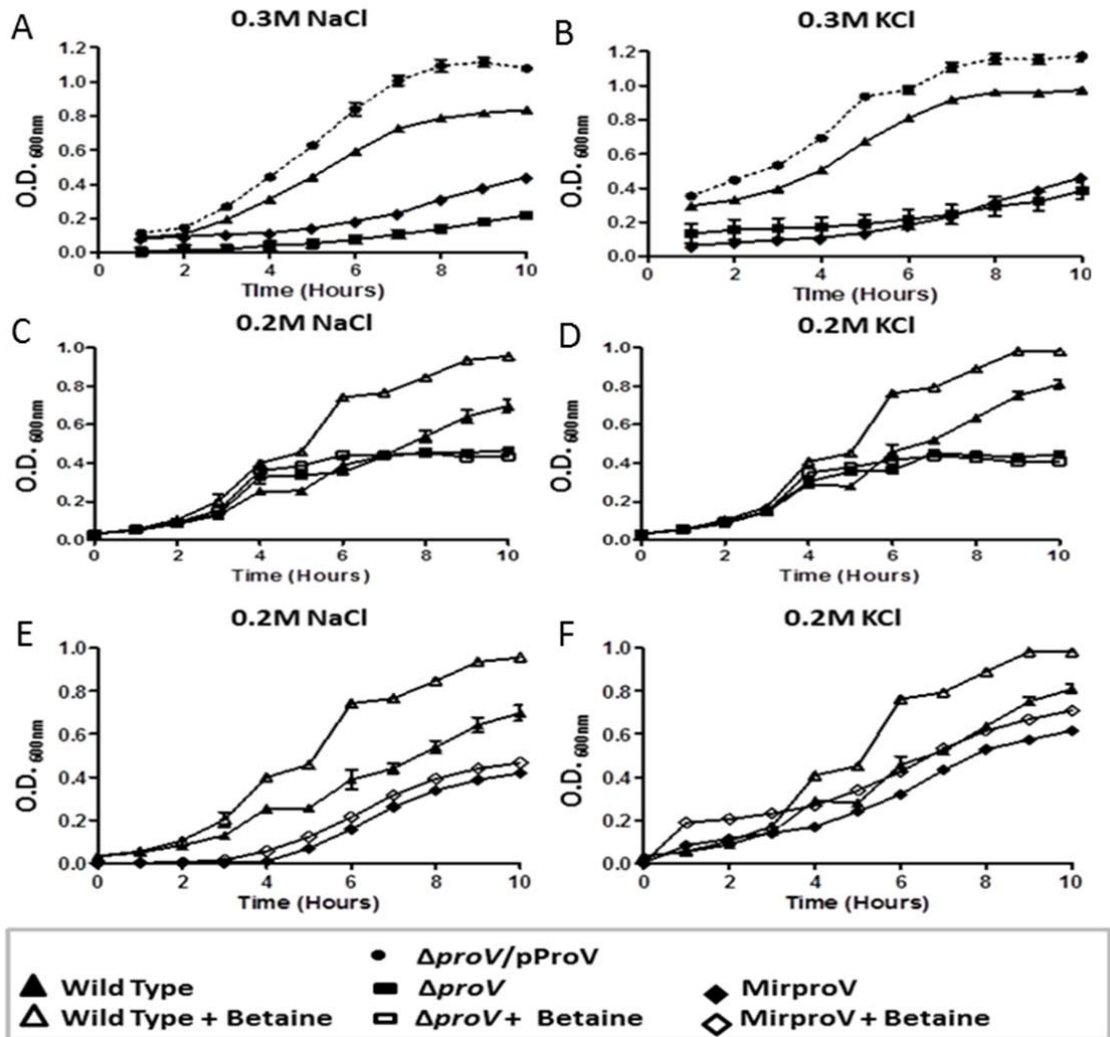
648

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

Growth curves of wild type (triangle), *proV* mutant (square), MirproV (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 three successive wells ± standard deviation (n=3).
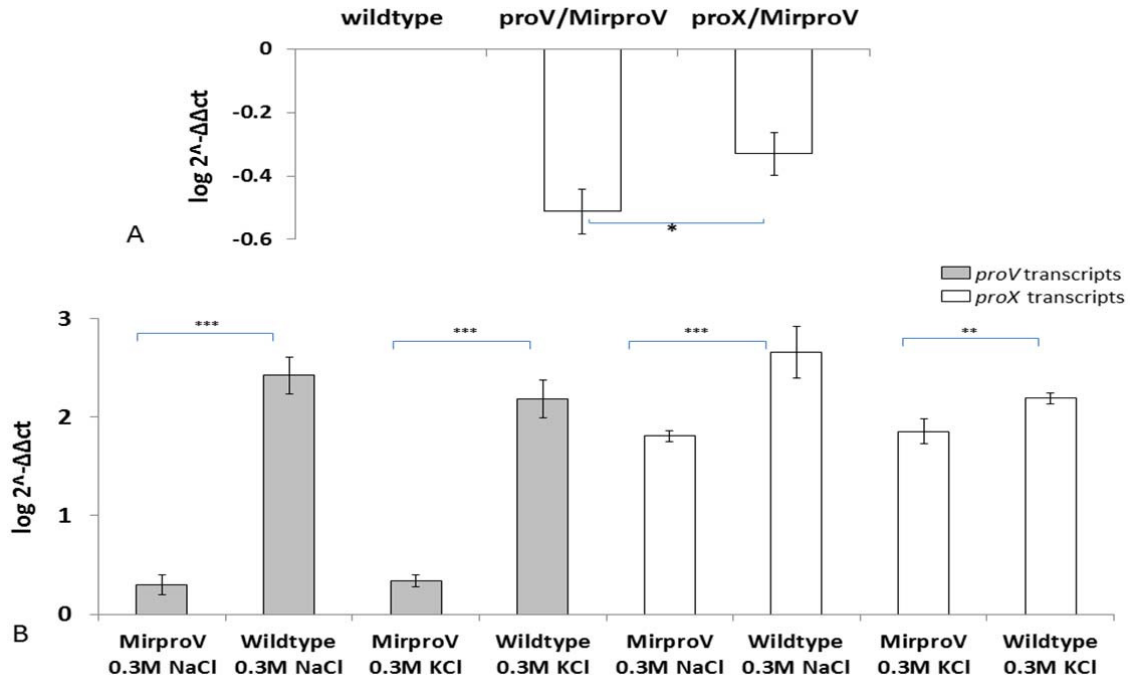
Figure 2. qRT-PCR analysis for both *proV* and *proX* transcripts in both wild type and MirproV

(A) qRT-PCR analysis of wild type and MirproV strains growth in M9 medium without supplement. Levels of *proV* and *proX* transcripts in wild type was set as calibrator (zero) and levels of transcripts in MirproV strain were calculated using the $2^{-\Delta\Delta ct}$ method; a significant drop of *proV* and *proX* transcripts was observed in MirproV strains. 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* (grey columns) and *proX* (open columns) genes in both wild type and MirproV grown in hyperosmotic media. The difference in *proV* transcripts between wild type and MirproV strains in presence of both 0.3 M NaCl and KCl is highly significant (*** *p* < 0.0001; grey columns); and the difference 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).

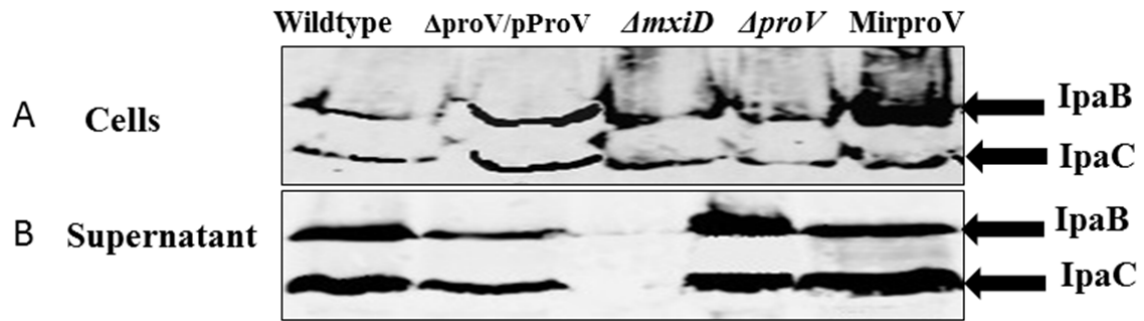All the results are means of three successive groups ± standard deviation (n=3).

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

S. sonnei wild type, the complemented strain Δ*proV/*PproV, *ΔmxiD*, 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.
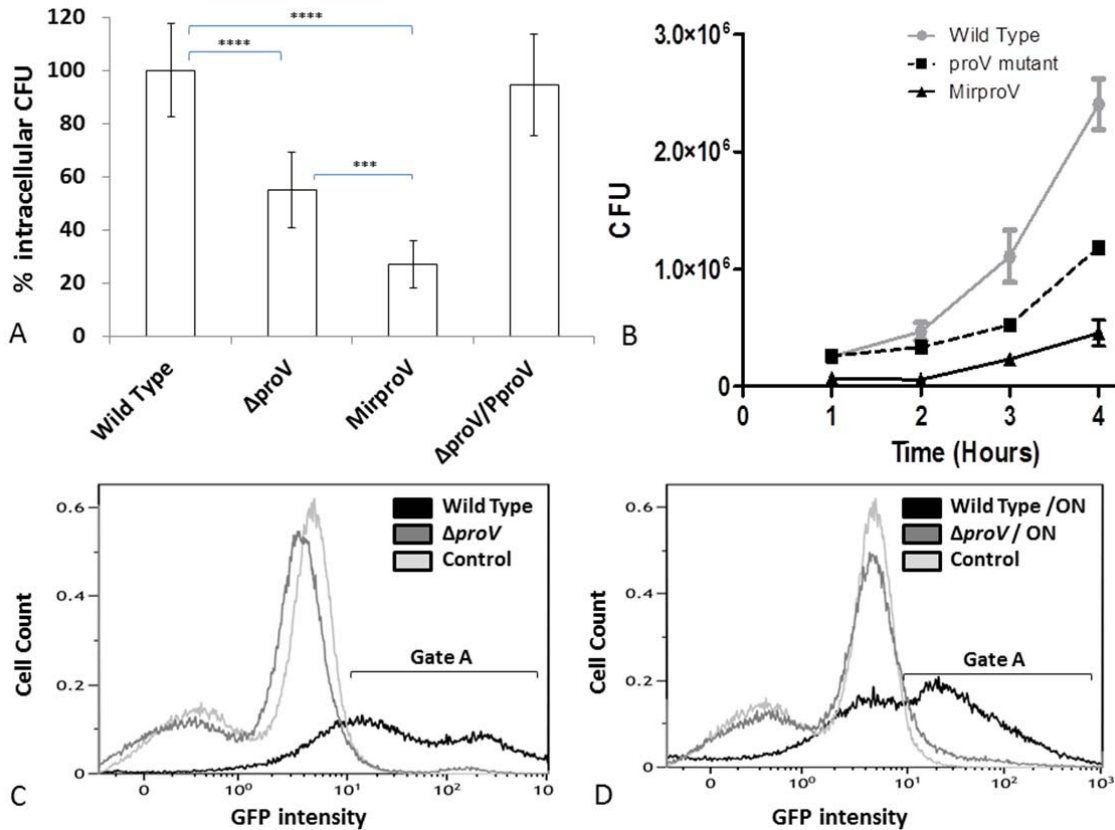
Figure 4. Testing the intracellular growth of the two mutation approaches *in vitro* using

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

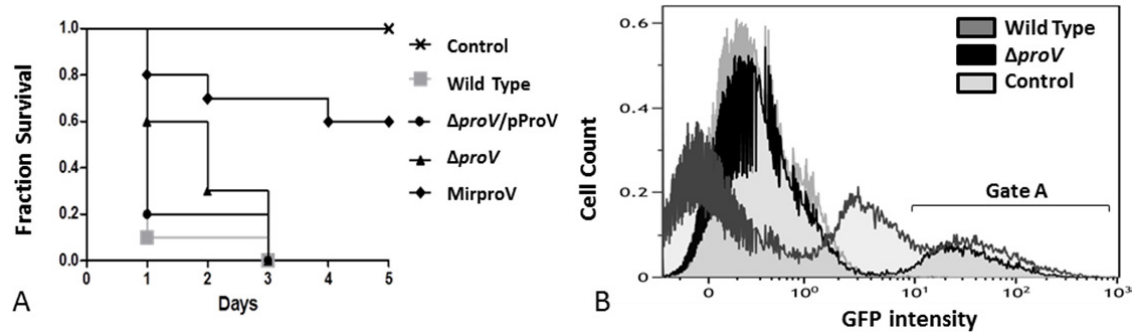overnight (D). Gate A depicts populations of cells emit EGFP signals.

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 (dark grey) 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.